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EVALUATION OF EFFECTS OF NEW ANTIFOULING SYSTEMS, ALTERNATIVE TO ORGANOTIN COMPOUNDS, ON BENTHIC MARINE INVERTEBRATES AT ECOSYSTEM, ORGANISMAL AND CELLULAR LEVEL

Thesis written with the financial contribution of RESIMIX s.r.l. (Brendola, Italy)

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ABSTRACT

Marine biofouling on anthropic submerged substrata is associated with major ecological and socioeconomic impacts worldwide. The most widely used antifouling systems are chemical ones represented by paints with a biocide, to which booster substances can be added. The latter are highly toxic chemical substances from agriculture (herbicides, fungicides, acaricides, wood preservatives) and pharmaceutical industry (bactericides, fungicides), these cause various ecological problems due to disruptive effects provoked on non-target organisms and depletion of coastal biocoenoses. In 2001, the paints including organotin compounds (TBT and TPT), which had the best performance and had been used worldwide for decades, were banned by International Maritime Organization (IMO) after the discovery of their severe impact on the oyster farms. As a consequence of the restrictions on the use of organotin-based paints, finding new antifouling systems has become a primary necessity. Therefore, the research was devoted to new eco-friendly formulations. In particular, physical antifouling systems have been recently introduced as more environmental-friendly alternative to the traditional chemical systems.

My scholarship has been entirely financed by RESIMIX s.r.l of Brendola, Vicenza (Italy). The university-enterprise collaboration aimed to develop a new eco-friendly paint. More in general, the research program of my PhD thesis focused on the implementation of new antifouling systems with low effects on benthic marine invertebrates. My PhD activity has been addresses the two main types of antifouling systems, i.e., chemical and physical. To determine and compare the effects of these new antifouling systems on both target species (ascidians and mussels) and non-target species (clams) the tasks have been developed at three study levels, i.e., ecosystem, individuals, and cells.

As regards of *chemical antifouling systems* I have investigated the significant differences in the ecological succession of hard-substratum community, by means of a series of biodiversity indexes, during at least one-year exposure to various RESIMIX paints and commercial copper-based paints. In addition, a comparative monitoring with antifouling paints has been considered together with the effects on settlement and metamorphosis of ascidian larvae and finally, the observation of the mechanisms of action in in vitro immunotoxicity assays on dominant bioindicators in benthic biocoenoses like tunicates, clams and mussels. From these preliminary but significant results about chemical antifouling systems, crucial questions have arisen regarding the continuous indiscriminate introduction of such biocides into the environment.

As regards *physical antifouling systems* I have been considered geotextiles (for protection from coastal erosion), and ultrasound (to prevent biofilm and disturb the larval settlement) reaching interesting results in both the field and the lab, which revealed the till now hidden downside of these systems.

SUMMARY

The present PhD thesis is structured as follows:

- **INTRODUCTION**. It addresses topics concerning the problem of biofouling in coastal areas, a brief history of chemical biocides for biofouling prevention and the development of antifouling systems (chemical and physical ones).

- **OBJECTIVES OF THE THESIS.** The aims of the research work are listed and briefly described.

- **MATERIALS AND METHODS.** A general overview of the methodologies employed in the thesis.

- **RESULTS CHAPTERS**. My PhD research activity has been divided in two tasks, i.e., testing both chemical and physical antifouling systems. It has been developed at three study levels, i.e., ecosystem, individuals, and cells. Each of the following tasks are presented in the form of scientific papers preceded by a comprehensive illustration of the content.

  TASK 1: Testing chemical antifouling systems

- Study at ecosystem level. See my technical report for the RESIMIX test paints and my research article: Cima F., Varello R., 2023. Potential disruptive effects of copper-based antifouling paints on the biodiversity of coastal macrofouling communities. *Environ. Sci. Pollut. Res*., 30: 8633–8646.

- Study at individual level. See my research article: Cima F., Varello R., 2022. Effects of exposure to trade antifouling paints and biocides on larval settlement and metamorphosis of the compound ascidian *Botryllus schlosseri*. *J. Mar. Sci. Eng.,* 10, 123.

- Study at cellular level. See my research article: Cima F., Varello, R., 2020. Immunotoxicity in ascidians: Antifouling compounds alternative to organotins - V. The case of dichlofluanid. *J. Mar. Sci. Eng.*, 8: 396. And also, my manuscript recently submitted to *Frontiers in Physiology* and currently under revision: Cima, F., Varello, R., 2023. Immunotoxic effects on target and non-target bivalve species after exposure to the antifouling copper(I) biocide: A comparative *in vitro* study between *Mytilus galloprovincialis* and *Ruditapes philippinarum*.

  TASK 2: Testing physical antifouling systems

I have evaluated the potentiality of physical systems, represented by geotextiles and ultrasound devices, which are at present used for preventing coastal erosion and biofouling settlement on ship's hulls, respectively.

- Nonwoven geotextiles (Study at ecosystem level). See my research article: Varello, R., Wetzel, MA., Cima, F., 2021. Two facets of geotextiles in coastal ecosystems: Anti-or profouling effects? *Mar. Environ. Res.*, 170, 105414.

- Ultrasonic generators (Study at individual and cellular level): See my research article: Varello, R., Asnicar, D., Boaga, J., Cima, F., 2023. Behavioural responses to ultrasound antifouling systems by adult solitary ascidians. *J. Mar. Sci. Eng*. *J.* 11, 1115.

❖ **CONCLUSIONS**. The results of the multifaceted work and future perspectives are here summarized and critically commented.

❖ **REFERENCES**

❖ **REPORT Ph.D. ACTIVITIES**

❖ **AKNOWLEDGMENTS**

1. INTRODUCTION

1.1 Marine biofouling

In the sea, submerged artificial substrates are rapidly covered by microorganisms and subsequently by algae and sessile invertebrates. This living covering forms a dynamic community, namely "biofouling" (Clare, 1996; Relini and Faimali, 2003; Delauney *et al*., 2010; Sánchez-Lozano *et al*., 2019). It is ubiquitous on hard substrata and causes damages to several human activities occurring in the marine environment. For example, it is a major problem for the shipping industry, because of increased frictional resistance, costs for hull maintenance and fuel consumption (Abbott *et al*., 2000; Almeida *et al.,* 2007). Biofouling is not only restricted to vessels, but can be also found on offshore structures, oil rigs and inside water-cooling pipes of power plants (Qian *et al*., 2000; Whomersley and Picken, 2003) (Fig. 1).

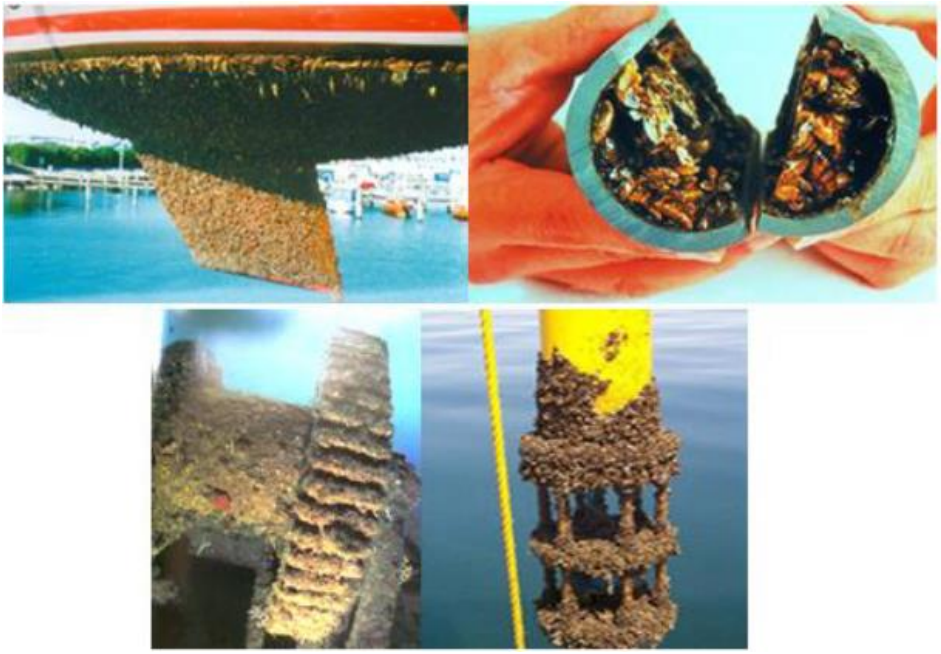

Fig. 1. Marine biofouling encrusting the hull of a vessel, waste pipes and submerged instrumentation (collection of free creative commons zero images).

Therefore, biofouling represents a severe global problem for marine artificial systems, causing worldwide extensive material and economic costs (Yebra *et al*., 2004; Schultz *et al*., 2011). Indeed, it can interfere with the activity of submerged equipment, increases the weight of submerged boat parts, and accelerates corrosion of various materials (Kohli, 2007; Schultz *et al*., 2011). The costs associated to the biofouling issue

also include money spent to clean and coat the ship's hull by the boat owners (Schultz *et al*., 2011; 2016).
Moreover, both biofouling on ship's hulls and spores and larvae from bilge waters contribute to the introduction of NIS (non-indigenous species) (Davidson *et al*., 2009). Commercial harbours are the first receivers for introduction of NIS, but recreational harbours may facilitate their secondary spread due to the intense traffic of small boats towards different marinas (Floerl *et al*., 2009; Marchini *et al*., 2015; Ferrario *et al*., 2017). The fouling community in the marinas was found to show a higher biodiversity than that found in commercial harbours, due to the lower level of pollution - that favours not only the more tolerant species as in the large ports -, variability of substrata and different routes of pleasure crafts (Connell, 2001; Holloway and Connel, 2002; Dafforn *et al*., 2009; Megina *et al*., 2016; Ferrario *et al*., 2017).
Furthermore, the biofouling growth has a negative impact on farmed species since it affects all the aquaculture structures (Willemsen, 2005; de Nys and Guenther, 2009; Dürr and Watson, 2010; Fitridge *et al*., 2012). All the structures submerged by the farmers are viable substrates for the settlement and development of a fouling community, in which the most common organisms are algae, hydroids, mussels and ascidians (Sarà *et al*., 2007; Guenther *et al*., 2010; Fitridge *et al*., 2012; Fernandez-Gonzalez and Sanchez-Jerez, 2017). The harmful effects to the artificial structures and stock species are numerous and can vary from deformation of cages and structures to the occlusion of the nets (Bannister *et al*., 2019).
The latter leads to less oxygen exchange and consequently increase of disease risk for fish in the finfish farms. In the shellfish cultures main problems are related to shell disruption, competition for food and space and recession of shell growth (Fitridge *et al*., 2012). The costs to solve the problem of biofouling for aquaculture industry is high, usually 20-30% of the total amount of management expenses (Claereboudt *et al*., 1994; Dürr and Watson, 2010). For Europe, the cost to solve the fouling-related problems amount up to 260 million euros per year and in general the biofouling can decrease the value of the products until the 90% (Dürr and Watson, 2010).

1.2 Development of biofouling

Marine biofouling can be divided in microfouling and macrofouling according to the organism size, despite the limit between the two categories is sometimes different (Callow and Callow, 2002; Relini and Faimali, 2003). In the process of biofouling formation on hard substrata, the microfouling usually occurs first, and is followed by macrofouling (Relini *et al*., 1974). Microfouling refers to the formation of an initial biofilm consisting of unicellular microorganisms such as bacteria and microalgae, whereas macrofouling refers to the development of plant and animals, and can be

subdivided into “hard-fouling”, represented by serpulids, barnacles, mussels and oysters, and “soft-fouling” including a series of taxa like algae, sponges, bryozoans (ctenostomes), hydroids and ascidians (Callow and Callow, 2002).

A linear “successional” model has generally stated the fouling process forming the community. In the temperate latitudes of the boreal hemisphere the ecological succession occurs as follows (Cima and Ballarin, 2008; 2013; Chambers *et al*., 2006; Yebra *et al*., 2004): i) early settlement of pioneer organisms in spring (April–May), ii) progressive extent of macrofouling coverage in summer (June–August), iii) competition and establishment of dominant taxa in autumn (September–November), and iv) reaching of a stable stage in early winter (December–January). The latter is a prolonged stage characterised by the highest number of species forming a relatively stable community that temporarily survives under the mild climatic conditions of the area. When harsh winter conditions are progressively established (February–March), the community loses this stable structure because most previous dominant species die and, in the following spring, a new community settles and grows up over remnants and concretions. This indeed represents a manifestation of seasonal phenomena termed “cyclical succession” due to the natural die-off of early colonists, as these species not only grow rapidly but also have short life spans (Shelford, 1930).

1.3 Main factors influencing the pattern of biofouling communities

The thickness of biofouling incrustation is related to several biotic and abiotic factors, all together driving the process of colonisation of the substratum. The most important environmental factors involved have been summarised by Cowie (2010) and include light, temperature, pressure, food supply and water current. The light is the main parameter that influences the structure of the fouling community. Since long time it was noticed that usually, the shaded undersides of surfaces often have more rich growth than vertical or unshaded surfaces (Visscher, 1928; Coe and Allen, 1937; Glasby and Connell, 2001). Temperature appears to be the principal factor limiting the geographical distribution of marine animals, and determining their reproductive periods (Woods Hole Oceanographic Institution, 1952). In general, fouling process is more massive in the temperate zone (Cao *et al*., 2011). Water current is an important factor for two main reasons: 1) it can prevent or interfere with the process of adhesion to the substratum and 2) it drives planktonic larval dispersion (Cowie, 2010). Moreover, biofouling is known to be more severe in shallow waters along the coast and in harbour basins, where a high supply of nutrients is available (Cao *et al*., 2011).

Many other factors of the substrata can affect the composition of this community, such as size (Keough, 1984), colour (James and Underwood, 1994), orientation (Glasby, 2001; Glasby and Connell, 2001) and movement (Glasby, 2001), surface material and

texture (James and Underwood, 1994), together with the various interactions between all these components (Marraffini *et al*., 2017).

1.4 Strategies of biofouling control and environmental impact

The problem of marine biofouling has long been recognised and various strategies have been applied to prevent it (Finnie and Williams, 2010), including UV irradiation, ultrasound, electric fields, foul-release polymeric coatings, and antifouling paints (Finnie and Williams, 2010; Guo *et al*., 2011, 2012; Martinelli *et al*., 2011; Richard *et al*., 2021). On the other hand, all these methods could interact with marine invertebrates and/or algae of the macrofouling community causing unexpected long-term disruptive effects on natural coastal ecosystems with a severe impact on organism survival and reproduction, food webs and, in some cases, human health.

1.4.1 Chemical methods

The most used method for prevention of biofouling is represented by the coating of the ship's hulls with antifouling paints (Almeida *et al*., 2007). Paints must have, in most cases, a broad spectrum of action to be able to counteract the more than 4000 biofouling species (Arai, 2009; Bai *et al*., 2013; Buskens *et al*., 2013). About 18 compounds are currently used as antifouling biocides (Yonehara, 2000). High concentrations of the most frequently used biocides have been found in marinas worldwide (Dahl and Blanck, 1996; Voulvoulis *et al*., 2000; Haglund *et al*., 2001; Konstantinou and Albanis, 2004; Eklund *et al*., 2008) and in the water column and sediments along shipping lanes (Strand *et al*., 2003). The widespread use of xenobiotic pesticides in antifouling paints has resulted in high levels of contamination in the environment and raised concerns about their effects on marine communities (Alzieu *et al*., 1986; Claisse and Alzieu, 1993; Thomas and Brooks, 2010; Dafforn *et al*., 2011).

1.4.1.1 From organotin to copper paints

The development of antifouling paints has an old origin, and is associated with the increase of the maritime traffic. Phoenicians and Carthaginians used copper sheets on their wooden ships, and later also coating containing sulphur and arsenic compounds, while Romans and Greeks introduced lead sheathing (Howell and Behrends, 2010). Grease, pitch, tar and other organic compounds were used on early wooden ships, until copper became the prominent antifouling agent (Readman, 2006; De Mora, 2009; Price and Readman, 2013; Deloitte MCS Ltd, 2016). Copper was patented as an antifouling agent as early as 1625 (Nurioglu *et al*., 2015), and copper-based paints

remained the most effective antifouling agent on the market until the organotin compound tributyltin (TBT) was introduced in the mid-1960s (De Mora, 2009; Deloitte MCS Ltd, 2016). TBT can be incorporated in resin-based paints used on all types of vessel surfaces (De Mora, 2009). Organotin was indiscriminately introduced as an antifouling compound for its high efficacy in preventing fouling settlement. However, beginning from 1980s a direct correlation with TBT contamination and severe effects on mollusc reproduction (imposex) and growth (shell thickness) was discovered in French oyster farms with serious economic repercussions (Voulvoulis, 2006). Consequently, the detrimental effects of TBT imposed on the aquatic environment and potential effect on humans led to its progressive ban on vessels less than 25 m in length since 2003 and on all vessels since 2008 (IMO, 2001).

1.4.1.2 Copper

After the international ban of organotin compounds by the International Maritime Organization (IMO) just beginning from 2003, copper formulations have again become the most widely used (Yebra *et al*., 2004; Willemsen, 2005). Copper compounds, include metallic copper, cuprous thiocyanate, and cuprous oxide, are the dominant active substances in antifouling paints (Voulvoulis *et al*., 2002; Yebra and Weinell, 2009; Cao *et al*., 2011).

Copper represents an essential micronutrient used in various metabolic processes, hence low concentrations of it are needed, but it starts to be toxic when it accumulates in tissues and exceeds the tolerance capacity of the organisms (Xie *et al*., 2005). Copper also adsorbs to particulate matter in the water column and accumulates in sediments (Thomas and Brooks, 2010). Indeed, high concentrations of dissolved copper are commonly found in shallow, near-coastal marine areas (Biggs and D'Anna, 2012). In certain poorly flushed basins and in crowded marinas copper accumulated and exceeded the Clean Water Act standard for this element, which is 3.1 µg/L in marine waters, therefore in 2021 US EPA announced intent to strengthen the regulation for reducing the copper impact. Furthermore, copper is worldwide recognised to facilitate tolerant aliens over the local species potentially changing the benthic community (Piola *et al*., 2009; Dafforn, 2017; Culver *et al*., 2021).

1.4.1.3 Booster biocides

After the ban of TBT, the number of paint formulations decreased, but new biocides have been introduced to increase the efficacy against a wider spectrum of organisms (Voulvoulis *et al*., 1999).

For example, some species of algae tolerate high concentrations of copper, making necessary to include additional biocides in the paint formulation, called "booster biocides" (Correa *et al*., 1996; Voulvoulis *et al*., 1999, 2006; Almeida *et al*., 2007; Manzo *et al*., 2008; Parks *et al*., 2010; Cima and Ballarin, 2012; Guardiola *et al*., 2012). A few 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, TCMTB, thiram, zineb, ziram), herbicides (diuron, Irgarol 1051) and insecticides (endosulfan) from agriculture (Konstantinou and Albanis, 2004; Zhou *et al*., 2006; Batista-Andrade *et al*., 2016). More examples include bactericides, anti-mould and anti-dandruff compounds from the polymer (chlorothalonil), leather (TCMS pyridine) and cosmetic-pharmaceutical (zinc pyrithione) industries, respectively (Voulvoulis *et al.*, 1999).

However, the environmental effects such as bioavailability, toxicity, and persistence of these biocides are not understood, as they have only been recently introduced and the problem of toxicity on several marine species remains (Terlizzi *et al.,* 2001; Maraldo and Dahllöf, 2004;). Recent studies have shown that, many of the booster biocides commonly employed are a threat to the marine environment (van Wezel and van Wlaardingen, 2004). Some boosters can accumulate to high levels, despite claims for rapid degradation, and have a biocidal effect on non-target marine organisms (Jacobson and Willingham, 2000; Ma *et al*., 2002; Sherrard *et al*., 2003; Konstantinou and Albanis, 2004; Bejarano *et al*., 2005; Braithwaite and Fletcher, 2005; Bellas, 2006; 2007; 2008; Mochida *et al*., 2010; Thomas and Brooks, 2010).

Some boosters are reported to be more toxic than TBT. As an instance, Bellas (2007) determined that Sea-Nine 211 had deleterious effects on the embryo-larval stages of the sea urchin, *Paracentrotus lividus*, despite its classification as a safe biocide (Braithwaite and Fletcher, 2005). Diuron and Irgarol 1051, which have been massively added to paint formulations, have been recently banned by many EU countries (Cresswell *et al*., 2006) due to increasing evidence of environmental toxicity (Munoz *et al*., 2010).

The use of booster biocides provides an interim solution and more effective antifouling strategies are required to combat marine fouling issues.

1.4.1.4 Paint matrix types

The efficacy and toxicity of an antifouling paint is not only determined by the type and combination of biocide(s), but also by the way in which the active substance(s) is incorporated into the product and consequently released upon use. The paint binder is the component that holds together the components of the paint matrix and, for this reason, is often considered the most important ingredient (Kill *et al*., 2006). In the

preventing action of settlement by organisms with strong adhesive capabilities, antifouling paints should contain molecules with biocidal action released at different times and concentrations depending on the matrix characteristics.

There are currently three main biocidal paint types (Zhou, 2015).

- Insoluble matrix paints (either contact leaching paints or hard paints) have a binder that does not erode when immersed in seawater (Fig. 2A). With time, the soluble biocidal molecules leach from the paint, leaving behind a depleted, porous matrix. As this depleted layer increases in thickness, the diffusion of biocides from the paint film decreases over time.
- Soluble matrix paints (either erodible or ablative paints) have a seawater soluble rosin binder. The paint erosion is controlled by the dissolution of both the binder and the biocidal molecules (Fig. 2B).
- Self-polishing paints are based on acrylate polymers, which undergo hydrolysis or ion exchange. The consequent continuous surface renewal yields a self-smoothing paint surface with a theoretically steady release of biocides over the lifetime of the paint (Fig. 2C).

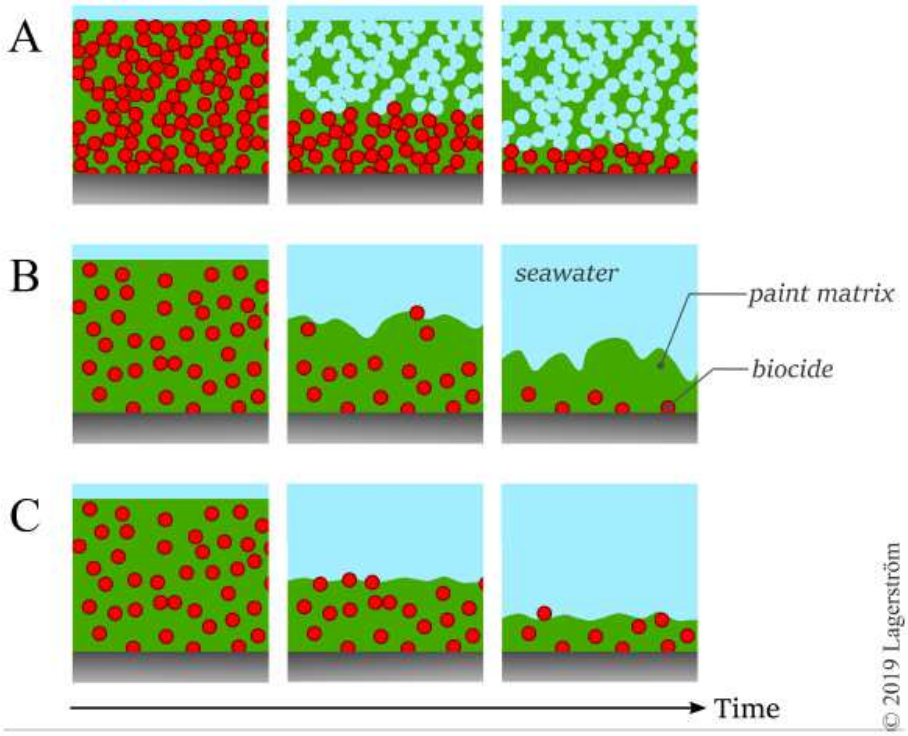

Fig. 2. Scheme of different paint types based on the matrix technology. Insoluble matrix (A), soluble matrix (B) and self-polishing (C), and the modality of release of biocide molecules over time (Lagerström, 2019).

1.4.2 Physical methods

The most challenging biofouling issue is how to control the fouling settlement and growth by non-toxic or environmentally friendly methods.
As an alternative to the antifouling paints, various physical and mechanical systems have been recently introduced. Some of these strategies to prevent fouling include geotextiles (Jackson *et al*., 2004), sound (Branscomb and Rittschof, 1984; Donskoy and Ludyanskiy, 1995; Guo *et al*., 2011; Kopel *et al*., 2011), vibration (Latour and Murphy, 1981; Nakagawa *et al*., 2006), ultrasound (Gavand *et al*., 2007), electrical micro-currents (Perez-Roa *et al*., 2008), microwave (Boldor *et al*., 2008), and aeration (Scardino *et al*., 2009; Bullard *et al*., 2010).

1.4.2.1 Geotextiles

Geotextiles are permeable fabrics which, when used in association with soil in geotechnical engineering works, have the ability to separate, filter, reinforce, protect, or drain (Ferreira Gomes, 2001). In the human history geotextiles were one of the first textile products utilised in roadway construction in Roman days to stabilize roadways and their edges (Mandal, 1990). Those textiles are distinguished by their constituent elements (fibres) and by their structure, resulting from the manufacturing process. Today, most of geotextiles are represented by synthetic fibres and can be grouped mainly into two major groups, i.e., woven and non-woven geotextiles.
Non-woven geotextiles are used in applications that require higher filtration capability than is obtainable with woven geotextiles, and large widths are necessary. The non-woven geotextiles are made of randomly oriented fibres connected in a flat structure. Synthetic polymers are the most used in the manufacture of geotextiles and related products (Ferreira Gomes, 2001). Three types of polymers are as raw materials, i.e., polyester, polypropylene and polyethylene (Arun Hegde, 2017).
The geosynthetic materials, despite being relatively new, had a fast development. The first application of a geotextile occurred in Florida in 1950 (Beckam and Mills, 1935; Barrett, 1966). The emergence of synthetic polymer started in later ‘60s with the development of polyolefins and polyester resins. In Europe, the application of woven geotextile fabrics (Gicot and Perfetti, 1982) dates from 1960 in the Netherlands and non-woven geotextiles in 1969 in France (Ferreira Gomes, 2001). After ‘70s, a great development of new materials occurred and in the ‘80s the term “geosynthetic” was introduced as a generic name for a variety of materials, such as geomembranes, geogrids, geocomposites, geotextiles and all related products. Between 1977 and 2002 many international conferences took place worldwide on geosynthetics. In 1983, the International Society of Geotextiles was established in Paris. Several specialist journals

appeared, in particular the “International Journal of Geotextiles and Geomembranes” from 1987 and “Geosynthetics International” since 1994 (Gicot and Perfetti, 1982; Veldhuijzen van Zanten, 1986; Ingold, 1994; Santvoort, 1994; Koerner and Soong, 2001; Ghosh, 2009; Adelman *et al*., 2012; Koerner, 2016; Shukla, 2017).

Over the last decades, nonwoven geotextile fabrics have been increasingly used in coastal and marine engineering (Lee and Douglas, 2012; Mitra, 2013; Oumeraci and Recio, 2017). They are widely used in civil and environmental engineering and construction projects, such as soil filtration (Palmeira *et al*., 1996), dyke construction (Koffler *et al*., 2008), and the general prevention of coastal erosion (Theisen, 1992). They have substituted other artificial materials like steel, stone and concrete usually employed for constructions in coastal areas. In some cases, such as protecting shores from erosion, large geotextile containers can be considered as preferential solutions when traditional materials are not acceptable (Heerten *et al*., 2000; Jackson *et al*., 2001; Restall *et al*., 2002; Tomlinson *et al*., 2003; Black and Mead, 2009) (Fig. 3).

Fig. 3. Examples of geotextiles employment in civil engineering to protect the coasts (collection of free creative commons zero images).

Nonwoven geotextile fabrics due to their physical, mechanical and hydraulic properties (i.e., ultraviolet (UV)- and chemical-resistant) have been introduced into coastal environments to prevent shelf erosion and to protect artificial submerged structures. Consequently, a specific industry arose that was involved in the manufacture of rolled erosion control products (RECP). The latter are used to mitigate short-term erosion and, in some cases, to enhance the long-term erosion control performance of established vegetative cover.

The surface structure of nonwoven geotextiles has a unique texture of polymers (Dassanayake and Oumeraci, 2012). They are made of long polypropylene (PP) and polyester (PET), fibres that are entangled or crimped in a fleece-like texture, which results in a highly irregular surface. Therefore, geotextile represent a potential settlement surface for species that naturally adhere on hard substrata.
Although geotextile materials have been used for several decades in aquatic environments, only a few studies mention biofouling on geotextile materials. These studies report that geotextile materials provide a good substratum for the settlement of a wide range of benthic species (Edwards, 2003; Jackson *et al*., 2004)

1.4.2.2 Ultrasound generators

The typical human range for audible sound is from 16 Hz to 20 kHz, every frequency that is included in this range is properly said sound, while frequencies below and above this range are called infrasound and ultrasound, respectively (Fig. 4).

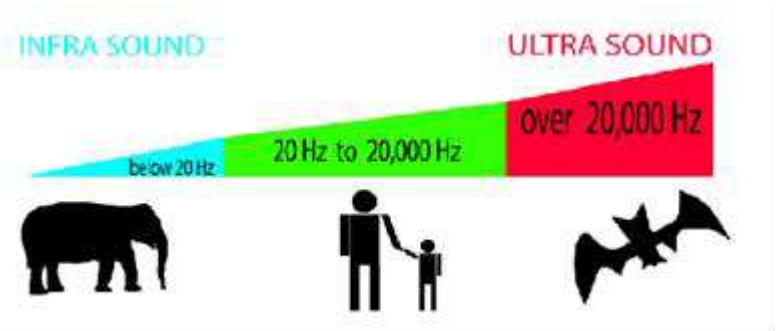

Fig. 4. Sound ranges from infrasound (<20Hz) to ultrasound (>20000Hz) (www.equipmentexplained.com).

Application to boats for antifouling purposes was suggested in the literature a few years ago (Guo *et al*., 2011) i.e., ultrasound waves are used for ballast water treatment (Holm *et al*., 2008; Estévez-Calvara *et al*., 2018). Nowadays, ultrasonic sound wave systems are sold by many companies as antifouling systems for boats. Generally, ultrasonic devices are composed of a generator and an amplifier, connected to a transducer, the latter internally fixed to the hull (Fig. 5) to make it vibrate at the desired frequency, generally between 28 and 40 kHz (Legg *et al*., 2015).

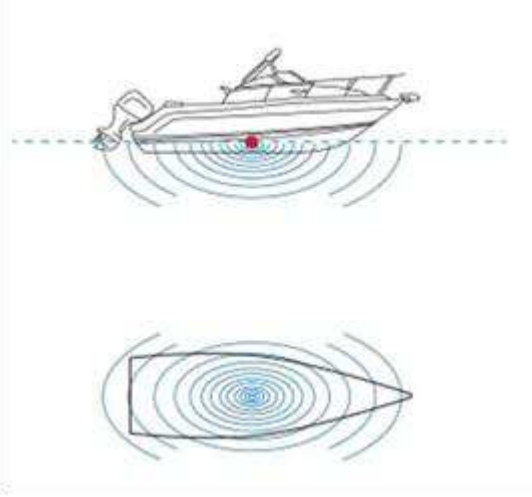

Fig. 5. Scheme of installation of an ultrasound device for a boat up to 8 meters (www.kunste.it).

In the case of higher-power ultrasound, cavitation appears to be the main mechanism that causes biofouling death and settlement inhibition. Cavitation consists in the phenomenon of formation and implosion of small bubbles during the propagation of ultrasound. These implosions generate shock waves that destroy the bonds between the biofouling and the surface where it is attached, causing its separation, or preventing its settlement (Ahmad *et al*., 2012; Aktij *et al*., 2020).

In the case of powers lower than the threshold that generates cavitation, the inhibition of biofouling could be due to the intense vibration of the hull or the masking of other sounds that could attract the biofouling (Legg *et al*., 2015).

Laboratory studies have shown that frequencies in the orders of tens of kHz efficiently kill barnacle larvae (Mori *et al*., 1969; Suzuki and Konno, 1970). In the field tests, ultrasound frequency ranging between 20 to 100 kHz was effective in keeping an area free of fouling marine organisms (Fischer *et al*., 1981). Recently, a relative systematic study of ultrasound on barnacle cyprid settlement and mortality was evaluated using three frequencies and various exposure intensity and the most effective frequency on settlement inhibition occurred at 19.5 kHz (Kitamura *et al*., 1995).

Likewise, ultrasound can be used on algae control. The effectiveness of ultrasound irradiation on algae removal was achieved at frequency of 40 kHz (Heng *et al*., 2009). Ultrasound frequency and intensity determine the removal efficiency of algae and the cavitation plays a significant role (Giordano *et al*., 1976; Ma *et al*., 2005).

2. OBJECTIVE OF THE THESIS

My PhD thesis focused on the implementation of new antifouling systems with low effects on benthic marine invertebrates. The colonial ascidian *Botryllus schlosseri* (Pallas, 1766), the Mediterranean mussel, *Mytilus galloprovincialis* (Lamarck, 1819) and the Manila clam, *Ruditapes philippinarum* (Adams and Reeve, 1850) were chosen as well-known filter-feeding bioindicators. The colonial ascidian *B. schlosseri* is widely represented and often dominant in the hard-substratum community of temperate areas, such as the Lagoon of Venice, and lives at the water/sediment interface. Moreover, the bivalves were chosen because they are dominant species of the trophic chain of the coastal ecosystem. *M. galloprovincialis* is a target species of antifouling control systems. It is a sessile species common on intertidal natural and artificial hard substrata, where it is a dominant species in the macrofouling community, and is an ideal sentinel organism for the water column. Instead, *R. philippinarum* represents a non-target species of antifouling control systems because it lives burrowing in the sandy-muddy bottoms.

2.1 Study of the effects of trade chemical and physical systems used as antifouling in coastal ecosystems

My project has been divided in two tasks, i.e., testing chemical and physical antifouling systems. To determine and compare the effects of these new antifouling systems on both target species (ascidians and mussels) and non-target species (clams) the tasks have been developed at three study levels i.e., 1) *ecosystem level* (evaluation of ecological succession by means of biodiversity indexes in selected areas of the Lagoon of Venice), 2) *individual level* (evaluation of alteration of both settlement capability and metamorphosis by ascidian larvae), and 3) *cellular level* (evaluation of effects on subcellular targets at sublethal concentrations on haemocytes of ascidians and bivalves).

2.2 RESIMIX collaboration for developing new eco-friendly antifouling paints

My project has been entirely financed by RESIMIX s.r.l., a company at Brendola in the province of Vicenza (Italy) that operated for more than 40 years in advanced materials for the building industry with products and technologies (paints, resins, adhesives, putties, primers, hardeners) for professionals and companies operating in civil and industrial construction, civil engineering, housing renovation, and artistic and

monumental restoration. A university-enterprise collaboration has been established on 6 May 2019 with the aim of developing innovative antifouling solutions not only for the nautical market but also for protection of submerged artifacts.

RESIMIX was committed to develop new water-based antifouling paints in order to zero emissions of volatile organic compounds (VOCs) greatly reducing the environmental impact of the compounds. On the other hand, my role consisted in monitoring and validating the mechanism of action of the paints produced by RESIMIX on coated wooden, fibreglass and steel panels submerged for at least one year in selected sites of the Lagoon of Venice.

In particular, the plan of the agreement was to produce new copper(I)-antifouling paints based on a long-term performing matrix containing the smallest possible amount of biocide working via a continuous direct contact with fouling organisms. The goal was to reduce the environmental impact with a low copper leaching from durable paints according to the EU regulations. RESIMIX has chosen copper oxide(I) because it is currently one of the biocides most present in antifouling paints and it is included in category 21 of the Biocidal Products Regulation (BPR, Reg. (EU) 528/2012) of European Commission (EC). BPR was adopted on 22 May 2012 and entered into force on 17 July 2012 with application from 1 September 2013. The aim of this Regulation was to determine the rules for both production and use of commercially available biocidal products, as well as to provide a protection from health and environmental risks. Today, all biocidal active substances require the approval of the Commission under this regulation before their introduction in the market. This regulation has determined 22 product types (PT) of biocidal products that are subdivided in four main categories: 1) *disinfectants*, 2) *preservatives*, 3) *pest control*, and 4) *other biocidal products*. The PT concerning antifouling biocides is “PT21-Antifouling products”, the designation of which is “these products aim to control the growth of fouling microorganisms, plant or animal species on vessels, aquaculture equipment or other construction used in water”.

3. MATERIALS AND METHODS

My project developed in fieldwork and laboratory experiments. The methodologies employed in the present thesis are listed and briefly described below.

3.1 Fieldwork

The study area, where the work has been conducted, is the Lagoon of Venice. This site is the largest coastal transitional ecosystem in the Mediterranean and, at the same time, one of the UNESCO World Cultural and Natural Heritage sites. It is characterised by a maze of channels (maximum depth exceeding 15 m), which cut across a large area of shallow waters (average depth of 1 m), fens and salt marshes. Today the total surface of the lagoon is 550 km^2: 390 km^2 of open lagoon including 40 km^2 of tidal channels, 70 km^2 of salt marshes, and 90 km^2 of fish farms. Three inlets (from North to South: Lido, Malamocco and Chioggia) connect the lagoon to the Adriatic Sea (Provincia di Venezia, 2009). The Lagoon of Venice is one of the most important Mediterranean Transitional Systems according to the 2000/60/EC Directive (WFD: Water Framework Directive) that aims to expand the scope of water protection to all waters, including inland waters, transitional and coastal waters, and groundwater, achieve "good status" for all waters, establish a "combined approach" of emission limit values and quality standards, get the citizen of the Community involved more closely, and streamline legislation. An integrated Weight-of-Evidence methodology to classify the quality of the water bodies of the Lagoon of Venice was developed and applied by integrating biological, physicol-chemical, ecotoxicological, and hydro-morphological data according to the guidelines of the European WFD (Micheletti *et al*., 2011).

The biology of the Lagoon of Venice is strongly affected by environmental parameters, primarily hydrological conditions and chemical properties of water, and also by human impacts such as fishing for clams (Pranovi and Giovanardi, 1994). The relevant biological events include seasonal phytoplankton blooms, exponential growth of macroalgae, invasion by non-indigenous species, fouling on ship roofs and the bilge waters of tourist and commercial boats.

It is an exceptional place to study biofouling, due to the high diversity and biomass of biofouling communities (also including a record number of non-indigenous species at Italian level). Studies of the fouling communities in the Lagoon of Venice are important because they 1) reveal long-term changes in the quality (pioneer, dominant, vicarious, allochthonous species) and quantity (species richness, settlement and covering ability, individual abundance) of the communities that are attributable to environ mental differences and 2) improve our knowledge of the interactions between the fouling

organisms and certain physicol-chemical factors of seawater such as pH, temperature, salinity.

- For the study at ecosystem level, the sampling area chosen was placed in two stations (with different hydrodynamic characteristics) in the Southern basin of the Lagoon of Venice, i.e., a mobile wharf along the Sottomarina channel (Lat 45° 14’ N; Long 12° 17’ E) near the port inlet of Chioggia and an abandoned mussel farm (Lat 45°13’ N; Long 12°15’ E) (Fig 6).

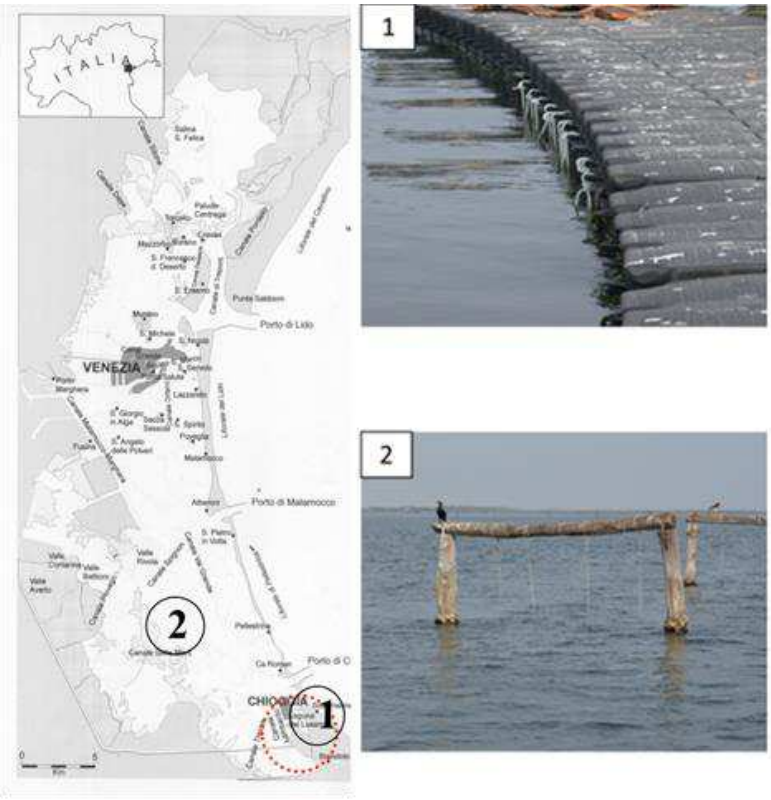

Fig. 6. Location of the mobile wharf (1) and the abandoned mussel farm (2) in the experimental site close to the Southern inlet (Chioggia) of the Lagoon of Venice, Italy.

- The experimental apparatus (Fig. 7) consisted of a series of units, formed of a single panel, deployed in the sampling station (Fig. 6). The substrata of the panels monitored during my PhD project were i.e., a series of 4 geotextiles and larch wood, fibreglass and stainless-steel panels coated with copper(I)-based antifouling paints. Substratum references have been represented by replicates of unpainted larch wood, fibreglass and stainless-steel panels.
  Each panel was tied at a depth of 50 cm to a thick nylon rope. The latter was maintained vertically in the water column by a brick as ballast at the bottom

end and was anchored to an eyehook on a mobile wharf at the top end. Each unit was arranged randomly at 60 cm from each other.

In particular, 1) the wooden, fibreglass and stainless-steel panels were 20 × 15, with a 1- cm-diameter hole where the rope passed through; 2) the panels of geotextiles (20 × 15 cm), were supported by a frame of Plexiglas with a thickness of 0.5 cm and a 1-cm-diameter hole for the rope on its upper side.

Regarding the monitoring activity, the experimental setup consisted of replicates (n=3) for each type of tested panel.

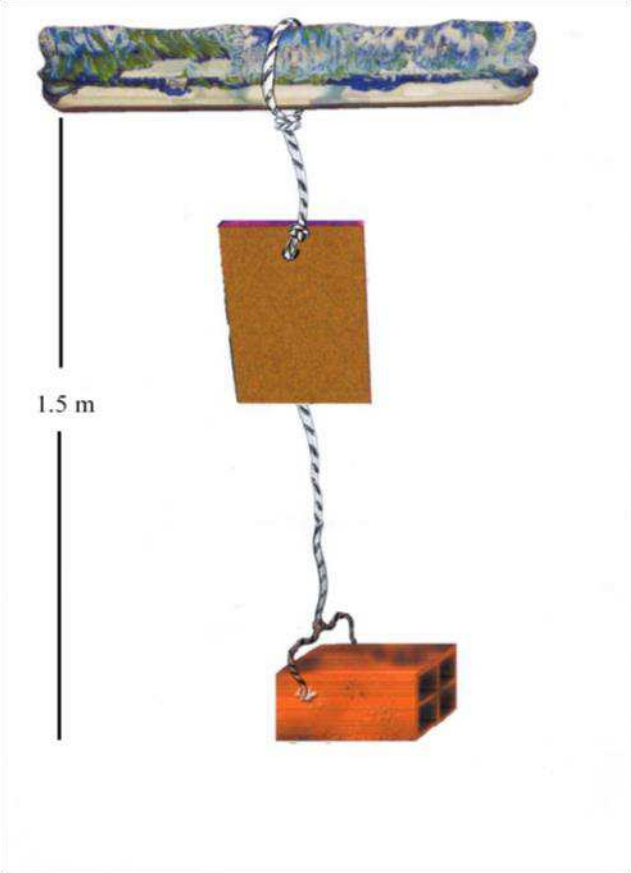

Fig.7. Scheme of one unit of the immersed panel systems at the sampling stations.

- The artificial substrata were submerged continuously for one year (from April to December/January), and the settlement by fouling organism was monitored monthly. In particular the monitoring of RESIMIX antifouling paints started in March (2020/2021 and 2022) and ended in December-January (2020/2021 and 2022).
- The analysis of the changes in ecological succession that occurred on commercial copper(I)-based paints and geotextiles were carried out with a series of biodiversity descriptors, i.e.,

i) *species richness*, i.e., was the total number of species, by month, present on all panels of the same type;

ii) *biocoenosis structure*, i.e., the percentage of coverage for each taxon, namely the set of species belonging to the same taxonomic group, in respect of the total coverage of the whole community (100%) on panels of the same type of substratum;

iii) *covering-abundance area*, i.e., a quantitative analysis (percent cover) of the settlement capacity of the various species on panel areas calculated from digital photos;

iv) *biomass*, expressed in g cm^2 of fresh weight of the living fouling organisms and determined by weighing the panels, after a rapid draining, with a portable electronic scale.

- Photographs of all panel's surfaces were taken with a Nikon Coolpix 995 digital camera (Nikon Corporation, Tokyo, Japan). As concerns the monitoring performed for the RESIMIX paints, only a qualitative analysis was performed based on the direct observations followed by photos collected in the field. The imaging analysis was performed using the Infinity Analyze Application v. 5.0.0 (Lumenera Co. 2002-2009).
- Taxonomic analysis: samplings of fouling organisms were collected from the panels and fixed in 5% formaldehyde in seawater for better species identification under a dissection binocular stereomicroscope Wild Heerbrugg with a 50x maximum magnification.
- Morphological analysis of the geotextiles' fibres, small samplings (0.5 × 0.5 cm) were observed and photographed under a Cambridge Stereoscan 260 scanning electron microscope (SEM) after critical point drying and gold scattering.

3.2 Laboratory experiments

- The study at individual level collected the results of investigations of the effects on ascidians (*Botryllus schlosseri*) larval viability, settlement and metamorphosis exposed to trade antifouling paints and worldwide employed biocides. I carried out larval toxicity assays to assess the effects of antifouling biocides and antifouling paints such on i) delay in larval metamorphosis, ii) malformations or iii) death, compared to unexposed larvae (controls).
- The study at cellular level, collected the results of *in vitro* immunotoxic assays of copper(I) ions on ascidian (*B. schlosseri*) and bivalve haemocytes (*Mytilus galloprovincialis* and *Ruditapes philippinarum*). A series of useful immune system biomarkers have been considered, i.e.,

i) *Trypan blue exclusion test* for LC_{50} evaluation;
ii) *Cell function assays*, i.e., cell adhesion assay, cell spreading index, cytoskeleton stability index (F-actin), phagocytosis index, lysosomal stability index (Neutral Red uptake assay), cytosolic Ca^{2+} content assay, apoptotic index (TUNEL reaction);
iii) *Cytochemical assays for evaluation of enzymatic index*, i.e., the hydrolase β-glucuronidase and acid phosphatase, phenoloxidase and the mitochondrial cytochrome-c oxidase (COX);
iv) *Oxidative stress assays*, i.e., glutathione content index and superoxide anion index.
These biomarkers are considered important for assessing the alterations in immune responses and determining the toxicities of pollutants and their interactions at both the cellular and subcellular levels (Alvarez and Friedl, 1992).

- Lastly, ultrasound effects on adult benthic invertebrates (the solitary ascidians *Ciona intestinalis*, *Ascidiella aspersa* and *Styela plicata*) have been studied in controlled laboratory conditions. I have evaluated the acoustic sensitivity and behavioural responses of adult ascidians to antifouling ultrasound systems, which are at present used for preventing larval settlement on ship's hulls.

3.3 Statistical approaches

Statistical analysis and graphical compilation were performed with the following test using R Software Environment (version 3.5.3) and DSAASTAT v. 1.1 2011:

- Dunnett's test for multiple comparisons
- Fischer's least significant difference (LSD) test
- X^2 test with Yates' p-value correction
- Analysis of variance (ANOVA)
- Nonmetric multidimensional scaling (NMDS)
- Permutational multivariate analysis of variance (PERMANOVA plus)
- Bray–Curtis similarity matrix and the Similarity Profile Routine (SIMPROF)

4. RESULTS CHAPTERS

The thesis addresses the two main types of antifouling systems, i.e., chemical and physical, through a series of experimental approaches aimed at quantifying effectiveness using a range of response variables and measurement methods. Some part of the work specifically addresses new antifouling solutions developed by an enterprise that also sponsors this PhD research.

4.1 TASK 1. Testing the effectiveness of chemical antifouling systems

4.4.1 Study at ecosystem level

I have investigated the ecological succession on wooden and stainless-steel panels painted with common copper(I)-based paints on the market – copper content ranging from 12 to 40% – developed with different technologies (self-polishing and insoluble matrix) and booster content.
This study monitored the ecological succession of macrofouling communities on panels immersed for 10 months in the southern basin of the Lagoon of Venice.
I statistically compared the development of macrofouling communities on the panels coated with copper(I)-containing antifouling paints with those on the reference (unpainted) and TBT-coated panels. To describe the effects on the communities, I used PERMANOVA and a series of biodiversity descriptors like species richness, coverage-abundance index, community structure, and Bray-Curtis similarity analysis on species presence/absence; these descriptors highlighted the disturbing activity of the antifouling paints that resulted in a negative selection of key species of the coastal community of hard-substratum. Green algae, bryozoans, and barnacles were the most tolerant taxa and a negative species selection occurred for sponges, serpulids, and ascidians.

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EFFECTIVE WASTE MANAGEMENT WITH EMPHASIS ON CIRCULAR ECONOMY

Potential disruptive effects of copper-based antifouling paints on the biodiversity of coastal macrofouling communities

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Abstract

The expanded use of copper(I)-based antifouling paints (AF) has increased copper leaching into coastal environments, requiring attention and legislative restrictions for potential long-term effects on benthic populations. The ecological succession of macrofouling communities was analysed on wooden and stainless steel panels coated with four copper(I)-based AF (Paints A–D) immersed for 10 months in the Lagoon of Venice. With the exception of Paint B, which contained only copper(I) compounds and was based on hard-matrix technology, the other paints were based on self-polishing matrices and various booster biocides. The booster content was a mix of TBT compounds for Paint A, dichlofluanid for Paint C, Irgarol 1051, and chlorothalonil for Paint D. The macrofouling communities appeared dissimilar to those on the reference uncoated panels as regard the species richness, the coverage areas, and the biocoenosis structure. Generally, green algae, bryozoans, and barnacles were the most tolerant taxa and a negative species selection occurred for sponges, serpulids, and ascidians. Paints A and D showed the highest performance, and Paint D also prevented molluscs on wood panels. Paints B and C rapidly decreased their efficiency, the first probably due to the insoluble matrix with the highest biocidal leaching rate, and the second due to the presence of a booster with low toxicity. Paint B also inhibited red algae and molluscs, but Paint C did not reveal significant differences in types of species settlements with reference panels.

