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RESEARCH REPORT

Ecotoxicity of hallachrome, an unusual 1-2 anthraquinone excreted by the infaunal polychaete *Halla parthenopeia*: evidence for a chemical defence?

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Abstract

Polychaetes play a prominent role in marine systems, but little is known about their secondary metabolites compared with other benthic taxa. In the present study, we investigated the toxicity of hallachrome, an unusual 1-2 anthraquinone identified from the skin of some polychaetes, including the Mediterranean infaunal species *Halla parthenopeia*. Under stress conditions, this worm releases a harmful purple mucus, whose noxious compounds were still unknown. We hypothesized that hallachrome also occurs in the purple mucus, giving rise to its color and toxicity. Soon after the production of the purple exudate, *H. parthenopeia* also secretes a harmless, transparent mucus, which pushes away the toxic one, suggesting protective functions for the worm itself. LC-MS and ^{1}H-NMR analyses confirmed the presence of the pigment hallachrome in the purple mucus. The average concentration of the pigment in the purple mucus was about 310 mg L^{-1}. Ecotoxicological bioassays on representative species of bacteria, protozoans, rotifers, crustaceans (*Artemia franciscana*) and polychaetes (*Dinophilus gyrociliatus*) revealed its severe toxic effects: LC_{50}/EC_{50} values ranged from 0.11-5.67 mg L^{-1}. Hallachrome showed higher toxicity for *A. franciscana* than other naturally occurring anthraquinones. Tests on encapsulated embryos of *D. gyrociliatus* evidenced the ability of a mucus layer to limit hallachrome diffusion, confirming the protective role of the transparent mucus. Given the information available on polychaetes anti-predator strategies, hallachrome cannot be considered a consumer deterrent. However its toxicity and wide range of activity suggest chemical defensive functions against potential competitors, parasites and/or pathogens.

Key Words: Anthraquinones; *Halla parthenopeia*; marine invertebrates; soft-bottoms; ecotoxicology

Introduction

Polychaetes comprise more than 15,000 described species of marine annelids and represent the major group of invertebrates in soft bottom communities. They include the Errantia (species with greater mobility) and most of the Sedentaria (species with less mobile lifestyles, and echiurids, annelids that lost segmentation) (WORMS, 2019). Given the wide range of body plans and life style and their prominent role in trophic interactions, only very few polychaetes are known to be toxic or venomous (Struck, 2017; Coutinho *et al.*, 2018). Most of them belong to the families Glyceridae and Amphinomidae, commonly known as “bloodworms” and “fireworms”, respectively (von Reumont *et al.*, 2014a; Struck, 2017). Bloodworms (*Glycera* spp.) have an eversible pharynx tipped with four hollow fangs which inject a complex proteinaceous venom into their invertebrate prey (von Reumont *et al.*, 2014b). Fireworms have tufts of white, sharp, harpoon-shaped chaetae to deter potential predators. In humans, stings of large-sized fireworms cause immediate painful burning sensation on contact, followed by the development of erythema, edema and paresthesia (Smith, 2002; Ottuso, 2013). It is still unknown whether the cause of these responses is mainly mechanical or chemical, but inflammation-inducing secondary metabolites (complanines) were isolated from the fireworm *Eurythoe complanata* (Nakamura *et al.*, 2008; von Reumont *et al.*, 2014b).

Besides, three other polychaete species secrete toxic substances: the echiurid *Bonellia viridis* (de Nicola Giudici, 1984), the lumbrinerid *Lumbrineris (Lumbriconereis) heteropoda* (Hashimoto and Okaichi, 1960), and the oenonid *Halla parthenopeia*. *B. viridis* produces copious green mucus containing a pigment named “bonellin”, a unique type of alkylated chlorin. Bonellin is highly toxic at a very low concentration

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for both eukaryotes and prokaryotes and protects *B. viridis* against benthic and microbic organisms widespread in the interstices where it lives (de Nicola Giudici, 1984). Nereistoxin (4-dimethylamino-1,2-dithiolane) is a strong neurotoxin isolated from *L. heteropoda* which causes neuromuscular blockade. It has been shown to have insecticidal activity and three chemically related synthetic insecticides have been developed and used commercially (e.g. cartap and bensultap; Coutinho *et al.*, 2018).

