Full Paper

Toxicity Assessment of Energetic Materials by Using the Luminescent Bacteria Inhibition Test

Thomas M. Klapötke,*[a] Regina Scharf,[a] Jörg Stierstorfer,[a] and Cornelia C. Unger[a]

Abstract: The luminescent bacteria inhibition test using *Aliivibrio fischeri* is a well-established method to determine the aquatic toxicity of soluble chemicals. More precisely, the effective concentration (EC_{50}) after 15 and 30 min is determined in this test. The inhibition of natural bioluminescence of these bacteria gives a first idea of the toxicity of compounds towards some aquatic organisms. It is a cost and time efficient experimental method, which does not involve animals. In this contribution, the experimental set up, comparability with other measurements, and results of recently described compounds is presented. Different types of energetic materials such as coordination (*e.g.* $[Cu(dtp)_3](ClO_4)_2$ and $[Fe(MTZ)_6](ClO_4)_2$), neutral (*e.g.* azidoethanol, 1- and 2-aminotetrazole) and ionic (*e.g.* polynitropyrazolates and PETNC salts) compounds were investigated and compared to commonly used materials, like RDX, ammonium perchlorate (AP) and azide salts. Furthermore, different substitution patterns and energetic functionalities such as azido-, nitro- and nitramino-groups were investigated.

1 Introduction

Strong research efforts are ongoing to find new energetic materials with superior energetic properties like higher performance, lower sensitivities, and better stabilities during the last decades [1]. However, not only physicochemical properties are important but also environmentally friendly substances are requested. Unfortunately, some commonly used explosives, such as hexogen (RDX) and 2,4,6-trinitrotoluene (TNT) have shown to be toxic depending on dose and organism [2]. Furthermore, waste streams of TNT, are a possible source for pollution of drinking water with TNT, dinitrotoluene, nitrotoluene, and acids [3]. Another example of how the ecosystem could be affected is that munitions manufacturing led to contamination of soils, sediments, and water with explosives such as TNT, RDX, and PETN [4]. Furthermore, lead is a highly poisonous metal whether it is inhaled or swallowed affecting the whole body and may even cause death in high concentrations [5]. Studies showed that at shooting ranges and military training grounds the maximum accepted concentration of lead (0.15 mg m^{-3}) [6] is often exceeded by far [1c, 7]. One possible reason is that lead is the main element of lead shots, which also contain variable amounts of tin, arsenic, and antimony [8]. The latter and also lead are replaced in the SINTOX primer composition, developed at the Dynamit Nobel AG [9]. Amongst others, this development provoked the research towards 'greener' primer compositions and pyrotechnics. Still, ammonium perchlorate is the commonly used oxidizer in solid rocket propellants. Furthermore, many pyrotechnic formulations contain perchlorates, since there is a lack of suitable alternatives. It is known that the per-

chlorate anion interferes with the thyroid function [10]. Therefore, there is need for new environmentally friendlier energetic materials.

Several tests have been implemented to determine the toxicity of compounds or ground waters utilizing plants, algae, fishes, mice, or water fleas [11]. However, most of these tests show disadvantages like huge test volume, long exposure periods, difficulties with the standardizations of the organisms, and subsequent low reproducibilities [12]. Comparing the toxicity levels of TNT and PETN in *Aliivibrio fischeri*, *Daphnia magna*, and *Pimephales promales*, as well as the LD_{50} value in rats, TNT is more toxic than PETN in every organism. Comparing the exposure periods, *Aliivibrio fischeri* (30 min) is superior to *Daphnia magna* (48 h) as well as *Pimephales promales* (96 h) and amongst them the only animal free test. Therefore, Luminescent Bacteria Inhibition Test provides a quick, simple, and reproducible possibility to test new energetic materials towards their environmental

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acceptability for aquatic organisms. Since the bioluminescent bacterium *Aliivibrio fischeri* is an excellent representative for aquatic life, it is used as indicator for groundwater pollution [13] and gained more attention in different research areas over the last years [14]. Bioluminescence is a form of chemiluminescence where light is released by a chemical reaction. The complex biochemical mechanism of the bioluminescent marine bacteria *Aliivibrio fischeri* is shown in Figure 1 [15]. In the system, three enzymatic complexes are involved: the Flavin Reductase (FMN Reductase), the Luciferase, and the Fatty Acid Reductase. In the first step flavin mononucleotide (FMN) is metabolized to its reduced form (FMNH$_2$) catalyzed by the FMN Reductase. The reduced flavin molecule is able to bind to the Luciferase and in combination with an aliphatic aldehyde and under consumption of oxygen the peroxihemiacetal complex L-FMNH−O−O−CHOH−R is formed. In the following step aliphatic acid is released and a singlet excited hydroxide complex (L-FMNH-OH)* is generated, which directly reacts to the hydroxide complex L-FMNHOH in the ground state under liberation of light in a chemically initiated electron exchange luminescence (*CIEEL*) mechanism (see ESI). The emitted light with a wavelength of 490 nm can be observed and measured by a photomultiplier [15–16].

