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Immunotoxicity in Ascidians: Antifouling Compounds Alternative to Organotins—V. the Case of Dichlofluanid

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Abstract: Dichlofluanid has long been employed as a fungicide in agriculture and has been massively introduced in antifouling paints for boat hulls over the last two decades. One of the most important toxic effects of antifoulants is represented by immunosuppression in marine invertebrates, which can be analysed in vitro with a number of short-term toxicity assays on haemocytes. Among bioindicators, the colonial ascidian *Botryllus schlosseri* is a useful candidate; it is a filter-feeding organism living in the water-sediment interface that is found worldwide and is sensitive to antifouling xenobiotics. Dichlofluanid adversely affects both immunocyte lines (phagocyte and cytotoxic lines) after exposure to sublethal concentrations. At 0.05 μM (16.65 μg/L), dichlofluanid induced haemocyte apoptosis and cell shrinkage with a decrease in both motility and phagocytosis. At the lowest concentration (0.01 μM, 3.33 μg/L), inhibition of pivotal enzymatic activities of phagocytes and cytotoxic cells occurred. At the highest concentration (0.1 μM, 33.3 μg/L), dichlofluanid increased glutathione oxidation, leading to stress conditions. The effects of dichlofluanid on immune defence responses are similar to those of organometal-based antifoulants (i.e., organotin compounds and zinc pyrithione), and its use in coastal areas requires attention.

Keywords: ascidians; antifouling paints; *Botryllus*; dichlofluanid; haemocytes; immunotoxicity

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1. Introduction

Fouling consists of a community of organisms that have settled and grown on natural and artificial hard surfaces submerged for a long period of time in aquatic environments [1–4]. Concretions can reach a thickness of centimetres and change the strength and solidity of a structure. In the case of boats, this solid accumulation represents a severe problem because even a millimetre of bacterial-algal film, namely, a biofilm, can cause a decrease in speed of up to 80% due to increased friction, resulting in an increase of 17% in terms of fuel consumption, which, in turn, contributes to global climate change. In addition, the problem of hull erosion caused by these organisms forces shipowners to more frequently clean their boats in storage docks, raising maintenance costs that have been estimated to be approximately 5.7 trillion dollars per year [5–7]. Since the 1960s, slow-release antifouling paints have been used worldwide, which appeared to be particularly efficient against the most frequently target organisms of fouling on boats, such as macroalgae, serpulids, barnacles and molluscs. From 1988 to 1993, an exponential increase in the use of paints occurred, in turn increasing the concentrations of various biocides in aquatic ecosystems without corresponding monitoring of the effects or assessments of environmental risks [8]. In the document "Pesticides 1998" from the Health and Safety Executive of the Pesticides Safety Directorate, 600 different antifouling mixtures were recorded, including 60 active ingredients represented by both main antifoulants, such as organotin and copper compounds, and

booster antifoulants, which are often used to increase the spectrum of action synergistically. After the international ban of organotin compounds such as tributyltin (TBT) and triphenyltin (TPT) by the International Maritime Organization (IMO) beginning from 2003, the number of paint formulations decreased, but new biocides have been introduced. A number of compounds have been specifically formulated as eco-friendly antifouling substances (i.e., Sea-Nine 211), but other compounds have been taken from uses in other human activities, such as fungicides (CuSCN, dichlofluanid, maneb, TCMTB, thiram, zineb, ziram), herbicides (diuron, Irgarol 1051) and insecticides (endosulfan) from agriculture. More examples include bactericides, anti-mould and anti-dandruff compounds from the polymer (chlorothalonil), leather (TCMS pyridine) and cosmetic-pharmaceutical (zinc pyrithione) industries, respectively [8]. Although these antifouling agents are less effective in comparison to organotin compounds—the average duration of these antifouling paints on boats is 2 years compared to 5 for the TBT-based paints—many of these compounds are of environmental concern for their potential widespread toxicity from different sources. High concentrations of biocides were measured in European estuarine and coastal areas, especially where intense nautical activity and low water turnover occurred, the latter of which increased the persistence in the coastal environments of these compounds [9–12]. However, concentrations measured in the coastal environments of many of these substances cannot be solely attributed to their antifouling use. Indeed, owing to agricultural use, they can easily reach the marine environment through leaching, which depends on the amount of precipitation during the period of massive use of pesticides in crops [13].

The fate of these biocides and the bioavailability and bioaccumulation rates throughout the food chain of marine ecosystems are closely linked to their breakdown velocity between water and sediments, since adsorption reduces the biocide concentration in the water column and represents the main pathway of accumulation in sediments [14–16]. Moreover, the mixture of various biocides present in antifouling formulations potentially causes unknown combined toxic effects. The study of the interactions of individual paint components on biological systems is therefore of great importance for discovering toxic effects, mechanisms of action and acceptable limit values according to the EU Water Framework Directive 2000/60/EC. This represents the first phase, together with a constant monitoring action, for the development of safeguard planning for fragile ecosystems.