The genus *Halla* (Errantia: Oenonidae) includes large infaunal burrowing polychaetes (up to 1 m long), with documented reports from the Mediterranean, Red Sea and Japan Sea. The Mediterranean specimens of *H. parthenopeia* live in soft bottom habitats at 10-50 m depth and is highly appreciated as fishing bait in France, Spain, Italy and Egypt (see Iori *et al.*, 2014). *H. parthenopeia* feeds mainly on bivalve molluscs and, when undisturbed, produces large amounts of a colourless mucus which stabilizes and keeps open its tubes in the sediment, facilitating also locomotion (Osman *et al.*, 2010; Iori *et al*., 2014). When disturbed, *H. parthenopeia* emits a purple mucus with severe toxic effects on representative species of bacteria, polychaetes, rotifers and brine shrimps (Iori *et al.*, 2014). To date, information on the biology of this species derives only from laboratory studies and none is available on the origin of the purple mucus toxicity. Just after the emission of the purple mucus, *H. parthenopeia* quickly releases large amounts of a transparent mucus that pushes away from its body the purple one (Iori e*t al.*, 2014). Given that mucus secreted by marine invertebrate could display different protective properties (Stabili *et al.*, 2009), the transparent mucus of *H. parthenopei*a could have defensive function against its own harmful purple exudate (Iori *at al.*, 2014).

In this paper we hypothesized that the purple mucus of *H. parthenopeia* contains a red pigment called hallachrome (7-hydroxy-8-methoxy-6-methyl-1,2-anthraquinone, $C_{16}H_{12}O_4$, molecular mass = 268.26; Prota *et al.*, 1971) as responsible for its colour and toxicity. Hallachrome was identified from the skin of *H. parthenopeia* (Prota *et al.*, 1971) and was synthesized by Cameron and collegues (1999), although its biological activity remains unknown. To isolate the purple mucus pigment, samples of exudate were submitted to extraction and purification, whilst the toxicity was assessed through six ecotoxicological assays. Four of these had been previously used to test purple mucus effects (Iori *at al.*, 2014): Microtox® (with the bacterium *Vibrio fischeri*), Rotoxkit® (with the rotifer *Brachionus plicatilis*), Artoxkit® (with the crustacean *Artemia franciscana*) and acute toxicity bioassays with juveniles of the polychaete *Dinophilus gyrociliatus*. Bioassays on *A. franciscana* are commonly used because oral acute toxicity and cytotoxicity of natural products are correlated to brine shrimp lethality (Parra *et al.*, 2001). *D. gyrociliatus* well represents small sized invertebrates and larvae/juveniles that may come into contact with the toxic purple mucus (Simonini *et al.*, 2011). In addition, the Toxi-ChromoTest™ (with the bacterium *Escherichia coli*) and an acute test with the ciliate *Euplotes crassus* were performed to extend the result obtained with the Microtox® to other microbes.

To investigate the transparent mucus function, we tested the ability of a mucous layer to limit the toxic effect of the purple mucus pigment exposing *D. gyrociliatus* embryos and juveniles to different concentrations of the pigment dissolved in seawater. Since this polychaete develops within a protective mucous capsule, the hatching rate (*i.e.*, the number of survived juveniles that emerge from capsules; Simonini and Prevedelli, 2003) was analyzed, expecting higher survivorship in protected embryos than in free-living juveniles.

Material and methods

Mucus collection

Detailed information on *H. parthenopeia* rearing procedures and mucus collection are reported by Iori and collaborators (2014). Briefly, the worms (25-40 cm in length) were collected by professional divers from sandy-muddy sea bottoms in the Ligurian Sea (North-western Mediterranean Sea). They were transported to the laboratory inside refrigerated containers (12-17 °C) and maintained in large, aerated plastic tanks filled with artificial seawater (Reef Crystal, Instant Ocean, 30-35 salinity) and 7 cm of sand. During the laboratory rearing, worms were periodically fed with live clams. They were removed from their tank the day before mucus collection and gently transferred in pairs into smaller plastic tanks (40×60×12 cm) filled with seawater. The transparent mucus produced during the night was removed. Then, worms were repeatedly stimulated (with plastic pliers or by transferring them to adjacent tanks) until they secreted the purple mucus, which was collected using plastic tweezers or pipettes and stored at 4 °C. The mucus was then centrifuged in 50 mL plastic vials at 4,000 rpm, and the clear supernatant water was discarded (Iori *et al.*, 2014).