Keywords Antifouling paints · Booster biocides · Copper(I) compounds · Macrofouling communities · Organotin compounds

Introduction

Large amounts of antifouling paints are employed worldwide on vessels and permanently submerged structures to preserve them and reduce the associated economic costs. However, the severe damage caused to coastal ecosystems by the leaching of the biocidal substances contained in these paints has been well known since the 1980s for organotin compounds (Champ and Seligman 1996).

Both paint efficacy and its potential for negative effects on the environment are related to content and release rate of the biocides. The latter is mainly controlled by the paint properties. The matrix is the most important component that incorporates the paint ingredients into the product. Apart from the biocide solubility, the matrix properties control both the manner and the rate at which the biocide is released into the water column (Kiil et al. 2006; Almeida et al. 2007). Therefore, the choice of the paint type, i.e., contact leaching or self-polishing paints, is based on the knowledge of the projected docking cycle and the speed and activity of the vessel, which determine the limitations of application. Contact leaching paints, also known as hard or long-life paints, have an insoluble matrix, which does not erode when immersed in seawater. Continuous biocide release is due to the high biocide concentration and, as the biocidal molecules are leached from the surface, microchannels are formed, which permit the release of biocide from deeper surfaces in the coating. Self-polishing paints are based on acrylic polymers, with which seawater reacts causing hydrolysis or ion exchange on the surface layer of the coating, allowing the biocide to be released into the water. The residual polymer backbone then becomes water-soluble and dissolves,

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exposing a fresh layer of active surface. The consequent constant and uniform surface renewal yields a self-smoothing paint surface with a steady release of biocidal molecules over the lifetime of the paint. Organotin copolymer paints, based on tributyltin methacrylate, were the first self-polishing antifouling coatings, in which the copolymer acted as both the paint matrix and biocide (Swain 1999).

After the total ban on organotin compounds — the main compound used was TBT — by the International Marine Organization — Marine Environment Protection Committee (IMO-MEPC) (1998) and, subsequently, by the European Commission (EC ordinance No. 782/2003, 14 April 2003), the paint industry developed substitutive formulations. Among them, numerous commercial tin-free paints are now available as effective replacement, which are based on copper-based compounds (Yebra et al. 2004). Copper(I) oxide (Cu_2O) and copper thiocyanate (CuSCN) are the main copper(I) compounds typically employed at concentrations between 20 and 40% (Environment Agency 1998; Brooks and Waldock 2009; Pérez et al. 2015). Although copper is the most important active compound of the antifouling paints that are being used, unlike the organotin paints, these copolymers do not generate sufficient biocide to be effective. Therefore, series of booster substances are incorporated in the formulations together with the principal copper-based biocide to increase the antifouling efficiency of the paints. The association with boosters has the role of increasing the antifouling activity and performance of the paints to a wide spectrum of target organisms tolerant to copper but have the potential to cause environmental damage, for which the risk evaluation is complex (Voulvoulis et al. 1999; Evans et al. 2000; Almeida et al. 2007; Ytreberg et al. 2021).

In all organisms, copper is an essential element because it plays a role in many enzymatic reactions. In mammals, copper has an important role in the formation of erythrocytes and in the development of bone, central nervous system (CNS) and connective tissue (Underwood 1977; WHO 1996). However, an excess of copper can cause severe metabolic imbalances in humans, since copper has a very complex toxicity mechanism (Goyer 1995; Ellingsen et al. 2014; Royer and Sharman 2020). It is absorbed along the gastrointestinal tract and accumulates in the liver, brain, kidney and heart. Copper increases the membrane permeability, resulting in cell lysis, and inhibits glutathione reductase, causing oxidative stress. Consequently, the maximum copper concentration permissible for human health in drinking water is 1.3 mg/l (NRC 2000). In natural environments, copper partly arises from the windblown dust and the volcanic eruptions (Nriagu and Davidson 1986), and partly from runoff waters of mineral deposits, the contribution of which is minimal since its concentration generally does not exceed 1 mg/l (Bergvist and Sundbom 1980; Davies and Bennett 1985; Purves 1985). Therefore, copper concentrations found in both freshwaters and coastal marine waters are of anthropogenic origin. The largest anthropogenic copper emissions into the environment (95%) are represented by extraction, production and processing of metals, wood and coal combustion, waste incineration, use of fertilisers, and antifouling paints (Nriagu 1979). In coastal marine waters, concentrations of copper higher than the mean natural concentration (0.003 mg l^{-1}) have been found in harbours and marinas due to the massive use of copper-based paints that began after TBT restrictions were enacted (Claisse and Alzieu 1993; Srinivasan and Swain 2007). The US EPA Clean Water Act (CWA) established that copper must not exceed the 3.1 µg/l limit in coastal waters (US EPA 2007), with regulatory compliance by 2022. Marine organisms that are able to accumulate high amounts of copper include different species of crustaceans and filter-feeding invertebrates such as bivalve molluscs (mussels, clams, oysters) and ascidians (NAS 1977). On the one hand as edible species, this bioaccumulation could represent a risk for human health, and on the other hand as key coastal ecosystem species, their survival and reproductive capability could be negatively affected causing significant changes in communities (Addison et al. 2008). In these taxa, copper inhibits fertilisation and causes embryotoxicity, larval mortality, metabolic impairment, neurotoxicity and immunotoxicity (Bellas et al. 2004; Brown et al. 2004; Zhang et al. 2010; Cima and Ballarin 2012; Fabbri et al. 2014).

The aim of the present study was to evaluate the disturbing effects of a series of copper(I)-based paints found easily on the market due to their widespread use on boat hulls on the macrofouling communities on hard substrata in the Lagoon of Venice. The effects of antifouling paints on the ecological succession of these communities and the coverage areas of the settling species were analysed for 10 months on a series of wooden and stainless steel panels permanently submerged in the intertidal zone. The results were compared with reference (uncoated panels) and TBT-coated panels using a series of biodiversity descriptors.

Materials and methods

Study site

The study site (i.e., the sampling station) was a mobile wharf consisting of joined plastic floats located along the Sottomarina channel (lat. 45° 14′ N, long. 12° 17′ E) near the port inlet of Chioggia (Venice, Italy) in the southern basin of the Lagoon of Venice. The study area is a low-boat-traffic zone that represents a subtype of euhaline waters and contains an unconfined microenvironment of the lagoon biome that is particularly rich in biodiversity. The basin has a tidal range > 50 cm and a depth < 1.5 m.

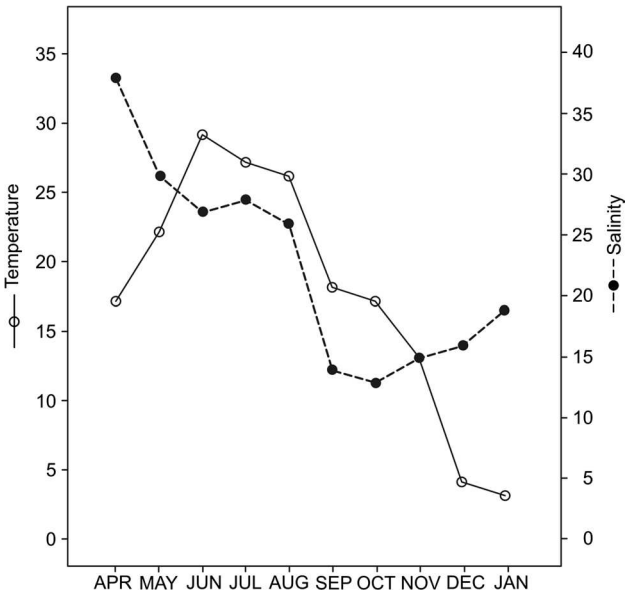


Fig. 1 Monthly trends in water temperature and salinity measured in the water column close to the study site over the course of the experiment

During the experiment (Fig. 1), temperature ranged between a minimum of 3.2 °C in January and a maximum of 30.0 °C in June. Salinity varied from 13.42 to 38.15 PSU depending on the rainfall trend. For the geographical location near the port inlet, the site, although with low hydrodynamicity, is greatly exposed to water circulation and tides that increased the oxygenation. Dissolved oxygen concentrations usually ranged from 7.03 mg l^{-1} in summer months to 7.92 mg l^{-1} in winter months (Irato et al. 2007).

Experimental setup

Twenty panels, each with an exposed surface of 20 × 15 cm, were deployed in the study site separately installed every 60 cm along the mobile wharf. Each panel was vertically anchored with a nylon rope to a brick basement on the bottom and to the floating pier on the top so that the panel remained constantly submerged with tides by approximately 50 cm never contacting the bottom. The close binding with the rope maintained the vertical orientation of the panels to limit rotation and exposition, which could influence fouling colonisation by shading or floating disturbance. Therefore, all panels had the same light-exposed colonisable area of 300 cm^2, which was considered for observations and analysis of fouling settlement.

The substrata of the panels were larch wood and SS-316 stainless steel. Four substratum references were represented by two replicates of unpainted wood and stainless steel panels, respectively. The remaining 16 panels were pairs of replicates of 8 wooden and 8 stainless steel panels coated with four ('Paints A–D') copper(I)-based antifouling paints designed for boat hulls (Table 1). On each treated panel, three coats of antifouling paint were given (40–50 µm in thickness per coat) over a basis of one coat of primer (15 µm in thickness) to improve the adhesion of the antifouling paint. On reference panels, primer coating was omitted.

The paints were chosen because of their widespread utilisation in the North Adriatic Sea. With the exception of Paint B, which contained only copper(I) compounds and was based on hard-matrix technology, the other paints were based on self-polishing matrices and differed due to the booster biocide additives they contained. In particular, Paint A contained a mix of organotin (TBT) compounds, Paint C contained a fungicide

Table 1 Copper-based antifouling paints used to coat the panels submerged at the sampling station

Paint		Biocides	Cu (wt%)	Matrix	Cu leaching rate (µg cm^{-2} d^{-1})	Use
A	Sigmaplane HB Antifouling	Cu_2O (28 wt%) TBT methacrylate (19 wt%) TBTO (0.5 wt%)	24.8	Self-polishing copolymers	6.25	Steel and wooden hull of fishing boats and cargo vessels more than 25 m in length
B	Sikkens Vinyl Antifouling 2000	Cu_2O (41 wt%)	36.41	Contact leaching (hard or insoluble)	25.53	Steel (not aluminium), wooden and polyester hull of sailing boats and yachts
C	Sikkens Self-polishing Antifouling 2000	CuSCN (20 wt%) Dichlofluanid (9 wt%)	10.4	Self-polishing copolymers	12.24	Steel, aluminium wooden, and polyester hull of sailing boats and yachts
D	Baseggio Sirena Antivegetativa Universale	Cu_2O (42 wt%) Chlorothalonil (7 wt%) Irgarol 1051 (1.1 wt%)	37.3	Self-polishing copolymers	ND	Steel, wooden and fibreglass hull of fishing boats

ND not determined, i.e., no information reported in manufacturer's datasheet or literature

member of the sulfamide class (dichlofluanid), and Paint D contained both an s-triazine herbicide (Irgarol 1051) and an organochlorine fungicide (chlorothalonil).

The panels were submerged from March to January and monitored monthly over a period of 10 months beginning in April. Photographs of the panel surfaces were taken with a Nikon Coolpix 995 digital camera (Nikon Corporation, Tokyo, Japan). All the operations were performed on the panels, which were immediately and gently air-exposed to avoid withering, drying up, and destroying collapse of biofoulers. The analysis of the changes in ecological succession that occurred on the coated panels in comparison with the reference panels was carried out with a series of biodiversity descriptors. The 'species richness' was the total number of species, by month, present on all panels of the same type. The 'coverage-abundance area' was a quantitative analysis (percent cover) of the settlement capacity of the various species on panel areas calculated from digital photos using the Infinity Analyze Application v. 5.0.0 software (Lumenera Co. 2002–2009). The 'biocoenosis structure' was the percentage of coverage for each taxon, namely the set of species belonging to the same taxonomic group, in respect of the total coverage of the whole community (100%) on panels of the same type.

Data analysis

Statistical analysis were performed using PRIMER 6 (PRIMER-E Ltd, Plymouth, UK), and the level of significance was set at $p < 0.05$ for all statistical tests. To investigate significant differences among the covering surfaces of each fouling species on the various types of paint coating, the measures of the areas (cm^2) per month were compared using permutational multivariate analysis of variance (PERMANOVA plus; Anderson 2001) considering one fixed factor, i.e., the type of paint coating, and one random factor, i.e., the monitoring month. All analyses were carried out using 9999 permutations. To test the hypothesis that the species composition of the community on different types of paint coating was significantly different, an agglomerative hierarchical cluster analysis using Bray–Curtis dissimilarity was used considering the presence/absence data of species (Kruskal and Wish 1978). Bray–Curtis, clustering was performed with the R package 'clustsig' (Whitaker and Christman 2015). Communities that are similar in terms of their species composition have been clustered together and represented through a dendrogram.

Results

On both the wooden and steel panels coated with antifouling paints, delays in the appearance of primary biofilms were observed, so these panels lacked the settlement of any kind of macrofouling organism until June or July. Generally, the settlement inhibition was more evident on wooden panels coated with the antifouling paints, which showed a less number of species and, in the case of Paints A and D, the absence of any recognisable ecological succession than the corresponding stainless steel panels.

Features of the panel colonisation in April (first observation) and January (last observation) are showed in Fig. 2. Every detail of the trends and fluctuations of the coverage areas (Figs. 3 and 4), those of species richness (Fig. 5), and those of the biocoenosis structure, the latter considered on the wooden panels as a case in point (Fig. 6), over the experimental immersion are reported for comparative considerations.

Reference panels

The early colonisation arose from a few species of pioneer organisms (Fig. 2), which appeared in spring and included green algae as *Lychaete pellucida* (Hudson) Wynne, 2017 and *Ulva intestinalis* Linnaeus, 1753, red algae as *Ceramium ciliatum* Ducluzeau, 1806, and small calcareous tubeworms (serpulids) as *Janua heterostropha* (Montagu, 1803).

The coverage extension occurred in the summer months by a higher number of species. They were represented by green algae (*U. intestinalis* and *Ulva rigida* Agardh, 1823), serpulids (*J. heterostropha* and *Hydroides dianthus* (Verrill, 1873)), bush-like bryozoans (*Bugula neritina* (Linnaeus, 1758) and *Bugulina stolonifera* (Ryland, 1960)), and benthic tunicates, i.e., ascidians (*Ascidiella aspersa* (Müller, 1776), *Molgula socialis* Alder, 1863, *Ciona robusta* Hoshino & Tokioka, 1967, *Styela plicata* (Lesueur, 1823), *Botryllus schlosseri* (Pallas, 1766), *Botrylloides leachii* (Savigny, 1816), *Diplosoma listerianum* (Milne Edwards, 1841)).

In autumn, a competition for the substratum occurred. The main species involved were green algae (*U. rigida*), red algae (*C. ciliatum*, *Gracilariopsis longissima* Steentoft, Irvine & Farnham, 1995 and *Polysiphonia sertularioides* (Grateloup) Agardh, 1863), bivalves (*Mytilus galloprovincialis* Lamarck, 1819), barnacles (*Amphibalanus amphitrite* (Darwin, 1854)), and spotted sponge areas of *Aplysina aerophoba* (Nardo, 1833), *Halichondria panicea* (Pallas, 1766), *Haliclona (Reniera) cinerea* (Grant, 1826), and *Leucosolenia variabilis* Haeckel, 1870. Ascidians persisted both with solitary (*C. robusta*, *S. plicata*) and colonial (*B. schlosseri*, *B. leachii*) species until winter, when significant areas of settlement were recognisable (Fig. 2).

Comparing the wooden panels with the stainless steel panels, the early colonisation appears more represented on steel panels but the following coverage trend is similar (Fig. 3) with the exception of the absence of bivalves on stainless steel panels (Fig. 4). The peak corresponding to the maximum species richness occurred from August to

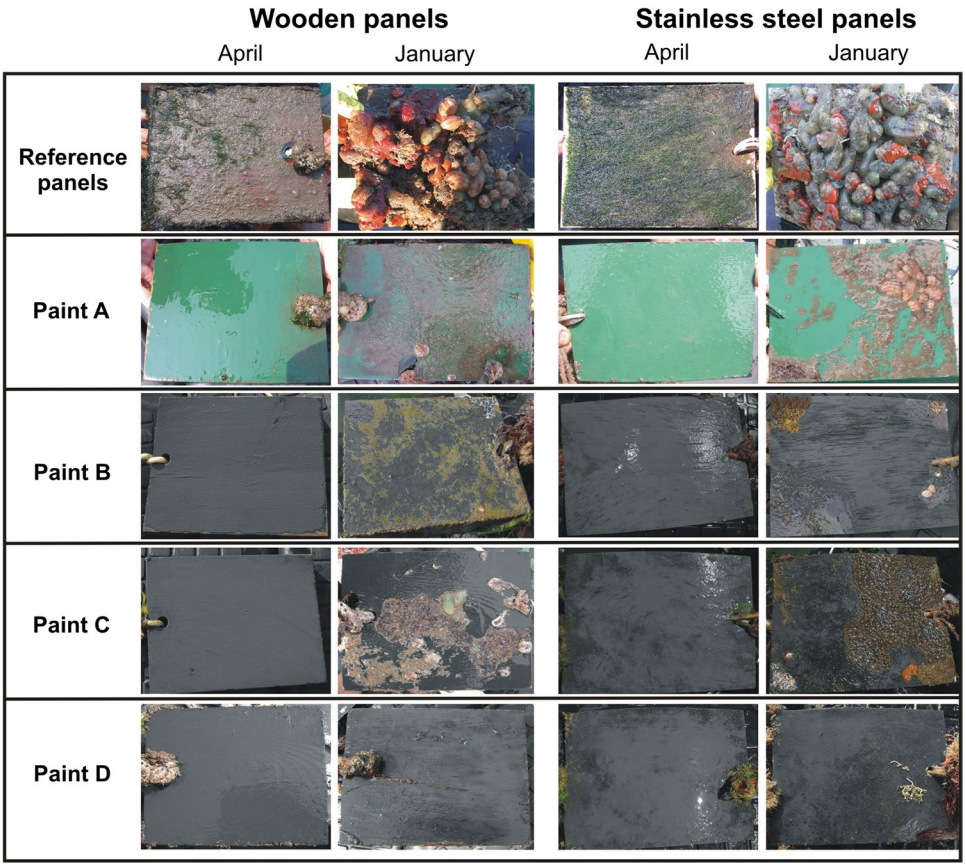


Fig. 2 Comparison between selected wooden (left) and stainless steel panels (right) at the beginning (i.e., April) and at the end (i.e., January) of the monitoring period in the Lagoon of Venice. For details on fouling composition, see Figs. 4 and 6

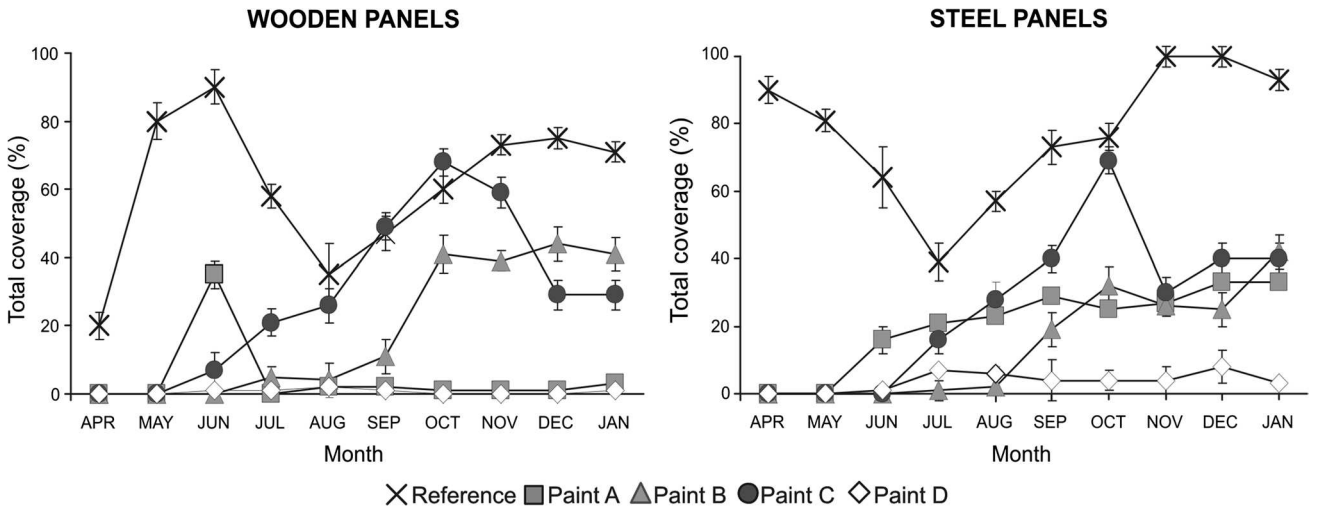
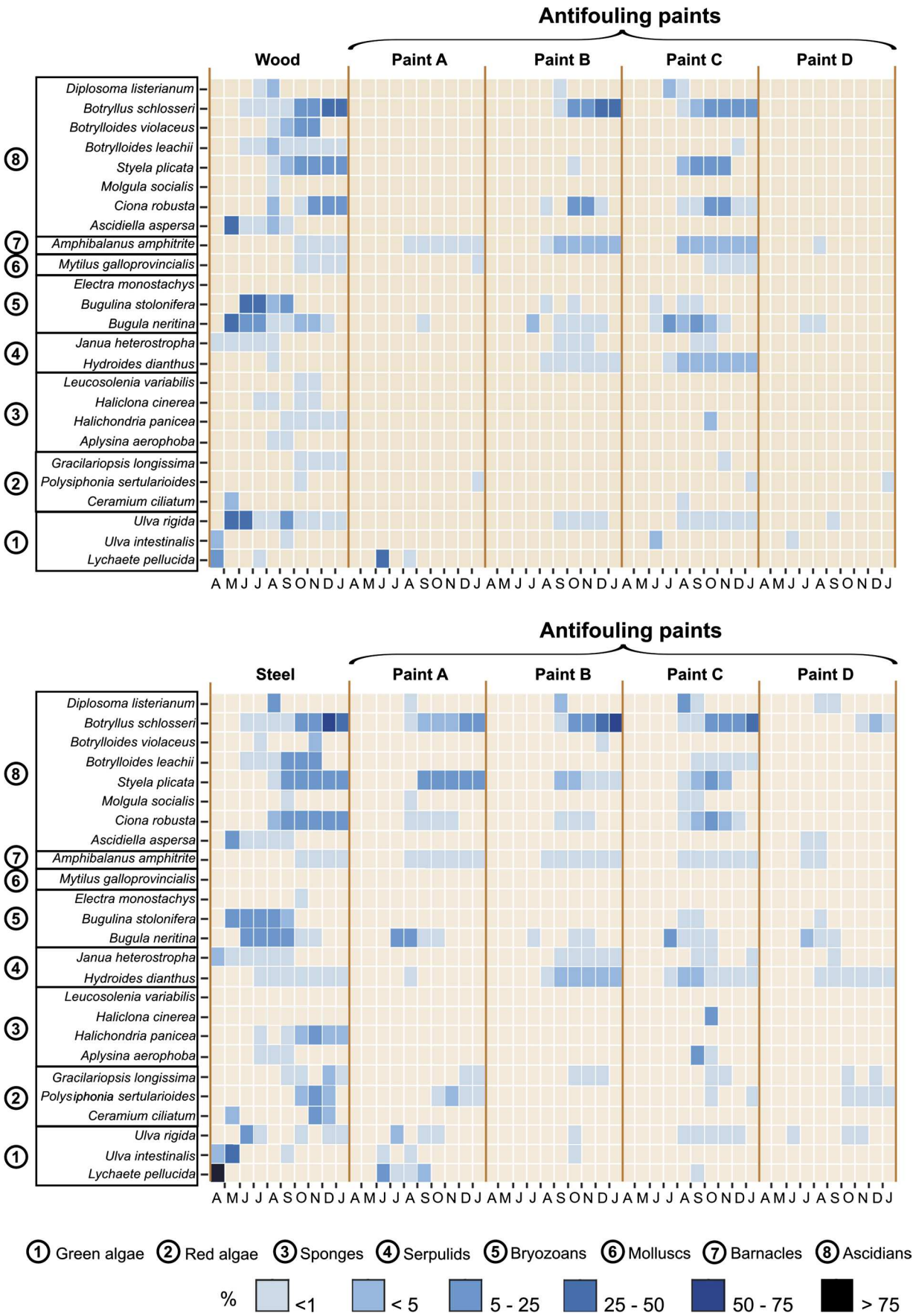


Fig. 3 Trend of percentage of total coverage dynamic monitored monthly as average ± standard deviation on replicates ($n = 2$) of the different panels

September on the wooden (value = 15) and stainless steel (value = 13) panels, respectively (Fig. 5), with biocoenosis structures formed of green algae, sponges, serpulids, bryozoans, and ascidians (Fig. 6).

Panels coated with antifouling Paint A

In June, the green alga *L. pellucida* appeared as the first pioneer species. Neither ecological succession (Fig. 3) nor


Antifouling paints
Wood
Paint A
Paint B
Paint C
Paint D
Diplosoma listerianum
Botryllus schlosseri
Botrylloides violaceus
Botrylloides leachii
Styela plicata
Molgula socialis
Ciona robusta
Ascidiella aspersa
Amphibalanus amphitrite
Mytilus galloprovincialis
Electra monostachys
Bugulina stolonifera
Bugula neritina
Janua heterostropha
Hydroides dianthus
Leucosolenia variabilis
Haliclona cinerea
Halichondria panicea
Aplysina aerophoba
Gracilariopsis longissima
Polysiphonia sertularioides
Ceramium ciliatum
Ulva rigida
Ulva intestinalis
Lychaete pellucida
8
7
6
5
4
3
2
1
A M J J A S O N D J A M J J A S O N D J A M J J A S O N D J A M J J A S O N D J A M J J A S O N D J
Antifouling paints
Steel
Paint A
Paint B
Paint C
Paint D
Diplosoma listerianum
Botryllus schlosseri
Botrylloides violaceus
Botrylloides leachii
Styela plicata
Molgula socialis
Ciona robusta
Ascidiella aspersa
Amphibalanus amphitrite
Mytilus galloprovincialis
Electra monostachys
Bugulina stolonifera
Bugula neritina
Janua heterostropha
Hydroides dianthus
Leucosolenia variabilis
Haliclona cinerea
Halichondria panicea
Aplysina aerophoba
Gracilariopsis longissima
Polysiphonia sertularioides
Ceramium ciliatum
Ulva rigida
Ulva intestinalis
Lychaete pellucida
8
7
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1
A M J J A S O N D J A M J J A S O N D J A M J J A S O N D J A M J J A S O N D J A M J J A S O N D J
1 Green algae 2 Red algae 3 Sponges 4 Serpulids 5 Bryozoans 6 Molluscs 7 Barnacles 8 Ascidians
% <1 < 5 5 - 25 25 - 50 50 - 75 > 75

◂**Fig. 4** Trend of the biodiversity descriptor 'coverage-abundance area' as the total percent area of each species measured monthly using photos of replicates ($n=2$) of the wooden (top scheme) and stainless steel (lower scheme) panels. Species are clustered in taxonomic groups (number in small ring)

stable biocoenosis structures (Fig. 6) were recognisable, and only the appearance of spotted areas colonised by organisms that are resistant to biocidal action, such as the red algae *P. sertularioides* and *G. longissima*, barnacles (*A. amphitrite*), and ascidians remained until the end of the observation period on stainless steel panels (Figs. 2 and 4). Generally, in the various studied months, the species richness was scarce (Fig. 5), with maximum values of 2 and 7 on the wooden and steel panels, respectively. PERMANOVA revealed significant negative effects for serpulid (*J. heterostropha*) and bryozoan (*B. stolonifera*) settlements on both wooden and steel panels (Tables 2 and 3). Negative effects were much more evident on the wooden panels than on the stainless steel panels due to the complete absence of the green alga *U. rigida*, the red alga *G. longissima*, serpulids (*H. dianthus*), sponges, and ascidians.

Panels coated with antifouling Paints B and C

On panels coated with Paints B and C, the observed ecological successions resulted in less coverage (Fig. 3) and less representative taxa (Fig. 6) compared with those observed on the reference panels. On all the panels, stable fouling organism coverage occurred beginning in July, when the biocide substance efficiency, which was higher in the first months of panel immersion, progressively decreased. Before July, only temporary organism settlements appeared, represented by the temporary presence of green algae or sponges and bryozoans. After July, the biocoenosis structures showed the presence of green and red algae, bryozoans, serpulids, barnacles, and ascidians. The latter, mainly including *C. robusta*, *S. plicata*, and botryllids, progressively extended their coverage areas until the winter months (Figs. 2 and 4).

Regarding Paint B, the peak species richness was observed in autumn (October), with maximum values of 7 and 9 on the wooden and stainless steel panels, respectively (Fig. 5). PERMANOVA showed significant inhibition of settlements of the red algae *G. longissima* and *P. sertularioides*, and of the ascidian *B. leachii* on both the wooden and stainless steel panels (Tables 2 and 3), and of the bivalve *M. galloprovincialis* on the wooden panels (Table 3).

Regarding Paint C, the peak species richness was observed in late summer (August–September), with maximum values of 9 and 12 on the wooden and stainless steel panels, respectively (Fig. 5). PERMANOVA did not reveal significant differences in types of species settlements between the coated and reference panels except for the late settlement of the serpulid *H. dianthus*, the bryozoan *B. neritina*, and the ascidian *B. schlosseri* on the stainless steel panels (Table 3). This exception was shared with Paint B and could be related to the scarce adhesion onto stainless steel panels, which caused a high leaching of the paints and a progressive increase of antifouling uncoated area favouring some taxa.

Panels coated with antifouling Paint D

Paint D showed strong antifouling action, as the macrofouling settlements on panels coated with these paints were transitory and very limited in both the coverage areas (Figs. 2, 3 and 4) and numbers of species (Fig. 5) throughout the entire observation period. The presence of species, mainly represented by macroalgae, bryozoans, and barnacles, never exceeded 1 month; often, primary biofilms again appeared and replaced the organisms that had previously settled. Therefore, the settlements appeared to be random and to lack any stabilised biocoenosis structure (Fig. 6). The species richness was much more limited on the wooden panels (maximum value = 1) than on the stainless steel panels (maximum value = 4) (Fig. 5). The PERMANOVA confirmed the negative effects of the paint on settlements of species belonging to serpulids, bryozoans, molluscs and ascidians (Tables 2 and 3).

Discussion

Modality and time of development of a biofouling community depend on climate, immersion site, and physico-chemical characteristics of both water column and substratum. A temporal succession, a seasonal succession, and a biotic succession of the principal taxa can be always distinguished (Redfield and Delvy 1952; Scheer 1945). In the present study, the ecological succession resulting from the monthly analysis of the reference panels from spring to winter was similar to those reported in previous research works confirming the trend of settlement and growth of macrofouling organisms of hard substrata in the Lagoon of Venice (Cornello and Manzoni 1999; Cornello and Occhipinti Ambrogi 2001; Cima and Ballarin 2013).

Generally, macrofouling was observed 2 months later on the panels treated with antifouling paints in comparison with the reference panels. The resulting areas of such settlements were much smaller in comparison with those of the reference panels, appearing as spotted distributions. As regard the entire period of monitoring, the mean percentages of coverage area reduction followed the order Paint D (98.79%) > Paint A (94.25%) > Paint B (71.84%) > Paint C (52.14%) on wood panels, and Paint D (94.4%) > Paint A

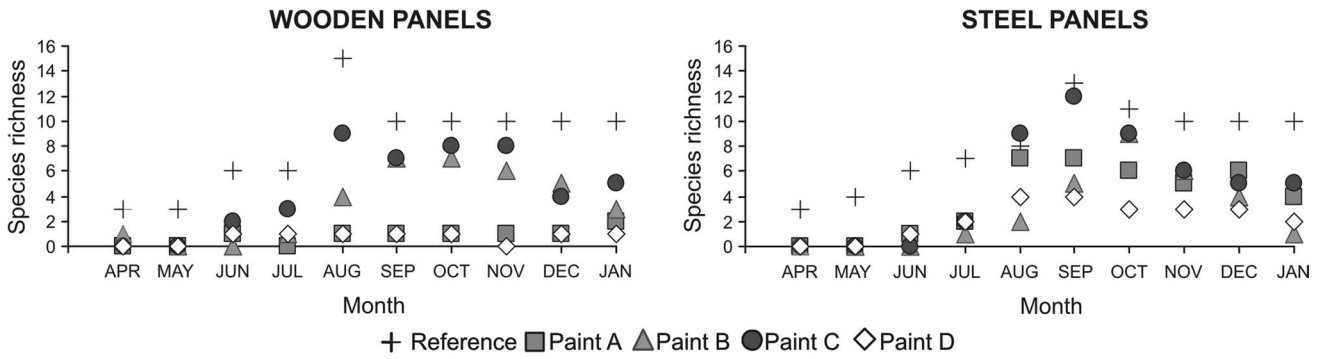

Fig. 5 Trend of the biodiversity descriptor 'species richness' considered as the total number of species found monthly on the replicates ($n = 2$) of the different panels

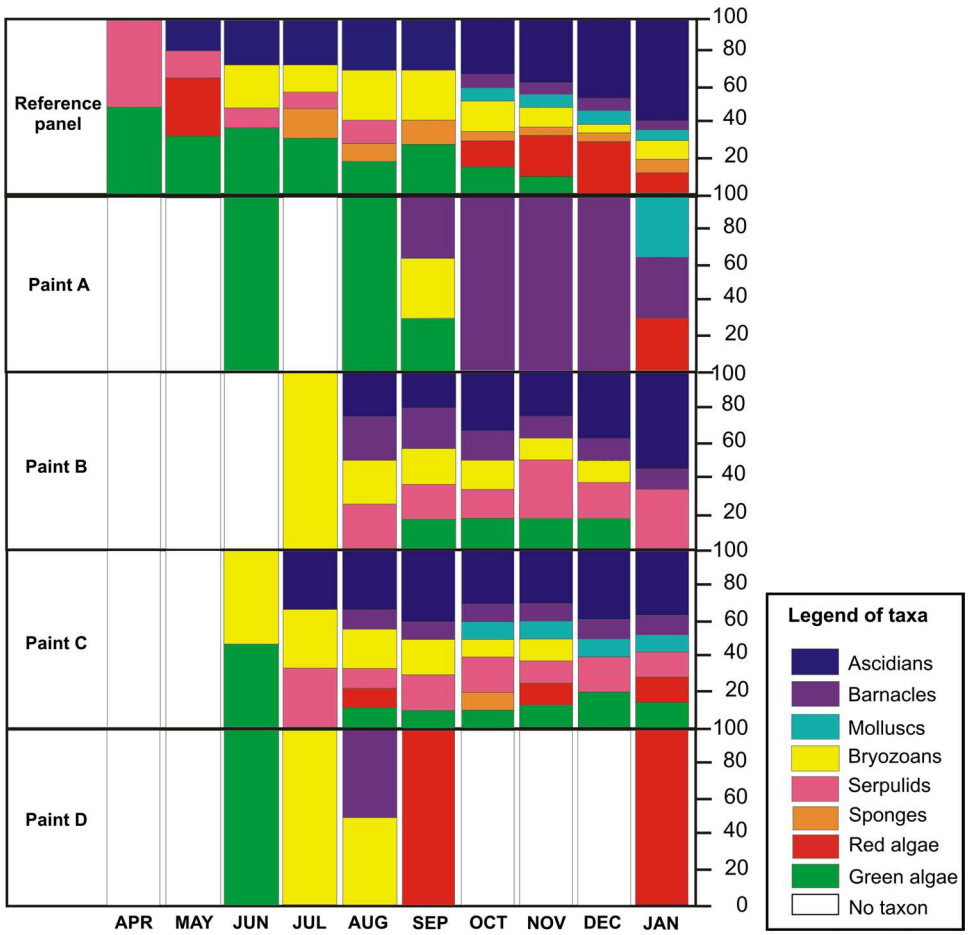

Fig. 6 Changes in the biodiversity descriptor 'biocoenosis structure' on the reference and paint-coated wooden panels throughout the experimental immersion in the Lagoon of Venice. The percent value of each taxonomic group is expressed as the total value obtained from the pool of two replicates

(71.27%) > Paint C (65.13%) > Paint B (54.9%) on stainless steel panels.

All antifouling paints significantly inhibited the settlement of the ascidian *A. aspersa* and the sponges *H. cinerea* and *L. variabilis* on the wooden panels and of the red alga *C. ciliatum* and the sponge *H. panicea* on the stainless steel panels. These results support previous negative effects of copper-containing antifouling paints observed on sponges (Rejeki et al. 2010), ascidians (Osborne et al. 2018), and red algae (Ytreberg et al. 2010).

The macrofouling communities observed on the various experimental panels coated with antifouling paints appeared dissimilar in taxa composition to those on the reference uncoated panels (Fig. 7). Paints A, B, and D generally inhibited the settlement of sponges. Both Paints A and D also inhibited serpulids and ascidians on wood

Table 2 PERMANOVA results of species coverage on wooden panels. For each species, pseudo-*F* values (indicated as *F*) and Monte Carlo *p*-values (indicated as $P_{(MC)}$) are reported for 'month', 'antifouling paint', and 'month x antifouling paint interaction'. Statistically significant effects ($p < 0.05$) are indicated in bold

Species	Month (MO)	Antifouling paint (AP)	MO × AP
Lychaete pellucida	$F_{(9.99)} = 1.411$ $P_{(MC)} = 0.292$	$F_{(8.99)} = 1.001$ $P_{(MC)} = 441$	$F_{(72.99)} = 1.479$ $P_{(MC)} = 0.249$
Ulva intestinalis	$F_{(9.99)} = 0.389$ $P_{(MC)} = 0.912$	$F_{(8.99)} = 0.928$ $P_{(MC)} = 0.497$	$F_{(72.99)} = 0.312$ $P_{(MC)} = 0.998$
Ulva rigida	$F_{(9.99)} = 3.944E-2$ $P_{(MC)} = 1$	$F_{(8.99)} = 4.728$ $P_{(MC)} = \mathbf{0.0003}$	$F_{(72.99)} = 7.3717E-2$ $P_{(MC)} = 1$
Ceramium ciliatum	$F_{(9.99)} = 7.6253E-2$ $P_{(MC)} = 0.999$	$F_{(8.99)} = 0.854$ $P_{(MC)} = 0.555$	$F_{(72.99)} = 0.126$ $P_{(MC)} = 1$
Polysiphonia sertularioides	$F_{(9.99)} = 1.137$ $P_{(MC)} = 0.415$	$F_{(8.99)} = 1.960$ $P_{(MC)} = 0.061$	$F_{(72.99)} = 0.182$ $P_{(MC)} = 1$
Gracilariopsis longissima	$F_{(9.99)} = 0.185$ $P_{(MC)} = 0.988$	$F_{(8.99)} = 2.324$ $P_{(MC)} = \mathbf{0.0259}$	$F_{(72.99)} = 0.139$ $P_{(MC)} = 1$
Aplysina aerophoba	$F_{(9.99)} = 0.267$ $P_{(MC)} = 0.970$	$F_{(8.99)} = 1.9756$ $P_{(MC)} = 0.063$	$F_{(72.99)} = 0.455$ $P_{(MC)} = 0.969$
Halichondria panicea	$F_{(9.99)} = 3.719$ $P_{(MC)} = \mathbf{0.025}$	$F_{(8.99)} = 0.9$ $P_{(MC)} = 0.531$	$F_{(72.99)} = 2.222$ $P_{(MC)} = 0.082$
Haliclona cinerea	$F_{(9.99)} = 2.6144E-2$ $P_{(MC)} = 1$	$F_{(8.99)} = 13.5$ $P_{(MC)} = \mathbf{0.0001}$	$F_{(72.99)} = 4.4444E-2$ $P_{(MC)} = 1$
Leucosolenia variabilis	$F_{(9.99)} = 3.9216E-2$ $P_{(MC)} = 1$	$F_{(8.99)} = 6$ $P_{(MC)} = \mathbf{0.0001}$	$F_{(72.99)} = 6.6667E-2$ $P_{(MC)} = 1$
Hydroides dianthum	$F_{(9.99)} = 0$ $P_{(MC)} = 0$	$F_{(8.99)} = 6.677$ $P_{(MC)} = \mathbf{0.0001}$	$F_{(72.99)} = 0$ $P_{(MC)} = 0$
Janua heterostropha	$F_{(9.99)} = 0.109$ $P_{(MC)} = 0.999$	$F_{(8.99)} = 2.711$ $P_{(MC)} = \mathbf{0.011}$	$F_{(72.99)} = 0.158$ $P_{(MC)} = 1$
Bugula neritina	$F_{(9.99)} = 0.195$ $P_{(MC)} = 0.989$	$F_{(8.99)} = 5.402$ $P_{(MC)} = \mathbf{0.0001}$	$F_{(72.99)} = 0.138$ $P_{(MC)} = 1$
Bugulina stolonifera	$F_{(9.99)} = 5.5438E-2$ $P_{(MC)} = 1$	$F_{(8.99)} = 3.812$ $P_{(MC)} = \mathbf{0.001}$	$F_{(72.99)} = 8.774\ E-2$ $P_{(MC)} = 1$
Mytilus galloprovincialis	$F_{(9.99)} = 0.424$ $P_{(MC)} = 0.889$	$F_{(8.99)} = 2.71$ $P_{(MC)} = \mathbf{0.0104}$	$F_{(72.99)} = 0.186$ $P_{(MC)} = 1$
Amphibalanus amphitrite	$F_{(}\ F_{(9.99)} = 0$ $P_{(MC)} = 0$	$F_{(8.99)} = 5.589$ $P_{(MC)} = \mathbf{0.0001}$	$F_{(72.99)} = 0$ $P_{(MC)} = 0$
Ascidiella aspersa	$F_{(9.99)} = 5.5099E-2$ $P_{(MC)} = 1$	$F_{(8.99)} = 1.676$ $P_{(MC)} = \mathbf{0.003}$	$F_{(72.99)} = 9.3668E-2$ $P_{(MC)} = 1$
Ciona robusta	$F_{(9.99)} = 1.566$ $P_{(MC)} = 0.250$	$F_{(8.99)} = 6.107$ $P_{(MC)} = \mathbf{0.0002}$	$F_{(72.99)} = 0.414$ $P_{(MC)} = 0.985$
Molgula socialis	$F_{(9.99)} = 5.8824E-2$ $P_{(MC)} = 0.999$	$F_{(8.99)} = 1$ $P_{(MC)} = 0.445$	$F_{(72.99)} = 0.1$ $P_{(MC)} = 1$
Styela plicata	$F_{(9.99)} = 31.803$ $P_{(MC)} = \mathbf{0.0001}$	$F_{(8.99)} = 5.900$ $P_{(MC)} = \mathbf{0.0001}$	$F_{(72.99)} = 22.147$ $P_{(MC)} = \mathbf{0.0001}$
Botrylloides leachii	$F_{(9.99)} = 3.9216E-2$ $P_{(MC)} = 1$	$F_{(8.99)} = 10.907$ $P_{(MC)} = \mathbf{0.0001}$	$F_{(72.99)} = 5.5729E-2$ $P_{(MC)} = 1$
Botrylloides violaceus	$F_{(9.99)} = 3.405E-2$ $P_{(MC)} = 0.999$	$F_{(8.99)} = 8.275$ $P_{(MC)} = \mathbf{0.0001}$	$F_{(72.99)} = 5.7884E-2$ $P_{(MC)} = 1$
Botryllus schlosseri	$F_{(9.99)} = 3.007$ $P_{(MC)} = 0.052$	$F_{(8.99)} = 4.869$ $P_{(MC)} = \mathbf{0.0003}$	$F_{(72.99)} = 0.806$ $P_{(MC)} = 0.718$
Diplosoma listerianum	$F_{(9.99)} = 1.371$ $P_{(MC)} = 0.314$	$F_{(8.99)} = 0.941$ $P_{(MC)} = 0.491$	$F_{(72.99)} = 1.146$ $P_{(MC)} = 0.439$

panels but only serpulids on steel panels, and Paint D prevented molluscs on wood panels as well. Paint B inhibited red algae and molluscs on wood panels. Overall, the most tolerant taxa to these antifouling paints were represented by green algae, bryozoans and barnacles, which were found on all panels. This observation confirms previous study on taxa with the most tolerance to copper, in particular the genus *Enteromorpha* (Correa et al. 1996), the bryozoan *B. neritina* (Piola and Johnston 2006), and the barnacle *A. amphitrite* (Qiu et al. 2005). Based on the dissimilarity of the communities, the comparison of the negative effects can lead to orders of antifouling efficiency

Table 3 PERMANOVA results of species coverage on stainless steel panels

Species	Month (MO)	Antifouling paint (AP)	MO × AP
Lychaete pellucida	$F_{(9.99)}=6.0166E-2$ $P_{(MC)}=0.999$	$F_{(8.99)}=0.878$ $P_{(MC)}=0.535$	$F_{(72.99)}=0.111$ $P_{(MC)}=1$
Ulva intestinalis	$F_{(9.99)}=5.4926E-2$ $P_{(MC)}=0.999$	$F_{(8.99)}=1.129$ $P_{(MC)}=0.347$	$F_{(72.99)}=0.100$ $P_{(MC)}=1$
Ulva rigida	$F_{(9.99)}=0.425$ $P_{(MC)}=0.893$	$F_{(8.99)}=1.170$ $P_{(MC)}=0.325$	$F_{(72.99)}=0.440$ $P_{(MC)}=0.978$
Punctaria latifolia	$F_{(9.99)}=0$ $P_{(MC)}=0$	$F_{(8.99)}=1$ $P_{(MC)}=0.445$	$F_{(72.99)}=0$ $P_{(MC)}=0$
Ceramium ciliatum	$F_{(9.99)}=5.2288E-2$ $P_{(MC)}=0.999$	$F_{(8.99)}=2.25$ $P_{(MC)}=\mathbf{0.035}$	$F_{(72.99)}=8.8889E-2$ $P_{(MC)}=1$
Polysiphonia sertularioides	$F_{(9.99)}=0.325$ $P_{(MC)}=0.944$	$F_{(8.99)}=2.051$ $P_{(MC)}=\mathbf{0.048}$	$F_{(72.99)}=0.100$ $P_{(MC)}=1$
Gracilariopsis longissima	$F_{(9.99)}=0.163$ $P_{(MC)}=0.995$	$F_{(8.99)}=2.117$ $P_{(MC)}=\mathbf{0.046}$	$F_{(72.99)}=8.874E-2$ $P_{(MC)}=1$
Aplysina aerophoba	$F_{(9.99)}=13.804$ $P_{(MC)}=\mathbf{0.0002}$	$F_{(8.99)}=1.242$ $P_{(MC)}=0.290$	$F_{(72.99)}=8.701$ $P_{(MC)}=\mathbf{0.0006}$
Halichondria panicea	$F_{(9.99)}=0.821$ $P_{(MC)}=0.619$	$F_{(8.99)}=3.330$ $P_{(MC)}=\mathbf{0.003}$	$F_{(72.99)}=0.759$ $P_{(MC)}=0.763$
Hydroides dianthum	$F_{(9.99)}=8.555$ $P_{(MC)}=\mathbf{0.001}$	$F_{(8.99)}=5.561$ $P_{(MC)}=\mathbf{0.0001}$	$F_{(72.99)}=1.330$ $P_{(MC)}=0.322$
Janua heterostropha	$F_{(9.99)}=0.811$ $P_{(MC)}=0.618$	$F_{(8.99)}=2.287$ $P_{(MC)}=\mathbf{0.032}$	$F_{(72.99)}=0.474$ $P_{(MC)}=0.964$
Bugula neritina	$F_{(9.99)}=6.495$ $P_{(MC)}=\mathbf{0.0034}$	$F_{(8.99)}=2.509$ $P_{(MC)}=\mathbf{0.016}$	$F_{(72.99)}=1.229$ $P_{(MC)}=0.388$
Bugulina stolonifera	$F_{(9.99)}=0.183$ $P_{(MC)}=0.991$	$F_{(8.99)}=7.084$ $P_{(MC)}=\mathbf{0.0001}$	$F_{(72.99)}=0.263$ $P_{(MC)}=0.999$
Electra monostachys	$F_{(9.99)}=5.8824E-2$ $P_{(MC)}=0.999$	$F_{(8.99)}=1$ $P_{(MC)}=0.451$	$F_{(72.99)}=0.1$ $P_{(MC)}=1$
Amphibalanus amphitrite	$F_{(9.99)}=0$ $P_{(MC)}=0$	$F_{(8.99)}=3.886$ $P_{(MC)}=\mathbf{0.0008}$	$F_{(72.99)}=0$ $P_{(MC)}=0$
Ascidiella aspersa	$F_{(9.99)}=5.4155E-2$ $P_{(MC)}=0.999$	$F_{(8.99)}=1.862$ $P_{(MC)}=0.079$	$F_{(72.99)}=9.2063E-2$ $P_{(MC)}=1$
Ciona robusta	$F_{(9.99)}=0.635$ $P_{(MC)}=0.745$	$F_{(8.99)}=6.819$ $P_{(MC)}=\mathbf{0.0001}$	$F_{(72.99)}=0.317$ $P_{(MC)}=0.998$
Molgula socialis	$F_{(9.99)}=1.313$ $P_{(MC)}=0.337$	$F_{(8.99)}=0.681$ $P_{(MC)}=0.708$	$F_{(72.99)}=0.697$ $P_{(MC)}=0.812$
Styela plicata	$F_{(9.99)}=1.423$ $P_{(MC)}=0.297$	$F_{(8.99)}=6.655$ $P_{(MC)}=\mathbf{0.0001}$	$F_{(72.99)}=0.296$ $P_{(MC)}=0.998$
Botrylloides leachii	$F_{(9.99)}=0.126$ $P_{(MC)}=0.997$	$F_{(8.99)}=3.086$ $P_{(MC)}=\mathbf{0.0047}$	$F_{(72.99)}=0.131$ $P_{(MC)}=1$
Botrylloides violaceus	$F_{(9.99)}=2.150$ $P_{(MC)}=0.125$	$F_{(8.99)}=1.246$ $P_{(MC)}=0.291$	$F_{(72.99)}=3.830$ $P_{(MC)}=\mathbf{0.012}$
Botryllus schlosseri	$F_{(9.99)}=7.785$ $P_{(MC)}=\mathbf{0.002}$	$F_{(8.99)}=2.617$ $P_{(MC)}=\mathbf{0.013}$	$F_{(72.99)}=1.0389$ $P_{(MC)}=0.520$
Diplosoma listerianum	$F_{(9.99)}=0.720$ $P_{(MC)}=0.677$	$F_{(8.99)}=1$ $P_{(MC)}=0.434$	$F_{(72.99)}=0.283$ $P_{(MC)}=0.999$

and consequent disruptive potentials on native community of the copper-based paints examined herein as follows: Paint D ≥ Paint A > Paint B > Paint C for wooden panels, and Paint B ≥ Paint D > Paint A > Paint C for stainless steel panels. A higher dissimilarity due to a higher performance of antifouling paints was in general present for communities on wooden panels than on stainless steel panels. The more extensive coverage of biofoulers on stainless steel panels than on wooden panels is far difficult to interpret. Different adhesion capability of antifouling paints on different types of substrata and the progressive wearing off of the paint coating must be considered, but other factors could be involved. For example, the surface of larch wood is rough even when it has been smoothed, and the roughness increases with the duration in the water because of the action of both swell and organisms. Rough and smooth

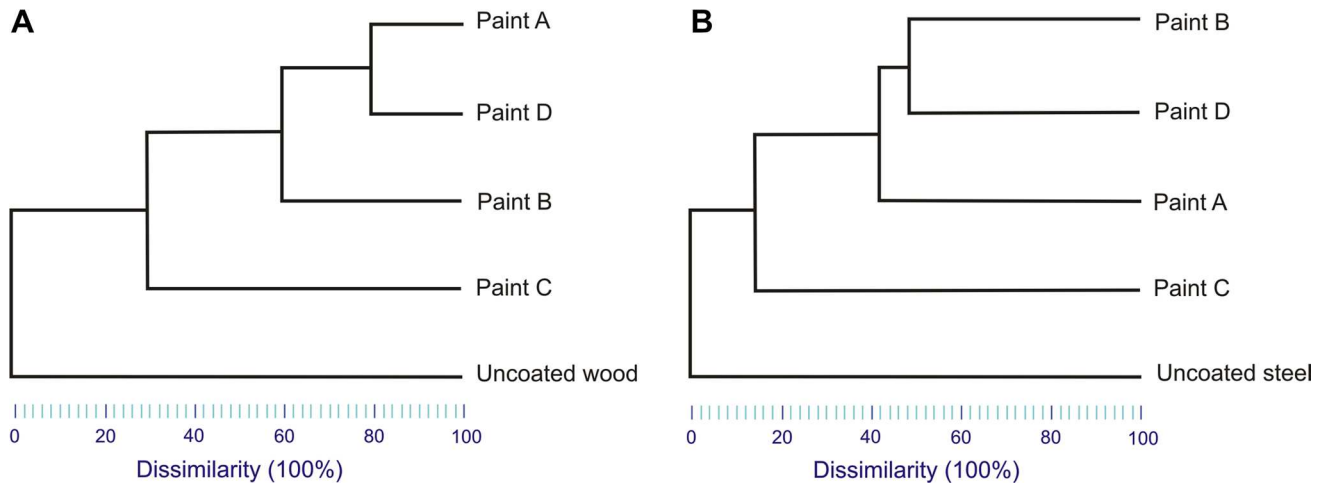

Fig. 7 Dendrograms obtained from cluster analysis using the Bray–Curtis percentage dissimilarity value of assemblages based on the presence or absence of taxonomic groups for all coating treatments. Clusters were obtained from pooled sets of species belonging to the same taxonomic groups settled on wooden (**A**) and stainless steel (**B**) panels throughout the 10-month experimental immersion in the Lagoon of Venice

substrata often show differences in composition of benthic communities in comparison with smooth substrata like metal or plastic panels. These differences could be due to the selection of the various types of potential biofoulers on the basis of their settlement capability on the different microtopography, wettability and nanostructures of the substrata (Johnson 1994; Commito and Rusignuolo 2000; Hirose and Sensui 2021). Moreover, a primary bacterial film on a metal surface immersed in natural waters could create electrochemical conditions which accelerate metal corrosion (Dexter 1993). Microbiologically influenced corrosion and marine biofouling are closely related to the biofilms in dictating the subsequent biofouler settlement (Li and Ning 2019), although the response of fouling organisms to materials is not universal and different taxa can be differently affected depending on physicochemical factors at the metal surface. As a result, metallic substrata are more susceptible to biofouling settlement than some non-metallic substrata such as fibreglass, wood and plastics (Pomerat and Weiss 1946).

