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Evaluation of the Sensitivity of Freshwater Organisms Used in Toxicity Tests of Wastewater from Explosives Company

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Abstract Explosives industries are a source of toxic discharge. The aim of this study was to compare organisms sensitivity (*Daphnia similis*, *Danio rerio*, *Escherichia coli* and *Pseudomonas putida*) in detecting acute toxicity in wastewater from two explosives, 2,4,6-TNT (TNT) and nitrocellulose. The samples were collected from an explosives company in the Paraiba Valley, São Paulo, Brazil. The effluents from TNT and nitrocellulose production were very toxic for tested organisms. Statistical tests indicated that *D. similis* and *D. rerio* were the most sensitive organisms for toxicity detection in effluents from 2,4,6-TNT and nitrocellulose production. The *P. putida* bacteria was the organism considered the least sensitive in indicating toxicity in effluents from nitrocellulose.

Keywords Explosives · Freshwater organisms · Sensitivity · Toxicity

Many explosives and components of explosive mixtures present toxic effects, among them 2,4,6-Trinitrotolueno (TNT) and nitrocellulose (Nipper et al. 2009; Stucki 2004). The effluents generated in nitrocellulose production posses high biochemical oxygen demand (BOD), the chlorinated compound measured as halogenated organic compounds (AOX), suspended solids (SS), mainly fiber, fatty acids, tannins, resin acids, lignin and their derivatives, sulfur (S) and sulfur compounds (Ali and Sreekrishanan 2001; Santos 2006). The effluents generated in TNT production possess free acids, and highly soluble dinitrotoluene sulfonates (DNTs), among other compounds, in its final composition (Nipper et al. 2009; Zhang et al. 2011). Moreover, the explosives compounds may undergo extensive transformation in aquatic systems, by microbial attack or abiotic mechanisms such as hydrolysis, oxidation, photo-transformation, among others (Nipper et al. 2009), exposing the aquatic organisms not only to energetic compounds released into the environment but also to their numerous transformation products.

Studies performed by Nipper et al. (2009) concerning the toxicity of TNT in various test specimens, among them *Pimephales promelas* and *Daphnia magna*, indicated toxicity in concentrations which varied from 10.6 to 16.3 and 19.4 to 52.9 µmoles L^{-1}, for each organism, respectively. Regarding nitrocellulose, the same author reported the absence of toxicity in various species of fish and invertebrates which were evaluated, and toxic effects to microalga *Pseudokirchneriella subcapitata*.

The species selection for ecotoxicological assays follows the criteria of economic and/or practical aspects, easy manipulation, sensitivity, wide geographic distribution, abundance, ecological importance and the relevance of the indicator organism for the purposes of the study to be carried out (Ronco et al. 2004). Other factors are the ease of obtaining these organisms in the study region and the ease of cultivation and testing. Due to the natural variation in the sensitivity of organisms and the toxicity of wastewater from explosives industries, this study compared the sensitivity of test organisms (*Daphnia similis*, *Danio rerio*, *Escherichia coli* and *Pseudomonas putida*) to detect acute toxicity in TNT and nitrocellulose effluents.

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Materials and Methods

One of the largest explosives industries in Latin America is in operation in Paraiba Valley, São Paulo state, Brazil. Effluents discharged by this industry drain into a tributary of the Paraiba do Sul River, which, in turn, flows into a river basin of great strategic importance to Brazil, like water supply. This industry produces two types of explosives: 2,4,6-TNT and nitrocellulose, in different plants.

TNT production by nitration of toluene is a three-stage process carried out in a series of reactors. The crude TNT formed during the third stage of nitration consists primarily of 2,4,6-TNT. This product is washed with hot water to remove the free acid, thus generating yellow water, the first effluent. The highly soluble sulfonates DNT (an undesired isomer) is then separated from the relatively insoluble TNT (a desirable product), resulting in red water generation, the second effluent. The production of nitrocellulose involves the following steps: (1) chemical purification of linter with alkaline pulping, NaOH (25 g L^{-1}) which generates the delignification effluent (420,000 L^{-1}/day), (2) bleaching with NaClO or Cl_2 to remove the remaining impurities from alkaline pulping, generating the bleaching effluent (360,000 L^{-1}/day) and (3) the stabilization process, generating the nitration effluent (2.46 million L^{-1}/day), which is rich in suspended solids. The process of production TNT and nitrocellulose occurs separately. The characteristics of the samples and the effluents collected are presented in Table 1.

