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Screening for antibacterial molecules in meiobenthic nematodes belonging to the Oncholaimidae family

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Abstract: Active substances such as antimicrobial peptides (AMPs) defined as antibiotics naturally produced by all living species, have already been characterized and identified from various marine organisms (fish, sponges, annelids, echinoderms, crustaceans, molluscs and tunicates) except from nematodes. In this study, we investigated the biochemical isolation of antibacterial substances from three free-living marine nematodes belonging to the Oncholaimidae family that dominated meiofauna of two coastal environments characterized by reduced and hypoxic sediments with high concentration of sulfides (Roscoff Harbour in France and Secca delle Fumose in Italy). There are no consensus sequences for AMPs which are even more diversified in the marine environment compared to the terrestrial one. A bioassay guided purification protocol was used since it constitutes the only method to find novel active peptides. Data showed the potential of two of the three nematodes species as interesting sources of small sized antibiotics. The third species showed an occasional epi-symbiotic association with filamentous bacteria, but singularly lacked antimicrobial activity. The lack of biological material did not allow the identification of the antimicrobial molecules.

Résumé: *Criblage de molécules antibactériennes chez les nématodes méio-benthiques appartenant à la famille des Oncholaimidae.* Les substances actives du type des peptides antimicrobiens (PAMs) définis comme des antibiotiques produits naturellement par toutes les espèces vivantes, ont déjà été caractérisées et identifiées chez divers organismes marins (poissons, éponges, annélides, échinodermes, crustacés, mollusques et tuniciers) à l'exception des nématodes. Dans cette étude, nous avons étudié l'isolement biochimique de substances antibactériennes à partir de trois nématodes libres marins de la famille des Oncholaimidae qui dominent la méiofaune de deux environnements côtiers caractérisés par des sédiments réduits et hypoxiques à forte concentration en sulfures (le port de Roscoff en France et la Secca delle Fumose en Italie). En effet, il n'existe pas de séquences consensus pour les PAMs. Ils semblent plus diversifiés dans l'environnement marin par rapport à l'habitat terrestre. Un protocole de purification guidé par un essai biologique a été utilisé car il constitue la seule méthode pour trouver de nouveaux peptides actifs. Les données montrent le potentiel de deux des trois espèces de nématodes comme sources intéressantes d'antibiotiques de petite taille. La troisième

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espèce a montré une association épi-symbiotique occasionnelle avec des bactéries filamenteuses, mais singulièrement ne présente pas d'activité antimicrobienne. Le manque de matériel biologique n'a pas permis l'identification des molécules antimicrobiennes.

Keywords: Antimicrobial peptides ● Free-living nematodes ● Marine worms

Introduction

There is a growing interest and demand of new compounds such as antimicrobial peptides (AMPs) during the last decades because of the emergence of multi-drug resistant bacteria (Mahlapuu et al., 2016; Kaur et al., 2019). AMPs are in the first line of innate immune defence of all organisms: they provide a rapid response to a broad spectrum of invading microorganisms (bacteria, fungi, viruses and parasites) and an alternative way to eliminate them (mostly by bacterial membrane disruption) with slow development of bacterial resistance, representing a potential class of new drugs (Maróti et al., 2011; Daphny et al., 2015). To date, marine peptides are largely unexplored compared to the number of identified terrestrial AMPs when considering the high species diversity in the ocean. Indeed there is a remarkable difference in the sampling effort between the terrestrial and marine habitats (with only 5% of marine living organisms screened for drug discovery) (Nalini et al., 2018; Pavlicevic & Maestri, 2020). Even if about 75% of the AMPs investigated come from the animal kingdom (Fig. 1), only two percent have been characterized and identified from marine organisms (fishes, sponges, annelids, echinoderms, crustaceans, molluscs and tunicates, except nematodes), suggesting that we may be facing the sheer tip of the iceberg of potential new compounds (Antimicrobial Peptides Database APD3, last access on 29 June 2020 (Wang et al., 2016)). Moreover, most of the investigation was focused on marine organisms of economic interest (shrimps, mussels, oysters ….). The major limitations for discovery and analysis of new substances from wild marine organisms (not issued from aquaculture) are the availability and the accessibility of bioactive material required to perform time and source consuming protocols, such as AMPs isolation and identification (Sperstad et al., 2011). Recent advances in technologies, sampling strategies and analytical techniques have enabled the finding of unique and structurally diverse biologically active substances from marine vertebrates and invertebrates, such as piscidins, polyphemusins and ALFs (Anti-Lipopolysaccharide Factors), conotoxins and myticusin, aurelin, pseudopterosins, BRICHOS AMPs, perinerin and hedistin (reviewed by Nalini et al., 2018; Wang et al., 2018; Bruno et al., 2019). The majority of investigated marine species so far, seems to contain one or more novel primary structures either species-specific or even confined to certain taxa (Tasiemski, 2008). The evolution of immune system genes (like AMPs) strictly depends on the evolutionary times that led to the whole marine diversity but also environmental abiotic and biotic factors that shaped this diversity (Rolff & Schmid-Hempel, 2016; Kaur et al., 2019). Therefore, marine AMPs uniqueness and diversification have presumably been associated with their evolution under the pressure of highly varying physicochemical conditions (temperatures, pH, pressure, salinity, *etc.*) and high density of bacteria notably proteobacteria, the bacterial family generating the most problematic drug resistances in human at the present time (Nalini et al., 2018).

Among these marine molecules are powerful compounds that have been proven to possess biological activities and potential beneficial uses in human health promotion or disease treatment (Wang et al., 2018).