The efficiency of both Paint A and Paint D was assessed by the fact that no ecological succession was recognisable as well as few taxa that were temporary resistant to biocidal action appeared several times with limited coverage. Paint D demonstrated lasting biocidal power, which was higher than that of the TBT-based Paint A because the taxa settlements always appeared to be random and never stabilised. This particular antifouling effect may be related to both the high copper(I) oxide content of the paint and the synergistic effect with boosters. Chlorothalonil is toxic to various aquatic invertebrates (Cima et al. 2008; Dumollard et al. 2017; Amara et al. 2018), and Irgarol 1051 is a photosynthesis-inhibiting herbicide used in agriculture and therefore able to withstand seaweed growth (Dahl and Blanck 1996; Hall et al. 1999; Johansson et al. 2012).

Regarding Paints B and C, in the first 3–4 months of the study period the few taxa settled with spotted areas forming not-well structured communities. However, beginning in the summer months the taxa settled on the panels coated with both paints showed increasing coverage areas on the antifouling-coated panels, suggesting a time-dependent loss of efficiency. The scarce effects observed in the case of Paint B – a unique paint based on contact-leaching technology with a high leaching rate and containing only copper(I) oxide without boosters – on species settlement and growth supported the importance of both the type of matrix with biocidal exposure and the co-presence of boosters for enlarging the performance of copper-containing paints. Antifouling life is not simply a function of product formulation, but also of the system application, the biocide package in the matrix, the biocide reservoir within the paint, and the biocidal leaching rate, the latter responsible of a shortening efficiency when it is too high (Ivče et al. 2020). With time, the soluble biocidal compounds are released from the contact leaching paints, leaving behind a depleted, porous matrix. As this depleted layer increases in thickness, the diffusion of biocides from the paint film decreases exponentially over time and effective life is limited (Zhou 2015). The low efficiency of Paint C could be related to the different copper(I) compound (CuSCN) contained in this paint type and/or its lower concentrations compared to those in Cu_2O-based paints. The presence of a less effective booster, such as dichlofluanid (Konstantinou and Albanis 2004), could also be considered as a cause of this result. The scarce antifouling

effect of Paint C is an example of the paradox regarding balancing the development of paints with low copper contents and compliance with EU Regulation 1143/2014 concerning the need to prevent the growth and minimise the transport of invasive alien species on hulls of pleasure craft (AMOG Consulting 2001).

Conclusion

Copper-based paints mainly differ in their copper(I) concentrations, presence of booster biocides, and type of matrix (i.e., contact leaching or self-polishing). Their commercial formulations must meet with two requirements, which might seem in contrast for the impossibility to develop targeted systems: to have the most efficient antifouling properties for organisms that settle on immersed structures and simultaneously be safe for non-target marine organisms (Rossini et al. 2019).

Copper compounds were considered ideal for use as antifoulants since the chemistry of copper changes in the marine environment, thereby affecting its bioavailability (and therefore its toxicity) by rapid organic complexation. A key factor in the environmental aspect of copper-based antifoulants is that the active ionic form of copper exists only briefly while it is on the coated surface. During that time, it performs its efficacy by impeding the settlement of fouling organisms. However, the co-presence of organic booster biocides and the type of polymeric matrix can significantly increase the performance of the paint. The synergistic effects of these compounds are still largely unknown together with their behaviour and fate in the coastal ecosystems and their potential to cause deleterious effects on various benthic organisms (Brooks and Waldock 2009).

Although antifouling products are regulated under the Biocidal Products Regulation in the EU (BPR, EU Regulation 528/2012) as regard their application, production, and marketing, most EU countries have not developed any restrictions or bans regarding the use of copper-based antifouling paints (Lagerström et al. 2020). Some countries have independently limited the emissions of copper from antifouling paints in their coastal waters, or the application of paints with copper biocides has been restricted only to recreational craft. Considering the wide use of these antifouling paints together with the continuous leaching of biocidal substances into the seawater column and sediments, the dangerousness of these xenobiotic compounds to coastal communities — in particular to fragile ecosystems such as those of the Lagoon of Venice — must be considered. In this study, results with copper-based paints revealed unpredictable effects on the settlement and growth of key species of macrofouling of hard-substrata, which have the potential to alter the native structure of the communities. Continue pollution of copper antifoulants can cause a reduction of diversity and has differential effects on recruitment of more tolerant non-indigenous species increasing the invader dominance (Dafforn et al. 2008; Piola and Johnston 2008). Consequently, greater control and monitoring of risk assessments before the introduction of so-called 'eco-friendly' copper-based formulations in commerce is critically essential to avoid long-term irreversible changes on benthic populations and coastal trophic networks. The problem of limiting the release of copper into the environment should be solved by researching new technologies at the matrix level. The amount of copper in the formulation of the traditional paint could be significantly reduced with a more resistant matrix useful to both prevent the rapid oxidation of copper and long-time maintain the antifouling action by contact with biofoulers. In such way, the antifouling action should be limited to the surfaces to be protected without leaching of biocides into the water column causing a minor impact on coastal ecosystems. An alternative system could be represented by electrochemical uptake and release of copper ions from natural seawater avoiding the continuous copper leaching from the matrix and minimising the environmental load of the biocide (Elmas et al. 2020, 2021).

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Data availability All data generated or analysed during this study are included in this published article.

Declarations

Ethical approval The authors followed all applicable international, national, and/or institutional guidelines for the care and use of animals.

Consent for publication Not applicable.

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4.1.2 Study at individual level

The environmental risk of antifouling compounds is a function of both their environmental concentration, their bioaccumulation in the trophic chains and the mechanism of action of their toxicity in both target and non-target species.

As an experimental model to evaluate the risks of new antifouling compounds, alternative to organotins through the study of their effects on larval settlement and metamorphosis on principal macrofouling taxa such as tunicates, I used the colonial ascidian *B. schlosseri*. This species is worldwide dominant in the soft-fouling of the hard-substratum benthic community and commonly reared in our laboratory, where the entire life-cycle can be followed in controlled experimental conditions.

I first studied in laboratory the settlement and metamorphosis ability of larvae in the presence of glass slides coated with various trade paints. All antifouling paints showed high performance, causing 100% mortality and metamorphic inhibition, with >75% not-settled dead larvae. In particular, paints containing ZnP + Sea-Nine 211, or CuSCN + Dichlofluanid, or Cu2O + Irgarol 1051 + Chlorothalonil prevented the larval adhesion. Paints containing Sea-Nine 211, TCMS pyridine + Diuron, or ZnP + Zineb + Endosulfan allowed the larval adhesion, but prevented metamorphosis. Paints containing metals and organometals like Cu(I) and TBT, partially allowed the adhesion and the metamorphosis, but killed the oozooids.

In a second series of experiments, I evaluated the effects of various biocidal concentrations on settlement and metamorphosis ability of exposed larvae. Results revealed that all antifouling biocides prevented the settlement of larvae and interfered with the metamorphosis causing developmental delays, malformations and mortality. All were lethal with the exception of TCMS pyridine and the herbicides Diuron and Irgarol 1051, which did not kill the larvae, but prevented their settlement on the substratum and inhibited their metamorphosis.

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Effects of Exposure to Trade Antifouling Paints and Biocides on Larval Settlement and Metamorphosis of the Compound Ascidian *Botryllus schlosseri*

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Abstract: To evaluate the effects of antifouling paints and biocides on larval settlement and metamorphosis, newly hatched swimming larvae of the compound ascidian *Botryllus schlosseri*, a dominant species of soft-fouling in coastal communities, were exposed to (i) substrata coated with seven antifouling paints on the market containing different biocidal mixtures and types of matrices and (ii) sea water containing various concentrations of eight biocidal constituents. All antifouling paints showed high performance, causing 100% mortality and metamorphic inhibition, with ≥75% not-settled dead larvae. All antifouling biocides prevented the settlement of larvae. The most severe larval malformations, i.e., (i) the formation of a bubble encasing the cephalenteron and (ii) the inhibition of tail resorption, were observed after exposure to metal and organometal compounds, including tributyltin (TBT) at 1 µM (325.5 µg L^{-1}), zinc pyrithione (ZnP) at 1 µM (317.7 µg L^{-1}), and CuCl at 0.1 µM (98.99 µg L^{-1}), and to antimicrobials and fungicides, including Sea-Nine 211 at 1 µM (282.2 µg L^{-1}) and Chlorothalonil at 1 µM (265.9 µg L^{-1}). The herbicides seemed to be less active. Irgarol 1051 was not lethal at any of the concentrations tested. Diuron at 250 µM (58.2 mg L^{-1}) and 2,3,5,6-tetrachloro-4-(methylsulphonyl)pyridine (TCMS pyridine) at 50 µM (14.8 mg L^{-1}) completely inhibited larval metamorphosis. These results may have important implications for the practical use of different antifouling components, highlighting the importance of their testing for negative impacts on native benthic species.

Keywords: ascidians; antifouling paints; *Botryllus schlosseri*; booster biocides; EC_{50}; fouling settlement; larval toxicity; metamorphosis; tunicates

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1. Introduction

Ascidians are the most common members of the urochordate subphylum. They are the closest relatives to vertebrates, and are the only chordates able to reproduce both sexually and asexually [1]. During the larval stage, known as the "tadpole" stage, they share with vertebrates the same body plan in the tail formed of a dorsal notochord and a tubular nervous system, both flanked by striated muscle. They are sessile occupants of hard substrata in coastal environments, where they often represent the dominant component of soft-fouling. As worldwide filter-feeding organisms living at the water–sediment interface, many solitary and compound species are considered useful bioindicators of various environmental pollutants. The developmental biology of ascidians with particular attention to both the larval stage, which is the dispersal and colonisation stage of the life cycle, and its metamorphosis have been studied extensively [2–5]. After a short dispersal period, during which the ascidian larvae actively swim, moving the tail according to a positive phototropism and a negative geotropism, the larvae select a substratum and attach to it following a photo- and geotropism reversal. They explore and contact the substratum by means of a series of rapid touches with the anterior area of the cephalenteron, which contains special sensory structures, namely "papillae". These structures, protruding from

the anterior epidermis and from which an adhesive substance is secreted, act as a control centre for the initiation of metamorphosis and are fundamental for larval settlement. Recently, many inducers of metamorphosis have been identified in papillae as specific gene expressions, growth factors, several neurotransmitters, and transient Ca^{2+} signals [6–8]. The temporary settlement with papillae is rapidly substituted by a stable settlement due to the protrusion of anterior blind-sac vessels, namely "ampullae", which expand onto the substratum and secrete a glue substance from an apical glandular epithelial. At the same time, tail resorption occurs with complete dismantling of the axial complex. Successively, metamorphosis continues inside the cephalenteron with the visceral rotation and the dismantling of other larval structures, such as ocellus and statocyst. Finally, the adult organ primordia complete the development, and the opening of both siphons occurs and the juvenile begins to filter-feed.

Generally, ascidians are pre-disposed to rapid and competitive colonisation of hard substrata. However, geographic invasion and the impact of fouling by ascidians on shipping, aquaculture, pleasure boating, oil and gas installations, and other industries are significant, with numerous species responsible for infesting anthropogenic structures [9]. Although the larval phase is short (24–48 h), the global spread is favoured by translocation via shipping since the hull fouling can be considered the most important vector. The massive ascidian fouling is potentially responsible for physical, economical, and ecological damage since it concerns the coverage of clean surfaces, loss of efficiency of submerged pipelines and harbour/industrial structures, infestation of shellfish and finfish aquacultures, increased fuel consumption during boat navigation, and decreased biodiversity in benthic communities.

For these reasons, beginning from the second half of the 1960s, antifouling compounds have been massively introduced in the formulation of paints to prevent the settlement of the most problematic foulers on submerged structures, such as ship's hulls and propellers, buoys, wharves, and platforms. The most successful antifouling paints at the time contained organotin compounds as biocides, mainly represented by tributyltin (TBT), triphenyltin (TPT), and their derivatives. These compounds proved to be harmful to the benthic marine communities, as they caused severe impacts on the oyster aquaculture and were persistent in the environment in the long term [10–13]. After the total ban on organotin compounds by the International Marine Organisation—Marine Environment Protection Committee (IMO-MEPC) in 1998, and subsequently by the Ordinance No. 782/2003, 14 April 2003, of the European Commission, the paint industry developed substitutive tin-free formulations. The new paint formulations mainly contained biocidal combinations of specific synthesis, e.g., Sea-Nine 211, or pesticides coming from the pharmacology industry (antimicrobials) or agriculture (herbicides, fungicides, insecticides). The aim of these formulations was not only to prevent the settlement of algal propagules and invertebrate larvae of macrofoulers, but also the formation of bacterial and microalgal microfilm, or "biofilm", from which the ecological succession of the hard-substratum community begins.

Because of this choice, a number of substances (Table 1) are at present in various commercial formulations of new-generation antifouling paints. The biocidal compounds play various roles, i.e., as alternatives to organotin compounds or as boosters, with the latter serving to increase the toxic performance of the antifouling paints towards a wider spectrum of fouling organisms. Before the introduction of these compounds in paint formulations, tests of acute and chronic toxicity were performed only on laboratory mammals and freshwater model fish. As a consequence, many new contaminants with potential accumulation and deleterious effects on coastal communities have been introduced worldwide from both direct and indirect pollution sources, which have followed the increase in productivity of agro-industrial, tourism, and commercial shipping sectors.

Table 1. Common biocidal substances used in formulations of antifouling paints in EU countries.

Chemical Name	CAS	Trademark(s)	Other Uses
zinc 2-pyridinethiol-oxide	13463-41-7	Zinc pyrithione, ZnP	Antimicrobial, fungicide in antidandruff shampoo, antiseborrheic, preservative in cosmetics
zinc N-[2(sulfidocarbothioylamino)ethyl]carbamodithioate	9006-42-2	Zineb, Metiram, Amarex, Polyram, Polycarbacin, Parzate, Dithane, Z-78	Fungicide
copper(I) oxide	1317-39-1	Cuprous oxide, Dicopper monoxide, Red copper oxide	Antimicrobial, fungicide, pigment, catalyst
copper(I) thiocyanate	1111-67-7	Cuprous thiocyanate, Copper sulfocyanide, Thiocyanic acid copper (I) salt	Antimicrobial, fungicide, paint additive
4,5-dichloro-2-n-octyl-4-isothiazolin-3-one	64359-81-5	Sea-Nine 211, DCOIT, Kathon 5287, C-9	Fungicide for sealants, PVC, and wood
2,4,5,6-tetrachloroisophthalonitrile	1897-45-6	Chlorothalonil, Bravo Daconil, Faber, Forturf, Nopcocide, Repulse, Termil, Tuffcide	Antimicrobial, fungicide, insecticide, acaricide
α,β-1,2,3,4,7,7-hexachlorobicyclo-[2.2.1]-2-heptene-5,6-bisoxymethylene sulfite	33213-65-9	Endosulfan, Benzoepin, BeositIndan, Sialan, Thiodan, Thiosulfan, Thionex, Thimul	Insecticide, acaricide
2-N-tert-butyl-4-N-cyclopropyl-6-methylsulfanyl-1,3,5-triazine-2,4-diamine	28159-98-0	Irgarol 1051, Cybutryne	Herbicide
3-(3,4-dichlorophenyl)-1,1-dimethylurea	330-54-1	Diuron, Duran, Dynex, Dichlorfenidim, Herbatox, Karmex, Telvar, Vonduron	Herbicide
2,3,5,6-tetrachloro-4-(methylsulphonyl)pyridine	13108-52-6	TCMS pyridine, Davicil, Dowco-282	Fungicide for leather and wood

Recently, toxic effects on aquatic organisms have arisen, with mechanisms of action involving various cell targets [14]. The risk assessment of these emerging contaminants in marine ecosystems is now a priority due to the continuous uncontrolled leaching from antifouling paints and the synergistic interactions, which could affect the primary production and the survival and reproduction of marine fish and invertebrates [15–18].

Organotin compounds and a few alternative biocides are known to provoke embryotoxicity in solitary ascidians such as *Styela plicata* and *Ciona intestinalis* [19,20]. These species are oviparous and both the spawned gametes and embryos might be exposed to biocides in the water column, with important effects on fertilisation and offspring. At present, no study has been performed on compound ascidians, which are ovoviviparous [21–23], and therefore, as a difference from solitary ascidians, only hatched larvae might be exposed. They can be considered a better model than the solitary ascidians for the evaluation of the effects on post-embryonic stages, settlement, and metamorphosis.

Botryllus schlosseri, commonly called the star ascidian, earns its name from the stellate colony formed by a group of asexually reproducing individuals. It is a cosmopolitan compound species, is easy to collect, and breed in aquaria. In peculiar ecosystems with transitional waters such as the Lagoon of Venice, during autumn, more than 90% of the community comprises solitary and colonial ascidians, predominantly botryllids (*Botryllus schlosseri* and *Botrylloides leachii*), forming a stable biocoenosis described as a "*Botryllus* community" [24]. The organism has recently emerged as a simple and important model species for morphogenesis, regeneration, allorecognition, and apoptosis and for studying a variety of biological problems, such as comparative immunobiology, sexual and asexual reproduction, stem cell differentiation, and regeneration [25–27].

In the present study, the compound ascidian *B. schlosseri* was used as an experimental model for the evaluation of the effects of (i) TBT-based and tin-free (alternative to TBT) antifouling paints and (ii) antifouling biocides on larval viability, settlement, and metamorphosis. The biocides considered have been distinguished into three groups: (i) metals and organometals, i.e., CuCl, TBT, and zinc pyrithione (ZnP); (ii) antimicrobials and fungicides, i.e., Sea-Nine 211 and Chlorothalonil; and (iii) herbicides, i.e., Irgarol 1051, Diuron, 2,3,5,6-tetrachloro-4-(methylsulphonyl)pyridine (TCMS pyridine). Experiments were performed in two steps by exposing larvae to substrata coated with seven trade antifouling paints containing mixtures of the above-reported biocides and two different types of matrices (contact-leaching or self-polishing), and to various concentrations of each type of biocide. Different antifouling performances and metamorphic abnormalities have also been considered, and the mechanisms of actions of larval toxicity have been proposed.

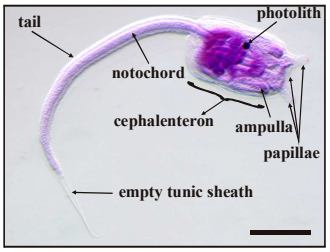
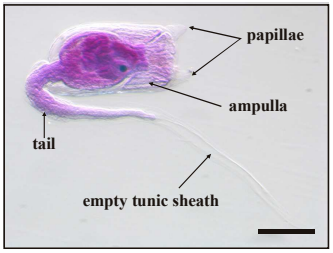
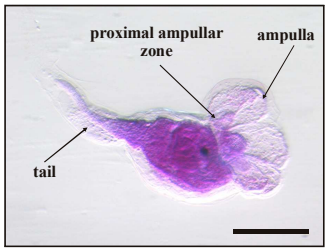
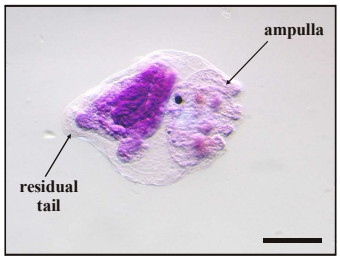
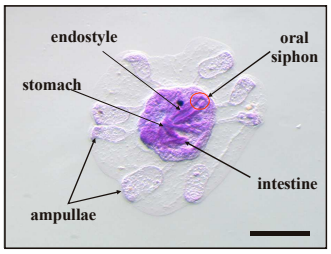
2. Materials and Methods

2.1. Larval Collection

B. schlosseri reproductive colonies were collected in spring (April–May) from the southern basin of the Lagoon of Venice and transferred to large aquaria, in which they were reared on glass slides in aerated filtered sea water (FSW) (salinity of 35 ± 1 psu, temperature of 19 ± 0.5 °C, pH 8.1). A semi-static system was used for animal maintenance, with seawater renewal every other day, and colonies were fed with Microbe-Lift®/Phyto-Plus B (Ecological Laboratories, Inc., Cape Coral, FL, USA) and microalgae (*Isochrysis galbana*). Newly hatched swimming larvae were easily identified under a dissection binocular stereomicroscope Wild Heerbrugg with 50× maximum magnification. They were immediately collected with a glass micropipette, counted, and temporarily transferred to a 30 mL glass-evaporating dish.

Five main stages were identified during metamorphosis, from the beginning of settlement to fully metamorphosed oozoids, as listed in Table 2.

Table 2. Main stages of the metamorphosis of *B. schlosseri* larva [1].

Metamorphosis Stages	
	**Stage 1** – Settled larva with three adhesive papillae – Initial resorption of tail from its distal region – No change in cephalenteron organs
	**Stage 2** – Tail resorption continues – A long tract of distal empty tunic sheath is recognisable – Protrusion of eight ampullae contacting the substratum
	**Stage 3** – Bell-shaped larva – Highly protruded ampullae – Two-thirds of tail resorption – Initial rotation of the cephalenteron organs
	**Stage 4** – Flattened larva with complete 180° rotation of organs – Ampullae radiating on the substratum – Almost complete tail resorption – Well-developed branchial pharynx – Enlarged stomach
	**Stage 5** – Oozoid – Complete expansion of ampullae over substratum – Marginal vessel formation – Siphon opening and filter-feeding activity – Presence of a primary bud from asexual reproduction

[1] In pictures, glutaraldehyde-fixed larvae stained with haematoxylin dye. Bar length: 200 µm.

2.2. Antifouling Paints

In the first series of experiments, seven (A–G) antifouling paints were tested (Table 3) with two replicates for a total exposure of 700 larvae. In each replicate, 50 swimming larvae were put into a glass-crystallising dish filled with 200 mL of FSW and with a 7.5 × 7.5 × 0.15 cm glass plate on the bottom that was previously coated with an antifouling paint. During exposure, the glass containers were covered outside and on the bottom with a black paper simulating a dark substratum, which favoured the settlement of larvae with positive geotropism and negative phototropism (Figure 1). The exposure occurred at

22 °C under artificial light until all larvae (100%) metamorphosed (48 h) in the reference glass-crystallising dishes, the latter without a painted plate on the bottom.

Table 3. Antifouling paints used to coat the glass plates for the assays of larval settlement and metamorphosis.

	Paint	Biocides	Matrix	Use
A	Sigmaplane HB Antifouling	Cu_2O (28%) TBT methacrylate (19%) TBTO (0.5%)	Self-polishing copolymers	Steel and wooden hull of fishing boats and cargo vessels more than 25 m in length (banned since 2003)
B	Marlin Velox TF	Zinc pyrithione (5–10%) Zineb (5–10%) Endosulfan (1–5%)	Contact leaching (hard or insoluble)	Propellers, shafts, and outdrives of fishing boats
C	Veneziani Propeller	CuSCN (7–10%) Zinc pyrithione (7–10%) Diuron (7–10%) Sea Nine 211 (1–3%)	Contact leaching (hard or insoluble)	Propellers, shafts, and outdrives of recreational craft
D	Veneziani Antialga	CuSCN (7–10%) Diuron (7.6%) Sea Nine 211 (2.7%)	Contact leaching (hard or insoluble)	Boattop of high-speed sailboats and powerboats
E	Sikkens Vinyl Antifouling 2000	Cu_2O (41%)	Contact leaching (hard or insoluble)	Steel (not aluminium), wooden, and polyester hull of sailboats and yachts
F	Baseggio Sirena Antivegetativa Universale	Cu_2O (42%) Chlorothalonil (7%) Irgarol 1051 (1.1%)	Self-polishing copolymers	Steel, wooden, and fibreglass hull of fishing boats
G	Veneziani Even Extreme 2 (reactive component)	TCMS pyridine (1–5%) Diuron (1–5%)	Self-polishing based on two-pack Biomatrix technology	Steel, wooden, and fibreglass hull of racing yachts

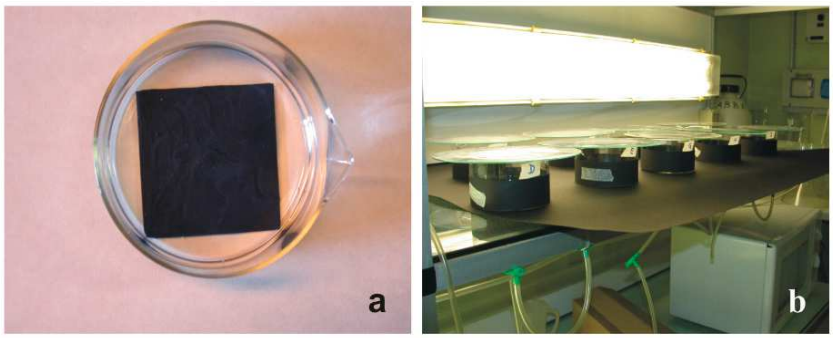


Figure 1. Experimental set-up for the evaluation of the settlement and metamorphosis ability of *B. schlosseri* larvae. (**a**) Glass-crystallising dish with a plate coated with an antifouling paint on the bottom. (**b**) Arrangement of the various glass-crystallising dishes in the thermostatic room at 22 °C.

2.3. Antifouling Biocides

In the second series of experiments, eight biocidal compounds were separately tested with three different concentrations and two replicates for a total exposure of 2400 larvae. For every replicate of biocidal concentration, 50 larvae were incubated in a glass-crystallising dish filled with 200 mL of biocidal solution in FSW. Concentration ranges of biocidal solutions were chosen based on previous studies of immunotoxicity in vitro on the haemocytes of this species [28–32]. The biocides considered were tributyltin (TBT) as monochloride (Sigma-Aldrich, Burlington, MA, USA), zinc pyrithione (ZnP, Sigma-Aldrich), copper(I) chloride (CuCl, purified, >99%, Sigma-Aldrich), Sea Nine 211 (Rohm & Haas, Philadelphia,

PA, USA), Chlorothalonil (Fluka), Irgarol 1051 (Riedel-de Haën GmbH, Seelze, Germany), Diuron (Sigma-Aldrich), and TCMS pyridine (Avecia, Manchester, UK).

Stock solutions were prepared at the nominal concentrations of 1 mM in FSW for CuCl; 10 mM in 95% ethanol for TBT, Sea Nine 211, Diuron, and TCMS pyridine; and 10 mM in dimethylsulfoxide (DMSO purum; >99%, Fluka Chemie Gmbh, Buchs, Switzerland) for ZnP, Chlorothalonil, and Irgarol 1051. They were freshly diluted to working concentrations in FSW as follows: 0.1, 1, 10 μM for TBT (corresponding to 32.5, 325.5, 3255 μg L^{-1}); 0.1, 0.5, 1 μM for ZnP (corresponding to 31.7, 158.5, 317.7 μg L^{-1} and 6.5×10^{-7}, 3.2×10^{-6}, 6.5×10^{-6} Zn wt%); 0.01, 0.1, 1 μM for CuCl (corresponding to 9.8, 98.99, 989.9 μg L^{-1} and 1.8×10^{-6}, 1.8×10^{-5}, 1.8×10^{-4} Cu wt%); 0.1, 1, 10 μM for Sea-Nine 211 (corresponding to 28.2, 282.2, 2822 μg L^{-1}); 0.1, 1, 10 μM for Chlorothalonil (corresponding to 26.5, 265.9, 2659 μg L^{-1}); 50, 100, 200 μM for Irgarol 1051 (corresponding to 12.6, 25.3, 50.6 mg L^{-1}); 100, 250, 500 μM for Diuron (corresponding to 23.3, 58.2, 116.5 mg L^{-1}); and 25, 50, 75 μM for TCMS pyridine (corresponding to 7.4, 14.8, 22.2 mg L^{-1}). In controls, larvae were incubated with FSW containing the maximum solvent concentration employed in the experiments with biocides, i.e., 0.02% DMSO or 0.01% 95%-ethanol. These concentrations had no effect on survival or metamorphosis. After all larvae (100%) unexposed to biocides (48 h at 22 °C) reached the oozoid stage, the number of living (motile) and dead (immotile) larvae were recorded, as well as the metamorphosis stage reached (developmental delay) and the presence of abnormalities under a Leica MZ16F stereomicroscope.

2.4. Statistical Analysis

Data are reported as the mean percentage ± standard deviation (SD). The statistical analysis was performed with IBM SPSS Statistics v. 25 software. The probit method was used to calculate the median lethal concentration (LC_{50}) and the median effective concentration (EC_{50}), the latter defined here as the toxicant concentration that reduced normal larvae by 50%, considering both the larval settlement and metamorphosis as endpoints and their 95% confidence intervals. Significant differences ($p < 0.05$) (i) between the control group and test concentrations, (ii) between the control group and test paints, and (iii) among the test concentrations or the paint groups were evaluated with one-way analysis of variance (ANOVA) followed by Fisher's least significant difference (LSD) test and Dunnett's multiple comparison test. In the case of values expressed as percentages, the raw data were analysed after arcsine transformation to achieve normality.

In the experiments of exposure to antifouling paints and biocides, a clustering analysis with average linkage between groups was used to obtain hierarchy dendrograms after a χ^2 test with Yates' p-value correction applied on the contingency table. Regarding the experiments of exposure to antifouling paints, the basic criterion of this test is to verify if the seven paints are significantly associated with different outcomes. Since it leads to rejecting the null hypothesis of independence between paints and outcomes, the clustering analysis can be applied to determine which paints can be considered similar and then grouped into clusters. In the case of the χ^2 test for the evaluation of the different outcomes related to the concentrations of the eight biocidal compounds, only Irgarol 1051 did not give significantly ($p = 0.999$) different outcomes at different concentrations. For all the other biocides, the different concentrations were significantly ($p < 0.001$) associated with different outcomes. For the combinations of biocides and concentrations, clustering analysis groups similar objects in clusters that are most homogeneous within them and most heterogeneous among them.

3. Results and Discussion

3.1. Effects of Antifouling Paints

In the experiments with plates coated with seven antifouling paints, larvae were placed inside glass-crystallising dishes filled with sea water and with a coated plate on the bottom. At the end of exposure, considered when all control larvae in filtered sea water metamorphosed forming completed filter-feeding oozoids (100% survival rate),

three effects were evaluated (Figure 2a): (i) complete metamorphosis with the oozoid formation, (ii) settled but dead larvae, and (iii) not-settled but free-floating dead larvae. These effects were also evaluated in three different conditions: (i) individuals floating in the sea water, (ii) individuals settled on the dish's glass bottom surrounding the coated plate, and (iii) individuals settled on the coated paint.

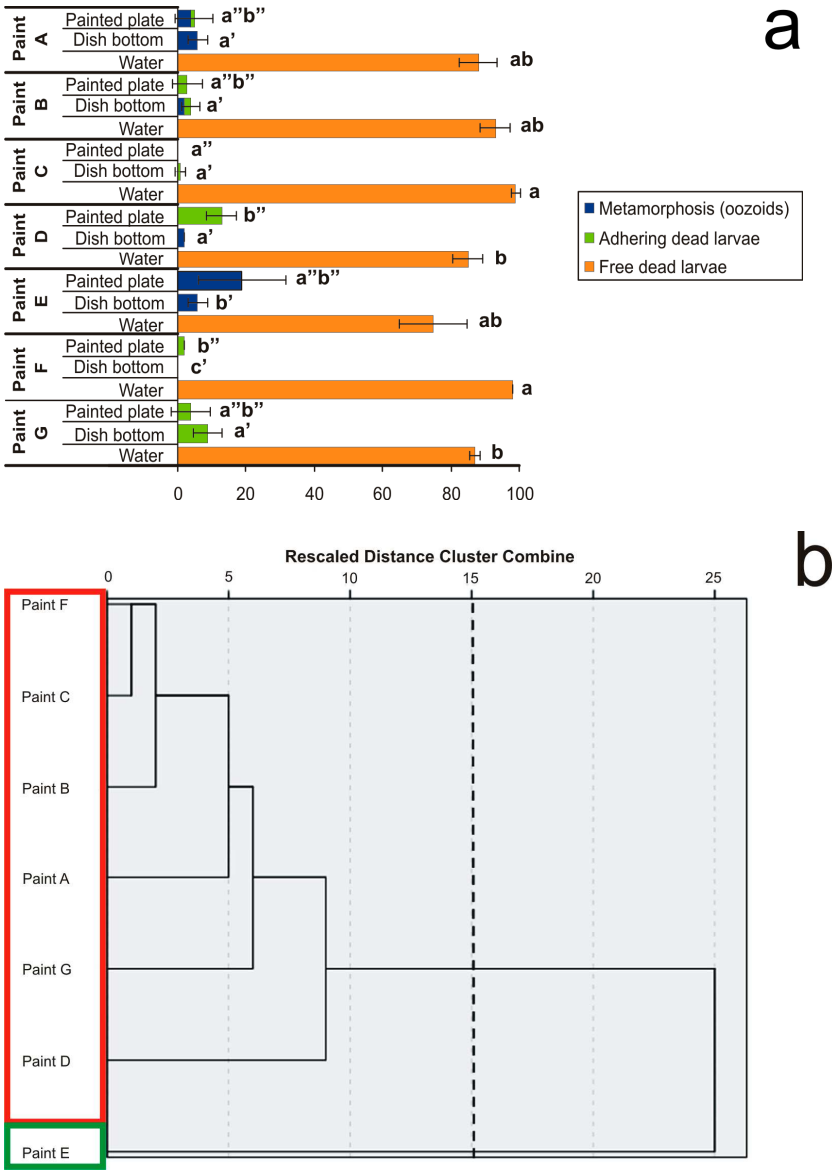


Figure 2. Effects of plates coated with seven antifouling paints on larval settlement and metamorphosis observed after exposure until all control larvae metamorphosed in the reference glass-crystallising dishes forming filter-feeding oozoids. (**a**) Percentages of three effects on metamorphosis, i.e., (i) complete metamorphosis with oozoid formation (blue), (ii) settled but dead larvae (green), and (iii) not-settled but free-floating dead larvae (orange). Larval mortality and abnormality were considered in three conditions: (i) individuals floating in the sea water ("water"), (ii) individuals adhering to the dish glass bottom peripheral to the painted plate ("dish bottom"), and (iii) individuals settled on the antifouling-coated plate ("painted plate"). Significant ($p < 0.05$) differences inside each of the three conditions obtained with Dunnett's test are expressed with different letters. (**b**) Dendrogram obtained from cluster analysis with average linkage between groups. The cutting point at 15 corresponds to the maximum margin showing two clusters bordered by different colours.

All antifouling paints demonstrated a remarkable performance because they caused both high mortality and inhibition of metamorphosis. Paints containing CuSCN + ZnP + Diuron + Sea-Nine 211 (Paint C) and Cu_2O + Chlorothalonil + Irgarol 1051 (Paint F) prevented larval adhesion. Paints containing ZnP + Zineb + Endosulfan (Paint B), CuSCN + Diuron + Sea-Nine 211 (Paint D), and TCMS pyridine + Diuron (Paint G) allowed minor larval adhesion, but prevented metamorphosis. Paints containing TBT (Paint A) or Cu_2O alone (Paint E) occasionally allowed adhesion and metamorphosis, but killed the oozoids. It is noteworthy that the antifouling components generally do not act only through direct contact—the presence of dead and partially metamorphosed larvae, both free-floating and adhering to the glass surrounding the painted plates, suggests a certain degree of leaching of the biocides from the antifouling paints. A large number of free-floating dead larvae were observed in all cases corresponding to $\geq$75% with small numbers of settled larvae, which died without completing metamorphosis (Figure 2a). Nevertheless, the analysis of variance did not permit us to clearly distinguish the different effects of the various antifouling paints.

From the hierarchy dendrogram obtained from the clustering analysis with average linkage between groups (Figure 2b), only two clusters emerged. These clusters represent the mean percentages of five variables, i.e., (i) free-floating dead larvae in sea water, (ii) oozoids on the dish glass bottom, (iii) dead larvae settled on the dish glass bottom, (iv) oozoids on the coated plate, and (v) dead larvae settled on the coated plate.

The first cluster only includes Paint E with 75% of free-floating larvae, 19% of oozoids on the coated plate, and 6% of oozoids on the dish glass bottom. The second cluster is represented by all the other paints with 91.7% of free-floating larvae, 3.8% and 2% of dead larvae settled on the coated plate and on the dish-glass bottom, respectively, and 1.7% and 0.7% of oozoids adhering to the dish glass bottom and to the coated plate, respectively. However, these results are difficult to interpret because the toxic effects are similar. The only relevant consideration is that Paint E, which showed a lower toxic effect than the other paints, is a unique antifouling paint without booster biocides and contains the highest amount of CuCl (41%). This concentration is similar to that of Paint F, which also contained Chlorothalonil and Irgarol 1051 as booster biocides and is based on self-polishing technology rather than on an insoluble matrix. In conclusion, the interactions of a variety of principal and booster biocides on one hand and different matrix technologies on the other hand are complex in commercial paints. The leaching rate of biocides from the paints, which is fundamental for the risk assessment, is often not reported in manufacturers' datasheets. It varies with the mixture of concentrations of biocides in the paint formulation and depends on the interaction with boosters and other additives; environmental abiotic parameters such as temperature, salinity, and pH; and the type of matrix [33,34].

Generally, the most commonly used approach for understanding the action of antifouling compounds is the direct exposure of embryos or larvae of marine invertebrates to various concentrations of a single biocide in sea water with controlled parameters, totally excluding the interaction of matrix, pigments, solvents, and other additives [35–41]. In this way, larval toxicity and the effects on metamorphosis can be clearly observed, elucidating the possible mechanisms of action at both cellular and subcellular levels.

3.2. Effects of Antifouling Biocides

At the end of the experiments, all control larvae in filtered sea water metamorphosed, forming complete filter-feeding oozoids (100% survival rate). The order of toxicity of the antifouling biocides assayed with the evaluation of the median lethal and median effective concentrations (LC_{50} and EC_{50}) able to cause mortality and inhibition of larval settlement/metamorphosis, respectively, in *B. schlosseri* (Table 4) is CuCl > ZnP > TBT > Chlorothalonil > Sea Nine 211 > TCMS pyridine > Irgarol 1051 > Diuron.

Table 4. Median lethal concentrations (LC_{50}) of antifouling biocides on *Botryllus schlosseri* larvae and median effective concentrations (EC_{50}) on larval settlement and metamorphosis as endpoints after 48 h exposure at 20–22 °C to antifouling biocides compared with data reported for the solitary ascidian *Ciona intestinalis*.

Biocide	Species	LC_{50}	EC_{50}
TBT	*B. schlosseri*	4.73 µM (1539 µg L^{-1})	0.08 µM (26 µg L^{-1})
	C. intestinalis	ND	0.02 µM (7.1 µg L^{-1}) [42]
ZnP	*B. schlosseri*	0.46 µM (146 µg L^{-1}, 3 × 10^{-6} Zn wt%)	0.06 µM (19 µg L^{-1}, 4 × 10^{-6} Zn wt%)
	C. intestinalis	ND	0.11 µM (35 µg L^{-1}, 7 × 10^{-6} Zn wt%) [43]
Copper(I) chloride	*B. schlosseri*	0.35 µM (34.6 µg L^{-1}, 2 × 10^{-6} Cu wt%)	0.04 µM (3.96 µg L^{-1}, 2 × 10^{-6} Cu wt%)
	C. intestinalis	ND	1.61 µM (159 µg L^{-1}, 1 × 10^{-3} Cu wt%) [44]
Sea-Nine 211	*B. schlosseri*	4.88 µM (1377 µg L^{-1})	0.25 µM (70 µg L^{-1})
	C. intestinalis	ND	0.15 µM (43 µg L^{-1}) [37]
Chlorothalonil	*B. schlosseri*	4.80 µM (1276 µg L^{-1})	0.23 µM (61 µg L^{-1})
	C. intestinalis	ND	0.16 µM (42 µg L^{-1}) [37]
Irgarol 1051	*B. schlosseri*	>200 µM (>50,674 µg L^{-1})	36 µM (9121 µg L^{-1})
	C. intestinalis	ND	>25.60 µM (>6486 µg L^{-1}) [37]
Diuron	*B. schlosseri*	214.96 µM (50,105 µg L^{-1})	64 µM (14,918 µg L^{-1})
	C. intestinalis	ND	ND
TCMS pyridine	*B. schlosseri*	34.99 µM (<10,392 µg L^{-1})	<25 µM (<7375 µg L^{-1})
	C. intestinalis	ND	ND

ND: not determined, i.e., no information reported in the literature. Reference numbers are placed in square brackets.

Both the LC_{50} and EC_{50} found for *B. schlosseri* are higher than the environmental concentrations reported in the literature, with the exception of ZnP and CuCl. The concentration ranges known in the sea water column are <0.02–31.7 µg L^{-1} for ZnP [45], 0.15–26 µg L^{-1} for CuCl [46,47], <0.001–3.3 µg L^{-1} for Sea Nine 211, <0.01–1.4 µg L^{-1} for Chlorothalonil, <0.001–1.7 µg L^{-1} for Irgarol 1051, and <0.001–6.7 µg L^{-1} for Diuron [18]. *B. schlosseri* appears to be more sensitive to both ZnP and CuCl than the solitary ascidian *Ciona intestinalis* [43,44]. On the contrary, *C. intestinalis* is about 4× and 1.5× more sensitive than *B. schlosseri* in terms of the reduction in larval settlement by 50% in the presence of TBT [42] and Sea-Nine 211 or Chlorothalonil [37], respectively, whereas the toxicity concerning Irgarol 1051 is similar [37]. Generally, CuCl and ZnP showed the highest toxicity, causing 50% larval mortality at 0.35 and 0.46 µM (34.6 and 146 µg L^{-1}), respectively, and reducing the larval settlement and metamorphosis by 50% at 0.04 and 0.06 µM (3.96 and 19 µg L^{-1}), respectively. This fact supports the hypothesis that the CuCl and ZnP concentrations usually employed in antifouling paints are too high for their goal and the continuous leaching of these compounds is of great concern considering their impact on coastal ecosystems, particularly the possible long-term negative effects on non-target benthic organisms. Therefore, restrictions and bans regarding the use of copper- and zinc-based antifouling paints are essential for the development of "eco-friendly" antifouling paints [48].

The clustering analysis with average linkage between groups was performed on 25 objects representing the linkages of biocides and test concentrations (Figure 3).

The hierarchy dendrogram obtained shows four clusters with mean percentages of six effects. The effects considered were (i) complete metamorphosis with the oozoid formation (blue, in the legend of Figure 3a), (ii) settled larvae with incomplete metamorphosis or metamorphic delay without abnormalities (green), (iii) incomplete metamorphosis without settlement of dead larvae with abnormalities (orange), (iv) not-settled dead larvae with abnormalities (violet), (v) not-settled dead larvae without recognisable abnormalities (black), and (vi) free-swimming larvae as the result of inhibition or severe delay of metamorphosis (yellow).

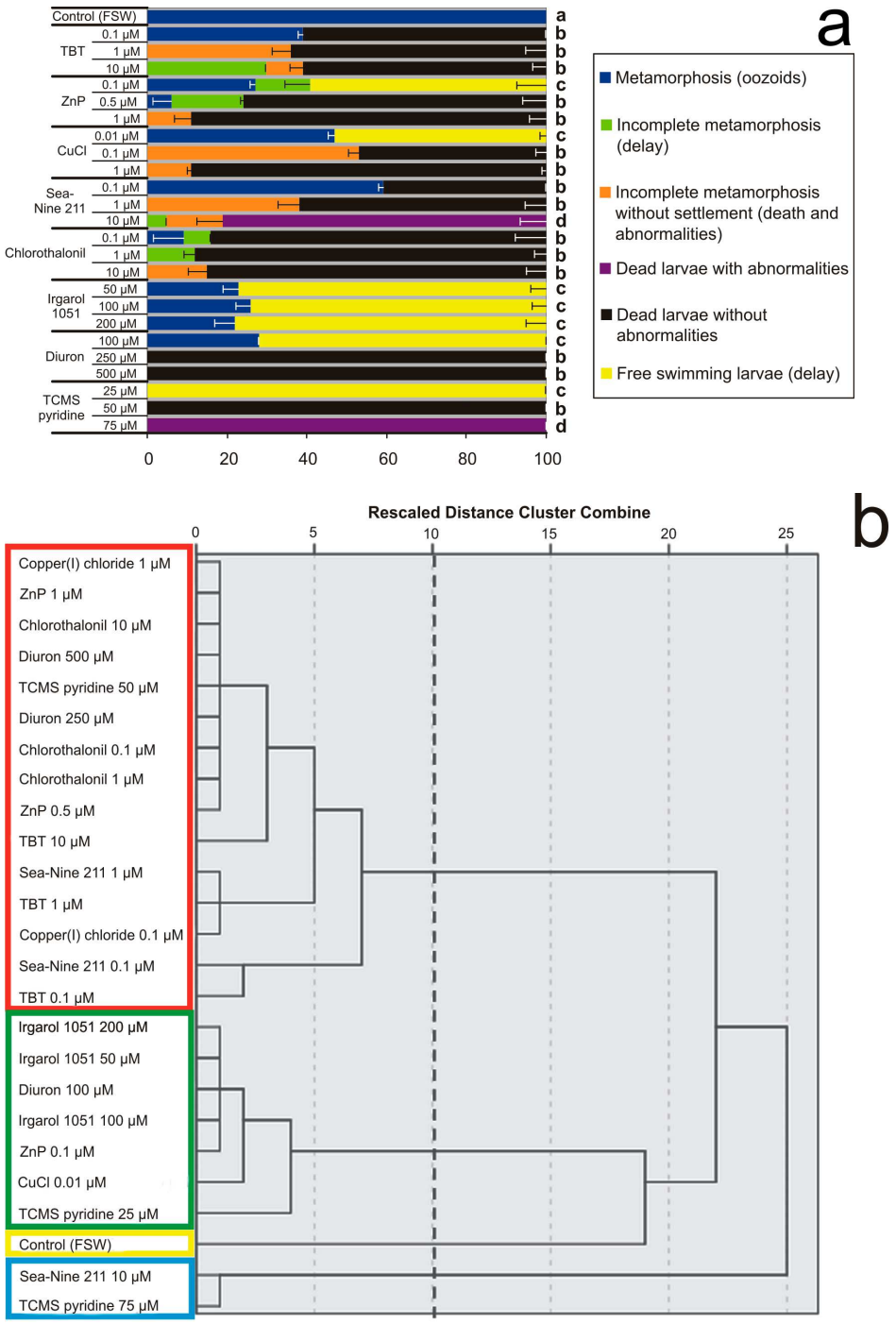


Figure 3. Effects of various concentrations of antifouling biocides in sea water on larval settlement and metamorphosis. (**a**) Percentages of larval mortality and abnormality observed after exposure until all control larvae in FSW completed the metamorphosis forming filter-feeding oozoids. Significant ($p < 0.05$) differences obtained with Dunnett's test are expressed with different letters. (**b**) Dendrogram obtained from cluster analysis with average linkage between groups. The cutting point at 10 corresponds to the maximum margin showing four clusters bordered by different colours.

