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A test battery approach for ecotoxicological evaluation of disinfectants prepared on the basis of sodium hypochlorite

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Abstract

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The research is related to the assessment of the overall sensitivity and applicability of many bioassays representing different trophic levels for the preliminary ecotoxicological testing of commercial disinfectants marked as SA (SAVO, Bochemie a.s., Czech Republic) and DoAm (Dom Amor, BOOS – Biologické substancie, Slovak Republic). Disinfectants were prepared based on sodium hypochlorite (NaOCl). SA contains only NaOCl while earthworm enzymes enrich DoAm. In both commercial products, the NaOCl content did not exceed 5%; pure NaOCl was used as a 10% solution as well. For bioassay, water organisms (*Vibrio fischeri, Desmodesmus subspicatus, Daphnia magna* and *Tubifex tubifex*) situated in various trophic levels were used. All the tests were confirmed as suitable for the determination of chlorine's adverse effects. Because the organisms' reactions to the tested disinfectants varied, they can be arranged in the following rank order of sensitivity: *V. fischeri* ≥ *D. subspicatus* >> *D. magna* >> *T. tubifex*. The toxicity of the tested substances (NaOCl, SA, DoAm) depends on the length of exposure, the species of the organism and FAC (free available chlorine) content. The effective concentrations of the tested products ranged from 0.13 to 8.18 µL L^{-1}, i.e., 0.014 to 0.26 mg L^{-1} of FAC. However, in the tests with *T. tubifex* and *V. fischeri* the toxic effect of NaOCl was the weakest; the tests with other two organisms confirmed this compound as the most toxic. Only for *T. tubifex* (96 hrs) did SA have a more adverse effect than DoAm.

Keywords

benthonic worms, bioassay, disinfectants, freshwater plankton, marine luminescent bacteria, sensitivity

Introduction

Experimental model systems and bioassays are widely used in ecotoxicology and environmental toxicology to provide information for risk assessment evaluation, as well as to register new chemicals and analyze their consequences and modes of action. Therefore, ecotoxicological models are used to identify, control, and monitor pollutant concentrations in air, water, soil, waste, etc. (Rajfur et al., 2016). We never provide a complete picture of the environmental quality as a single bioassay and should, therefore, establish a generic, cost-effective, and quantitative test battery. To avoid potential inconsistencies when using single species toxicity assays, a better scheme would be to conduct a suite or series of procedures using ecologically appropriate specimens. The benefit of a check battery methodology would provide high sensitivity and selectivity, high environmental significance, and an integrative understanding of the results and response mechanisms (Davoren et al., 2005; Repetto, 2013). To reduce the use of vertebrates in ecotoxicity analysis, the use of the

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research battery method would also satisfy consumer standards. Scientific interest in this type of study is meant to explore both terrestrial (including vegetables) and water ecosystems. The use of invertebrate species, microorganisms and plants that are more abundant than vertebrate organisms is still not great (Repetto, 2013). In this situation, the most promising option is the use of less susceptible species and animals not shielded by regulations for governing animal experiments. Bacteria, fungi, algae, plants, and invertebrates are included in such tests. When vertebrates, such as fish, amphibians, reptiles, birds, and mammals, are used for research, the method recommends the early stages of their development or *in vitro* experiments.

Chlorination is the traditional chemical method used in drinking and waste-water disinfection before disposal into distribution networks (Pignata et al., 2012). Chlorine residual activity protects water against contamination throughout the distribution system and the environment. Chlorination is associated with the ability to kill pathogens and is considered as a secure disinfectant procedure. Already low levels of residual chlorine in drain waters can be harmful to aquatic life (Berninger da Costa et al., 2014; Kim et al., 2013; Sapone et al., 2016). In contrast, dissolved chlorine dissociates into hypochlorite and hypochlorous ions, which penetrate the membranes of cells and can contribute to the development of genotoxic, mutagenic, or carcinogenic by-products (DBPs) (Villanueva et al., 2015; Li et al., 2015). For reduction or elimination of DBPs adverse effects, research needs to pay attention to the use of alternative disinfectants, as well as observe their adverse effects on living organisms and any potential harmful effects on human health (Hrudey, 2009).

