Comparative toxicity of chlorine and peracetic acid in the biofouling control of Mytilopsis leucophaeata and Dreissena polymorpha embryos (Mollusca, Bivalvia)

A. Verween a, *, M. Vincx a, S. Degraer b

a Ghent University, Biology Department, Marine Biology Section, Krijglaan 281/S8, B-9000 Gent, Belgium
b Management Unit of the Mathematical Model of the North Sea, Royal Belgian Institute of Natural Sciences, Gulledelle 100, 1200 Brussels, Belgium

ABSTRACT

Chlorination is the most common antifouling procedure, but the search for alternatives is ongoing. Although concentrations that kill adults will also be effective against larvae, it is advisable to evaluate the toxicity of any candidate toxicant against the combatable life stage. For mussels, the earliest life stages are the most vulnerable ones and thus may require the lowest doses biocides. Since the period of larval presence is restricted to a couple of months, a pointed dosage of biocides during this period will be as effective as a continuous dosage throughout the year. This study reports on the lethal acute toxicity of sodium hypochlorite and peracetic acid to 4 h old embryos of Mytilopsis leucophaeata and Dreissena polymorpha. Chlorination was found effective against M. leucophaeata from a concentration of 0.6 mg/l onwards, even at short exposure times. Commercial peracetic acid showed to be a very good alternative in both species although the most appropriate level still has to be determined.

Scientific relevance: This paper gives valuable information on the increasing biofouling problem of the invasive bivalve M. leucophaeata and the possible control and avoidance of its biofouling. Instead of acting in a reactive way, this is combating the fouling problem after mussels have settled, we are looking at the problem in a proactive way. Next to the common antifouling procedure of chlorination, also peracetic acid is considered as an alternative. The procedures were tested for M. leucophaeata and the zebra mussel, D. polymorpha. This makes comparison between both species more easy.

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1. Introduction

Mussels are the most dominant fouling organisms in the cooling water circuits of coastal power plants throughout the world (Fisher et al., 1984; Jenner et al., 1998; Rajagopal et al., 2003). Control of mussel fouling in existing cooling water systems can potentially be achieved by several strategies. Control can be accomplished by killing larvae before they settle. Alternatively, biofouling can also be prevented if mussels are prevented from settling by creating an environment in which settlement is postponed. Finally, control can be exerted by killing the adult mussels already present in the system.

The general trend at the moment is towards a tougher legislation to better protect public health and the environment from overuse and misuse of biocides. In 1996, the EU Directive on integrated pollution prevention and control (IPPC) (Directive 96/61/EC) was adopted, demanding that all companies considered to be a nuisance would act according to permit conditions based on Best Available Techniques (BATs) at least by 2007. In essence, the IPPC Directive is about minimizing pollution from various industrial sources throughout the European Union. In 2000, the Water Framework Directive (Directive 2000/60/EC) was formulated, being the most substantial piece of water legislation ever produced by the European Commission. The Directive will provide the major driver for achieving sustainable management of water in all the Member States for many years to come and will clearly have future impacts on biocide restriction. Although chemical concentrations that kill adults will also be effective against larval life stages, it is thus advisable to evaluate the toxicity of any candidate toxicant against the life stage for which the treatment will be used to ensure that appropriate (minimal) levels of chemicals are used (Fisher et al., 1994). It is generally assumed that veligers are more sensitive to toxicants than adults because the natural mortality rate at the veliger stage is high (Sprung, 1993; Stoeckel and Garton, 1993).
Bayne et al. (1976) also stated that the earliest stages in the life cycle of a bivalve are the most vulnerable ones. This was already proven for *Mytilopsis leucophaeata* (Conrad, 1831), where the vulnerability of life stages rapidly increases with age (Verween et al., 2007b) and for *Dreissena polymorpha* (Pallas, 1771) (Wright et al., 1996).

Within this strategy, pediveligers seem the most appropriate life stage to combat in controlling mussel fouling. They already settled on the substrate, which makes it easy to kill or harm the pediveliger by dosing biocides but they are not yet as "untouchable" as adult mussels, which have a very hard, protective shell. Therefore, most certainly a lower concentration of biocides will be needed to remove this early biofouling. However, no manuscripts concerning the biofouling control of mussels by combating the pediveliger stage have been published yet, because it is very difficult to cultivate them in densities high enough for toxicological testing.