The mixture effluent (Mix_{TNT}) was prepared in the laboratory by adding 77 % and 23 % of yellow and red water, respectively, according to the flow data reported by the industry. The mixture effluent (Mix_{NC}) was prepared in the laboratory by adding equal parts of the three effluents from nitrocellulose production, according to the flow data reported by the plant. The experimental conditions of ecotoxicological assays are presented in Table 2.

The tests with daphnia and fish were conducted in accordance with Brazilian guidelines published by the Brazilian Association of Technical Standardization (ABNT), the department responsible for the technical normalizing in the country.

In all experiments a preliminary test was carried out to determine if the effluent was toxic and to define the concentration range to be employed in the definitive tests. The experiments were conducted with five concentrations plus the control. The effluents were diluted with the cultivation water used by each organism. The test organisms used in the toxicity tests underwent sensitivity control by periodic tests using reference substances. Results obtained showed minimum variability of results, thus proving that the tests could be trusted.

Table 1 Description of samples taken

Effluents	Abbreviations	Samples number	Volume	Stored
Yellow water	Yw	12	5L^{-1} individually on each sampling	−18°C in a freezer for no more than 2 months—(ABNT 2006, 2004)
Red water	Rw			
Yw + Rw*	Mix_{TNT}			
Delignification	Dl			
Bleaching	Bl			
Nitration	Nt			
Dl + Bl + Nt**	Mix_{NC}			

* and **—produced in the laboratory from the collected effluents

Table 2 Summary of experimental conditions of ecotoxicological tests

Organisms	*D. similis*	*D. rerio*	*E. coli*	*P. putida*
Methodology	Immobility static test with neonates—NBR 12713/04 (ABNT 2004)	Lethality test -NBR 15088/06 (ABNT 2006)	Bacterial growth inhibition test - Slabbert (1986) with modifications	
Illumination	Photoperiod of 16:8 light:dark	Photoperiod of 16:8 light:dark	Without illumination	
Temperature	20 ± 2°C	25 ± 2°C	37°C	26°C
Replicas	4	3	3	
Test duration	48 h	48 h	6 h	
Results	EC 50 (%)	LC 50 (%)	IC 50 (%)	
Calculation	Trimmed Spearman–Karber (Hamilton et al. 1977)	Trimmed Spearman–Karber (Hamilton et al. 1977)	ICP program (the inhibition concentration program) version 2.0 (1993)	

EC effective concentration—is the average concentration which causes 50 % of maximal response, *LC* lethal concentration is the average concentration that will kill 50 % of the test organisms, *IC* inhibition concentration—concentration that inhibits 50 % of organism growth

In the bacterial growth inhibition test proposed by Slabbert (1986) a modification was employed, related to the increase of the volume of samples and dilution water added to the test containers. The concentrations prepared for the tests varied between 12.5 % and 87.5 %. This methodology presents a limitation, and is not suitable for strongly colored effluents that require large dilutions of the sample, which would generate errors in the test. The Gram-negative bacteria, *E. coli* strain, was obtained from pre-existing cultures at the Wastewater Treatment and Environmental Laboratory of the School of Engineering of Lorena, University of São Paulo. The *P. putida* strain was obtained from the Bacterial Culture Collection of the Oswaldo Cruz Foundation, Rio de Janeiro, Brazil. Positive and negative controls were applied to test the reliability of the results in the experiments of bacterial growth inhibition.

In all ecotoxicological tests for any of the organisms used, the pH of the samples was adjusted to 7.0 ± 0.2 immediately before analysis. Physicochemical parameters were analyzed using Standard Methods (APHA 2005), except for true color, which had its characteristics altered by the procedure mentioned in this method. Thus, it was found necessary to search for another methodology for the realization of true color analysis. It was: the CPPA method (1975) (Table 3). The colored effluents Rw, Mix TNT, Dl and Mix NC, were a problem in tests under a spectrophotometer, due to the innumerous dilutions which had to be done.