Sodium hypochlorite (NaOCl), a very useful and low-cost biocide, is one of the most widely used chemicals for water disinfection (Mohammadi, 2008; Binetti and Attias, 2009; Amin et al., 2013). Hypochlorite is a highly destructive, selective oxidant that reacts easily with all biomolecules. It can oxidize nucleotides, inactivate enzymes, and electron transport systems, as well as disrupts cell membranes and fragment proteins, which eventually lead to cell death (Hidalgo et al., 2002). Halogenated DBPs can either generate reactive oxygen species in cells, thereby reducing cellular glutathione levels, or affect cellular antioxidative damage to cellular proteins and DNA. Epidemiological investigations have shown a higher probability of cancers in people who drink tap water with high DBPs level (Gibbons and Laha, 1999). Recently, the investigation of this problem has also focused on its adverse reproductive and developmental effects (Ton et al., 2012).

Sodium hypochlorite disinfectants are used in households as well as in industry, healthcare, and agriculture. They are used as algicides and molluscocides for cooling water in power stations (Luttrell, 2001) and for biofouling reduction (Dobbs, 2005; Berninger da Costa et al., 2014). Kim et al. (2013) explained its effect on three species of seawater by the interaction between NaOCl toxicity and the residual amount CO_2, and Berninger da Costa et al. (2014) presented the influence of NaOCl on freshwater cladocerans (*Ceriodaphnia silvestrii, Daphnia similis*), midge larvae (*Chironomus xanthus*) and fish (*Danio rerio*).

Environmental exposure can occur directly using sodium hypochlorite or through the formation of hypochlorite from chlorine applied into the water (Binetti and Attias, 2009). Despite the versatility and extensive use of hypochlorite commodities, few data on their ecotoxicological effects are available. NaOCl is known to be low in toxicity to avian wildlife, yet highly toxic to freshwater fish and invertebrates (Sapone et al., 2016; US EPA, 1991; Añasco et al., 2008). However, the US EPA Quality Criteria for Water (US EPA, 1986) introduced the acute LC50 values for total residual chlorine (the sum of free and combined chlorine) for 43 freshwater species in 28 genera, data for benthonic organisms and algae, mainly for short-term toxicity, did not appear so frequently. Recently, valid ecotoxicological data for fish, daphnia, benthos, and algae are not yet established, and only supportive information is provided (Binetti and Attias, 2009). *In vivo* and *in vitro* antimicrobial activity investigations could be found in the study by Mohammadi (2008).

Sodium hypochlorite (CAS No. 7681-52-9 and EC No. 017-011-00-1) hydrolyses in water and yields hypochlorous acid (HOCl) and hypochlorite ions (OCl^-). In water is gradually depleted due to the oxidation of inorganic and organic compounds (Mohammadi, 2008). Despite serious health impact, NaOCl is used extensively due to its antibacterial and strong oxidation properties. The formed by-products (DPBs) (Sapone et al., 2016) in the cells irreversibly oxidize the -SH groups, bind to the amino groups of the proteins, thereby forming carcinogenic chloramines, and damage the overall metabolic functionality of the organism (Mohammadi, 2008). Until now, studies conducted on NaOCl have mostly dealt with its potency against pathogenic and invasive species (Dobbs, 2005; López-Galindo et al., 2010). Recently, *in vitro* experiments have shown that halogenated DBPs induce higher cytotoxicity (Zhai et al., 2014) or more significant development toxicity (Yang and Zhang, 2013) than the used disinfectants.