Although killing larvae before they settle seems a less ideal control measure than controlling the pediveliger stage, it is a pathway worthy to be examined. Hence, knowledge on the presence of mussel larvae provides a basis for an ecologically and economically proper use of these detrimental chemicals (Relini, 1984). To prevent new biofouling by *M. leucophaeata*, a pointed dosage of biocides during the period of larval presence will be as effective as a continuous dosage throughout the year (Verween et al., 2007a).

The antifouling procedure most favored by operators at northwestern European coasts and estuaries consists of a continuous low-level or intermittent chlorination with hypochlorite (Jenner et al., 1998; BREF, 2000; Rajagopal et al., 2003). Chlorination is also considered as Best Available Technique (BAT) in industrial cooling water systems within the cooling requirements of the industrial process (IPPC, 2000). According to the BAT-guidelines, successful conditioning regimes use at least 0.2 mg/l free oxidants in their system. However, concerns have risen about the detrimental effect of chlorination on the environment (Taylor, 2006). Thus, hypochlorite might not be the ideal antifoulant against mussel larvae, spurring the search for alternatives. As an alternative biocide, peracetic acid is suggested. The residual products after treatment are water, oxygen and a low residual content of organic constituents that are easily biologically degraded into carbon dioxide (Yeghiaian and Christo, 1992).

*M. leucophaeata*, the brackish water mussel, is a mytiliform bivalve and a typical brackish water species (Boettger, 1932) with very broad tolerances. The species is becoming an important biofouling species, rapidly expanding in Europe (Rajagopal et al., 1994, 1995; Verween et al., in press). The zebra mussel *D. polymorpha* is a temperate to subtropical freshwater species and possibly the most famous fouler, with an extremely rapid distribution throughout the Great Lakes in North America. Both species are Dreissenidae (Mollusca, Bivalvia, Veneroida).

Since *D. polymorpha* and *M. leucophaeata* both have a life phase dependant tolerance to toxicants, increasing with age, this study specialised on the embryonic life, the most vulnerable phase in their life cycle. The specific aims of this study were to investigate the vulnerability of *M. leucophaeata* and *D. polymorpha* larvae to different concentrations and contact times with chlorine and peracetic acid. The chlorine concentration used to control mussel larvae will as such be optimized and the efficacy of peracetic acid as an alternative biocide will be tested. The peracetic acid commercial formulate DEGACLEAN® 150 was supplied by Evonik company (Cristiani, 2006).

2. Material and methods

2.1. Brood stock

Before the start of the spawning season (Verween et al., 2005), approximately 200 adults of each species were collected from the river Schelde in Antwerpen (B) (*M. leucophaeata*) and lake Blaarmeersen in Gent (B) (*D. polymorpha*) and maintained in the laboratory in a flow-through brood stock tank at a temperature lower than that measured in the field (12 ± 1 °C) to prevent spawning of ripe animals (Stanczyzkowska, 1977; Stoeckel et al., 2004). Water collected from the field site was used and mussels were fed ad libitum (Helm et al., 2004) with live micro-algae, being the flagellate *Isochrysis galbana* Parke (3 × 10⁶ cells ml⁻¹) (Guillard, 1975).

2.2. Spawning and fertilization

Spawning was induced by placing the rive mussels individually into 50 ml beakers containing artificial brackish water (salinity 8) (Instant Ocean®, Aquarium Systems, France) for *M. leucophaeata* and natural freshwater, treated with UV-light (salinity 0) for *D. polymorpha*, both at a temperature of 20 °C. When the siphons were extracted, fluvoxamine was injected near the inhalant siphon (Ram et al., 1993; Fong, 1998). Fluvoxamine is the most powerful spawning inducer in any bivalve (Fong, 1998). Thirty minutes after this injection, the water was changed with fresh aerated water. Males began releasing sperm within 30–60 min, while females began releasing ova within 60–90 min. Once spawning was detected, adults were placed in new beakers with water without fluvoxamine. The water was changed every 30 min until no further animals started spawning. Bayne (1965) stated that sperm and eggs of *Mytilus edulis* should be less than 1 h old for fertilization. To ensure that eggs would be exposed to viable sperm, mussels were induced to spawn in two batches. The second batch was exposed to fluvoxamine 1–1.5 h after the first batch. In this way, we induced an overlap of females, spawning in the first batch, and males, spawning in the second batch (Stoeckel et al., 2004). Fertilization occurred with eggs and sperm from at least three individuals. More information concerning the spawning procedures can be found in Verween et al. (2007b).

