Ecotoxicity of Biocides (Chemical Disinfectants) - Lethal and Sublethal Effects on Non-target Organisms

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The aim of this paper was to assess de ecotoxicity effects of four biocides, used as chemical disinfectants for swimming pool water treatment, on non-target organisms such as Daphnia magna and Heterocypris incongruens crustaceans. The tests showed a significant difference of sensitivity between species. The biocides, at the recommended disinfection doses, induced a very toxic or toxic effect on Daphnia magna, based on their mortality percentage. On the contrary, the observed ostracods lethal effects were generally less than 50% mortality and no significant growth inhibitions were observed. Overall, the ostracods had a higher tolerance to subacute doses, including disinfection doses of 20 mg/L (potassium mono persulfate), 5 mg/L (sodium dichloroisocyanurate), 8 mg/L (trichloroisocyanuric acid 90%) and 7 mg/L (trichloroisocyanuric acid 87-88%). In the context of a continuous biocides market expansion, the paper highlighted that our research proposed testing methods to obtain fast and accurate ecotoxicity data for a sustainable biocide production / authorization and also for an environmental hazard assessment.

Keywords: biocide, ecotoxicity, crustaceans, ostracods

Biocide have a large application in different human activities like food industry, medicine, farming, water treatment, household products and cosmetics. Biocides have been designed for specific targets, so they have been classified in function of their specificity in: pesticides (such as fungicides, insecticides, algaecides, molluscicides, miticides and rodenticides) and antimicrobial (such as bactericides, antibiotics, antivirals, antifungals, anti-protozoals, germicides, antiparazites, spermicides) [1]. Unfortunately, their uncontrolled use and an inefficient wastewater treatment (due to the fact that the wastewater treatment plants have not been designed to remove those chemical compounds) made possible a high occurrence of biocides in freshwaters. These compounds have been found in wastewater effluents, sludge, natural waters, sediments and even in drinking water [2, 3].

In Europe, the market of biocides is in expansion even higher than the antibiotics market. In spite of a Biocides Regulation 528/2012, which covers the authorization process and placing of biocide products on market, the exact amount of biocide production [4], usage and environmental presence is not very well under control. Therefore, the biocides must be tested for their toxic efficacy, human and environmental safety, control of their residues and degradation products.

According to Guidance on the *Biocidal Regulation: Volume IV Environment, Part A Information Requirements*, the biocides risk assessment needs to be evaluated for their aquatic effects in different compartments and in multispecies experimental settings [5]. The evaluations, respectively the quantification of the predicted of no effects concentrations - PNEC values, are based on the most sensitive taxonomic group considering the short term or long term effects. Generally, it has been shown that biocides generate acute toxicity on vertebrate and invertebrate organisms (such as cytotoxic, genotoxic and endocrine disruptor effects) [2] and photosynthetic system inhibition in case of algae or aquatic plants [6]. In spite of the fact that disinfection agents are biodegradable, the chlorination process, biological methylation or photo oxidation, could transform the biocides in more toxic and persistent compounds leading to resistance in microbial communities [2, 7, 8] that can affect the swimmers or consumers and subsequently the freshwater ecological status [9]. In addition, since the biocides have been used as chemical disinfectants for water disinfection such as swimming pool water, drinking water, wastewater, the disinfection practice increased the concern of toxic effects on non-target organisms.

It was reported that microorganisms [10, 11] and invertebrates have been frequently used in environmental toxicity evaluation of chemicals [12]. Among the invertebrates, *Daphnia magna* crustaceans have been the most used indicator of the effects of xenobiotics on primary consumers in freshwater ecosystems [13-16]. Also, is known that benthic invertebrates are more susceptible to dissolved and undissolved pollutants than pelagic biota, and may serve as an environmental imbalance long term marker [17-20].

The paper aim was to assess the toxic effects generated by four antimicrobial biocides (used in swimming pool water disinfection) on two type of non-target aquatic organisms-*Daphnia magna* and *Heterocypris incronguens*.