Pigment extraction

Nearly pure hallachrome can be selectively extracted from *H. parthenopeia* by direct immersion of live specimens in chloroform (Prota *et al.*, 1971). Accordingly, to isolate purple mucus pigment, aliquots of centrifuged purple mucus (50-100 mL) were placed in a separating funnel containing 150-300 mL of chloroform (Carlo Erba reagents). The next morning, the resulting red chloroform extract was removed and the procedure was repeated three times until the solvent assumed a pale pink colour. The residual mucus was almost uncoloured with brownish debris: it was collected and placed for four hours in a rotary evaporator (Rotavapor Buchi R-200) in order to remove chloroform. A test to assess the potential toxicity of the residual mucus was performed with the same procedure adopted for the fresh transparent mucus (Iori at al., 2014). Juveniles of *D. gyrociliatus* were exposed for two weeks to experimental solutions with concentrations ranging between 10 and 500 $g \times L^{-1}$ (1 and 50%) of residual mucus. The residual mucus was not toxic. *D. gyrociliatus* fed on the residual mucus, survived,

grew up to sexual maturity and reproduced successfully.

Red chloroform extracts were pooled, filtered and concentrated using a rotary evaporator, obtaining a dry, purple pigment. This one was chromatographed on a 3x40 cm glass column filled with silica gel (0.06-0.20 mm, Carlo Erba reagents) using toluene/ethyl acetate (60:40, v/v) as mobile phase. On concentration, the red band gave purple crystals, which were insoluble in distilled water and seawater.

LC-MS/MS and FT-NMR analyses

Known aliquots of the purified pigment were dissolved in acetonitrile (HPLC grade, Carlo Erba reagents) and analysed by an Agilent 1200 series HPLC (Agilent Technologies) consisting of a vacuum degasser, an autosampler, and a binary pump equipped with a RP C18 analytical column (4.6×150 mm, 5 µm particle size, Agilent Zorbax Eclipse plus). Acidified water (0.1% formic acid v/v) and acidified acetonitrile (0.1% formic acid v/v) were used as mobile phases A and B, respectively. The mobile phase was programmed as follows: column flow, 0.300 mL min^{-1}; stop time, 22.00 min; post time, 8.00 min; timetable, 0 min, 3% B; 2min, 3% B; 12 min, 100% B; 20 min, 3% B; injection volume, 10.0 µL.

For LC-MS/MS analysis, the Agilent 1200 LC was coupled to an Agilent 6310A ion trap equipped with an electrospray interface in negative and positive ion mode. In negative mode, the ion trap scanned over the 50–800 *m/z* range at 13,000 $u \times s^{-1}$ during separation and detection. The maximum accumulation time for the ion trap was set at 10 ms, the target count was set at 8,000, and the compound stability was set at 100%. The optimum values of the ESI-MS parameters were as follows: capillary voltage, 3.5 kV; drying gas temperature, 320 °C; drying gas flow, 10.0 $L \times min^{-1}$; nebulising gas pressure, 32.0 psi. In positive mode, the ion trap scanned over the 50–800 *m/z* range at 13,000 $u \times s^{-1}$ during separation and detection. The maximum accumulation time for the ion trap was set at 6 ms, the target count was set at 60,000, and the compound stability was set at 100%. The optimum values of the ESI-MS parameters were as follows: capillary voltage, 3.5 kV; drying gas temperature, 320 °C; drying gas flow, 1.0 $L \times min^{-1}$; and nebulising gas pressure, 32.0 psi. Samples were further analyzed using Agilent Q-TOF Accurate Mass G6520A system. NMR spectra were also obtained at 200 MHz using a FT-NMR instrument. NMR spectra were recorded in DMSO-d_6 solutions and chemical shifts reported are in δ (ppm) scale relative to tetramethylsilane as external standard.

Ecotoxicological bioassays

Known aliquots of the purified pigment were dissolved in the carrier solvent dimethylsulfoxide (DMSO, Carlo Erba reagents) to obtain two concentrate solutions (10^4 and 10^3 $mg \times L^{-1}$). The experimental solutions employed in the bioassays were obtained diluting the two initial ones in artificial sea water (salinity 30-35), reaching different concentrations tested as treatments. In each test, at least five treatments were considered. The concentration of the solvent was adjusted and maintained the same in each treatment.

The Microtox® liquid phase test is an acute toxicity bioassay based on the reduction of the bioluminescence activity of *V. fischeri* after its exposure (15 min) to a toxic matrix. The test was carried out by ARPA Daphne (a reference centre for environmental control of coastal marine ecosystem) using the Microtox M500 analyser (Modern Water) according to ISO 11348-3:2007 protocol, with concentrations of pigment ranging from 0.1 to 10 $mg \times L^{-1}$. The concentration of DMSO was 1‰, far below the No Observed Effect Concentration (NOEC) (1% for Microtox, Pagnout *et al.*, 2006).