2.3. Static acute toxicity tests

Four hours after fertilization, embryos were added in a random order to the test solutions at a concentration of ±10 embryos/ml in a 50 ml glass cylindrical vial, already containing the test solution. The variation in the concentration of embryos in the various test solutions was minimized by keeping the embryo suspension well mixed with a plunger and using a high precision automatic pipette. The organisms were not fed during the test, because they do not feed during embryonic development into D-shaped larvae (first 72 h) (Honkoop, 1999).

Standardized static acute toxicity tests were conducted on 4 h old embryos of *M. leucophaeata* and *D. polymorpha* as recommended by the American Society for Testing and Materials (ASTM, 1999). Mussel embryos were tested for four different chlorine concentrations (0.2, 0.4, 0.5 and 0.6 mg/l) and five different peracetic acid concentrations (0.75, 1.5, 3, 6 and 9 mg/l) at different exposure times (Tables 1 and 2). The chlorine test solution was prepared from sodium hypochlorite and brackish or freshwater (according to the tested species). Test solutions were prepared on the day of the experiment and stored in a volumetric flask (Rajagopal et al., 2002a). In the experimental setup, Degaclean® was used, a biocide based on peracetic acid, containing it at 15%. Test concentrations mentioned in the manuscript are the peracetic acid concentrations, prepared from Degaclean® and brackish or freshwater. The range of experimental concentrations was chosen on the basis of literature on chlorination of adults (Rajagopal et al., 2002a,b, 2003) and preliminary research with Degaclean® for peracetic acid (Scheider, 2002). Temperature and salinity were kept...
constant in each of the experimental treatments. An appropriate mix of aerated water and sodium hypochlorite or Degaclean was used to create the desired test solutions just prior to the start of the experiments. All experiments were conducted in a temperature-controlled room at the same continuous light conditions. Vials were covered to keep out extraneous contaminants and bacteria and to minimize evaporation. Start and residual chlorine concentrations were tested using highly precise colorimetric Microquant® chlorine tests.

As a universal control for all experiments, embryos were exposed to artificial water with the same characteristics (20 °C and salinity 8 for M. leucophaeata; 20 °C and salinity 0 for D. polymorpha) as that in which fertilization occurred and similar to the field conditions, at the beginning of larval presence in the water column (Verween et al., 2005). Three replicates of each treatment and of controls were used for all toxicity tests.

All embryos were counted directly after the elapsed time period by means of a binocular microscope; a distinction was made between alive and dead embryos by the presence or absence of ciliary movement, either inside the translucent shell or on an extended velum (ASTM, 1999). For each test chamber in each treatment, the mortality A was calculated as follows (Stephan, 1977):

\[ A = 100 \left( \frac{N - B}{N} \right) \]

with B the number of moving larvae at the end of the test and N the total number of larvae.

For each test chamber in each treatment, other than the controls, the mortality rate was corrected using Abott's formula (Abott, 1925):

\[ E = \frac{100(A - M)}{(100 - M)} \]

with E being the mortality rate, adjusted for the controls and M the average mortality in the control treatments.

2.4. Statistical analysis

Homogeneity of variance and normality were tested using Levene's and Shapiro–Wilk's W-test respectively. Although in the majority of test cases ANOVA assumptions were not fulfilled (not even after arccusine-transformation) statistical differences on raw data were examined by multiple analysis of variance (ANOVA) (SAS 9.1). The large sample size allows the statistics to follow a normal distribution (Central Limit Theorem) (Sokal and Rohlf, 1981). Statements of significant differences were based on accepting \( P < 0.05 \).

