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First data on the effect of Aluminium intake in *Chamelea gallina* of exploited stocks in the Southern Adriatic Sea (Central Mediterranean Sea)

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ABSTRACT

The striped venus clam *Chamelea gallina* is an important shellfish resource in coastal area of Mediterranean Basin, playing a fundamental role in the ecosystems. In the last two decades there has been a reduction in the stocks of this bivalve with several mortality events along Adriatic Sea due to pollutants. Aiming to evaluate the effects on clams of the anthropogenic increase of some metals in the sea, we focused on aluminium. In particular, we tested whether Al could affect mucus secretion, since mucins are involved in protection from pathogens and toxicants and transport of alimentary particles and pseudofeces. Aluminium was administered in the form of aluminium chloride at concentrations of 25, 50 and 200 μM. Histochemical and lectin-histochemical - analyses were used to evaluate *in situ* qualitative and quantitative variation of the muciparous secretions produced by both the gills and foot epithelia. Treatments resulted in quali-quantitative alterations in the secretions of both gills and foot epithelia, in particular reduction of glycosylation resulting in a general decrease of acidic residuals and of mannosylated, glycosaminylated and fucosylated chains. This can result in a decrease of acidity and viscosity of mucus and thus it could affect its functionality and the survival of the animals.

1. Introduction

The striped venus clam, *Chamelea gallina* (Linnaeus, 1758) is a bivalve mollusc species of biocenosis of fine well-sorted sand (Pérès, 1967; Montefalcone et al., 2021) present in Mediterranean Basin and in Black Sea, and widespread along the east Atlantic coasts from Morocco to the British Isles, and Norway (Carducci et al., 2020). This species is a very important commercial resources in the Atlantic (Gaspar et al., 2004; Delgado et al., 2015) and many Mediterranean areas, such as Cadiz and Valencia in Spain, along the Italian coastal areas of Sardinia, in the Adriatic and the Thyrrenian seas, and in the Black Sea (Deval, 2001; Baeta et al., 2021). The harvesting is carried out through hydraulic dredges on shallowest soft-bottoms, included between 2 and 12 m of depth in the Mediterranean Sea (Morello et al., 2005). The commercial importance, as well as the interesting dietetic properties due to their low lipid and cholesterol contents (Orban et al., 2007), have contributed to an increase of the consumption of venus clams, with an increasing in the global landings, although with a inhomogeneous trends among geographical areas (Baeta et al., 2018, 2021).

In the Italian fishing market, the economic yield of the venus clam accounts for about 12 million of euros (DGPEMAC, 2019), and the Adriatic Sea represents the main exploited area for the harvesting of clams and other shellfishes. However, a long-term demographic declines of the Adriatic populations together with mass-mortality events have been recorded starting from the end of 1990s (Romanelli et al., 2009; Scarcella and Cabanelas, 2016). The causes of decline are the combination of several impacts both due to the fishing exploitation and environmental drives, which often are unknown (Froglia, 2000; Morello et al., 2005). In this worrying context, the growing interest of national institutions in mitigating the socio-economic impacts on the Italian shellfish sector has led to the implementation of new clam stock management measures (Carlucci et al., 2015). In particular, the Minimum Conservation Reference Size (MCRS) of *C. gallina* has been reduced from 25 mm total length (TL) [Council Regulation (CE) No. 1967/2006, 2006 of the European Community (EC)] to 22 mm TL [Delegated Regulation (UE) No. 2016/2376, 2016 of

Abbreviations: Fuc, fucose; Gal, galactose; GalNac, N-acetylgalactosamine; GlcNac, N-acetylglucosamine; Glc, glucose; MαM, methyl-α-mannopyranoside; Man, mannose; Neu5Ac, N-Acetylneuraminic acid; TACT, N, N', N''-triacetylchitotriose

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the European Union (EU) and Commission Delegated Regulation (EU) 2020/3, 2020]. Although this regulation tries to ensure a trade-off between the maintenance of the stock productivity within the limits of the biological sustainability of the species (Bargione et al., 2021), to understand the influence of other human and environmental impacts on the mortality of *C. gallina* is required. Therefore, new investigations on the interactions between *C. gallina* and other potential stressors should be performed addressing future regulations towards the framework of the Ecosystem-based Management approach (Long et al., 2017).

Although the fishery is one of the most anthropogenic stressors impacting on the population dynamics of the striped venus clam (Morello et al., 2005), other human and environmental pressures can induce negative impacts and mass mortality events on the *C. gallina,* with a consequent decline of the populations (Milan et al., 2019). Several factors, likely acting in synergic way, seem to contribute to striped venus clam mass mortality. For instance, extremely variations in water temperature, salinity and pH driven by the climate change (Ezgeta-Balić et al., 2011; Matozzo et al., 2012; Monari et al., 2011; Sordo et al., 2021), as well as the effects of phytoplankton blooms (e.g., red tides and gelatinous floating masses), can have negative impacts on larval recruitment of the striped venus clam (Romanelli et al., 2009). These stresses could affect the immune system of the striped venus clam, with an increase in infectious diseases (Matozzo et al., 2012; Milan et al., 2016a,b). In addition, the role of microbiota hosted in the *C. gallina* seem to be relevant in the mortality events when chemical stresses compromising the immune pathways and defence mechanisms of the bivalve (Milan et al., 2019).

The impacts due to the chemical pollution are also relevant, representing an emerging warning for the status of *C. gallina* and its human food consumption (Visciano et al., 2015). Indeed, bivalves accumulate various classes of contaminants in different tissues such as those of gills and digestive gland. In particular, the gills of these animals represent the first contact with environment, and the first site of uptake and accumulation of water-soluble pollutants such as heavy metals. In the Manila clam, *Ruditapes philippinarum*, arsenite and arsenate induce oxidative stress in the digestive gland (Ji et al., 2015). A metabolomic analysis showed that both chemical forms of As induce immune stress, as shown by the increase in branched-chain amino acids (valine, leucine and isoleucine) and osmotic stress (Ji et al., 2015). Besides, when heavy metals are in form of nanoparticles, absorption by the gills and the digestive gland induces lysosomal perturbation and oxidative stress (Canesi et al., 2012). Mucus secretion proved a good marker for pollution in both bivalves and gastropods (David and Fontanetti, 2009; Cutuli et al., 2021). In particular, mucus from gills has a number of important functions, such as modulating exchanges of water, salts, food and toxicants between the environment and the body, particles trapping and transport and formation of pseudofeces. These are also used to expel toxicants and prevent tissue accumulation (e.g., David and Fontanetti, 2009). Thus, changes in mucus secretion and composition as those induced by toxicants can result in altered functional conditions with possible consequences on bivalve health and survival.