Experimental part

Materials

All tested biocides products were chemical disinfectants used for swimming pool water disinfection in Romania (table 1). The products were noted as biocide 1, biocide 2, biocide 3 and biocide 4. Biocide 1 was based on potassium mono persulfate (or potassium peroxymonosulfate) an oxidizing agent that releases free sulfate radicals and free hydroxyl radicals with high activity in water less toxic than chlorine disinfection. This substance is being used for disinfection, veterinary hygiene, food and animal feeds and drinking water sanitation [21].

Biocide 2 was based on sodium dichloroisocyanurate dihydrate (or sodium troclosene, sodium 3,5-dichloro-2,4,6-

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Table 1
CHEMICALS INFORMATION [22]

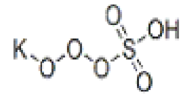
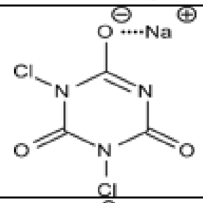
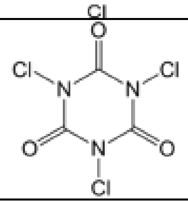
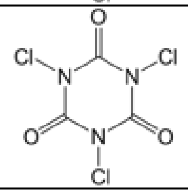
Biocide products	Active substances	CAS number	Dose for water treatment (mg/L)		Chemical formula 2D
			Shock dose	Maintenance dose	
Biocide 1	Potassium mono persulfate / potassium peroxymonosulfate 100%	70693-62-8	20	12	
Biocide 2	Sodium dichloroisocyanurate / troclosene 100%	51580-86-0	3 - 5	1.8 – 3.6	
Biocide 3	Trichloroisocyanuric acid / simclosene 90% and aluminum sulfate 3%, copper sulfate 2%, boric acid 1.9%	87-90-1	-	8	
Biocide 4	Trichloroisocyanuric acid / simclosene 87-88% and aluminum sulfate 3-4%, copper sulfate 1-2%, boric acid 2%	87-90-1	-	7.14	

Table 2
TOXICITY METHODS

Non-target organisms	Method	Materials	Method adjustment	Toxicity end points
Daphnia magna	ISO 6341 OECD 202 Acute toxicity (24h and 48h)	Daphtoxkit F magna *	To avoid the vaporization and absorption in tested solutions, each concentration was individually tested	Mortality / immobilization % $EC_{50\ -\ 24h,\ 48h}$ (mg/L)
Heterocypris incongruens	ISO 14371 Subacute toxicity (6 days)	Ostracodtoxkit F *		Mortality % Growth inhibition % $EC_{50\ -\ 6\ days}$ (mg/L)

EC_{50} – effective concentration for 50% of organisms; * kits supplied by MicroBiotests Inc. Belgia

trioxo-1,3,5-triazinan-1-ide) with many applications (based on chlorine release) as cleansing agent, biocide, industrial deodorant and also as disinfectant agent in water sanitation (drinking water or swimming pools) [23]. The active substance from biocides 3 and 4 is trichloroiso-cyanuric acid (1,3,5-trichloro-1,3,5-triazinane-2,4,6-trione) an organic compound used as disinfectant (also based on chlorine release), algaecide and bactericide especially in pools water treatment and also as a bleaching agent in textile industry, in civil sanitation, in animal husbandry and fisheries, fruit and vegetable preservation and others [24, 25]. All the active substances from the studied biocide products are listed as High Production Volume (HPV) chemicals.

The laboratory tests were performed using the reagents / growth media supplied by MicroBiotests Belgia [26, 27] and using the infrastructure, equipment and logistic support of Biotests Laboratory of National Research and Development Institute for Industrial Ecology ECOIND.

Toxicity methods

The ecotoxicity tests were performed on two type of aquatic organisms: *Daphnia magna* (water flea, planktonic crustaceans) and *Heterocypris incongruens* (ostracods, benthonic crustaceans). The organisms were selected according to Biocides Regulation requirements and also to cover two different aquatic compartments: water and sediment. Another reason of this selection was that *Heterocypris incongruens* and *Daphnia magna* are common species that inhabit shallow seasonal pools and small water bodies [28].

According to standardized procedure [29, 30] (table 2) the *Daphnia magna* neonates were exposed to a various concentrations of biocides during 24h and 48h in darkness at 20°C. The mortality / immobilization results were analyzed on the stage of a light table. The data were used to calculate 50% effect threshold (EC_{50} at 24h and 48h) with the MBT Daphnia Regtox Program (HILL model).

