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The effects of a copper-based antifouling paint on mortality and enzymatic activity of a non-target marine organism

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Abstract

Antifouling paints are used on a wide range of underwater structures in order to protect them from the development of fouling organisms. The leaching of the toxic substances from the matrix of the paint causes toxic effects not only to the fouling organisms but also on other "non-target" biota. The present study addresses the impact of the antifouling paint Flexgard VI-II on brine shrimp nauplii selected as convenient test organisms. The surface to volume (S/V) concept developed by Persoone and Castritsi-Catharios (1989) was used to determine S/V-LC_{50s} for the test biota exposed to PVC test panels of 400–1000 mm^2 surface coated with the antifouling paint in test vessels containing 20 ml seawater. Total ATPase and Mg^{2+}-ATPase were also analyzed for coated surface areas inducing less than 50% mortality in the brine shrimp nauplii. The calculated S/V-LC_{50}(24 h) was 24.6 mm^2/ml, which shows the high toxic character of the antifouling paint. Decreased enzymatic activities were noted in the brine shrimp nauplii exposed to test panels of 50 and 100 mm^2 in 20 ml seawater. The present study indicates that the "surface to volume" concept is an interesting methodology that can be applied with both lethal and sublethal effect criteria for the determination of toxic stress from leaches of painted surfaces.

Keywords: Copper; Antifouling; Non-target species; *Artemia*; ATPase; Aquaculture

1. Introduction

Copper-based antifouling paints have regained increased attention in replacement of triorganotin antifouling paints such as TBT-paints. The latter paints indeed were found to have toxic effects on non-target organisms, which were fully recognized in the 1980's, when oyster farming in the bay of Arcachon in France nearly collapsed (Alzieu et al., 1986; Michel and Averty, 1999).

In the fish farming industry, antifouling paints are used on the nets of fish cages in order to prevent the attachment and growth of fouling organisms. Fouling of the nets eventually reduces water flow through the cage and as a result impacts on the water flow and the amount of dissolved oxygen. In addition, the cages suffer from increased structural fatigue and the fouling communities are also a possible source of diseases (Hodson et al., 2000).

The aim of the present study is to examine the potential toxic effects of a copper-based antifouling paint on non-target marine organisms. Brine shrimp nauplii (instar II and III) were exposed to panels coated with antifouling paint and the effects on mortality and enzymatic activities of the organisms were determined. *Artemia* nauplii were selected as test organisms because homogenous populations can be obtained for the experiments in a short period of time without the need for stock culturing of the test biota. The "surface to volume" concept originally worked out by Persoone and Castritsi-Catharios (1989) with brine shrimp, as representatives of non-target organisms, has been applied in this study with the same non-test species, to determine the toxic impact of coatings releasing specific chemicals. The results are expressed in surface to volume (S/V) units, i.e. the area of painted surface which affects or kills 50% of the tests organisms in 1 ml of test medium (S/V-LC_{50}) within a particular exposure period. As a

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sublethal effect criterion total ATPase and Mg^{2+}-ATPase enzymatic activities were also determined. Membrane enzymes such as ATPases, which carry out ion transport with parallel energy production, are well characterized and the decrease of their activity can be used to measure the toxic impact of chemicals (Machera et al., 1996; Cotou et al., 2001).

2. Materials and methods

2.1. Antifouling paint

The commercial copper-based antifouling paint Flexgard VI-II produced by the company Flexabar was used for the determination of the S/V-LC_{50} (24 h) and the ATPase enzymatic activities. This antifouling paint is used for the treatment of cage nets in aquacultures and has a latex matrix, which contains 18% cuprous oxide (Cu_2O) as the active ingredient (15% metallic copper). It also contains chlorothalonil (2,4,5,6-terachloroisopthalonitrile) which is a booster biocide for the control of copper resistant fouling organisms such as algal slimes (Thomas et al., 2001).

2.2. Seawater

Artificial seawater Instant Ocean® of 36‰ salinity and pH ≈ 8 was used both for hatching of *Artemia* cysts and the performance of the assays.