The Toxi-ChromoTest™ (EBPI, 2016) is a microplate toxicity bioassay based on the ability of toxicants dissolved in liquid matrices to inhibit bacterial growth and *de novo* synthesis of the inducible enzyme β-galactosidase. The test is performed on a highly permeable mutant of *E. coli*, which is highly sensitive to a wide spectrum of toxic substances (Reinhartz *et al.*, 1987). After the exposure (incubation period 90 min), a chromogenic substrate that measures β-galactosidase activity is added. If the sample is toxic, bacteria will not grow, thus no synthesis of β-galactosidase and colour development will occur; if the sample is non-toxic, bacteria will grow, synthesize the enzyme and a distinctive blue colour will appear quickly. The toxicity of the sample can be assessed by a simple visual qualitative evaluation of the colour obtained, or quantitatively by spectrophotometry analysis using a plate reader at a wavelength of 610 nm (the peak of absorption of the chromogenic substrate). In this study, Toxi-ChromoTest™ 96-well plates were set up according to producer guidelines (EBPI 2016) and the quantitative method was used to asses the toxicity of the pigment (concentration ranging from 250 to 0.03 $mg \times L^{-1}$ prepared by serial dilutions; microplate reader: Synergy HTX HTX Multi-Mode). Preliminary analysis evidenced that also the purple pigment absorbs at 610 nm. Consequently, appropriate blanks were prepared by diluting the pigment in the reaction mixture lacking bacterial suspension. The DMSO concentration was 2%, which was lower than the EC_5 (3.1%). The EC_{50} of $HgCl_2$ (positive control) was 0.20 $mg \times L^{-1}$ (95% confidence intervals [c.i. 95%]=0.18-0.22 $mg \times L^{-1}$).

E. crassus is a common inhabitant of healthy *D. gyrociliatus* cultures. The exposure to the purple mucus causes the slowdown of ciliary beat followed by cellular lysis and death (Iori *et al.*, 2014; Simonini, unpublished). A motility test was set up starting from the procedure proposed by Gomiero and collaborators (2013) for *E. crassus* mortality test. Five ciliates were picked up from the culture using a micropipette and placed onto a glass slide containing a depression filled with the experimental solution. For each treatment, ten slides-replicates were prepared and maintained in a wet chamber to avoid excessive evaporation. After two hours of exposure, ciliates were checked individually and were considered motionless if they did not show any ciliary beat during 5 s. Concentrations of pigment ranged from 0.06 to 2 $mg \times L^{-1}$. The NOEC for DMSO was 1‰ (1,000 $mg \times L^{-1}$), which was higher than the

concentration of the solvent used in the experiments (200 mg×L^{-1}).

The Rotoxkit M® bioassay uses newborns emerging from cysts of *B. plicatilis* that were exposed to concentrations of pigment ranging from 0.025 to 0.5 mg×L^{-1}. Rotoxkit M® was run for 24 h and was conducted according to the standard operating procedure (Microbiotests Inc) by ARPA Daphne. The concentration of DMSO (50 mg×L^{-1}) was considered non toxic for *B. plicatilis* (Marcial *et al.*, 2005).

The Artoxkit M® test uses instar II–III nauplii of *A. franciscana*. The concentrations of pigment tested ranged from 0.6 to 40 mg×L^{-1} (DMSO concentration = 5‰). At concentrations of 25 mg×L^{-1} or higher, the pigment precipitated in few hours after its addition to the seawater. The motility of *A. franciscana* was checked 24 h after the exposure to the experimental solutions, according to the Artoxkit M® standard operating procedure (Microbiotests Inc). The sensitivity of instars to DMSO and $K_2Cr_2O_7$ (PanreacQuimica, analytical grade) was also assessed. The NOEC for DMSO was 6.7‰ (6700 mg×L^{-1}), which was higher than the concentration of the solvent used in the experiments. The 24-h EC_{50} for $K_2Cr_2O_7$ was 35.0 mg L^{-1} (c.i. 95%=30.5-40.2 mg×L^{-1}), consistent with that certified by the supplier (MicroBioTests inc., 24-h EC_{50} = 30.9 ppm, c.i. 95%= 26.7-35.6 mg×L^{-1}).