Daphnids, algae, benthonic organisms, and bacteria are often used to study chemicals toxicity in aquatic ecosystems due to their high vulnerability, short time of generation, and ease of handling. Invertebrates, with considerable respect to aquatic crustaceans, are still at the center of attention as research species due to the need to develop non-mammalian tests for chemicals, heavy metals, and pesticides toxicity evaluation, as well as assessing the risk posed by environmental pollutants that have affected aquatic ecosystems (Ferrao-Filho et al., 2010; Wojtal-Frankiewicz and Frankiewicz, 2011). Due to their species diversity and abundance, algae very well reflected the ecological status of surface waters and

are therefore often used in biomonitoring of water quality (Bilous et al., 2012). Underwater sediment composition is important to the protection of an aquatic environment. Assessment and monitoring of sediments quality and benthos vitality are an integral part of the evaluation of water quality. To evaluate the quality and vitality of benthos as well as the development of measure to remedy the situation, is appropriate to use a battery of tests that cover a wide variety of organisms (Davoren et al., 2005).

This research is a contribution to choosing adequate endpoints for specific toxicity experiments as well as evaluating the exposure in an aquatic environment at various trophic levels. The primary purpose was to establish an ecotoxicological battery with various organisms as indicators capable of detecting the effects of disinfectants prepared based on sodium hypochlorite (NaOCl). The tests used included immobilization of the cladoceran *Daphnia magna*, inhibition of bioluminescence in the marine bacterium *Vibrio fischeri*, growth inhibition of the alga *Desmodesmus subspicatus*, and mortality of the *Tubifex tubifex* benthonic worms.

Materials and methods

Disinfectants

The following commercial products based on sodium hypochlorite (NaOCl) marked as SA and DoAm were investigated concerning their ecotoxicological effects. SA (SAVO, Bochemie a.s., Bohumín, Czech Republic) contains only NaOCl; while DoAm (Dom Amor, BOOS – Biologické substancie, Košice, Slovak Republic) contains both NaOCl and earthworm enzymes. The content of active ingredients (CEI) of both products did not exceed 5%. The toxicity of sodium hypochlorite (NaOCl) (10% solution) was also determined. Due to chlorine volatility only fresh samples were used, and the dissipation tests were conducted before the experiments. Free available chlorine concentration (FAC) (Table 1) was determined according Añasco et al. (2008). Doses of the disinfectants used in the tests were determined from preliminary experiments performed in the laboratory according to the corresponding guide for each selected organism (Table 2).

Table 1. Content of active ingredients – CEI (%) and concentration of free available chlorine – FAC (mg L^{-1}) in tested substances (Fargašová, 2017)

Product	CEI (%)	FAC (mg L^{-1})
SA	5	39.90
DoAm	5	32.21
NaOCl	10	108.86

Free available chlorine (FAC) was monitored during the experimental period twice a day in each sample, and according to these values, the disinfectant concentrations were adjusted in order to provide a constant level (Sapone et al., 2016). Under these conditions, FAC concentration at the end of the experiments did not decrease below 90%.

Algal growth inhibition test

An algal growth inhibition test was performed follow

Table 2. Concentrations of SA, DoAm and NaOCl used during the tests for individual tested organisms expressed as FAC concentrations in mg L^{-1} and volume of solution µL L^{-1} (concentrations were selected on the basis of preliminary tests)