Dichlofluanid (*N*-{[dichloro(fluoro)methyl]sulphanyl}-*N*′, *N*′-dimethyl-*N*-phenylsulphuric diamine, CAS registry number: 1085-98-9, molecular formula: $C_9H_{11}C_{l2}FN_2O_2S_2$, molar mass: 333.22 g/mol) is a biocide belonging to the sulphamide group that has been massively (>20% [17]) introduced over the last decade into a number of new antifouling paint formulations for boat hulls. As reported in the PubChem database (https://pubchem.ncbi.nlm.nih.gov/), it is a fungicide, used in agriculture beginning from 1965 as a potent inhibitor of spore germination; it is active via leaf and fruit contact against different cryptogams and particularly effective for pome defence from rust, brown spots, scabs, *Gloeosporium* sp., and various agents that cause storage diseases, such as *Botrytis* sp. and *Alternaria* sp. Although this compound has recently been without regulatory approval for use in agriculture in the EU according to EC Regulation 1107/2009 for its high toxicity in birds, earthworms and honeybees, it is still widely employed in various countries; e.g., in Australia. Regarding aquatic life, its estimated bioconcentration factor (BCF) of 72 (±14) in fish (*Lepomis macrochirus*) and its high octanol/water partition coefficient (3.7 at pH 7.0) suggest that dichlofluanid tends to associate with particulate matter [18], forming strong bonds with clay sediments rather than sandy sediments [19], and the adsorption increases as the pH increases with a releasing factor from the polluted sediments corresponding to less than 1% [20]. For these reasons, it may moderately bioconcentrate in aquatic organisms [21]. It is highly unstable both in the water phase and in the soil, where it rapidly undergoes hydrolysis and photodegradation [22–25], resulting in the derivatives *N*, *N*-dimethyl-*N*′-phenyl-sulphanilamide (DMSA), dichlorofluoromethane, *N*-(dichlorofluoromethylthio) aniline and aniline, but it is more persistent in marine sediments (Figure 1). The principal degradation product, DMSA (CAS registry number: 4710-17-2), has very low toxicity in the aquatic environment [26,27]. Although biocidal products, including antifouling paints, are regulated under the EU Biocidal Products Directive "BPD" 98/8/EC in Europe and the use

of dichlofluanid as an antifouling agent ("Product-type 21") is still approved by the EU regulation number 528/2012, ECHA/BPC/120/2016, restrictions on dichlofluanid-based paints show a fragmented patchwork of national directives. Only Sweden, Denmark and The Netherlands do not permit its use on boats <25 m [28].

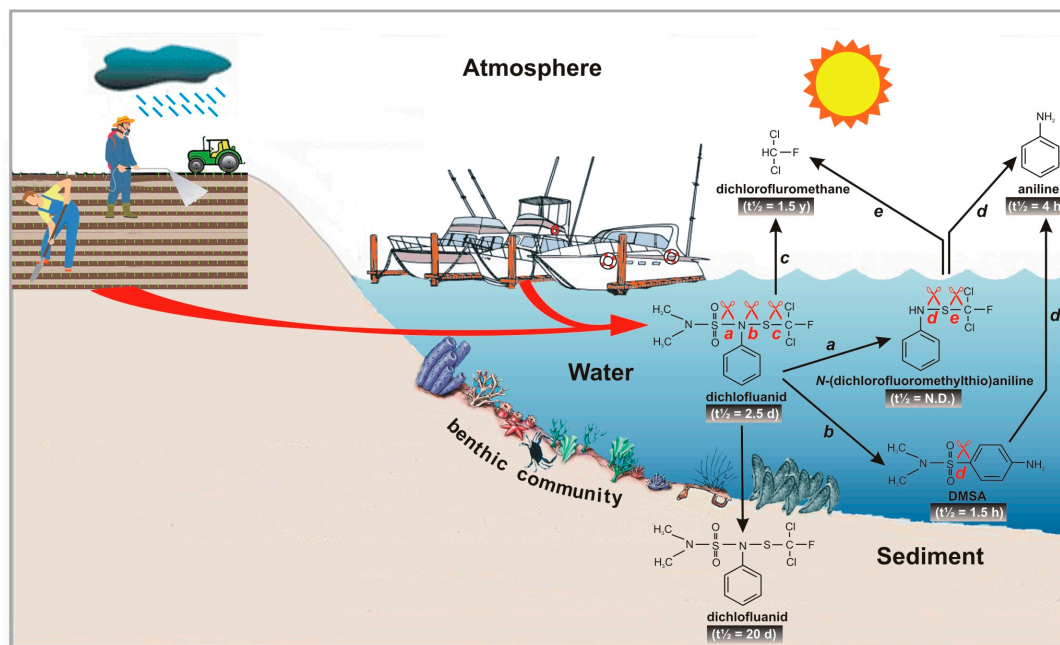

Figure 1. The main sources of environmental pollution of dichlofluanid (in agriculture as a fungicide and from boats as an antifoulant) and the biogeochemical fate of dichlofluanid and its derivatives. Black arrows show the degradation pathways by photodegradation, hydrolysis and biotic and abiotic anaerobic processes in seawater, sediments and air. Half-times are indicated below each product (N.D.—not determined). Bond cleavages are represented by shears and letters. Note the rearrangement of the *N-N*-dimethyl sulphonyl group in the *para* position after bond cleavage from dichlofluanid to DMSA.

The concentration of dichlofluanid in the coastal waters of Greece (<284 ng/L, corresponding to 0.85 nm, [29]) is lower than that measured in Spain (600 ng/L, corresponding to 1.8 nm, [30]). In England, the water column was found to be pollutant-free, unlike the sediments, which showed high concentrations ranging from <0.1 to 688.2 g/kg [31]. The environmental fate in marine ecosystems and the bioaccumulation potential in trophic networks remain unknown; therefore, the massive use of this compound requires attention. The concentration in the water column is also subject to fluctuations. It is higher during the months following the painting of the hulls and during the nautical season, and shows a decrease in the laying up period, since a significant amount of biocide remains in the sediments [31–33]. Toxicity data for aquatic species reported in EU regulation number 528/2012, ECHA/BPC/120/2016 only regards freshwater fish and invertebrates. It must be considered that the presence of this compound in the coastal sediments and water column could expose benthic marine animals to stress conditions, particularly their immune system, which first undergoes alterations that limit the defence capabilities against microorganisms and other xenobiotic compounds, resulting in both a decrease in survival capacity and long-term negative effects on coastal communities.