### Table 1

<table>
<thead>
<tr>
<th>Exposure time (h)</th>
<th>0.2 mg/l</th>
<th>0.4 mg/l</th>
<th>0.5 mg/l</th>
<th>0.6 mg/l</th>
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<tr>
<td>30' ± 15'</td>
<td>M/D</td>
<td>M/D</td>
<td>M/D</td>
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<tr>
<td>1 h ± 15'</td>
<td>M/D</td>
<td>M/D</td>
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<tr>
<td>2 h ± 30'</td>
<td>M/D</td>
<td>D</td>
<td>M/D</td>
<td>M/D</td>
</tr>
<tr>
<td>3 h ± 30'</td>
<td>M/D</td>
<td>M/D</td>
<td>M/D</td>
<td>M/D</td>
</tr>
<tr>
<td>4 h ± 30'</td>
<td>M/D</td>
<td>M/D</td>
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### Table 2

<table>
<thead>
<tr>
<th>Exposure time (h)</th>
<th>0.75 mg/l</th>
<th>1.5 mg/l</th>
<th>3 mg/l</th>
<th>6 mg/l</th>
<th>9 mg/l</th>
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<tr>
<td>15'</td>
<td>M/D</td>
<td>M/D</td>
<td>M/D</td>
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<tr>
<td>30' ± 15'</td>
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<td>1 h ± 15'</td>
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<td>2 h ± 30'</td>
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<tr>
<td>3 h ± 30'</td>
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<td>4 h ± 30'</td>
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### 3. Results

All control treatments in the different experiments showed a survival percentage >80%. This number lies above 71.8%, i.e. the boundary recommended by the ASTM (1999).

#### 3.1. Chlorine dependent vulnerability

Overall two-way ANOVA showed no significant difference in mortalities of 4 h old embryos at different exposure times (\( F = 0.91; \text{df} = 4; P = 0.464 \)) but did indicate a highly significant difference in mortalities at different concentrations (\( P < 0.001 \)). Although no statistically significant difference in mortality between species was detected (\( F = 0.33; \text{df} = 1; P = 0.569 \)), a different trend could be distinguished.

#### 3.1.1. M. leucophaeata

Adjusted mortality in M. leucophaeata 4 h old embryos was very low in the chlorine concentration range 0.2–0.5 mg/l (max 8 ± 2.1%), merely independent of the exposure time (two-way factorial ANOVA: \( F = 2.4; \text{df} = 4; P = 0.085 \)). A significantly higher adjusted mortality was detected at concentration 0.6 mg/l (\( P < 0.001 \)), with average value of 87.9 ± 4.5% (Fig. 1). This higher concentration was only tested at short exposure times, i.e. 30 min and 1 h.

#### 3.1.2. D. polymorpha

Toxicity tests with D. polymorpha showed no clear effect of concentration on adjusted embryonic mortality (two-way main effects ANOVA: \( F = 0.3; \text{df} = 2; P = 0.714 \)) with very low average mortalities, ranging between 7.0 ± 0.3% and 39.1 ± 11.5%. However, a significant effect of exposure time (\( P < 0.001 \)) was registered with high exposure times, 3 and 4 h, showing significantly higher adjusted embryonic mortality (average 30.1 ± 6.8%) as compared to the shorter exposures (average 8.8 ± 12%) (Fig. 1).

#### 3.2. Peracetic acid dependent vulnerability

Overall two-way ANOVA showed no significant effect of exposure time on adjusted mortality of 4 h old embryos (\( F = 1.07; \text{df} = 5; P = 0.382 \)). Peracetic acid concentration however did indicate a significant difference in adjusted mortality (\( P < 0.01 \)). No significant difference in sensitivity between species was found (\( F = 0.01; \text{df} = 1; P = 0.949 \)); both showed a very low survival rate at the tested concentrations.

#### 3.2.1. M. leucophaeata

Adjusted mortality rates in M. leucophaeata were very high; even at the lowest concentration (0.75 mg/l), an average mortality of 93.6 ± 1.5% was found (Fig. 2). This concentration was only tested at exposure times of 2 h and more. From 3 mg/l on, adjusted mortality above 98% was detected, even at an exposure time of only 15 min. No significant effect of concentration (\( F = 0.88; \text{df} = 4; P = 0.484 \)) or exposure time (\( F = 0.58; \text{df} = 5; P = 0.718 \)) was found.