The first cluster ("a", in Figure 3a; yellow frame, in Figure 3b) is represented by the unique effect observed in the control, i.e., 100% complete metamorphosis and survival. The second cluster ("b", red frame) has 15 objects, represented by all concentrations of TBT and Chlorothalonil, and the middle concentrations of ZnP, CuCl, Sea Nine 211, Diuron, and TCMS pyridine. In this cluster, 76.5% of dead larvae without abnormalities prevail, followed

by 11.5% incomplete metamorphosis without settlement, 7.5% complete metamorphosis, and 4.5% incomplete metamorphosis with settlement. The third cluster ("c", green frame) includes seven objects represented by all Irgarol 1051 concentrations, and the lowest concentrations of ZnP, CuCl, Diuron, and TCMS pyridine, with 73.3% free-swimming larvae, 24.7% completed metamorphosis, and 2% incomplete metamorphosis of settled larvae without abnormalities. The fourth cluster ("d", cyan frame) contains two objects, represented by the highest concentrations of Sea Nine 211 and TCMS pyridine, with 90.5% dead larvae with abnormalities, 7% incomplete metamorphosis without settlement, and 2.5% incomplete metamorphosis with settlement.

These results show that although all biocides are able to prevent larval settlement on the substratum and provoke severe metamorphic malformations, the herbicides Diuron and TCMS pyridine at their lowest concentrations and Irgarol 1051 at all concentrations are not lethal.

3.3. Larval Abnormalities

The larval abnormalities of the experiments reported in Section 3.2 were analysed under a light microscope (Figure 4). The most severe morphological effects in metamorphic development have been observed after exposure to 1 and 10 µM TBT (Figure 4a), Sea-Nine 211 and Chlorothalonil, 1 µM ZnP, and 0.1 µM CuCl (Figure 4c). In these cases, the initial metamorphosis was evident with the protrusion of ampullae and tail resorption at various degrees, but cephalenteron always appeared to be encased by an anomalous large bubble filled with a colourless fluid with small, scattered cells. Organotin compounds, zinc, copper, and Chlorothalonil are known to cause significant changes in hydromineral fluxes and membrane permeability, mechanisms that maintain osmotic homeostasis [49–52]. The bubble formation in *B. schlosseri* larvae could be the result of the disruption of osmotic control systems due to the direct action on lipid composition and/or ion transport across the plasma membranes. Regarding the tail resorption, TBT, Sea-Nine 211, and Chlorothalonil caused an abnormal extensive detaching of spherical-shaped cells throughout the entire tail length. These compounds are known to directly and indirectly inhibit a series of enzymes. In particular, Ca^{2+}-ATPase is inhibited via biocide interaction with calmodulin [53]. This pump inhibition increases intracellular Ca^{2+} concentrations, which in turn provoke the depolymerisation of cytoskeletal components [54] and induce cell apoptosis [55]. The incomplete tail resorption with the persistence of apoptotic cells dissociated from the axial complex indicates the absence of phagocytosis, which is usually carried out by motile phagocytes able to engulf all dead cells and tissue debris [56], confirming the immunosuppressive activity of these biocides [28,29].

At 0.5 µM ZnP (Figure 4b) and 1 µM CuCl (Figure 4d), the cephalenteron was not encased in a bubble. In the case of ZnP, protrusion of ampullae occurred, but not the tail resorption. The formation of proximal and median bulges of detached cells was recognisable along the tail, probably due to the inhibition of phagocytosis [32]. In the case of CuCl, tail resorption appeared to be nearly completed, but no protrusion of ampullae occurred and the cephalenteron organs underwent a massive regression, recognisable by the appearance of large vacuolated cells instead of the organ primordia.

Many heavy metals are essential to the growth of marine organisms. Low concentrations of copper ions stimulate metamorphosis in ascidians [57], but at high concentrations, metal ions become toxic. Metals can significantly decrease the synthesis of ATP and negatively alter the metabolic activity causing cell death because they act as uncouplers of oxidative phosphorylation or via opening pores in the mitochondrial membrane [58].

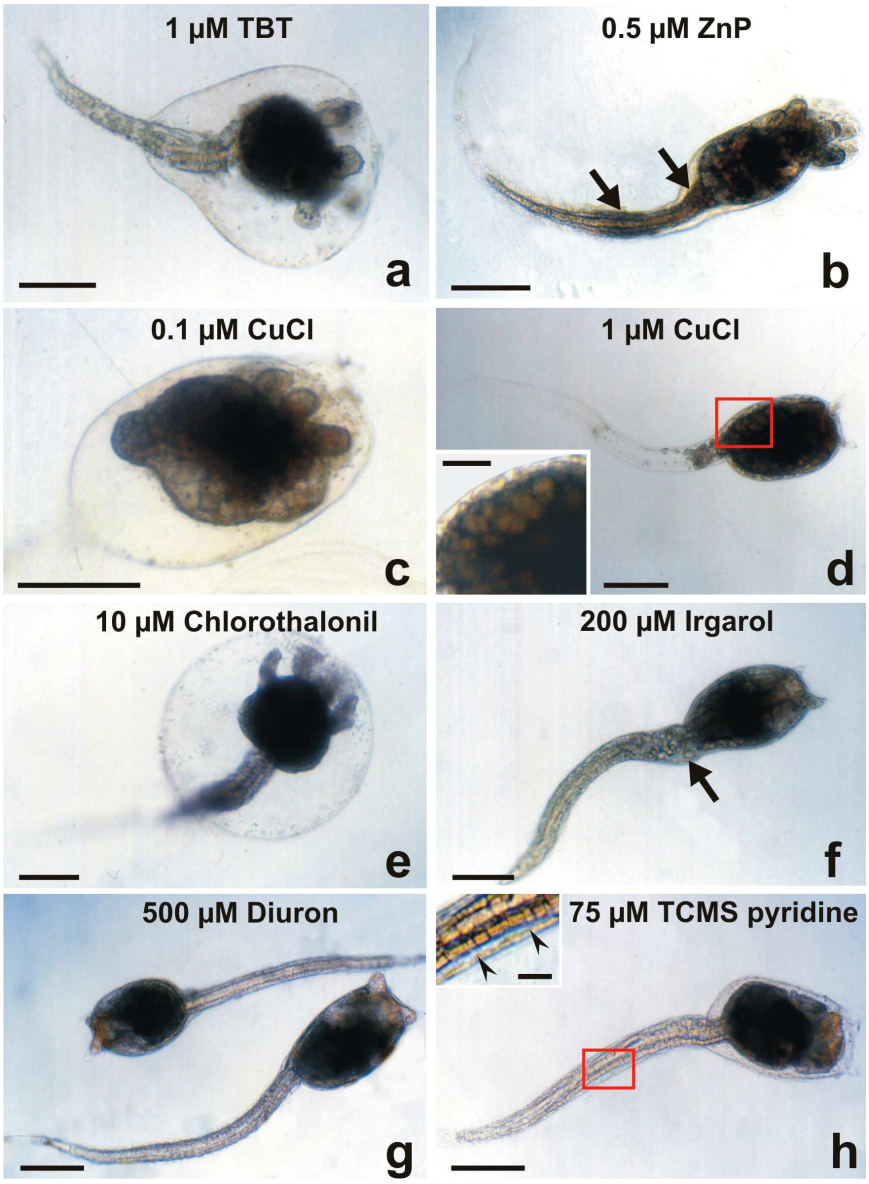


Figure 4. Larval abnormalities in *Botryllus schlosseri* after exposure to antifouling biocides. (**a**) Protruded ampullae inside an anomalous bubble encasing the cephalenteron, and extensive spherical-shaped cells detaching from the tail tissues. (**b**) Protruded ampullae and tail with proximal and median bulges (*arrows*). (**c**) Advanced metamorphosis (protruded ampullae and tail resorption) inhibited by the formation of a bubble encasing the whole body. (**d**) Tail resorption and inhibition of cephalenteron metamorphosis due to extensive organ regression forming vacuolated cells (inset in (**d**)). (**e**) Protruded ampullae inside a bubble encasing the cephalenteron, and normal tail resorption. (**f**) Detaching of cells from the proximal tail zone (*arrow*). (**g**) Absence of metamorphosis. (**h**) Initial protrusion of ampullae, and separation of the lateral tail muscles from the epidermis by large intercellular spaces (*arrowheads* in inset in (**h**)). Bar length: 200 µm in (**a**–**h**); 30 µm inset in (**d**); 40 µm inset in (**h**).

Regarding the herbicides Irgarol 1051 (Figure 4f), Diuron (Figure 4g), and TCMS pyridine (Figure 4h), the metamorphosis was completely inhibited. The larvae did not swim or settle because they were dead only in the cases of 250 µM Diuron and 50 µM TCMS pyridine. As a minor difference from Diuron, an initial protrusion of ampullar rudiments was observed after exposure to TCMS pyridine. These compounds are known to cause disturbances in the mitochondrial respiratory chains and induce cell apoptosis

due to severe oxidative stress [59,60]. After the exposure to 500 μM Diuron (Figure 4g), larvae were without evident malformations but immotile. The absence of signs of tail resorption indicates an alteration of communication with the cephalenteron. It could be triggered through the abnormal activation by xenobiotics of stress-related signal transduction pathways, such as the stress-inducible protein HSP90, which in turn activates the enzyme nitric oxide synthase (NOS). The consequent NO production is responsible for the metamorphosis repression in ascidians, as observed in *Boltenia villosa* larvae, which failed in tail resorption and protrusion of the ampullar rudiments [61]. Cell dissociation occurred in the proximal region of the tail of a small number of larvae after exposure to the highest concentration (200 μM) of Irgarol 1051 (Figure 4f). An extensive separation of the lateral tail muscles from the caudal epidermis by intercellular spaces was recognisable after exposure to 75 μM TCMS pyridine (inset of Figure 4h). These events could be related indirectly to HSP90/NO activation and directly to sudden increases in intracellular Ca^{2+} concentrations. The latter induce both apoptosis through the activation of endonucleases, triggering DNA fragmentation [30,31], and the loss of Ca^{2+}-dependent cell adhesion molecules such as cadherins, causing extensive tissue detachment [62,63].

Finally, it must be emphasised that in all cases of absence of tail resorption, the increase in intracellular calcium ions might cause extensive depolymerisation of the microfilaments of the cells of the caudal epidermis. In such a way, the F-actin responsible for contractions that provide the driving force in tail resorption [2] failed.

4. Conclusions

The experiments carried out on the free-swimming larvae of the compound ascidian *B. schlosseri* provide new evidence for better understanding the effects of trade antifouling compounds on settlement ability, metamorphic development, and viability, arranging them in an order of decreasing toxicity: metals and organometals > antimicrobials and fungicides > herbicides. These results confirm the high toxicity of TBT, ZnP, and copper(I)-based antifouling compounds, but also reveal that Sea-Nine 211 and Chlorothalonil, recently introduced by "green chemistry" in new antifouling paint formulations as TBT alternatives, are effective in causing larval death and abnormalities. Moreover, the herbicides Irgarol 1051, Diuron, and TCMS pyridine, although introduced in paint formulations only to prevent the settlement and growth of algae, showed toxicity towards animals. Since these compounds have been in commerce for decades, and, consequently, widespread in the marine environment, a real risk for the coastal biocoenoses appears to be undeniable. In many cases, their partitioning between water and sediment, modality and half-time of biotic and abiotic degradation, speciation, binding to various ligands, formation of complexes, environmental fate, and bioaccumulation in the trophic chains are not yet well known.

The effects of exposure to antifouling paints are much more difficult to interpret than those of exposure to antifouling biocides directly introduced in the sea water. The latter approach is preferable because it can be better controlled and not affected by the modality of leaching of a biocidal mixture from paints, which could vary with various environmental parameters and also determine a network of synergistic effects with booster biocides, other paint additives, and types of matrices. Paints with a contact-leaching matrix are more resistant to abrasion and rubbing than those with a self-polishing matrix, but they tend to oxidise with time, decreasing the biocidal release. By comparing the biocidal release rates from contact-leaching and self-polishing paint technologies, although the initial value of the latter is low, a constant release soon occurs throughout the lifetime of the paint.

Therefore, according to the Biocides Directive (98/8/EC) [64], more assays of acute and chronic toxicity on various target and non-target marine organisms should be performed before new potential pollutants enter the market, in order to prevent the same errors that already occurred with TBT in both the ecological and economical fields due to the deterioration of environments and habitats.

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4.1.3 Study at cellular level

Immunotoxicology is the study of the effects of xenobiotic substances on the immune system of an organism. The principal role of the immune system is to recognise elements that do not belong to the body (non-self) from those that are (self) (Peakall *et al*., 1992). When a non-self element such as foreign cells, exogenous microorganisms (bacteria, viruses, fungi, parasites, etc.) or xenobiotic substances (chemicals, pesticides, etc.) enters the body, it triggers the immune response (Peakall *et al*., 1992; Galloway and Depledge, 2001).

Since 1990s, in vitro toxicity assays have been used on haemocytes of marine invertebrate species (Smith *et al*., 1992; Seibert *et al*., 1994; Auffret *et al*., 1997; Nusetti *et al*., 1998; Pipe *et al*., 1999; Coteur *et al*., 2001; Matozzo *et al*., 2002 a,b; Matozzo and Marin, 2005; Pagano *et al*., 2017), in which useful sentinel species, namely, bioindicators, emerged. They are mainly represented by cosmopolitan organisms that are most affected by changes in environmental conditions, the functional responses of which are closely indicative of environmental quality (Anderson, 1988).

For my study I carried out in vitro immunotoxicity assays on the haemocytes of the star tunicate *Botryllus schlosseri* (Pallas, 1766). *B. schlosseri* is a colonial ascidian and it is a worldwide filter-feeding organism living in the water-sediment interface. Moreover, I carried out immunotoxicity assays on bivalves to compare the effects on immune responses by pivotal species of the coastal community, i.e., a target species dominant in the macrofouling community like the Mediterranean mussel, *Mytilus galloprovincialis* (Lamarck, 1850) and a non-target species like the Manila clam, *Ruditapes philippinarum* (Adams and Reeve, 1850). These bivalves were chosen in this study as well-known filter-feeding bioindicators because they are dominant species of the trophic chain of the coastal ecosystem and are edible molluscs.

Immunomarkers are used to evaluate the effects on the immune system of xenobiotic substances by performing in vitro laboratory assays. The biomarkers that I have been considered was LC_{50}, cell adhesion assay, cell spreading index, cytoskeleton stability ondex (F-actin), phagocytosis index, lysosomal stability index (Neutral Red uptake assay), cytosolic Ca^{2+} content assay, apoptotic index (TUNEL reaction assay); enzymatic indexes of the hydrolase β-glucuronidase and acid phosphatase, phenoloxidase and the mitochondrial cytochrome-c oxidase (COX); glutathione content index and a histochemical assay for superoxide anion production.

In the first study, I carried out immunotoxicity assays on *B. schlosseri* I considered the sulfamide Dichlofluanid, a fungicide that was recently introduced as a low-impact

biocide in the trade antifouling paints (Thomas, 2001). Dichlofluanid adversely affected 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. In conclusion, the effects of dichlofluanid on immune defence responses are similar to those of organometal-based antifoulants (i.e., TBT and ZnP), and its use in coastal areas requires attention.

In the second study, I used copper(I) chloride for a comparative analysis of the effects on two bivalve species. Copper(I) is the biocide most commonly used in antifouling paints today. It is insoluble and does not degrade in sea water, but dissociates to form Cu^{+} ions, which quickly oxidize to Cu^{2+} (Voulvoulis *et al*.,1999; Cima and Ballarin, 2012). For this reason, a specific copper(I) chelator (i.e., bathocuproine) was used to distinguish the effects of copper(I) from those of copper(II) (White *et al*., 2001).

The results obtained showed that copper(I) adversely affects both immunocyte lines (hyalinocytes and granulocytes) after exposure to sublethal concentrations. After exposure, the haemocytes of *M. galloprovincialis* and *R. philippinarum* revealed altered stability of lysosomal membranes, reduced phagocytic capacity, inhibition of enzymatic activities, decrease in reduced glutathione content and significant increase of apoptotic features beginning from concentrations between 0.5 and 1 µM. The comparison of responses to the exposure to the various concentrations assayed generally shows that the immune system of *M. galloprovincialis* is less sensitive than *R. philippinarum* confirming the potential harmful effects of antifouling biocides on the survival of non-target pivotal species of the coastal community. The paper has been recently submitted to *Frontiers in Physiology.*

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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

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].

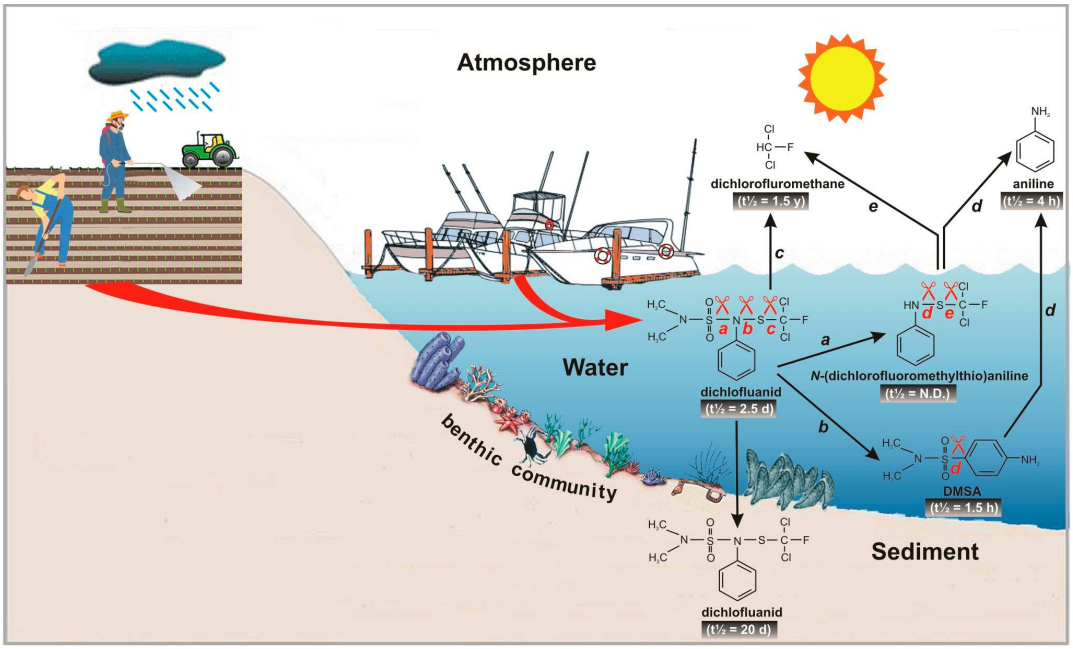

Figure 1. The main sources of environmental pollution of dichlofluanid (in agriculture as a fungicide and from boats as an antifoulant) and the biogeochemical fate of dichlofluanid and its derivatives. Black arrows show the degradation pathways by photodegradation, hydrolysis and biotic and abiotic anaerobic 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) 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™ (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).

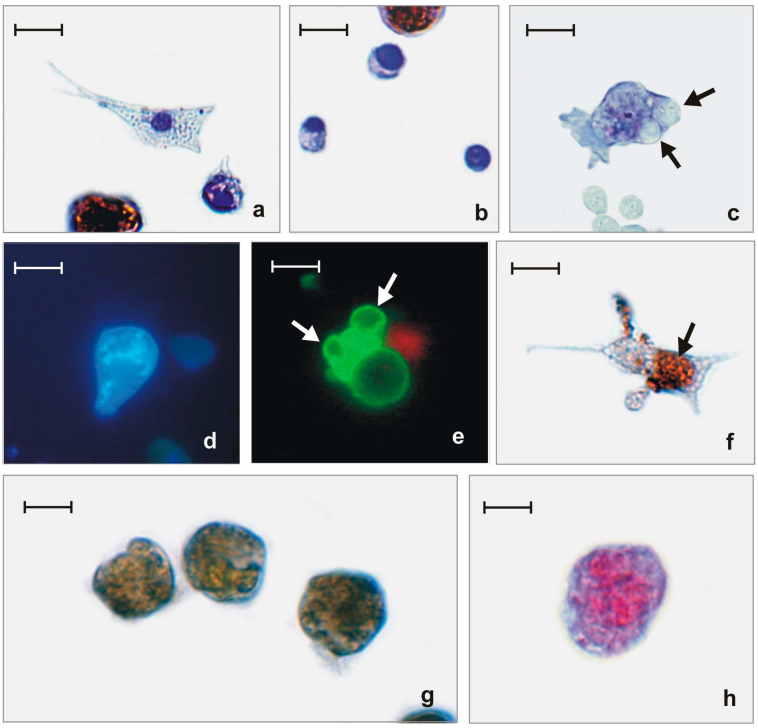

Figure 2. Haemocytes of *Botryllus schlosseri*. (**a**) Glutaraldehyde-fixed phagocyte stained with Giemsa dye showing long pseudopodia. (**b**) Three cells of the same type after exposure to 0.1 μM (33.3 μg/l) dichlofluanid. Note the cell shrinkage and the roundish shape due to the loss of pseudopodia, 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**).

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).

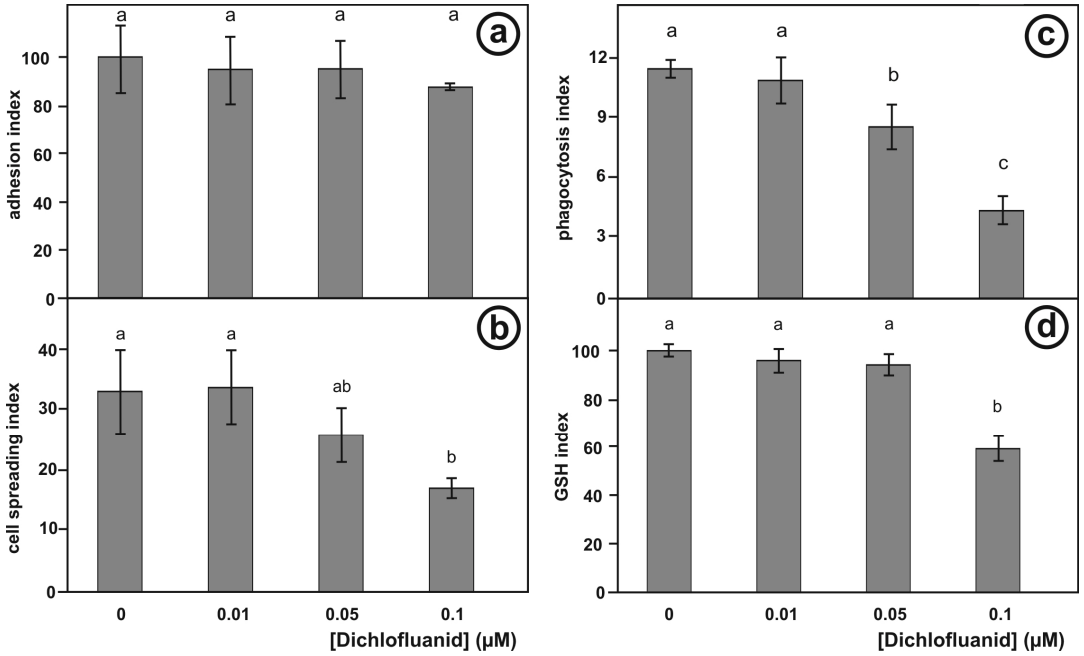

Figure 3. Adhesion index (**a**), cell spreading index (**b**), phagocytosis index (**c**) and GSH index (**d**) in the presence of various concentrations of dichlofluanid. Control (0) refers to the absence of dichlofluanid. The results are expressed, in the case of the adhesion index, as the number 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.

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).

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).

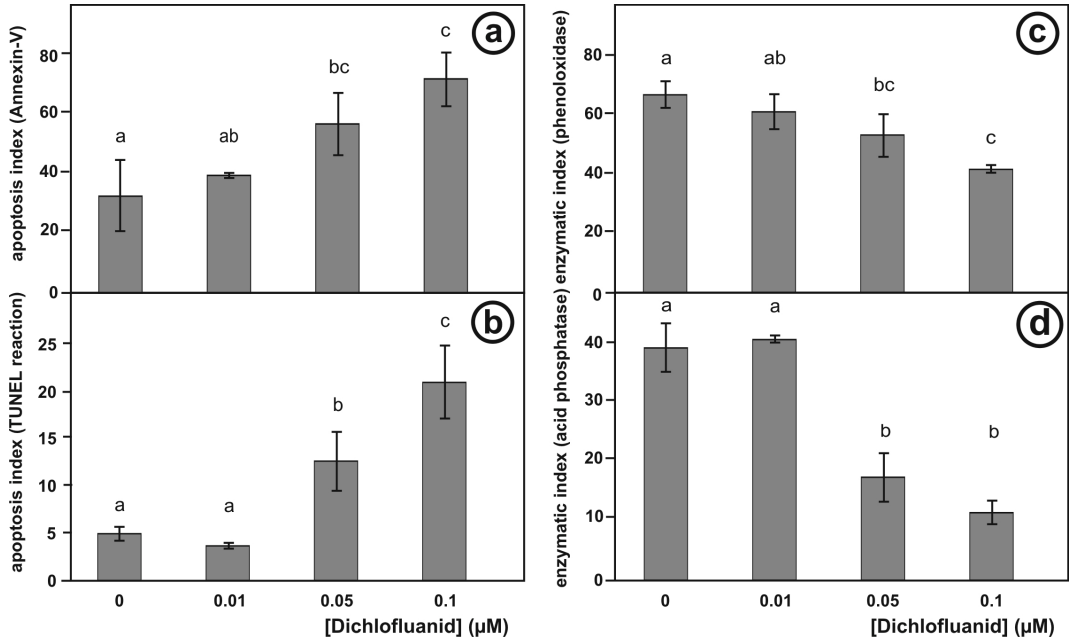

Figure 4. Indexes of early (**a**) and late (**b**) apoptosis evaluated with the annexin-V assay and TUNEL reaction, respectively, and enzymatic indexes of phenoloxidase (**c**) and acid phosphatase (**d**) activities of the cytotoxic and phagocytic lines, respectively, in the presence of various concentrations 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.

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 4,800 and 8,200 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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Immunotoxic effects of exposure to the antifouling copper(I) biocide on target and nontarget bivalve species: A comparative *in vitro* study between *Mytilus galloprovincialis* and *Ruditapes philippinarum*

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Keywords: Antifouling paints; Bivalves; Copper(I) chloride; Hemocytes; Immunotoxicity; Mollusks; *Mytilus galloprovincialis*; *Ruditapes philippinarum*

Abstract

Edible bivalves constitute an important bioresource from an economic point of view, and studies on their immune responses to environmental xenobiotics are crucial for both the preservation of biodiversity and economic reasons. The worldwide diffusion of copper(I)-based antifouling paints has increased copper leaching into coastal environments and its potential impact on both target and nontarget organisms. In this study, immunotoxicity assays were carried out with short-term (60 min) cultures of hemocytes from the bivalves *Mytilus galloprovincialis* – a mussel dominant in the macrofouling community – and *Ruditapes philippinarum* – a clam dominant in the soft-sediment community – exposed to CuCl to compare the toxic effects on their immune responses. The LC_{50} values were similar, 40 µM (3943.2 µg l^{-1}) for the mussel and 44 µM (4337.52 µg l^{-1}) for the clam. In both species, apoptosis occurred after exposure to 1 µM (98.9 µg l^{-1}) CuCl, the concentration able to significantly increase the intracellular Ca^{2+} content. Biomarkers of cell morphology and motility revealed microfilament disruption, a significant decrease in yeast phagocytosis and lysosome hydrolase (β-glucuronidase) inhibition beginning from 0.5 µM (49.5 µg l^{-1}) CuCl in both the mussel and clam. The same concentration of CuCl affected biomarkers of oxidative stress, as a significant decrease in reduced glutathione content in the cytoplasm and inhibition of mitochondrial cytochrome-c oxidase (COX) were detected in both species. Comparison of the biomarkers showed that clam is more sensitive than the mussel regarding alterations to the lysosomal membrane and ROS production, which supports the potential harmful effects of antifouling biocides on the survival of nontarget pivotal species in the coastal community.

1 Introduction

Antifouling paints are widely used to prevent organisms from settling to ship hull surfaces. Among all the different solutions proposed over the years, coatings with tributyltin (TBT) have been the most effective (Yebra et al., 2004). However, since the 1980s, many environmental problems have been reported and correlated with TBT diffusion in coastal ecosystems, such as shell malformations and imposex in mollusk species, the latter of which represents the acquisition of male sex organs by females leading to reproductive failure and a marked population decline (Bryan et al., 1986; Gibbs and Bryan, 1986; Coelho et al., 2006; Bigatti et al., 2009; Gibbs, 2009; Strand et al., 2009). In addition, the endocrine-disrupting agent TBT has proven to be extremely toxic to a number of aquatic

organisms, particularly to those in sensitive early life stages (Thain and Waldock, 1986; Fent, 1996; Lignot et al., 1998).

Since 2001, when organotin compounds were banned worldwide by the International Maritime Organization, copper-based release technologies have been developed by the chemical marine paint industry and have progressively become the most widely used replacements for TBT-based coatings (Trentin et al., 2001; Voulvoulis et al., 2002; Yebra et al., 2004; Willemsen, 2005; Jones and Bolam, 2007). Copper compounds, including metallic copper, cuprous thiocyanate, and cuprous oxide, are the dominant active substances in currently used antifouling paints (Voulvoulis et al., 2002; Yebra and Weinell, 2009; Cao et al., 2011). Copper is an essential micronutrient used in various metabolic processes; hence, low concentrations of copper are needed, but it starts to be toxic when it accumulates in tissues and exceeds the tolerance capacity of organisms (Xie et al., 2005). Speciation studies have indicated that in coastal waters, most copper is bound to organic ligands (Voulvoulis et al., 1999), and it can form lipophilic complexes with organic compounds, e.g., dithiocarbamates, increasing their toxicity by means of synergistic effects (Bonnemain and Dive, 1990). Consequently, the copper present in surface waters is mostly bound to organic materials with a high molecular weight and tends to settle in sediments. Only a limited amount of the total dissolved copper is bioavailable in the water column and, therefore, harmful to aquatic organisms. Dissolved copper can persist in the water column as Cu(II) or Cu(I) ions, where the latter has higher biocidal activity but could be easily oxidized to Cu(II). Anoxic conditions in sediments favor the stability of the reduced form, representing another long-term source of Cu(I) (Thomas and Brooks, 2010). High concentrations of dissolved copper have been commonly found in shallow, near-coastal marine areas (Biggs and D'Anna, 2012). The concentration of copper ions in marine sediments can be up to three times higher than that in the water column (Brooks and Waldock, 2009).

In vitro experiments are well suited to quickly screen various classes of xenobiotics and provide a significant amount of information about their mechanisms of toxicity (Carballal et al., 1997a,b; Pipe et al., 1997; Wootton et al., 2003; Cao et al., 2007; Donaghy et al., 2009; De Vico and Carella 2012; Matozzo et al., 2012; Mosca et al., 2013; Messina et al., 2014; Pagano et al., 2016; Jiang et al., 2017). Functional responses have been used in the field of ecotoxicology as biomarkers or stress indexes to assess the effects of pollutants on environmental quality (Anderson, 1988). In particular, since the immune system plays a fundamental role in organism survival, biomarkers have been found in marine invertebrates such as bivalves for immunotoxicity studies of antifouling biocides (Cima et al., 1999). These organisms have been demonstrated to be very sensitive to various xenobiotics. They are sessile or mobile, have a high filtration rate, may accumulate a wide range of contaminants in their tissues, play an important ecological role in aquatic environments and are a widespread commercial product (Goldberg, 1975).

Mussels of the genus *Mytilus* have been successfully tested worldwide in a number of field studies as sentinel organisms to examine the effects of xenobiotics in marine coastal waters (Goldberg, 1975; Kraak et al., 1992; Da Ros et al., 2000; Broeg and Lehtonen, 2006; Cravo et al., 2009; Zorita et al., 2007). In recent decades, various species of benthic filter feeder mollusks, such as clams (i.e., *Ruditapes* sp.) have been tested in both field and laboratory studies as indicators of the water-sediment interface (Goulletquer and Heral, 1997; Bebianno et al., 2004; Blaise et al., 2002; Matozzo and Gagné, 2016).

The Mediterranean mussel *Mytilus galloprovincialis* (Lamarck, 1819) and the Manila clam *Ruditapes philippinarum* (Adams and Reeve, 1850) were chosen in this study as well-known filter-feeding bioindicators because they are dominant species in the trophic chain of the coastal ecosystem and are

edible mollusks. The ingestion of organisms collected from contaminated sites represents a potential risk for human health. These species are also widely used for baseline experimental bioassays and differ in their behaviors and habitats in coastal ecosystems. *M. galloprovincialis* is a sessile species that cement itself to hard substrata using byssus threads. It is common in the intertidal zone, where is dominant in the macrofouling community, and is an ideal sentinel organism for the water column. It is a target species of antifouling control systems (Parisi et al., 2022). Negative effects from copper(I)-based paints on the settlement of this species together with those of serpulids, bryozoans and ascidians were previously observed on steel and wood panels immersed for 10 months (Cima and Varello, 2023). *R. philippinarum* is a mobile bivalve that locomotes through sediments using an enlarged, muscular foot. It is a nontarget species of antifouling control systems because it burrows in sandy-muddy bottoms. It is native to Japan, Korea, and the Philippines, from which it has been successfully imported since 1983 for human consumption in the Lagoon of Venice (northern Adriatic Sea) (Donaghy et al., 2009).

Alterations in immunosurveillance have been reported for bivalve mollusks exposed to metals (Cheng and Sullivan, 1984; Cheng, 1988; Pipe et al., 1999) and xenobiotics (Fries and Tripp, 1980; Alvarez and Friedl, 1992; Beckmann et al., 1992; Coles et al., 1994; Cima et al., 1998). Xenobiotics may alter the functional parameters of mollusk hemocytes, such as viability (Alvarez and Friedl 1992), phagocytosis (Fries and Tripp, 1980; Anderson, 1988; Cima et al., 1998), aggregation (Auffret and Oubella, 1997), adherence (Chen and Bayne, 1995), lysosomal enzyme activity (Cima et al., 1999), and lysosomal membrane stability (Lowe et al., 1995; Grundy et al., 1996). Although copper is considered one of the least toxic biocides, previous studies have shown its potential to cause various damages to marine organisms and compromise their immune responses, development and survival (Bao et al., 2011; Kiaune and Singhasemanon, 2011; Cima and Ballarin, 2012; Tornero and Hanke, 2016). In the present study, a comparative evaluation of the toxic effects of Cu(I) ions on some functional responses of hemocytes from cultures of *M. galloprovincialis* and *R. philippinarum* was conducted. Additionally, a series of useful immune system biomarkers were considered.

2 Materials and methods

2.1 Animals and experimental plan

M. galloprovincialis and *R. philippinarum* were collected along the southern coast of the Lagoon of Venice (near Chioggia, Italy) and immediately transferred to the laboratory. Organisms acclimated for one week before exposure to Cu(I) ions were reared in aquariums filled with filtered seawater (FSW, 35 psu, pH 8.1), which was changed every other day. They were kept in thermostatic rooms at 19 °C, and the water temperatures in the experimental tanks were maintained at constant values using electronic thermostats. The bivalves were fed with Microbe-Lift®/Phyto-Plus B (Ecological Laboratories, Inc., Cape Coral, FL, USA) and microalgae (*Isochrysis galbana*). The collected bivalves were carefully checked for shell damage (damaged animals were not used in the experiments), and epibionts (i.e., barnacles and algae) were removed from the mussel shells. The experiments were performed outside the period of sexual maturity for mussels (winter to late spring, Da Ros et al., 1985) and clams (late spring to summer, Meneghetti et al., 2004) to reduce the potential for additional stress related to spawning during the experiments and to favor the energy balance towards the growth rate rather than the reproductive rate.

2.2 Copper(I)

Copper(I) chloride (purified, >99%, code 22433-2) was purchased from Aldrich Chem. Co., Milwaukee, WI, USA, as a water-soluble form of copper(I). Various nominal concentrations were

obtained from a freshly prepared 10 mM stock solution in FSW, i.e., 0.05, 0.1, 0.5, and 1 µM, corresponding to 4.9, 9.8, 49.5, and 98.9 µg l^{-1} (0.9×10^{-5}, 1.8×10^{-5}, 0.9×10^{-4}, and 1.8×10^{-4}% by weight of Cu). Working solutions were always freshly prepared by dissolving drop-by-drop stock solutions in FSW to avoid the formation of precipitates and appeared clear for the entire experimental time. Bathocuproine (2,9-dimethyl-4,7-diphenyl-1,10-phenanthroline sulfonate; 96% pure, code 140910, Aldrich) was used at a concentration of 1 µM as a specific chelator of Cu(I) ions (White et al., 2001) to distinguish between the effects of Cu(I) and Cu(II) ions in the medium.

2.3 Hemocyte collection

Hemolymph was collected from the anterior adductor muscle with a 1-ml plastic syringe in the presence of 0.38% Na-citrate in FSW, pH 7.5, as an anticlotting agent. Once collected, the hemolymph was placed into a 1.5 ml Eppendorf tube and immediately centrifuged at 780 × g for 10 min. The pellet was resuspended in FSW and temporarily stored on ice. Sixty microlitres of hemocyte suspension was placed in the centre of a culture chamber or on a Superfrost Plus slide (Thermo Scientific, Gerhard Menzel B.V. & Co. KG, Braunschweig, Germany) depending on the assay. Culture chambers were set up with a Teflon ring (15 mm internal diameter and 1 mm thick), glued to the centre of a siliconized glass slide, and then smeared with Vaseline to be covered with a coverslip (Cima, 2010). Hemocytes were left to adhere to the glass substratum (in the case of the culture chambers, the glass substratum was represented by the coverslip after keeping the chambers upside down) for 30 min at room temperature. Culture chambers were used only for the LC_{50}, lysosome stability index, phagocytosis index and glutathione index assays. For each experiment, pools of hemocytes from various animals were used.

2.4 Trypan Blue exclusion test for LC_{50} evaluation

This assay (Strober, 2015) was employed to determine the median lethal concentration (LC_{50}), which is the compound concentration that is lethal for 50% of the cultured cells exposed for the experimental time (60 min). After adhesion, hemocyte monolayers were incubated with CuCl at 0.1, 1, 10, 20, and 50 µM, corresponding to 9.89, 98.99, 989.97, 1979, and 4929 µg l^{-1}, respectively. At the end of exposure, the contaminant was discharged by two washes with FSW, and the hemocytes were incubated for 5 min with 0.25% Trypan Blue in FSW. This vital dye is excluded from functional/vital hemocytes but is retained in the cytoplasm of any senescent/dead hemocyte with altered plasmalemma permeability (Gorman et al., 1997). Observations, counting and micrographs of the hemocytes were performed directly on the culture chambers with an Olympus CX31 light microscope (LM) equipped with a Lumenera Infinity 2 digital camera and Infinity Capture Application v. 5.0.0 software (Lumenera Co. 2002–2009, Ottawa, ON). The percentage of dead hemocytes was determined by counting the number of cells with blue-colored cytoplasm, i.e., those unable to exclude the dye, divided by the total number of cells. For the LC_{50} evaluation, the probit method (SPSS 11.0, SPSS Corp., Chicago, IL, USA) was performed. Based on the results obtained, the concentrations used in the subsequent acute toxicity experiments were considered sublethal.

2.5 Cell function assays

2.5.1 Cytoskeleton stability index (F-actin content)

After adhesion and exposure to various concentrations of the contaminant, hemocytes 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 phosphate-buffered saline (PBS, 1.37 M NaCl, 0.03 M KCl, 0.015 M KH_2PO_4, 0.065 M Na_2HPO_4, pH 7.4). The monolayers were washed with PBS for 10 min and then

permeabilized with 0.1% Triton X-100 in PBS for 5 min at 4 °C. Hemocytes were then washed with PBS for 5 min and incubated for 30 min at 37 °C in 3.5 µl of Acti-stain^{TM} 488 Phalloidin (Cytoskeleton, Inc.) in 500 µl of PBS in the dark. Phalloidin binds in a specific and stable way to F-actin (microfilaments) but not to G-actin (monomeric), providing evidence of microfilament distribution in the cytoplasm. Finally, after rinsing with PBS, the slides were mounted and observed under the LM equipped with an amplified fluorescence by transmitted excitation of radiation (AFTER) LED fluorescence module (Fraen Corp., Corsico, Milan, Italy), with excitation at 470 nm. The cytoskeleton stability index was established by evaluation of the percentage of hemocytes with fluorescent cytoplasm due to the presence of nondepolymerized microfilaments with respect to the total hemocytes.

2.5.2 Phagocytosis index

After adhesion, hemocyte monolayers were exposed to a yeast suspension (*Saccharomyces cerevisiae*; yeast:hemocyte ratio of 10:1) in FSW containing the four nominal concentrations of the contaminant reported above. After incubation for 60 min at 25 °C, the slides were washed four times with renewed FSW to remove the nonphagocytized yeast cells. The hemocyte monolayers were fixed with 1% glutaraldehyde in PBS plus 1% sucrose. After washing with PBS, the monolayers were stained with 10% Giemsa dye in distilled water for 10 min, observed with an LM after several washes with distilled water and mounted with Acquovitrex aqueous mounting medium (Carlo Erba, Milan). The percentage of hemocytes with engulfed yeast cells with respect to the total was counted in 10 optical fields at a magnification of 1000× (Cima et al., 1995).

2.5.3 Lysosomal stability index

Lysosomal membrane stability was assessed following a modified method of Lowe and collaborators (1995). A stock solution of neutral red (NR) (0.4%) was prepared in FSW, and the working solution was obtained by diluting 10 µl of the stock solution with 5 ml of FSW. After exposure of the hemocytes, 60 µl of NR working solution was added to each chamber. After 10 min, living hemocytes were observed under LM at 1000×. The lysosomal stability index is expressed as the percentage of hemocytes showing dye loss from lysosomes into the cytosol, which appeared reddish-pink.

2.5.4 Cytosolic Ca^{2+} index

Sustained increases in cytosolic Ca^{2+} were revealed as dark-blue precipitates by Von Kossa's substitution method (Callis, 2002). Glutaraldehyde-fixed monolayers were immersed in 5% silver nitrate and exposed to ultraviolet light for 15 min. Cells were then rinsed with distilled water, incubated for 2–4 min in a 5% aqueous sodium thiosulfate solution, washed with distilled water, mounted in Acquovitrex, and observed under the LM at 1000×. The cytosolic Ca^{2+} index is expressed as the percentage of hemocytes containing precipitates.

2.5.5 Apoptotic index

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, hemocytes 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 with 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 permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate for 2 min at 4 °C. The monolayers were then washed with PBS two times for 2 min each time and incubated for

60 min at 37 °C with the reagents of the in situ Cell Death Detection, POD kit (Sigma–Aldrich/Roche), containing FITC-labeled dUTP nucleotides and deoxynucleotidyl transferase (TdT). By means of TdT, labeled nucleotides bind to broken DNA strands at the free 3'-OH ends, which are as numerous as the DNA fragmentation that has occurred in apoptotic nuclei. At the end of incubation, the monolayers were washed three times with PBS, and the fluorescence signal was visualized by incubating the hemocytes for 30 min at 37 °C with sheep anti-FITC antibody Fab fragments conjugated with horseradish peroxidase (CONVERTER-POD) provided in the same kit. The monolayers were finally washed three times with PBS and incubated for 10 min at room temperature in the peroxidase substrate (5 mg of 3-3'-diaminobenzidine (DAB, Sigma) dissolved in 200 µl of dimethyl sulfoxide plus 5 µl of H_2O_2 in 10 ml of PBS). The percentage of hemocytes with brown-colored nuclei compared to the total was taken as the apoptosis index.

2.6 Cytoenzymatic assays to evaluate the enzymatic index

2.6.1 β-Glucuronidase

This enzyme (EC no. 3.2.1.31) is well represented inside the lysosomes of bivalves (Cima et al., 1999). It is a type of glycosidase that catalyses the hydrolysis of β-D-glucuronic acid residues from the nonreducing ends of mucopolysaccharides. After adhesion and exposure to various concentrations of the contaminant, hemocytes were fixed in 0.1% glutaraldehyde and then washed with sodium acetate buffer (0.1 M, pH 5.5) for 10 min and then incubated for 2 h at 37 °C in the following incubation mixture: 4 mg of naphthol AS-BI β-glucuronide (Sigma–Aldrich) dissolved in 250 µl dimethylformamide (DMF), 400 µl of solution A (0.4 g new fuchsin, Fluka), 2 ml of 36% HCl, 8 ml of distilled water, 400 µl of solution B (4% $NaNO_2$ in distilled water) and 20 ml of 0.1 M sodium acetate buffer, pH 5.2 (Hayashi et al., 1964). After incubation, the hemocytes were washed with sodium acetate buffer for 10 min and mounted. The enzymatic index is expressed as the percentage of hemocytes showing red-stained (positive) sites of enzyme activity.

2.6.2 Cytochrome-c oxidase (COX)

Cytochrome-c oxidase (COX, EC no. 7.1.1.9) is an enzyme in the mitochondrial respiratory chain, and its activity, which is related to mitochondrial functionality, is required for adenosine-5'-triphosphate (ATP) production (Cima et al., 2008). After adhesion and exposure to various concentrations of the contaminant, hemocytes were fixed in 0.1% glutaraldehyde and then washed with 0.1 M sodium acetate buffer (pH 5.5) for 10 min and then incubated for 2 h at 37 °C in the following incubation mixture: 9 ml of 0.1 M acetate buffer and 0.2% DAB in 1% $MnCl_2$ containing 0.01% H_2O_2 (Novikoff and Goldfischer, 1969). The enzymatic index is expressed as the percentage of hemocytes showing brown-stained (positive) sites of enzyme activity.

2.7 Oxidative stress assays

2.7.1 GSH index

After adhesion and exposure to the various concentrations of the contaminant, living hemocytes 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) (Cookson et al., 1998). After at least two washes with FSW to eliminate the background fluorescence of the fluorochrome not bound to GSH in the cytoplasm, hemocytes were immediately observed under a LM equipped with an AFTER LED fluorescence module with excitation at 365 nm (UV). In this assay, the positive hemocytes appeared

with blue fluorescence in the dark field, and the GSH index was obtained by calculating the percentage of fluorescent cells compared to the total, the latter of which were counted in the bright field.

2.7.2 Superoxide anion index

After exposure to the contaminant, glutaraldehyde-fixed cells were incubated in 1 mg ml^{-1} nitro blue tetrazolium (NBT; Sigma-Aldrich) in PBS for 2 h at 37 °C and then washed with PBS according to the method of Kim and collaborators (2009). Superoxide anions lead to the reduction of NBT by cleaving the tetrazolium ring to formazan, which precipitates as blue granules. After further washing with distilled water, the slides were mounted with Acquovitrex (Carlo Erba) for observation with a LM. The superoxide anion index is defined as the percentage of hemocytes containing dark blue spots of precipitated formazan.

2.8 Data analysis

Each experiment was replicated three times ($n = 3$), and the results are expressed as the average ± SD. The percentages of positive cells were evaluated by counting the hemocytes (at least 200 cells for each monolayer) in 10 optic fields at a magnification of 1000× (0.21 mm view field diameter). Values were normalized to control in FSW (100%). Data from the treated and control hemocytes were compared using one-way ANOVA followed by Dunnett's test for multiple comparisons with DSAASTAT v. 1.1 2011 (Onofri, 2007). Differences were considered statistically significant when $p < 0.05$.

3 Results and Discussion

Bivalves have an important ecological role and are widespread in ecosystems and as commercial products worldwide (Goldberg, 1986). Hemolymph contains two main types of circulating cells, i.e., hyalinocytes and granulocytes (Carballal et al., 1997; Cima et al., 2000), which play key roles in the fundamental functions of organisms. They are involved in shell repair, digestion, excretion, and the immune response. Toxic effects due to interactions with xenobiotics can potentially impact the survival of organisms (Matozzo et al., 2001). The immunotoxic effects induced by Cu(I) in bioindicator species of water quality that belong to biofouling (mussel) or not (clam) have been considered in short-term *in vitro* assays. The indexes of immunotoxicity used as biomarkers showed that Cu(I) exposure had various effects on functional hemocyte responses.

The LC_{50} values of CuCl were determined to be 40 µM (3943.2 µg l^{-1}) for *M. galloprovincialis* and 44 µM (4337.52 µg l^{-1}) for *R. philippinarum*. Therefore, the concentrations used in the ecotoxicological assays here can be considered sublethal because they are all far below the LC_{50} values.

3.1 Effects on cell morphology

Xenobiotics can act on cytoskeleton proteins that ensure cell motility based on shape changes, cytosol fluidity and pseudopodium formation. Actin microfilaments consist of F-actin that assembles from globular monomeric actin or G-actin according to a model of dynamic instability that requires energy such as ATP (Romet-Lemonne and Jégou, 2021). Even in bivalve hemocytes, microfilaments are responsible for the formation of the cellular projections involved in cell motility and phagocytosis processes, such as pseudopodia and lamellipodia (Olabarrieta et al., 2001).