After the discovery in 1989 of cecropin P1 (Lee et al., 1989), the first nematode AMPs (from the parasite *Ascaris suum*), efforts were mostly focused on the terrestrial genetic model *Caenorhabditis elegans* (reviewed by Bruno et al., 2019). Later, several groups of AMPs were identified in nematodes: defensin-like antibacterial factors (ABFs, about 6500 Da), caenopores (9000-10000 Da), caenacins (CNCs, 4000-6000 Da) and neuropeptide-like (NLPs, 5000-6000 Da) (reviewed by Tarr, 2012). The minority of these peptides, mainly found in *Caenorhabditis* and *Ascarididae* species (such as *C. elegans, C. briggsae, A. suum, A. lumbricoides* and *Toxocara canis*), were purified from crude extracts of worms: they were identified by using inverse genetic and/or by screening

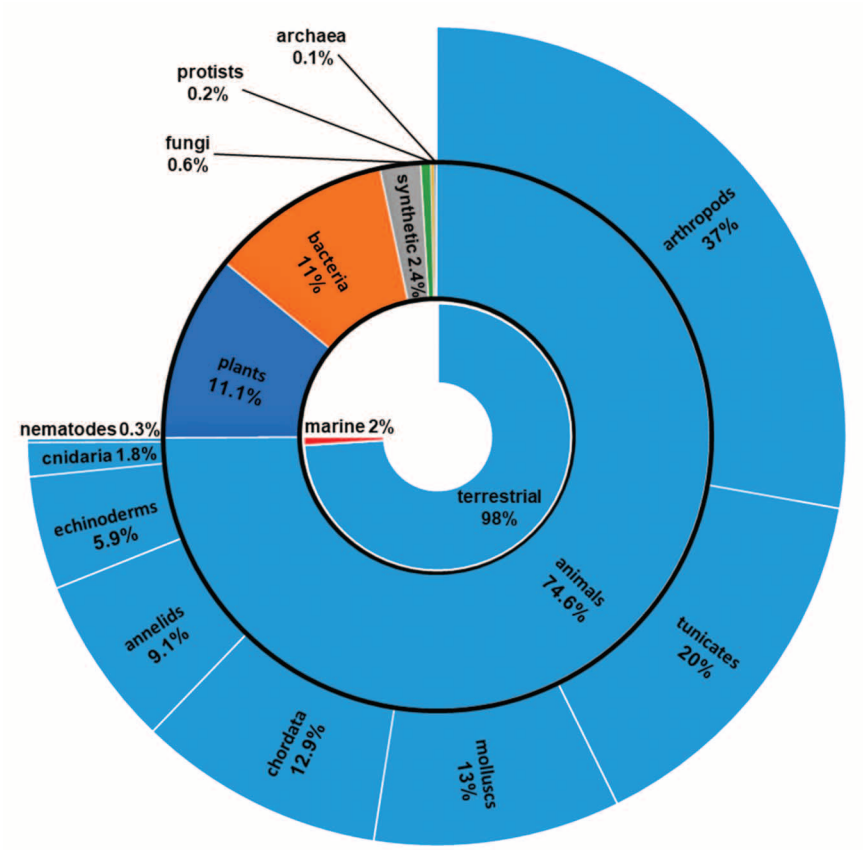

Figure 1. Distribution of antimicrobial peptides through the living. Multiple pie chart representing the percentage of AMPs found in the six kingdoms (central circle), the distribution of AMPs per animal group (external circle) and per their host environment (internal circle). Data obtained by using the Antimicrobial Peptides Database (APD3, last access on 29 June 2020 (Wang et al., 2016)).

omic databases. Sequences of already known AMPs are blasted to genome or transcriptomic databases to pick up homologues in other species. This “in silico” approach does not allow the discovery of new substances and is successful on closely related species (reviewed by Bruno et al., 2019). For these reasons, biochemical purification remains the only way to discover new compounds with the disadvantage to be time consuming and to require large amount of biological material (Sperstad et al., 2011).

Several studies demonstrated that many marine invertebrates have evolved via a variety of physical and chemical defence mechanisms, for instance antimicrobial metabolites (Faulkner, 2000). Meiobenthic nematodes, representing 60-90% of meiobenthos in marine ecosystems, may reach abundances of more than 90% in marine environments characterized by reduced and hypoxic conditions, with high concentrations of sulphide (black anoxic zones of the sediment) and a plethora of bacteria (Zeppilli et al., 2017). Therefore, it seems reasonable to assume that marine nematodes could produce still undiscovered bioactive substances, as AMPs (Bulgheresi, 2011; Heip et al., 1985).

Moreover, because nematodes and arthropods are the most successful animals for adapting almost all environmental conditions over the planet with a very old but common ancestry (forming the group of Ecdysozoa), we might expect that marine nematodes display a diversity of bioactive substances, as high as that found for crustaceans (Aguinaldo et al., 1997). To date, in crustaceans (mostly farmed species), there are 15 distinct AMP families: some of them were found in all crustaceans studied (such as the ALFs) and others specific to certain lineages (like the penaeidins, restricted to penaeid shrimp) with a remarkable diversity of marine peptides in the structural and genetic composition compared to terrestrial counterparts (Destoumieux et al., 1997; Rosa & Barracco, 2010).

Here three species of marine meiobenthic nematodes inhabiting the sediments of the “black zone” in the Gulf of Naples (Italy) and in Britany (Roscoff, France) were used for searching new

antibacterial compounds. Biochemical purification and identification of novel AMPs produced by these tiny species were investigated with the expectations to find them in a large number.

Materials and methods

Source of AMPs

The "Antimicrobial Peptide Database" (APD3 by Wang et al., 2016, http://aps.unmc.edu/AP/main.php) was used to list the number of AMPs already discovered in different kingdoms and phyla. According to statistical data in APD3, at the date of 29 June 2020, there were a total of 3201 peptide sequences that have been reported to exhibit antimicrobial activities. The percentages used to build the pie charts were obtained using the following equation: number of AMPs belonging to the selected group/ 3201 × 100.

Study areas and nematode collection

Two sites were selected to collect meiobenthic nematodes from the black zone, characterized by anoxic and sulphide-rich sediments, in which they are supposed to abound (harbour of Roscoff in France and "Secca delle Fumose", Gulf of Naples in Italy) (Bellec et al., 2019 & 2020; Donnarumma et al., 2019; Appolloni et al., 2020).

In the old harbour of Roscoff (48°43′34.20″N-3°58′50.53″W, France), the sediment samples were collected manually at low tide. The top black layer of the sediment (< 5 cm) was sieved (0.5 mm, sieve mesh size) in the field and quickly brought to the laboratory, where live nematodes were sorted under a stereomicroscope (M125; Leica, Wetzlar, Germany).

In Naples, samples of sediment were collected by scuba-diving operators from a degassing structure offshore of the Campi Flegrei caldera, "Secca delle Fumose" (40°49'23''N-14°05'15''E, Italy). The samples were kept at 4°C and quickly brought to the laboratory. In the lab, live nematodes were sorted under a stereomicroscope (SMZ800; Nikon Corporation, Tokyo, Japan).