Aluminium (Al) is the third most abundant element in Earth's crust (Wilson, 2011) after oxygen and silicon, and naturally wide present as complex with these two in the aluminosilicate and oxide minerals (Namieśnik and Rabajczyk, 2010). Despite its abundance, Al dissolved concentration in the Mediterranean basin is of few micrograms per litre in the water column (Caschetto and Wollast, 1979; Ghani, 2015); higher concentrations are found in the sediments and are from few tens to one hundred micrograms per litre (Ghani, 2015). This metal is widely used in everyday life as packing, drugs, antiperspirant, herbicides, objects used in road traffic, civil engineering, etc., exposing people and the environment to its contamination (Schmitz, 2006). Excessive dosages of Al have been shown to cause toxic effects in several organisms, both terrestrial and aquatic, in fresh- and seawater (Rosseland et al., 1990; Jones and Ryan, 2016; Monaco et al., 2017; Capriello et al., 2019; Parrino et al., 2021; Katalay et al., 2022; Chelyadina et al., 2022). In human, Al is also correlated with several diseases like neurocognitive (Bolla et al., 1992) and neurodegenerative diseases such as dialysis encephalopathy, Parkinson, Alzheimer, and amyotrophic lateral sclerosis (Campbell, 2002; Bjorklund et al., 2018; Jones et al., 2017). Molluscs are known to accumulate Al in tissues (Kádár et al., 2001). However, information regarding the alterations induced by Al on mucous secretions of bivalves is still scarce, and little is known about changes in mucus composition.

Keeping all the previous as reference, in this study we investigated the alterations caused by Al contamination on the mucous secretion of the *C. gallina* coming from exploited stocks in the Southern Adriatic Sea (Central Mediterranean Sea).

2. Materials and methods

Striped venus clams of commercial size (above 2.5 cm) were sampled in Margherita di Savoia (BT), an area in the Southern Adriatic Sea characterized by the occurrence of fishing grounds exploited with hydraulic dredges (Fig. 1). The sampling was carried out on January 20th, 2022, far from the reproductive phase and the warmest period of the year with the aim of minimizing stress (Monari et al., 2007a; Guglielmi et al., 2023). The individuals were immediately transported into the laboratory for housing and treatments.

2.1. Housing and treatments

Four groups of twenty clams each were acclimatised for two days in artificial saltwater (salinity 35‰) in four different aerated ten litres-tanks at 16 °C in a controlled temperature chamber. Each group was daily fed with 50 ml of the alga *Tetraselmis suecica*. Total water changes and pH measurements were performed daily. After the acclimatization period and under the same conditions, different dosages (25 μM, 50 μM, 200 μM) of aluminium chloride ($AlCl_3 \times 6H_20$, Carlo Erba Reagents S.A.S, Val de Reuil, France) were administered for three days to three experimental groups. The fourth group was used as a control (CTRL). In the treated groups, Al salt administration occurred in conjunction with the administration of algae.

After three days, five specimens per tank were measured. Mean length and weight for each group are given in Table 1. Subsequently, these specimens were sedated with magnesium chloride (Ross and Ross, 2009), fixed in formalin, and cut along the foot-umbo axis (Howard, 2004). Samples of gills and foot were dehydrated and processed for paraffin inclusion (e.g., Mastrodonato et al., 2017). Sections were serially cut at 5 μm and mounted on slides for processing with conventional and lectin histochemistry.

2.2. Histochemical staining and lectin binding

For conventional histochemistry, sections were rehydrated and stained with Periodic-Acid Schiff (PAS), Alcian Blue pH 2.5 (AB), and high iron diamine (HID) (e.g., Petraccioli et al., 2013). Counterstain with Carazzi's hematoxylin was also performed. Stains allowed to identify the presence of glycoconjugates (PAS), acidic carboxylated and/or sulphated glycans (AB), and sulphated carbohydrates (HID), respectively.

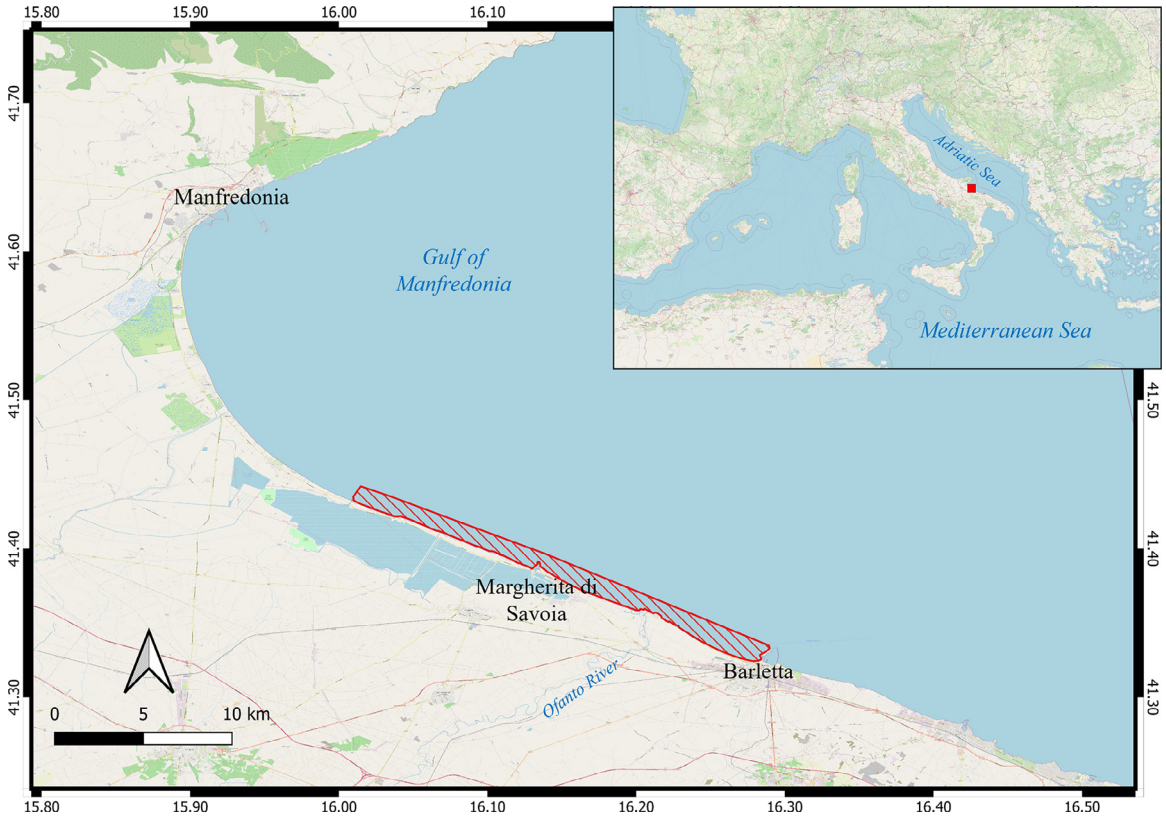

Fig. 1. Fishing ground exploited with hydraulic dredges in the Co.Ge.Vo. (Clam Management Consortium) of Barletta. The sampling area is located between the town area of Margherita di Savoia and the mouth of the Ofanto river.