Organisms	SA (mg L^{-1} (FAC))	SA (µL L^{-1} (V))
D. subspicatus	0.012; 0.014; 0.016; 0.018; 0.02; 0.022; 0.024; 0.028; 0.032	0.3; 0.35; 0.4; 0.45; 0.5; 0.55; 0.6; 0.7; 0.8
V. fischeri	0.8; 1.2; 1.6; 2.0; 2.4; 2.8; 3,2; 3.6; 4.0	20; 30; 40; 50; 60; 70; 80; 90; 100
D. magna	0.04; 0.06; 0.07; 0.08; 0.09; 0.12; 0.14; 0.16	1.0; 1.5; 1.75; 2.0; 2.25; 3.0; 3.5; 4.0
T. tubifex	0.08; 0.12, 0.16; 0.2; 0.24; 0.28; 0.32; 0.36; 0.4	2.0; 3.0; 4.0; 5.0; 6.0; 7.0; 8.0; 9.0; 10.0
	DoAm (mg L^{-1} (FAC))	DoAM (µL L^{-1} (V))
D. subspicatus	0.01; 0.011; 0.013; 0.015; 0.016; 0.018; 0.019; 0.023; 0.026	0.3; 0.35; 0.4; 0.45; 0.5; 0.55; 0.6; 0.7; 0.8
V. fischeri	0.65; 1.0; 1.3; 1.6; 1.9; 2.25; 2.6; 2.9; 3.2	20; 30; 40; 50; 60; 70; 80; 90; 100
D. magna	0.03; 0.05; 0.06; 0.065; 0.07; 0.1; 0.11; 0.13	1.0; 1.5; 1.75; 2.0; 2.25; 3.0; 3.5; 4.0
T. tubifex	0.6; 0.1; 0.13; 0.16, 0.19; 0.23; 0.26; 0.29; 0.32	2.0; 3.0; 4.0; 5.0; 6.0; 7.0; 8.0; 9.0; 10.0
	NaOCl (mg L^{-1} (FAC))	NaOCl (µL L^{-1} (V))
D. subspicatus	0.008; 0.009; 0.0093; 0.01; 0.01035; 0.011; 0.016; 0.022; 0.027	0.07; 0.08; 0.085; 0.09; 0.095; 0.1; 0.15; 0.20; 0.25
V. fischeri	2.18; 3.27; 4.36; 5.45; 6.54; 7.63; 8.72; 9.81; 10.9	20; 30; 40; 50; 60; 70; 80; 90; 100
D. magna	0.055; 0.06; 0.065; 0.076; 0.008; 0.09; 0.093; 0.1	0.5; 0.55; 0.6; 0.65; 0.7; 0.75; 0.8; 0.85; 0.9
T. tubifex	0.055; 0,109; 0.16; 0.19; 0.22; 0.25; 0.27; 0.33; 0.38	0.5; 1.0; 1.5; 1.75; 2.0; 2.25; 2.5; 3.0; 3.5

FAC, free available chlorine; V, correspondent volume of compound solution.

ing the OECD Guideline 201 (2011). The green alga, *Desmodesmus subspicatus* Brinkmann (SAG 86.81), was obtained from the Department of Hydrobiology, Microbiology and Toxicology of the Water Research Institute, Bratislava. The assays were carried out in Erlenmeyer flasks (750 mL) with 250 mL of an algal growth medium with various nutrients and microelements and disinfectant solutions in required concentrations. The control contained only sterile algal growth medium. 1–4 mL of *D. subspicatus* culture in exponential growth phase was injected directly into the tested solutions to ensure a final algal concentration of 104 cells mL^{-1}. The algae grew in axenic conditions and were illuminated continuously with a constant light (22.1 µmol s^{-1} m^{-2}) and maintained at a temperature of 24 ± 2 °C with permanent shaking (143 rd min^{-1}). After 72 hrs of incubation, culture growth was determined microscopically by counting the cell number. Toxic response was expressed as growth inhibition (I), and then calculated as the reduction of the algal growth rate caused by the test substance according to the control. SA, DoAm and NaOCl were applied in concentrations presented in Table 2.

Vibrio fischeri bioluminescence test

The bioluminescence test using marine Gram-negative bacteria *Vibrio fischeri* NRRL-B-11177 of the Vibrionaceae family (distributed by Dr. Lange) was carried out with a Dr. Lange LUMIStox system according to the European standard DIN EN ISO 11348-2 (2009). Bacteria were purchased in freeze-dried form from Strategic Diagnostic Inc. (SDI, Newark, DE, USA) and reconstituted by rehydration. Reconstituted bacteria were exposed to the tested samples for 15 and 30 min. at an incubation temperature of 15 °C. Light production was directly proportional to the bacterial population's metabolic activity, and any suppression of enzymatic activity caused a corresponding decrease in bioluminescence. The test provides a measure of sub-lethal response (Parves et al., 2006; Bayo et al., 2009a, b). The concentrations of samples (mg L^{-1} FAC) which produced a 50% decrease in light intensity after exposure are designated as EC50 values (Effective Concentrations). Acute toxicity, determined as luminescent inhibition, was related to non-toxic control. Tests with *V. fischeri* were arranged in concentrations presented in Table 2.