Morphological observation/identification of the collected nematodes

We sorted live worms under stereomicroscope and we easily identified 3 different morphotypes, 2 belonging to the genus Oncholaimus (here reported as *Oncholaimus* morpho1 and *O.* morpho2) and one morphotype of Metoncholaimus (identified as *Metoncholaimus albidus*). A set of nematodes, up to 20 for each morphotype was sampled and analysed for verifying that all the nematodes belonged to the same species. We performed a detailed microscopical identification, by optical microscopy and scanning electronic microscopy (SEM). For each morphotype several nematodes were mounted on slides using the formalin–ethanol/glycerol technique (Vincx, 1996) and observed using a Leica DM IRB microscope and a Zeiss AxioZoom microscope, each equipped with live camera (Image-Pro and Zen software, respective).

A set of nematodes, between 2 and 19 for each morphotype, was post-fixed in 0.8% osmium tetroxide 20 h at 4°C and then dehydrated through an ethanol series. Nematodes were desiccated with a critical-point dryer (CPD 300; Leica, Wetzlar, Germany) and then mounted on a specimen stub. They were gold-coated using an SCD 040 (Blazers Union, Blazers, Liechtenstein). Observations were made with a Quanta 200 MK2 microscope (FEI, Hillsboro, OR, USA) and the xT microscope software (FEI). Scanning electron micrographs were used for morphological identification.

Rearing conditions

Some individuals, 240 of *M. albidus* (from Roscoff, July 2017) and 160 of *O.* morpho2 (from Naples, November 2017) were maintained in glass petri dishes (H 25 mm, diam. 150 mm) containing sterilized oxygenated seawater (Instant Ocean at a salinity of 33), at 18°C and with natural light. The worms were fed weekly with 0.5 g of a ground commercial dried baby crop (HiPP Biologique, France).

Crude extracts of nematodes

Two methods were used depending on the number of collected specimens. Only animals collected at a relatively large scale (> 20) were experimentally challenged with bacteria to potentially increase their AMP production.

O. morpho1 and *M. albidus* (Roscoff 2016): only 20 nematodes of each species were sampled and identified. Worms were immediately frozen in liquid nitrogen and grounded in a Potter-Elvehjem homogenizer; 20 µL of Phosphate Buffered Saline (PBS Euromedex 10X, 0.1 M of pure water, pH7.6) were used to collect the very small amount of crude extract.

O. morpho2 (Naples 2016): 400 worms were sorted out, one-half (200 specimens) were incubated in filtered sea water (unchallenged samples) and the other 200 individuals (challenged samples) were

incubated in the same water supplemented with a culture of environmental bacteria isolated from local marine sediments (in order to induce the synthesis of antibacterial substances by the worms). After 4 hours, both sets were frozen in liquid nitrogen, homogenised by using prefilled bead (1.4 mm ceramic) tubes and weighed (0.0763 g and 0.1703 g, respectively wet weight of challenged and unchallenged samples). The samples were finally suspended in 1 mL of PBS (0.1 M of pure water, pH 7.6), determining the final concentration 1.1379 $g.mL^{-1}$ and 1.097 $g.mL^{-1}$, respectively challenged and unchallenged samples.

Microorganisms

Bacteria used for the antibacterial assays. Gram positive: *Micrococcus luteus IFO12708*.
Gram negative: *Escherichia coli K-12 strain D31* and *Aeromonas hydrophila*.

The human pathogenic strains (*E. coli*, *M. luteus* and *A. hydrophila*) were cultivated at 37°C in Luria-Bertani (LB Broth Lennox, Athena ES) medium under shaking at 140 rpm and maintained on Luria-Bertani agar at 37°C.

Microorganisms used for the bacterial challenge. A spoon of sediment from the site of "Secca delle Fumose" was first incubated 6 hours in 10 mL of liquid Zobell medium (4 g Bacto Proteose Peptone (BD Biosciences), 1 g Bacto Yeast Extract (BD Biosciences), 23.4 g NaCl, 1.5 g KCl, 1.2 g $MgSO_4$ x $7H_2O$, 0.2 g CaCl x 2 H_2O, in 1 L of pure water) at 28°C under shaking condition (140 rpm), to stimulate bacterial growth. 2 mL of the supernatant were incubated overnight at room temperature stirring (140 rpm). Then this sediment slurry was centrifuged (4000 x g, 10 minutes at room temperature) and the supernatant was eliminated. The bacterial pellet was re-suspended in 10 mL of filtered sea water (0.20 µm) and the $O.D._{600}$ (Optical Density at 600 nm) was measured. The sample was then diluted in filtered sea water (0.20 µm) to obtain a final concentration of 2×10^9 colony-forming units (CFU).mL^{-1}. 1 mL of this bacterial culture was added to 1 mL sea water filtered containing the nematodes: after 4 hours of incubation at room temperature, nematodes were transferred in cryotube and immediately frozen in dry liquid nitrogen.

Antibacterial solid plate assay

The antibacterial activity of the crude extracts was assayed by a solid growth inhibition assay using the bacterial strains listed before as previously described (Tasiemski et al., 2000). One colony of bacteria was grown in 3 mL of LB medium overnight at 37°C under agitation (140 rpm) to an O.D.600 of 0.4. The culture was then diluted: 230 µL in 50 mL of LB agar (0.15% (w/v) in LB), placed in petri dishes and stored at 4°C.

10 µL of extract of *M. albidus* or *O.* morpho1 were plated on the nutrient agar containing bacteria and incubated at 37°C overnight.

In the case of *O.* morpho2, 3 µL of pre-purified extract (1.1379 $g.mL^{-1}$ and 1.097 $g.mL^{-1}$, challenged and unchallenged concentrations respectively) were directly spotted on the agar plates. Following overnight incubation, the diameters of the growth inhibition zones were measured (diameter in mm) and correspond to the antibacterial activity of the crude extract.

Biochemical purification of the antibacterial substances

The protocol is optimized for a peptide extraction of AMP in biological samples (Sperstad et al., 2011; Tasiemski et al., 2014).

Prepurification steps. The crude extracts of *O.* morpho2 (1.1379 $g.mL^{-1}$ and 1.097 $g.mL^{-1}$, respectively challenged and unchallenged samples) were brought to pH 3.5 using 1 M HCl (acid protein precipitation) and centrifuged (8000 x g, 20 min, 4°C), then the supernatants were pre-purified by solid-phase extraction on a 12 cc C18 Sep-Pak Vac cartridge (2 g, Waters Associated) equilibrated in acidified water (0.05% trifluoroacetic acid). Elution steps were performed with 10, 60 and 80% of acidified acetonitrile (ACN) and the fractions eluted with 60% of ACN were lyophilized (1.8 mg and 2.5 mg, respectively challenged and unchallenged samples) by speed vacuum and reconstituted with HPLC pure water (108 and 150 µL respectively), obtaining the final concentration of 0.0167 $mg.\mu L^{-1}$. The fractions eluted with 60% of ACN (3 µL) were then submitted to the purification steps.