Table 1
Mean length and weight with relative standard deviations of the experimental groups of striped venus clams.

Treatment	Mean length (mm) ± S.D.	Mean weight (g) ± S.D.
CTRL	26.6 ± 3.2	5.74 ± 1.74
25 μM	27.0 ± 1.9	5.89 ± 0.90
50 μM	24.2 ± 1.3	4.44 ± 0.59
200 μM	27.2 ± 0.8	5.94 ± 0.33
Tot.	26.2 ± 2.2	5.53 ± 1.1

Nine lectins conjugated with fluorescein isocyanate (FITC) were tested to identify glycosidic chains of the mucins. Details of lectins used, their concentrations and specificity of binding to a given sugar are shown in Table 2. Sections were deparaffinized and incubated for one hour at room temperature with FITC conjugated lectins in appropriate buffer: PNA, WGA, SBA, UEA-I, AAA, in 4-2-hydroxyethyl-1-piperazineethane-sulfuric acid (HEPES) and ConA, SNA, MAA-II and LTA in 0.1M phosphate-buffered saline (PBS). Further details about lectin protocols are in Scillitani et al. (2011). Sections were then washed in the same buffer and mounted in Fluoromount aqueous mounting medium. For PNA and SBA, binding experiments were performed also after desulphation as in Scillitani and Mentino (2015).

All chemicals were from Sigma-Aldrich Inc (St. Louis, MO, USA); lectins were from Vector Laboratories (Burlingame, CA, USA) except for AAA and MAA-II from US biological (Salem, MA, USA) and their effectiveness was tested on tissues of molluscs known to bind to the cited lectins as previously demonstrated (Petraccioli et al., 2013; Accogli et al., 2017). Negative-binding control experiments were performed with the buffer alone or in presence of the corresponding inhibiting sugar (Table 2).

For a quantitative analysis of stain intensity, five bright-field images were captured for each stain, sample and experimental condition using a Nikon Eclipse E600 microscope equipped with DA-Fi3 microscope-camera (Nikon Instruments SpA, Campi Bisenzio, FI, Italy). Ten to twenty cells per image were selected and their intensity of staining was estimated by computing Optical Density (OD) values after processing for colour deconvolution (Ruifrok and Johnston, 2001) to separate colour channels for the stain, the counterstain, and the background as in Mastrodonato et al. (2020). Similarly, for lectin-binding five images per stain, sample and condition were taken with the same microscope equipped with an epifluorescence device under 495 nm light emission. Ten to twenty cells per image were selected and their intensity of staining was estimated by computing the corrected total cell fluorescence, CTFC on greyscale images (McCloy et al., 2014). Image analysis was performed with the ImageJ package (Rasband, 1990) implemented with colour deconvolution plugin (Landini et al., 2021).

2.3. Statistical analyses

Mean OD or CTFC values from each staining, cell type and condition were compared by one-way analysis of variance (ANOVA) followed by resampling through a 10.000-iteration bootstrap of the F-stat values (e.g., Carlucci et al., 2022). Differences between means by post-hoc pairwise comparisons were tested by Tukey's Honestly-Significant-Difference (THSD) (Accogli et al., 2017). Statistical significance was set at $p < 0.01$. Statistical computations were generated by the Real Statistics Resource Pack plugin for Excel (Release 4.3) (Zaiontz, 2018).

Table 2
FITC-Lectins used with related binding specificity, dilution, and inhibiting sugars used in negative controls experiments.

Lectin	*Source*	*Binding sites*	Dilution (μg/mL)	Inhibiting sugar
PNA	*Arachis hypogaea*	Galβ1,3GalNAc	10	0.2 M Gal
SBA	*Glycine max*	GalNAc	20	0.2 M GalNAc
WGA	*Triticum vulgaris*	(GlcNAcβ1,4)n	20	0.01 M TACT
LTA	*Tetragonolobus purpureus*	L-Fucα1,6GlcNAc; L-Fucα1,2Galβ1,4[LFuc1,3]GlcNAcβ1,6R	20	0.2 M L-Fuc
UEA-I	*Ulex europaeus*	Fucα1,2	10	0.2 M L-Fuc
AAA	*Aleuria aurantia*	Fucα1,6GlcNAc-βNAsn; Fucα1,3,Fucα1,4	10	0.2 M L-Fuc
SNA	*Sambucus nigra*	Neu5Acα2,6Gal/GalNAc	20	0.2 M Neu5Ac
MAA-II	*Maackia amurensis*	Neu5Acα2,3Galβ1,4GlcNAc	20	0.02 M Neu5Ac
ConA	*Canavalia ensiformis*	D-Man, D-Glc	20	0.1 M MαM

3. Results

3.1. Housing and treatments

No specimens in CTRL group died during the three days of experimentation. A percentage mortality of 35% (7/20) was recorded in specimens treated with 25 μM concentration and 70% (14/20) in both 50 μM and 200 μM $AlCl_3$ treated specimens, respectively. During the last day of treatment, a cloud of mucus enveloped one specimen in the 50 μM group and three specimens treated at 200 μM. A total of 11 males and 9 females was analysed.

3.2. Histochemical staining and lectin binding.

The foot of clams presented two kinds of mucous secreting cells, interspersed in the surface epithelium and clustered in pluricellular glands in the subepidermal area, respectively, as shown in Fig. 2.a–b.

Histochemical stains of the foot and plots of OD are in Fig. 3. PAS positivity was seen only in the subepidermal glands (Fig. 3.a). A progressive reduction of stain intensity was observed from the CTRL groups to the treatments (Fig. 3.b–d). Alcianophily as indicated by AB staining was observed in both the subepidermal glands group, as well as in the glycocalyx of surface cells in the CTRL group (Fig. 3.e). with moderate decrease in the treated with 25 μM and 50 μM (Fig. 3.f–g), becoming massive with those at 200 μM (Fig. 3.h). Similarly, HID staining indicating the presence of sulphated glycans was observed in both the subepidermal glands and the surface cells and their glycocalyx (Fig. 3.i). As observed for the other stains, its intensity decreased from the CTRL to the treatments in which its intensity appeared constant among different concentrations (Fig. 3j–l).

The results of lectin-histochemistry experiments on the foot are resumed in figures and plots of CTFC intensity in Fig. 4. PNA, SBA, LTA, UEA-I, SNA, and MAA-II never bound. PNA and SBA did not bind even after desulfation. Only WGA, AAA, and ConA linked to the tissues. WGA-binding was negative in the subepidermal glands, and strongly positive in the surface cells and the glycocalyx of CTRL (Fig. 4.a), resulting negative in treated specimens (Fig. 4.b–d). In the CTRL, AAA was negative in the subepidermal glands, and weakly positive in the surface cells and the glycocalyx (Fig. 4.e). In the treatments, binding intensity of glycocalyx did not change whereas the intensity of subepidermal cells decreased at 200 μM (Fig. 4.f–h). ConA was positive in both the subepidermal glands and the glycocalyx of CTRL and reduced in treatments (Fig. 4.i–l).