The metabolic activity of the bacteria is directly proportional to the light production and any inhibition of enzymatic activity due to toxicant causes a corresponding decrease in bioluminescence. The value at which the luminescence of *Aliivibrio fischeri* is reduced by 50% at its specific concentration is determined and is defined as EC$_{50}$ (effective concentration). This concentration is determined after 15 min and 30 min, respectively, for various energetic materials such as RDX, ammonium perchlorate, and several more recently synthesized neutral and ionic compounds, as well as complexes.

2 Experimental Section

2.1 Test Compounds

Commercially available compounds were used as supplied without further purification. The purity of in-house synthesized materials was determined using elemental analysis.

2.2 Measurement

Liquid dried luminescent bacteria of the strain *Aliivibrio fischeri* NRRL-B-11177 obtained by HACH LANGE GmbH (Düsseldorf, Germany) were used for the luminescent bacteria inhibition test.

Prior to the measurements a 2% NaCl stock solution was prepared using HPLC-grade water to ensure optimal salt conditions for the bacteria. The tested compounds of known weight are diluted in this stock solution and after complete solvation, as well as setting the pH value to 6–8, were adjusted to a final volume. A dilution series was prepared out of this test solution referring to DIN 38412L34, L341. The dilutions range from 1:2 to 1:32. Due to the low solubility of RDX in water RDX was first dissolved in acetone and then diluted in 2% NaCl stock solution to obtain a 1% (vol%) acetone concentration for each dilution. A 1% acetone concentration in the control of the measurement showed a negligible effect on the bacteria [17]. Our resulting values for RDX are consistent with literature values [18].

The measurements were performed on a *LUMIStox 300* spectrometer obtained by HACH LANGE GmbH (Düsseldorf, Germany), were DIN EN ISO 11348-2, which is similar to ASTM method D5660, was used as a guideline [19]. The samples were incubated at 15 °C and the luminescence was tested in the beginning of each experiment and after 15 min and 30 min. During the whole measurement, the temperature must be kept at this temperature within a range of ±0.3 °C. Each dilution step was measured twice. To calculate the correction factor of a non-toxic control two bacteria suspensions with 1% NaCl were measured at the beginning of each measurement. The toxicity data with the inhibition were used to fit a straight line, and therefore to calculate the EC$_{50}$ value. For details of the calculation see ESI. When the inhibition of a compound did not reach the 10% limit, the EC$_{50}$ reported "≫" for the highest measured.

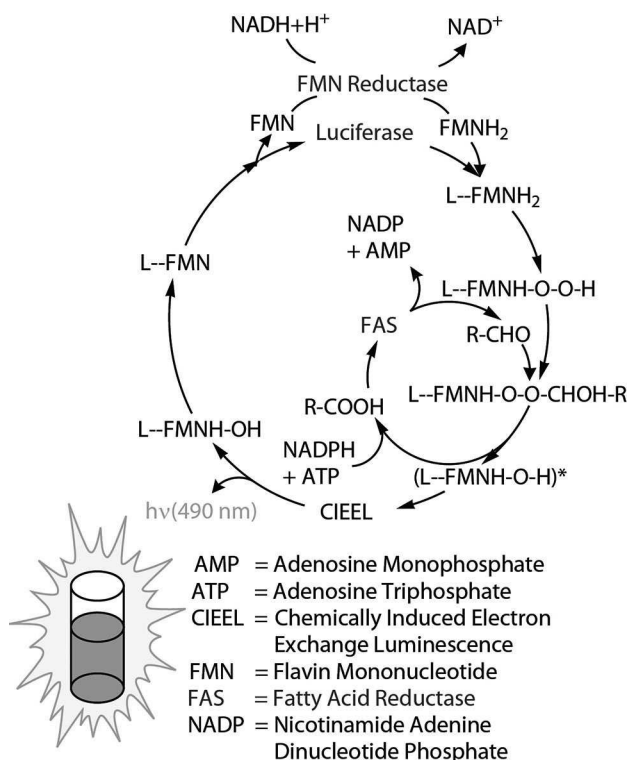**Figure 1.** Schematic overview of the biochemical light emitting pathway of the bioluminescent bacteria *Aliivibrio fischeri* and *Photobacterium*.