Purification steps. The following HPLC steps were carried out on a Perkin Elmer series 200 HPLC system with a variable wavelength detector. The column effluent was monitored by absorbance at 225 nm (absorption wavelength of peptide bond).

First step. The active prepurified extracts (showing antimicrobial activity) were subjected to reversed-phase high-performance liquid chromatography (RP-HPLC), on a Sephasyl C18 column (250 × 10.0 mm, model US5C183-250/100, Interchim). The elution was performed with a biphasic gradient consisting of 5-65% ACN in acidified water for 90 min and 65 80% for 30 min, at a flow rate of 1 $mL.min^{-1}$. The fractions corresponding to absorbance peaks were collected in

polypropylene tubes, dried, reconstituted in HPLC grade water, and tested (3 µL) for a second screening by agar diffusion method against *E. coli* (as above).

Second step. The two active fractions were pooled and further separated on a C18 column (250 × 2.1 mm, model 218TP52, Vydac) with a biphasic gradient consisting of 5-25% ACN in acidified water for 20 min, 25-45% for 60 min and 45-80 for 10 min at a flow rate of 1 mL.min^{-1}. The fractions were collected and treated as above, then tested by agar diffusion method against *E. coli* and *M. luteus* (as above).

Mass spectrometry. The purity assessment of active fractions was carried out by mass spectrometry analyses (UltraFlex II MALDI-TOF/TOF instrument, Bruker Daltonics, Bremen, Germany) in the range 700-4000 Dalton, using matrix αcyano-4-hydroxycinnamic acid (HCCA, 10 mg dissolved in 1 ml of ACN/0.1% TFA in water (7:3, v/v)) optimized for peptide adsorption and flexAnalysis software (version 3.4, Bruker Daltonics).

Results

Morphological identification/description of the meiobentic Nematodes

The Nematodes found in both sites were largely dominated by specimens belonging to the family Oncholaimidae but the abundance of each species was not constant from one year to the other (Table 1) (Bellec et al., 2019; Baldrighi et al., 2020).

These nematodes are characterized by a relatively large size for the meiofauna (up to 8 mm) which allows their identification by stereomicroscope. We easily discriminated two Oncholaimidae morphotypes (one belonging to the genus *Oncholaimus* and one to genus *Metoncholaimus*) in Roscoff sediments and another different morphotype of *Oncholaimus* in Naples samples. The free-living marine nematodes species sampled in Roscoff harbour and in Naples (Fig. 2) have been identified as three species belonging to the family Oncholaimidae, *Metoncholaimus albidus* (Bastian, 1865) and two newly recognized species belonging to the *Oncholaimus* genus (Fig. 3).

A description is presently being prepared for the two *Oncholaimus* morphotypes not yet illustrated (Zeppilli personal communication), so in this study they will be referred as *Oncholaimus* morpho1 and *O.* morpho2. The genus *Oncholaimus* (Smol et al., 2014) is characterized by: left ventrosublateral tooth largest, monodelphic-prodelphic females with antidromously reflexed ovary, well developed demanian system, terminal ducts and pores present in variable number or absent in virgin females, diorchic males, spicules short, gubernaculum absent, tail short. *O.* morpho1 (from Roscoff) main features are short cephalic setae (2-1-2), 12 lines of cervical double and single setae and a very short and truncated tail (Fig. 3A-B). The species sampled in Naples, *O.* morpho2, is characterized by a cloacal aperture, surrounded by setae and a conical papilla on the tail (Fig. 3C-D).

The other species reported in this study, *M. albidus* is characterized by long spicules, the presence of a gubernaculum, and a well-developed demanian system with single uvette and double monoliform terminal (Bellec et al., 2019). Details of the *M. albidus* specimens sampled in Roscoff are reported in Bellec *et al*., 2019. Interestingly, during our sampling we observed the presence of filamentous bacterial ectosymbionts on numerous individuals of *M. albidus*, as also recently described by Bellec *et al.* (Fig. 3E).

At the Roscoff Harbour, 20 individuals of *O.* morpho1 were found during July 2016; only 8 individuals were found in July of the following year (July 2017) at exactly the same site and the same date (Table 1). By contrast, 20 individuals of *Metoncholaimus albidus* were found the first year of sampling while they were very abundant the second year of sampling at the Roscoff Harbour (Table 1). In Naples, only *O.* morpho2 was observed and essentially during the first sampling in November 2016. Unfortunately, only 13 individuals (compared to more than 400 in the previous year) were found from the second sampling at the same site after a week of intensive sorting.

For *M. albidus* and *O.* morpho2, breeding attempts have been performed in the laboratory in order to have enough biological material to successfully isolate and identify active substances without any success: for

Table 1. Number of nematodes individuals sampled in Roscoff and Naples (sampling not performed are represented by /).

Location	Nematodes species	July 2016	Nov. 2016	July 2017	Nov. 2017
Roscoff Harbour	*Metoncholaimus albidus*	40	/	220	/
	Oncholaimus morpho1	40	/	8	/
Naples	*Oncholaimus morpho2*	/	600	/	13