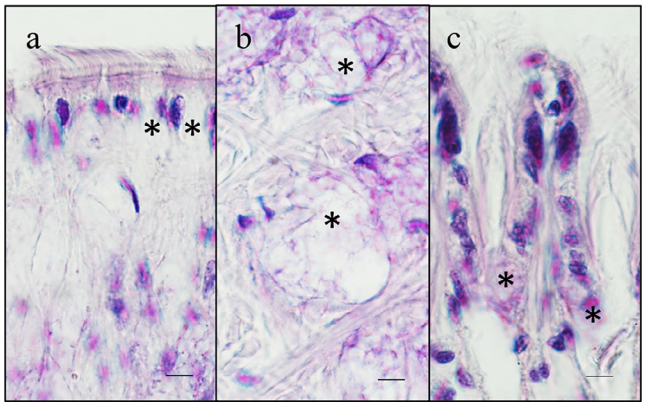

Fig. 2. (a) Mucus secreting cells interspersed in the surface epithelium and (b) pluricellular glands in the subepidermal area. (c) Detail of a gill showing the pair of mucus secreting cells located at the base of the branchial filament. Hematoxylin and eosin stain; 5 μm bar scale.

The gills present branchial filaments with some apical ciliated cells and a pair of mucous cells per side, a lateral one next to the ciliated cells and an abfrontal one located at the base of the filament (Fig. 2.c). Abfrontal cells were observed more frequently than lateral ones, so the intensity of stain or lectin binding was evaluated only for the former. Histochemical stains and OD plots are in Fig. 5. The mucous cells of the gills resulted always PAS-negative, and few were AB-positive only in CTRL, and weakly positive or negative in the treatments (Fig. 5.a–d). They resulted strongly positive to HID in CTRL, and weakly positive or negative in the various experimental conditions (Fig. 5.e–h). Besides, the gills of two specimens treated at 200 μM showed morphological alterations of branchial filaments, with a total degeneration of the gill tissues (Fig. 6). Lectin-binding to mucous cells was observed only for WGA and AAA (Fig. 7).

CTFC intensity of FITC-WGA-binding reduced already significantly in the treatments at 25 μM (Fig. 7.a–d), whereas in AAA decreased massively only in the treatments at 200 μM (Fig. 7.h).

3.3. Statistical analyses

Results of statistical analyses are reported for OD values in Table 3 and CTFC values in Table 4 and are resumed graphically in Figs. 3.m–o, 4.m–o, 5.i,j, and 7.i,j. The ANOVA analyses always resulted in significant differences. THSD post-hoc tests

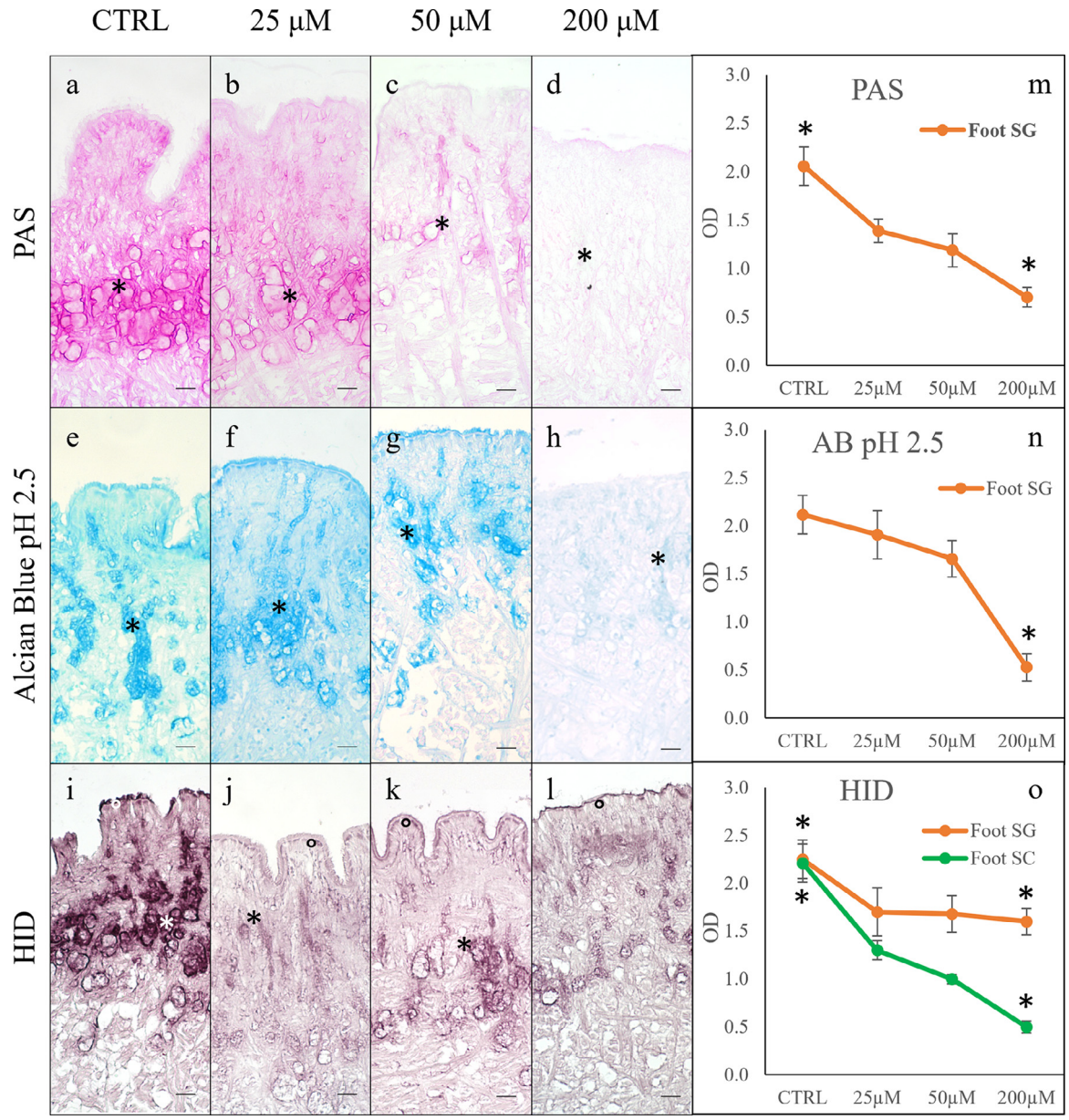

Fig. 3. (a) PAS is positive in the subithelial glands in the CTRL foot; (b–d) PAS positivity is progressively reduced in the treated with increasing concentrations; (e–h) Alcian Blue pH 2.5 shows like PAS; (i) strongly positive HID in the subepithelial glands of CTRL specimens; (j–l) HID positivity is maintained even in the treated under different experimental conditions, but with a lower intensity than CTRL. 20 µm scale bar. (m–o) OD values observed in the three experimental conditions. SG: subepidermal glands; SC: surface cells. Asterisks (*) indicate values significantly diverging from the others ($p < 0.01$) in THSD post-hoc tests.