The colonial ascidian *Botryllus schlosseri*, a benthic filter-feeding organism widely represented and often dominant in the hard-substratum community of temperate areas, such as the Lagoon of Venice [34], is a good bioindicator that lives at the water/sediment interface, and has been indicated to be sensitive in in vitro immunotoxicity studies to various biocides, such as organotin compounds [35,36], Sea-Nine 211, chlorothalonil [37], diuron, TCMS pyridine [38], copper(I), Irgarol 1051 [39] and zinc pyrithione [40]. To highlight the potential immunotoxic effects, short-term cultures of haemocytes

exposed to various concentrations of dichlofluanid were carried out. Effects on two immunocyte cell lines present in the haemolymph, represented by cytotoxic cells and phagocytes [41], were described through the analysis of results (toxicity indexes) from a series of functional assays considering the lowest observed effect concentration (LOEC) values as endpoints, i.e., the lowest concentrations of a substance that had a statistically significant adverse effect.

2. Materials and Methods

2.1. Animals

Colonies of *B. schlosseri* were collected in the Lagoon of Venice, transferred to 5 × 5 cm glass slides and reared in an aerated aquarium filled with filtered seawater (FSW), which was changed every other day. They were kept in thermostatic rooms at 19 °C and fed with Microbe-Lift®/Phyto-Plus B (Ecological Laboratories, Inc., Cape Coral, FL, USA) and microalgae (*Isochrysis galbana*).

2.2. Biocide

Dichlofluanid was purchased from Merck (Pestanal® 45433) as a white powder that is insoluble in water (1.3 mg/L) and soluble in organic solvents (200,000 mg/L) at 20 °C. Therefore, a stable stock solution (10 mM, 3.33 g/L) was obtained by dissolving the powder in 95% ethanol and storing at room temperature in the dark. Working sublethal solutions employed in acute toxicity assays, i.e., 0.01 μM (3.33 μg/L), 0.05 μM (16.65 μg/L) and 0.1 μM (33.3 μg/L), were obtained by diluting the stock solution in FSW (25 °C, pH 8.1, 35 psu). In controls, dichlofluanid was omitted, and 0.01% of 95% ethanol was added to the FSW.

2.3. Haemocyte Cultures

Haemolymph was collected with a glass micropipette from the marginal vessels of the colonial tunic with a thin needle of tungsten in the presence of 0.38% sodium citrate in FSW at pH 7.5 as an anticoagulant solution to prevent haemocyte clotting and then transferred into a 1.5 mL Eppendorf tube. The cell suspension was centrifuged at 780× *g* for 10 min, and the pellet was resuspended and diluted in FSW to obtain a final suspension of 10^7 cells/mL. In the culture chambers, which were formed by a Teflon ring (15 mm in diameter and 1 mm thick) glued with silicone sealant on a glass slide, 60 μL of the haemocyte suspension was placed, and a thin layer of solid Vaseline was smeared on the surface of the Teflon ring to hold a coverslip that closed the chamber. Then, the culture chambers were turned upside down for 30 min to allow the cells to adhere to the coverslip surface.

In all assays with biocide, the exposure time of the short-term cultures was 60 min, according to previous experiments with other antifoulants. The toxicity assays briefly described in this paper were performed following methods and procedures reported in detail elsewhere [41–43].

2.4. Trypan Blue Exclusion Test for LC_{50} Evaluation

This assay was employed to determine the median lethal concentration (LC_{50}), i.e., the compound concentration that is lethal for 50% of the cultured cells exposed for the experimental time. After adhesion, haemocyte monolayers were incubated with 10, 25, 50, 75, 100, 150 or 250 μM (3332, 8330, 16,660, 24,990, 33,320, 49,980, or 83,300 μg/L, respectively) dichlofluanid. At the end of exposure, the contaminant was eliminated by 1 or 2 washes with FSW, and the haemocytes were incubated for 5 min with 0.25% trypan blue in FSW. This vital dye is excluded from functional/vital haemocytes but is retained in the cytoplasm of any senescent/dead haemocyte with altered plasmalemma permeability. Observations, counting and micrographs of the haemocytes were performed directly on the culture chambers with an Olympus CX31 light microscope (LM) equipped with a DV Lumenera Infinity 2 and Infinity Capture Application software version 5.0.0 (Lumenera Co. 2002–2009). The percentage of dead haemocytes was determined by counting the number of blue-coloured cells; i.e., those unable to exclude the dye from the cytoplasm, on the total number of cells. For the LC_{50} estimate, the probit

method (SPSS 11.0, SPSS Corp., Chicago, IL, USA) was performed. On the basis of the results obtained, the concentrations used in the subsequent acute toxicity experiments can be considered sublethal.

2.5. Cell Functional Assays

2.5.1. Cell Adhesion Assay

After adhesion to the coverslips, the haemocyte monolayers were incubated with dichlofluanid, and after washing thoroughly with FSW, were immediately observed with a LM to determine the number of living haemocytes able to adhere firmly even after exposure to the contaminant (adhesion index) and compared with the cells counted in absence of contaminant and adhering on poly-L-lysine-coated (50 μg/mL, Sigma, Hong Kong, China) slides used as reference controls (100% adhesion).