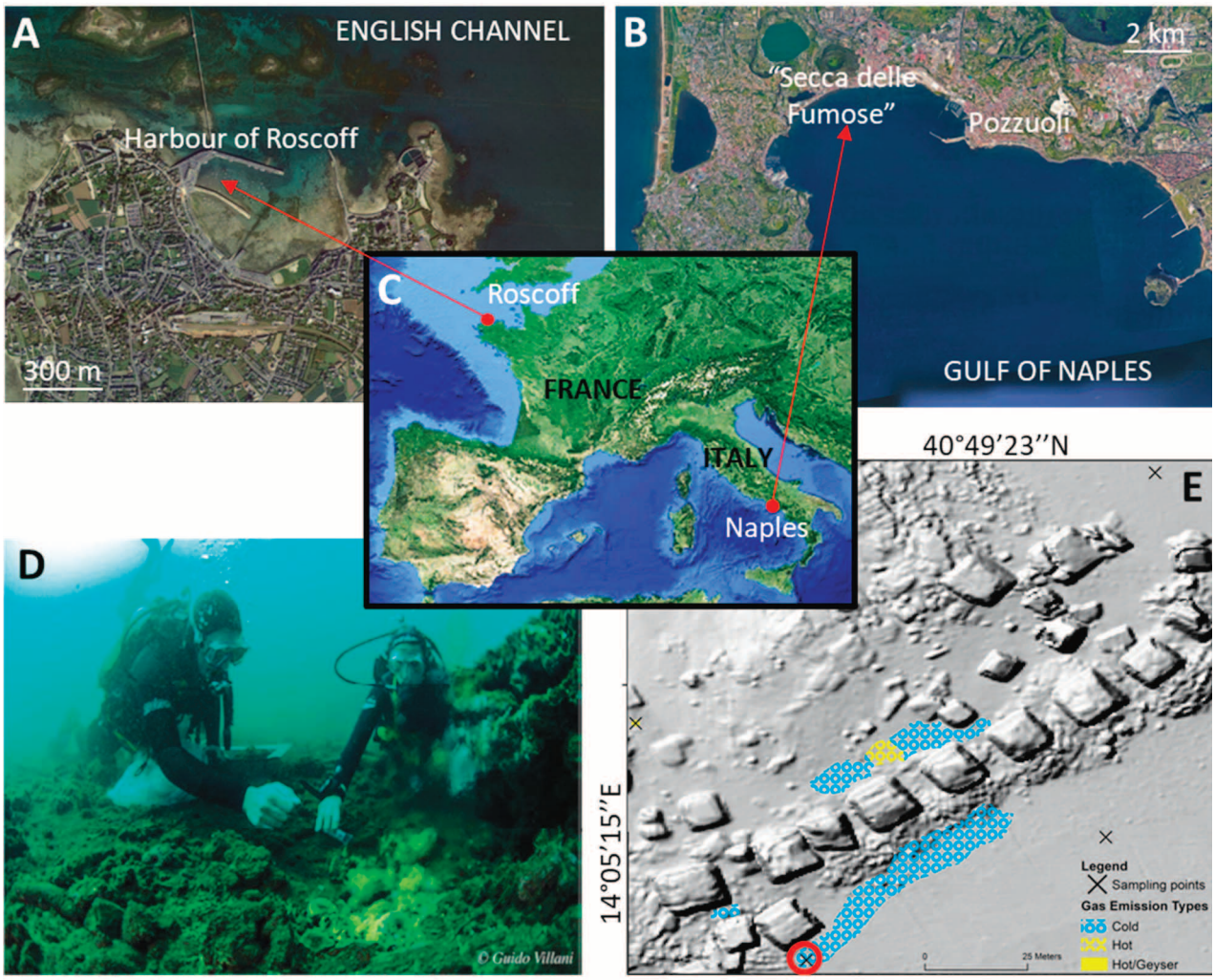

Figure 2. Location of the sampling areas. Satellite image of the sampling areas. **A.** Roscoff harbour (48°43′34.20″N and 3°58′50.53″W). **B.** "Secca delle Fumose", Gulf of Naples (40°49'23''N and 14°05'15''E). **C.** Geographical location of the sites. **D.** Scuba-diving operators at the shallow-vent zone, collecting anoxic and sulphide-rich sediments (forming a yellow carpet type layer on the top of the sediments; @ Guido Villani). **E.** Map of the sampling sites in the study area ("Secca delle Fumose") and digital elaboration of seafloor geomorphology (© Luca Appolloni); the red circle represent the sampling spot where practically the totality of *O.* morpho2 was collected (Baldrighi et al., 2020).

both species, we observed a constant decrease of the number of individuals per petri dishes, until the complete loss of the worms after about two months, and without any detection of juveniles (Fig. 4).

Differential antibacterial activities of the crude extracts from Metoncholaimus albidus*,* Oncholaimus *morpho 1 and 2*

For the three nematodes species, the crude extracts were tested for their antibacterial activities by solid plate assay against Gram positive (*M. luteus*) and Gram negative (*E. coli or A. hydrophila*) bacteria (Fig. 5A & B).Under the tested conditions, only crude extracts of *O.* morpho 1 (10 µL, half part of the extract obtained from 20 worms) and O. morpho2 (3 µL at concentration of 1.1379 g.mL^{-1} and 1.097 g.mL^{-1}, respectively challenged and unchallenged samples) inhibit both the growth of the Gram negative bacteria (*E. coli* and *A. hydrophila*).

The extract from *O.* morpho1 is more active against *M. luteus* than *O.* morpho2. *O.* morpho1 extract displayed antimicrobial effect against both tested strains, showing substantial inhibition areas (both about 13 mm of diameter). No activity was observed with the *M. albidus* extract (using 10 µL, half part of the extract obtained by 20 worms), against the tested bacteria.

Thanks to the relatively large number of *O.* morpho2 individuals collected during the first mission