To compare organism sensitivity, data obtained in acute toxicity tests with TNT and nitrocellulose effluents were submitted to a statistical test proposed by Barros Neto et al. (2007) and USEPA (1985). The test takes into account the reliable limits obtained for the EC50 or LC50 values according to Eq. (1):

$$G = (\log(UL_{(1)} \div EC50_{(1)}))^2 + (\log(UL_{(2)} \div EC50_{(2)}))^2 \quad (1)$$

where $UL_{(1)}$ = upper confidence interval for test 1, $UL_{(2)}$ = upper confidence interval for test 2, $EC50_{(1)}$ = Effective concentration for test 1, $EC50_{(2)}$ = Effective concentration for test 2.

Then,

$$H = 10^G \quad (2)$$

$$Z = EC50\,\text{greater} \div EC50\,\text{smaller} \quad (3)$$

If Z > H: There is significant value difference amongst the values of EC50.

Results and Discussion

The results of acute toxicity tests and statistical analyses of the effluents from TNT and nitrocellulose production are presented in Table 4.

In Table 4 its can observe that the effluents analyzed were extremely toxic for tested organisms, except nitration and bleaching effluents that were the least toxic potential. The effluents evaluated also caused chronic toxicity in alga *P. subcaptata*. Barreto-Rodrigues et al. (2009), upon evaluating yellow water effluents produced by the same industry evaluated in this study, using *E. coli* and *P. subcaptata*, detected a toxicity of 89.2 % and 8.5 %, respectively. Santos (2006), utilizing *Artemia salina*, detected toxicity in the effluents of the alkaline pulping of linter and bleaching. The concentrations of 30 % and 60 % of these effluents were lethal to 100 % of microorganisms.

The species comparison shows that the differences in sensitivity between species were statistically significant in 14 of 13 comparisons. Therefore the species *D. similis*, *D. rerio*, *E. coli* and *P. putida* (in declining order of sensitivity) can be used as a tool for predicting toxicity to the effluent.

Martins et al. (2007) showed that the sensitivity of organisms can occur due to the compounds to which they are exposed. This author evaluated the sensitivity of *D. magna* and *D. rerio* using a wide variety of products and chemicals in various chemical categories. The results indicated that both organisms are equally sensitive to

Table 3 Analyzed parameters and their respective limits of quantification

Parameters	Quantification limit	Units	Technical method	Equipment
pH	1.0		pH-meter	pH-meter Marte MB-10
Chemical oxygen demand (COD)	5.0	mg L^{-1}	Colorimetry/closed flow digestion	UV/Vis Spectophotometry U-2000 Hitachi
Total dissolved solids (TDS)	2.0	mg L^{-1}	Electrode	Hanna Instruments HI991300
True color		HU	CPPA (1975)	Centrifuge and UV/Vis Spectophotometry U-2000 Hitachi

Table 4 Acute and chronic toxicity test results for TNT production effluents with respective confidence intervals

Effluent	YW	RW	MIX_{TNT}	DEL	BLE	NTR	MIX_{NC}
D. similis	0.65	0.30	0.52	0.23	A	22.69	2.64
EC50 (%) 48 h	(0.59–0.71)	(0.2–0.5)	(0.42–0.65)	(0.20–0.28)		(20.02–25.72)	(2.34–3.01)
D. rerio	0.47	0.35	0.74	2.29	B	83.17	4.42
LC50 (%) 48 h	(0.42–0.52)	(0.27–0.45)	(0.71–0.78)	(2.15–2.43)		(78.92–87.65)	(4.14–4.70)
P. putida	1.74	NR	1.8	NR	73.11	C	E
EC50 (%) 6 h	(1.61–2.09)		(1.78–2.32)		(71.11–74.25)		
E. coli	1.98	NR	0.72	NR	36.6	D	2.4
EC50 (%) 6 h	(0.97–2.08)		(0.66–0.83)		(35.17–37.93)		(1.50–3.36)
P. subcaptata	0.2	0.10	0.10	0.1	0.2^{F}	50	0.2^{G}
NOEC (%)							
96 h							