Acute toxicity tests with *Daphnia magna*

Acute toxicity test conditions (24, 48 hrs) conformed to the OECD Guideline 202 (2004). Cladoceran (*Daphnia magna*) neonates less than 24-hrs old originated from a laboratory culture from the Department of Hydrobiology, Microbiology and Toxicology of the Water Research Institute, Bratislava. Ten neonates were kept for 24 or 48 hrs in 30 mL of water solutions in a 75 mL glass beaker. *D. magna* were maintained at 20 ± 1 °C in the de-chlorinated tap water (72.6 mg L^{-1} Ca, 17.7 mg L^{-1} Mg; free Cl < 0.02 mg L^{-1}; pH = 7.48 ± 0.13) with a dissolved oxygen concentration greater than 6.0 mg L^{-1}. All tested solutions containing the newborns were kept without feeding and aeration in darkness. The measured effect was death, which was defined as immobilization for 15 s after stimulation by bright light and shakeup. The tests with *D. magna* were arranged in concentrations presented in Table 2. Data were analyzed by probit analysis to calculate the slope of the curves, and the EC50 values with 95% confidence intervals (CI) were determined.

Acute toxicity tests with *Tubifex tubifex*

The worms *Tubifex tubifex* used for the tests, were obtained from the Department of Zoology, SAS, Bratislava, Slovakia. For this test, ten *T. tubifex* worms of the same age group, similar size, and in their first reproductive periods were added to 60 mL of sample with 15 g of artificial sediment (Arrate et al., 2004). Before the tests, the worms were washed in de-chlorinated tap water (72.6 mg L^{-1} Ca, 17.7 mg L^{-1} Mg; free Cl ± 0.02 mg L^{-1}; pH = 7.48 ± 0.13) for 24 hrs and after that used in the tests (ASTM, 2010). The organisms were kept at a room temperature of 23 ± 2 °C, a photoperiod of 12/12 hrs light/dark, without food and aeration. The tests lasted 24 and 96 hrs, after which the dead organisms were counted. Criteria for death are immobility or lack of reaction to a mechanical stimulus. Worms that died during the experiment were removed to prevent microbial infection and counted as dead. Mortality was expressed as LC50 value with 95% confidence interval (CI). Concentrations of disinfectant solutions were selected based on the preliminary tests and are presented in Table 2.

Statistical analysis

In a completely randomized design, all experiments were set up in three replications each with five parallels and included a control in tap water or cultivation medium. Quality control data were considered acceptable according to control charts and other established criteria. Results were evaluated as inhibition concentration IC50 (algae), effective concentration EC50 (bacteria and *Daphnia*) and lethal concentration LC50 (worms) values and their 95% confidence intervals (CI) by probit analysis or as average values with their standard deviations (SD). Fisher's exact test was used to distinguish significant differences in the survival of the organisms between the control and disinfection treatment. For all evaluated inhibition concentrations, the free available chlorine content (FAC) was also determined. Calculation and statistical data analysis were performed using the statistic package ToxCalc™ v5.0 software.

Results and discussion

All results obtained using the respective protocols de-

Table 3. Concentrations of free available chlorine FAC (mg L^{-1}) to correspondent volume of compound solution (in µL L^{-1}) and their 95% confidence intervals CI (mg L^{-1} FAC × 10^{-2}) expressed as FAC/V ± (CI) values determined at IC50 (alga *D. subspicatus*), EC50 (bacteria *V. fischeri* and cladoceran *D. magna*) and LC50 (worms *T. tubifex*) values (mg L^{-1} FAC or µL L^{-1} of volume) of the tested disinfectants (SA, DoAm, NaOCl)