AMP = Adenosine Monophosphate
ATP = Adenosine Triphosphate
CIEEL = Chemically Induced Electron Exchange Luminescence
FMN = Flavin Mononucleotide
FAS = Fatty Acid Reductase
NADP = Nicotinamide Adenine Dinucleotide Phosphate

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3 Results and Discussion

Table 1. Toxicity data of common energetic materials and ionic compounds after 15 min and 30 min of incubation and their considered toxicity level after 30 minutes [less toxic (−), toxic (+), very toxic (+ +) [18]].

	EC_{50} (15 min) [g L^{-1}]	EC_{50} (30 min) [g L^{-1}]	Toxicity level
NaN$_3$	0.25	0.18	+
NaIO$_4$	0.77	0.65	+
KIO$_4$	0.89	0.68	+
KClO$_3$	> 2.49	> 2.49	−
KBrO$_3$	> 2.49	> 2.49	−
KIO$_3$	≫ 2.53	≫ 2.53	−
NH$_3$OHCl	0.59	0.22	+
NH$_4$N$_3$	0.26	0.15	+
NH$_4$NO$_3$	10.49	6.39	−
NH$_4$ClO$_4$	14.58	11.13	−
NH$_4$IO$_4$	0.58	0.48	+
NH$_4$N(NO$_2$)$_2$	7.25	4.50	−
RDX	0.33	0.24	+

Table 1–Table 8 summarize the toxicity data of several neutral and ionic compounds like ammonium, hydroxylammonium, sodium and potassium salts. Most of the compiled compounds are useful energetic materials or potential precursors. An important factor when measuring the toxicities of energetic materials is the water solubility of the substances. To increase the water solubility RDX was first dissolved in acetone and then diluted to get a 1% acetone solution. The EC_{50} value of RDX after 15 min incubation ($EC_{50} = 0.327$ g L^{-1}) fits well with the value of $EC_{50} = 0.322$ g L^{-1} given in the literature [18]. Nevertheless, it was not possible to obtain a concentration high enough to determine the EC_{50} values of pentaerythritol tetranitrate (PETN), cyclotetramethylene tetranitramine (HMX), 2,4,6,8,10,12-hexanitro-2,4,6,8,10,12-hexanitrohexaazaisowurtzitane (CL-20) and 2,2-dinitroethene-1,1-diamine (FOX-7). For classification of the toxicity, the compounds with EC_{50} values lower than 0.10 g L^{-1} are categorized as very toxic (+ +) while compounds with EC_{50} values between 0.10 g L^{-1} and 1.00 g L^{-1} are rated as toxic (+) and above 1.00 g L^{-1} as less toxic (−) to the marine bacteria *Aliivibrio fischeri* after 30 min incubation time [18].

Table 2. Toxicity data of neutral and ionic energetic materials **1 a**–**7 a** after 15 min and 30 min of incubation and their considered toxicity level after 30 minutes [less toxic (−), toxic (+), very toxic (+ +) [18]].

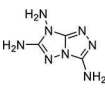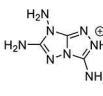|	1a	2a	3a	4	5	6a	7a
EC_{50} (15 min) [g L^{-1}]	> 1.63	> 5.93	14.08	7.23	6.69	≫ 1.60	≫ 1.61
EC_{50} (30 min) [g L^{-1}]	3.92	11.63	4.35	4.58	3.87	≫ 1.60	≫ 1.61
Toxicity level	−	−	−	−	−	−	−

Table 3. Toxicity data of neutral and ionic energetic materials **8 a**–**15 a** after 15 min and 30 min of incubation and their considered toxicity level after 30 minutes [less toxic (−), toxic (+), very toxic (+ +) [18]].
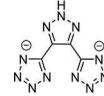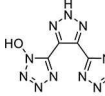

	8a	9a	9b	10	10a	10b	11a	12a	12b	13a	14	14a	14b	15a
EC_{50} (15 min) [g L^{-1}]	1.17	1.63	4.21	0.60	0.39	1.73	0.32	0.25	2.03	0.33	0.13	0.75	≫ 1.58	> 1.04
EC_{50} (30 min) [g L^{-1}]	0.58	0.33	3.68	0.49	0.18	1.03	0.24	0.10	0.89	0.19	0.07	0.35	≫ 1.58	3.78
Toxicity level	+	+	−	+	+	−	+	+	−	+	+ +	+	−	−

