Identification, caractérisation et fonctions des peptides antimicrobiens chez les vers extrémophiles

Université de Lille

Laboratoire: Evolution, Ecologie, Paléontologie (EEP), UMR CNRS 8198

Ecole Doctorale Sciences de la Matière, du Rayonnement et de l'Environnement

THESE

Présentée et soutenue par Renato Bruno le 10/12/2020, pour obtenir le grade de Docteur en Sciences et Technologies, dans la spécialité Géoscience, Ecologie, Paléontologie, Océanographie

devant la commission d'examen :

Mme. Christine BRAQUART-VARNIER - Université de Poitiers - Rapporteur

M. Jean MARY - Sorbonne Université - Rapporteur

Mme. Priscille BRODIN - Institut Pasteur de Lille - Examinateur

M. Michael LAFOND - Aix-Marseille Université - Examinateur

M. Bruno FRANZETTI - Université Grenoble Alpes - Président

Mme. Daniela ZEPPILLI - IFREMER - Encadrante

Mme. Aurélie TASIEMSKI - Université de Lille - Directrice de thèse

PIONEER

2017-2020

TOTAL FOUNDATION

Acknowledgements

Over the course of the past 3 years, several people have contributed to the works included in this thesis. I am grateful to each one of these relentless co-operators.

I want to thank my professors and colleagues at my hometown university who paved the way that led me up to this point. In particular, I am thankful to Prof. Roberto Sandulli who involved me in the PIONEER project, giving me the opportunity to get in touch with Aurélie and Daniela, respectively my supervisor and co-supervisor. Thanks to them for believing in me since the beginning when no one would do so.

I am deeply grateful to Aurélie, for opening this opportunity and her guidance throughout these 3 years. In spite of the distance, Daniela has always been present at the most important moments of my thesis, always encouraging and supporting me.

My PhD experience would not have been the same without the patient and constant presence of Céline. I feel profoundly thankful for what she has done throughout these years.

I am also very grateful to all the people I met in Lille. The SPICY team welcomed me warmly since the very first days.
I am also glad to have spent valuable time with all the members of the lab EEP. The atmosphere was always relaxed and chill.
I am particularly grateful to Romuald (and his improbable French lessons), Christophe Van Brussel and Christophe Calarnou for their friendship and their help with French bureaucracy, and Alessandro & Natasha for their support during these years. I also want to thank Claudia, Laura, Nicolas, Leslie, Thomas and Marina: the lab would not be the same without this folk.

I want to thank the “Equipe CGIM” at the Institut Pasteur of Lille, where I spent the last year of my thesis. Everyone made me feel like one of the group, they supported me and helped me until the end, and we also shared lots of fun and exciting moments.

I am deeply grateful to Marc Maresca for hosting me at his lab in Marseille, he and Hamza, they made me feel at home. I have learned a lot from them in such a short time.

All my co-authors have been impeccable partners. I want also to thank all the members of my PhD committee and the members of the jury for taking part to and providing valuable feedback on my work.

Many important people also played a role in the backstage of this academic scenario. My family, first of all, always supported me. They are still trying to understand what a PhD is exactly, but their unconditional love and support has helped me since the earliest days. This thesis is dedicated to them.

A special thanks to Melissa, who have sustained and endured me for a long time during this journey, always believing in my potential and listening for my boring complaints.
I am deeply grateful to my housemates, especially Andrea and Rodrigo ("my younger brothers") for helping me feel like at home, for making the experience more enjoyable and much more.
I want to thank also Daniela L. and Marilù, with whom I shared some difficulties and some joys of this experience.
Last but not the list, the old friends from my hometown: I am blessed for having them on my side unconditionally.

The list of people included in these pages is not meant to be exhaustive.

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ABSTRACT

There has been a growing interest in and demand of new compounds such as antimicrobial peptides (AMPs) during the last decades because of emerging Multi Drugs Resistant bacteria. 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. They also contribute to symbiostasis in vertebrates and invertebrates by controlling, shaping, and confining the symbiotic microflora in specific anatomical compartments (gut, bacteriomes, skin).