Organisms		
	SA	
D. subspicatus	72-hrs: 0.02/0.52 (1.9–2.4)	
V. fischeri	15 min : 0.021/0.53 (1.9–2.2)	30 min : 0.028/0.70 (2.7–2.9)
D. magna	24-hrs: 0.10/2.76 (10–11)	48-hrs: 0.08/2.51 (9–10)
T. tubifex	24-hrs: 0.26/8.18 (24–29)	96-hrs: 0.17/4.20 (16–18)
	DoAm	
D. subspicatus	72-hrs: 0.02/0.61 (1.8–2.1)	
V. fischeri	15 min: 0.019/0.61 (1.8–2.1)	30 min: 0.027/0.84 (2.6–2.8)
D. magna	24-hrs: 0.10/2.76 (8–9)	48-hrs: 0.07/2.47 (6–7.5)
T. tubifex	24-hrs: 0.26/8.18 (24–29)	96-hrs: 0.19/5.86 (18–21)
	NaOCl	
D. subspicatus	72-hrs: 0.014/0.13 (0.9–1.5)	
V. fischeri	15 min: 0.085/0.78 (7.5–8.9)	30 min: 0.093/0.84 (8.6–9.4)
D. magna	24-hrs: 0.09/0.77 (7–10)	48-hrs: 0.07/0.65 (6–8)
T. tubifex	24-hrs: 0.24/2.25 (23–26)	96-hrs: 0.22/1.99 (21–23)

scribed above were evaluated according to an ecological risk assessment framework which involves the examination of risks from natural, human and industrial activities. The toxicity of disinfectants, based on NaOCl, was calculated as the LC50 concentration for algal growth inhibition, EC50 concentration for daphnids immobilization, or reduction of bacteria bioluminescence and LC50 concentration for mortality of benthic worms (Table 3).

One of the most sensitive organisms used to determine the disinfectants' toxic effects in our tests was the green alga *D. subspicatus*. Only 0.014 mg L^{-1} of free available chlorine (FAC) (0.13 µL L^{-1}) of NaOCl solution inhibited growth up to 50%. Although sodium hypochlorite produces in water, similarly to Cl_2, hypochlorous acid, its toxic effect on algae was stronger than that reported for chlorine by Junli et al. (1997). These authors determined for the algae *Chlamydomonas, Phorimidum, Ulothrix* and *Microphorumidium* considerably low toxic levels of Cl_2 ranging from 3 to 5 mg L^{-1} of FAC. However, for long-term toxicity (28 days), Binetti and Attias (2009) determined for algal biomass production in microcosm the EC50 values for free available chlorine (FAC) as low as 2.1 µg L^{-1}.

Based on the above-mentioned results, the toxicity of the tested products containing sodium hypochlorite to *D. subspicatus* decreased in the following rank order: NaOCl >> SA = DoAm. After 72 hrs of application, the commercial disinfectant SA had the same algicidal effect as DoAm. The inhibitory effect of pure NaOCl was more than 10 times higher than that of both commercial products (Table 3).

Our study confirmed that the green alga *D. subspicatus*, ranked at the beginning of the food chain, is very sensitive to chlorinated disinfectants. This is consistent with the results obtained by Rav-Acha et al. (1995) and Junli et al. (1997) who indicated the disinfection effects of Cl_2 to *Chlorella* sp. and 8 different algal species (the active substance of Cl_2 in water, just like in NaOCl, is hypochlorous acid). Obtained results confirmed that the green alga *D. subspicatus* is a good test model that may be used for the determination of toxic characteristics of chlorinated disinfectants.