Table 4. Toxicity data of neutral and ionic energetic materials **16–22 a** after 15 min and 30 min of incubation and their considered toxicity level after 30 minutes [less toxic (−), toxic (+), very toxic (+ +) [18]].
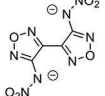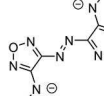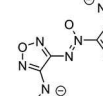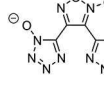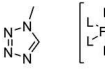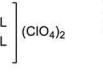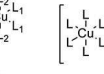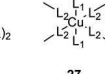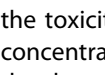
	16	16a = NO3−	17a	18	19a	20a	21a	22a
EC$_{50}$ (15 min) [g L^{-1}]	5.01	3.56 **a**	2.97	0.59	2.88	0.09	1.19	5.73
EC$_{50}$ (30 min) [g L^{-1}]	4.84	3.36 **a**	1.82	0.55	2.80	0.07	0.71	5.42
Toxicity level	−	−	−	+	−	+ +	+	−

Table 5. Toxicity data of energetic ligands and complexes **23–27** after 15 min and 30 min of incubation and their considered toxicity level after 30 minutes [less toxic (−), toxic (+), very toxic (+ +) [18]].

	23	24 L = 23	25 L1 = 23 L2 = ClO3−	26 L = 23	27 L1 = 23 L2 = BrO3−
EC50 (15 min) [g L^{-1}]	31.51	2.62	0.19	0.53	0.29
EC50 (30 min) [g L^{-1}]	5.45	1.66	0.19	0.13	0.21
Toxicity level	−	−	+	+	+

have the series of dilution be in the range of the above-mentioned categorization. Still, the toxicity according to EC$_{50}$ values is relative and can be more or less toxic at higher or lower concentrations. To get a detailed impression on the toxicity, the EC$_{50}$ values should be measured in various concentrations and compared to other organisms in further development. This categorization is also common amongst other scientific fields, such as antibiotics research, furthermore, labelling following the globally harmonized system (GHS) is possible [20]. The GHS refers to three acute toxicity classification categories. Therefore, they recommend determining a fish 96 hour LC$_{50}$, a crustacea species 48 hour EC$_{50,}$ and/or an algal species 72 or 96 hour EC$_{50}$. Substances classified according to the criteria are categorized as 'hazardous to the aquatic environment' as it follows:

- ≤ 1 mg L^{-1} Acute 1
- $> 1 - \leq 10$ mg L^{-1} Acute 2
- $> 10 - \leq 100$ mg L^{-1} Acute 3

The toxicity measurements of commercially available salts like potassium chlorate, bromate, and iodate as well as

Therefore mainly the EC$_{50}$ value after 30 min incubation will be discussed. The concentration of the compounds was mainly chosen to be over 3 g L^{-1} in the stock, in order to

Table 6. Toxicity data of energetic ligands and complexes **28–33** after 15 min and 30 min of incubation and their considered toxicity level after 30 minutes [less toxic (−), toxic (+), very toxic (+ +) [18]].
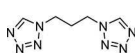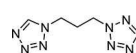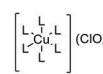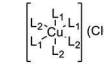
	28	29	30	31 L = 28	32 L = 29	33 L1 = 30 L2 = H2O
EC$_{50}$ (15 min) [g L^{-1}]	13.90	0.81	0.36	0.44	0.64	0.34
EC$_{50}$ (30 min) [g L^{-1}]	10.30	0.79	0.36	0.35	0.44	0.28
Toxicity level	−	+	+	+	+	+

Table 7. Toxicity data of neutral and ionic energetic materials **34a–40a** after 15 min and 30 min of incubation and their considered toxicity level after 30 minutes [less toxic (−), toxic (+), very toxic (+ +)] [18]].
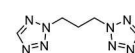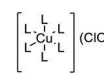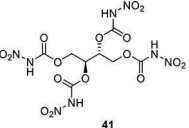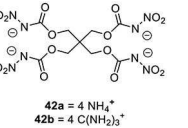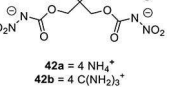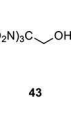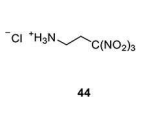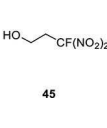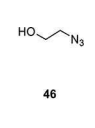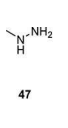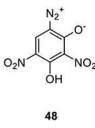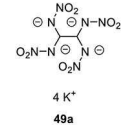
	34a	35a	36	36a = K$^+$	37a	38a	39a = K$^+$ / 39b = Na$^+$, H$_2$O		40a
EC$_{50}$ (15 min) [g L^{-1}]	< 0.10	0.27	0.27	1.21 **a**	0.70	0.61	0.75 **a**	0.60 **b**	2.86
EC$_{50}$ (30 min) [g L^{-1}]	< 0.08	0.20	0.19	0.95 **a**	0.43	0.30	0.74 **a**	0.58 **b**	1.42
Toxicity level	+ +	+	+	+	+	+	+	+	−

Table 8. Toxicity data of neutral and ionic energetic materials **41–49a** after 15 min and 30 min of incubation and their considered toxicity level after 30 minutes [less toxic (−), toxic (+), very toxic (+ +)] [18]].