NR not realized, *A* sensitivity up to the concentration tested of 0.05 %, *B* sensitivity up to the concentration tested of 0.01 %, *C* WITHOUT inhibition up to the concentration tested of 87.3 %, *D* without inhibition up to the concentration tested of 80 %, *E* WITHOUT inhibition up to the concentration tested of 50 %. *F* and *G* 100 % of inhibition up to the tested concentration

metals and pesticides, and less sensitive to solvents. Gellert (2000) reports that fish are not very sensitive to compounds such as AOX, present in effluents from pulp and paper industry and nitrocellulose production. According to Slabbert and Venter (1999) fish and microcrustaceans are sensitive organisms for evaluating toxicity in effluents from paper mills. In the present study, *D. similis* and *D. rerio* were efficient indicators of toxicity of the effluents tested.

The toxicity of these effluents (TNT and nitrocellulose) is related mainly to two factors: the composition of effluents, mentioned above, and their physical–chemical characteristics (Table 5). It was observed that the pH of yellow water, Mix_{TNT} and nitration effluents was very acidic. On the other hand, the pH of delignification and bleaching was very basic. But all of them were adjusted to neutrality before the toxicity tests. The content of TDS and COD in the effluents was very high; TDS causes general osmotic instabilities and water regulation problems in aquatic organisms, and the COD, high oxygen consumption. After neutralizing the pH, measurements of the TDS content in the effluents were not taken. To evaluate the neutralizing pH effect on toxicity tests, simplified tests were realized, using *D. similis.* These experiments indicated that in general, the effluents from the production of TNT and nitrocellulose were less toxic than the original effluents without neutralizing the pH.

During the tests with fish, the DO content was monitored. It was seen that during the course of the tests there was a reduction in DO concentration. Even so, the DO stayed above 4.0 mg L^{-1} in the effluents from TNT production. In the effluents from delignification, nitration and mixture (nitrocellulose), the DO was quite reduced, hitting zero at the end of the tests, mainly in the greatest concentrations of the effluents. These reductions in DO content show the presence of reducing substances in the effluents and unfavorable life conditions for the fish.

The comparison of the results obtained in this study with Brazilian legislation which deals with the classification of water bodies, CONAMA 357/2005, indicated that the pH in the effluents of TNT production and nitrocellulose was not in agreement with the quality standards to be heeded in this resolution, which is from 5 to 9, except red water and Mix $_{NC}$ which were in agreement with the standards. TDS

Table 5 Physicochemical characteristics of TNT and nitrocellulose effluents

Effluent	pH	TDS (ppm)	COD (mg L^{-1})	Color (HU)
Yelow water	0.9 ± 0.5	18,100 ± 20	389 ± 39	2,083 ± 20
Red water	7.4 ± 0.2	27,866 ± 13	27,364 ± 132	360,416 ± 36
Mix TNT	1.3 ± 0.3	ND	8,471 ± 127	84,611 ± 24
Delignification	12.4 ± 0.2	27,730 ± 10	24,535 ± 456	137,500 ± 49
Bleaching	9.3 ± 0.2	6,850 ± 7	78.0 ± 3.1	ND
Nitration	0.8 ± 0.4	16,730 ± 30	85.0 ± 18.6	ND
Mix NC	7.4 ± 0.3	14,300 ± 20	9,197 ± 172	90,200 ± 25
CONAMA 357/2005	5–9	500		75
*Class 2				

ND not done, *TDS* total dissolved solids, *COD* chemical oxygen demand; **Class 2* waters: preponderant use for human consumption after conventional treatment and color content also greatly exceeded the recommendation in this resolution, indicating that the release of these effluents without adequate treatment could cause a great impact on the quality of water in the receiving body. Moreover, the high COD concentrations would exert a great demand for oxygen in the waters, which could lead to anoxic conditions, depending on the flow from the receiving body. The Brazilian resolution, CONAMA 430/2011, which deals with conditions and standards of effluent releases, states that the effluent must not cause or potentially cause effects which would be toxic to aquatic organisms in the receiving body. In this study it was shown that all the effluents evaluated are toxic and thus need to have their impact potential evaluated before release.