D. gyrociliatus is a small annelid (max adult length 1 mm) with a short life cycle (5 days from zygote to hatching, 6 days from hatching to sexual maturity at 24 °C; Simonini and Prevedelli 2003) that can be easily cultured in the laboratory and is very sensitive to various toxic compounds (Reish and Gerlinger, 1997; Marcheselli *et al.*, 2010), including secondary metabolites (Simonini *et al.*, 2011). Adult females reproduce semicontinuously and lay transparent mucous capsules containing small eggs, that develop into dwarf males, and larger eggs, from which females originate. Only females give rise to free living populations and are used in bioassays. The capsule (wall thickness 20-50 µm) limits the stage of dispersion, permits matings among siblings and protects developing embryos. Indeed, under optimal conditions, hatching rates and juvenile survival (*i.e.*, percentage of females which become sexually mature) are both very high, reaching 92-96% and 98-100% respectively (Simonini and Prevedelli, 2003; Marcheselli *et al.*, 2010). The acute toxicity (96h) bioassays with newborn *D. gyrociliatus* were performed according to the ASTM E1562-00 protocol, using the same strain of Iori and collaborators (2014). For each experiment, about 500 juveniles collected approximately 8-12 h after hatching were randomly placed in 10 mL bowls and exposed to the experimental solutions, which contained the pigment in concentration ranging between 0.025 and 0.3 mg×L^{-1}. The concentration of the solvent was 300 mg×L^{-1}. The ability of the mucous capsule of *D. gyrociliatus* to limit pigment toxic effects was assessed starting from newly laid eggs, which were obtained as well as the acute test. After adults removal, eggs in each bowl were counted and randomly assigned to an experimental treatment. *D. gyrociliatus* egg capsules tenaciously adhere to the glass of bowls, so the culture seawater can be completely replaced with the experimental solutions at the same concentrations used in the test with juveniles (and vice versa). Capsules were not touched or removed until the end of the test to avoid any mechanical stress. Consequently, the number of eggs presents in each treatment was different, varying from 24 to 39. Developing embryos were daily checked during the following 96 hours through transparent capsules. After 4 days, bowls content was again substituted with culture seawater. To obtain information on hatching rates in different treatments, emerging juveniles were counted and moved in another bowl for the subsequent 48 h.

Additional tests were performed to check the sensitivity of *D. gyrociliatus* juveniles to the solvent (DMSO) and to $Cu(NO_3)_2$ (PanreacQuimica) as reference toxicant. The 96-h LC_{50} for DMSO was 16,300 mg×L^{-1} (1.63%): no lethal effects were observed at the concentration of 2,000 mg×L^{-1}, which was about 7 times the concentration of the solvent used in the experiments. The 96-h LC_{50} value for copper was 0.10 mg×L^{-1} (c.i. 95%=0.09-0.11 mg×L^{-1}), similar to that reported in literature (Reish and Gerlinger 1997; Iori *et al*., 2014).

Data analysis

EC_{50} values of tests with *V. fischeri* were calculated using Microtox® and Toxi-chromotest™ calculation programs and procedures.

The trimmed Spearman-Karber method was used to obtain LC_{50}/EC_{50} (median lethal/effective concentration) values and their relative 95% c.i. in tests with *E. crassus, B. plicatilis*, *A. franciscana* and *D. gyrociliatus*. Mortality correction was not necessary because the control mortality was always less than 10%. Hatching rates of treatments and controls in experiments with *D. gyrociliatus* egg capsules were compared using Fisher's exact test.

Results

LC-MS and NMR analyses of the purified pigment

Overall, 230 mL of purple mucus were obtained from 18 specimens of *H. parthenopeia*. After chromatographic purification, the concentration of the red band obtained gave 71 mg of crystalline pigment. Thus, the average concentration of the pigment in the purple mucus was about 310 mg×L^{-1}.

In the positive-ion-mode, MS and MS/MS mass spectra of the pigment displayed predominant protonated molecular ion ($[M+H]^+$) at *m*/*z* 269 and major daughter ion at *m*/*z* 254. In the negative-ion-mode, MS and MS/MS mass spectra displayed deprotonated molecular ion ($[M-H]^-$) at *m*/*z* 267 and major daughter ion at *m*/*z* 252. ESI-MS^- *m/z*: Exact mass calculated for $C_{16}H_{11}O_4$ $[M-H]^-$: 267.0657, found: 267.0654; ESI-MS^+ *m/z*: Exact mass calculated for $C_{16}H_{13}O_4$ $[M+H]^+$: 269.0814, found: 269.0816.