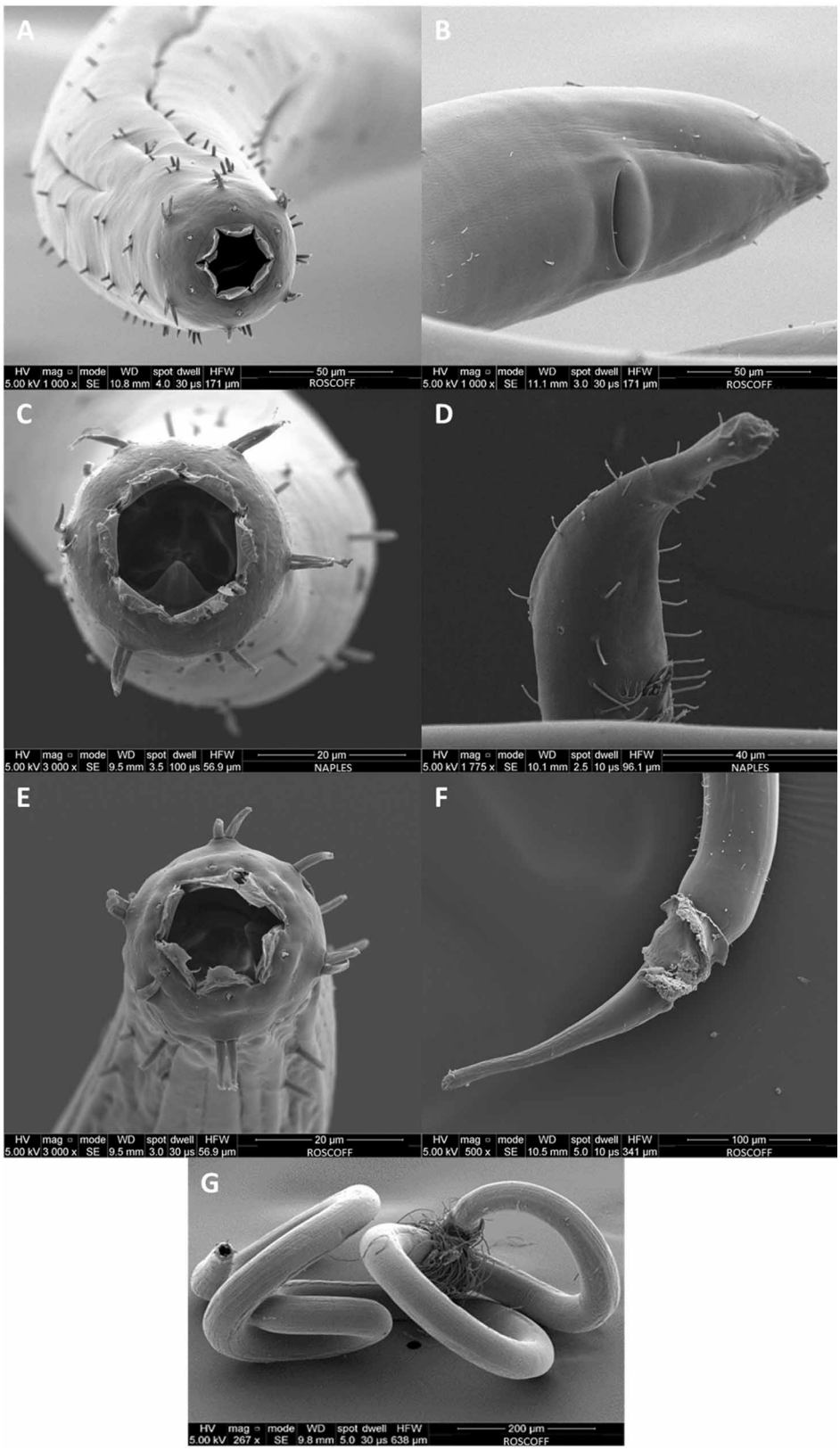

Figure 3. *Oncholaimus* morpho1 (Roscoff), *Oncholaimus* morpho2 (Naples) and *Metoncholaimus albidus*. SEM images of heads (anterior view) and tail regions. **A.-B.** *Oncholaimus* morpho1. **C.-D.** morpho2. **E.-F.** *M. albidus*. **G.** SEM image of *M. albidus* specimen associated with filamentous bacteria.

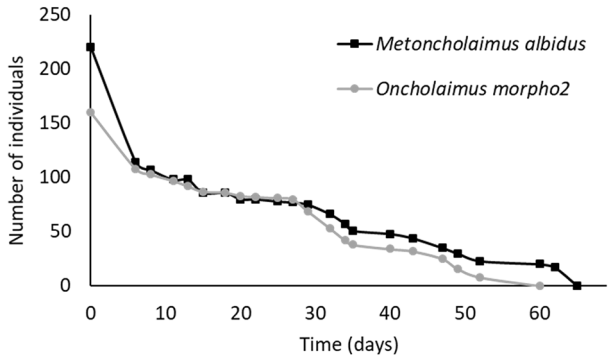

Figure 4. Survival curve of *Metoncholaimus albidus* and *Oncholaimus* morpho2 maintained in the laboratory.

in Naples, bacterial challenges were performed with a mix of bacteria collected from the field. Extracts from challenged and unchallenged worms exhibited bacterial growth inhibition activities against the tested strains, without strong differences (Fig. 4B). In particular, *E. coli* was the most sensitive to both extracts (9-10 mm growth inhibition diameters), while a slight activity was observed against *M. luteus* (4-5 mm growth inhibition diameters).

AMP purification from O. morpho2

After the screening, two candidates appeared interesting to go further into the purification of AMPs: *O.* morpho1 and 2 (Fig. 5). Unfortunately, even though *O.* morpho1 produced antibacterial substances, it was not possible to perform a biochemical purification of the active substances from this species, due to the limited amount of material from the first sampling of nematodes. A large-scale sampling was then planned but the unexpected random distribution of Oncholaimidae in the field resulted in an unfruitful sampling (see before).

In order to detect and isolate the substances responsible for the antimicrobial activities, the crude extracts from *O.* morpho2 challenged and unchallenged individuals were submitted to a purification by RP-HPLC chromatography. As evidenced by the two chromatograms (Fig. 6A & B), challenged extract presents two additional peaks (red rectangle in Fig. 6A). We assumed that the two peaks (eluted at 34% and 35% ACN) were related to the enhanced production of antimicrobial substances by stimulated samples. Our hypothesis was confirmed by the results of the antimicrobial assay that was