The freshwater cladoceran *D. magna*, which was also presented as a susceptible organism, had after 24 and 48 hrs of disinfectants application approximately 5.0 and 4.5 times, respectively, lower sensitivity than alga *D. subspicatus* after 72 hrs. *D. magna* sensitivity to chlorine was similar to that of *Ceriodaphnia dubia* with 24-hrs LC50 of 0.12 mg Cl L^{-1} (Manning et al., 1996) and fell within the range of 0.076–0.160 mg L^{-1} of chlorine reported for *D. magna* in 24-hrs tests (US EPA, 1994; Emmanuel et al., 2004). In the Safety Data Sheet (SDS) No. 106 (Anonymus, 2015), it is presented for 24 hrs *D. magna* tests with the NaOCl solution the LC50 value in the range of 0.07–0.7 mg L^{-1} FAC, and this corresponds with our results. For the commercial products DoAm and SA, it was observed that after 24 and 48-hrs of application the immobilization of *D. magna* reached 50% in concentrations 2.76 and 2.47 or 2.51 µL L^{-1}, respectively. In these concentrations, the content of free available chlorine did not exceed 0.1 mg L^{-1}. From the results obtained, water zooplankton is very sensitive to disinfectants based on NaOCl, too. Both tested commercial products (SA, DoAm) rapidly increased the experimental animals' immobilization in very low concentrations, and this is consistent with the study by Emmanuel et al. (2004). The acute toxicity effect of DoAm on *D. magna* immobilization was higher than that of SA after 48 hrs application.

The freshwater tubificid sludge worms *T. tubifex*, which are not as popular as *D. magna* in toxicity tests, also showed sufficient sensitivity to chlorinated substances. As reported by Leynen et al. (1999), only

a few individuals of Tubificidae worms (mixture of *T. tubifex*, *Limnodrilus hoffmeisteri, Limnodrilus ukedemianus*) did not respond to the presence of NaOCl at a concentration of 0.5 mg L^{-1} of FAC for the duration of 1 hr. Conversely, during our test, 50% mortality of *T. tubifex* was observed after 24 and 96 hrs in the presence of pure NaOCl in concentrations as low as 2.25 and 1.99 µL L^{-1}, or 0.24 and 0.22 mg L^{-1} FAC, respectively. Even if these LC50 values are low, they are approximately 16–17 times higher than LC50 values determined for alga *D. subspicatus* in standard 72 hrs toxicity tests and approximately 2.6–3.1 times higher than those for *D. magna*. For a benthonic environment, are *Chironomus* sp. larvae used as a model organism more frequently than *T. tubifex* worms. The LC50 value for the FAC effect on these larvae was presented as many times higher than those obtained during our tests for the *T. tubifex* worms' mortality. Sun et al. (2007) presented for the chironomid 4th instar larvae the 24 hrs LC50 value for FAC effect as 0.41 mg L^{-1}. The 1st instar larvae were most sensitive and their LC50 value was 1.78 times lower than that for 4th instar larvae.

The luminescent bacteria test is a simple, rapid method for monitoring the toxicity of water samples. The test is based on changes in the light output of luminescent bacteria, and is measured in a photometric device. While under suitable conditions the amount of emitted light is constant, the exposure to toxicants quickly diminishes the light intensity, and this phenomenon is proportional to the toxicant concentration (Parvez et al., 2006). Chlorine gas (Cl_2) and sodium hypochlorite (NaOCl) added to drinking water effectively inactivate bacteria in 20 min. at concentrations of free available chlorine from 0.03 to 0.06 mg L^{-1} at pH levels ranging from 7.0 to 8.5 and a temperature of 4–22 °C (Anonymus, 1992). In the case of our toxicity tests performed by bioluminescent bacteria *V. fisheri*, the tolerance to DoAm and SA after 15- and 30-min. incubation was nearly the same as that for alga *D. subspicatus*, however, for NaOCl *V. fischeri* was about 6 times less sensitive than algae. According to Bulich (1979), the EC50 values for the 30 min. *V. fischeri* bioluminescent test, was still lower, and half reduced light intensity was observed in the FAC concentration of 0.005 mg L^{-1}. This low concentration did not correspond with the results of Bayo et al. (2009a, b), and was not confirmed during our bioluminescent tests either. Bayo et al. (2009a, b) confirmed that significant correlations were obtained between toxicity values and total carbon, total inorganic carbon, total nitrogen, chlorine, and pH, and that these parameters markedly influence chlorine toxicity. In contrast, total organic carbon, chemical oxygen demand, electrical conductivity and turbidity had no effect on toxicity formation. Toxicity increased with the Cl_2:NH_4^+ ratio at a higher chlorine concentration released from combined chlorine. Regression models provided a good fit for effective concentration (EC50) as a function of total carbon and total nitrogen, after 5, 10, and 15 min. of exposure. All these conditions influence chlorine toxicity and could explain the high variability between results introduced for various subjects by various authors.