Phalloidin is a toxin produced by *Amanita phalloides* that selectively binds to microfilaments, blocking their depolymerization and therefore stabilizing them (Dancker et al., 1975). Therefore, phalloidin can be used in laboratory assays when conjugated with fluorochromes to mark F-actin. These assays highlight the disappearance of cytoskeletal microfilaments due to their disassembly provoked by toxic substances. After exposure to CuCI, a highly significant decrease ($p < 0.001$) in fluorescence beginning from a concentration of 0.5 µM (49.5 µg l^{-1}) was found in both *M. galloprovincialis* and *R. philippinarum* (Figure 1A). Data on the effects of xenobiotics on the actin cytoskeletons of bivalves are generally scarce. The effects of exposure to Cu(II) ions have been well documented on only *M. galloprovincialis* hemocytes. Fagotti and collaborators (1996) first reported a cytoskeleton F-actin damage effect at concentrations of 5 µg l^{-1}. Lethal and sublethal concentrations of Cu(II) ions from $CuCl_2$ dissociation in water (3.17×10^5 to 12.72×10^5 µg ml^{-1}) (Gómez-Mendikute and Cajaraville, 2003) and 0.02, 0.2, 1, 1.5 and 2 mg l^{-1} (Katsumiti et al., 2018), caused deleterious effects on F-actin. According to these authors, the harmful effects consist of the irregular organization of the cytoskeletal filaments of F-actin, which are poorly arranged and lead to decreased phagocytosis activity.

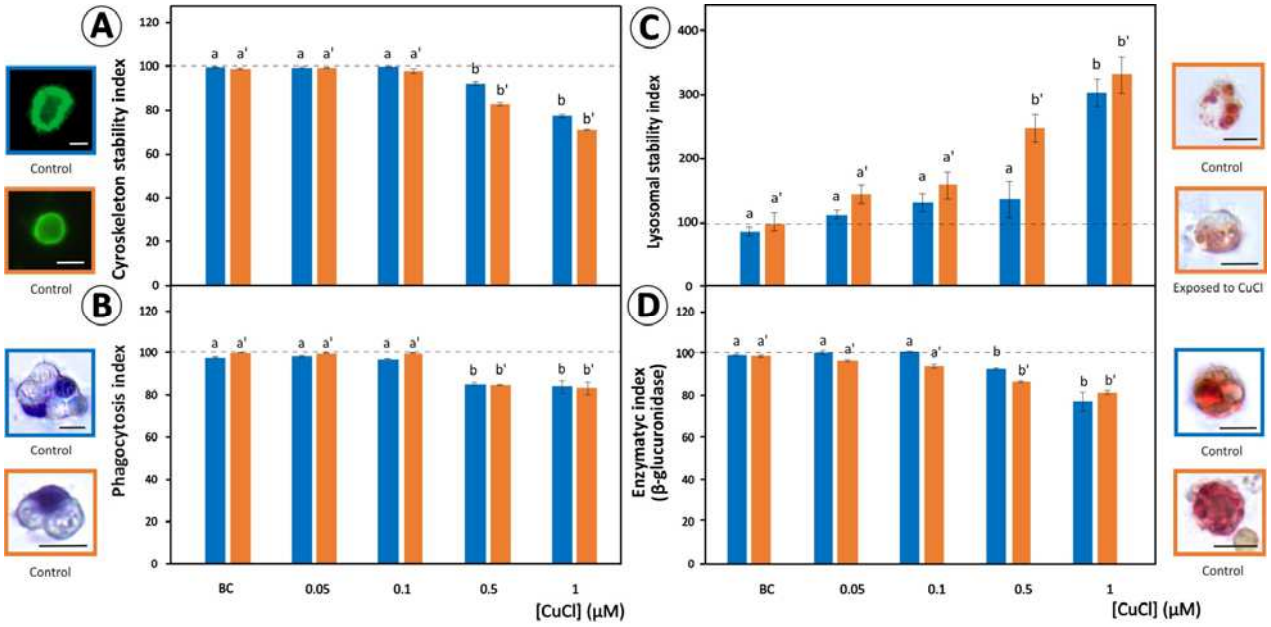


FIGURE 1. Effects of CuCl on the cell morphology and phagocytosis capability of *M. galloprovincialis* (blue bars and frame pictures) and *R. philippinarum* (orange bars and frame pictures) hemocytes. Cytoskeleton stability indexes of F-actin content (A), phagocytosis index (B), lysosomal stability index (C) and enzymatic index of β-glucuronidase (D) in the presence of various concentrations of CuCl. Controls (100%, dotted line) in FSW. BC, the specific copper(I) chelator bathocuproine (1 µM) plus 1 µM CuCl. Data are shown as the mean ± s.d. $n = 3$. Different letters on histograms indicate significant ($p < 0.05$) differences for the pairwise comparison in *M. galloprovincialis* (a to b) and *R. philippinarum* (a' to b'). Scale bars in pictures: 5 µm.

3.2 Effects on phagocytosis

In the cell-mediated innate immune responses of bivalves, phagocytosis is the main defence mechanism against pathogens and foreign materials (Cheng, 1981). This process involves the fusion of lysosomes with phagosomes containing the phagocytic material and then the release of the lysosomal hydrolases contained within (Cima et al., 2000).

The phagocytosis index describes the ability of hemocytes to phagocytize target particles such as yeast cells. These target cells are easily phagocytized by control (unexposed) hemocytes, which retain their original size but withdraw pseudopods to assume a spherical shape.

In aemocyte cultures exposed to CuCl, the percentages of *M. galloprovincialis* and *R. philippinarum* cells that engulfed the target yeast cells significantly decreased beginning from a concentration of 0.5 μM (49.5 μg l^{-1}) ($p < 0.001$) (Figure 1B). Similar results have been reported after bivalve hemocyte exposure to Cu(II) ions beginning from concentrations of 50 μg l^{-1} for *M. galloprovincialis* (Torres-Duarte et al., 2019) and 60 μg l^{-1} for *R. philippinarum* (Matozzo et al., 2001). These authors interpreted these observations by analyzing the relationship between the changes in phagocytic activity and alterations in cytoskeleton organization.

During phagocytosis, lysosomes have an important role because lysosomal hydrolases degrade foreign material engulfed inside heterophagic vacuoles. The hemocytes of bivalve mollusks may accumulate high levels of metals, mainly in the lysosomes (Pauley and Nakatini, 1968; Viarengo et al*.,* 1981; Moore, 1990; Bordin et al*.,* 1996). Alterations in the integrity of lysosomal membranes can lead to the release of such enzymes into the cytosol, resulting in damage to the cell itself (Lowe et al*.,* 1995). For these reasons, the stability of lysosomal membranes can be evaluated as a useful biomarker of possible cellular damage through their capacity to retain the NR basic vital dye (Matozzo et al., 2001). NR is a cationic dye that can enter viable cells by pinocytosis or passive diffusion across the plasma membrane and accumulate in acidic intracellular compartments such as lysosomes (Coles et al., 1995). Healthy cells retain NR in their lysosomes that appear with a red-stained content. Cells that exhibit lysosomal membrane alterations display an entirely red cytosol, since the dye diffuses outside the lysosomes. Severe alterations in NR uptake have been reported in bivalve hemocytes of the genera *Mytilus*, *Dreissena* and *Ruditapes* collected from animals exposed to cadmium, lead and tributyltin (Coles et al., 1995; Giamberini and Pihan, 1997; Matozzo et al., 2002). These alterations to lysosomal membranes might be attributable to the high levels of metals that can accumulate in lysosomes, leading to the inactivation of Mg^{2+}-ATPase, a proton pump that maintains the acid gradient inside the organelles. Dysfunction of this pump allows NR to pass from the inside of the lysosome to the cytosol. Alternatively, the accumulated metals can cause lipid peroxidation, leading to the destabilization of lysosomal membranes (Matozzo et al., 2001).

In the present study, a significant ($p < 0.001$) increase in the release of NR towards the cytoplasm from lysosomes was observed in the presence of 1 μM (98.9 μg l^{-1}) and 0.5 μM (49.5 μg l^{-1}) CuCl (Figure 1C) in *M. galloprovincialis* and in *R. philippinarum*, respectively. The results of previous studies utilizing this assay demonstrated a significant increase in toxicity by Cu(II) ions to *M. galloprovincialis* hemocytes, starting from a concentration of 1 mg l^{-1}, which corresponds to a concentration of 7.43 μM (Katsumiti et al., 2018).

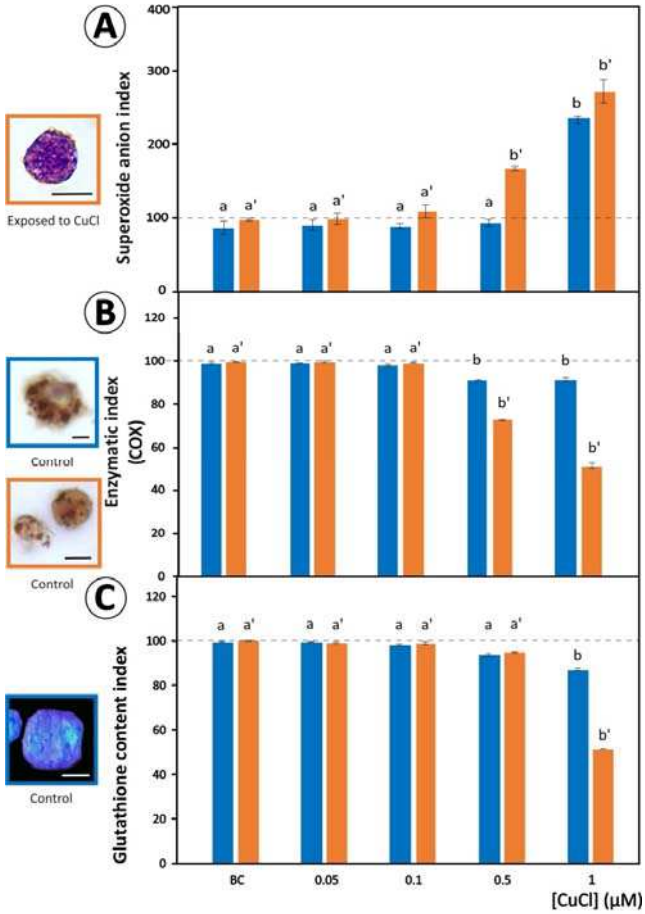
β-Glucuronidase is a lysosomal enzyme that hydrolyses carbohydrate polymers. It is widespread in bivalve mollusks, and its activity is used as a biomarker of xenobiotic toxicity (Cima et al., 1999). The cytoenzymatic assay (Hayashi et al., 1964) allows the *in situ* localisation of the activity of this enzyme. β-Glucuronidase acts on the synthetic substrate naphthol AS-BI β-glucuronic acid by hydrolysing it. This reaction generates a product that binds with the diazonium salt pararosaniline, forming an insoluble red precipitate that makes the occurrence of the reaction identifiable within the cell (Cima, 2017). After exposure to CuCl, a significant ($p < 0.001$) decrease (Figure 1D) in the *in situ* enzyme activity was observed in the hemocytes beginning from a concentration of 0.5 μM (49.5 μg l^{-1}).

3.3 Oxidative stress

The actions of the lysosomal transport chain and lytic enzymes on the degradation of xenobiotics produces reactive chemical species (Galloway and Depledge, 2001), which are unstable and may have strong oxidizing actions on other cellular structures (Livingstone, 2001; Valavanidis et al., 2006). Reactive oxygen species (ROS) are molecules such as hydrogen peroxide (H_2O_2), the hydroxyl radical ($^{\cdot}OH$), the superoxide anion radical ($O^{\cdot -}_2$) and the peroxyl radical ($ROO^{\cdot}$) that are generally produced during basal cell metabolism. However, a small percentage (2-3%) of ROS can leave the compartments where these reactions occur and cause oxidative damage to various molecules essential for cell viability, such as phospholipids, proteins and nucleic acids. Therefore, cells develop a series of molecules and enzymes with antioxidant capacity that are able to react with ROS and prevent oxidative damage. Some xenobiotics may cause increased ROS production, exceeding the antioxidant capacities of the cell. The imbalance between the production and neutralization of ROS in the cell is called oxidative stress, and has become an important evaluation parameter in environmental toxicology (Kaloyianni et al., 2009). Consequently, changes in the activities of antioxidant molecules and enzymes are useful indexes in the evaluation of the toxicity of an environmental contaminant. In the hemolymph of *M. galloprovincialis*, extensive oxidative stress was indicated by various immunohistochemical parameters after exposure to cadmium, and a significant increase in the production of ROS, followed by oxidative damage identified by DNA fragmentation, protein carbonylation and lipid peroxidation, has been reported (Koutsogiannaki et al., 2014).

The superoxide anion radical is a main type of ROS produced in oxidative stress induced by xenobiotics and is the precursor of most ROS. Under normal conditions, the superoxide anion radical plays a positive role because it is a mediator in oxidative chain reactions (Bus and Gibson, 1982), as it can reduce cytochrome c in the intermembrane space of mitochondria (Wegerich et al., 2013). The mitochondrial matrix contains a specific form of superoxide dismutase (SOD), which then eliminates the formed superoxide anion radical (Turrens, 2003), but an excess of this radical due to xenobiotics can overpower its scavenging activity. Increased mitochondrial reactive oxygen species production, superoxide anions included, is responsible for the disruption and dysfunction of (COX), which is the terminal oxidase in the mitochondrial electron transport chain and is pivotal for ATP synthesis (Srinivasan and Avadhani, 2012). In the present paper, intracellular superoxide anion production significantly ($p < 0.001$) increased after exposure to 1 μM (98.9 μg l^{-1}) and 0.5 μM (49.5 μg l^{-1}) CuCl (Figure 2A) in *M. galloprovincialis* and in *R. philippinarum*, respectively. The effects on ROS production by xenobiotics often show contradictory results depending on the exposure conditions used.

As an example, in previous experiments exposing *M. galloprovincialis* hemocytes to $CuCl_2$ for 24 h, a significant decrease in intracellular superoxide anion production was reported (Gómez-Mendikute and Cajaraville, 2003). Most ROS are very reactive, have a very short lifetime and decompose very quickly even in the absence of external agents. In this scenario, the presence of low levels of superoxide anion radicals found might be considered in connection with a long exposure time to xenobiotics. Nevertheless, the progressive increase in oxidative damage is related to self-sustaining and amplifying actions triggered by ROS. A free radical can give up its unpaired electron to a nonradical, which, in turn, becomes a free radical capable of extending and propagating oxidative damage through a chain process. Therefore, the formation of a free radical often represents the initial, triggering event of the production of various molecules capable of damaging the normal redox state of the cell (Zorov et al., 2006).



411

412 **FIGURE 2.** Oxidative stress induced by CuCl in the hemocytes of *M. galloprovincialis* (blue bars and frame pictures)
413 and *R. philippinarum* (orange bars and frame pictures). Superoxide anion index (A), enzymatic index for COX (B) and
414 GSH index (C) in the presence of various concentrations of CuCl. Controls (100%, dotted line) in FSW. BC, the specific
415 copper(I) chelator bathocuproine (1 µM) plus 1 µM CuCl. Data are shown as the mean ± s.d. $n = 3$. Different letters on
416 histograms indicate significant ($p < 0.05$) differences for the pairwise comparison in *M. galloprovincialis* (a to b) and *R.*
417 *philippinarum* (a' to b'). Scale bars in pictures: 5 µm.

418 The enzymatic index of COX was evaluated by determining the percentage of positive cells
419 containing mitochondria that turned brown in the presence of the active enzyme. After exposure to
420 CuCl, this enzyme was significantly ($p < 0.001$) inhibited (Figure 2B) beginning from a
421 concentration of 0.5 µM (49.5 µg l^{-1}) in both species, indicating oxidative stress induced by the
422 overproduction of ROS. This result is in agreement with previous observations on *R. philippinarum*
423 hemocytes exposed to $CuCl_2$, which revealed an increase in the activity of COX at a concentration of
424 60 µg l^{-1} and a decrease in enzymatic activity at the highest concentration of 110 µg l^{-1}. These
425 apparently opposite behaviors can be explained as two ways to address oxidative stress. Initially, an
426 increase in the energy need of cells occurs with treatment of up to 60 µg l^{-1} $CuCl_2$, then the energy
427 demand decreases when cell damage is excessive at the highest xenobiotic concentration (Matozzo et
428 al., 2001).

429 Among molecules with antioxidant capacity, reduced glutathione (GSH) is a cytoplasmic tripeptide
430 present in all animal cells. Its protective function against oxidative stress is due to the presence of a
431 thiol group, which is reactive with ROS, decreasing their toxicity to the cell. By reducing other
432 molecules, GSH is converted into its oxidized form, GSSG, which is able to react with electrophilic
433 oxidants through a redox reaction. Thus, cell survival depends on the GSH/GSSG ratio. Glutathione

can be considered an important biomarker that signals oxidative stress in the cell because the amount of GSSG depends on stressful conditions accompanied by the high production of ROS. When the cells are exposed to xenobiotics, the enzymes used to reduce GSSG to GSH, such as glutathione reductase and glutathione transferase are unable to convert the large amount of the oxidized form into the reduced form, and the cell undergoes oxidative stress (Liu et al., 2022). Chlorobimane allows the evaluation of GSH content in hemocytes, as the cytoplasm emits blue fluorescence when excited by UV light. This assay showed a significant ($p < 0.001$) decrease (Figure 2C) in the number of fluorescent cells beginning from a concentration of 0.5 µM (49.5 µg l^{-1}) CuCl in both *M. galloprovincialis* and *R. philippinarum*, suggesting that this contaminant can alter the oxidation state of cytoplasmic GSH with an increase in ROS production.

3.4 Induction of apoptosis

The phenomenon of apoptosis, or programmed cell death, is characterized by cell shrinkage and nuclear pyknosis and is accompanied by alterations in the integrity of the plasma membrane in the absence of inflammatory responses (Elmore, 2007). This irreversible phenomenon is considered to be related to both alterations in the homeostasis of cytosolic Ca^{2+} (Marinovich et al., 1996) and the induction of oxidative stress (Kannan and Jain, 2000).

The degree of chromatin fragmentation, i.e., the last event before cell blebbing, was evaluated by means of the TUNEL reaction. Significant increases in the number of *M. galloprovincialis* and *R. philippinarum* cells with fragmented DNA were observed after exposure to 1 µM (98.9 µg l^{-1}) CuCl (Figure 3A). At this concentration, the hemocytes became small, shrank, and underwent blebbing.

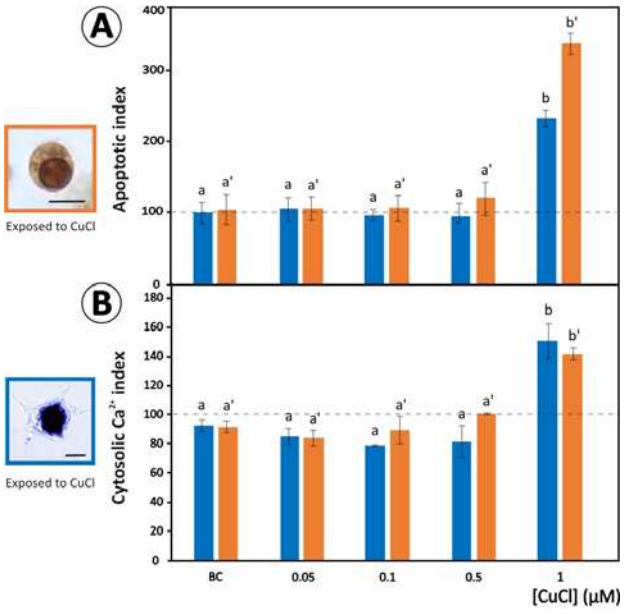


FIGURE 3. Apoptosis induced by CuCl in hemocytes of *M. galloprovincialis* (blue bars and frame pictures) and *R. philippinarum* (orange bars and frame pictures). Apoptotic index (A) and cytosolic Ca^{2+} index (B) in the presence of various concentrations of CuCl. Controls (100%, dotted line) in FSW. Bc, the specific copper(I) chelator bathocuproine (1 µM) plus 1 µM CuCl. Data are shown as the mean ± s.d. $n = 3$. Different letters on histograms indicate significant ($p < 0.05$) differences for the pairwise comparison in *M. galloprovincialis* (a to b) and *R. philippinarum* (a' to b'). Scale bars in pictures: 5 µm.

Recently, evidence from several species of mollusks has supported the hypothesis that the induction of apoptosis in hemocytes is a cellular response to exposure to environmental pollutants such as

herbicides, insecticides and drugs. As an example, the gastropod *Lymnaea stagnalis* responds to exposure to various toxic substances by activating an apoptosis program involving lectins and increased ROS production (Russo and Madec, 2007). In particular, regarding the effects of Cu(II), a dose-dependent increase in apoptosis induced by oxidative stress has also been reported in the hemocytes of the bivalve *Perna canaliculus* after *in vitro* exposure (Nguyen et al., 2018).

At the same CuCl concentration able to induce apoptosis, a sustained increase in cytosolic calcium was observed in both *M. galloprovincialis* and *R. philippinarum* (Figure 3B). This event consequently gives rise to a series of Ca^{2+}-dependent toxic mechanisms in the cell, such as cytoskeleton disassembly, calmodulin inhibition, and endonuclease activation (Orrenius et al., 2003). These alterations could be caused directly by the binding of metal particles to proteins, followed by protein denaturation, or indirectly by a change in calcium metabolism due to oxidation of the thiol groups of cytoskeletal proteins by the increased presence of ROS. Viarengo and collaborators (1994) assumed that impairment of Ca^{2+} homeostasis observed in the hemocytes of *Mytilus edulis* is the consequence of a direct effect of the metal on Ca^{2+} channels. However, heavy metals, including copper, have affinities for the SH groups of proteins such as Ca^{2+}-ATPase. Inactivation of this protein could lead to imbalances in calcium homeostasis and increased cytosolic Ca^{2+}, causing severe alterations in the cytoskeleton and affecting important functions such as adhesion, motility and phagocytic capacity (Viarengo et al., 1993; Matozzo et al., 2001).

4 Conclusion

In the literature, data on the immunotoxic effects of copper to mussel and clam hemocytes have only considered Cu(II). A comparison of the toxic effects observed with Cu(I) in this study with previously reported results on the same specific biomarkers after bivalve hemolymph exposure to Cu(II) is summarized in Table 1.

From these data, although incomplete, regarding the differences in the mechanism of immunotoxicity at the subcellular level, Cu(I) shows significant detrimental effects on the cytoskeleton, stability of the lysosomal membranes, and increases in cytosolic Ca^{2+} at much lower concentrations than Cu(II). These different behaviors suggest the potential enhanced toxicity on molecular events by copper ions in the lowest oxidation state (Beswick et al., 1976; McBrien, 1980). The absence of toxic effects in the presence of bathocuproine, a Cu(I) chelator, confirms that toxicity is due to the presence of Cu(I) ions, and not Cu(II), in the medium, possibly due to its oxidation.

A comparison of the responses after exposure to various Cu(I) concentrations of the two bivalves reveals that the immune system of *M. galloprovincialis* appears more resistant than that of *R. philippinarum.* In particular, the toxic effects of ROS production and lysosomal membrane permeability alterations were higher in *R. philippinarum*. In a previous comparative study on three species of marine invertebrates (*Carcinus maenas*, *Patella vulgata* and *M. edulis*), *M. edulis* was shown to be the most resistant species in a series of hemolymph assays that assessed differential sensitivity after exposure to $CuCl_2$ for 7 days (Brown et al., 2004).

Both *M. galloprovincialis* and *R. philippinarum* have been proven to be good sentinel organisms of biofouling and benthic habitats, respectively, because they can provide fundamental information on the actions of xenobiotics released into the environment from antifouling paints. Moreover, Cu(I) bioaccumulation not only can cause immunodepression but also other potential harmful effects – reduced adaptability and survival capability, and reproductive failure – on nontarget pivotal species of bivalves of the coastal community living in the water-sediment interface, such as clams. These

effects might progressively deplete the clam population, endanger trophic chains and, no less important, have consequences on human health due to Cu(I) and Cu(II) biomagnification in the edible tissues.

TABLE 1. Comparison of toxic effects, evaluated *in vitro*, of Cu(II) and Cu(I) ions on clam and mussel hemocytes.

Biomarker	*R. philippinarum* Cu(II) [(1)]	*R. philippinarum* Cu(I)	*M. galloprovincialis* and *M. edulis* Cu(II)	*M. galloprovincialis* Cu(I) [(6)]
Cytoskeleton stability index (F-actin content)	-	49.5 µg l^{-1} ↓ (0.5 µM)	12.72 × 10^5 µg ml^{-1} ↓ (9.46 × 10^5 µM) [(2)]	49.5 µg l^{-1} ↓ (0.5 µM)
Phagocytosis index	10 µg l^{-1} ↓ (0.07 µM)	49.5 µg l^{-1} ↓ (0.5 µM)	50 µg l^{-1} ↓ (0.31 µM) [(3)]	49.5 µg l^{-1} ↓ (0.5 µM)
Lysosomal stability index	110 µg l^{-1} ↑ (0.81 µM)	49.5 µg l^{-1} ↑ (0.5 µM	1 mg l^{-1} ↑ (7.43 µM) [(4)]	98.9 µg l^{-1} ↑ (1 µM)
Cytosolic Ca^{2+} index	-	98.9 µg l^{-1} ↑ (1 µM)	135.8 µg l^{-1} ↑ (1 µM) [(5)]	98.9 µg l^{-1} ↑ (1 µM)
Apoptotic index	-	98.9 µg l^{-1} ↑ (1 µM)	-	98.9 µg l^{-1} ↑ (1 µM)
Enzymatic index (β-glucuronidase)	-	49.5 µg l^{-1} ↓ (0.5 µM)	-	49.5 µg l^{-1} ↓ (0.5 µM)
Enzymatic index (COX)	60 µg l^{-1} ↑ (0.45 µM)	49.5 µg l^{-1} ↓ (0.5 µM)	-	49.5 µg l^{-1} ↓ (0.5 µM)
GSH index	-	49.5 µg l^{-1} ↓ (0.5 µM)	-	49.5 µg l^{-1} ↓ (0.5 µM)
Superoxide anion index	60 µg l^{-1} ↓ (0.45 µM)	49.5 µg l^{-1} ↑ (0.5 µM)	-	98.9 µg l^{-1} ↑ (1 µM)

↑, increased and ↓, decreased effect relative to controls.

[(1)]Matozzo et al., 2001; [(2)]Gómez-Mendikute et al., 2003; [(3)]Torres-Duarte et al., 2019; [(4)]Katsumiti et al., 2018; [(5)]Viarengo et al., 1994; [(6)]the present work

5 Data availability statement

The original contributions presented in the study are included in the article, further inquiries can be directed to the corresponding author.

6 Author contributions

FC: conceptualization, funding acquisition, writing, review and editing. RV: investigation, formal analysis, writing. All authors contributed to the article and approved the submitted version.

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9 Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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4.1.4 Technical report for the RESIMIX antifouling test paints

INTRODUCTION

From 2001, copper-based release technologies became the most widely used for antifouling paints replacing the TBT-based coatings (Trentin *et al*., 2001; Voulvoulis *et al*., 2002; Yebra *et al*., 2004; Willemsen, 2005; Jones and Bolam, 2007). Nowadays, copper-based antifouling paints are still the preferred alternative for leisure boaters (Karlsson *et al*., 2010) because they present a much-reduced risk compared to TBT (Hall and Anderson, 1999). Cuprous oxide was the first biocide marketed for large scale industrial production of antifouling paints (Cima and Ballarin, 2012). A content of 30–40% Cu2O (w/w) is typical in antifouling coatings, making copper(I) one of the major constituents of the paint as it exhibits antifouling activity against organisms such as barnacles, tube worms and most of algal fouling species (Lagerström and Ytreberg, 2021). Frequently in antifouling paints present on the market, cuprous oxide is in combination with booster compounds, to increase the effectiveness and target spectrum of antifouling coatings because macroalgae have a relatively high tolerance (Voulvoulis *et al*., 1999, 2006; Almeida *et al*., 2007; Parks *et al*., 2010; Cima and Ballarin, 2012; Guardiola *et al*., 2012).

The environmental impact depends on leaching rate of Cu from antifouling paints. It varies according to the structure of the paint formulation and environmental conditions (Valkirs *et al*., 2003; Schiff *et al*., 2004), e.g., water temperature, pH, salinity, copper content, formulation and age of the paint (Woods Hole Oceanographic Institution, 1952; Srinivasan and Swain, 2007; Ytreberg *et al*., 2017; Lagerström *et al*., 2018). Molecules with biocidal action included in antifouling paints are released at different times, mechanism and concentrations depending on the matrix in which they are incorporated to counteract the settlement of organisms. Currently, paints with modern self-polishing copolymer (SPC) technology occupy 80% of the antifouling paint on the market. In SPC, biocides are incorporated in an acrylic or methacrylate polymer matrix. The release mechanism of the biocides is based on the dissolution of the matrix by hydrolysis in seawater, which gives a considerable smoothness to the surface, thus reducing friction and, therefore, the resistance to the ship's motion. The release rate of substances can be controlled based on the degree of polymerisation and the polymer chains' hydrophilic properties (Yebra, 2004). cuprous oxide is the main anti-fouling agent applied in self-polishing antifouling coatings (Detty *et al*., 2014; Fu *et al*., 2019; Paz-Villarraga *et al*., 2022) and the amount of copper present in an antifouling paint varies typically between 12 and 40 wt % or more (Pérez *et al*., 2006). Reducing the amount of copper without decreasing the overall effectiveness of the paint is a research topic that has been taking into consideration since more than 20 years ago

(Vetere *et al*., 1997; Pérez *et al*., 2003, 2006; Peres *et al*., 2014). In consideration of these concerns, the aim of RESIMIX has been to produce new copper(I)-antifouling paints based on a long-term performing matrix containing the smallest possible amount of biocide working via a continuous direct contact with fouling organisms. The goal was to reduce the environmental impact with a low copper leaching from durable paints according to the EU regulations.

EXPERIMENTAL DESIGN AND SAMPLING

The sampling area chosen is placed in two stations in Chioggia with different hydrodynamic characteristics: a *mobile wharf* along the Sottomarina channel (Lat 45° 14′ N; Long 12° 17′ E) and an *abandoned mussel farm* (Lat 45°13′ N; Long 12°15′ E). The mobile wharf was a structure located 30 m from the docks, a site far from boat traffic and with limited hydrodynamics. For mobility, the wharf had panels that were always submerged following the tide fluctuations without contacting the bottom.
The experimental apparatus consisted of a series of units deployed in the study site separately installed every 60 cm. Each unit is formed of a single panel was vertically anchored with a nylon rope to a brick basement on the bottom and to the floating pier on the top so that the panel remained constantly submerged with tides by approximately 50 cm never contacting the bottom. Each panels had an exposed surface of 20 × 15 cm. The close binding with the rope maintained the vertical orientation of the panels to limit rotation and exposition, which could influence fouling colonisation by shading or floating disturbance. Therefore, all panels had the same light-exposed colonisable area of 300 cm2. Larch wood, fibreglass and SS-316 stainless-steel panels furnished by RESIMIX represented the substrata. The experimental design was performed with triplicate panel units for a useful statistical analysis. Substratum references have been represented by replicates of unpainted larch wood, fibreglass and stainless-steel panels. The other replicates were represented by wooden, fibreglass and stainless-steel panels coated with a series of samples of antifouling paints designed for boat hulls over a primer coating, which was omitted on reference uncoated panels, except for stainless-steel.

Panel monitoring started in March (2020/2021 and 2022) and the species settlement and the ecological succession have been investigated monthly. Photographs of the panel surfaces were taken with a Nikon Coolpix 995 digital camera (Nikon Corporation, Tokyo, Japan).

OBSERVATIONS ON PAINT PERFORMANCE

During the first year, I considered three copper(I)-based antifouling paints manufactured by RESIMIX with different matrix technology. Copper(I) oxide was purchased from Urai Spa (Assago, Italy). The fourth paint contained metal copper. As regard the matrix technology, three paints were based on an insoluble matrix (*Racecare*, *Redcare* and *Cuprocare*) and one on a self-polishing matrix (*Sailing Pro*) (Table 1).

Table. 1. RESIMIX and trade antifouling paints used in the first monitoring year

RESIMIX PAINTS	Cu %	BOOSTER	MATRIX
RACECARE	Cu_2O	graphite	Hard technology (epoxy resin)
REDCARE	Cu_2O	-	Hard technology (epoxy resin)
CUPROCARE	Cu^0	-	Hard technology (epoxy resin)
SAILING PRO (F58)	38% Cu_2O	-	Self-polishing (rosin)

At the end of the one-year monitoring, it was possible to establish that the self-polishing paint (*Sailing Pro*) was the best one in preventing biofouling settlement, whereas the worst one was a paint based on an insoluble matrix plus graphite (*Racecare*), the latter added to reduce the ship's hull friction. In less than 5 months the fouling covered 100% of surface. *Racecare* (Fig. 8a) revealed the worst performance since the ecological succession followed that on the reference panels. *Redcare* and *Cuprocare* (Fig. 8b,c) resulted in a fairly similar coverage, with a dominance of serpulids compared to other taxa.

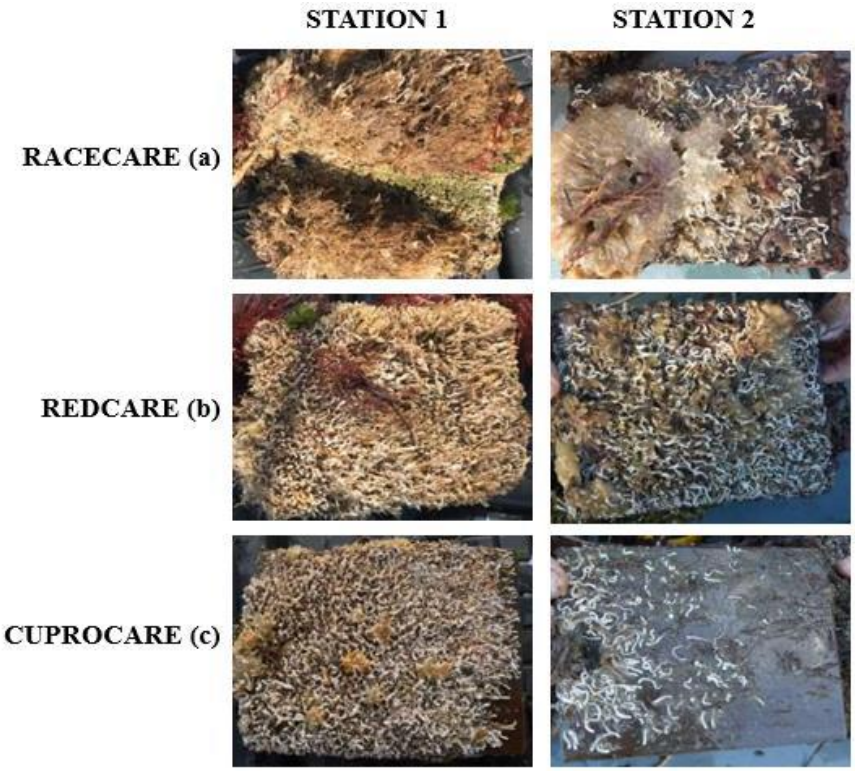

Fig. 8. Examples of settlement of macrofouling on wood panels coated with a) *Racecare*; b) *Redcare*, and c) *Cuprocare*.

Sailing Pro demonstrated lasting biocidal performance. It has demonstrated since the first months of immersion, a greater antifouling effect that remained until the end. However, already at the third month of immersion, in some places more exposed of all replicates such as the corner of the panels, the paint detached and left place to the primer below. This behaviour was found more in the station with less hydrodynamics. Then, in the zones free from antifouling, serpulids settled in station 1 (on the left of Fig. 9) and bryozoans and barnacles in station 2 (on the right of Fig. 9).

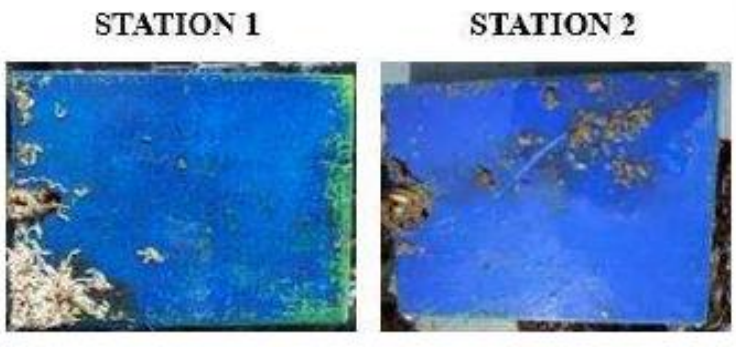

Fig. 9. Examples of antifouling activity of paint *Sailing pro* with 38% of copper (I) oxide: station 1 (low hydrodynamics); station 2 (highest hydrodynamics).

This high antifouling effect may be related to both the high copper(I) oxide content of the paint and the type of matrix. On the other hand, it appeared fundamental to strongly reduce the current biocide content (38% of copper(I) oxide) – the professional antifouling paints on the market usually contain 30-40% – for limiting the copper ion leaching into the environment according to the Biocidal Products Regulation N° 528/2012 of the European Chemicals Agency (ECHA).

During the second year, in agreement with RESIMIX, the choice was addressed to self-polishing technology and other four alternative formulations of Sailing Pro have been considered as regards both the percentage of biocide ranging between 5 – 10% and type of matrix (Table 2): 1) 5% of copper(I) oxide in a rosin matrix, 2) 10% of copper(I) oxide in a rosin matrix, 3) 10% of copper(I) oxide in a water paint (namely *Sailing Pro ECO*) with acrylate polymers, and 4) 10% of copper(I) oxide in a combined matrix (rosin plus acrylate polymers).

Table. 2. RESIMIX antifouling paints used in the second monitoring year.

RESIMIX PAINTS	Cu %	BOOSTER	MATRIX
SAILING PRO (F53)	10% Cu_2O	-	Self-polishing (rosin)
SAILING PRO (F54)	5% Cu_2O	-	Self-polishing (rosin)
SAILING PRO ECO (F21)	10% Cu_2O	-	Self-polishing water paint (acrylate polymers)
SAILING PRO (F58 mod)	10% Cu_2O	-	Self-polishing (rosin plus acrylate polymers)

In this case, fibreglass panels were chosen as a substratum, because this material better represented the commonest ship's hulls than wood. The performance of the antifouling paints monitored appeared almost equal. In the first months of immersion, a greater antifouling effect was observed in the station with highest hydrodynamics confirming that the difference in hydrodynamics is decisive for the formation of biofilm on the substrata, which is the basis for the development of the ecological succession. *Sailing Pro* with 10% of copper(I) oxide and a rosin matrix demonstrated the greater antifouling effect that remained until the end of the monitoring. In some spots on the panel surface, especially on panels in the station with highest hydrodynamics (on the right of Fig. 10), the paint left place to the primer below. In these zones free from antifouling, serpulids settled in station 1 (on the left of Fig. 10) and bryozoans and red algae in station 2.

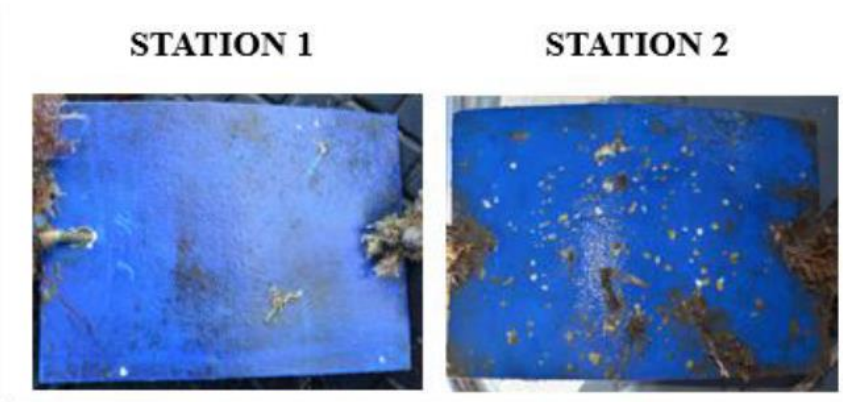

Fig. 10. Examples of antifouling activity of paint *Sailing pro* with 10% of copper(I) oxide in a rosin matrix: station 1 (low hydrodynamics); station 2 (highest hydrodynamics).

Sailing Pro with 5% of copper(I) and a rosin matrix showed a less performance. In the monitoring of the station with lowest hydrodynamics (on the left of Fig. 11) the panels showed a scanty coverage of biofilm mixed with green algae. However, when the panel was rinsed two or three times by rapid submersion in seawater the biofilm disappeared. On the contrary, in station 2 (on the right of Fig. 11) the greater hydrodynamics maintained the panels clean.

Although this paint obtained excellent results it does not fully meet the goal of this study. The matrix still contains rosin and therefore is not yet a completely eco-friendly paint.

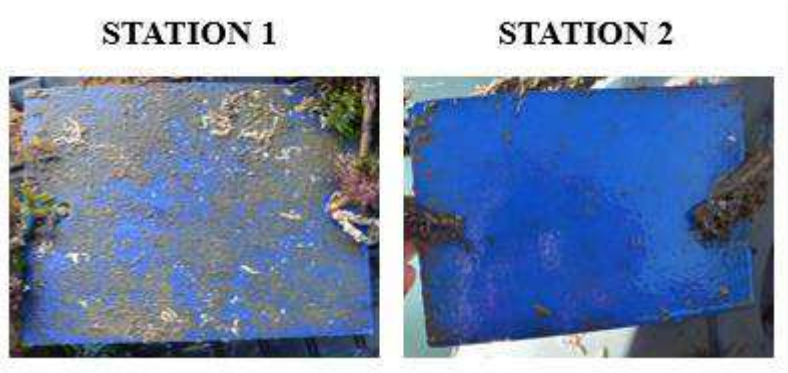

Fig. 11. Examples of antifouling activity of paint *Sailing pro* with 5% of copper(I) oxide in a rosin matrix: station 1 (low hydrodynamics); station 2 (highest hydrodynamics).

During the third year, to achieve the aim of the PhD project, that is, to develop a formulation of *Sailing Pro* with a lower content of biocidal product compared to competing products, it was decided to continue next year the monitoring with a water paint with acrylate polymers (*Sailing Pro ECO*) containing 5% copper(I) oxide. Stainless-steel panels were added to the fibreglass ones to extend the performance study on the type of substratum of ship's hulls. I monitored the new *Sailing Pro* versions in water

paints of acrylate polymers, namely *Sailing Pro ECO* (Table 3): 1) 1,3% of colloidal silver (40,000 ppm) solution on fibreglass; 2) 1,3% of colloidal silver (40,000 ppm) solution plus 2% eugenol on fibreglass; 3) 5% copper(I) oxide plus 2% eugenol on both fibreglass and stainless-still. Contemporary, I also continued the monitoring of *Sailing pro ECO* with 10% of copper(I) oxide, and Sailing Pro with 10% of copper(I) oxide in a combined matrix (rosin plus acrylate polymers).

Table. 3. RESIMIX antifouling paints used in the third monitoring year.

RESIMIX PAINTS	Cu %	BOOSTER	MATRIX
SAILING PRO ECO (F21)	10% Cu_2O	-	Self-polishing; (water paint, acrylate polymers)
SAILING ECO (F21.2)	5% Cu_2O	2% eugenol	Self-polishing; (water paint, acrylate polymers)
SAILING ECO (F23)	1,3% of solution at 40000 ppm of colloidal silver	-	Self-polishing; (water paint, acrylate polymers)
SAILING ECO (F25)	1,3% of solution at 40000 ppm of colloidal silver	2% eugenol	Self-polishing (water paint, acrylate polymers)
SAILING PRO (F58 mod)	10% Cu_2O	-	Self-polishing (rosin plus acrylate polymers)

Eugenol was proposed as a booster to inhibit the settlement of copper-tolerant green algae in particular the genus *Enteromorpha* (Pérez *et al*., 2015). It was purchased from Leuviah Srl (Catania, Italy). Eugenol have antiseptic, antimicrobial and insecticidal properties (Enan, 2001; Vazquez *et al*., 2001; Chaieb *et al*., 2005). The degradation of eugenol in environment by photolysis and biodegradation (Rabenhorst, 1996; Isman, 2000; Amat *et al*., 2005; Kadakol *et al*., 2010) indicates its non-toxic and antifouling characteristics. The U.S. Food and Drug Administration (FDA) considers eugenol as "generally recognized as safe" (GRAS), and allows the use of eugenol in food (Morales-Cerrada *et al*., 2021). In 2015, Pérez and collaborators found that the incorporation of thymol, eugenol and guaiacol into marine paints showed clear antifouling activity at tested concentrations. In this paper, the paint with both copper(I) and eugenol showed similar performance against green algae to the paints containing 16% copper(I). Similar antifouling results were achieved with both copper(I) and eugenol-based coatings containing 90% less copper than traditional copper-based coatings (García *et al*., 2015). In 2022, Tong and co-workers reported a polydimethylsiloxane (PDMS) elastomer with the modified eugenol and coumarin with antifouling properties.

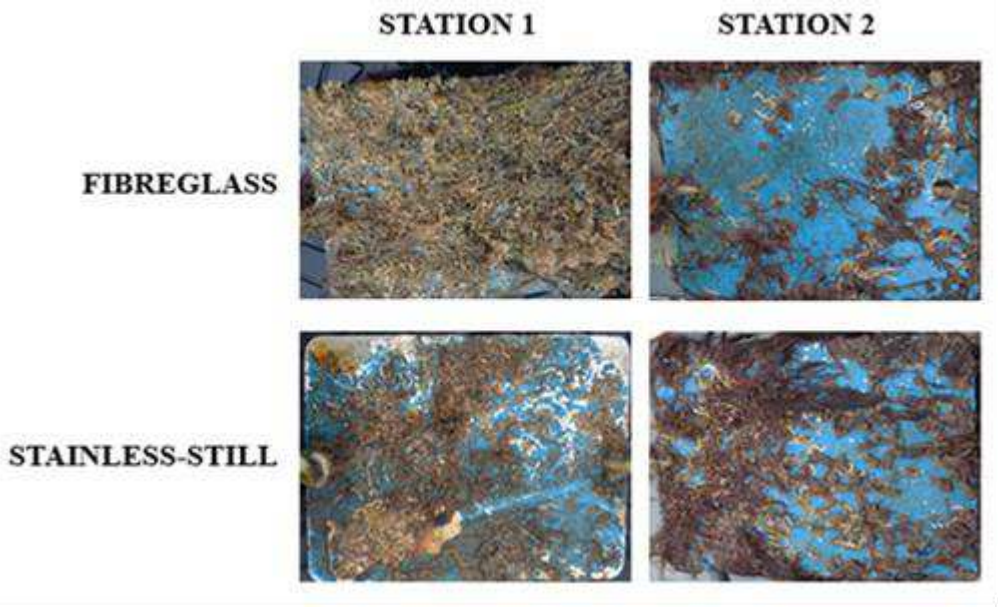

Fig. 12. An example of antifouling activity of paint Sailing pro with 5% copper(I) oxide plus 2% eugenol in a water paint with acrylate polymers: station 1 (low hydrodynamics); station 2 (highest hydrodynamic).

As regards the RESIMIX paint with 5% copper(I) oxide plus 2% eugenol (Fig. 12), the more extensive coverage of biofoulers observed on stainless-steel panels than on fiberglass panels, is far difficult to interpret and probably depends on primer adhesion with steel substratum. Primer and paint became a single layer that peeled from the steel panel, leaving the surface completely exposed (Fig. 13).

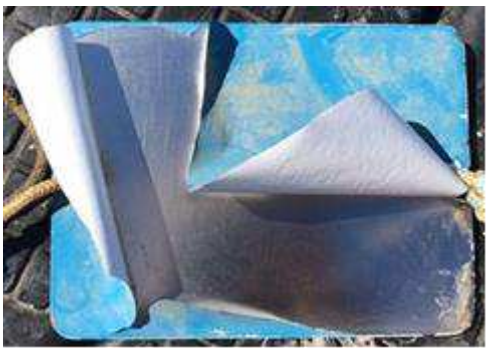

Fig. 13. Example of problems of adhesion between primer and steel panel.

These panels were promptly replaced but the problem also occurred in the following month when in some panels the same extensive detachment was observed. Probably, there was a technical problem during the panel preparation. The steel surfaces of the panels were oiled before cutting and probably the surface was still greased when the primer was spread hindering the adhesion between primer and substrate. However, the panels that did not have the problem of “peeling” confirmed that metallic substrata are more susceptible to biofouling settlement than some non-metallic

substrata such as fibreglass, wood and plastics as previously reported (Pomerat and Weiss, 1946).

RESIMIX also planned to test a new silver-based paint, for the excellent bactericidal properties of this metal in a colloidal form, which could inhibit the biofilm formation on panels. Silver is one of the most widely studied bactericide because of its well-known antimicrobial properties (Searle, 1919; Sotiriou and Pratsinis, 2010; Levard *et al*., 2012). Silver is used to coat catheters to retard biofilm development (Riley *et al*., 1995; Morones *et al*., 2005). Recently, silver nanoparticles are reported to be effective in deactivating the microorganisms (Dai and Bruening, 2002). RESIMIX employed colloidal silver purchased from 2B Minerals S.r.l. (Campogalliano, Italy). Results (Fig. 14) revealed that both colloidal silver and eugenol were ineffective as regards the antifouling activity. The panels painted with these biocides added alone or together with the copper(I) oxide, did not increase the antifouling effects of the copper itself, but even showed a high biofouling coverage on both sites.

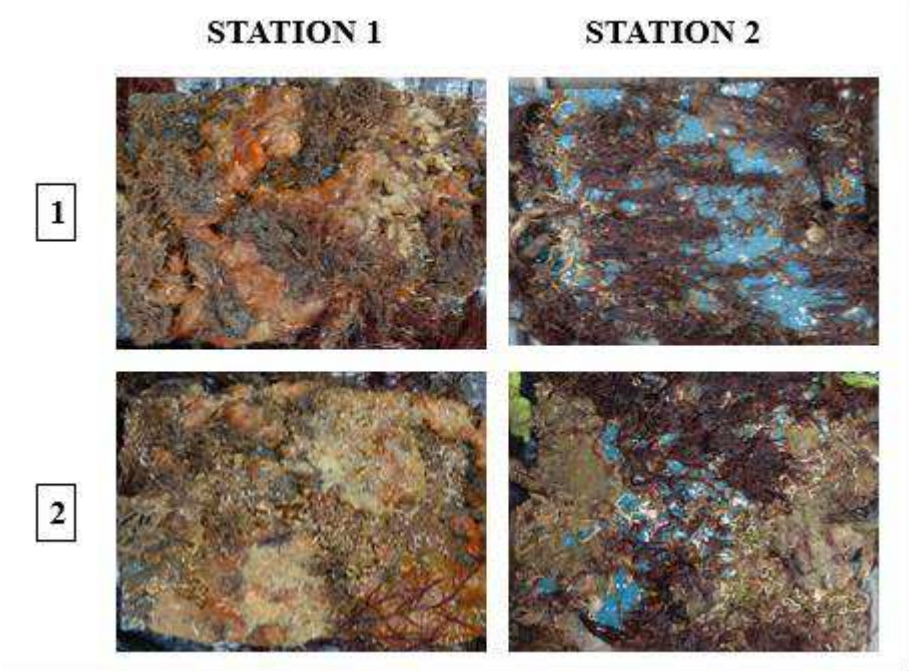

Fig. 14. Examples of antifouling activity of Sailing Pro ECO paint with (1) 1,3% of colloidal silver (40,000 ppm) solution and (2) 1,3% of colloidal silver (40,000 ppm) solution plus 2% eugenol; station 1 (low hydrodynamics); station 2 (highest hydrodynamic).