2.5.2. Cell Spreading Index

After adhesion, the monolayers were incubated with various concentrations of dichlofluanid, and then washed in FSW. The haemocytes were fixed in a solution of 1% glutaraldehyde plus 1% sucrose in FSW at 4 °C for 30 min, washed for 10 min with 0.1 M phosphate-buffered saline (PBS: NaCl 8 g/L, KCl 0.2 g/L, KH_2PO_4 0.2 g/L, Na_2HPO_4 1.15 g/L, pH 7.2), stained with a 10% aqueous solution of Giemsa dye (Fluka) for 5 min and finally mounted with Acquovitrex (Carlo Erba) on glass slides for observation with a LM to count the percentage of cells with an amoeboid shape on the total number of cells.

2.5.3. Phagocytosis Index

After adhesion, haemocyte monolayers were exposed to a yeast suspension (*Saccharomyces cerevisiae*; yeast: haemocyte ratio of 10:1) in FSW containing various concentrations of the contaminant. After incubation for 60 min at 25 °C, the slides were washed 4-5 times with renewed FSW to remove the non-phagocytised yeast cells; haemocytes were glutaraldehyde-fixed, stained with Giemsa dye as described above and finally observed with a LM. The percentage of haemocytes with engulfed yeast cells with respect to the total was counted.

2.5.4. Apoptotic Index

Early apoptosis was detected with annexin-V, a protein that has a high affinity for the phospholipid phosphatidylserine, which is exposed on the outer side of the plasmalemma when the cell begins apoptosis. After the haemocytes adhered to the coverslips and were exposed to various concentrations of the contaminant, living monolayers were incubated in the dark for 15 min in 60 μL of solution from an Annexin-V-FLUOS staining kit (Sigma-Aldrich/Roche), obtained by adding 20 μL of annexin-V conjugated with fluorescein isothiocyanate (FITC) to 1 mL of FSW. After two washes with FSW, the monolayers were mounted with FluorSave^{TM} (Calbiochem), a reagent that slows fluorescence fading, and living cells were immediately observed under a blue light excitation source (450–490 nm) from the LM equipped with an LED fluorescence module Amplified by Fluorescence Excitation of Radiation Transmitted (AFTER, Fraen Corp. s.r.l., Milan, Italy). The plasma membranes with exposed phosphatidylserine appeared with green fluorescence. The early apoptosis index was determined by counting the percentage of cells with the green-fluorescent plasma membrane compared to the total number of cells in the clear field.

Late apoptosis was detected with the TUNEL reaction, which labels fragmented DNA within the nuclei. After adhesion and exposure to various concentrations of the contaminant, haemocytes were fixed in a 4% paraformaldehyde solution containing 0.1% glutaraldehyde, 1.7% NaCl and 1% sucrose for 30 min at 4 °C, washed for 10 min in PBS and incubated in methanol plus 5% H_2O_2 for 30 min at room temperature to block endogenous peroxidase. The monolayers were washed with PBS for 10 min and then permeabilised with 0.1% Triton X-100 in 0.1% sodium citrate for 2 min at 4 °C. They were then washed with PBS two times for 2 min each and incubated for 60 min at 37 °C in the in

situ Cell Death Detection, POD kit (Sigma-Aldrich/Roche), containing FITC-labelled dUTP nucleotides and deoxynucleotidyl transferase (TdT). By means of TdT, labelled nucleotides bind to broken DNA strands at the free 3′-OH ends, which are as much numerous as DNA fragmentation has occurred in apoptotic nuclei. At the end of incubation, the monolayers were washed three times with PBS, and the fluorescent signal was converted by incubating the haemocytes for 30 min at 37 °C with sheep anti-FITC antibody Fab fragments conjugated with horseradish peroxidase (CONVERTER-POD) provided by the same kit. The monolayers were finally washed three times with PBS and incubated for 10 min at room temperature in the peroxidase substrate, represented by 5 mg of 3-3′-diaminobenzidine (DAB, Sigma) dissolved in 200 µL of dimethyl sulphoxide plus 5 µL of H_2O_2 in 10 mL of PBS. The percentage of haemocytes with brown-coloured nuclei compared to the total describes the late apoptosis index.

2.6. Cytochemical and Cytoenzymatic Assays

2.6.1. Glutathione Content

After adhesion and exposure to various concentrations of the contaminant, living haemocytes were washed with FSW and then incubated for 10 min at 37 °C in a 40 µM chlorobimane (Sigma-Aldrich) solution in FSW, which was obtained from a 20 mM stock solution in 95% ethanol. Chlorobimane has a high affinity for reduced glutathione (GSH) [44]. After at least two washes with FSW to completely eliminate the background fluorescence of the fluorochrome not bound to GSH in the cell cytoplasm, haemocytes were immediately observed with a LM under a UV excitation source (365 nm). In the assay, the positive haemocytes appeared with bright blue fluorescence in the dark field, and the content of GSH was obtained by calculating the percentage of fluorescent cells compared to the total, the latter of which were counted in the clear field.

2.6.2. Phenoloxidase

Among oxidative enzyme activities, phenoloxidase was chosen as the enzyme typical of the cytotoxic line. After adhesion and exposure to the contaminant, the monolayers were glutaraldehyde-fixed and then washed with PBS. They were incubated for 60 min at 37 °C in a saturated solution of β-(3,4-dihydroxyphenyl)-L-alanine (L-DOPA, Sigma-Aldrich) in PBS, washed with distilled water and mounted with Acquovitrex to be observed with the LM. The enzymatic index corresponds to the fraction of haemocytes with positive sites stained brown-black compared to the total.