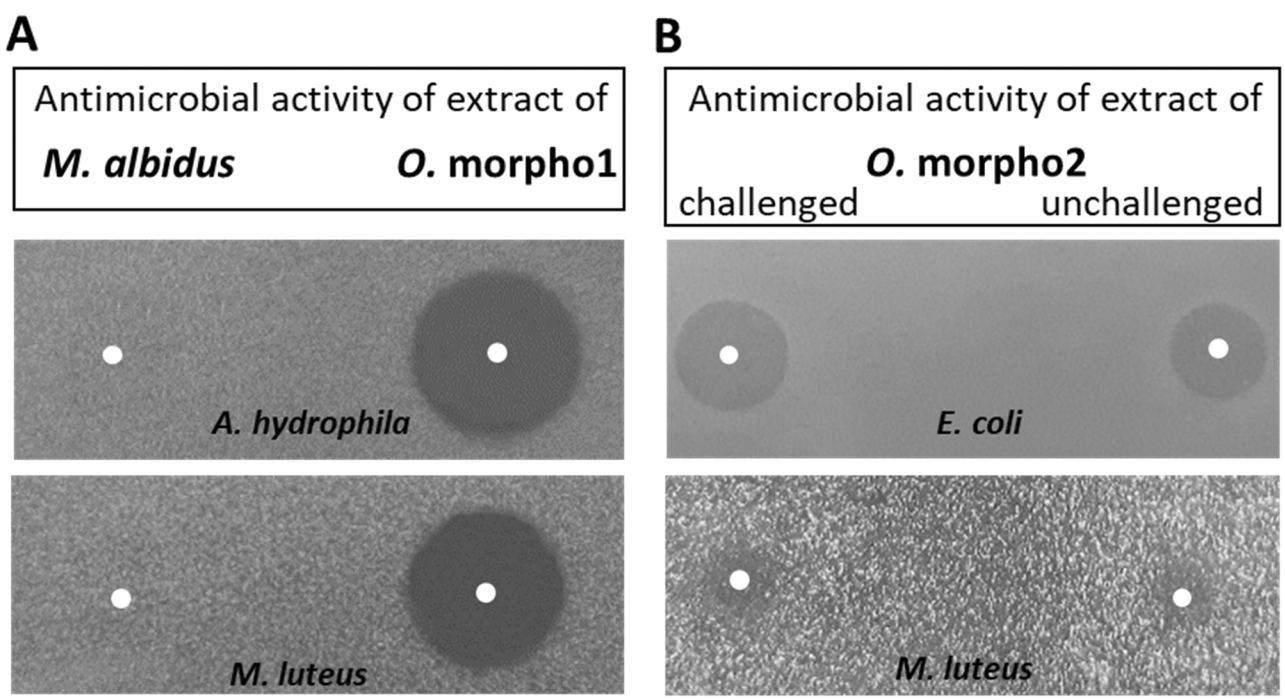

Figure 5. Antimicrobial activity of *Metoncholaimus albidus* and *Oncholaimus* morphotypes. Antibacterial solid plate assay. **A.** *M. albidus* and *O.* morpho1 crude extracts against *M. luteus* and *A. hydrophila.* **B.** Challenged and unchallenged pre-purified extracts from *O.* morpho2, against *E. coli* and *M. luteus*. The white dots indicate the position of the extract on the agar plates. The diameter (in mm) of the growth inhibition zones was determined.

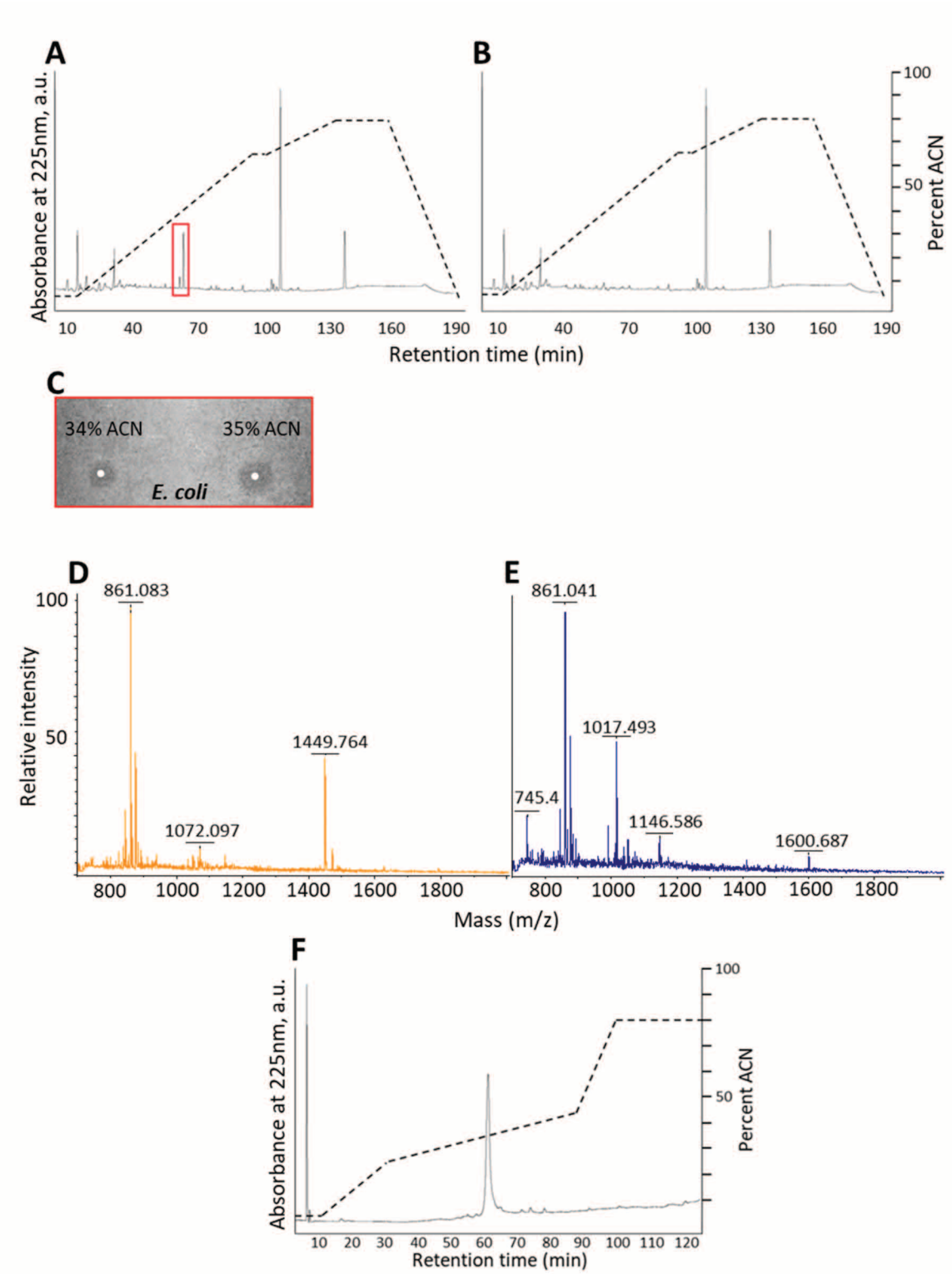

Figure 6. AMP purification. The extracts of (**A**) challenged and (**B**) unchallenged nematodes, eluting at 60% acetonitrile (ACN) upon solid phase extraction was loaded onto a C18 column (250x10mm, Sephasyl). Elution was performed with a biphasic gradient of acetonitrile in acidified water (dotted line) and absorbance was monitored at 225 nm. (**C**) Each individually collected fraction was tested for its antimicrobial activity against *E. coli* (Red rectangle in A): The fractions (eluted at 34% and 35% ACN) containing antimicrobial active substance were analysed by MALDI TOF-MS (**D-E**), pooled and further purified by additional RP-HPLC purification step (**F**).

performed on the fractions derived from each collected peak of the chromatograms (against *E. coli*). Antibacterial activity was detected only in the fractions corresponding to the two peaks, showing 5-6 mm growth inhibition diameters (Fig. 6C).

