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Toxic effects of new antifouling compounds on tunicate haemocytes I. Sea-Nine 211™ and chlorothalonil

Francesca Cima [a], Marcantonio Bragadin [b], Loriano Ballarin [a,*]

[a] *Dipartimento di Biologia, Università di Padova, via Ugo Bassi 58/B, 35131 Padova, Italy*
[b] *Dipartimento di Scienze Ambientali, Università di Venezia, Italy*

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Abstract

After the definitive ban on tin-based antifouling substances, new organic compounds have recently been introduced in antifouling paint formulations, as either principal or booster biocides. In most cases, previous risk assessment of these biocides has been inadequate so that their possible effects on aquatic ecosystems is a matter of great concern.

We studied the effects of two new organic biocides often associated in paint formulations, Sea-Nine 211™ (4,5 dichloro-2-*n*-octyl-4-isothiazoline-3-one) and chlorothalonil (2,4,5,6-tetrachloroisophthalonitrile), on haemocytes of the compound ascidian *Botryllus schlosseri* exposed for 60 min to various concentrations (from 0.1 to 10 μM) of the xenobiotics. This species had previously proved to be a good bioindicator of organotin compounds.

Both compounds, at concentrations of 1 and 10 μM, altered the morphology of phagocytes, and these changes were closely related to disrupting effects on cytoskeletal components. At the same concentrations, phagocytosis, which requires cytoskeletal modifications for pseudopod formation, was severely hindered.

Both compounds were able to induce apoptosis of *Botryllus* blood cells, probably as a consequence of severe oxidative stress related to the reported decrease of intracellular reduced glutathione (GSH) content. In the case of Sea-Nine 211™, a substantial increase in intracellular Ca^{2+} and a negative effect on Ca^{2+}–ATPase activity may also be involved in the activation of the cell death machinery. Cytochrome-*c*-oxidase was also significantly inhibited by the two biocides, indicating perturbation of the mitochondrial respiratory chain. Isodynamic mixtures of Sea-Nine 211™ and chlorothalonil were used to evaluate the occurrence of interactions between the two compounds. Results suggest the combined action of partial additivity when cell-spreading and cytochrome-*c*-oxidase activity were considered, and were indicative of antagonism in the case of the GSH depletion.

On the whole, our results indicate that short-term *in vitro* exposure of haemocytes to high concentrations of Sea-Nine 211™ and chlorothalonil provokes a marked reduction in haemocyte functionality, higher than or comparable to that of TBT. These assays of acute toxicity stress the immunosuppressive potential of these compounds, which, although counterbalanced by their short half-life in the marine environment, can lead to biocoenosis dismantling through rapid bioaccumulation by filter-feeding non-target benthic organisms.

Keywords: *Botryllus*; Haemocytes; Sea-Nine 211™; Chlorothalonil; Phagocytes; Immunotoxicity

1. Introduction

New-generation antifoulants are mainly represented by copper and copper derivatives – Cu_2O, CuSCN – used to replace organotin compounds, but organic and organometallic biocides of new synthesis or coming from either the pharmacology industry – bactericides – or the agriculture – herbicides, fungicides, insecticides – have been recently introduced in paint formulations to increase their performance against a wider spectrum of fouling organisms. As a consequence, more than 10 xenobiotics (Table 1) are now used worldwide as principal and/or booster biocides in antifouling paints for the preservation of submerged structures like boat hulls and helixes, buoys and moles from the settlement of various aquatic sessile organisms (Voulvoulis et al., 1999; Ranke and Jastorff, 2000; Konstantinou and Albanis, 2004). Their efficacy also depends on the type of matrix that is generally represented by rosin – hard matrix paints – or copolymers – self-polishing matrix (Omae, 2003; Yebra

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* Corresponding author. Tel.: +39 049 8276198; fax: +39 049 8276199.
E-mail address: loriano.ballarin@unipd.it (L. Ballarin).

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Table 1
Alternative/booster biocidal substances used in formulations of new antifouling paints at present on the market in EU Countries[a]

Chemical name	Trademark	Other uses
Cu_2O, CuO, CuSCN, Cu	None	Fungicide
Zinc 2-pyridinethiol-oxide	Zinc pyrithione, ZnP	Bactericide, fungicide in antidandruff shampoos
2-Methylthio-4-ter-butylamino-6-cyclopropylamino-s-triazine	Irgarol 1051	Herbicide
4,5-Dichloro-2-*n*-octyl-4-isothiazolin-3-one	Sea-Nine 211, Kathon 5287, C-9	None
3-(3,4-Dichlorophenyl)-1,1-dimethylurea	Diuron, Karmex, Telvar	Herbicide
2,4,5,6-Tetrachloroisophthalonitrile	Chlorothalonil, Bravo, Daconil, Termil, Tuffcide	Fungicide
N-Dimethyl-*N*-phenylsulphamide	Dichlofluanid	Fungicide
2,3,5,6-Tetrachloro-4-(methylsulphonyl)pyridine	TCMS pyridine	Leather preservative
2-[Benzothiozolylthio]methylthiocyanate	TCMTB	Fungicide, wood preservative
Manganous ethylenebis[dithiocarbamate]	Maneb, Manzate, Dithane	Fungicide
bis(Dimethylthiocarbamoyl) disulfide	Thiram, TMTD, Thiurad, Thiosan	Fungicide
Zinc dimethyldithiocarbamate	Ziram, Methasan, Zimate, Zirberk, Karbam White	Fungicide
Zinc ethylenebis[dithiocarbamate]	Zineb, Parzate, Dithane, Z-78	Fungicide
α,β-1,2,3,4,7,7,-Hexachlorobicyclo-[*2.2.1*]-2-heptene-5,6-bisoxymethylene sulfite	Endosulfan, Benzoepin, Thiodan	Insecticide, acaricide