2.6.3. Acid Phosphatase

Among the hydrolytic enzymes, acid phosphatase was chosen for its presence in the lysosomes of the phagocytic line. After adhesion, exposure to the contaminant and glutaraldehyde fixation, a simultaneous azo-coupling detection method was used to detect enzymatic activity. Monolayers were kept for 60 min at 37 °C in 0.05% naphthol AS-BI phosphate (Sigma-Aldrich), previously dissolved in dimethylformamide, in buffered hexazonium-p-rosaniline (0.1 M Na-acetate buffer, pH 5.2). After incubation, several washes with distilled water were carried out, followed by mounting with Acquovitrex for observation with the LM. The enzymatic index was calculated by counting the percentage of cells with positive sites stained red compared to the total number of cells.

2.7. Statistical Analysis

Each experiment was replicated three times (n = 3), and the results are expressed as the averages ± SD. According to the various assays, the number (adhesion index) or the percentage (other assays) of positive cells was estimated by counting the haemocytes (at least 200 cells for each monolayer) in 10 optic fields at a magnification of 1000 × (0.21 mm viewfield diameter). Data of the treated and control haemocytes were compared using one-way ANOVA followed by Dunnett's test for multiple comparisons with DSAASTAT v. 1.1 2011 [45]. Differences were considered statistically significant when $p < 0.05$.

3. Results

3.1. Effects on Cell Viability and Morphology

On the basis of the viability assays performed with increasing concentrations of dichlofluanid, the LC_{50} value was not obtained because was >250 µM, a concentration beyond which the corresponding high amount (2.5%) of 95% ethanol as a solvent could interfere with the responses of the haemocytes in the functional assays. The maximum concentration used in all toxicity assays was 0.1 µM. At this concentration, dichlofluanid did not seem to cause detachment of the various types of haemocytes (Figure 2) from the substrate or their cytolysis, since the number of adhering cells per coverslip did not significantly decrease (Figure 3a).

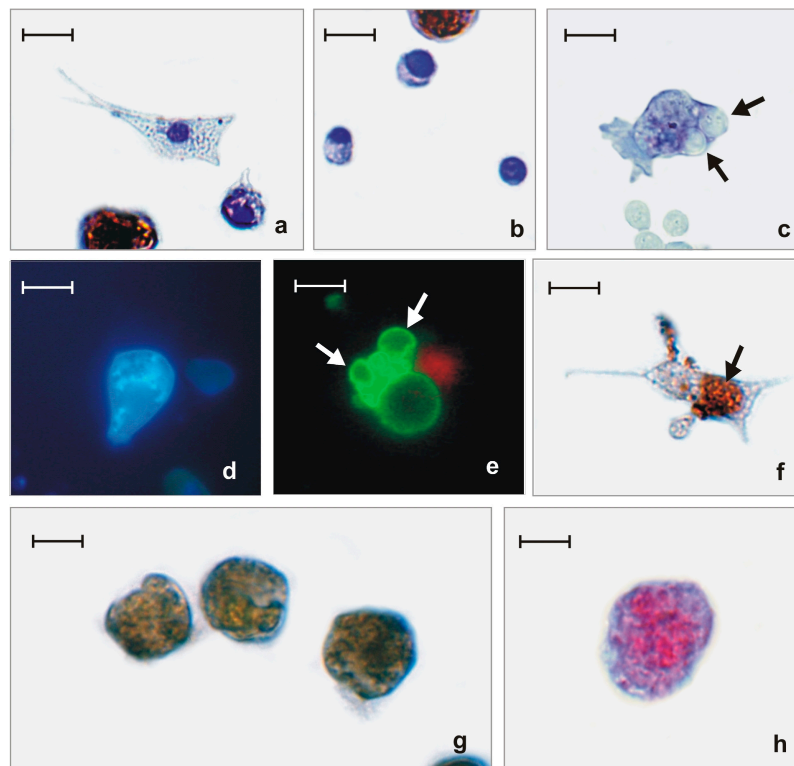

Figure 2. Haemocytes of *Botryllus schlosseri*. (**a**) Glutaraldehyde-fixed phagocyte stained with Giemsa dye showing long pseudopodia. (**b**) Three cells of the same type after exposure to 0.1 µM (33.3 µg/L) dichlofluanid. Note the cell shrinkage and the roundish shape due to the loss of pseudopodia, indicating damage to both the pump proteins and cytoskeleton, respectively. (**c**) Fixed large phagocyte stained with Giemsa dye showing yeast cells engulfed inside phagosomes (*arrows*) after the phagocytosis assay; a cluster of yeast cells is present below. (**d**) Untreated living phagocyte containing GSH in the cytoplasm. (**e**) Living phagocyte positive to the annexin-V reaction after exposure to 0.05 µM (16.6 µg/mL) dichlofluanid. Note the cytoplasmic protrusions, namely, blebs, which are a typical feature of early apoptosis (*arrows*). (**f**) Glutaraldehyde-fixed phagocyte positive to the TUNEL reaction after exposure to 0.05 µM (16.6 µg/mL) dichlofluanid. Note the brown labelled nucleus typical of late apoptosis with DNA fragmentation (*arrow*). (**g**) Glutaraldehyde-fixed cytotoxic cells (morula cells) positive for phenoloxidase activity. (**h**) Glutaraldehyde-fixed large phagocyte with vacuoles positive for acid phosphatase. Bar length: 5 µm in (**a**,**b**); 3.5 µm in (**c**); 10.5 µm in (**d**); 11.5 µm in (**e**); 7 µm in (**f**); 4 µm in (**g**); and 3 µm in (**h**).