The inspection of the ^{1}H-NMR spectrum of the pigment in DMSO-d_6 (Fig. 1) showed the presence of characteristic signals of three different types of aromatic protons at δ 8.49, 7.90 and 7.59 ppm (each integrating for 1H). Furthermore, signals of one aromatic OH were present at δ 9.83 ppm.

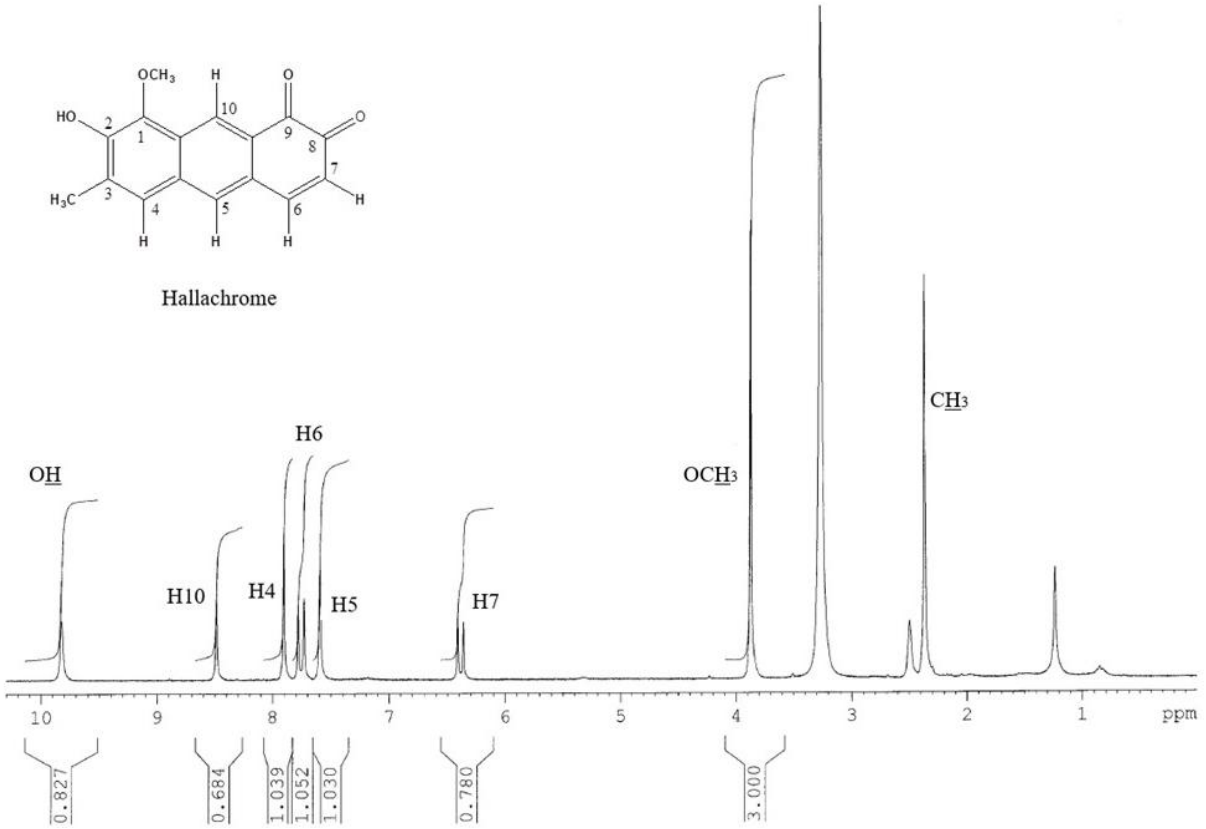

Fig. 1 ^{1}H-NMR spectrum of the purified pigment extracted from the purple mucus of *H. parthenopeia*, with the structure of hallachrome.

Additionally, the ^{1}H-NMR showed the presence of two *cis* olefinic protons (doublets at 7.76 and 6.38 ppm, J = 10.2 Hz), and two signals each integrating for 3H at 3.87 (Ar-OCH_3) and 2.34 (Ar-CH_3) ppm respectively (Fig. 1).

Bioassays

The emitted luminescence of *V. fischeri* was reduced by exposure to the pigment (Microtox®, $EC_{50\ 15\ min}$=0.88 mg×L^{-1}) as well as the synthesis of β-galactosidase in *E. coli* (Toxi-chromotest™, $EC_{50\ 90\ min}$=1.40 mg×L^{-1}) (Table 1).