Following the positive results of the monitoring, during the first year, of the *Sailing Pro* paint with 38% of copper (I) oxide, this paint has been modified compared to the previous one to increase its performance. Acrylic resin has been added to the original rosin matrix and the percentage of biocide was reduced to 10% to make it more eco-friendly. The monitoring was followed for two years (2021-2022). At the end of 2021, in the low hydrodynamic station (upper left of Fig. 15) the surface of the panels showed

low biofilm coverage. In some places of the panel surface, the paint disappeared after a rinse removing biofilm, leaving only the primer. Instead, in station 2 (upper right of Fig. 15), few red algae appeared on the panel surface.

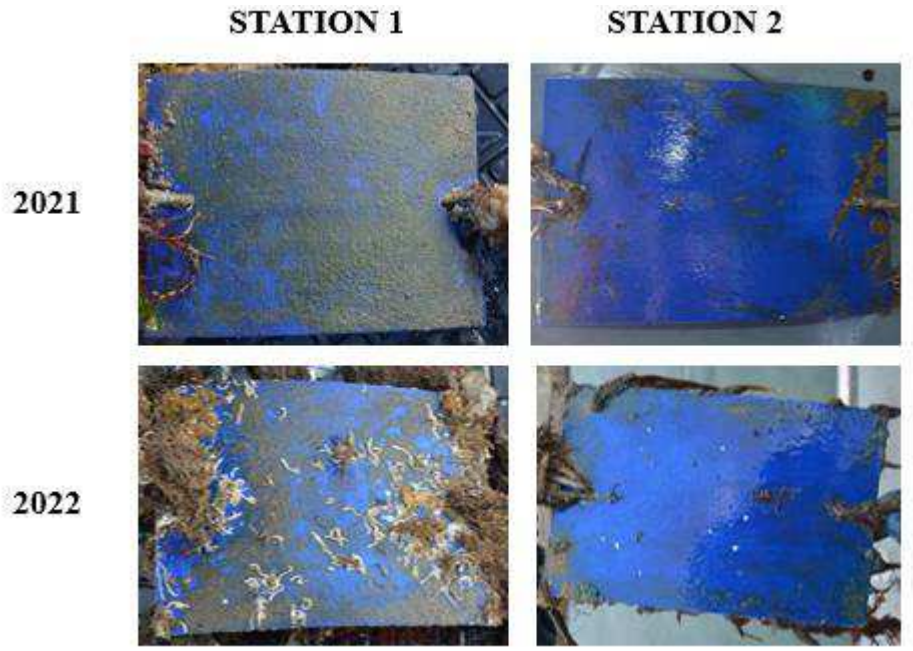

Fig. 15. Examples of antifouling activity of paint Sailing Pro ECO in a combined matrix (rosin plus acrylate polymers) monitored for two years (2021-2022): station 1 and station 2 (low/ highest hydrodynamics, respectively).

Predictably, the results obtained at the end of the second year of monitoring (2022) at station 1 (bottom left of Fig. 15), showed a decrease in paint performance. In some places, where before primer was only present, a year later I found a partial coverage by serpulids. On the other hand, in the station 2 (bottom right of Fig. 15), the situation appeared different. The panels showed a minor coverage represented by red algae.

Sailing Pro ECO with 10% of copper(I) oxide was developed by RESIMIX with acrylate polymers forming a water paint. This paint fully satisfies the goal of RESIMIX. It contains a low percentage of biocidal and a matrix without rosin. In the station 1 (upper left of Fig. 16), the panels showed a removable coverage of biofilm mixed with green algae. In the station 2 (upper right of Fig. 16), the panels showed a surface free from fouling.

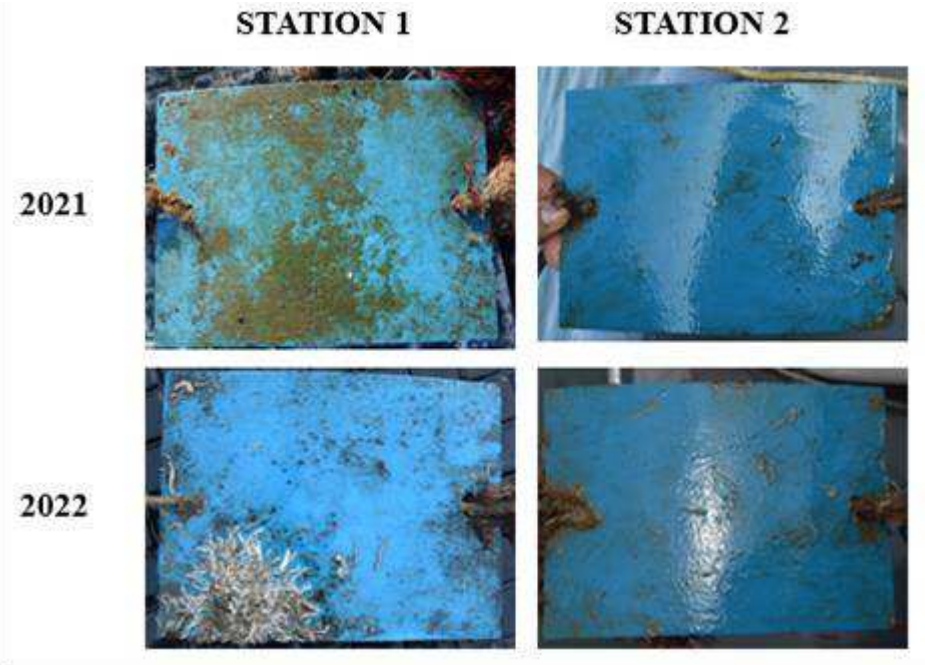

Fig. 16. Examples of antifouling activity of paint Sailing Pro ECO with 10% of copper(I) oxide in a water paint with acrylate polymers monitored for two years (2021-2022): station 1 and station 2 (low/ highest hydrodynamics, respectively).

Given the excellent results obtained, the monitoring continued for another year (2022). In the low hydrodynamic station (bottom left of Fig. 67), an increase of coverage by serpulids occurred. In the station 2 (bottom right of Fig. 16), the situation was very different because the surface of the panels was almost completely free of fouling, except for a few spots of green algae and serpulids.

RELEASE RATES OF COPPER LEACHING FROM RESIMIX PAINTS

The release rate of copper from an antifouling coating into the water column is dependent on several physical and biological factors (e.g., hydrodynamics, temperature, pH and salinity), as well as the presence of biofilm on the coating surface (Ferry and Carritt, 1946; Thomas *et al*., 1999). The release rate is also affected by the copper content of antifouling. Excessive amount of copper ions released and accumulation in sediments and water column will cause big impacts on ocean ecosystems. Some countries and regions have restricted the use of Cu2O or limited its dosage in formulations (Chambers *et al*., 2006; Detty *et al*., 2014). Different methods have been used for measuring release rates of anti-fouling paints. The most common approach is to immerse in artificial seawater (ASW) painted rotating cylinders or dishes placed on a shaker table (Valkirs *et al*., 2003; Karlsson and Eklund, 2004; Schiff *et al*., 2004; Ytreberg *et al*., 2010). Therefore, the final part of this project consisted to

measure the release rates of trade anti-fouling paints (Bluefish, Marlin and Venox, AEMME Colori) and some RESIMIX antifouling paints (Table 4) in laboratory.

Table. 4. Antifouling paints used for measuring the copper release rates.

PAINTS	Cu (%)	BOOSTER	MATRIX
A	22% Cu_2O (BLUEFISH)	2% Zineb	Self-polishing (rosin)
B	19% Cu_2O (VENOX)	5,4% Zineb	Self-polishing (rosin plus acrylate polymers)
C	5% Cu_2O	-	Self-polishing (water paints, acrylate polymers)
D	5% Cu_2O	2% Eugenol	Self-polishing (water paints, acrylate polymers)
E	10% Cu_2O	-	Self-polishing (water paints, acrylate polymers)
F	10% Cu_2O	-	Self-polishing (rosin)

The leaching behaviours of antifouling paints were studied in ASW at a salinity of 35‰. Two coats of paint were applied onto a 33,17-cm^2 square area of 3-mm thick glass dishes previously coated with primer and allowed to dry for 24 h. Finally, the paint thickness was about 0.2 mm. The painted dishes were then transferred in a beaker containing 1 l of ASW. For each paint, two replicates were considered. The beakers were closed with a bottle cap to prevent evaporation, maintained in the dark to prevent growth of photosynthetic organisms and placed on a shaker table (50 oscillations per minute) to simulate seawater movement. The copper leaching assay was performed for three months at the temperature at 19 °C. After 24 h, 48 h, 96 h, 1 week, 2 weeks, 3 weeks, 4 weeks, 2 months and 3 months, 10 ml water sample were collected from each beaker in glass vials and the copper release (µg l−1) was measured (Fig. 17) with a Copper High Range portable photometer (HI-96702, HANNA Instruments). In addition, the average release rate (µg cm^{-2} day^{-1}) (Fig. 18) was calculated using varying start times (d0, 4, 7, 14, 21, 30, 60 and 90) according to the method of Lindgren and collaborators (2018).

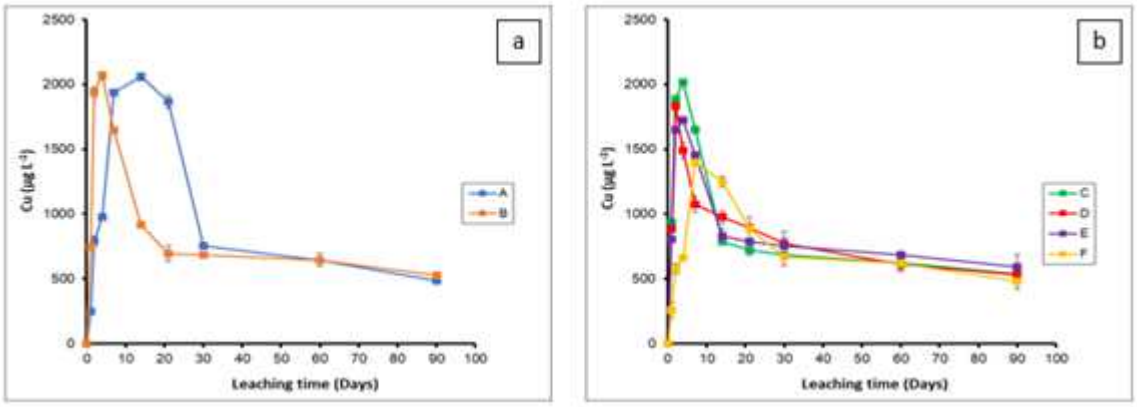

Fig. 17. Diagrams showing cumulative release copper (µg l^{-1}) from paints with different copper content during 90 days; (a) trade copper(I)-based paints (A,B), and (b) RESIMIX paints (C-F).

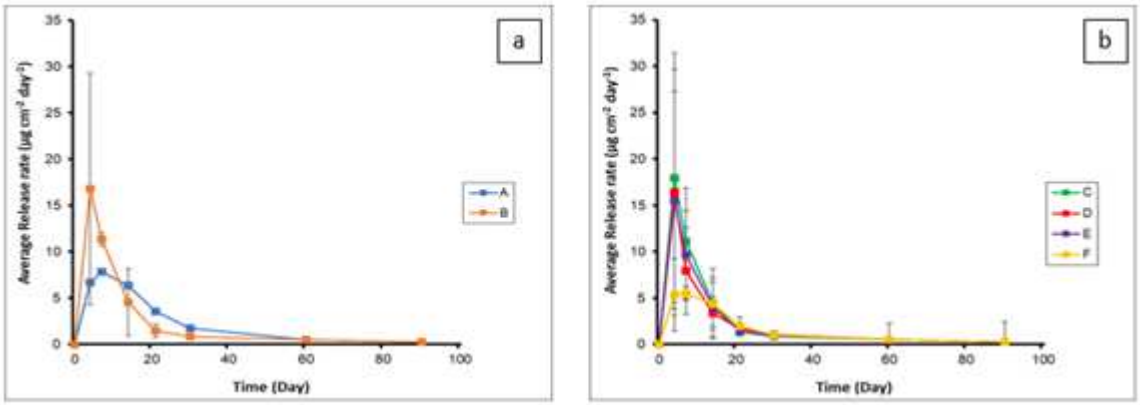

Fig. 18. Diagrams showing the average release rates (µg cm^{-2} day^{-1}) between the different measurement points ± standard deviation; (a) trade copper(I)-based paints (A,B) and (b) RESIMIX paints.

Overall (Fig. 17-18), the release of copper followed a similar pattern over time in all the copper paints. The variations in release were in general high prior to d14. Between d14 and d21, the release was more constant followed by a substantial reduction between d21–90.

Comparing rosin-based paints (A and F) the maximum release generally occurred further forward in time than other types of paints. In paint A, the release peak occurred at d14 and, in paint F, at d7, between d21 and d30 a significant decrease in the concentration of copper released in ASW was observed, mainly as regards paint A. On the other hand, in paint F the decrease in copper releasing is less pronounced. Finally, from d30 to d90 a constant low release was observed.

Sailing Pro ECO paints (C, D, and E) showed the same trend over time. Paints D and E reached the release peak after d2 whereas paint C reached it after d4. Then, the release began to decrease but although it occurs more gradually for paints C and E, a clear decline was observed in paint D. These data supported the observations of the

progressive efficacy loss made during the monitoring of the panels immersed in the Lagoon. Indeed, paint D did not show positive results as the surface of the panel was covered by biofouling.

As regards the release of copper from paint B, a unique paint with mixed matrix (rosin plus acrylate polymers), a similar trend to that of the paint C (water paints, acrylate polymers) was observed. Both paints reached their release peak after d4 . Then a clear decrease occurred up to 14 days and another decrease, but more gradual, between d14 and d21 was observed. Finally, from d21 to d90, a much smaller decrease was observed. A common observation in all paints has been that from d30 to d90 the decrease in copper release occurred in the same way.

Table 5. Average release rates of copper as µg cm^{-2} day^{-1} ± standard deviation (days 14–60, n = 2) by the trade paints compared with RESIMIX Sailing Pro paints.

PAINTS	Cu (%)	BOOSTER	MATRIX	Cu release rate (d14–60, µg cm^{-2} day^{-1})	Total released Cu (d0–90, µg cm^{-2})
A	22% Cu_2O (BLUEFISH)	2% Zineb	rosin	1.94 ± 0.97	294.24 ± 7.54
B	19% Cu_2O (VENOX)	5.4% Zineb	rosin plus acrylate polymers	0.94 ± 0.39	297.41 ± 6.63
C	5% Cu_2O	-	water paints (acrylate polymers)	1.40 ± 0.85	296.50 ± 6.63
D	5% Cu_2O	2% Eugenol	water paints (acrylate polymers)	1.09 ± 0.42	273.44 ± 13.26
E	10% Cu_2O	-	water paints (acrylate polymers)	0.98 ± 0.34	279.77 ± 11.76
F	10% Cu_2O	-	rosin	1.15 ± 0.56	205.00 ± 11.46

From the total release of copper on the d90 (Table 5), the paint F was the one that released less than all, i.e., 205.00 ± 11.46 µg cm^{-2}, and supporting the previous observations carried out during the monitoring on panels in Lagoon. Indeed, the panels painted with paint F was immersed in seawater for two years because this paint had already shown a good antifouling performance in the previous monitoring year.

Finally, the average release rate (µg cm^{-2} day^{-1}) at different measurement points, d0–4, d4–7, d7–14, d14–28, d28–60, and d60–90 has been calculated. The highest average release rates of copper were found for trade paint A (BLUEFISH) with the highest copper content. These results are in agreement with Ding and collaborators (2018)

which affirm that, the higher the copper(I) content in the paint, the higher the rate of copper(I) release.

CONCLUSIONS

Copper-based paints monitored in these studies mainly differ in their copper(I) concentrations, presence of booster biocides, and type of matrix. A greater antifouling effect was observed in the highest hydrodynamic station confirming that hydrodynamics acts by increasing the self-polishing of the matrix preventing biofilm formation.

The order of efficacy of RESIMIX antifouling paints can be established as follows: 10% of copper(I) oxide in a water paint with acrylate polymers (*Sailing Pro ECO*)/copper(I) oxide in a combined matrix / 38% of copper(I) oxide in a rosin matrix > 10% of copper(I) oxide in a rosin matrix > 5% of copper(I) oxide in a rosin matrix > copper(I)oxide plus eugenol > colloidal silver/colloidal silver plus eugenol > *Cuprocare* > *Redcare* > *Racecare*.

The best monitored paints that achieved our purpose are the *Sailing Pro ECO*, with 10% of copper(I) oxide, which maintained a good antifouling performance for two years regardless of the type of matrix, i.e., water paint or rosin.

According to the observations made during these three monitoring years, my suggestions to RESIMIX to continue the research for an eco-friendly paint, could be 1) maintaining the percentage of copper(I) between 5 and 10% 2) employing a water matrix, and 3) trying other types of substrata. In this way, the company could become more competitive on the market.

4.2 TASK 2. Testing the effectiveness of physical antifouling systems

Physical antifouling systems have been introduced in recent years in relation to the development of a more environmentally friendly approach rather than the chemical systems. For this project, I focused my studies on two types, i.e., the nonwoven geotextiles and the ultrasonic generators.

4.2.1 Nonwoven geotextiles (Study at ecosystems level)

Data about nonwoven geotextile fabrics collected in the framework of a previous research (my own bachelor degree thesis) were here statistically analysed. In a 10-month experiment, the colonisation of macrofouling organisms on different substrata based on polypropylene (PP), polyester (PET) or high-density polyethylene (HDPE) fibres was investigated in the Lagoon of Venice and compared with the colonisation on wood as a reference substratum until a “stable stage”, formed of the maximum number of species belonging to dominant taxa, was reached. Geotextile affected both ecological succession settlement and growth in different ways by preventing biofouling settlement (HDPE fabrics) or favouring dominant species (PP fabrics). For these two-faceted aspects that potentially cause different long-term impacts on the biodiversity of resident communities, the use of geotextile fabrics as alternative antifouling systems to biocide-based paint formulations or as profouling systems for restoration of degraded ecosystems has been discussed. In all cases, the communities displayed unique properties, such as a delay of the settlement of pioneer species, an initial disturbance to serpulids settlement, absence of barnacles, selection of dominant taxa (ascidians and, to a lesser extent, bryozoans and molluscs), and changes in the percentages of various taxa forming the community structure.

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Two facets of geotextiles in coastal ecosystems: Anti- or profouling effects?

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ABSTRACT

Nonwoven geotextile fabrics have physical, mechanical and hydraulic properties useful in coastal protection as an alternative to natural stone, slag, and concrete. In a 10-month experiment, the colonisation of macrofouling organisms on different substrata based on polypropylene (PP), polyester (PET) or high density polyethylene (HDPE) fibres was investigated in the Lagoon of Venice, Italy – an environment with temperate transitional waters with high biodiversity – and compared with the colonisation on wood as a reference substratum, because of its occurrence in artificial structures at the study location, until a stable stage was reached in the development of the macrofouling community. Geotextile fabrics showed implications for community development. They affected both ecological succession in different ways by disturbing biofouling settlement and growth (HDPE fabrics) or favouring species which become dominant (PP fabrics). For these two-faceted aspects that potentially cause different long-term impacts on the biodiversity of resident communities, the use of geotextile fabrics as antifouling or as profouling systems for restoration of degraded ecosystems is discussed. In all cases, the communities displayed unique properties, such as differences in the settlement of pioneer species, an initial disturbance to serpulid settlement, absence of barnacles, selection of dominant taxa (ascidians), and changes in the percentages of various taxa forming the community structure. Given the increasing interest in geotextile materials for employment in various marine developments and industries, these results could represent first lines of evidence to inform decision-making to minimise/modify biofouling, and/or predict the use of artificial substrata as habitats by marine organisms.

1. Introduction

Many marine algae and invertebrates depend on hard substrata for the settlement of their propagules and larvae. Both natural and artificial hard substrata are made of different materials. Natural hard substrata are not only represented by rocks, shingle and coral reef but also by shells, exoskeletons and teguments of other organisms. Artificial hard substrata include concrete, slag, steel piling, synthetic plastics, and wood, and offer relatively stable habitats but are often rare in natural areas compared with soft sediments (Taylor and Wilson, 2003). On the other hand, artificial substrata such as seawalls, steel and wood pilings, jetties, groynes, and pontoons are common habitats today along many coasts and estuaries of industrialised countries, where up to 60% of the shoreline is obstructed (Wetzel et al., 2014). Anthropogenic structures provide habitable spaces for many benthic organisms and can be colonised very rapidly, e.g., beginning from 1 to 2 weeks under favourable environmental conditions in the temperate zone (Scheer, 1945; Anderson and Underwood, 1994; Moreau et al., 2008), often causing severe damage to submerged structures (Connell, 2001; Atilla et al., 2003; Bulleri and Airoldi, 2005; Dobretsov et al., 2013). This phenomenon is of high economic interest because biofouling represents a large problem worldwide (Alberte et al., 1992; Holm, 2012). Biofouling on ships' hulls (Callow and Callow, 2002) and on surfaces of coastal structures, marine industries and hydrotechnical infrastructures is a major economic and technical problem with international efforts in the development of ocean engineering. Biofouling does not only increase static and hydrodynamic loading but also affects corrosion characteristics and impedes underwater inspection and maintenance (Callow and Edyvean, 1990; Flemming et al., 2009).

Geotextiles represent new synthetic materials that have been introduced into coastal environments to prevent shelf erosion and to protect artificial submerged structures. They are ultraviolet (UV)- and chemical-resistant and are widely used in civil and environmental engineering and construction projects, such as soil filtration (Palmeira et al., 1996), dyke

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construction (Koffler et al., 2008), and the general prevention of erosion (Theisen, 1992). As an alternative material in aquatic engineering, geotextiles can replace natural stone, slag, or concrete materials. In some cases, such as protecting shores from erosion, large geotextile containers can be considered preferential solutions when traditional materials are not acceptable (Heerten et al., 2000a,b; Jackson et al., 2001; Restall et al., 2002; Tomlinson et al., 2003; Black and Mead, 2009). In particular, nonwoven geotextile fabrics have been increasingly used in coastal and marine engineering over the last decades (Lee and Douglas, 2012; Mitra, 2013; Oumeraci and Recio, 2017), representing in turn a potential settlement surface for species that naturally occur on hard substrata.

The surface structure of nonwoven geotextiles has a unique texture of polymers that is unlike any texture found on natural hard substrata as a result of their production process (Dassanayake and Oumeraci, 2012). They are made of long polypropylene (PP), polyester (PET), or high-density polyethylene (HDPE) fibres that are entangled or crimped in a fleece-like texture, which results in a highly irregular surface. The fibre geometry allows for efficient hydraulic and drainage performance, whereas the heavy mass of the fabric allows for a high level of toughness and damage resistance (Carneiro et al., 2018a). Because of their texture, these textiles could affect organism settlement and the biodiversity of the resident community in coastal ecosystems, preventing biofouling settlement or favouring species which could become dominant. Therefore, the impact on ecosystem biodiversity caused by geotextiles requires attention.

The aim of the present study was to investigate the differences in the ecological succession trends of the hard-substratum community in the southern basin of the Lagoon of Venice on four nonwoven geotextile fabrics based on PP, PET and HDPE. The selective effects of different polymer compositions and surface properties on both settlement and growth capability of fouling species were considered on the basis of significant change in biodiversity, substratum coverage and biocoenosis structure. The macrofouling communities that these textiles support have been compared with communities developing on wood. Natural hard substrata are not present in the Lagoon of Venice, but some artificial ones, like wooden piles (namely 'bricole'), have been 'naturalised' in this ecosystem by their being permanently immersed in the Lagoon waters over a longtime - in some cases, for centuries - and colonised by a complex benthic community which has been previously widely studied (Occhipinti-Ambrogi et al., 1988; Sacchi et al., 1998; Sconfietti et al., 2003; Corriero et al., 2007; Cima and Ballarin, 2013). Thus, wood has been chosen as an artificial-substratum reference for its prevalent presence in various artificial submerged structures throughout the Lagoon of Venice (Ceccato et al., 2013). Experiments were conducted on panels permanently submerged for ten months. The ecological succession was monitored monthly from biofilm formation to the reaching of a stable stage of the development of the community on the wooden panels (Scheer, 1945; Railkin, 2004; Wahl, 2009).

2. Materials and methods

2.1. Study site

The study site was a mobile wharf consisting of large plastic floats located in the southern basin of the Lagoon of Venice along the Sottomarina channel (Lat. 45° 14′ N, Long. 12° 17' E), in a zone with low boat traffic representing a subtype of the euhaline, not confined microenvironment of the lagoon biome connected to the port inlet of Chioggia (Venice, Italy) (Fig. 1). The basin has a tidal range >50 cm and a depth <1.5 m. The plastic floats never contact with the bottom because the average height of the low waters is 30 cm. Temperature usually ranges between a minimum of 5.6 °C in February and a maximum of 29.9 °C in August. Salinity varies from 28.1 to 38.1 PSU depending on the rainfall trend (database of the Hydrobiological Station of Chioggia, https://chioggia.biologia.unipd.it/en/the-database/parameters-of-lagoon/). For the geographical location near the port inlet, the site is greatly exposed to water circulation and tides that increase the oxygenation. Dissolved oxygen concentrations usually range from 7.03 mg l^{-1} in August to 7.92 mg l^{-1} in February (Irato et al., 2007).

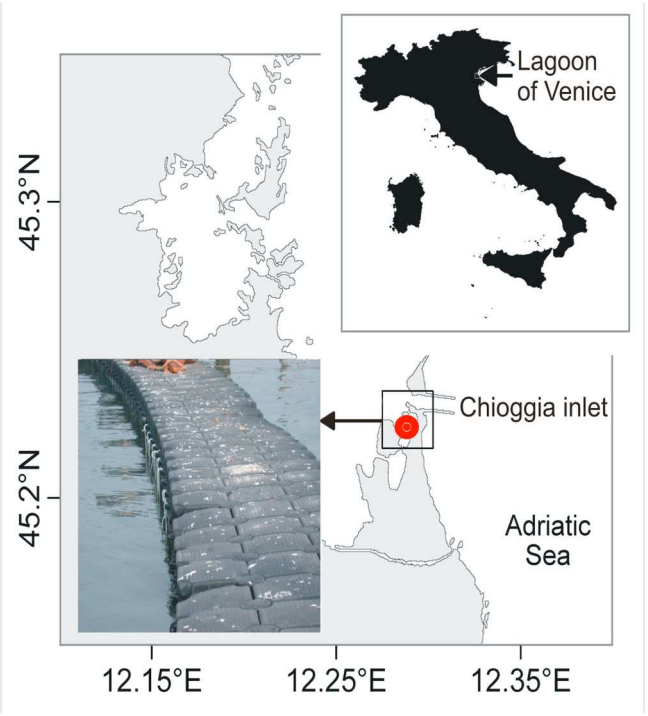

Fig. 1. Location of the mobile wharf (photo) in the experimental site close to the southern inlet (Chioggia) of the Lagoon of Venice, Italy.

2.2. Substrata

For this experiment, four different nonwoven geotextile fabrics furnished by Naue GmbH & Co. KG (Espelkamp-Fiestel, Germany) were used, represented by 'black HDPE', 'white PP & white PET', 'coloured (i.e., recycled) PP & PET', and 'white PP', showing different textures and engineering applications (Table 1). For their long-term abrasion resistance, recent special applications include i) rock revetments to prevent erosion to structures of coastal and estuarine defence from waves or currents and ii) sand container bags for visible and invisible shore protection and construction of artificial reefs, groynes, and breakwaters. For the morphological analysis of the fibres, small samplings (0.5 × 0.5 cm) of the geotextiles were observed and photographed under a Cambridge Stereoscan 260 scanning electron microscope (SEM) after critical point drying and gold scattering (Fig. 2).

Seasoned wood panels of larch (*Larix decidua*) were chosen as a reference substratum. Larch lumber is one of the best materials for naval, diving and hydraulic buildings, such as piles, bridges, wharves and pipelines, because it is water resistant and long lasting. It is heavy, compact, elastic and durable. It has high resistance to decay in water and was used, to a large extent, to build the foundations of the cities of Venice and Chioggia. Because of the presence of very marked parallel veins, the surface of larch wood is rough even when it has been smoothed, and the roughness increases with the duration in the water because of the action of both swell and organisms.

2.3. Experimental setup

Twenty experimental units were deployed in the study site, 16 of which with the four geotextiles considered in this study, represented by four replicates for each type, and 4 with larch wood as a reference

Table 1
Nonwoven monolayer geotextile fabrics employed in the present study.

Trade-name	Composition	Feature	Technical specifications	Applications
DEPOTEX R305	black HDPE	staple, needle-punched smooth fibres 30 μm in diameter	- highly UV-resistant - mass per unit area: 300 g m^{-2} - thickness: 2.7 mm - water permeability (VIH50): 5.5 × 10^{-2} m s^{-1}	protection of landfill geomembranes
SECUTEX GRK4C	white PP & white PET	staple, needle-punched, hot-calendared smooth fibres 30 μm in diameter	- mass per unit area: ≥250 g m^{-2} - thickness: 1.4 mm - VIH50: 5.5 × 10^{-2} m s^{-1}	road and dyke construction
SECUTEX R404	coloured PP & PET	mixture of recycled staple PP smooth fibres 30 μm in diameter, needle-punched and crimped fibres with PET fibres 40 μm in diameter and furrowed by longitudinal grooves	- mass per unit area: 400 g m^{-2} - thickness: 3.6 mm - VIH50: 7.5 × 10^{-2} m s^{-1}	sand-filled containers in barrier systems
SECUTEX R601	white PP	staple, needle-punched smooth fibres 30 μm in diameter	- mass per unit area: 600 g m^{-2} - thickness: 5 mm - VIH50: 3.0 × 10^{-2} m s^{-1}	drainage, separation and filtration

replicate-group. Each unit was formed of a single panel tied at a depth of 50 cm to a thick nylon rope. The latter was maintained vertically in the water column by a brick as ballast at the bottom end and was anchored to an eyehook on a mobile wharf at the top end. The wharf (Fig. 1) was a structure located 30 m from the docks, a site far from boat traffic and with limited hydrodynamics. For mobility, the wharf had panels that were always submerged following the tide fluctuations without contacting the bottom. Each unit was arranged randomly at 60 cm from each other. The wooden panels were 20 × 15 × 2.5 cm in size, with a 1-cm-diameter hole where the rope passed through. The panels of geotextiles, each forming a free surface of 20 × 15 cm with variable thicknesses, were supported by a frame of Plexiglas with a thickness of 0.5 cm and a 1-cm-diameter hole for the rope on its upper side. The close binding with the rope maintained the vertical orientation of the panels to limit rotation and exposition, which could influence fouling colonisation by shading or floating disturbance. Therefore, all panels had the same light-exposed colonisable area of 300 cm^2.

The units were constantly immersed from spring (April) to winter (January) as artificial substrata for settlement by fouling organisms. The ecological succession was monitored monthly on the light-exposed side of the panels. Photographs of the air-exposed panel surfaces were taken with a Nikon Coolpix 995 digital camera (Nikon Corporation, Tokyo, Japan). Samplings of small fouling organisms (0.5–10 mm in length) were collected from the panels and fixed in 5% formaldehyde in seawater for better species identification under a dissection binocular stereomicroscope Wild Heerbrugg with a 50x maximum magnification. From the analysis of the photos, data were analysed according to previously developed methods and parameters for studying the macrofouling community of hard substrata in the lagoon ecosystem (Cima and Ballarin, 2008, 2013), which include tests for differences in both biodiversity indexes and rates of changes in biomass. In particular, the biotic data were expressed by means of four descriptors of biodiversity: i) *species richness*, i.e., the total species richness (total number of species) and mean species richness (average number of species ± standard deviation) by month present on all panels of the same type; ii) *biocoenosis structure*, i.e., the percentage of coverage for each taxon, namely the set of species belonging to the same taxonomic group, in respect of the total coverage of the whole community (100%) on panels of the same type of substratum; iii) *covering-abundance area*, i.e., a quantitative analysis (percent cover) of the settlement capacity of the various species on areas calculated in photos using the Infinity Analyze Application v. 5.0.0 software (Lumenera Co. 2002–2009); iv) *biomass*, expressed in g cm^{-2} of fresh weight (FW) of the living fouling organisms and determined by weighing the panels, after a rapid draining, with a portable electronic scale. All the operations on air-exposed panels occurred immediately and gently *in situ* to avoid withering, drying up and destroying collapse of biofoulers.

Data on the physical and chemical parameters of seawater, such as temperature, pH, and salinity (Supplementary Fig. S1), were collected monthly using a Cyber Scan XS PC 300 Waterproof Hand-held pH/Conductivity//Temperature Metre (Euthech Instruments Pte Ltd, Singapore, www.eutechinst.com). Conductivity values were subsequently converted to practical salinity unit values (PSU) in relation to the thermal properties of seawater (IOC et al., 2010).

2.4. Data analysis

Statistical analysis and figure compilation were performed using R software (version 3.1.2; R Core Team, 2014), and the level of significance was set at $p < 0.05$ for all statistical tests. The data on species richness and coverage area were analysed with analysis of variance (ANOVA) with a repeated measures design considering interactions among the predictors 'time' (month) and 'type of substratum'.

To investigate significant differences among the covering surfaces of each fouling species on the various substrata, the measures of the areas (cm^2) per month were compared using permutational multivariate analysis of variance (PERMANOVA plus; Anderson, 2001) considering one fixed factor, i.e., the type of substratum, and one random factor, i.e., the monitoring month. All analyses were carried out using 9999 permutations.

To test the hypothesis that the species composition of the community on different substrata was significantly different and changed over time, a Bray–Curtis similarity matrix (Kruskal and Wish, 1978) was calculated with presence/absence of species using raw data (untransformed). A hierarchical cluster analysis and the Similarity Profile Routine (SIMPROF; Clarke et al., 2008) were used to identify significant differences between species assemblages. Analysis of similarity percentages (SIMPER; Clarke, 1993) was used to rank the species that contributed most to the average Bray-Curtis dissimilarities between the communities identified by the SIMPROF procedure. Bray-Curtis, clustering, and SIMPROF calculations were performed with the 'simprof' function in the R package 'clustsig' (Whitaker and Christman, 2015), and the SIMPER procedure was performed using the 'simper' function from the R package 'vegan' (Oksanen et al., 2018). The results of these analyses were plotted in a classification dendrogram.

The hypothesis stating that the biomass development on different nonwoven geotextiles differs significantly depending on the type of surface was tested using a combination of statistical methods. First, we observed that the biomass development on the different substrata was differentiated into two sections: (i) the increase in biomass at the start of the experiment and (ii) a more or less static or decreasing phase, which occurred later in the year after the biomass development had reached its

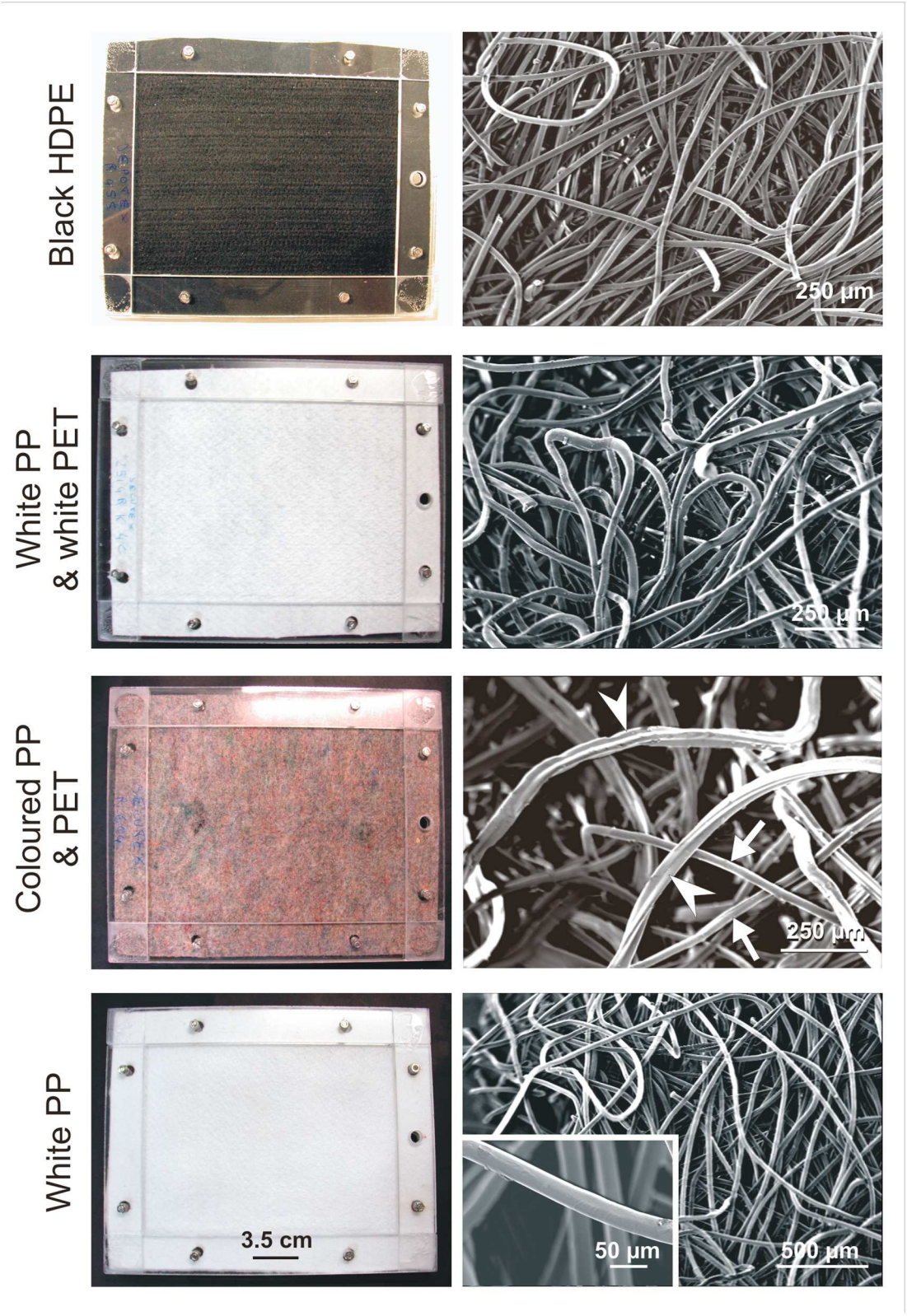

Fig. 2. Panels of geotextiles fixed inside a plexiglass frame (left) and detailed structure and microtopography of polymer fibres with SEM (right). Note that for the 'coloured PP & PET', two types of fibres formed the crimped felt, i.e., smooth PP fibres (arrows) and larger furrowed PET fibres (arrowheads).

peak (change point). The substratum-specific change points were identified using the method of Davies (2002). This method tests for a non-zero difference in slope parameters of a segmented relationship and is also suitable for small sample sizes. With this procedure, change-points were calculated as points where the linear regression coefficients shift from one stable regression relationship to a different one using the package 'segmented' (Muggeo, 2015). Data were expressed as means of FW with standard deviations. To allow

identification of statistical differences between the change-points we used bootstrapping. In brief, from the biomass values collected on each substratum each month, the average value from four replicates was chosen and the change-point was calculated. This was repeated 100 times for each substratum, which resulted in a dataset of 100 randomly collected change-point values per substratum. These bootstrap generated values which were then used to test the hypothesis stating that different substrata showed a different change-point using one-way

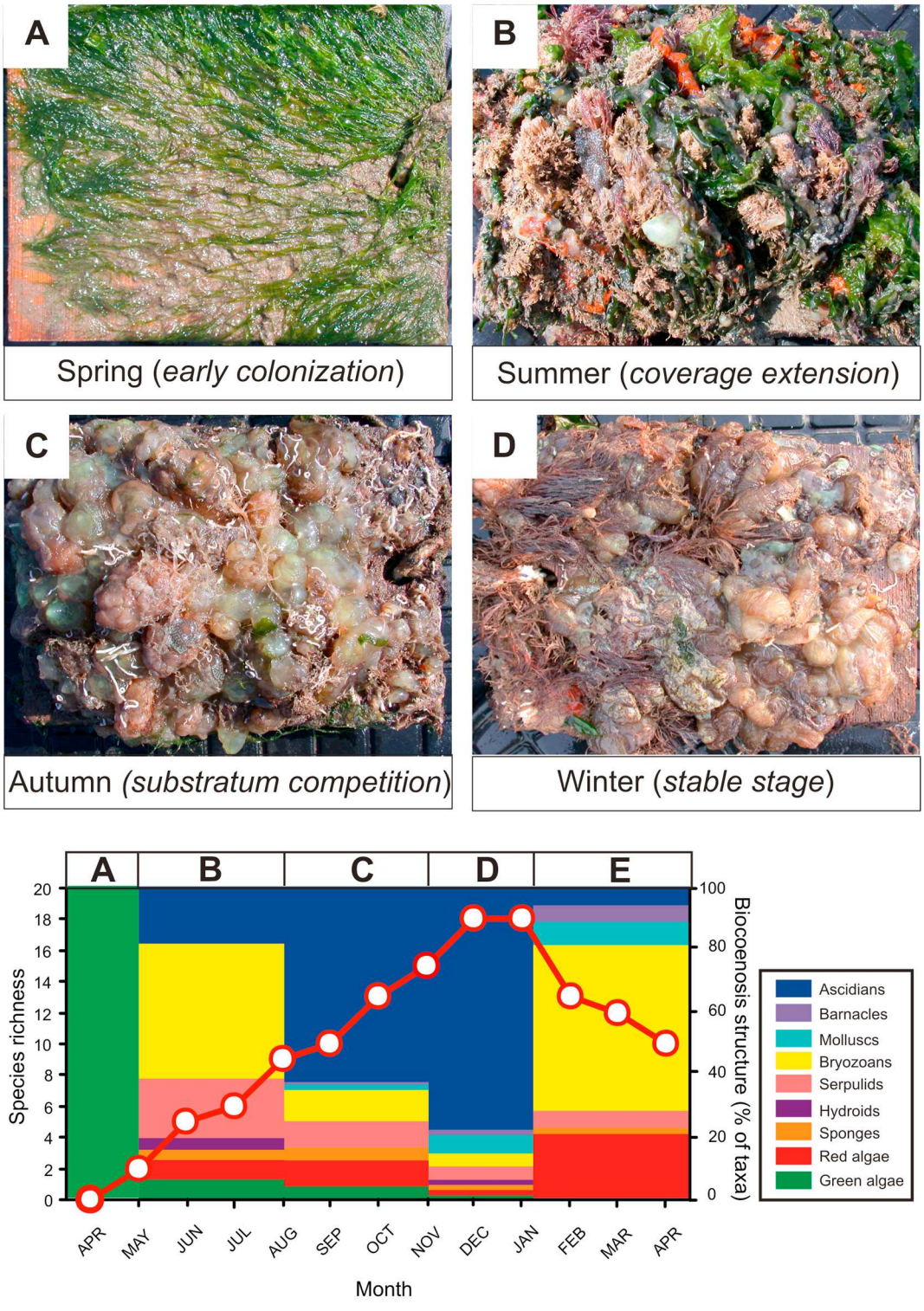

Fig. 3. Main seasonal phases (A–D) of ecological succession on selected wooden panels throughout the experimental immersion in the Lagoon of Venice. In the graph, example of typical trends of the biodiversity descriptors 'species richness' (solid line) and 'biocoenosis structure' (bar plots) during an 1-year experimental immersion (April 2005–April 2006), showing the phases (A–E) of ecological succession. After the reaching of a 2-month stable community between late autumn and winter called 'stable stage' formed of the maximum number of species belonging to dominant taxa (D), in late winter and early spring - when harsh winter conditions are established - a reduction in the number of species followed by a dismantling of the previous biocoenosis structure occur (E).

Analysis of Variance (ANOVA). The latter was carried out with month as random factor, followed by a Tukey Honest Significant Differences test (Tukey HSD) for post-hoc pairwise comparison.

3. Results

3.1. Changes in species richness and covering-abundance area

In the Lagoon of Venice, the ecological succession of the community on wooden panels (Fig. 3) occurred regularly with the same main phases over seasons observed in the previous monitoring campaigns on wooden and steel hard substrata (Cima and Ballarin, 2008, 2013): i) early settlement of pioneer organisms in spring (April–May), ii) progressive extent of macrofouling coverage in summer (June–August), iii) competition and establishment of dominant taxa in autumn (September–November), and iv) reaching of a stable stage in early winter (December–January). The latter is a prolonged stage characterised by the highest number of species forming a relatively stable community that temporarily survives under the mild climatic conditions of the area. When harsh winter conditions are progressively established (February–March), the community loses this stable structure because most previous dominant species die and, in the following spring, a new community settles and grows up over remnants and concretions. This indeed represents a manifestation of seasonal phenomena termed 'cyclical succession' due to the natural die-off of early colonists, as these species not only grow rapidly but also have short life spans (Shelford, 1930).

The ANOVA analysis of change in species richness and coverage area on geotextiles by macrofouling (Table 2) revealed that significant differences in species richness only depended on month by month (i.e., seasonality), whereas the coverage area of biofouling depended significantly on both month and type of geotextile. In particular, at the end of the experimental period 'black HDPE' showed a significant decrease in the coverage area in comparison with both the other geotextiles and wooden panels (Supplementary Fig. 2S). The trends and fluctuations of species richness and covering-abundance area of each species on the various experimental substrata over the course of the experiment are reported in detail in Figs. 4 and 5, respectively. Total coverage dynamic on different substrata is described in Fig. 6. Results from these figures reveal that in spring, one month after immersion, all of the substrata were covered to a large degree with a biofilm - a fine mixture of inorganic sediment and organic substance entrapping microorganisms such as bacteria and microalgae - with the exception of the wooden substrata, the latter showing a higher coverage area (60%) of the green macroalga *Ulva intestinalis* Linnaeus, 1753 as a pioneer species. In the subsequent month, only ascidian species had largely colonised the geotextile substrata, represented by the solitary species *Ciona robusta* Hoshino & Tokioka, 1967 and *Ascidiella aspersa* (Müller, 1776) and the colonial species *Botryllus schlosseri* (Pallas, 1766). In summer, species richness showed very rapid growth on all of the substrata, from 1 to 3 species in May to 4 to 8 species in July. The red alga *Gracilariopsis longissima* Steentoft, Irvine & Farnham, 1995 first occurred on all of the substrata, and unlike the wooden panels, the geotextile panels did not display settlement of green algae during this period. The dominant animal species in the summer months were the solitary ascidians *A. aspersa*, *C. robusta*, and *Styela plicata* (Lesueur, 1823) and the colonial ascidians *Botrylloides leachii* (Savigny, 1816) and *B. schlosseri*. In particular, the solitary ascidians *A. aspersa* (coverage areas: 18.2% on wood, 87.4% on 'black HDPE') and *C. robusta* (coverage areas: 4% on 'black HDPE', 20% on 'coloured PP & PET') and the bryozoan *Bugulina stolonifera* (Ryland, 1960) (coverage areas: 7% on 'black HDPE', 15% on wood) appeared on all of the substrata. The latter two species remained, with changing coverage, throughout the experiment. Late in autumn and initial winter, new taxonomic groups were observed such as barnacles (*Balanus* sp.) only on all wood replicates, and molluscs (*Mytilus galloprovincialis*, *Ostrea edulis*, *Patella caerulea*) on all of the substrata. The number of species increased and the controls reached a stage of competition for the substratum preceding a stage of monopolisation of space by dominant species. The stable stage was completed on controls in December–January, with ascidians as the dominant species in the community and, in general, a constant increase in the number of species with the exception of the 'coloured PP & PET' and 'black HDPE' geotextiles. The latter also showed a decrease in coverage areas (35%) with respect to those of both the other geotextiles (70%) and wood (50%).

In general, among the approximately 24 species - 6 macroalgae and

Table 2
ANOVA on total species richness and coverage area of macrofouling. Statistically significant effects ($P < 0.05$) of the variables are indicated in bold.