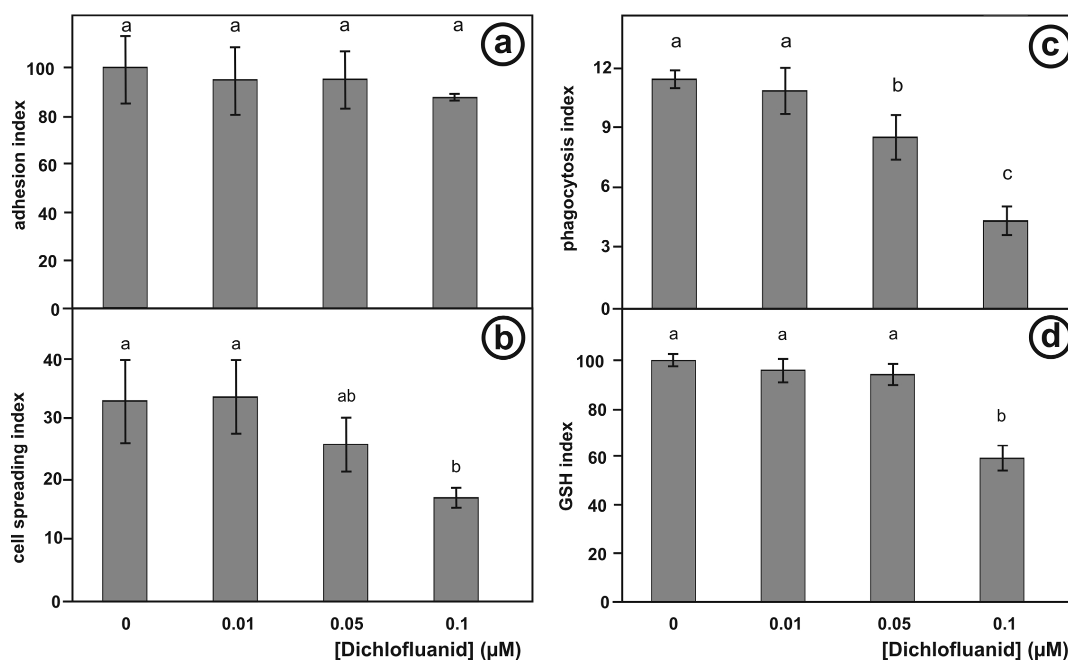

Figure 3. Adhesion index (**a**), cell spreading index (**b**), phagocytosis index (**c**) and GSH index (**d**) in the presence of various concentrations of dichlofluanid. Control (0) refers to the absence of dichlofluanid. The results are expressed, in the case of the adhesion index, as the number of living haemocytes adhering to the substrate after exposure to the dichlofluanid, and in the other cases, as the mean percentage of cells with amoeboid shape (cell spreading index), containing engulfed yeast cells (phagocytosis index) and positive staining (GSH index), ± SD (n = 3) compared to the total number of cells. Significant ($p < 0.05$) differences are expressed with different letters.

The cells of the phagocytic line are motile and particularly active when it comes to non-self recognition and uptake. Therefore, the phagocytes show varied morphologies (Figure 2a) characterised by the presence of numerous pseudopods that are involved in amoeboid movements, allowing these cells to rapidly move for chemotaxis on substrates and engulf the foreign material. After exposure to dichlofluanid, a significant decrease in the percentage of amoeboid phagocytes was observed at a concentration of 0.05 μM (Figure 3b). The phagocytes that suffered from the presence of the contaminant in the medium underwent volume shrinkage and withdrew their pseudopods, resulting in a spherical motionless shape (Figure 2b). The phenomenon is irreversible because even after repeated washes with FSW, the cells did not recover their original morphology.

3.2. *Effects on Phagocytosis*

The phagocytosis index represents the ability of cells to phagocytise target particles *in vitro*. This parameter is closely related to cell morphology and chemotactic ability. Following the ingestion of yeast cells, the phagocytes increased in size, acquired a spherical shape and contained numerous large phagosomes with the ingested yeast cells (Figure 2c). In the controls, the maximum yeast uptake was reached after 60 min (the average phagocytosis index was 11 ± 0.4), but in the assays with dichlofluanid, the percentage of cells that actually phagocytised the target particles significantly decreased beginning from a concentration of 0.05 μM (the average phagocytosis index was 8 ± 1.03) (Figure 3c).

3.3. *Effects on the GSH Content*

Exposure to dichlofluanid resulted in a significant decrease in the number of chlorobimane-stained haemocytes (Figure 2d), suggesting that the contaminant altered the oxidation state of the cytoplasmic content of GSH beginning from the highest concentration employed (0.1 μM) (Figure 3d).

3.4. Apoptosis Induction

Dichlofluanid showed an inductive role in apoptosis, which was revealed through phosphatidylserine exposure on the outside of the plasmalemma in haemocytes treated in the affinity assay for annexin-V (Figure 2e), beginning from the lowest concentration of contaminant employed (0.01 μM) (Figure 4a). At this concentration, the haemocytes also developed spheroid cytoplasmic protrusions, namely, apoptotic blebs (Figure 2e).