2.3. Test panels

The test panels, which were used as substrates for the antifouling paint, were cut out of white PVC sheets, sandblasted and coated with the paint 24 h before the exposure. The application of the paint was done with a thin brush and in such a way, that the brush was slipping on the surface of the panel, leaving at the end, a small amount of paint that created a thin layer. The panels were left to dry in a dark place without air circulation in order to avoid photooxidation of the paint.

2.4. Test vessels

Fifty milliliter glass test vessels were used for the determination of the surface to volume mortality and 200-ml plastic non-toxic vessels were used for the determination of ATPase enzymatic activities.

2.5. Test organisms

Dry *Artemia* cysts were kindly supplied by the company Inve Hellas. *Artemia* II–III nauplii were hatched, following the experimental protocol of the ARC-toxicity test worked out by Vanhaecke and Persoone (1984).

2.6. Determination of the surface to volume mortality

The determination of the S/V-LC_{50} (24 h) was based on the method of Persoone and Castritsi-Catharios (1989). Ten nauplii were exposed in five replicates in test vessels containing 20 ml seawater, to seven different panel areas coated with the antifouling paint. The surface of the painted panels ranged from 400 to 1000 mm^2. The experiments were carried out in a temperature controlled room at 23 ± 1 °C, under continuous illumination. All the test vessels were placed on a shaker with slow horizontal movement and left there for 24 h. After this period, the number of dead nauplii was counted in each test vessel and the mortality data vs. panel areas were used for the calculation of the S/V-LC_{50} (24 h), at the aid of a Probit Analysis Program (Weber, 1993).

2.7. Determination of ATPase enzymatic activities

The enzymatic assays were based on the method of Cotou et al. (2001) and were carried out subsequent to the mortality experiments. Twelve sets of 100 nauplii were exposed for 24 h to the two coated panels in which less than 50% mortality had been recorded in the previous experiment (i.e. 50 and 100 mm^2 surface) as well as to a non-coated control panel.

For each of the two panels, 2×4 replicates with 100 nauplii each were used for the determination of total ATPase and Mg^{2+}-ATPase. After the exposure period the nauplii of four replicates were pooled and transferred to a sieve with a nylon mesh for rinsing with distilled water to remove excess salts. The nauplii were then put in an Eppendorff tube for homogenization with 0.1 ml of 0.1 M imidazole buffer, pH 7.2. The homogenate was washed twice with 0.1 ml of a solution of 0.8 M NaCl and 0.2 M KCl. The enzymatic reactions were initiated in the first pool of four replicates by addition of 0.2 ml of a solution of 0.125 M imidazole buffer (pH 7.2), 0.025 M $MgCl_2$ and 0.0125 M Na_2ATP. The second pool was given the same treatment and the same reaction solution, supplemented with 0.00125 M ouabain. The two pools were incubated for one hour at 37 °C after which 3 ml of 1.6 N H_2SO_4 was added to stop the reactions. A reagent kit (Merkotest 3331, method without deproteinization) was used for the estimation of the amount of inorganic phosphate set free from ATP. The presence of ouabain, which is a specific inhibitor of Na^+/K^+-ATPase, allows the determination of Mg^{2+}-ATPase activity. The remaining four replicates were also pooled and used for the determination of total protein concentration according to Bradford (1976) with crystalline bovine serum albumin as standard.

ATPase activities are given as μmol PO_4^{3-} per mg protein per hour. The statistical analysis of the results

Table 1
Mortality of *Artemia* nauplii exposed to increasing surfaces of coated panels

Test panel area (S) mm^2	S/V ratio ($V = 20$ ml)	Total number of exposed organisms	Number of dead organisms	% Mortality
400	20	49	16	32.65
500	25	46	28	60.87
600	30	46	27	58.70
700	35	46	40	86.96
800	40	50	42	84.00
900	45	49	47	95.92
1000	50	47	44	93.62
Control	0	24	1	4.17

Table 2
ATPase activities (mean ± sem) estimated in *Artemia* nauplii exposed to two coated panels of different surface and in control organisms

Test panel area (mm^2)	Enzymatic activity (μmol PO_4^{3-}/mg protein/h)	
	Total ATPase	Mg^{2+}-ATPase
Control	41.4 ± 2.3^c	25.5 ± 2.9^c
50	21.9 ± 1.9^a	15.2 ± 0.6^a
% Inhibition	47.0	40.3
100	29.8 ± 6.0^b	21.0 ± 3.4^b
% Inhibition	28.1	17.6

Mean values with different letter in superscript are significantly different ($P < 0.00$).

was performed with the Anova and Multiple Range tests using the statistical package Statgraphics.