(not shown) indicated that in most cases mean values of CTRL were significantly higher than those of the treatments. In the same tests, values of the treatments at 200 µM often resulted significantly lower than the others.

4. Discussion

The decline in abundance of *C. gallina* stocks in the Adriatic Sea requires new information for the implementation of management regulations because multiple stressors negatively affect the population dynamics of this resource (Grazioli et al., 2022). Besides fishing activity, other cumulative impacts could act in synergistic manner (Furlan et al., 2019). For example, the reduction in the extent of habitats (sandy and muddy-sandy bottoms) for the venus clam has already a negative impact on its survival, but further risks may arise from this condition of habitat degradation. Indeed, the confinement of the resource in areas that are progressively shallower (2–5 m) and closer to the coastline (Carlucci et al., 2015) could increase the exposure of organisms to the risk of contamination by chemical pollutants, which are mainly diffused in shallower waters by rivers and anthropogenic discharges along the coastline (Spagnoli et al., 2021). In this framework, these first data on the negative effect by Aluminium intake on the *C. gallina*, should be considered in the fishery management plans based on the EBM approach, addressing an effort in studies on the occurrence and diffusion of pollutants in exploited fishing grounds.

Being filtering and sedentary organisms, clams can provide information about the conditions of the surrounding environment. In fact, *C. gallina* is generally recognized as a low tolerant bivalve species. Evaluations conducted on the haemocytes present in the haemolymph of the clam showed a susceptibility to anoxic phenomena (Matozzo et al., 2005; Monari et al., 2005), as well as stress induced by high salinity (Matozzo et al., 2007; Monari

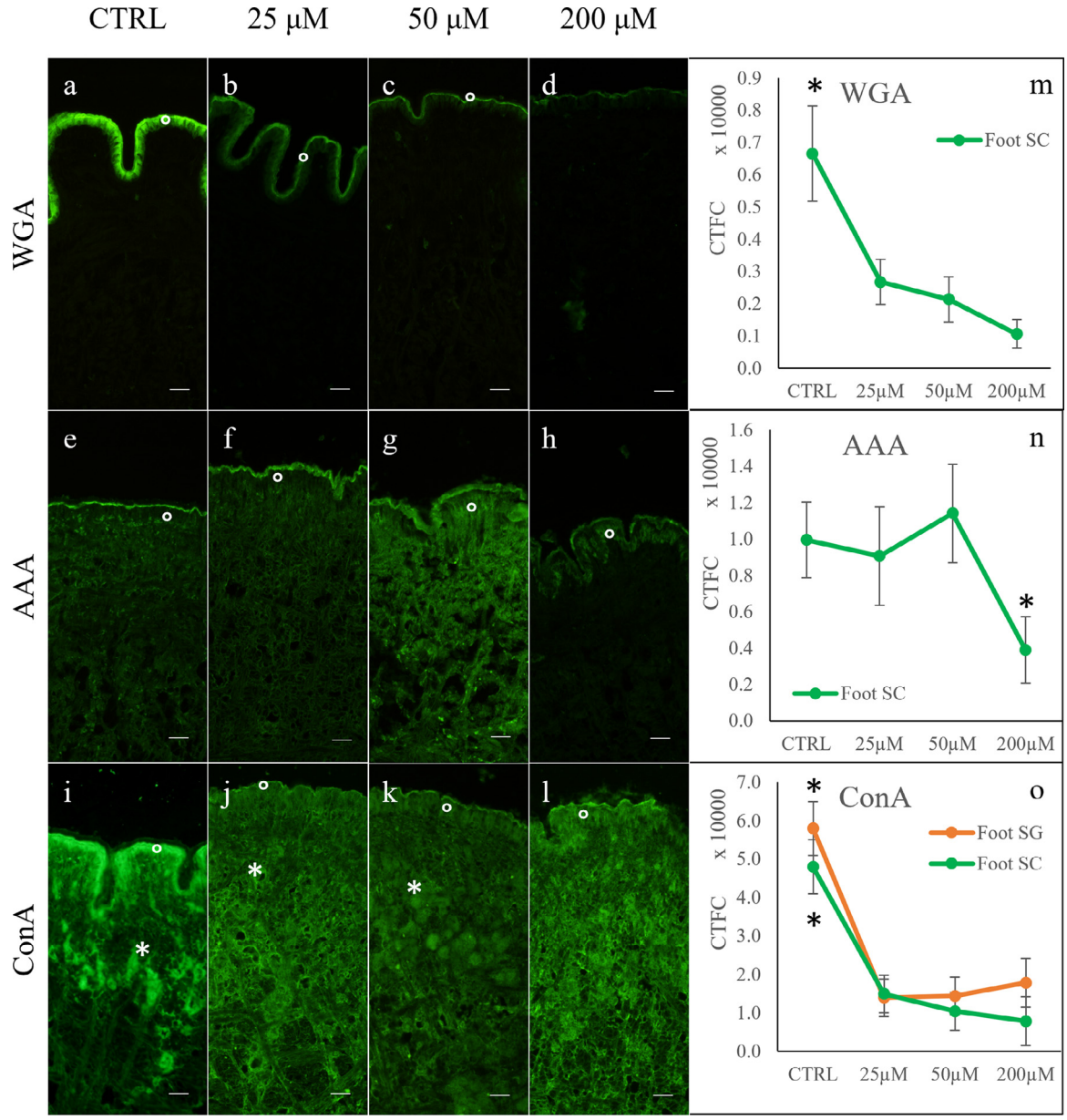

Fig. 4. (a) WGA is strongly positive in the superficial cells of the foot of the CTRL while in the treated (b–d) the binding with this lectin is lower; (e–h) The binding of AAA lectin is weakly positive in the foot glycocalyx and in the foot superficial cells, both in the CTRL and in the treated ones; (i–l) ConA shows a binding affinity with the subepithelial glands of the foot and with the superficial cells. The intensity is high in the CTRLs group, while it tends to decrease in the treaties. 20 µm scale bar. (m–o) Binding intensity of WGA, AAA, and ConA lectins as indicated by mean CTFC values. SG: subepidermal glands; SC: surface cells. Asterisks (*) indicate values significantly diverging from the others ($p < 0.01$) in THSD post-hoc tests.

et al., 2007b) and at high temperatures, above 25 °C (Monari et al., 2007a). Although spectrometric analyses have been carried out on metalloids present in *C. gallina* for human consumption (Gedik and Ozturk, 2019), ecotoxicological studies that highlight the *in situ* effects of pollutants on tissues are rare, such as the study on the effects of flame retardants on the gonads of the striped venus clam (Angioni et al., 2013). Being the mucus a barrier that modulates the exchanges across the integument, as well as the interaction with the microbiota and the entrapping and transport of food particles and pseudofeces, it would be expected that alterations of mucins induced by environmental stress, such as pollutants, would have consequences on the cited functions. In the present work, Al treatments resulted in quali-quantitative modification of mucins secreted by the foot and the gill filaments.