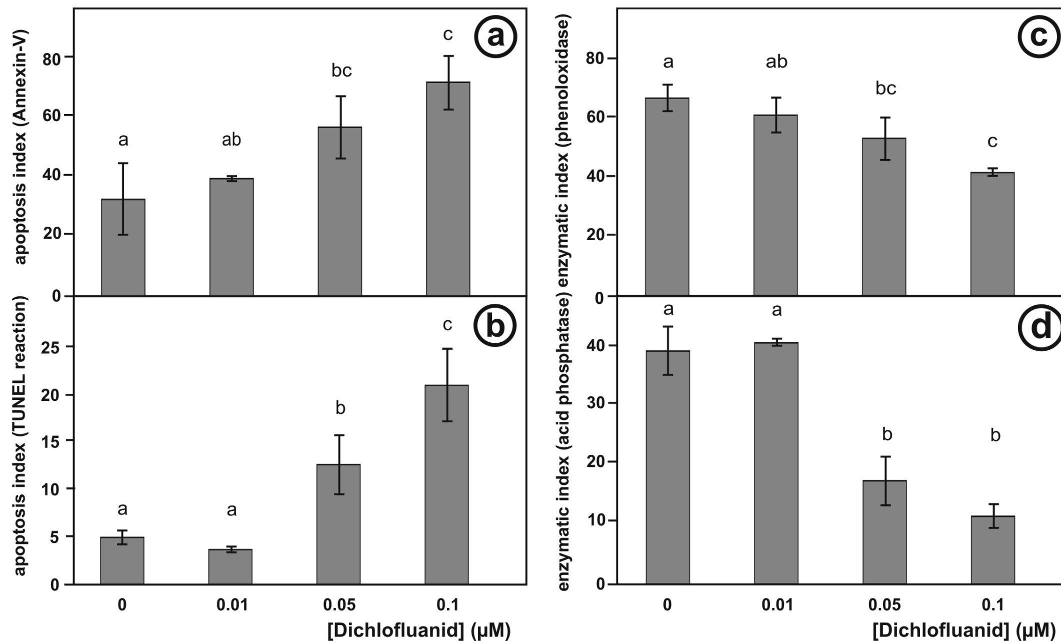

Figure 4. Indexes of early (**a**) and late (**b**) apoptosis evaluated with the annexin-V assay and TUNEL reaction, respectively, and enzymatic indexes of phenoloxidase (**c**) and acid phosphatase (**d**) activities of the cytotoxic and phagocytic lines, respectively, in the presence of various concentrations of dichlofluanid. Control (0) refers to the absence of dichlofluanid. The results are expressed as the mean percentage of cells with positive staining ± SD (n = 3) compared to the total number of cells. Significant ($p < 0.05$) differences are expressed with different letters.

In the TUNEL reaction assay, extensive damage to chromatin and DNA cleavage into oligonucleosomal-length DNA fragments was investigated (Figure 2f). In this case, the reaction revealed a significant increase in the number of apoptotic cells with fragmented DNA in the nucleus after exposure to 0.05 μM dichlofluanid (Figure 4b).

3.5. Effects on Enzymatic Activities Involved in Immune responses

The activity of phenoloxidase is typical of the cytotoxic line of circulating haemocytes in the ascidian haemolymph. In the vacuoles of morula cells of *B. schlosseri*, it was revealed through the transformation in melanin of L-DOPA, used as an endogenous substrate, as a brown-black product of the reaction inside positive cells (Figure 2g). In the morula cells exposed to dichlofluanid, considerable alteration of the activity of this enzyme was detected as a significant decrease in the enzymatic index from the lowest concentration of dichlofluanid (0.01 μM) employed (Figure 4c).

For the hydrolytic activity of the phagocytic line, acid phosphatase activity was chosen, which is well represented in both lysosomes and phagosomes (Figure 2h). The enzyme index showed a significant decrease after exposure to concentrations of 0.05 μM dichlofluanid (Figure 4d).

4. Discussion

With the introduction of new antifouling compounds in paint formulations for the bottoms and hulls of boats, the study of their possible effects on the marine environment developed at the same rate in the last three decades. Ecological interest is based on the fact that the survival and reproductive capacities of animals are closely dependent on the health of their immune system. For this reason, environmental ecotoxicological studies are often focused on immunotoxicity assays to understand the mechanisms of action of xenobiotics at the molecular, cellular and systemic levels. Since the 1990s, in vitro toxicity assays have been used on haemocytes of marine invertebrate species [46–55], in which useful sentinel species, namely, bioindicators, emerged. They are mainly represented by organisms that are most affected by changes in environmental conditions, the functional responses of which are closely indicative of environmental quality [56].

As a filter-feeding benthic organism living at the water column-sediment interface, the colonial ascidian *B. schlosseri* has proven to be particularly sensitive to immunotoxicity assays with organotin compounds, and after their retirement from the market, to a number of alternative antifouling compounds. This study completes the series of immunotoxicity analyses of such biocides with dichlofluanid by evaluating the effects of this xenobiotic on some functional parameters of haemocytes. Cell viability, adhesion to the substrate, phagocytosis, oxidative stress, enzymatic activities, etc., are considered important biomarkers for assessing the alterations in immune responses and determining the toxicities of pollutants and their interactions at both the cellular and subcellular levels [57].