The pigment inhibited the ciliary beat of *E. crassus* ($EC_{50\ 2\ h}$=0.29 mg×L^{-1}) (Table 1). After two more hours, all cells were lysed at 0.5 mg×L^{-1}.

B. plicatilis was the second most sensitive organism (Rotoxkit®, $LC_{50\ 24\ h}$= 0.18 mg×L^{-1}), while lethal effects on *A. franciscana* were observed for higher concentrations (Artoxkit®, $LC_{50\ 24\ h}$=5.67 mg×L^{-1} [Table 1]; $LC_{100\ 24\ h}$=10 mg×L^{-1}).

The pigment was very toxic for *D. gyrociliatus* juveniles ($LC_{50\ 96\ h}$ = 0.11 mg×L^{-1}) (Table 1). The $LC_{100\ 96\ h}$ was 0.20 mg×L^{-1}, while all juveniles survived in the control group.

When encapsulated embryos of *D. gyrociliatus* were exposed to the pigment, no young hatched after 96 h of exposure to 0.30 mg×L^{-1}, but the percentage of hatching found in the treatment with 0.10 mg×L^{-1} of pigment (93%, n=28) was not significantly different from that of embryos (94%, n=36; Fisher test: p>0.56) and juveniles (survivorship: 100%, n=50, Fisher test: p>0.12) control groups.

Discussion

LC-MS spectra and mass estimates, and results of ^{1}H-NMR analysis of the pigment purified from the purple mucus of *H. parthenopeia* are consistent with characteristics and structure of hallachrome reported by Prota and collaborators (1971). Bioassay results support the hypothesis that hallachrome is the secondary metabolite responsible for the toxicity of the purple mucus. The test battery evidenced the harmful effects of hallachrome on all the organisms considered and highlighted its broad-spectrum of activity on marine invertebrates. The mortality of *D. gyrociliatus* and *B. plicatilis*, and the ciliary motility of *E. crassus* were the most sensitive endpoints. The bioluminescence emission of *V. fisheri* and the β-galactosidase synthesis of *E. coli* were intermediate, while the mortality of *A. franciscana* was the least sensitive endpoint. This pattern of test sensitivity was observed also for the purple mucus (Table 1). Tests on *D. gyrociliatus* embryos, which develop within a mucous capsule, revealed that hallachrome concentration correspondent to the LC_{50} of new-born

Table 1 Median lethal/effective concentration (LC_{50}/EC_{50}) and confidence intervals (c.i. 95%) obtained for hallachrome in ecotoxicity biossays. The confidence intervals do not overlap. Reference toxicity data for purple mucus (Iori *et al*., 2014) are also provided in the last column. * test not performed in previous experiments (Iori *et al*., 2014)

Taxon	Species	Hallachrome (present experiment) [mg×L^{-1}]		Purple mucus LC_{50} or EC_{50} [mg×L^{-1}]
		LC_{50} or EC_{50}	C.I. 95%	
Bacteria	*Vibrio fischeri*	0.88	0.40-1.90	16,200
Bacteria	*Escherichia coli*	1.40	0.96-2.40	*
Ciliophora	*Euplotes crassus*	0.29	0.27-0.32	*
Rotifera	*Brachionus plicatilis*	0.18	0.16-0.20	8,100
Crustacea	*Artemia franciscana*	5.67	4.06-7.93	76,000
Polychaeta	*Dinophilus gyrociliatus*	0.11	0.10-0.12	700

free-living *D. gyrociliatus* had no effects on hatching. This suggests that a mucous layer could limit the diffusion of hallachrome, reducing its harmful effects. Further experiments on the diffusion rate of the hallachrome through the transparent mucus produced by *H. parthenopeia* should be performed to confirm if its release represents a physiological defence of the worm against its toxic purple mucus.