The mucous cells in the foot of *C. gallina* presented the typical arrangement of Veneridae (Eble, 2001; Calabrò et al., 2005). Besides, stain reactivity was observed also in the glycocalyx of the epidermal cells, but its intensity was not evaluated quantitatively because it was difficult in the image analysis to separate it from the surrounding stained structures.

PAS-positivity indicates the generic presence of glycoconjugates, in particular those with carbohydrates having 1,2-glycols. Alcianophily and intense HID staining indicate the presence of acidic glycoproteins and GAGs rich in sulphates, respectively. Acidic mucins are commonly secreted by bivalves (e.g., Davies and Hawkins, 1998; Eble, 2001; Calabrò et al., 2005) and contribute to mucus mechanical properties, such as protection, viscosity, and interactions with the microbiota (e.g., Denny, 1983).

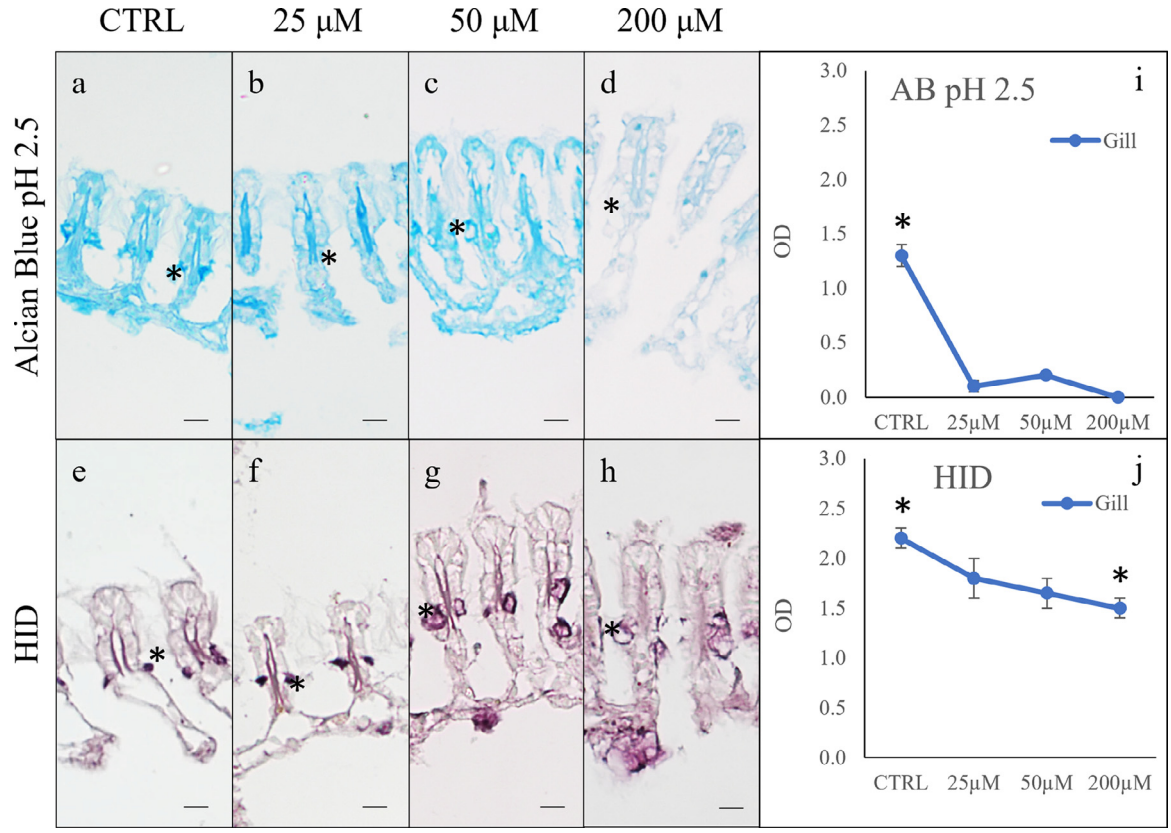

Fig. 5. (a–d) Alcian Blue pH 2.5 is positive in the CTRLs and weakly positive or negative in the treated products; (e–h) HID is strongly positive in the controls and in the treated at $AlCl_3$ concentrations of 25 μM, and is reduced in the treated at higher concentrations. 10 μm scale bar. (i–j) Stain intensity of AB pH 2.5 and HID as indicated by mean OD values. Asterisks (*) indicate values significantly diverging from the others ($p < 0.01$) in THSD post-hoc tests.

Table 3
Histochemical stains of gills and foot: mean values of OD (standard deviations in brackets), results of F-statistics (with degrees of freedom between and within groups) and estimated probability. Abbreviations: bg, between groups; d.f., degrees of freedom; F, value of F-statistics; OD, optical density; P, probability; wg, within groups.

Stain	Organ	Mean OD (±S.D.)				F (d.f. bg, wg)	P
		Ctrl	25 μM	50 μM	200 μM		
PAS	Foot SC	–	–	–	–	–	–
	Foot SG	2.058 (±0.202)	1.390 (±0.120)	1.188 (±0.170)	0.704 (±0.100)	20 388.420 (3, 6472)	0.000
	Gills	–	–	–	–	–	–
AB pH 2.5	Foot SC	–	–	–	–	–	–
	Foot SG	2.117 (±0.221)	1.908 (±0.253)	1.658 (±0.191)	0.528 (0±.143)	18 705.317 (3, 5841)	0.000
	Gills	1.300 (±0.112)	0.100 (±0.051)	0.200 (±0.023)	–	125 466.034 (2, 4436)	0.000
HID	Foot SC	2.211 (±0.231)	1.305 (±0.143)	1.134 (±0.051)	0.531 (±0.063)	25 875.338 (3, 5977)	0.000
	Foot SG	2.250 (±0.202)	1.710 (±0.250	1.680 (±0.190)	1.600 (±0.140)	3257.9725 (3, 6174)	0.000
	Gills	2.208 (±0.121)	1.807 (±0.242)	1.650 (±0.153)	1.500 (±0.114)	4848.539 (3, 5977)	0.000

In the treatments, a general decrease of intensity of the three stains was observed, in relation to increase of $AlCl_3$ concentration, as a possible consequence of an augmented secreting activity to expel aluminium. Anyway, the patterns of decreasing secretion differ among staining and cell types. In particular, in the surface cells sulphation decreases progressively, whereas in the subepidermal glands it decreases from CTRL to 25 μM Al but higher concentrations seem not to have further effects.