Index	Source	df	SS	MS	F	P
Species richness	Month	8	100.8	12.6	8.972	**0.000**
	All substrates	4	16.7	4.2	1.174	0.336
	Geotextiles	3	16.6	5.5	1.520	0.227
	Month*Wood	8	128	16	2.97	0.072
	Month*Black HDPE	8	101.44	12.68	7.19	**0.006**
	Month*Wt.PP & Wt.PET	8	288.78	36.10	13.06	**0.000**
	Month*Col.PP & PET	8	239	29.87	25.31	**0.000**
	Month*White PP	8	155	19.38	45.00	**0.000**
	Wood*Black HDPE	1	16.05	16.05	1.336	0.264
	Wood*Wt.PP & Wt.PET	1	0.2	0.2	0.013	0.907
	Wood*Col.PP & PET	1	2	2	0.098	0.757
	Wood*White PP	1	5.5	5.5	0.439	0.517
	White PP*Wt.PP & Wt.PET	1	8	8	0.451	0.511
	White PP*Col.PP & PET	1	0.88	0.88	0.040	0.843
	White PP*Black HDPE	1	2.72	2.72	0.199	0.661
	Wt.PP & Wt.PET* Col.PP & PET	1	3.55	3.55	0.139	0.713
	Wt.PP & Wt. PET*Black HDPE	1	20.05	20.05	1.173	0.294
	Col.PP & PET*Black HDPE	1	6.72	6.72	0.315	0.582
Coverage area	Month	8	33,226	41,532	5.341	**0.000**
	All substrates	4	10,839	27,098	2.151	0.092
	Geotextiles	3	10,024	33,415	2.817	**0.044**
	Month*Wood	8	145,795	18,224	1.71	0.233
	Month*Black HDPE	8	467,458	58,432	5.16	**0.016**
	Month*Wt.PP & Wt.PET	8	426,709	53,338	29.21	**0.000**
	Month*Col.PP & PET	8	400,490	50,061	6.11	**0.010**
	Month*White PP	8	537,104	67,138	45.26	**0.000**
	Wood*Black HDPE	1	60,522	60,522	4.456	**0.040**
	Wood*Wt.PP & Wt.PET	1	14,463	14,463	0.105	0.749
	Wood*Col.PP & PET	1	43,569	43,569	0.352	0.561
	Wood*White PP	1	41,705	41,705	0.276	0.606
	White PP*Wt.PP & Wt.PET	1	105,288	105,288	0.79	0.386
	White PP*Col.PP & PET	1	170,529	170,529	1.429	0.249
	White PP*Black HDPE	1	964,683	964,683	7.344	**0.015**
	Wt.PP & Wt.PET* Col.PP & PET	1	7827	7827	0.073	0.789
	Wt.PP & Wt. PET*Black HDPE	1	432,570	432,570	3.669	0.073
	Col.PP & PET*Black HDPE	1	324,023	324,023	3.109	0.096

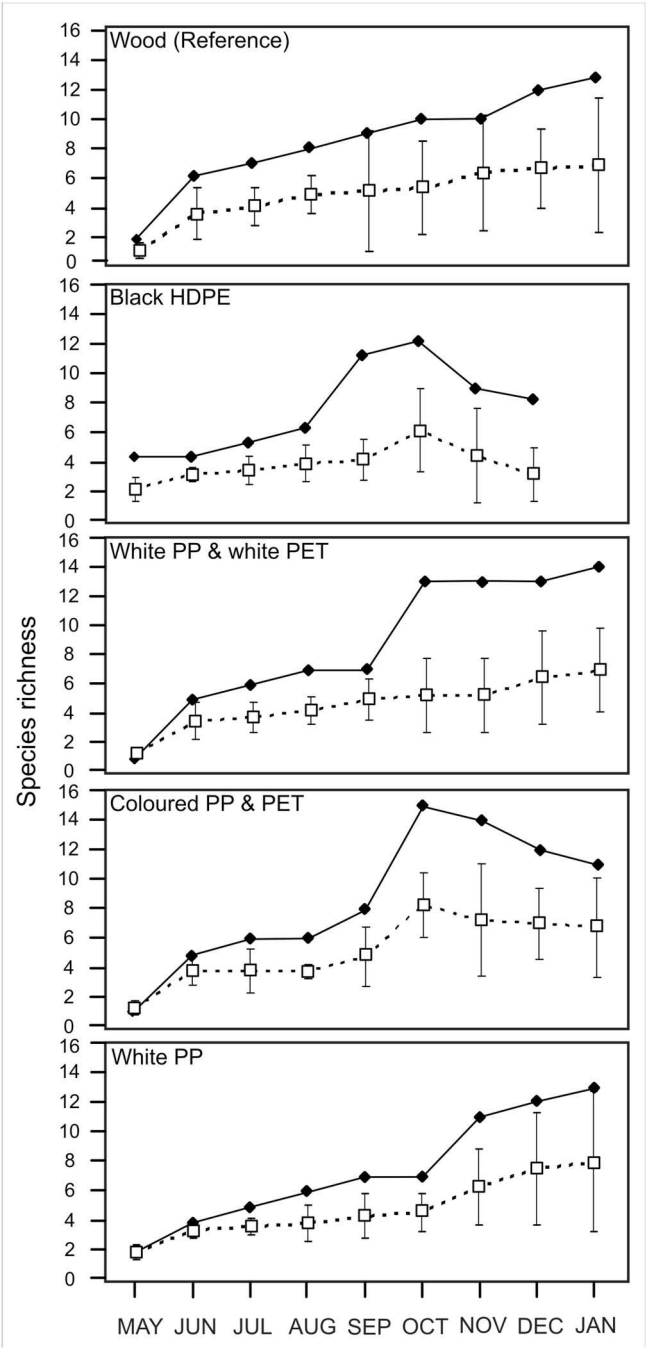

Fig. 4. Trend of the biodiversity descriptor 'species richness' as the total number of species (solid line) and mean number of species ± standard deviation (dashed line) found monthly on replicates (n = 4) of the five different substrata.

18 invertebrates - considered, PERMANOVA data analysis (Supplementary Table S1) showed that for the ascidians *C. robusta* and *S. plicata*, the coverage depended significantly on both time (month) and type of substratum. For the ascidian *B. leachii* and the barnacles (*Balanus* sp.), the coverage depended significantly only on the substratum factor. For red algae (*Ceramium ciliatum* Ducluzeau, 1806; *G. longissima*), sponges (*Haliclona (Reniera) cinerea* (Grant, 1826)), serpulids (*Filograna* sp.), bryozoans (*Bugula neritina* (Linnaeus, 1758), *B. stolonifera*), and the ascidians *A. aspersa* and *Diplosoma listerianum* (Milne Edwards, 1841), the coverage depended significantly only on the time factor.

3.2. Changes in biocoenosis structure

For the description of the community, species were clustered in taxonomic groups, i.e., green algae (or chlorophyta), red algae (or rhodophyta), sponges (or porifera), hydroids (or hydrozoan cnidarians), serpulids (or serpulid polychaetes), bryozoans, molluscs, barnacles (or cirriped crustaceans), and ascidians (or benthic tunicates). In the various seasonal phases of ecological succession, the biocoenosis structure on the different substrata is expressed using stacked bars reporting the percentages of coverage of the taxonomic groups (Fig. 7). Promotion and inhibition of the settlement and growth of different species of fouling organisms was observed on the different substrata. On all of the geotextiles, the settlement of barnacles did not occur, and a delay in the settlement of serpulids was observed. 'Black HDPE' also inhibited the settlement of sponges and molluscs and disturbed that of the dominant ascidian *C. robusta*, which was less represented than on the other substrata. Conversely, 'white PP' favoured a brief (one month) settlement of hydroids, and both the mixed PP & PET geotextiles favoured the settlement of molluscs and ascidians.

The SIMPROF analysis indicated the presence of three clusters representing significantly different fouling communities (Fig. 8). The community from cluster A consisted of the samples from all of the substrata in May (spring), i.e., the pioneer assemblage of *U. intestinalis*, *B. schlosseri*, and *B. leachii*. Cluster B contained the samples from June to September (summer) from all of the substrata, including the community on 'white PP & white PET' from the month of November (autumn). Cluster C included the communities on all of the substrata from October to January (competition stage and stable stage), except for that on the 'white PP & white PET' substratum from November. These results showed that changes in community composition, based on the presence or absence of species, reflected seasonal changes involving the same taxonomic groups. The subsequent analysis of similarity percentages (Table 3) showed that the species contributing most to the differences between cluster A and cluster B were the bryozoan *B. stolonifera* and the ascidian *C. robusta*, each of which contributed 12.8% to the observed differences between the clusters. These species appeared in June on all of the substrata. The ascidians *A. aspersa* and *S. plicata* contributed to 11.5 and 8.3%, respectively, of the differences between these clusters. The differences between clusters B and C were mainly due to the bryozoan *B. stolonifera*, with a 4.5% contribution, and *A. aspersa*, with a 3.4% contribution to the observed differences.

3.3. Differences in biomass among substrata

By comparing the biomass values measured on all of the substrata, the wood substratum showed the highest biomass, with a mean value of 6.3 ± 0.51 g FW cm^{-2}, and the 'black HDPE' had the lowest value of 3.3 ± 0.11 g FW cm^{-2}.

Biomass increased on all of the substrata from the start of the experiment in April to the substratum-specific peaks (change points) (Fig. 9), and differences in the rates of biomass accumulation and decline, and the timing of peak biomass were evaluated. The community on the reference material (wooden panels) reached the biomass peak in October. As regards geotextiles, the biomass peak was reached the earliest (June) on the 'black HDPE' fabric, followed by the 'coloured PP & PET' and 'white PP' fabrics (July) and the 'white PP & white PET' fabric (August). With the exception of 'black HDPE', biomass stabilises over the last six months most likely for the extensive coverage of solitary ascidians on these panels (Fig. 5). Differences in the change points of biomass development were highly significant (F (4, 495) = 114.7, $p <$ 0.001), and the post hoc test indicated that significant differences were present among all of the change points, except for the comparison of biomass development between 'coloured PP & PET 'and 'white PP.'

In the initial growth phase, the increase in biomass was significant on all of the substrata and showed a similar pattern, except for one significant difference between the wooden substratum and the 'white PP'

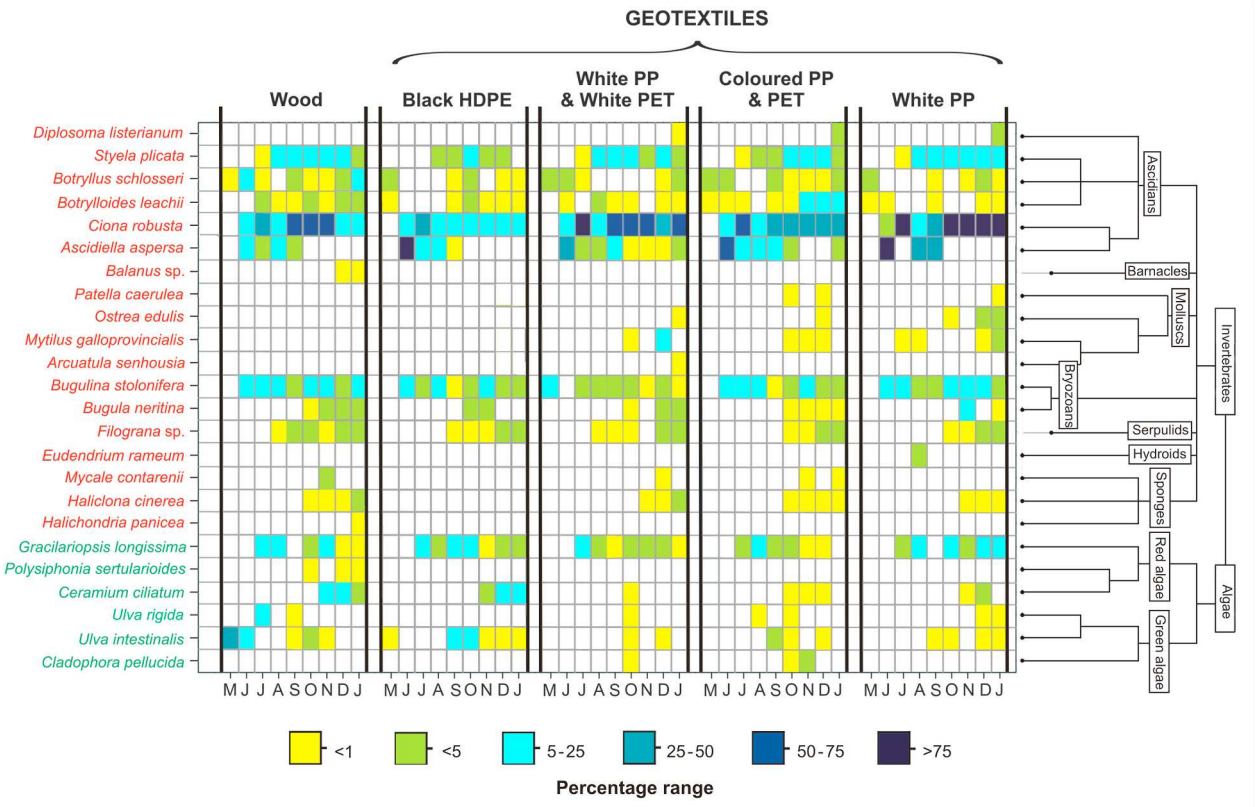

Fig. 5. Trend of the biodiversity descriptor 'covering-abundance area' as the total percent area of each species measured monthly using photos of replicates (n = 4) of the five different substrata. In the dendrogram of the similarities of the 24 species included in the analysis, species are clustered in taxonomic groups (right).

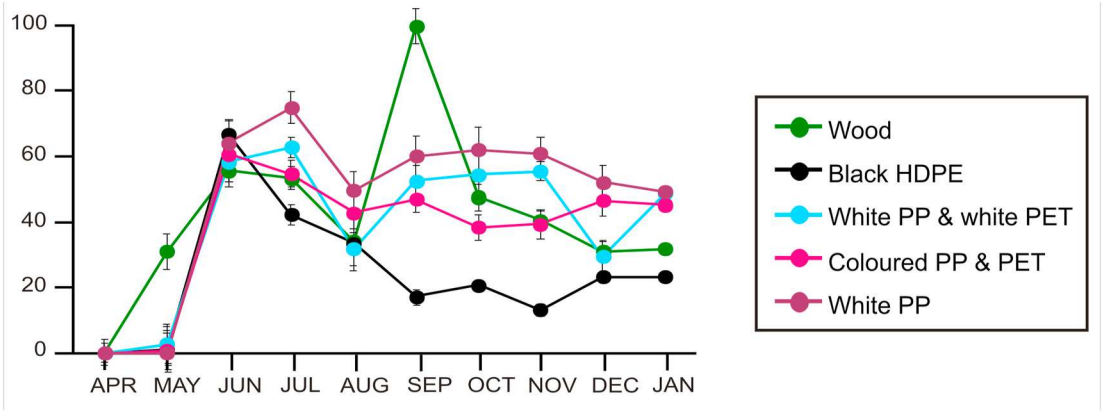

Fig. 6. Trend of total coverage dynamic monitored monthly as average ± standard deviation on replicates (n = 4) of the five different substrata.

fabric. After the biomass change point, only the wooden substratum and the 'black HDPE' fabric showed a significant decrease in biomass, whereas the other geotextile substrata showed only a small decrease in biomass, which was not significant.

4. Discussion

The present study represents the first approach to long-term experimental monitoring, in an open-air natural laboratory rich in biodiversity such as the Lagoon of Venice, of the ecological succession of fouling communities on different nonwoven geotextile fabrics with various engineering applications, including prevention of shore and artefact erosion. These geotextiles showed to interfere with the settlement and growth of various macrofouling organisms, and potentially could cause changes in the composition, extension and structure of the hard-substratum natural community in the long term. Benthic communities found on artificial materials may differ from those found on natural substrata in many ways (Guerra-García, 2004; Marzinelli et al., 2009; Andersson et al., 2009). Differences occurred within both the taxonomic groups and the individual amounts, supporting a potential selective effect of the various substrata (Glasby, 1999), which could be added to differences in exposure time and different hydrodynamic conditions (Burt et al., 2009). The recruitment of benthic species colonising submerged hard substrata and the consequent type of community depend, to a large extent, on the microrelief and microtopography of the surface itself (Berntsson et al., 2000; de Nys and Steinberg, 2002). For example, rough substrata can support a completely different benthic community than smooth surfaces (Johnson, 1994; Commito and Rusignuolo, 2000).

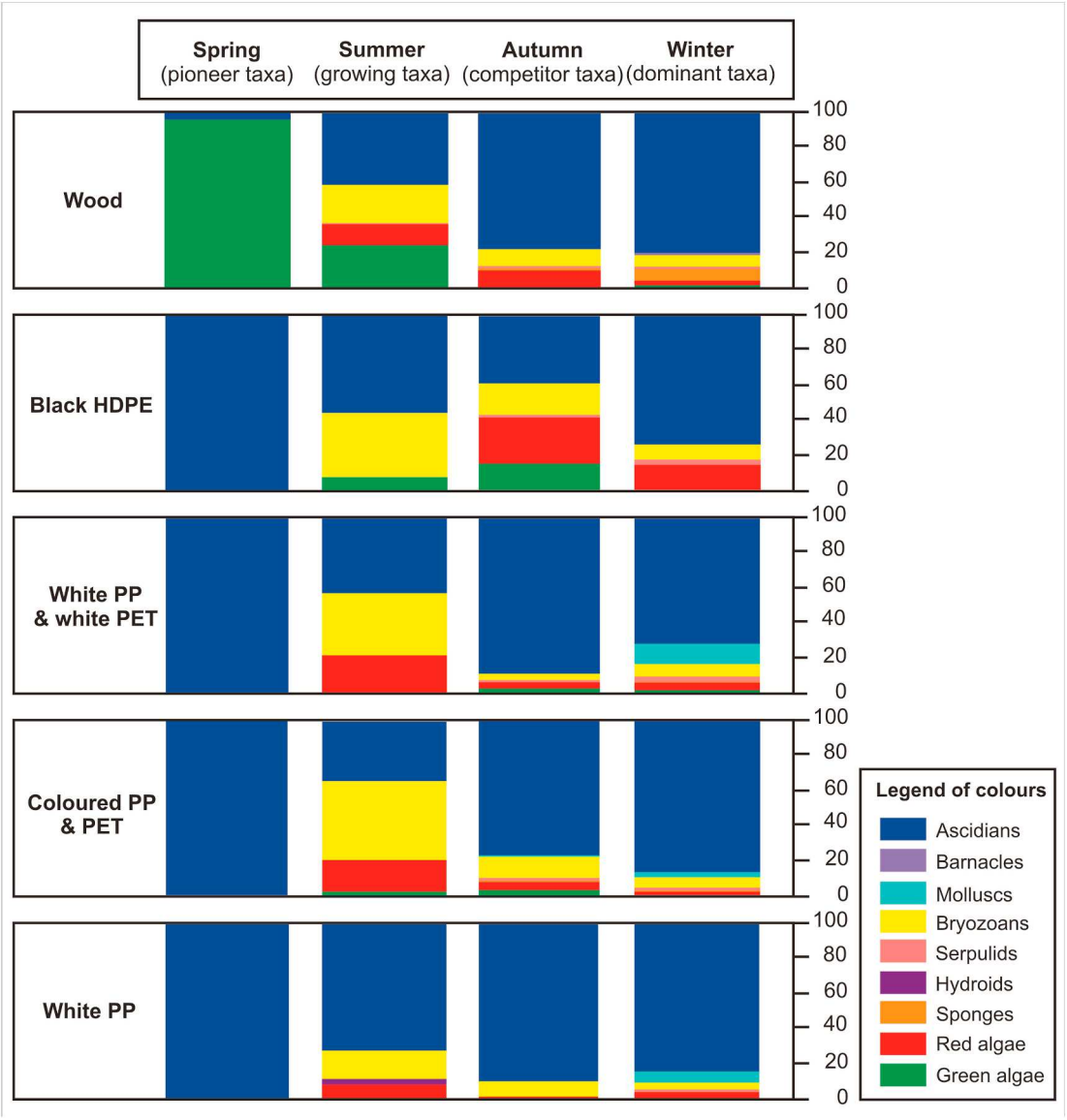

Fig. 7. Changes in 'biocoenosis structure' during the main phases of ecological succession on the various substrata. Percent value of coverage of each taxonomic group expressed as the total from the pool of four replicates.

The species communities found on geotextile fabrics were usually substantially different from those on natural reefs (Edwards, 2003), and considerable differences were observed between the fouling communities on woven and nonwoven substrata (Jackson et al., 2007; Wetzel et al., 2011). Jackson et al. (2005) also observed that barnacles made up approximately 90% of the coverage on woven textiles and that nonwoven textiles were mainly covered by red algae (>90%); thus, the geotextile type can influence the type of fouling community.

Our monitoring and analysis of the 10-month community growth revealed selective effects of nonwoven geotextile fabrics on species richness, covering-abundance area, biocoenosis structure, and biomass development with different potential impacts on the biodiversity of the resident community. The results support that nonwoven geotextile fibres can change biofouling settlement in a selective manner and, in some cases, favour species that increase rapidly in terms of number of individuals and strongly compete for the substratum, becoming dominant.

From the point of view of potential applications in biofouling prevention, geotextiles could represent a new tool as an 'antifouling system' alternative to the dangerous biocide mixtures employed worldwide in antifouling paints. The latter have the potential to disrupt aquatic communities by releasing pollutants with deleterious effects on non-target organisms (Ranke and Jastorff, 2000; Cima and Ballarin, 2008; Manzo et al., 2014). Therefore, in the last decades the research of new eco-friendly systems to prevent fouling settlement on artificial structures has become a primary requirement for the safeguarding of the coastal ecosystems. As regards the biomass trend and the structure/change of the community, all nonwoven geotextiles employed in the present study reduced fouling to some extent compared with wood. An order of effectiveness for the geotextiles in disturbing fouling growth can be established as follows: 'black HDPE' > 'coloured PP and PET' > 'white PP and white PET' > 'white PP'. The inhibitory activity of geotextiles is principally mechanical since the fuzzy surfaces of the geotextiles prevent larval and propagule settlement with continuous micromovements of the polymer fibres (Wetzel et al., 2011). This mechanism of action is not comparable with the real 'antifouling action' of biocide-based paints because it leads to a selection of species on the basis of a balance between the preference of the substratum and the disturbing action of the geotextile components. With respect to wood, geotextiles in general prevent the settlement of green algae as first colonists, initially block the settlement of serpulids and totally inhibit that of barnacles (all), green

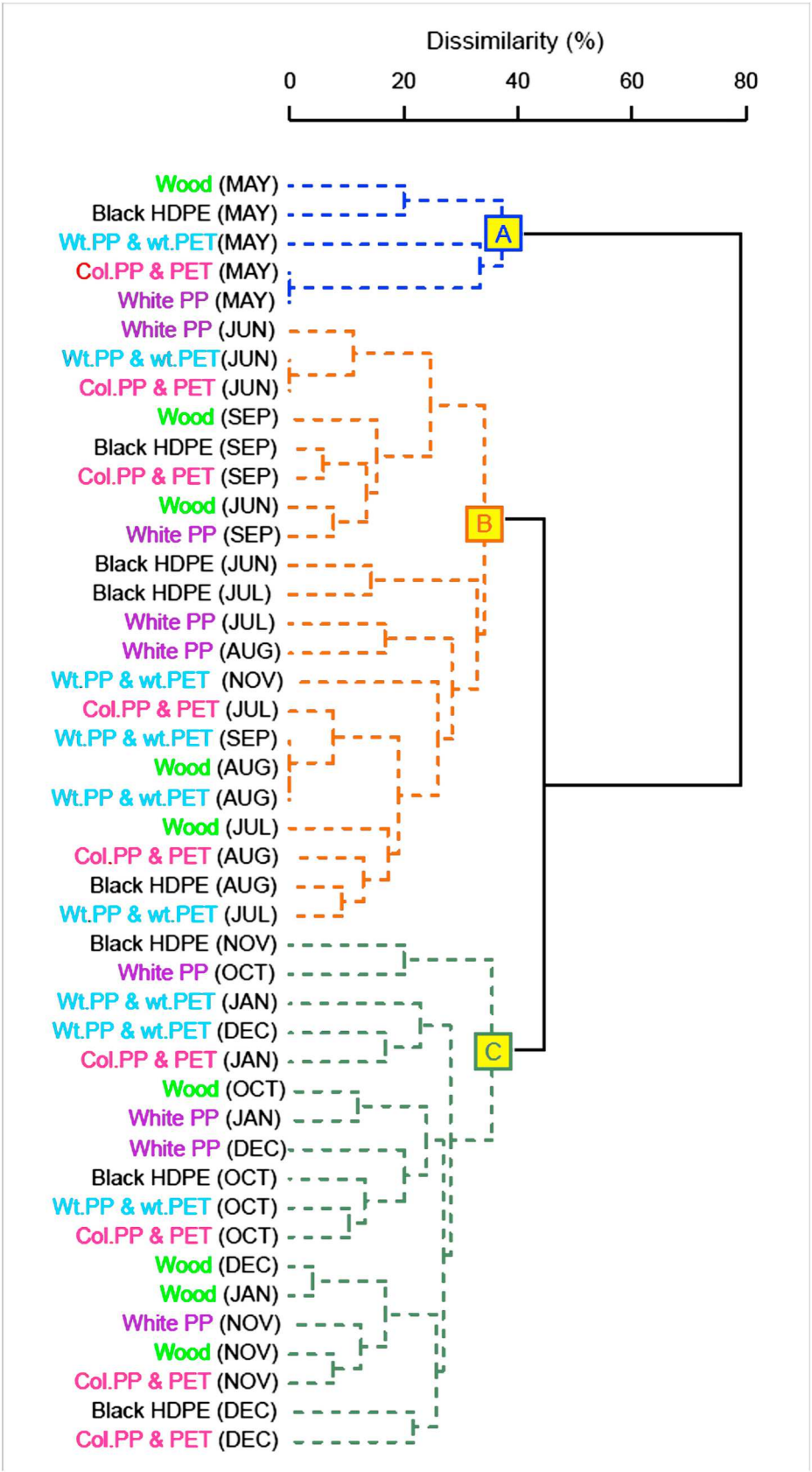

Fig. 8. Cluster analysis using Bray-Curtis dissimilarity values of presence/absence data. Significantly different clusters (A to C) were identified using SIMPROF procedure. For the species that contributed the most to the differences between clusters (SIMPER analysis), see Table 3.

Table 3
SIMPER analysis results showing the contribution (%) of the species responsible for the differences observed between the three clusters identified in the SIMPROF procedure (see Fig. 8). Only the topmost six values that contributed to the differences are shown.

Species	A – B	A – C	B – C
Red algae			
Gracilariopsis longissima	0.8	6.9	8.1
Serpulids			
Filograna sp.		7.4	
Bryozoans			
Bugula neritina		5.8	
Bugulina stolonifera	12.8	7.4	12.8
Ascidians			
Ascidiella aspersa	11.5		11.5
Ciona robusta	12.8	7.4	12.8
Botryllus schlosseri	0.7		7.9
Styela plicata	8.3	7.4	8.3

Cluster A: spring pioneer assemblage; cluster B: summer assemblage; cluster C: autumn and winter assemblages.

algae ('white PP' and 'black HDPE'), sponges and molluscs ('black HDPE'). This order most likely results from the differences in the material properties. Different species have different surface quality requirements. In particular, early colonists are very sensitive to the physico-chemical properties of the substratum (Wahl, 1989). The effects due to polymer composition, colour, texture, microtopography and tangle of fibres together with their thickness and capacity to move passively under various hydrodynamic conditions are worth considering in future studies. Initial recruitment was apparently not substratum specific because significant differences in the presence or absence of pioneer species were not observed between different substrata, with the exception of *U. intestinalis* on wood panels, and because the first biomass development on all of the substrata followed a similar pattern. This was likely due to the initial conditions of ecological succession that were shared by all of the substratum types, depending on the nutrient availability and the trend of blooms and dispersal of typical pioneer species, which were the eurythermal species of early spring in the Lagoon of Venice (Sconfietti and Marino, 1989; Libralato et al., 2002). However, the differences in biomass were particularly pronounced after the peak was reached. The substratum material was the most important factor and explained 57% of the biomass differences after the substratum-specific change point was exceeded. On the HDPE geotextile, the coverage of bacterial biofilm was abundant throughout the monitoring period, supporting the facts that the macrofouling biomass was particularly low and the panel surface never appeared fully covered. The polymer composition of this material might be responsible for the differences observed with respect to the other geotextiles employed. The influence of the chemical nature of substratum materials on the properties of fouling communities is well known (Pawlik, 1992; Bergey, 2008), and HDPE compounds in particular have been successfully used to prepare bioactive surfaces that can reduce biofouling (Yu et al., 2011). Ascidian larvae significantly prefer more hydrophobic substrates to hydrophilic ones (Sensui and Hirose, 2020; Hirose and Sensui, 2021). HDPE shows weak wettability as an effect of its hydrophobic character but, as a difference with the other geotextiles, has a low adhesion property due to the lack of a functional group (Conceição et al., 2019). 'Black HDPE' geotextiles might be suitable for fouling reduction measures on human-made structures, thereby enhancing their maintainability and decreasing maintenance costs. However, before employing this geotextile in coastal ecosystems with this purpose the underlying mechanism of action in fouling reduction should be better understood together with duration of the effects on the fouling growth in multi-year studies.

From the point of view of potential applications, geotextiles could find new employment as 'profouling systems' for habitat and

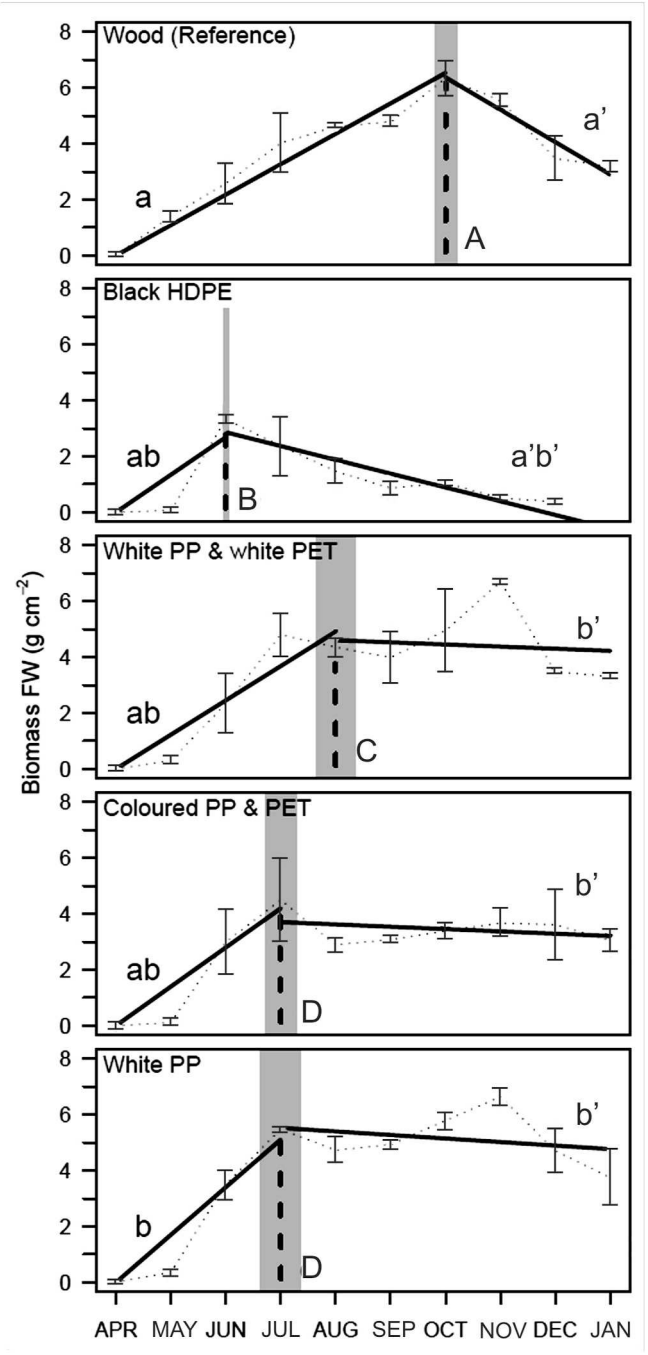

Fig. 9. Differences in biomass development monitored monthly on the five different substrata. The mean of four replicates with standard deviations is shown throughout the dotted line. The calculated change points, i.e., the substratum-specific peak of biomass expressed in g cm^{-2} of fresh weight (FW) is indicated by the dashed lines, and their corresponding confidence intervals are indicated by the grey areas. Different letters indicate significant differences for the pairwise comparison of the slopes before the change points (a to b, left), the position of the change points (A to D, middle), and the slopes after the change points (a' to b', right) among the five substrata.

microhabitat enhancement. Together with shore protection, they could play a key role in restoration of degraded and fragmented ecosystems by supplying alternative substrata which favour species settlement. In terms of substratum selection, bivalve molluscs settled on PP and PET geotextiles. This was in agreement with a previous study of oyster larval settlements on various plastic materials, where PP represented a good substratum that was also superior to other plastics, such as PVC (Taylor

et al., 1998). Tunicates found the 'white PP' geotextile to be particularly suitable for their settlement. In particular, the most favoured species was the dominant tunicate *C. robusta*, which is typical of the Lagoon of Venice (Brunetti et al., 2015), where it was first described by Lazzaro Spallanzani in the 18th century (Gibin, 1997). It is now considered among the most damaging of invasive species in the world by the effect of natural spread of the larvae and of human-related transportation of larvae through bilge waters and adults on boat keels (Shenkar et al., 2018). Also the didemnid ascidian *D. listerianum*, although not invasive in the Lagoon of Venice, appear to settle and grow easily on geotextiles according to the previous failed approaches to eradicate the invasive *Didemnum vexillum* based on geotextile fabrics to protect artificial structures in harbours and mussel farms of New Zealand (Coutts and Forrest, 2007). Therefore, it must be considered that a long-term and extensive use of geotextiles worldwide could negatively affect local biodiversity and develop on artificial substrates such as plastic, a high selection of invasive species by acting as a collector for larvae (Pinochet et al., 2020).

In conclusion, geotextiles act by selecting species, and modifying the biocoenosis composition with potential long-term impact on coastal ecosystems. They represent technical surfaces that are capable of exerting only a partial and temporary physical defence against the settlement of organisms and must not be considered as eco-friendlier barriers that are alternatives to antifouling paints in biofouling control. On the other hand, these materials provide - by paying close attention to fibre dislodgement, long-term fabric durability and abrasion resistance to avoid a potential contribution to plastic pollution in the marine environment (Dias et al., 2017; Carneiro et al., 2018b) - a good substratum for a wide range of benthic species (Jackson et al., 2004). In some conditions, they could enhance biodiversity and productivity at a local scale in depauperated areas and contribute to overall regional productivity (Edwards, 2003; Jackson et al., 2005). At present, artificial structures account for a great deal of the potential negative impacts of increasing connectivity, facilitating the spread of non-native species and contributing to biotic homogenisation. The ecological engineering of artificial habitats aims to test alternative materials and designs to encourage the colonisation of more diverse or more natural communities (Rinkevich, 2020). Consequently, the study of the influence of the substratum on the settlement of various organisms of the hard-substratum macrofouling community represents an essential tool to choose a geotextile for the most appropriate application in a variety of coastal marine ecosystems. Further efforts should be made to better clarify the effective role of the type of substratum on species selection by monitoring the macrofouling settlement capability on monthly-renewed nonwoven geotextile panels for limiting the interference of accumulation of high amounts of biofilm and sediment, which could progressively change the original characteristics of the fabric surface.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.marenvres.2021.105414.

Credit author statement

Varello Roberta: Original draft preparation. Visualization, Investigation. Wetzel Markus A: Software, Validation, Data curation, Formal analysis, Validation. Cima Francesca: Conceptualization, Methodology, Writing – review & editing.

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4.2.2 Ultrasonic generators (Study at individual and cellular level)

Acoustic devices have been specifically introduced to prevent biofilm formation and larval settlement on ship's hulls. They are present in the market as "eco-friendly" antifouling systems, but there is no evidence that they are. For these reasons, I was interested in applications of ultrasonic devices used as antifouling systems.

In my study, common species of solitary ascidians have been chosen, i.e., *Ascidiella aspersa* and *Ciona intestinalis* (order Enterogona) and *Styela plicata* (order Pleurogona), which were carried out with a series of behavioural experiments concerning the siphon reactivity soon after and during various exposure times to two ultrasound frequencies (30 and 35 kHz).

Two types of responses were considered, i.e., the squirt response (SqR: fast closure of both siphons and body contraction "Squirt") due to the stimulation of vibration sensors in the inner epithelium of the siphon and along the rim, and the crossed response (CrR: closure of the atrial siphon, no squirt) due to the stimulation of mechanoreceptors represented by sensory cells of the coronal organ along the oral velum and the branched tentacles considered homologues of the hair cells of the vertebrate internal ear (Rigon *et al*., 2013).

The number of the two types of responses to ultrasound was evaluated for 5 min after an exposure for 10 s on 20 individuals per species and the statistical analysis was performed considering their size clustering. In another series of experiments, individuals were continuously exposed to 30 kHz for 5 hours and their behaviour (e.g., events of SqR, CrR, opening of a single siphon, long-term closing or opening of both siphons) was monitored every 30 min and compared to that in total absence of external stimuli. Finally, an assay of response to a mechanical stimulus was performed by touching the oral siphon every 30 min during 5 hours of continuous exposure at 30 kHz. This study demonstrated that a progressive muscle fatigue occurred supporting the hypothesis of a severe weakening of both particle selection ability during the filter-feeding activity and defence reaction towards natural predators endangering the adult survival.

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Behavioural Responses to Ultrasound Antifouling Systems by Adult Solitary Ascidians

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Abstract: Ultrasonic antifouling devices are installed globally on a variety of vessel types and are marketed as an environmentally friendly method for biofouling control. The aim of this study was to examine the effects of ultrasound on adults of three species of common solitary ascidians (*Ciona intestinalis*, *Ascidiella aspersa* and *Styela plicata*). After a brief (10 s) exposure to two ultrasound frequencies (30 and 35 kHz), alterations in the frequency of siphon closing events and the length of time the siphons remained closed/open were observed. The results revealed that ascidians are able to perceive ultrasound, showing frequency-dependent behavioural responses that vary depending on the species and size of individuals involving both tactile receptors and an acoustic system homologous to the vertebrate inner ear. Continuous (5 h) 30 kHz exposure caused other types of responses, the most interesting of which was the long-term opening of the oral siphon, indicating a lack of reactivity to mechanical stimuli. This effect suggests a stress condition that could lead to increased vulnerability to predators and filter-feeding impairment. Therefore, knowledge of the acoustic sensitivity of sessile marine species appears to be essential for better understanding the potential effects of noise pollution on marine ecosystems.

Keywords: ascidians; marine invertebrates; noise pollution; sea squirts; tunicates; ultrasound

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1. Introduction

The natural environment is characterised by a specific sound environment [1]. Animals use sound to communicate with conspecifics for reproduction, foraging, predation or escape from predators [1,2]. For many marine animals, sound is the most important sensory modality, and rising levels of noise pollution pose a risk to marine species that rely on sound [2–4]. Shipping, naval operations, offshore construction, seismic exploration, oceanographic research and fishing activities contribute to increasing anthropogenic noise pollution [5].

The first and most extensive studies on the effects of marine noise on animals have focused on the most iconic marine mammals [2,6,7]. Noise impacts on marine mammals range from physiological (e.g., shifts in hearing sensitivity and elevated stress hormone levels) to behavioural effects [8–10]. Southall and collaborators (2008) [7] reported that marine mammals might develop behavioural disorders when they are exposed to noise above 230 dB over a 24 h period. Regarding other marine vertebrates, the threshold audiogram measures for several species of fish [11,12] show that they cannot detect sound up to several tens of kHz. Thus, ultrasonic frequencies are well above the hearing ranges of almost all fish species. Although few studies have been carried out on diving seabirds and marine reptiles, many of these species appear susceptible to noise exposure [13–16].

On the other hand, research focused on the impact of sound on marine invertebrates is scant [17,18]. The great variety that is observed in the sensory organs of the various species suggests that, among these, there are differences in the auditory threshold and behavioural responses [19]. Invertebrates are more sensitive to the particle motion associated with sound rather than to sound pressure. They often use various types of mechanoreceptors and statocysts [18]. The component of sound related to particle motion is damped within a short distance from the source, whereas vibrations can propagate over great distances into the substratum and cause motion in overlying water particles that therefore can be perceived by benthic invertebrates [20]. Pacific oyster, *Magallana gigas*, exhibits transient valve closures in response to sound frequencies of 10 to <1000 Hz in a frequency-dependent manner, with maximum responses occurring between 10 to 200 Hz [21]. Lobster, *Nephrops norvegicus*, shows postural responses at frequencies between 20 and 120 Hz due to the perception of particle motion and not sound pressure [22]. Cephalopods are the most studied marine invertebrates, and their hearing relies on the presence of statocysts with sensitivity to frequencies beyond 400 Hz [23]; e.g., behavioural responses in squid, *Doryteuthis pealeii*, to stimuli can occur up to 1000 Hz [24].

Most studies have investigated the impact of anthropogenic sound on motile species of invertebrates such as squids [25] and decapod crustaceans [26,27]. Recently, it was discovered that the sounds produced by boats could have a negative impact on the settlement and metamorphosis of the larvae of various fouling organisms [2,16,28]. Every hard substratum submerged in the sea represents a new habitat that is rapidly colonised by a biofilm, followed by the settlement of a macroscopic community of marine organisms. This process is known as biofouling, which causes severe damage to ships' hulls and submerged structures [29]. Since the 1980s, physical antifouling systems have been introduced to various types of vessels to protect hulls from biofouling during long mooring periods [30–33]. These systems focus on changing the physical properties of submerged surfaces or water hydrodynamics to remove settled organisms without using antifouling paints, which release chemical biocides into the environment [34]. Therefore, these devices are considered 'eco-friendly' antifouling systems [35], but there is no evidence to prove this. Guo and collaborators [36–38] observed the most effective inhibition of settlement in cyprid larvae of *Amphibalanus amphitrite* after a 23 kHz ultrasound treatment in comparison with other higher ultrasound frequencies (63 and 102 kHz). An ultrasound treatment operating at 20 kHz was able to kill barnacle larvae within 45 s [39]. This has also been confirmed by other studies, which have shown that frequencies of 20–22 kHz prevent fouling settlement [39,40].

The potential impact of ultrasound devices on coastal marine ecosystems has not been assessed. In particular, it is important to investigate the stress that sessile species might suffer. Motile organisms may respond to auditory disturbances with evasive behavioural responses [41,42], whereas sessile species cannot move or hide to minimise the effects of such disturbances. Among the benthic species of the coastal community, tunicates are the dominant taxon of the so-called 'soft-macrofouling' [43]. The first observations concerning the effects of anthropogenic noise on behaviour in adult tunicates have been reported by White and collaborators [44]. The authors exposed 48 specimens of the solitary ascidian *Styela plicata* for 8 s to three separate stimuli measured from the position (150 mm) of the ascidians in the tank, i.e., a recording of a boat motor (sound pressure level, the SPL peaked at 82–70 dB and a frequency of 100 Hz), a song recording (the SPL peaked at 81–57 dB and a frequency of 100 Hz) and a water current to simulate turbulence. Individuals showed an increase in the frequency of siphon closure when compared with the control.

The present study is the first to determine the behavioural effects of ascidians after exposure to the frequencies used by antifouling ultrasound systems commonly seen on the market. The responses to ultrasound have been investigated in adults of three species of solitary ascidians, i.e., *Ciona intestinalis*, *Ascidiella aspersa* and *Styela plicata*, considering two types of siphon responses (Figure 1). Various studies have shown that the siphon area is the most sensitive to tactile stimulation [45–48]. Primary sensory cells are located within

a sensory field on the inside of the oral siphon between the velum and the rim [49–51]. They are hydrodynamic and vibration sensors sensitive to touch. Their stimulation evokes the squirt response (SqR), a strong, synchronous contraction of both siphons that causes a violent ejection of water from both the oral and the atrial siphon. Secondary sensory cells are mechanoreceptors located at the base of the oral siphon along the velum and the branched tentacles, where they are arranged in one row or in a few rows and described as the 'coronal organ' as a whole [52–56]. For their morphology and development, these sensory cells, flanked by supporting cells, are considered homologues of the hair cells of the vertebrate internal ear and lateral line system [57,58]. Like hair cells, they are mechanoreceptors that mediate vibrational and fluid-low sensing, which allow hearing and vibrational sensing [59]. Their stimulation, as a consequence of the mechanical stimulation of the tentacles, evokes the crossed response (CrR), a contraction of the atrial siphon while the oral siphon stays open [60,61].

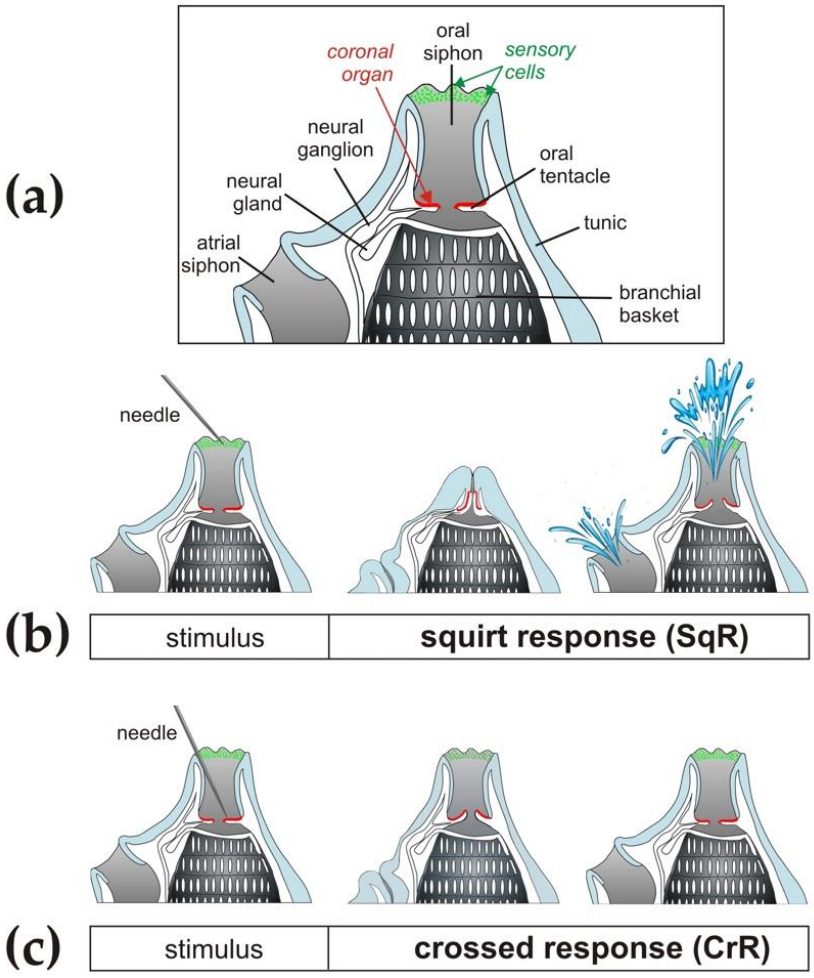

Figure 1. Schematic drawing of the two main types of siphon responses to mechanical stimuli in ascidians according to the behavioural responses described in detail by Mackie and collaborators [60]. (**a**) General schematic morphology of siphons with the location of sensory cells. (**b**) Squirt response (SqR), i.e., fast closure of oral and atrial siphons and body contraction with ejection of water from both siphons due to stimulation of primary sensory cells in the inner epithelium of the siphon and along the rim. (**c**) Crossed response (CrR), i.e., closure of the atrial siphon due to the stimulation of secondary sensory cells along the coronal organ.

2. Materials and Methods

2.1. Animals

Three species of solitary ascidians (Figure 2) common in the intertidal and temperate zones were used for this study: *Ciona intestinalis* (Linnaeus, 1767), *Ascidiella aspersa* (Müller, 1776) and *Styela plicata* (Lesueur, 1823). The first two belong to the order Phlebobranchia, while *S. plicata* belongs to the order Stolidobranchia [62]. Individuals of *A. aspersa* and *S. plicata* were collected in the southern basin of the Lagoon of Venice (near Chioggia), while wild individuals of *C. intestinalis* were furnished by the Biological Station in Roscoff (France) and transported by air in an appropriate container. Once in the laboratory, ascidians were acclimated for one week (mortality less than 10%) and kept in aquaria inside thermostatic chambers under controlled conditions (temperature of 12 °C and salinity 35‰) in seawater filtered through 0.45 µm filters (FSW). The aquaria were kept well ventilated by oxygenators and replaced every 48 h, and the ascidians were fed with unicellular algae (1:1 ratio of *Dunaliella* sp. and *Tetraselmis* sp.). Before the experiment, the length of each individual was measured along the antero–posterior axis of the body, which is parallel to the endostyle and passes between the oral siphon and the digestive tract [63]. For *S. plicata* and *A. aspersa*, measurements were performed with a calliper. However, *C. intestinalis* individuals were placed in a glass Petri dish that was marked with a graphic scale of 1 cm on the bottom. When the animal was relaxed, a photo was taken, and then the size measure was obtained with the Infinity image analysis software Analyze Application v. 5.0.0 (Lumenera Co., Ottawa, Canada, 2002–2009). The body length can reach up to 20 cm in *C. intestinalis*, 13 cm in *A. aspersa* and 8 cm in *S. plicata* and is age-related [62]. However, it could also depend on other environmental and physiological conditions, such as nutrient availability, filtration rate and reproductive period.

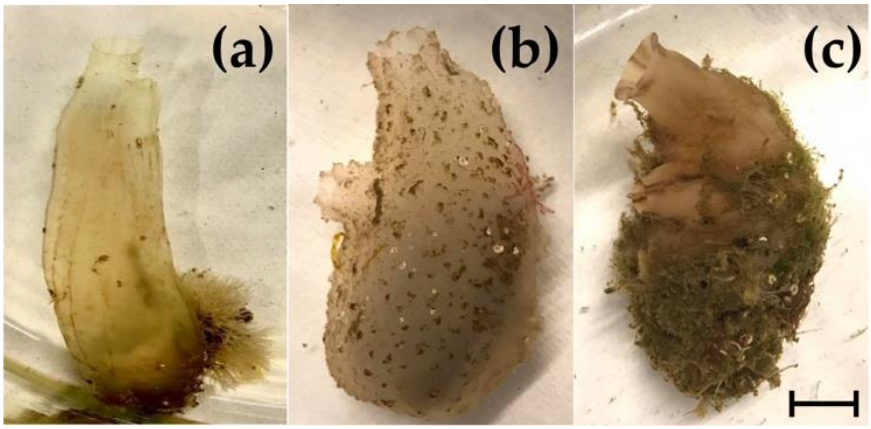

Figure 2. Solitary ascidians employed in the present study. Vase tunicate, *Ciona intestinalis* (**a**); fluted sea squirt, *Ascidiella aspersa* (**b**); pleated sea squirt, *Styela plicata* (**c**). Relative dimensions of the individuals have been maintained. Bar length: 1 cm.

2.2. Ultrasound Devices

For this experiment, two types of continuous frequencies were used: (i) 30 kHz (90% amplitude), produced by a Sonoplus sonicator mini20 (Bandelin Electronic GmbH & Co. KG, Berlin, Germany); (ii) 35 kHz (90% amplitude), produced by a GN20Pro ultrasonic cleaning rod (Shenzhen Baryon Acoustic Technology Co, Ltd., Shenzhen, China).

The frequency of the ultrasound devices was validated with a hydrophone HydroMoth 1.0.0 (Open Acoustic Devices, https://www.openacousticdevices.info, accessed on 3 April 2023) with a sensitivity of 94 dB SPL@1 kHz. The recorded main frequencies of operation for the Sonoplus sonicator mini20 and the GN20Pro ultrasonic cleaning rod were 29.4 kHz and 35.1 kHz, respectively (Figure 3). Sonoplus sonicator mini20 and GN20Pro ultrasonic cleaning rod produced an environmental noise of 71.1 and 74.8 dB, respectively,

which was measured with a Curconsa SL720 portable sound level phonometer (Shenzhen Putest Instrument Co., Ltd., Shenzhen, China).