The obtained results suggested wide differences among the sensitivity of the tested organisms on the evaluated products. The determined active concentrations of the tested products varied from 0.13 to 8.18 µL L^{-1} or from 0.014 to 0.26 mg L^{-1} of FAC. The most sensitive to all three tested products were *V. fischeri* and *D. subspicatus* and the least benthonic worms *T. tubifex*. Based on the obtained results, the following rank order of sensitivity can be arranged: *V. fischeri* ≥ *D. subspicatus* >> *D. magna* >> *T. tubifex*.

In terms of toxic effects intensity for the individually tested organisms and exposure time, the evaluated compounds can be arranged in the following rank orders according to the FAC concentrations:
V. fischeri (15, 30 min): DoAm ≥ SA >> NaOCl
D. subspicatus (72-hrs): NaOCl > DoAm = SA
D. magna (24-hrs): NaOCl ≥ DoAm = SA
D. magna (48-hrs): NaOCl = DoAm > SA
T. tubifex (24-hrs): NaOCl > SA = DoAm
T. tubifex (48-hrs): SA > DoAm > NaOCl.

From these rank orders, it is evident that besides the organism species and FAC (free available chlorine) content in the tested compounds, the time of exposure can also play an important role. While NaOCl for *T. tubifex*, was determined as the most toxic product after 24 hrs of exposure, its toxicity was the lowest from all the tested disinfectants after 96 hrs. The dependency of NaOCl effectiveness on time exposure was not confirmed for *D. magna* and *V. fischeri*.

Despite apprehensions about the formation of toxic by-products which are usually associated with the use of chlorinated substances (Emmanuel et al., 2004), chlorine (Cl_2) and its derivatives are still widely applied; thanks to their excellent disinfecting properties (Amin et al., 2013). Since the dechlorinating process removed residual chlorine, a positive correlation between toxicity values and chlorine concentrations confirmed the formation of toxic disinfection by-products. Some of these substances were proven to be carcinogenic in humans and animals (Bayo et al., 2009a, b).

The tests and obtained results indicate adverse toxic effect of all three tested products on water organisms at very low concentrations. Therefore, handling them should be careful since they represent a severe threat after entering the environment.

For most of the organisms used during the presented experiments, big differences between the chlorine toxic values are introduced in previous literature. For example, while the US EPA *Ambient Water Quality Criteria for Chlorine* (1984) presents a total residual chlorine (TRC) value of 27.66 µg L^{-1} for *D. magna*, Singleton and Bio (1989) presented this value as low as 17 µg L^{-1}, and both the US EPA (1994) and Emmanuel et al. (2004) indicate the 24-hr value for chlorine

in the range 0.076–0.160 mg L^{-1}. The same inconsistency was also found for other organisms used here in the presented tests. The reported results rely on the hypochlorite decomposition end-product to which the analysis relates, i.e., total chlorine, active chlorine, and the like. Quite often, when the authors present the results for chlorine, it is hard to determine whether they are for free active chlorine, combined chlorine or total residual chlorine. Comparing these different results is questionable, therefore we strictly presented the type of chlorine used for toxicity determination and compared only related research in the discussion.

Conclusions

The aim of these tests was: (1) to evaluate the overall tolerance and functionality of each organism and endpoint in the test battery, and (2) to assess the toxicity for each endpoint. The obtained results confirmed the variations between the sensitivity of the selected water endpoints and the tested products. However, the bacteria *V. fischeri* and algae *D. subspicatus* were more sensitive to free active chlorine when used as model subjects for tests with plankton, the benthonic worms *T. tubifex*, expressed the lowest sensitivity. All subjects used were found to be highly vulnerable to chlorine and could be recommended as appropriate for toxicity bioassay. Both commercial products, DoAm and SA, have comparable effects with NaOCl and are suitable for water disinfection in equivalent concentrations.

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