The benthic ostracods *Heterocypris incongruens* (table 2) were directly exposed to an artificial contaminated sediment and their growth or mortality percentage were detected and compared to control results [31]. After six days in darkness at 25°C, the biocides effects were evaluated. The ostracods handling and mortality analyze were made using a MOTIC stereomicroscope type DM-143 FBGG-B with incorporated camera. For ostracods length measurements a stereomicroscope M205FA from

Leica Microsystems (Schweiz) A (objectives 1x -work distance 61.5 and 2x - work distance 20.1) with image processing software was used. The results were processed using Microsoft EXCEL Program and EC_{50} base on mortality percentages at 6 days were analyzed. Standard deviation values were calculated for the mean length of ostracods.

The range of tested concentrations of each biocide was established according to the used doses and toxicity method requirements. At least five concentrations for each biocide were tested per each non-target organisms: biocide 1 -0.25 to 50 mg/L; biocide 2 -0.05 to 12.5 mg/L; biocide 3 and 4 -0.01 to 20 mg/L. The tests were performed in replicates for each tested concentration and control to ensure statistically relevant results.

Results and discussions

Toxicity effect induced to Daphnia magna

During the first 24h of biocide intoxication the neonates of planktonic crustaceans, *Daphnia magna*, showed an increased acute sensitivity expressed by percentages of immobilization / mortality (fig. 1). Lethal effects of 100% were observed at the lowest concentrations of 12 mg/L for biocide 1; 1.8 mg/L for biocide 2; and 0.1 mg/L in case of biocides 3 and 4. Effects in control limits (0-10% mortality) were observed for 0.5 mg/L - biocide 1 (5%) and 0.05 mg/L in case of biocides 3 and 4 (10%) (fig. 1).

Biocide 2 induced 15% mortality at the lowest tested concentrations of 0.05 mg/L. Water treatment doses of all studied biocide products highlighted total mortality of *Daphnia magna*. The same sensitivity of *Daphnia magna* to biocides (triclosan and triclorocarban -similar with the studied compounds) was showed also by other studies [3].

For instance, the results obtained for the biocide 2 (sodium dichlorizocyanurate), biocides 3 and 4 (trichloroisocyanuric acid) showed a different effect compared with biocide 1 (potassium mono persulfate). Biocide 1, based on the release of active oxygen was less toxic for *Daphnia magna* compared to active chlorine based biocides (fig. 2). The value of EC_{50} for the first 24h of exposure was higher than EC_{50} after 48h, which showed a shock effect. The EC_{50} at 24h were in the range of 0.058 mg/L to 2.54 mg/L. The EC_{50} at 48h showed the followed values: 10.45 mg/L for biocide 1, 0.19 mg/L for biocide 2, 0.068 mg/L for biocide 3 and 4. Similar results for biocide 3 and 4 were obtained because of insignificant difference in concentrations of active substances that have not influenced the toxicity.

According to Regulation (EU) 286/2011 for modification of Regulation (CE) 1272/2008 [32], the biocide 1, 2 and 4 were classified as *very toxic* ($EC_{50-48h} \leq 1$ mg/L, risk phrase H 400 - *very toxic for aquatic life)*. Biocide 1 based on potassium mono persulfate was classified as *toxic* (1 mg/L< $CE_{50-48h} \leq 10$ mg/L, risk phrase H 411 *-toxic for aquatic life, with long term effects)*. The same class of toxicity were identified for Daphnis in literature. The toxicity values for biocide 1 ranged in the interval 2.5 -5.3 mg/L, for biocide 2 were between 0.11 - 0.42 mg/L and for biocide 3 and 4 were between 0.16 -0.21 mg/L (fig. 2) [33].

Toxicity effects induced to Heterocypris incongruens

The *direct contact* sediment toxicity tests were carried out on neonates of benthic crustaceans *Heterocypris incongruens*. The bioassays could be considered a subacute test, because the testing period covers six days and growth of organisms is an evaluation criterion of toxicity. The ostracods revealed lethal effects in the range of 0-43% for biocide 1; 6-100% for biocide 2; 13-46% for biocide 3; 13-40% for biocide 4 (fig. 3).