	41	42a = 4 NH$_4^+$ / 42b = 4 C(NH$_2$)$_3^+$		43	44	45	46	47	48	49a
EC50 (15 min) [g L^{-1}]	0.87	≫ 2.02 **a**	2.86 **b**	0.29	< 0.10	0.002	8.70	6.00	0.01	> 15.07
EC50 (30 min) [g L^{-1}]	0.87	≫ 2.02 **a**	1.42 **b**	0.22	< 0.10	0.001	8.55	5.89	0.01	> 15.07
Toxicity level	+	−	−	+	+ +	+ +	−	−	+ +	−

ammonium nitrate and perchlorate showed almost no toxicity of the salts towards the bacteria because no inhibition of luminescence was observed with concentrations of 2.5 g L^{-1} and an incubation time of 30 min. Besides, the highest toxicity is observed for the azide anion followed by the periodate anion, whereas ammonium nitrate and dinitramide led to higher EC$_{50}$ values as seen in Table 1 and Figure 2. Similar toxicity trends against *Aliivibrio fischeri* were observed for the azide, nitrate and dinitramide salts of 1,4-
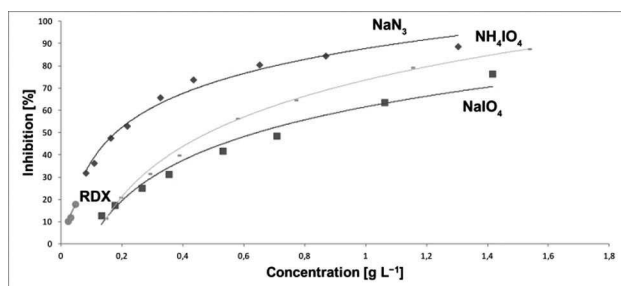
Figure 2. Diagram of the inhibition of some common energetic salts and RDX after 30 min of incubation.

dimethyl-5-aminotetrazole in the literature [18]. The perchlorate anion showed no effect on the luminescence of the bacteria. Studies showed toxicity of the perchlorate anion towards vertebrates, which probably only results from the interaction with the sodium/iodide symporter [21].

Nonetheless, also for the hydroxylammonium cation a negative effect on the toxicity was observed. The primary explosives dipotassium 1,5-di(nitramino)tetrazolate (**1a**) [22] and dipotassium 1,1′-di(nitramino)-5,5′-bitetrazolate (**2a**) [22] as well as copper(I) 5-nitrotetrazolate (DBX-1) [23] are potential lead-free replacements for lead azide (Table 2).

Since DBX-1 is nearly insoluble in water no EC$_{50}$ value could be determined. Therefore, the precursor of DBX-1 sodium 5-nitrotetrazolate (**3a**) [23b] was measured. With EC$_{50}$ values higher than 3.9 g L^{-1} all of these compounds are classified as not toxic towards the marine bacteria *Aliivibrio fischeri* [18]. Further salts of 5-nitrotetrazole were mentioned so far, *e.g.* the guanazinium (EC$_{50}$ (30 min) = 0.10 g L^{-1}), the guanidinium (EC$_{50}$ (30 min) = 0.78 g L^{-1}), the aminoguanidinium (EC$_{50}$ (30 min) = 2.65 g L^{-1}) and the 1,4-dimethyl-5-aminotetrazolium (EC$_{50}$ (30 min) = 3.61 g L^{-1}) salts [18].

Another evaluated variation affecting the tetrazole scaffold, listed in Table 2 as well, are the 1- and 2-amino as well as the nitramino substituted derivates **4**–**7a**. These show EC_{50} values much higher than 1.00 g L^{-1}, therefore are classified as non-toxic against *Aliivibrio fischeri* [24].