Hallachrome was the only 1,2-anthraquinone natural product characterized until the late '90's. More recently three other 1,2-anthraquinones, the pigment sinapiquinone and rufoolivacins C and D, were extracted from mushrooms of the genus *Cortinarius*, but they have a biaryl skeleton very different from the hallachrome structure (Gao *et al.*, 2010; Bai *et al.*, 2013). While 1,2-anthraquinones are rare, mono-aryl 9,10-anthraquinones are very common. They occur (both free or as glucoside) in a large number of plants, fungi (e.g. Mueller at al., 1999), and in some animals, including polychaetes of the genus *Tomopteris* (Francis *et al.*, 2014). Some 9,10-anthraquinones show weak to moderate toxicity for *A. franciscana* (Gao *et al.*, 2010). For instance, the 24-h LC_{50} of the emodin estimated by bioassay on the brine shrimp is about 43 mg×L^{-1} (159 µM; Ayo *et al.*, 2007). Moreover, the 24-h exposure to 10 mg×L^{-1} of physcion (35 µM), 1-hydroxy-6,8-dimethoxy-3-methyl anthraquinone (34 µM) and 6,8-dimethoxy-citreorosein (34 µM) induced the mortality of 41%, 39% and 37% of brine shrimp nauplii, respectively (Gao *et al.*, 2010). Our Artoxkit tests suggest that these anthraquinones are less toxic than hallachrome ($LC_{50\ 24\ h}$ = 21 µM) for *A. franciscana*. In the bioassay with *D. gyrociliatus* hallachrome ($LC_{50\ 96\ h}$ = 0.41 µM) was more toxic than copper ($LC_{50\ 96\ h}$ = 1.59 µM), which is among the most harmful metals for polychaetes (Reish and Gerlinger, 1997). In addition to the severe toxicity, hallachrome is also very abundant in the purple mucus, where it reaches a concentration 2-3 orders of magnitude higher than the one toxic for the organisms here assessed. Even today the dermal contact with hallachrome is quite common among fishermen using *H. partenopeia* as bait (Iori *et al.,* 2014). Yet, no cases of human harm after contact with *H. parthenopeia* have been reported, apart from strong and persistent stainings of the skin. In fact, given the difficulty with which hallachrome crosses the thin mucus capsule of *D. gyrociliatus*, it seems unlikely that it could overcome the cornified layer of human skin. However further experiments on the toxicity of hallachrome for mammals should be performed to exclude the possibility that this substance is harmful to humans. It would be advisable for those who manipulate these worms (including divers who collect *H. parthenopeia* from seabed and sellers) to wear disposable gloves as a precautionary measure to avoid excessive exposure to hallachrome toxicity.

The ecological significance of this toxin is still unclear. Despite the harmful effect and concentration of hallachrome in the purple mucus, this exudate did not deter fish feeding in palatability experiments with the ricefish *Oryzias melastigma*, and it is normally ingested by the seabass *Dicentrarchus labrax* and the gilthead seabream *Sparus aurata* when worms are used as fish baits (Iori *et al.*, 2014). Indeed, oenonids may avoid predators living in tubes within the sediment and feeding preferably during the night (Saito *et al.*, 2004). These strategies are often observed in palatable worms lacking chemical and mechanical deterrents against predators (Kicklighter and Hay 2006). This pattern indicates that hallachrome cannot deter predation. On the other hand, analogies in terms of modality of emission, high toxicity and range of activity with the bonellin produced by echiurids (de Nicola Giudici, 1984) suggest that hallachrome can defend *H. parthenopeia* from potential competitors, parasites and/or pathogens, including the microflora colonizing worms' tube and body wall. Further experiments with test species that co-occur in the same habitat of *H. parthenopeia* (or actually

represent a threat to the worm) are required to confirm that this unusual 1-2 anthraquinone could act as a chemical defense.

Cimino and collegues (1985) found hallachrome also in the body of another popular fishing bait, the lumbrinerid *Scoletoma (Lumbrineris) impatiens*, which belongs to one of the most specious family of polychaetes (Messina *et al.*, 2005). Both *H. parthenopeia* and *S. impatiens* contain also 1,2,7-trihydroxy-8-methoxy-6-methylanthracene (TMMA), which yields hallachrome by oxidation and is considered its biogenetic precursor (Cimino *et al.*, 1985). Perhaps, hallachrome and related compounds may also occur in other mobile polychaete species, but their ecological role cannot be predicted from chemical characterization alone. Interestingly, TMMA has never been found in the purple mucus of *H. parthenopeia*, but it occurs in a relatively large amount in *S. impatiens,* which does not secrete purple exudates.

The few toxic or venomous polychaetes known to date show different lifestyles and possess a wide range of diversely acting biomolecules (Nakamura *et al.*, 2008; von Reumont *et al.*, 2014b). These studies are relatively rare (Coutinho *et al.*, 2018), perhaps because sheltered lifestyles make them difficult to observe on the field. However, given the diversity of polychaete species future ecological, chemical and toxicological investigations supported by the application of novel methods (i.e. Next Generation Sequencing and -omic analyses, von Reumont *et al.*, 2014) should likely uncover other venomous or toxic worms.

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Conflict of Interest

On behalf of all authors, the corresponding author states that there is no conflict of interest.

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