The highest biocide concentrations showed more than 30% mortality on tested organisms. Effects in control limits (0-10%) were observed in case of 0.25 and 1 mg/L biocide 1, 0.25 mg/L biocide 2, and 0.1 mg/L biocide 3 and 4. Because of reduced toxic effects (<50%) in case of biocide

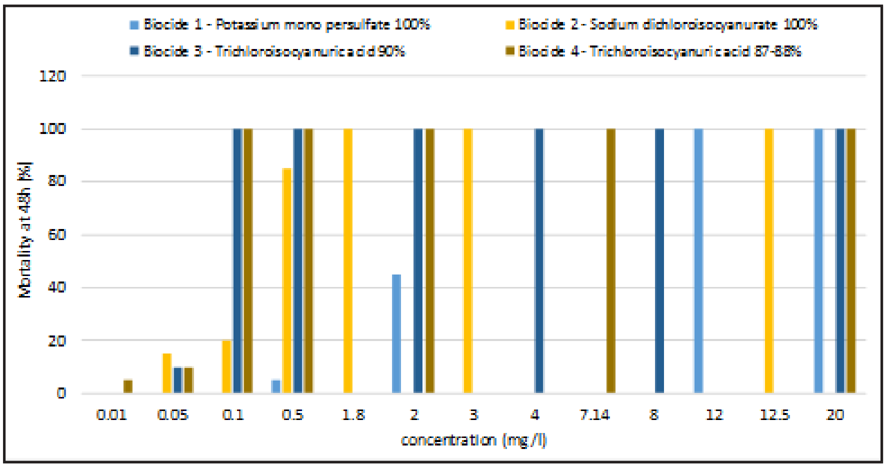


Fig. 1. Lethal effects of biocides on *Daphnia magna* at 48h

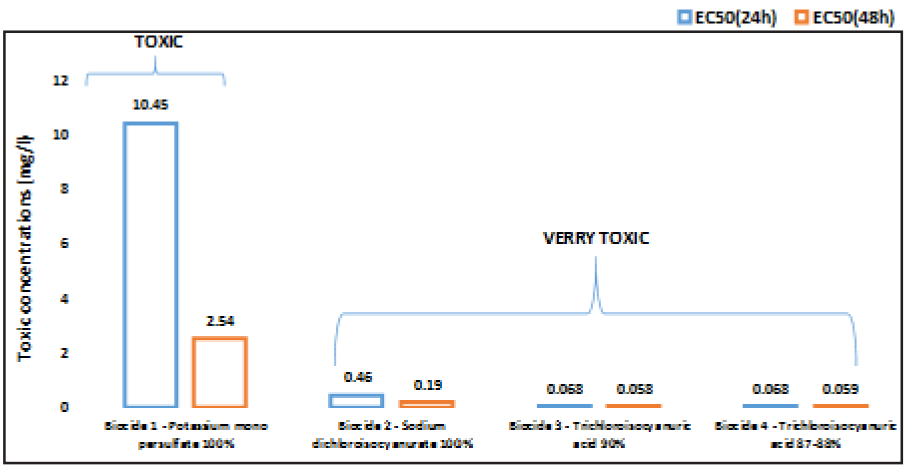


Fig. 2. EC_{50} of biocide 1, 2, 3 and 4 on *Daphnia magna*

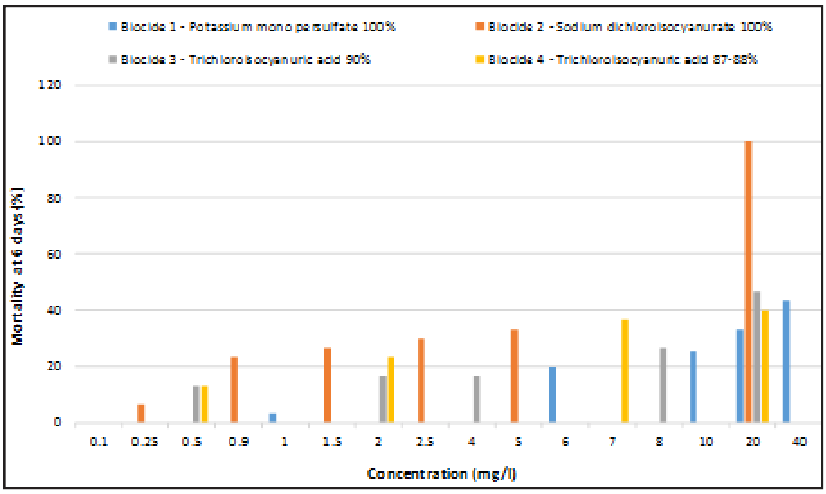


Fig. 3. Lethal effects (%) of biocide on *Heterocypris incongruens*

1, 3 and 4, the EC_{50} calculation was not possible, but it was estimated that EC_{50} could be more than 40 mg/L for biocide 1 and more than 20 mg/L for biocide 3 and 4. EC_{50} at 6 days for biocide 2 was 7.70 mg/L (classified as *toxic* for ostracods). The concentrations used by producer in pools water treatment highlighted 26-33% mortality on ostracods (fig. 3).

Heterocypris incongruens revealed a great resistance to tested biocides concerning the sub lethal effects. Growth inhibition was measured using the length of exposed ostracods compared with the control growth at 6 days. The ostracods mean length and the growth inhibitions values (%) are presented in table 3.

The toxic effects on organism's growth were less than 30% in the range of 0.1-20 mg/L for all tested biocides. Moreover, the smallest concentrations reveled an increasing growth of tested ostracods, this results being embedded in the control limits of ±10% (fig. 4 - 7). For the concentrations