[a] Cima et al. (2004) modified.

et al., 2004). In spite of their increasing use, the previous risk assessment of these biocides has been inadequate. In most of cases, only limited amounts of data are available regarding their toxic effects on marine organisms and on the possible synergic interactions which may enhance their toxicity (Evans et al., 2000; Van Wezel and van Vlaardingen, 2001, 2004; PAN Pesticides Database, http://www.pesticideinfo.org; Toxicology Data Network, http://toxnet.nlm.nih.gov; Extension Toxicology Network, http://extoxnet.orst.edu), so that their possible effects on aquatic ecosystems and organisms are a matter of great concern.

Sea-Nine 211^{TM} and chlorothalonil are two of the most widespread alternative antifoulants, as they have a short half-life (Konstantinou and Albanis, 2004), which minimises their impact on marine life and reduces the risk of bioaccumulation. They are commonly added to paint formulations as principal and/or booster biocides, often associated.

Sea-Nine 211^{TM} (4,5-dichloro-2-*n*-octyl-4-isothiazolinone), also known as Kathon 5287, C-9, and DCOI, was introduced specifically as an antifoulant in 1996 by Rohm & Haas (Philadelphia, PA, USA). It is usually present in commercial paints as a main or booster biocide at concentrations of 1–3% and is registered as highly toxic by U.S. EPA Pesticide Production Information System since 2002. Until now, its use has not been regulated in the world, with the exception of United States (Compiled List of U.S. Registered Active Ingredients, Pesticide Action Network North America, February 12, 2005), Australia (Record of Approved Active Constituents for Chemical Products, National Registration Authority for Agricultural and Veterinary Chemicals (NRA), June 12, 2001), and New Zealand (Agricultural Compounds and Veterinary Medicines (ACVM) Database on Currently Registered Pesticides, Ministry of Agriculture and the Environment, June 17, 2002). Since it has been introduced quite recently into the environment, few studies have been carried out on its fate and degradation rate in seawater. Although stable when inside the paint matrix, it has half-lives in sterile buffered aqueous solutions at pH 5, 7, and 9 of 216 h, >720 h, and 288 h, respectively (Shade et al., 1994), but is quickly degraded by microorganisms, with a half-life of less than 1 h in the aquatic environment and about 24 h in sediments, independently of the presence of oxygen (Jacobson and Willingham, 2000). Once degraded, its metabolites are about 100,000 times less toxic than the original compound (Jacobson and Williams, 2000). Its toxic effects, although poorly documented, are directed towards a wide variety of fouling organisms, such as bacteria (Fernàndez-Alba et al., 2002), fungi, algae (Miller and Lovegrove, 1980; Vasishtha et al., 1995; De Nys et al., 1996; Burridge and Gorski, 1998; Willemsen et al., 1998), briozoans, tunicates and diatoms (Harrington and Willingham, 1993), within a concentration range of 1–30 μg/l (Ranke and Jastorff, 2000). Table 2 reports the principal toxicity studies on marine animals. Until now, studies of the *in vitro* toxicity of this compound have only been performed on fish cells (Okamura et al., 2002) and rat liver mitochondria (Bragadin et al., 2005).

Chlorothalonil (2,4,5,6-tetrachloro-1,3 benzenedicarbonitrile or 2,4,5,6-tetrachloroisophthalonitrile), also known with various trade names including Bravo, Clorthalonil, DAC-2787, Daconil 2787, Echo, Exotherm Termil, Forturf, Mold-Ex, Nopcocide N-96, Ole, Pillarich, Repulse, Sweep, Tuffcide, Vanox, is an aromatic halogen compound, a member of the chloronitrile chemical family. It is present in antifouling paints, mainly as a booster biocide, but, differently from Sea-Nine 211^{TM}, it has been first in use as a broad-spectrum organochlorine fungicide in agriculture, silviculture, urban settings, originally against *Alternaria solani*. It is also employed in other industrial applications as a preservative for paints and adhesives. Therefore, its presence in seawater is partly due to soil leaching by drainage waters from inland areas. It was commercially introduced in 1969 as a General Use Pesticide (GUP) classified by the U.S. EPA in the toxicity "class II - moderately toxic", due to its potential for eye irritation. Until 1998, it was the major compound used on lawns and crops in both United States and Canada. How-