The toxicities of different hydroxyl ammonium (Figure 3) and ammonium bitetrazolates were determined (Table 3). Bis(hydroxylammonium) 5,5′-bitetrazole-1,1′-dioxide (**8a**, TKX-50) [25], bis(hydroxylammonium) and bis(ammonium) 5-(1-oxidotetrazolyl)-tetrazolate (**9a** and **9b**) [26], bis(hydroxylammonium) and bis(ammonium) 5-(2-oxidotetrazolyl)-tetrazolate (**10a** and **10b**) [27] and bis(hydroxylammonium) 5,5′-bitetrazole 1,2′-dioxide (**11a**) [28] are potential replacements for the secondary explosive RDX. All of these salts as well as the neutral compound 5-(1H-tetrazolyl)-2-hydroxytetrazole monohydrate (**10**) [27] were tested by the luminescent bacteria inhibition test. The ammonium salts **9b** and **10b** showed with EC_{50} values of 3.68 g L^{-1} and 1.03 g L^{-1}, respectively, low toxicities towards the marine bacteria. However, the exchange of the non-toxic ammonium cation with the hydroxylammonium cation significantly increases the toxicity of the bitetrazolate salts. With EC_{50} values in the range of 0.10–0.58 g L^{-1} after 30 min incubation, the compounds are classified as toxic. Nevertheless, for **8a** (EC_{50} (30 min) = 0.58 g L^{-1}) and **9a** (EC_{50} (30 min) = 0.33 g L^{-1}) lower toxicities than for RDX (EC_{50} (30 min) = 0.24 g L^{-1}) were observed. The EC_{50} value for the neutral bitetrazole **10** (EC_{50} (30 min) = 0.33 g L^{-1}) is in between the hydroxylammonium salt **10a** and the ammonium salt **10b**. As **10a** is more toxic than **9a** and **11a** is more toxic than **8a**, it seems that a substitution at 2-position results in higher toxicity values compared to toxicity values of the derivatives with substitution at 1-position. Still, they are less toxic compared to the divalent hydroxylammonium (**12a**) and ammonium (**12b**) salts of the unsubstituted bitetrazole. The monovalent ammonium 1,5-bistetrazole was described to have an EC_{50} value of 0.84 g L^{-1} after 30 minutes of incubation against *Aliivibrio fischeri*, which is in a comparable range as the divalent **12b** (EC_{50} = 0.89 g L^{-1}) [18].
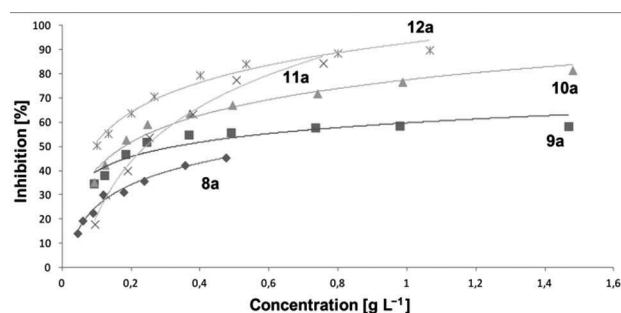
Also the toxicities of the hydroxylammonium salts of 3,3′-dinitro-5,5′-bi-1,2,4-triazole-1,1′-diol (**13a**, MAD–X1) [29] and 5,5′-diamino-4,4′-dinitramino-3,3′-bi-1,2,4-triazole (**14a**) [30] have been investigated. Both compounds are toxic to aquatic life with EC_{50} values of 0.19 g L^{-1} (**13a**) and 0.35 g L^{-1} (**14a**). The neutral compound 5,5′-diamino-4,4′-dinitramino-3,3′-bi-1,2,4-triazole [30] is classified as very toxic (EC_{50} (30 min) = 0.07 g L^{-1}). The thermally stable nitrogen-rich aromatic cations have been investigated, too. Toxicity measurements for 4,4′,5,5′-tetramino-3,3′-bi-1,2,4-triazolium dinitramide (**15a**) [31] as well as 3,6,7-triamino-7H-[1,2,4]triazolo[4,3-b][1,2,4]triazolium nitrate (**16a**) [32] and its neutral analogue 3,6,7-triamino-7H-[1,2,4]triazolo[4,3-b][1,2,4]triazole (**16**) [33] showed EC_{50} values higher than 3.36 g L^{-1} and are therefore low toxic for marine organisms.

The compounds 4,5-bi-(1H-tetrazol-5-yl)-2H-1,2,3-triazole (**17**) and 4,5-bi-(1-hydroxytetrazol-5-yl)-2H-1,2,3-triazole (**18**) combine the advantages of the triazole and tetrazole heterocycles by forming energetic and thermally stable molecules [34]. For toxicity measurements the ammonium salt of **17** as well as the neutral compound **18** were investigated. While bis(ammonium) 4,5-bi-(1H-tetrazol-5-yl)-2H-1,2,3-triazolate (**17a**) is less toxic to *Aliivibrio fischeri* (EC_{50} (30 min) = 1.82 g L^{-1}) a decrease of luminescence is observed for **18** (EC_{50} (30 min) = 0.55 g L^{-1}) (Table 4).