#### 3.2.2. D. polymorpha

Four hours old D. polymorpha embryos still showed a low tolerance to peracetic acid at its lowest tested concentration (0.75 mg/l) with an average adjusted mortality rate of 89.6 ± 3.4% (Fig. 2). At this concentration only exposures more than 2 h were tested. From 1.5 mg/l on, adjusted mortality rate is almost 100%, with mortality ranging between 94.1 ± 1.0% and 100%. At 1.5 mg/l, exposures above 1 h were tested, but from 3 mg/l on, even exposures as low as 15 min were almost 100% deadly to the embryos. Two-way main effects ANOVA confirmed this difference (\( P < 0.05 \)).
with a significant lower adjusted mortality at concentration 0.75 mg/l. No significant effect of exposure time was detected ($F = 1.44; df = 5; P = 0.239$).

4. Discussion

The likely release of chlorination byproducts (CBPs) in the effluent stream in the process of chlorination – such as organohalogens, chlorobromoform and phenols – and their possible damage to the aquatic life in the natural environment have raised concern in the use of this technology. Scientific literature on this topic only provides a limited understanding in the chemical dynamics of the effluent stream, leaving room for speculation (Taylor, 2006). Another important disadvantage of chlorination is the species-dependent tolerance to hypochlorite; some species, even from the same phylum, are far more resistant to chlorination than others. Chlorination efficiency is further temperature-dependent: if the water temperature is low (<15°C), the time required for effective chlorination will be prolonged (Jenner and Janssen-Mommen, 1993). As an alternative biocide, peracetic acid is suggested. In the commercial formulation as Degaclean® 150, it is an equilibrium mixture of peracetic acid, hydrogen peroxide, acetic acid and water (Scheider, 2002). In surface water, peracetic acid is hydrolyzed into acetic acid and hydrogen peroxide, both easily biodegradable (Cristiani, 2005). The product Degaclean® 150 is further considered very effective over a wide pH and temperature range and features a low corrosive effect in comparison to other commercial peracetic acid products (Scheider, 2002).

4.1. Vulnerability to chlorine

Chlorination is universally accepted as an efficient biocide because of its broad-spectrum activity, easy availability and simple applicability (Bidwell et al., 1999). Its cost effectiveness makes chlorination the most favored antifouling procedure worldwide. According to BAT which is developed to control adult mussel fouling, successful conditioning regimes use at least 0.2 mg/l free oxidants in their system. However, our experimental data show that embryos of *M. leucophaeata* and *D. polymorpha*, being the most vulnerable phase in their life cycle, only show low mortality at this concentration. At the moment, before the implementation of BAT, still a total residual chlorine level of 2 mg/l is used to control mussel fouling in Europe during breeding periods, while during non-breeding periods considerably lower chlorine levels (0.2–0.5 mg/l) are used (Jenner et al., 1998; Rajagopal et al., 2003). Embryonic *M. leucophaeata* were very resistant to chlorination at concentrations 0.2–0.5 mg/l, independent of the exposure time. This indicates that a concentration of 0.2 mg/l chlorine to combat the existing fouling in the system will not avoid new biofouling. Larvae entering the system, even when chlorination is ongoing, will survive and thus will have an opportunity to settle into the system and as such create new biofouling. The biofouling problem of *M. leucophaeata* will thus never be completely solved with the
strategy of continuous low-level or intermittent chlorination with hypochlorite at proposed doses. A concentration of 0.6 mg/l however was lethal to most embryos, even at a very short exposure of 30 min. Since larvae enter the cooling water system continuously during the breeding period, indeed a higher concentration than that recommended in the BAT-document (min. 0.6 mg/l) should be applied during this period.