3. Results

Table 1 gives the surfaces of the tested coated panels, the surface to volume ratio which this represents in 20 ml seawater, the total number of organisms exposed in each test vessel, the number of dead organisms and the percentage mortality. The S/V-LC_{50} (24 h) calculated from these data was 24.6 mm^2/ml with 21.7–26.9 mm^2/ml as 95% confidence limits.

Total ATPase and Mg^{2+}-ATPase are shown in Table 2, indicating that both enzymatic activities were inhibited by the leaching of chemical compounds from the two coated panels. The inhibition was statistically significant at the 95% confidence level in comparison to the control. In addition, the enzymatic activity of both ATPases differed significantly between the two experimental panels, being higher for the larger panel.

4. Discussion

The copper-based antifouling paint used was found to be substantially toxic to *Artemia* nauplii. The calculated S/V-LC_{50} (24 h) was indeed as low as 24.6 mm^2/ml. The toxic effect of this paint is due to the continuous release of cuprous (Cu^+) ions in the medium of the test vessel. These ions oxidize rapidly in cupric (Cu^{2+}) ions, which is the prevalent form of dissolved copper in the marine environment (Hall and Anderson, 1999). When the Cu^{2+} ions enter in the cells, they are reduced to cuprous ions, which have a great affinity for sulphydryl groups (Viarengo et al., 1996). ATPase enzymes contain such -SH groups which explains the inhibition of their activity. The binding of copper ions to the -SH groups of the enzymes causes structural changes which prevent ion transport (Trieff, 1980; Ay et al., 1999). The slight increase of the two enzymatic activities with the increase of the coated surface of the panels may be due to the activation of repairing mechanisms (Ay et al., 1999) to prevent the damaging of the enzymes from the higher copper concentrations.

Total ATPase activity is the sum of the enzymatic activities of Na^+/K^+-ATPase and Mg^{2+}-ATPase (Machera et al., 1996). Na^+/K^+-ATPase is generally accepted as a membrane pump responsible for the transport of Na^+ and K^+ ions across the cell membrane. In *Artemia* nauplii it serves to regulate the intracellular composition of cells bathed by hemolymph (Ewing et al., 1974; Cotou et al., 2001). Mg^{2+}-ATPase participates in the oxidative phosphorylation and ensures the transport of Mg^{2+} ions across the cell membrane. These ions are important for the integrity of the cell membrane (Cotou et al., 2001). The inhibition of the activity of the ATPases noted in the test vessels containing panels coated with the antifouling paint points to a general failure of osmoregulation in the brine shrimp, which leads to their death when the organisms are exposed to larger coated panels. These findings also shows the potential toxic effects of antifouling paints on marine organisms with osmoregulation similar to that of *Artemia* such as e.g. other crustaceans or teleost fish (Ewing et al., 1974; Holliday et al., 1990).

Considering the results of this study based on the "surface to volume" determination of toxicity of coated surfaces, we believe that the S/V-LC_{50} concept is an appealing method for the determination of the potential toxicity of antifouling paints in combination with biochemical markers such as the Mg^{2+}-ATPase activity proposed by Machera et al. (1996). Furthermore, the parallel decrease in the activity of Na^+/K^+-ATPase enzymes, shown in other studies (Viarengo et al., 1996; Ay et al., 1999) and Mg^{2+}-ATPase demonstrated in the present investigations, could also be used for the identification of toxic stress caused by copper, as proposed by Cotou et al. (2001).

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