The effluent from nitrocellulose production can cause considerable damage if released untreated, due to its high biochemical oxygen demand, total dissolved solids, chlorinated compound measured as halogenated organic compounds, suspended solids, mainly fiber, fatty acids, tannins, resin acids, lignin and their derivatives, sulfur and sulfur compounds (Talmage et al. 1999; Tadros et al. 2000). Cellulose, cellulose nitrate, contaminated acid waters from the verification and purification steps, alcohol, ether solvents and waste material from the refining and processing steps, can also be found in wastewaters from nitrocellulose production (Nipper et al. 2009).

Ryon et al. (1984) concluded that nitrocellulose seems to produce significant effects on the abiotic environment when its effluent is released. Because of its fibrous nature, nitrocellulose tends to cover the benthic habitat, perhaps even robbing oxygen from some bodies and filling the interstitial spaces used as a shelter for invertebrates and periphyton. This potential for habitat alteration is an additional component of the apparent lack of environmental degradation of nitrocellulose. When compared to the low toxicity of the compound, this alteration of habitat becomes a significant aspect for regulatory control. Another point is that the residual content of chlorine in the bleaching effluent could be the cause of this toxicity. Santos (2006), upon evaluating the content of residual chlorine in the aforementioned effluent generated by the same industry evaluated in this study, observed concentrations of 3,100 mg L^{-1} de Cl.

TNT companies generate large volumes of water containing organonitrogens. The toxicity of their effluent is also related to the presence of several other compounds considered toxic. Apart from TNT and its major by-products, 2.4-dinitrotoluene (2.4 DNT) and 2.6-dinitrotoluene (2.6 DNT), its effluent contains carbonate, sulfate and sodium sulfite. Yellow water contains not only TNT, but also various impurities like residual nitric acid, sulfuric acid and trinitrobenzoic acid, among others. Red water production is the result of washing TNT with a solution of sodium sulfite (Na_2SO_3) which in turn reacts with the asymmetric forms of TNT. According to Barreto-Rodrigues et al. (2009) the yellow water effluent contains approximately 156 mg L^{-1} of soluble TNT.

Amongst the possibilities of TNT effluent treatment is incineration (Lewis et al. 2004), the method which is presently employed by the industry which generates the effluent evaluated in this study. To treat yellow water, Barreto-Rodrigues et al. (2009) suggests a reductive process with iron of carbon steel, because it provokes modifications in the effluent of yellow water, permitting it to be treated by physicochemical or biological means. Of all the oxidative processes, the author still considers the fenton processes as being those most appropriate, because the effluent already presents a good level of Fe^{+2} and pH. Due to high toxicity of TNT red water to microorganisms and the organic and inorganic compounds, Zhang et al. (2011) recommends a cost-effective preprocess before biological treatment, like activated coke (AC) from lignite, obtaining the removal of COD, besides compounds like 2.4-dinitrotoluene-3-sulfonate (2.4-DNT-3-SO_3) and 2.4-dinitrotoluene-5-sulfonate (2.4-DNT-5-SO_3).

In the case of effluents from the production of nitrocellulose, Santos (2006) suggests that the effluent of alcaline pulping (delignification effluent) be treated by using a combination of chemical and biological processes. These treatments remove, to a great extent, color, TOC, COD, BOD and toxicity. For the bleaching effluent, sand filtering and activated vegetable carbon are recommended.

The effluents from TNT and nitrocellulose production were very toxic for tested organisms. The results obtained in the present study and some other studies mentioned herein, demonstrated that all organisms have a valued role in monitoring and controlling impacts in aquatic environments. Consequently, a battery of toxicity tests should be performed, with organisms of different levels of the ecosystem, since the advantages of some tests may complement the limitations of others. The comparison of sensitivity performed in toxicity tests with effluent from TNT and nitrocellulose production shows that *D. similis*, *D. rerio*, *E. coli* and *P. putida* were efficient in toxicity indication. However, the microcrustacean and the fish were highly sensitive in detecting effluent toxicity and distinguishing the levels of their toxicity. Besides their toxic potential, effluents from the production of TNT and nitrocellulose also possess physicochemical characteristics which do not comply with the Brazilian standards of effluents, CONAMA 430/2011. Considering the toxicity detected in the effluents from TNT and nitrocellulose production, it is essential that there be a treatment before they are released into the receiving body, the reason being that these can cause great impacts on the aquatic community.

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