Scarce data in the literature suggest moderately toxic activity of dichlofluanid since LC_{50} values between 4800 and 8200 g/L (14 and 25 μM, respectively) have been reported in 48-h exposure assays of four species of shellfish [58]. At first notice, dichlofluanid appears to confirm its low toxicity with regard to the observed effects on the viability of *B. schlosseri* haemocytes. As with other biocides previously tested on this species, such as diuron, TCMS pyridine [38] and Irgarol 1051 [39], the LC_{50} value was not established. Dichlofluanid has an LC_{50} that is higher than the concentrations found in polluted areas, and the concentrations used in the ecotoxicological assays on haemocytes can be considered sublethal. Nevertheless, it must be considered that this low lethality does not exclude the ability of dichlofluanid to cause important physiological effects on adaptive responses; knowledge of the mechanism of action of pollutants in living organisms remains indispensable for developing descriptive and predictive models in ecotoxicology and for appropriate risk assessment [59].

Although dichlofluanid causes severe alterations in the cytoskeleton supported by the irreversible withdrawal of pseudopods and the spherical shape of the cells, it does not interact with focal adhesions to the substrate since the cells do not detach. Similar behaviours are shared with other antifouling compounds, such as organotin compounds [60,61], Sea-Nine 211 and chlorothalonil [37], diuron, TCMS pyridine [38] and Irgarol 1051 [39]. Cytoskeletal alterations immobilise haemocytes and negatively affect many functional activities of immune cells, such as chemotaxis [62,63] and phagocytosis [64–66], potentially resulting in immunosuppression [67,68]. It is therefore likely that, also in this case, the effects observed on yeast phagocytosis might be due to a limitation in cell motility as a result of contaminant interactions with cytoskeleton proteins.

In addition, the remarkable cell shrinkage observed could be related to an alteration of the osmotic control systems of the cell resulting in water loss from both the cytoplasm and the internal compartments. This is often one of the first events that occur when a cell starts apoptosis [69]. The latter initially reveals an alteration of integrity and permeability of the plasma membrane, followed by a decrease in the size of the cell and condensation of the nuclear DNA. Like all other antifoulants previously assayed in vitro on *B. schlosseri* haemocytes, dichlofluanid is also able to induce apoptosis at low concentrations following the typical morphological changes of this event, such as the initial exposure of phosphatidylserine on plasmalemma [70,71], the formation of blebs [72], and finally, the fragmentation of nuclear DNA [73]. In the case of the organotin compounds, which are the most studied antifouling substances regarding their toxic mechanism of action in cells, the induction of

apoptosis in the haemocytes of this species [74] has been associated with the altered homeostasis of cytosolic calcium ions [75,76] and the induction of oxidative stress [77].

Organotin compounds [77], Sea-Nine 211, chlorothalonil [37] and TCMS pyridine [38] induce oxidative stress in *B. schlosseri* haemocytes, causing a significant decrease in reduced glutathione. The latter is a tripeptide (γ-glutamyl-cysteinyl-glycine) found in all eukaryotic cells with concentrations ranging from 0.5 to 10 mM, which is a non-enzymatic reducing agent that acts as an electron donor [78]. It is an antioxidant and detoxifying agent of electrophilic xenobiotics protecting the cell from many toxins, including free radicals, which can cause apoptosis [79,80]. Under normal conditions, glutathione is present in the cytoplasm in its reduced form (GSH); the percentage of its oxidised form (GSSG) can be considered a measure of the toxicity status of the cell [81]. Without protection from GSH against oxidative risks, the cell might be severely damaged or die. This supports the hypothesis that, also in the case of dichlofluanid, the significant GSH loss observed in the exposed haemocytes could be an indirect cause of apoptosis induction.

Cytoenzymatic assays showed that dichlofluanid inhibits important enzymatic activities of *B. schlosseri* immunocytes involved in the main defence responses. Phenoloxidase, which is typical of the morula cells belonging to the cytotoxic line, is involved in inflammatory reactions against pathogens penetrating into the haemolymph [82,83] and in rejection reactions between genetically incompatible colonies that have come into contact to avoid sharing their circulatory systems [84]. The oxidation of polyphenols contained inside the vacuoles of morula cells by activated phenoloxidase triggers a cascade of reactions in situ that produces many reactive oxygen species (ROS), the release of which into the medium causes the rapid death of foreign (non-self) cells [85]. Acid phosphatase is a common hydrolytic enzyme in the phagocytic line [41]. In mammalian macrophages, its activation occurs within lysosomes when they merge with phagosomes and is involved in killing pathogens, non-self cells, and self cells that have been altered by viruses or are carcinogenic or senescent, through the safe digestion of foreign or modified nucleic acids [86,87]. In *B. schlosseri* phagocytes, dichlofluanid inhibits both enzymatic activities at the lowest concentration assayed, which are lower than those reported for all previously tested antifoulants, including organotin compounds. This means that dichlofluanid can adversely affect both immunocyte lines and cause stress conditions that are able to compromise immune defence responses.

In conclusion, this colonial ascidian has proven, once again, to be an excellent bioindicator for the pollution of antifouling compounds in coastal marine ecosystems. In vitro assays on cell functionality have allowed us to define toxicity indexes on haemocytes of this species that provided pivotal information about the potentially immunosuppressive impact of dichlofluanid: this xenobiotic has been shown to be one of the most toxic antifouling biocides of those examined to date, with toxicity comparable to organometals such as organotin compounds and zinc pyrithione. From these preliminary but significant results, crucial questions arise regarding the indiscriminate introduction to the environment. Without a risk assessment, biocide substances could later be revealed to be dangerous contaminants with unpredictable long-term consequences on marine ecosystems; therefore, a contribution to the selection/formulation of more eco-friendly antifouling systems must be encouraged.

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