A lower diversity of saccharidic residuals evidenced by lectin-binding was observed in respect to other species (e.g., Calabrò et al., 2005). Three lectins only bound, indicating the presence of mannosylated and/or glycosylated residuals (ConA), as well as of glycosaminylated and/or sialylated (WGA) and fucosylated (AAA) residuals.

WGA lectin binds to glycosaminylated and/or sialylated residuals. The presence of sialic acid in molluscs is questioned (e.g., Bolognani Fantin et al., 1969; Menghi et al., 1991; Robledo et al., 1997; Calabrò et al., 2005). Anyway, in *C. gallina* the subepidermal cells are not WGA-positive whereas they are strongly alcianophylic, suggesting that in these cells sialic acid is lacking. On the contrary, in the surface cells co-distribution of WGA-binding and alcianophylic residuals in CTRL and their parallel decrease in treatments could indicate the presence of sialic acid, even if

Table 4
Lectin-binding of gills and foot: mean values of CTFC (standard deviations in brackets), results of F-statistics (with degrees of freedom between and within groups) and estimated probability. Abbreviations: bg, between groups; CTFC, corrected total cell fluorescence; d.f., degrees of freedom; F, value of F-statistics; P, probability; wg, within groups.

Lectin	Organ	Mean CTFC (±S.D.)				F (d.f. bg, wg)	P
		Ctrl	25 μM	50 μM	200 μM		
AAA	Foot SC	9955.927 (±2073.488)	9067.853 (±2709.533)	11 427.123 (±3283.242)	3882.722 (±1830.111)	2523.354 (3, 5842)	0.000
	Foot SG	–	–	–	–	–	–
	Gills	28 794.411 (±4936.824)	27 906.692 (±4300.981)	30 730.219 (±5642.149)	10 987.101 (±2925.254)	6212.027 (3, 6023)	0.000
WGA	Foot SC	6659.949 (±1480.219)	2679.523 (±696.224)	2139.272 (±597.786)	1063.559 (±440.899)	11 723.293 (3, 6015)	0.000
	Foot SG	–	–	–	–	–	–
	Gills	16 151.239 (±1349.855)	5067.273 (±1212.781)	2789.292 (±603.678)	1965.432 (±831.258)	64 927.786 (3, 6368)	0.000
ConA	Foot SC	47 972.88 (±6989.197)	14 941.077 (±4794.219)	10 415.86 (±5120.114)	7845.2644 (±6814.184)	16 425.481 (3, 7023)	0.000
	Foot SG	57 972.877 (±7023.724)	13 941.077 (±4912.335)	14 415.860 (±4798.213)	17 845.264 (±6357.222)	22 648.707 (3, 5987)	0.000
	Gills	–	–	–	–	–	–

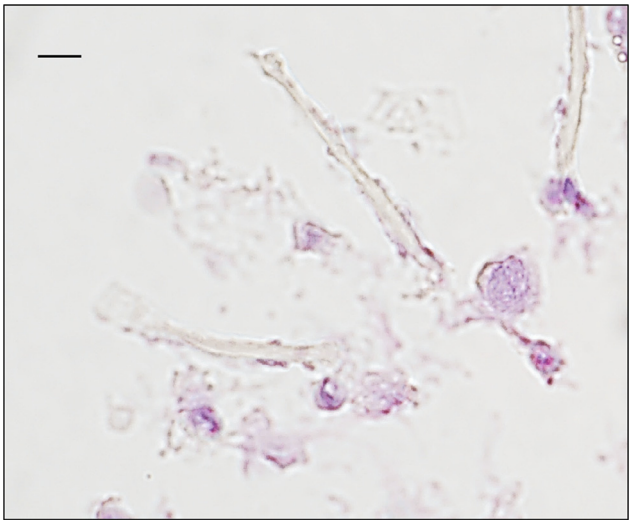

Fig. 6. Detail of branchial filament of specimen treated at 200 μM $AlCl_3$ concentrations. A total degeneration of the gill epithelium is evident. 5 μm scale bar.

other acidic carbohydrates, such as N-acetylmuramic acid (Bolognani Fantin and Ottaviani, 1990), could be responsible of WGA-positivity as well (Jarroll et al., 1989). Glucosaminylated residuals are common in O-linked saccharidic chains and can provide attaching sites for sulfates or muramic-N-acetylgalactosamine dimers (Menghi et al., 1991; Esko et al., 2009).

AAA-lectin detects fucosylated residuals that are seen only in the surface cells and their glycocalyx. They could be involved in mucus viscosity, possibly affecting the adhesion of the foot to the substrate as well as modulate interactions with the microbiota, inhibiting the attack of pathogens on one side and favouring the settlement of symbionts on another (Nizet and Esko, 2009).

ConA positivity indicated mannosylated and/or glycosylated residuals in the saccharidic chains of both subepidermal and surface mucous cells, as well as in the glycocalyx of the epidermal cells. After a massive decrease from CTRL to 20 μM Al the values did not change significantly. A possible explanation is that the mannosylated residuals in the treatments are in glycans that are not secreted, but have structural and regulative functions, such as those in the N-linked glycans, that present a mannosylated core (Esmail and Manolson, 2021). Thus, their amount could be not affected by the increase of secretion.

The muciparous cells on branchial filaments of *C. gallina* are arranged similar to what has been reported for other bivalves, with the abfrontal cells outnumbering the lateral ones (e.g., Foster-Smith, 1975; Davies and Hawkins, 1998). Histochemical results indicated in the secretion from the abfrontal cells the presence of sulfated GAGs, and PAS-negativity indicated the absence of neutral mucins. Acidic GAGs, both carboxylated or sulfated, are reported for several bivalves (Ahn et al., 1988; Beninger and St-Jean, 1997; Davies and Hawkins, 1998; Eble, 2001), although co-present with neutral mucins, absent in *C. gallina*. Lectin-histochemistry indicated that in the mucins there are glycosaminylated (WGA-binding) and fucosylated residuals (AAA-binding). Ahn et al. (1988) in the mucous cells of the gills of the mussel *Mytilus edulis* suggested the presence of sialic acid that cannot be excluded in *C. gallina*, even if WGA-binding could be due also to N-acetylmuramic acid, as previously discussed. As observed for the foot, sulfated and fucosylated residuals are important for the viscosity of the mucus and thus for the entrapping of food particles or pseudofeces. $AlCl_3$ treatments resulted in a reduction of CTFC intensity, even if with differing patterns in WGA and AAA.

A general trend of progressive reduction in staining/binding was observed in the $AlCl_3$ treatments.