The effectiveness of chlorination against adult zebra mussels is very well known (Jenner, 1985; Rajagopal et al., 2003). Effects of chlorine on the larval stages of *D. polymorpha* have not been widely investigated and the few researches conducted showed very contradictory results. In one study, veligers were still alive after 1 h exposure to 7.5 mg/l chlorine (Wilhelm, cited in Clarke, 1952) while Klers et al. (1993) found 100% mortality even at chlorine concentrations as low as 0.5 mg/l within 2 h. Sensitivity tests of different life stages of *D. polymorpha* to commercial molluscicides showed a successive decrease of sensitivity for each developmental stage through the adult, although chlorine was not specifically tested (Fisher et al., 1994). Also in this study, 4 h old *D. polymorpha* embryos reacted in a different way to chlorine. Only long exposure times of 3 and 4 h showed a significant increase of mortality, even at low concentrations. Mortality however stayed very low, never exceeding 50%. Even at a concentration of 0.6 mg/l, lethal to *M. leucophaeata* larvae, almost no *D. polymorpha* larvae died. This pattern is opposite to the adult mussels, where resistance of *M. leucophaeata* to different chlorine concentrations was much higher than other mussel species such as *D. polymorpha* and *M. edulis* (Rajagopal et al., 2002b). A possible explanation can be found in the environment where adult *D. polymorpha* have been sampled: lake Blaarzeemen is a small, almost completely enclosed lake, difficult to compare with the Great Lakes or rivers where test species were collected for other toxicity experiments (Wallner et al., 1993; Wildridge et al., 1998; Rajagopal et al., 2003). Its narrow biotope can have modified the species tolerances, ending in a high tolerance to chlorine. Differences in sensitivity to molluscicides among life stages have however also been observed in studies with related bivalves, following different trends (Roosenburg et al., 1980; Watling, 1982; Stongen and Nielsen, 1991, all in Fisher et al., 1994).

4.2. Peracetic acid dependent vulnerability

Peracetic acid is commonly used as disinfectant for the control of harmful micro-organisms in raw sewage and sewage effluent (Raymond, 1995), such as surface attached, slime-forming bacteria (Briñez et al., 2006; Meylheuc et al., 2006; Gram et al., 2007) and viruses (Jolivet-Gougeon et al., 2006; Martin et al., 2007). At first sight, peracetic acid seems a very good alternative for chlorination but the product is – as most alternatives of chlorination – less cost effective. The range of tested concentrations was chosen on basis of preliminary research on mussel larvae, in which a dosage of 40 mg/l Degaclean°C during 15 min per day was proposed. Considering that Degaclean°C contains peracetic acid at 15%, this concentration corresponds to 6 mg/l as peracetic acid.

Both *M. leucophaeata* and *D. polymorpha* embryos show a very low resistance to peracetic acid, even at concentrations as low as 0.75 mg/l where tested exposure augmented 2 h. At 3 mg/l however, a 15 min-exposure is already lethal to 95% of all embryos for *D. polymorpha* and to more than 98% for embryonic *M. leucophaeata*. Because of the high costs of continuous dosage, initially an intermittent chemical dosage was preferred. This study indicates that a dosage as low as 0.3 mg/l during 15 min is as efficient as the proposed 6 mg/l in combating new mussel fouling by *D. polymorpha* or *M. leucophaeata*. Thus for conditions like the ones in the test system, concentrations can even be lowered considering future biocide restrictions and still be as effective against mussel fouling.

These results also leave room for a strategy of longer dosing at a lower dosage, wherein a higher percentage of incoming mussel larvae will be killed: 1.5 mg/l during 1 h is economically equal to a pointed dosage of 15 min at 6 mg/l, but will combat a larger amount of incoming larvae.

5. Conclusion

Since chemical minimization strategies require the use of the smallest amount of chemical possible, candidate molluscicides should be tested on the life stage targeted for control (Fisher et al., 1994). Within the framework of this strategy, sodium hypochlorite is a good biocide against new *M. leucophaeata* biofouling, although minimal concentration should be 0.6 mg/l, and thus not 0.2 mg/l as recommended by the BAT-guidelines to control adult mussel fouling (IPPC, 2000). No clear effect of chlorine was found on *D. polymorpha* embryos, possibly because of the isolated origin of the adult individuals, used as brood stock. Peracetic acid, here tested as the commercial product Degaclean°C, however is a very good alternative from an ecological point of view for both species, but economically it can be disadvantageous. However, the fact that proposed concentrations are well above the lethal level for both species leaves room for a more cost effective strategy.

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References