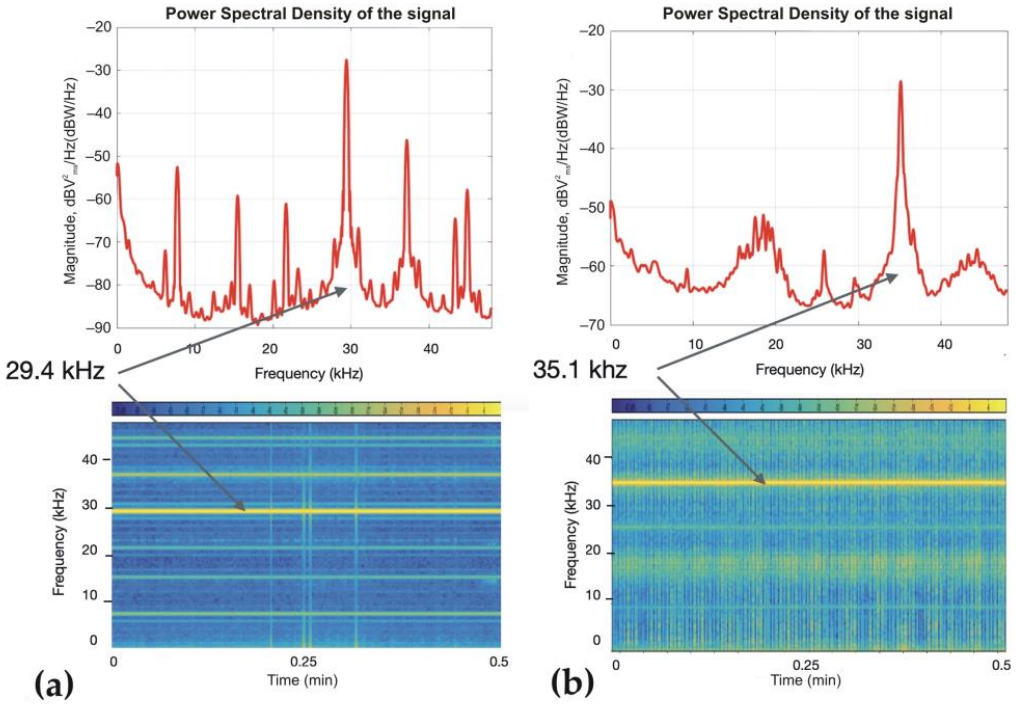

Figure 3. Characteristics of ultrasound exposures produced by two devices. (**a**) Power spectral density and spectrogram of the Sonoplus sonicator mini20 recording over time. It is a continuous signal with mixed frequency content but with a main excited frequency at 29.4 kHz. (**b**) Power spectral density and spectrogram of the GN20Pro ultrasonic cleaning rod recording over time. It is a continuous signal with a main frequency content of 35.1 kHz.

2.3. Experimental Setup

The experiments were conducted in a soundproof thermostatic chamber with a low basal noise range (30–35 dB).

The experimental apparatus (Figure 4) consisted of a sound-absorbing polystyrene tank with a wall thickness of 40 mm to avoid sound wave diffraction from the walls and external vibrational interference. The tank was filled with 10 L FSW (temperature 12 °C, salinity 35‰ and density 1.0375 g cm^{-3}) in the absence of both oxygenators and food particles. Each individual was placed inside a beaker to keep the body in a vertical position with both siphons clearly visible and facing upwards.

Exposure to ultrasound occurred at a distance of 150 mm from the tip of the transducer, which was submerged at 20 mm and 12 mm for Sonoplus and GN20Pro, respectively.

The number of SqRs and CrRs after exposure to ultrasound was evaluated for 5 min after exposure to either 30 or 35 kHz for 10 s. For each frequency, 20 individuals per species were considered. At the beginning of the experiments, the ascidians were kept quiet until both siphons continuously stayed open. The oral siphon was then gently touched with a dissection needle, with the time of rapid closing and subsequent opening responses of both siphons (SqR) noted to confirm the animal reactivity. Only animals that promptly reacted to this stimulus were considered. The exposure to ultrasound began after waiting for the siphons to reopen, and the observations of responses were carried out for 5 min. During this time range, the number of SqR and CrR events was collected.

Figure 4. A visual representation of the ultrasound tank set up. The walls of the tank were composed of polystyrene (40 mm in thickness). The tank was filled with 10 L seawater. Externally, mobile support allowed for the tip of the device to be held in place. The tank housed an ascidian individual placed in a glass beaker at 150 mm from the tip of the device.

In two additional series of experimental assays, eight individuals per species were continuously exposed to 30 kHz for 5 h. Before the exposure trial, background siphon behaviour in the absence of stimuli was recorded for 5 h. The videos were recorded by attaching a GoPro Hero 5 Black digital camera, Full HD 1080p resolution, version 2.70 (GoPro, Inc., San Mateo, CA, USA) to a support in a vertical position over the animals. For each species, four specimens were analysed simultaneously, each one separately kept in an upright position in a 50 or 10 mL beaker. The GoPro was set to timelapse recording mode, taking one photo per second. These videos allowed us to measure the average frequency of SqRs in the total absence of external stimuli, including not only accidental impacts on the apparatus and the consequent movement of water but also the reflex responses due to suspended particulates that could touch the sensory cells.

During the first experimental exposure to ultrasound assays, the response behaviour (e.g., events of SqRs, events of CrRs, long-term opening of the oral siphon and long-term closing or opening of both siphons) was monitored by splitting the observations into 30 min sections and was matched with that of specimens in total absence of external stimuli (control).

In the second experimental exposure assays, a collection of immediate responses to a mechanical stimulus (e.g., long-term closing of both siphons, weak and slow SqR events and long-term opening of both siphons) was performed by touching the oral siphon with a dissection needle every 30 min during 5 h of continuous exposure at 30 kHz and was matched with that of unexposed specimens (control).

In all experiments, the temperature was constantly monitored and kept at 12 $\pm$ 1 °C for the entire duration of the trials.

2.4. Statistical Analysis

Regarding the first series of experiments, each experiment was replicated with twenty individuals per species (n = 20), and the results are expressed as the averages $\pm$ SD. The statistical analysis of the number and type of responses (SqR and CrR) collected for 5 min after exposure for 10 s was performed considering the individual size clustering (i.e., size classes) using the statistical program R Software Environment, version 3.5.3 [64]. The threshold for statistical significance was set at $p < 0.05$. Differences in the responses to the

ultrasonic frequencies among size classes were investigated using nonmetric multidimensional scaling (NMDS) to graphically show differences or similarities among size classes. This allowed us to obtain, for all species tested, a distribution in three size classes with good scores of stress values (<0.2) [65], i.e., minor class (small individuals), intermediate class (medium-sized individuals) and major class (large individuals). Comparisons of SqRs and CrRs among size classes within the single exposure and within the same frequency were analysed with a glm with Poisson distribution (calculating *p*-values by using the likelihood ratio chi-square), followed by a post hoc test performed using the emmeans function (package emmeans [66]) to assess differences among the classes.

For the third experiment carried out with eight individuals per species ($n = 8$), to check for a statistical difference between control and treatments in the behavioural responses investigated, a permutational analysis of variance (PERMANOVA, with 9999 permutations, applying square root of dissimilarities, using the adonis2 function of the vegan package [67]) was performed on the entire dataset.

3. Results

3.1. Siphon Responses in the Absence of Stimuli

Ascidians in normal conditions keep their siphons open for a long time to favour filter-feeding activity. An SqR occasionally occurs, which consists of the rapid closure of both siphons, followed by equally rapid reopening and water ejection to promote the expulsion of faecal pellets from the atrial siphon and clean the branchial pharynx through the oral siphon. In the case of the observations carried out for 5 h to establish the background siphon behaviour in the absence of stimuli, the average frequency of a recorded SqR was 2.94 per h ± 0.09 for *C. intestinalis*, 2.7 per h ± 0.11 for *A. aspersa* and 5.7 per h ± 0.06 for *S. plicata*.

3.2. Siphon Responses after Exposure to a Brief Ultrasound Input

In these experiments, the number of SqR and CrR events was collected within a period of 5 min after a 10 s ultrasound input. Within the 30 kHz frequency assay, no differences were found among species in either the SqR (Chisq = 4.426, Df = 2, *p*-value = 0.1094) or the CrR (Chisq = 5.0582, Df = 2, *p*-value = 0.07973). Within the 35 kHz frequency assay, no differences were found among species in the SqR (Chisq = 3.398, Df = 2, *p*-value = 0.1829), whereas significant differences were highlighted in the CrR (Chisq = 7.4175, Df = 2, *p*-value = 0.02451).

3.2.1. *Ciona intestinalis*

On the basis of the distribution of responses to ultrasound with an NMDS analysis (Figure 5a,c), for both frequencies considered, a clear subdivision into three size classes of the treated individuals occurred, corresponding to minor (2–6 cm), intermediate (6–7.5 cm) and major (7.5–10 cm) classes.

After exposure to the 30 kHz ultrasound (Figure 5b), both the SqR and CrR were not dependent on the size of the animals (Chisq = 0.68301, Df = 2, *p*-value = 0.7107 for SqR and Chisq = 0.67236, Df = 2, *p*-value = 0.7145 for CrR). After exposure to a 35 kHz ultrasound (Figure 5d), the SqR was not dependent on the size of the animals (Chisq = 0.2574, Df = 2; *p*-value = 0.8792). Conversely, in the case of the CrR, the size of the individuals significantly influenced the response (Chisq = 6.8377, Df = 2, *p*-value = 0.03275), with a significant difference between the smaller and intermediate group (emmeans post hoc, *p*-value = 0.0304).

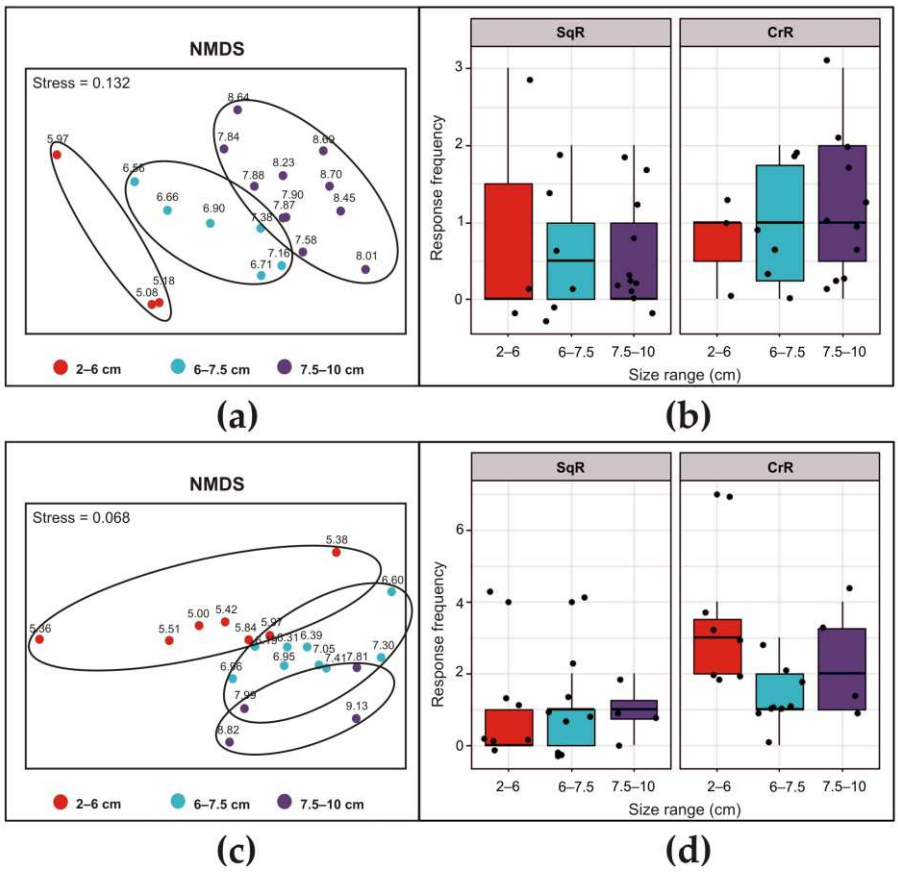

Figure 5. Siphon responses of *Ciona intestinalis* after exposure to ultrasonic input at 30 kHz (**a**,**b**) and 35 kHz (**c**,**d**) for 10 s. NMDS of the average frequency of responses of 20 individuals showing differences among the three size classes (**a**,**c**). Boxplots showing the differences in sea squirt response frequency (SqR on the **left** and CrR on the **right**) among the three size classes of animals exposed to ultrasound frequencies. Above the boxplot, each point represents the value of a single specimen (**b**,**d**).

3.2.2. *Ascidiella aspersa*

The NMDS analysis of the distribution of responses to stimuli with ultrasound led to three size classes. Because different animals were used and size uniformity could not be achieved, the three size classes were slightly different for the two exposure frequencies (Figure 6a,c). In particular, the dimensional ranges were determined to be 2–6 cm at 30 kHz and 2–5 cm at 35 kHz for the minor class, 6–8 cm at 30 kHz and 5–6.5 cm at 35 kHz for the intermediate class, and 8–10 cm at 30 kHz and 6.5–10 cm at 35 kHz for the major class. At 30 kHz (Figure 6b), the size of the individuals significantly influenced the SqR (Chisq = 6.2493, Df = 2, p-value = 0.04395). However, the post hoc analysis did not detect differences among the three groups. Similarly, in the case of CrR, a significant size-dependent effect was found (Chisq = 7.606, Df = 2, p-value = 0.0223), but the post hoc analysis following the glm did not reveal differences among the three groups. At 35 kHz (Figure 6d), the size of the individuals significantly influenced the SqR (Chisq = 7.373, Df = 2, p-value = 0.02506), although the post hoc analysis did not detect differences among the three groups. The CrR was not dependent on the size of the animals (Chisq = 1.2289, Df = 2; p-value = 0.5409), but it was significantly different compared to that of *S. plicata* (emmeans post hoc, p-value = 0.0222).

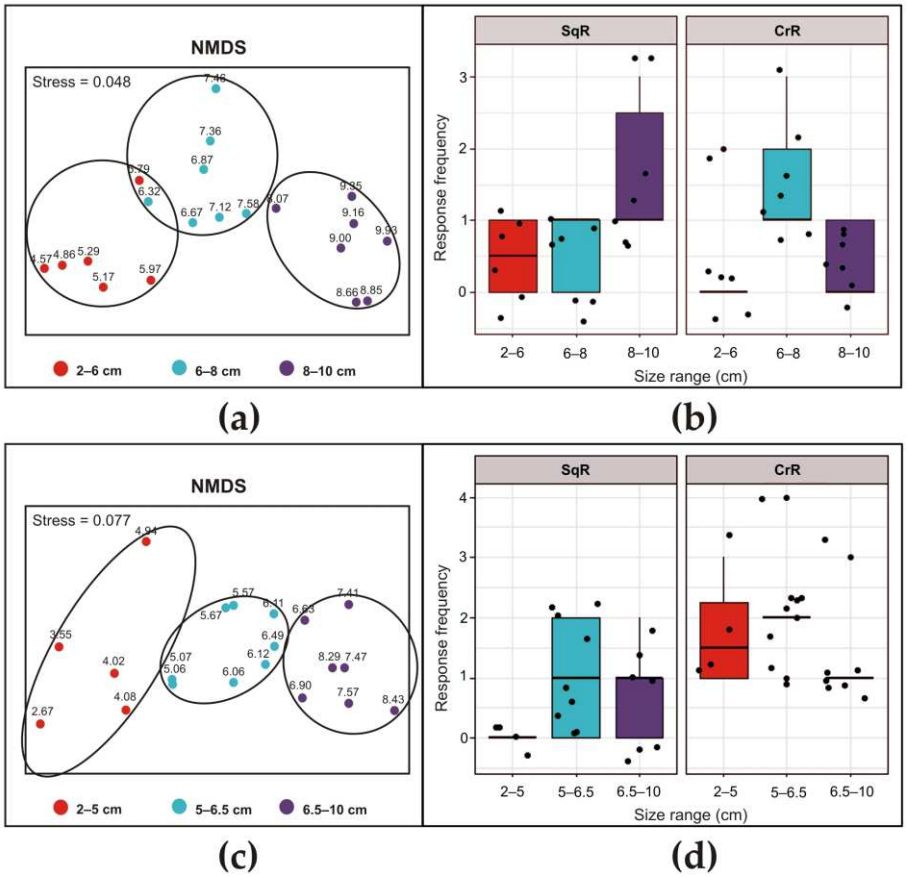

Figure 6. Siphon responses of *Ascidiella aspersa* after exposure to ultrasonic input at 30 kHz (**a**,**b**) and 35 kHz (**c**,**d**) for 10 s. For details, see the legend in Figure 5.

3.2.3. *Styela plicata*

The size classes determined via the NMDS analysis (Figure 7a,c) were the same for the two exposure frequencies, i.e., minor (2–5 cm), intermediate (5–7 cm) and major (7–10 cm).

At 30 kHz (Figure 7b) and 35 kHz (Figure 7d), both the SqR and CrR were not dependent on the size of the animals (Chisq = 1.6818, Df = 2, p-value = 0.4313 for SqR and Chisq = 0.69316, Df = 2, p-value = 0.7071 for CrR at 30 kHz; Chisq = 0.53625, Df = 2, p-value = 0.7648 for SqR and Chisq = 1.2635, Df = 2, p-value = 0.5316 for CrR at 35 kHz).

3.3. Siphon Responses during Continuous Ultrasound Exposure

During the 5 h of exposure to the 30 kHz ultrasound, the responses were reported by splitting the observations into ranges of 30 min to highlight changes across time. Differences among species were observed throughout the exposure. The most remarkable behaviour was the long-term opening of both siphons, which occurred after 150 min of exposure in all species.

C. intestinalis was the species with the highest number of responses (n = 304) during the 5 h of exposure to ultrasound (Figure 8a). The main type of response was the long-term opening of the oral siphon. The responses of this type were almost double (n = 158) those observed in the other two species (n = 75 in *A. aspersa* and n = 81 in *S. plicata*). Moreover, the number of long-term closures of both siphons was a behavioural response that, in *C. intestinalis*, was approximately three times greater (n = 52) than in *A. aspersa* (n = 18) and *S. plicata* (n = 15). On the other hand, the episodes of the long-term opening of both siphons were similar to those observed in *S. plicata* (n = 19 and n = 21 in *C. intestinalis* and *S. plicata*, respectively) but were approximately half of those of *A. aspersa* (n = 48); *A. aspersa* proved to be the species with the greatest number of this type of response (Figure 8b). Unlike other species, the episodes of long-term closure of both siphons were observed in *C. intestinalis*

throughout the exposure time and were very numerous (n = 52) (Figure 8a). *S. plicata* showed a greater number of episodes of CrR (n = 14) than *C. intestinalis* and *A. aspersa* (n = 5) during the whole period of ultrasound exposure (Figure 8c).

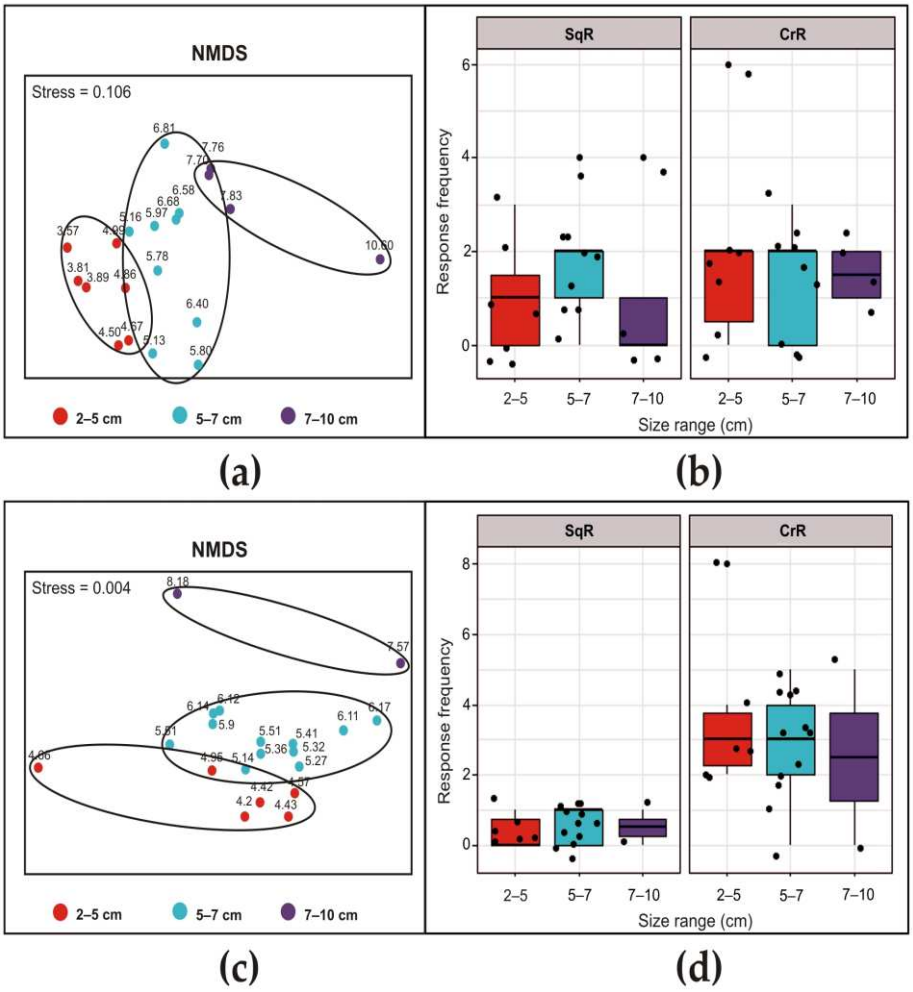

Figure 7. Siphon responses of *Styela plicata* after exposure to an ultrasonic input at 30 kHz (**a**,**b**) and 35 kHz (**c**,**d**) for 10 s. For details, see the legend in Figure 5.

3.4. Responses of the Oral Siphon to a Mechanical Stimulus during Continuous Ultrasound Exposure

C. intestinalis, *A. aspersa* and *S. plicata* responded in a similar way to the mechanical stimuli carried out by touching the rim of the oral siphon with a needle every 30 min within 5 h of ultrasound exposure (Figure 9). The behavioural modes were only directly annotated and not recorded on the camera to time the response speed. For all species, the PERMANOVA revealed a statistically significant effect of the condition of exposure (*C. intestinalis*: $F_{1,18}$ = 49.028, R^2 = 0.73146, p-value < 0.001; *A. aspersa*: $F_{1,18}$ = 64.395, R^2 = 0.78154, p-value < 0.001; *S. plicata*: $F_{1,18}$ = 64.395, R^2 = 0.69653, p-value < 0.001).

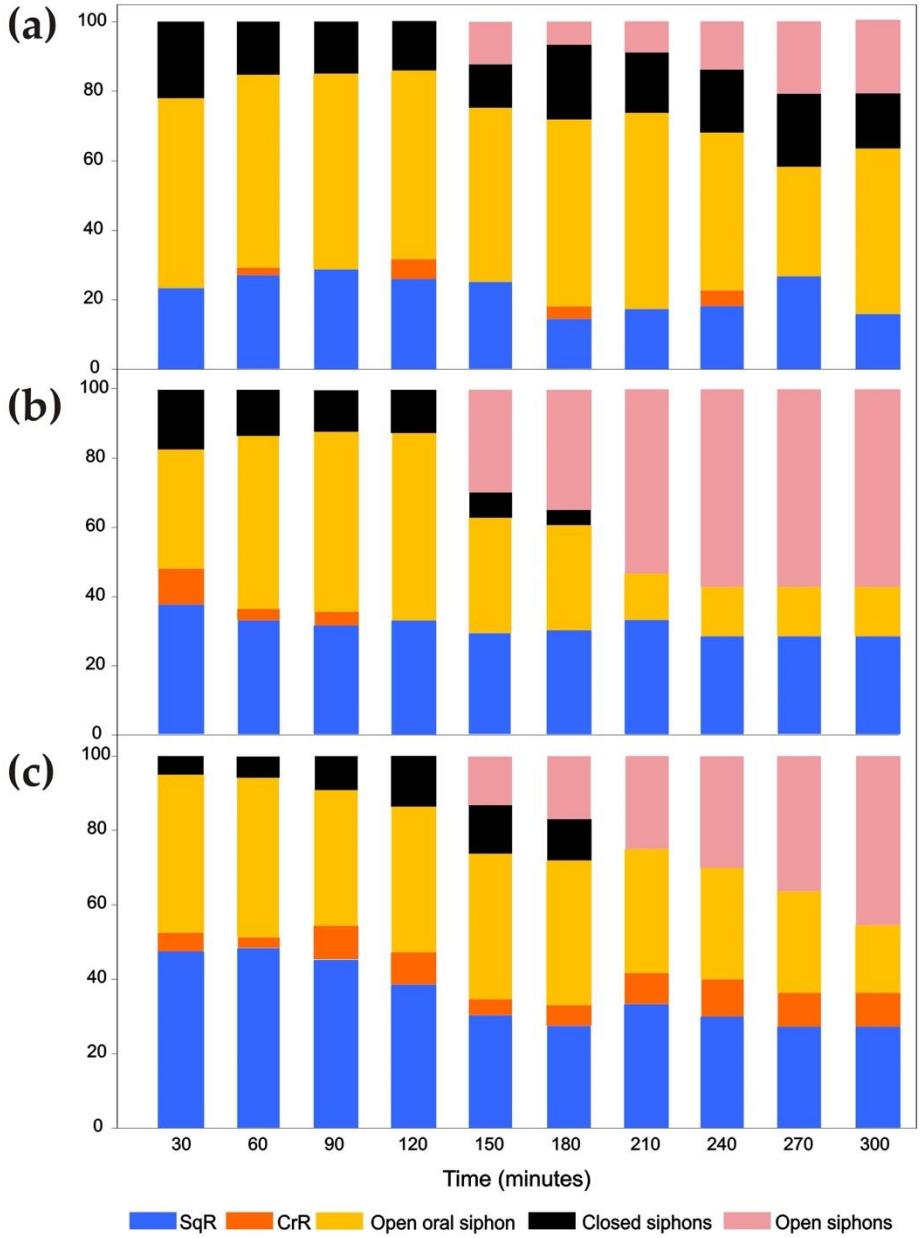

Figure 8. Percent of responses observed every 30 min during 5 h of exposure to 30 kHz ultrasound in *Ciona intestinalis* (**a**), *Ascidiella aspersa* (**b**) and *Styela plicata* (**c**). The average results for eight specimens of each species are shown.

In the first 30 min for all species, the episodes of SqR occurred more slowly than in the controls not exposed to ultrasound. Approximately the same number (~20) of the prolonged closing responses of both siphons was detected. Beginning from 150 min, siphons were observed to remain open despite the mechanical stimulus, and this response prevailed over the other types of response. This lack of response to the stimulus was mostly presented by *A. aspersa* beginning at 120 min.

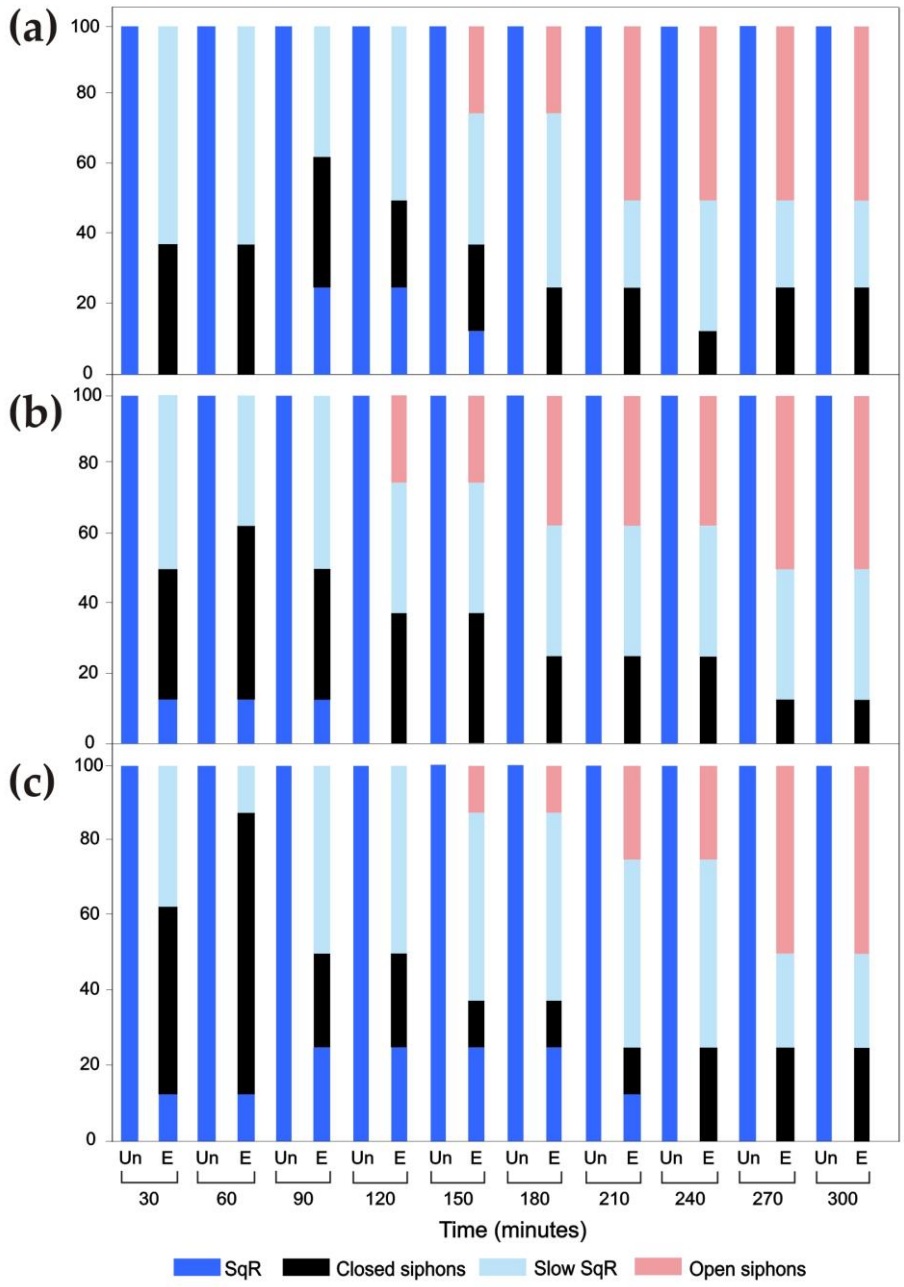

Figure 9. Percent value of responses to a mechanical stimulus of the oral siphon observed every 30 min for 5 h in normal conditions (Un, unexposed specimens) and during continuous exposure to 30 kHz ultrasound (E, exposed specimens) in *Ciona intestinalis* (**a**), *Ascidiella aspersa* (**b**) and *Styela plicata* (**c**). The average results of eight specimens for each species are shown. Note that responses in normal conditions are SqRs only (100%).

4. Discussion

This study represents the first to investigate behavioural effects in adult ascidians after exposure to the frequencies created by new-generation ultrasonic antifouling systems. The siphon responses that were observed in the short-term ultrasonic exposure experiment at frequencies of 30 and 35 kHz were both SqRs and CrRs. SqRs are behavioural responses that occur even at rest, but in the case of sound stimuli, they are significantly more numerous and intense and are explained by the stimulation of the epidermal sensory cells of the rim of the oral siphon, probably due to acoustic pressure, as observed in the case of noise [44].

CrRs were the most interesting responses from an acoustic point of view since they are caused by the direct stimulation of the coronal organ by sound vibrations. All species tested showed high sensitivity, especially at the highest frequency. Regarding the distribution based on the size class, the highest number of CrRs was observed at 35 kHz in the minor class of *C. intestinalis*. It is possible that juvenile forms have a greater number of sensory cells in the coronal organ, similarly to what occurs in the inner ear of mammals, which tends to lose sensory cells and consequently hearing ability with age [68].

In the experiments in which individuals were continuously exposed to 30 kHz for 5 h, a variety of behavioural responses occurred. In addition to SqRs and CrRs, events of the long-term opening of the oral siphon and the long-term closing or opening of both siphons were observed. Particularly in *A. aspersa* and *S. plicata*, a greater number of episodes of siphon closure were more concentrated in the first part of the exposure. Then, a new behaviour appeared from two and a half hours onwards, represented by the long-term opening of both siphons. Unlike the other two species, in *S. plicata*, more episodes of CrR during 5 h of exposure were observed, confirming the previous behavioural responses observed after short exposure. This feature of prevailing CrRs may reflect the difference in the extent of the coronal organ. *S. plicata* has the largest number of tentacles, up to 60, compared to 25–45 in *C. intestinalis* [69] and 16–31 in *A. aspersa* [70].

The long-term opening of the siphons observed during the continuous exposure to the 30 kHz ultrasound could be interpreted as stimulus-specific habituation [71]. However, by changing the nature of the stimulus, such as a physical contact (i.e., the mechanical touch of the oral siphon), a new response did not occur. Both the siphon and body stopped reacting, and the SqRs disappeared. This alteration in behavioural response could be due to muscle fatigue, which functions similarly to chronic treatment, as a generation of stress caused, in this case, by continuous siphon responses to ultrasound stimuli. Somatic, smooth muscles are particularly abundant around the siphons, especially the oral siphon, where they form a siphonal sphincter [50] innervated by cholinergic nerves [72]. Keeping the siphons open or the occurrence of a limited closing capacity could make the animals more vulnerable to predators or parasites and to branchial damage caused by large food particles that enter the pharynx with the water flow and cannot be expelled by the ejection reaction [60,73]. Thus, the constant opening of siphons has potentially negative consequences on filter-feeding activity and survival. Recently, in other filter-feeding organisms such as bivalves, valve closure and reduced gill function have been considered protective responses when they are stressed by exposure to pollutants [74] and anthropogenic noise [21,75]. This behaviour had the positive result of less pollutant accumulation but the negative outcome of reductions in feeding and growth [76].

5. Conclusions

Increasing attention has recently been given to marine noise pollution with regard to both its sources and its damage to marine mammals. Nevertheless, knowledge of the acoustic sensitivity of various marine species, fish and invertebrates to the anthropogenic sound frequencies that are released into the environment is essential to better understand the effects that noise pollution might have on marine organisms. Although the responses of marine organisms to sound are complex, appearing to be both species- and sound-specific, the continuous emission of ultrasound from antifouling devices over time such as that occurring in harbours and marinas—where ships and boats are moored for a long time—could cause stress in local benthic animals with unpredictable negative consequences on populations and ecosystems. From the results obtained in this preliminary study based on frequencies used by acoustic antifouling systems, it has been possible to observe for the first time how ascidians are able to perceive ultrasound. Various behavioural responses of their siphons, varying according to species and the size of the individuals, were observed. The most important features appear to be the involvement of the coronal organ, which is based on axonless hair cells resembling those of the vertebrate acusticolateralis system, and

the establishment of stress conditions able to cause muscle fatigue, both of which warrant further in-depth studies.

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5 CONCLUSIONS

In 1962, Rachel Carson publishes her renowned book *Silent Spring*, bringing for the first time the attention of public opinion and the scientific community on the massive use of the famous pesticide DDT in the American countryside and its negative effects on ecosystems (Carson, 2002).

In the last decades, the focus on the environment and its protection has involved various sectors, such as research, economic policy and social communication. Among the multidisciplinary studies, those concerning the analysis of substances produced by man and the effects resulting from their uncontrolled release into ecosystems are a primary concern. In particular, the importance of marine ecosystem conservation arisen in recent years is based on protection of habitats, communities and food chains, and restoring the productivity of the coastal areas.

My PhD thesis deals with an important and urgent environmental issue, i.e., the management of biofouling, which is a problem of increasing interest worldwide, being also addressed by global programs of the United Nations (IMO, 2011 – GloFouling program). In particular, my project focused on the implementation of new antifouling systems with low impact on benthic marine invertebrates at ecosystem, organismal and cellular levels. Until now, the most used systems in the world have been represented by antifouling paints, which caused various ecological problems due to disruptive effects provoked on non-target organisms and consequent depletion of coastal biocoenoses. In the last years, worldwide research shifted attention to the development of alternative antifouling eco-friendly systems that can be divided into chemical and physical categories.

Chemical antifouling systems are represented by paints with a biocide, to which booster substances – many of which are pesticides – can be added and represent potential long-term environmental damage (Alzieu *et al*., 1986; Claisse and Alzieu, 1993; Thomas and Brooks, 2010; Dafforn *et al*., 2011). Copper compounds were considered ideal for use as antifoulants since the chemistry of copper changes in the marine environment, thereby affecting its bioavailability (and therefore its toxicity) by rapid organic complexation. A key factor in the environmental aspect of copper-based antifoulants is that the active ionic form of copper exists only briefly while it is on the coated surface. During that time, it performs its efficacy by impeding the settlement of fouling organisms. However, the co-presence of organic booster biocides and the type of polymeric matrix can significantly increase the performance of the paint (Correa *et al*., 1996; Voulvoulis *et al*., 1999, 2006; Almeida *et al*., 2007; Manzo *et al*., 2008; Parks *et al*., 2010; Cima and Ballarin, 2012; Guardiola *et al*., 2012).

I have investigated the ecological succession on wooden and stainless-steel panels painted with common copper(I)-based paints on the market developed with different technologies (self-polishing and insoluble matrix) and booster content. This study monitored the ecological succession of macrofouling communities on panels immersed for 10 months in the southern basin of the Lagoon of Venice. Results with copper-based paints revealed unpredictable effects on the settlement and growth of key species of macrofouling of hard-substrata, which have the potential to alter the native structure of the communities. A series of biodiversity descriptors highlighted the disturbing activity of the antifouling paints that resulted in a negative selection of key species of the coastal community of hard-substratum. Green algae, bryozoans, and barnacles were the most tolerant taxa and a negative species selection occurred for sponges, serpulids, and ascidians.

The university-enterprise collaboration, of which I was the beneficiary, aimed to develop a new eco-friendly copper-based formulation. After three years of monitoring of numerous RESIMIX's samples in different hydrodynamic sites of the Lagoon of Venice, good, long-term qualitative results have been reached with a copper(I)-based formulation (*Sailing Pro ECO*, with 10% of copper(I) oxide), where the biocide concentration and leaching were lower than in the trade formulations, and the self-polishing technology of the matrix was represented by an innovative water paint with acrylate polymers. It also maintained a good antifouling performance for two years. During the last monitoring some problems with adhesion between primer and steel substratum were encountered. RESIMIX should improve the primer formulation for a correct adhesion to this and other types of substrata.

Moreover, comparison with commercial antifouling paints has been considered together with the effects on settlement and metamorphosis of ascidian larvae. Results revealed that all antifouling biocides prevented the settlement and interfered with the metamorphosis causing developmental delays, malformations and mortality on *B. schlosseri* larvae. All were lethal with the exception of TCMS pyridine and the herbicides Diuron and Irgarol 1051, which did not kill the larvae, but prevented their settlement on the substratum and inhibited their metamorphosis.

As reported in the PubChem database (https://pubchem.ncbi.nlm.nih.gov/) Dichlofluanid is a fungicide for agriculture and wood preservation that, in the last two decades, has been introduced as a booster in antifouling paints due to its rapid degradation to compounds with reduced toxicity (Toxicity data for aquatic species reported in EU regulation number 528/2012, ECHA/BPC/120/2016). Despite the dichlofluanid is probably one of the least toxic organic biocides it is able of causing various damages to non-target marine organisms and compromises their survival, embryonic development, and metamorphosis (Nishiuchi and Yoshida, 1972; Martin *et al*., 1995; Aoki *et al*., 2000; Lee *et al*., 2013). Many aspects of its toxicity are still

unknown. For this reason, I have carried out a series of *in vitro* assays to evaluate the immunotoxicity of this composed in *B. schlosseri*. Results obtained with a series of biomarkers revealed that it is one of the most toxic antifouling biocides of those examined to date, with toxicity comparable to organometals such as organotin compounds and zinc pyrithione. Its high ability to adversely affect the immune system could promote the onset of diseases and decrease tolerance to other xenobiotics and, finally, the survival capability.
In addition, similar assays have been carried out on bivalves after exposure to various concentrations of copper(I) chloride. These experiments allowed to compare the effects on immune responses by pivotal species of the coastal community, i.e., a target species dominant in the macrofouling community like *M. galloprovincialis* and a non-target species living in soft sediments like *R. philippinarum*.
From these preliminary but significant results about chemical antifouling systems, crucial questions have arisen regarding the continuous indiscriminate introduction of such biocides into 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. Therefore, according to the Biocides Directive (98/8/EC), more assays of acute and chronic toxicity on various target and non-target marine organisms should be performed before marketing of new potential pollutants, in order to prevent the same errors that already occurred with TBT in both the ecological and economical fields due to the deterioration of environments and habitats.

Physical antifouling systems have been introduced in recent years in relation to the development of a more environmentally friendly approach rather than the chemical systems (Latour and Murphy, 1981; Branscomb and Rittschof, 1984; Donskoy and Ludyanskiy, 1995; Jackson *et al*., 2004; Nakagawa *et al*., 2006; Gavand *et al*., 2007; Boldor *et al*., 2008; Perez-Roa *et al*., 2008; Scardino *et al*., 2009; Bullard *et al*., 2010; Guo *et al*., 2011; Kopel *et al*., 2011). I considered geotextiles (as new hard substrata used for coastal erosion protection), and ultrasound (to prevent biofilm and disturb the larval settlement) reaching interesting results in both the field and the lab, which revealed the till now hidden downside of these systems.
Geotextile fabrics used to prevent coastal erosion (Theisen, 1992) showed implications for community development (Palmeira *et al*., 1996; Ferreira Gomes, 2001; Koffler *et al*., 2008; Lee and Douglas, 2012; Mitra, 2013; Oumeraci and Recio, 2017). They affected both ecological succession settlement and growth in different ways by preventing biofouling settlement or favouring dominant species. For these two-faceted aspects that potentially cause different long-term impacts on the biodiversity of

resident communities, the use of geotextile fabrics as alternative antifouling systems to biocide-based paint formulations or as profouling systems for restoration of degraded ecosystems must be carefully considered. The study of the influence of the substratum on the settlement of various organisms of the hard-substratum macrofouling community represents an essential tool to choose a geotextile for the most appropriate application in a variety of coastal marine ecosystems. For the increasing interest in geotextile employments in various marine activities and industries, these results could represent first lines of evidence to inform decision-making to minimise biofouling and/or predict the use of artificial substrata as habitats for marine organisms.

In addition, I was interested also in applications of *ultrasonic devices* used as antifouling systems (Guo *et al*., 2011). These acoustic generators have been recently introduced as eco-friendly antifouling systems (Legg *et al*., 2015) to prevent biofilm formation and larval settlement on ship's hulls but nothing is still known about the effects on benthic adult invertebrates. My study demonstrated that a progressive muscle fatigue occurred in ascidian siphons supporting the hypothesis of a severe weakening of both particle selection ability during the filter-feeding activity and defence reaction towards natural predators. These results represent the first evidence of disturbing activity of ultrasound on benthic marine invertebrates. This study could continue by deepening the observations on morphological damage in the acoustic sensory organ of ascidians (coronal organ) and stress responses after prolonged exposure to ultrasound.

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7 REPORT OF Ph.D. ACTIVITIES

❖ PUBLICATIONS

2020

1) Cima F., **Varello R., 2020**. Immunotoxicity in ascidians: Antifouling compounds alternative to organotins - V. The case of dichlofluanid. *J. Mar. Sci. Eng.,* 8: 396. (doi:10.3390/jmse8060396) (MDPI, IF [2018]: 1.732)

2021

2) Varello, R., Wetzel, M. A., Cima, F., **2021**. Two facets of geotextiles in coastal ecosystems: Anti-or profouling effects*? Mar. Environ. Res*, 170: 105414. (doi.org/10.1016/j.marenvres.2021.105414) (Elsevier; IF [2021]: 3.737).

2022

3) Cima F., and **Varello R., 2022**. Effects of Exposure to Trade Antifouling Paints and Biocides on Larval Settlement and Metamorphosis of the Compound Ascidian *Botryllus schlosseri*. *J. Mar. Sci*. *Eng.* 10, 123. (doi:10.3390/jmse10020123) (MDPI, IF [2021]: 2.744).

2023

4) Cima F., and **Varello R., 2023**. Potential disruptive effects of copper-based antifouling paints on the biodiversity of coastal macrofouling communities. *Environ. Sci. Pollut. Res*., 30: 8633–8646. (doi:10.1007/s11356-021-17940-2) (Springer; IF [2021]: 5.190).

5) Varello, R., Asnicar, D., Boaga, J., Cima, F., **2023**. Behavioural responses to ultrasound antifouling systems by adult solitary ascidians. *J. Mar. Sci. Eng*. 11, 1115. (doi.org/10.3390/jmse11061115) (MDPI, IF [2021]: 2.744).

6) Cima F., and **Varello, R., 2023**. Immunotoxic effects on target and non-target bivalve species after exposure to the antifouling copper(I) biocide: A comparative *in vitro* study between *Mytilus galloprovincialis* and *Ruditapes philippinarum*. *Frontiers in physiology*. Under review.

❖ MEETINGS

1) 7th International Conference on Pollution Control & Sustainable Environment. March 02, 2020 Rome (Italy). INTERNATIONAL CONGRESS PROCEEDINGS (poster): Varello R.,

Wetzel M.A., Cima F. (2020). Two faces of geotextiles in coast ecosystems: A matter of anti- or profouling effects. *Environ. Pollut. Clim. Change*, 4: 44-45.

2) 8th International Conference on Environmental Management, Engineering, Planning and Economics (CEMEPE 2021) and SECOTOX conference. July 20-24, 2021 Thessaloniki (Greece). *INTERNATIONAL CONGRESS, POSTER*: Varello R. and Cima F. "*Potential disruptive effects of copper-based antifouling paints on the biodiversity of coastal macrofouling communities*".

3) ECSA 58 - EMECS 13: Estuaries and coastal seas in the Anthropocene – Structure, functions, services and management. 6-9 September 2021. Online Live and On-demand. *INTERNATIONAL CONGRESS, POSTER:* Varello R. and Cima F. "*Selective potential of geotextiles on marine fouling settlement*".

4) 22th Meeting of the Società Italiana di Immunobiologia Comparata e dello Sviluppo (SIICS), February 16-18, 2022, Padova (Italy). NATIONAL CONGRESS (talk): Varello R. and Cima F. "*Immunotoxic effects of a new-generation antifouling biocide in a compound ascidian*".

5) 5th Scientific Retreat of the Department of Biology – University of Padova, 26-27 May, 2022 (Padova, Italy). DEPARTMENTAL SCIENTIFIC RETREAT (poster and elevator pitch): Varello R. and Cima F. *"Potential disruptive effects of copper-based antifouling paints on the biodiversity of coastal macrofouling communities".*

6) 81th Congress of the Unione Zoologica Italiana (UZI), 20-23 September 2022 (Trieste, Italy). NATIONAL CONGRESS (poster): Varello R. and Cima F., "*Effects of geotextiles for coastal erosion control on marine fouling settlement*".

❖ COURSES

1) PhD School Course. Prof. Marta Giacomello (University of Padova, Italy): 4 lessons (February-March 2020.), "*Perspectives on fluorescence microscopy*".

2) Eduopen.org Course. Prof: Alessandra Bianchi, Manuela Cattelan, Giorgia Callegaro. Online Course: 35 hours; 6 modules (April 2020), "*Probabilità e statistica*".

3) PhD School Course. Prof. Alberto Barausse (University of Padova, Italy): 16 hours (30, 31 March – 13, 14, 20, 21, 27, 30 April 2021), "*Ecological modelling basics*".

4) Online course. Dr Marco Chierici (University of Insumbria, Italy): 6-7 May 2021, "*Il software R - corso avanzato*".

5) PhD School Course. Prof: Ballarin L., Brivio M., de Pittà C., Sandrelli F., Venier P. (University of Padova, Italy): 10 hours (24-25-26 May 2021), "*Invertebrate physiology: from development to immunity*".

6) PhD School Course. Prof. Maddalena Mognato (University of Padova, Italy): 7 June 2021, "Humans in a dish: the potential of organoids in modelling diseases. "*Organoids as a 3D-model to study the DNA-damage response pathway*".

7) Summer school at University of Pavia: 28 June - 2 July 2021, "*Monitoring marine alien species in ports with the SERC protocol*".
8) Online course. Dr Damiano Preatoni (University of Insumbria, Italy): 7-9 July 2021, "*Il software R - corso base*".
9) PhD School Course. Prof Tamas Szekely (University of Bath, UK): 4 h (25-26 November 2021), "*Sexual conflicts*".
10) PhD School Course. Prof Paola Venier (University of Padova, Italy): 2 h (22 June 2022), "*Adaptive potential and successful strategies within the invisible majority*".
11) Course financed by the Excellence Project of the Department of Biology, University of Padova. Prof Pedro Martinez (University of Barcelona, Spain): 6 h (12-13-14 September 2022), "Topic: a) *Gene Regulatory Networks in animals*; b) *Genomic analysis of development (with a focus on marine invertebrates)*; c) *The stem cell niche concept in animals (with a focus on marine invertebrates)"*.
12) PhD School Course. Prof. Chiara Romualdi (University of Padova, Italy): 4 h (26-27 September 2022), "*Notes on statistical methods for biological data*".

❖ TUTORED STUDENTS (BD theses)

1) Pesce A., 2019-2020. "*L' impatto ambientale del biocida diclofluanide negli ecosistemi marini costieri*". Elaborato di Laurea in Biologia, Università degli Studi di Padova.
2) Rizzi A., 2020-2021. "*L'impatto ambientale di biocidi antivegetativi a base di Cu(I) sugli ecosistemi marini costieri*". Elaborato di Laurea in Biologia, Università degli Studi di Padova.
3) Citton A., 2021-2022. "*Effetti di ultrasuoni utilizzati come sistemi antivegetativi sulle risposte comportamentali di ascidie solitarie*". Elaborato di Laurea in Scienze Naturali, Università degli Studi di Padova.
4) Carollo F., 2021-2022. "*Immunotossicità di Cu(I): evidenze nel bivalve Mytilus galloprovincialis*". Elaborato di Laurea in Scienze Naturali, Università degli Studi di Padova.

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