In the freshwater bivalve *Anodonta cygnea* experimentally exposed to Al^{3+} Kádár et al. (2001) observed a reduction in the mean duration of the shell opening, accumulation in the kidney, the digestive gland and the gills and an increase of Al in the pseudofeces. Kádár et al. (2001) hypothesize that mucus is important in trapping Al since glycoproteins are known to bind to it (e.g., Powell and Thompson, 1993). Thus, the reduction of mucus in *C. gallina* could be due to increased secretion in the attempt to expel the excess of Al. At higher concentration in some animals gill functionality was compromised by epithelial exfoliation with consequent loss of cilia. This alteration was already observed in specimens of *Venerupis pullastra* and *Mytilus galloprovincialis* exposed to organic and inorganic contaminants (Carballeira et al., 2011; Maisano et al., 2017). The patterns of reduction of sulfates, fucosylated and glycosaminylated residuals anyway were somewhat dissimilar from the general trend of mucus reduction and among them, suggesting that Al treatments affected the composition of the saccharidic chains in glycoproteins and GAGs and thus the mechanical and immunological properties of mucus. As

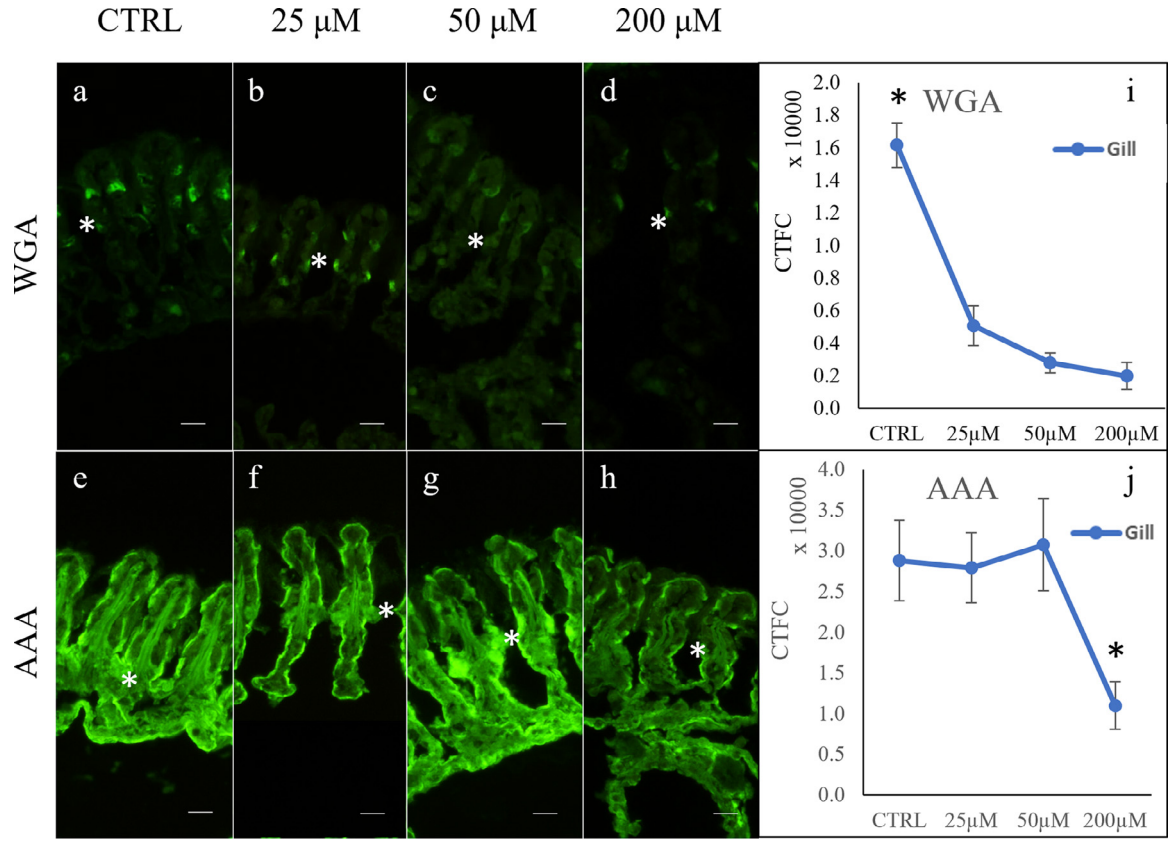

Fig. 7. (a–d) The mucus present in the secreting cells of the branchial filaments is positive for WGA. However, positivity is significantly reduced between the CTRL and the different experimental conditions. (e–g) The secreting cells placed on the branchial filaments have a secretion that binds strongly with the AAA. The bond intensity is appreciable both in the CTRL and in the treated at 25 and 50 Mm; (h) the intensity, on the other hand, is reduced in the treated to 200 µM; 10 µm bar scale. (i–j) Binding intensity of WGA, and AAA lectins as indicated by mean CTFC values. Asterisks (*) indicate values significantly diverging from the others ($p < 0.01$) in THSD post-hoc tests.

an example, reduction of sulfated and fucosylated residuals could result in loss of viscosity and thus of the ability to bound and entrap particles, whereas alteration of glycosaminylated residuals could result in alteration of their protective capabilities against pathogens.

In the Adriatic Sea, several human cumulative impacts are distributed along the western and eastern coasts, such as fishery, naval traffic, tourism, urban development, industrial discharges, and oil and gas extraction (Coll et al., 2010; Farella et al., 2020). Several of these activities contribute to the sediment contamination with metals and, in the Adriatic Italian region, a number of rivers input nutrients and other chemical contaminants (Spagnoli et al., 2021).

Therefore, considering the importance of the striped venus clam plays in ecosystems, reducing the load of nutrients, creating, and modifying habitats (Vaughn and Hoellein, 2018), and in the national fishery, they are excellent indicators both for environmental monitoring (Zuykov et al., 2013) and for human health (Robledo et al., 2019). Assays of mucins such as the one presented here can be a good biomarker.

5. Conclusions

Preliminarily, we can conclude that high dosages of $AlCl_3$ affect the quantitative expression of glycans in the mucous secretion, reducing its acidity and increasing the viscosity of the mucus itself, with effects on the health of the animal. Similar to what observed in gastropod molluscs (McDermott et al., 2021), it would be interesting to characterize the gene sequences of secretory mucins, to understand their structure, functions, and responses to certain pollutants in search of stress indicators.

CRediT authorship contribution statement

Marco Vito Guglielmi: Conceptualization, Formal analysis, Investigation, Writing – original draft, Writing – review & editing. **Daniela Semeraro:** Formal analysis, Investigation. **Pasquale Ricci:** Investigation, Writing – original draft. **Maria Mastrodonato:** Conceptualization, Formal analysis, Writing – review & editing. **Donatella Mentino:** Formal analysis, Investigation. **Roberto Carlucci:** Project administration, Writing – review & editing. **Francesco Mastrototaro:** Conceptualization, Writing – review & editing. **Giovanni Scillitani:** Conceptualization, Writing – original draft.

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.

Data availability

Data will be made available on request.

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