The energetic nitrofurazans 3,3′-dinitramino-4,4′-bifurazan (**19**), 3,3′-dinitramino-4,4′-azobifurazan (**20**), 3,3′-dinitramino-4,4′-azoxybifurazan (**21**) and bi(1-oxidotetrazolyl)-furazan (**22**) as well as their salts are also possible RDX replacements [35]. For toxicity assessment, the ammonium salts of these compounds (**19a**, **20a**, **21a**, and **22a**) were tested. The bifurazan salt **19a** and the bi(tetrazolyl)-furazan salt **22a** show both low toxicities, while the azo-bridged compounds possess moderate (**21a**) to high toxicities (**20a**) towards aquatic life. Unfortunately, also the thermal stabilities and sensitivities of most of the furazan compounds are worse than of RDX.

The 1-methyl-substituted tetrazole **23** is as comparably non-toxic as the salts of amino-, nitro- and nitramino-substituted tetrazoles **1a**, **3a**, **4**, **5**, **6a** and **7a**. Also, the iron(II) complex **24** of **23** remains non-toxic according to this test [36]. Its toxicity drops towards the classification of toxic compounds upon complex formation using copper(II)metal, regardless if the used anion was chlorate (**25**), perchlorate (**26**), or bromate (**27**), as listed in Table 5 [36–37]. Due to the toxicity of the copper(II)metal towards microorganisms [38], similar results were observed for the copper(II)complexes **31**–**33**, which were more toxic compared to their used ligands only (Table 6). Those propyl-linked bitetrazoles (**28**–**30**) have a little variation in their substitution pattern, but the EC_{50} values are in the range of 0.36 g L^{-1} (**30**) to 10.30 g L^{-1} (**28**) [39]. Whereby, the 2,2-substituted is the most toxic, followed by 1,2-substitued and the 1,1-substituted is the non-toxic tetrazole. This trend was also observed for the bitetrazoles around TKX-50 (**8a**–**11a**).

Figure 3. Diagram of the inhibition of the hydroxylammonium salts of bitetrazoles **8a**–**12a** after 30 min of incubation.

Pyrazoles are depicted in Table 7 and form a class of substances, which gained more attention in the energetic community recently. Their concentration depending inhibition is shown in Figure 4. According to former studies, pyrazoles, in general, are biologically active, whereby they inhibit several enzymes and led to centrilobular necrosis of the liver as well as the thyroid and adrenals in both rats and mice [40]. There were also investigations on 3-nitropyrazoles, which showed an effect on bacterial infections, but the acute toxicity against mice, rats, or dogs was relatively low [41]. In addition, we recently published a study on high performing dinitropyrazoles including the aquatic toxicities [42]. They also include a comparative study of aquatic toxicity and the mutagenic potential of BDNAPM [1c, 43]. Further effort is part of our ongoing research.

The synthesis of meso-erythritol tetranitrocarbamate (**41**) started from corresponding sugar alcohol by an economically benign two-step synthesis [44]. Primary nitrocarbamates form a new class of energetic materials with good detonation performances and lower sensitivities than the commonly used nitrate ester explosive PETN. During the toxicity measurements, moderate inhibition of luminescence was observed for compound **41** ($EC_{50} = 0.87 \, g \, L^{-1}$) which is in comparison to RDX still less toxic. The PETN analogous PETNC (**42**), which is synthesized from the same starting material as PETN, is not water soluble itself [45]. Therefore the aquatic toxicity of its ammonium (**42a**) and guanidinium (**42b**) salts were determined. Both showed no toxic effect towards *Aliivibrio fischeri* (Table 8) [46].

An important building block for the synthesis of oxygen-rich energetic compounds is the 2,2,2-trinitroethanol (**43**). It is formed by a simple Henry reaction starting from trinitromethane and formaldehyde [1c, 47]. In contact with nucleophiles and bases, it decomposes into its starting materials. When measuring the toxicity of the alcohol **43** and its decomposition products by the luminescent bacteria inhibition test an EC_{50} value of $0.22 \, g \, L^{-1}$ was determined. Therefore, **43** has to be classified as toxic. The toxicity of another water-soluble trinitroalkyl compound, trinitropropylammonium chloride (**44**), is lower and in the

range of very toxic compounds ($EC_{50} < 0.10 \, g \, L^{-1}$) [48]. A further trinitroalkyl substituted compound, (bis(2,2,2-trinitroethyl)-hydrazodicarboxylate), was reported to show high aquatic toxicity against *Aliivibrio fischeri* (EC_{50} (30 min) = 0.02 mg L^{-1}) [18]. With a fluorodinitroethyl moiety attached to an ethanol backbone, as in **45**, the toxicity drops further to $0.001 \, g \, L^{-1}$ [49]. This high toxicity value is consistent with former measurements using gram positive bacteria such as *Staphylococcus aureus* and *Intestinal bacillus* [50]. Adding 2-azidoethanol (**46**) to the row of aliphatic alcohols, which are important in the field of energetic materials, especially for propellants, it's the least toxic compound towards *Aliivibrio fischeri*. It is also relatively low in toxicity compared to ionic azide compounds NaN_3 and NH_4N_3 and also to monomethylhydrazine (**47**) [51]. The trend of covalent azides being less toxic compared to their ionic representatives continues according to our ongoing research.