MALDI-TOF mass spectrometry analysis detected that the two active fraction samples (Fig. 6D-E) are characterized by low-molecular-weight (around 1000 m/z), what may correspond to AMPs of approximately 10 amino acids. The two fractions were pooled and further purified by a supplementary RP-HPLC chromatography (Fig. 6F). The antibacterial activities were lost after this step due to the too low quantity of material.

Discussion

Currently, a growing interest in research is devoted to marine invertebrates as promising sources for the discovery of novel and unique compounds having a plethora of activities (antimicrobial, antiviral, antifungal, *etc*.) and applications (Tasiemski et al., 2014; Rajanbabu et al., 2015; Bruno et al., 2019). Herein, a preliminary investigation using marine nematodes crude extracts clearly demonstrated for the first time their inhibitory activity. These marine worms produce still uncharacterized compounds exhibiting promising bioactivities (probably AMPs), deserving further investigations. Different nematodes species can produce several classes of AMPs (see introduction) as natural response to microbial (bacterial, viral, fungal and yeast) attack (Tarr, 2012). In sulfide-rich black mud, marine organisms are permanently in close contact with very high densities of microbes (Zeppilli et al., 2017): relying on a broad-spectrum defence, such as AMPs release, means protection from a biotic factor of external environmental, reducing the number of constraints to face. More investigations are required to better define the environmental selective pressures driving the evolution of defence mechanism (antimicrobial compounds and/or epibiosis) by different organisms (Harder, 2009; McFall-Ngai et al., 2013). Information on antimicrobial molecules from marine nematodes may shed light on the evolutionary origin and history of these defences in nematodes and in the taxon Ecdysozoa.

Nowadays, AMPs from nematodes were identified exclusively in terrestrial species (such as *C. elegans* and *A. suum*) mostly by genetic “in silico” approaches based on already known sequences issued from peptide purification (reviewed by Bruno et al., 2019). Our previous work on worms notably on annelid polychaetes provided evidence that marine invertebrates inhabiting harsh habitats constitute interesting sources of novel and unique AMPs (reviewed by Bruno et al., 2019). The AMP from the extreme Pompeii worm was patented for its potential use in human antibiotherapy. We also demonstrated the role of annelid AMPs in the innate immunity as well as in the control of their bacterial symbionts. The same procedure of AMP purification than the one used for annelids was then applied to the three species of Oncholaimidae presented here. Because they inhabit hostile habitat (sulfide rich, reduced and hypoxic sediment), we expected novel and unique sequences and/or structural motifs from these marine nematodes as observed for annelids sharing the same kind of habitats (Tasiemski et al., 2014). Oncholaimidae being described as major constituent of the biomass of the meiofauna at hydrothermal vent sites (Zeppilli et al., 2015), we also expected a large quantity of individuals what is a prerequisite for a successful bioassay guided purification assay (*i.e.* to obtain at the end of the purification enough molecule for the amino acid sequencing/identification of the peptide). The two samplings at exactly the same site and at the same season revealed in fact a completely random (patchy) distribution (almost all or nothing) of the three species of Roscoff and of Naples (Bellec et al., 2019; Donnarumma et al., 2019; Bellec et al., 2020) while other species such as the marine annelid *Capitella sp.* known to be an opportunistic species (Gamenick et al., 1998), inferred to habitats enriched in sulfides was observed within each sampling for both sites. To increase the quantity of biological material, attempts to rear the nematodes according to the protocol used for *Capitella* in the laboratory (Boidin-Wichlacz et al., unpublished data) were performed without any breeding success and a complete loss of the nematodes after 2 months.

A first screening of the antibacterial activities from the crude extracts was anyway performed for each species. Data showed that the crude extract from *Metoncholaimus albidus* did not display any antibacterial activities against the tested bacteria. To date, the biological role of immune molecules in marine host-symbiont association is a burgeoning field (Bulgheresi, 2011; Brinkmann et al., 2017). Recently, the key involvement of AMPs in the control/establishment of the ectosymbiontic communities was described in marine invertebrates from sulfide-rich environments, such as *Alvinella pompejana* and *Rimicaris exoculata* (Tasiemski et al., 2014; Le Bloa et al., 2020). Besides antimicrobials produced by marine organisms, it has been however

shown that host-associated epibiotic bacteria inhibit the growth and attachment of co-existing bacterial species or new epibiotic colonizers competing for the same niche (Harder, 2009). Therefore, we hypothesised the unexpected lack of antimicrobial activity in *M. albidus* as a result of a too low amount of biological material available but also to the presence of the epibiotic bacteria which may act as a substitute to prevent pathogenic infections.

Only the two *Oncholaimus* morphotypes referred as *O.* morpho1 (average length of 8 mm) and O. morpho2 (average length of 6 mm) (species in course of description, D. Zeppilli personal communication) showed antibacterial activities against *E. coli, A. hydrophila* and *M. luteus.* Because *O.* morpho2 from Naples was the species from which we had the higher amount of material, biochemical purification optimized for the search of AMPs was performed on this species. After a precipitation step and a two-step purification by RP-HPLC of the Sep Pack prepurified extract and analyses by mass spectrometry, data showed the presence of active molecules at the molecular size ranges around 1000 to 1600 m/z only in the bacterial challenged nematodes. Unfortunately, the very low quantity of extract did not allow to purify further the molecules and to identify them by amino acid sequencing. A second sampling of this relatively abundant species in 2016 was then planned the following year without any success. To date, there is no description of Ecdysozoa AMPs of a such small molecular weight (see introduction). Among the invertebrates including marine organisms, small sized AMPs (around 10 amino acids) have been only characterized in molluscs, annelids and echinoderms: Peptide 7 (865 Da, from the marine snail, *Rapana venosa*), Paracentrin 1 (1251 Da, from the sea urchin, *Paracentrotus lividus*) and Urechistachykinin I and II (respectively 1177 and 984 Da, from the echiuroid worm, *Urechis unicinctus*) (Dolashka et al., 2011; Schillaci et al., 2014; Sung et al., 2008).

Since there are no transcriptomic or genetic databases for the three nematode species studied here, a reverse genetic approach using degenerated primers designed from the amino acid sequences of small AMPs (such as those listed above), may be investigated in order to identify the AMPs of the present work even if the best strategy would be to get much more specimens from another sampling to finalize the identification of the bioactive molecules by bioassay-guided purification.

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