of biocides used regularly for water treatment, the following effects were observed: 20% growth inhibition for biocide 1 and 2 (at 20 mg/L respectively 5 mg/l), 16 % for biocide 3 (at 8 mg/L, fig. 3) and 8% in case of biocide 4 (at 7 mg/L, fig. 4). Ostracods tolerance to pollutants was also reported in previous studies on benthic invertebrate [18, 34, 35]. Considering that *Heterocypris incongruens* prays the small organisms like *Daphnia magna*, the negative effects of biocide obtained for Daphnis could induce imbalances in ostracods communities [36].

Table 3
OSTRACODS MEAN LENGTH AND GROWTH INHIBITION

Biocide and tested concentration (mg/L)	Control mean length - µm (0h)	Control mean length - µm (6 days)	Mean length in test - µm (6 days)	Growth inhibition (%)
Biocide 1 - Potassium mono persulfate 100%				
0.25	214±10.51	619±8.58	629±57.74	-2.57
1			629±37.63	-2.54
6			577±35.73	10.31
10			556±20.19	15.58
20			539±19.10	19.72
40			443±28.17	43.35
Biocide 2 - Sodium dichloroisocyanurate 100%				
0.25	214±10.51	619±8.58	709±27.94	-22.22
0.9			647±77.84	-7.13
1.5			626±25.56	-1.70
2.5			616±26.12	0.58
5			393±7.31	19.56
Biocide 3 - Trichloroisocyanuric acid 90%				
0.1	249±10.49	493±34.41	523±13.5	-7.23
0.5			507±48.87	-5.42
2			497±11.26	-1.60
4			469±17.52	9.81
8			455±13.02	15.89
20			420±8.64	29.89
Biocide 4 - Trichloroisocyanuric acid 87-88%				
0.1	249±10.49	493±34.41	494±63.12	-0.25
0.5			497±30.32	-1.59
2			498±30.92	-1.97
7			474±63.49	7.90
20			425±10.24	27.95

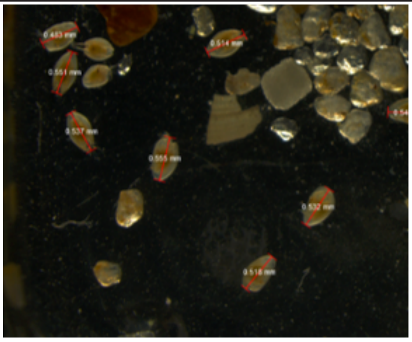


Fig. 4. Control at 6 days (1X, 2mm)