6-Diazonium-3-hydroxy-2,4-dinitrophenolate (**48**) is a derivative of the commercially used primary explosive 2-diazonium-4,6-dinitrophenolate (DDNP). Chemical and physical studies as well as detonation calculations showed similar or even better properties than DDNP [52]. However, toxicity measurements of the benzene derivative **48** revealed a high toxic effect on the marine bacteria (EC_{50} (30 min) = $0.01 \, g \, L^{-1}$).

1,1,2,2-Tetranitraminoethane (**49**) was first synthesized in 1988 as an intermediate for the synthesis of CL-20 [53]. However, **48** itself and the salts thereof are already energetic materials with high oxygen content, high density, and high thermal stability. Toxicity measurements of the potassium salt of **49** (**49a**) [54] showed even at high concentrations negligible effects on the luminescence of the bacteria ($EC_{50} > 15.07 \, g \, L^{-1}$). Therefore, compound **49a** is more than 50 times less toxic to the bacteria *Aliivibrio fischeri* than RDX.

4 Conclusion

The toxicities of several energetic neutral and ionic compounds as well as complexes have been tested using the luminescence bacteria inhibition test. Some trends according the EC_{50} values are discussed. Even though the median effective concentration just shows a point estimate from a dose response curve and the toxicity might vary in higher or lower concentrations. Even to an order of magnitude. During the measurements of salts, a minor toxic effect of the ammonium, potassium, and sodium cations was found, whereas the hydroxylammonium cation showed considerable toxicity. For the azide and periodate anion on the one hand high aquatic toxicities were observed, where on the other hand the perchlorate anion led to low toxicity values against *Aliivibrio fischeri*.

For the primary explosives measured (**1a** and **2a**) and the sodium salt **3a**, a precursor for the synthesis of DBX-1, hardly no toxicities towards the marine bacteria were ob-
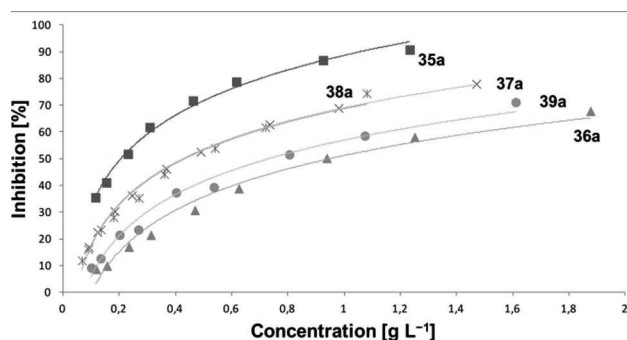

Figure 4. Diagram of the inhibition of the potassium salts of pyrazoles **35a–39a** after 30 min of incubation.

served. Also, most of the secondary explosives revealed good to excellent properties regarding the toxicity to aquatic life. For the intensively investigated secondary explosives **8 a** (TKX-50, $EC_{50} = 0.58\,g\,L^{-1}$) and **13 a** (MAD–X1, $EC_{50} = 0.19\,g\,L^{-1}$) EC_{50} values similar to RDX ($EC_{50} = 0.24\,g\,L^{-1}$) were observed (for EC_{20} and EC_{80} values see ESI).

Furthermore, trends which were observed and are under constant investigation:

- The tetrazole moiety revealed to have a low toxic effect on the marine bacteria *Aliivibrio fischeri*. Thereby, the 2-substitution showed the higher impact on the toxicity than 1-substitution.
- Adding an azo-coupling increases the aquatic toxicity dramatically at least for furazans **19–22**.
- The nitramino functionality mainly has no toxic effect, especially for the potassium salt **49 a**, which possesses four of those functionalities. It exhibits a value higher than $15.07\,g\,L^{-1}$ a very low toxicity.
- There is a trend that covalent azides are less toxic compared to their ionic representatives.
- The pyrazole scaffold represents a relatively toxic unit, no matter how many nitro-groups they carry. This is also indicated by the effect that C–C connected dipyrazoles drop in their toxicity towards the marine bacteria.
- For the trinitroalkyl and the fluorodinitroethyl moiety very toxic effects were observed. The second one is significantly more toxic
- The aquatic toxicity of complexes is mainly dominated by the chosen metal, as the toxicity of the free ligands often differ more.

Further attempts towards the comparability of the aquatic toxicity of energetic materials using *Aliivibrio fischeri* and other biological assays is ongoing research within our group.

Acknowledgements

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