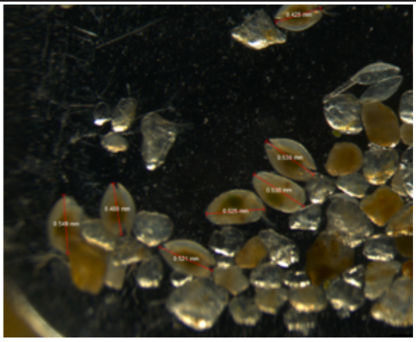


Fig. 5. Biocide 3 (8 mg/L) at 6 days (1X, 1mm)

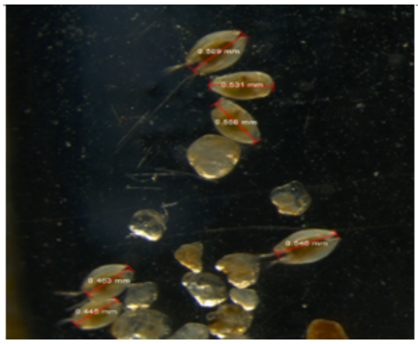


Fig. 6. Biocide 4 (7 mg/L) at 6 days (1X, 1mm)

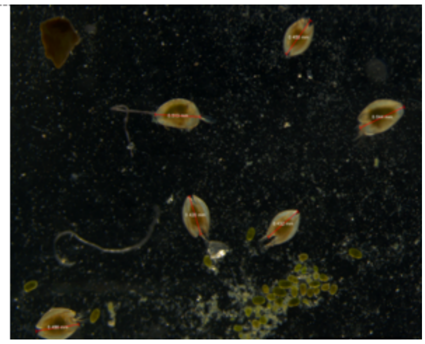


Fig. 7. Biocide 4 (0.5 mg/L) at 6 days (1X, 1mm)

Conclusions

The lethal and sublethal effects of four biocides (chemical disinfectants) from the Romanian market were evaluated on non-usually targeted aquatic organisms. Biocides toxicity was analyzed on two crustacean species (*Daphnia magna* and *Heterocypris incongruens*) from pelagic and benthic compartments of the aquatic ecosystem. The tests showed a significant difference of sensitivity between *Daphnia magna* and *Heterocypris incongruens.* Considering the percentage of mortality, the biocides induced a *very toxic* or a *toxic* effect on *Daphnia magna.* A 100% mortality of Daphnis was observed in case of the concentrations recommended by producer in swimming pool water treatment. Contrary to *Daphnia spp*., the ostracods lethal effects were less than 50% for biocide 1(potassium mono persulfate) and 3 and 4 (trichloro-isocyanuric acid). The exposure of ostracods to biocide 2 (dichloroisocyanurate dehydrate) reveled *toxic* effects. Also, no significant growth inhibition was observed in the tested concentrations range. Recommended doses reveled an ostracods mortality in range of 26-33% and a growth inhibition between 8 to 20%, indicating tolerance at tested concentrations. No significant toxicity differences were observed between biocide 3 and 4, the percentage of active substances being insignificant. The toxicity magnitude was in accordance to concentration increasing for the both species. The laboratory tests create the most unfavorable situation for biocide exposure, but in the field the majority of biocide are biodegradable, their degradation compounds could be harmful for another organism. In order to avoid the toxic effects of the biocide doses (recommended by the producer) on small crustaceans, the receiving water should assure a dilution factor of about 100 in case of biocide 1 and 2 and 200 for biocide 3 and 4. The ecotoxicity evaluation strategies on non-target organisms are not yet in trend with the continuous expansion of biocide market.

The European Commission is working since 2008 to create a *Sustainable Consumption and Production and Sustainable Industrial Policy Action Plan* that should improve the environmental performance of products and increase the demand for more sustainable goods and production technologies by 2030. In this context, the paper highlighted that the Romanian laboratory could perform the tests required to obtain ecotoxicity data for a sustainable biocide production / authorization and also for environmental hazard assessment.

Acknowledgments: The present research was financially supported by the Romanian National Nucleu Program (code PN 18-05 02 01).

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Manuscript received: 12.08.2018