Most of them (about 75%) come from animals among which only 2% of them belong to marine organisms. Marine AMPs are unique and structurally diverse presumably because they have evolved under the pressure of highly varying physicochemical conditions and high density of bacteria notably proteobacteria, the bacterial family generating the most problematic drug resistances in human at the present time.

The recent discovery of abundant and well-adapted worms in several extreme marine environments (polar, hydrothermal, abyssal, polluted, etc.), in co-occurrence with a large number and variety of bacteria, provided the opportunity to study an interesting source of unknown molecules with high antimicrobial potential.

In this context, the goal of this PhD was to expand the current knowledge on marine worms AMPs from extreme environments, analyzing how the external factors of worms habitat affect the structure and bioactivity of the peptides.

The manuscript starts with a general introduction explaining the concept of eco-immunology and why AMPs (in particular AMPs produced by extremophilic worms) were used as model to study eco-immunology. It is followed by a description of AMPs main features (their source, structure, composition, mode of action, biological activities, etc.). It ends with a review (published in Marine Drugs) that gives an overview on the state of the art about the different structures and functions of AMPs in worms (annelids and nematodes) to highlight the wide diversity and originality of their primary structures, that presumably mimics the highly diverse life styles and ecology of worms.

The first chapter of this thesis describes the search of new groups of AMPs from three species of marine meiobenthic nematodes inhabiting the anoxic sediments. Biochemical purification and identification of novel AMPs produced by these tiny species were investigated, providing evidences that two of the three Oncholaimidae nematodes sp. constitute interesting sources of small sized antibiotics. The limitations of applying biochemical method to such small animals, not raised in the lab with an unexpectedly random distribution were also discussed.

The second chapter examines the unique case of three members of BRICHOS-AMP family, polaricin, arenicin and alvinellacin from polychaetes living highly distinct habitats (polar, temperate and hot chimneys of hydrothermal vents respectively). We studied their adaptation to varying abiotic (thermal and pH variations) and biotic factors (environmental bacterial communities), providing a clear evidence of the adaptation of the biological activities to the environmental bacteria and the influence of the temperatures and the pH on the natural selection of AMPs. Because the number of disulfide bridges of the AMPs increases with the harshness of the worm habitat, we performed the same study with the AMPs devoid of disulfide bonds, showing their involvement in the thermal and pH stability of the peptides.

In the third chapter, the roles of BRICHOS domain from alvinellacin precursor were investigated. We recombinantly produced it and provide evidences of a chaperone-like function in the external immunity of worms, helping them to face extreme habitats.

From this thesis, we can conclude that extreme marine nematodes and annelids constitute valuable sources of promising bioactive substances, possessing peculiar characteristics (such as uncommon structure, pH- and thermo-tolerance). Moreover, they represent a remarkably attractive model to study AMPs evolution, as actors of worms' immune defence in extreme and fluctuating environmental conditions.

RESUME

L'intérêt et la demande de nouveaux composés tels que les peptides antimicrobiens (PAMs) se sont accrus au cours des dernières décennies en raison de l'émergence de bactéries multi-drogues résistantes.

Les PAMs sont en première ligne de la défense immunitaire innée de tous les organismes : elles apportent une réponse rapide à un large spectre de micro-organismes envahissants (bactéries, champignons, virus et parasites) et un moyen alternatif de les éliminer (principalement par la rupture de la membrane bactérienne) avec un développement lent de la résistance bactérienne, représentant une classe potentielle de nouveaux médicaments. Ils contribuent également à la symbiose chez les vertébrés et les invertébrés en contrôlant, modelant et confinant la microflore symbiotique dans des compartiments anatomiques spécifiques (intestin, bactériomes, peau).

La majorité des PAMs ont été trouvées chez les animaux (environ 75%) dont seulement 2 % appartiennent à des organismes marins. Les AMP marines sont uniques et structurellement diverses, probablement parce qu'elles ont évolué sous la pression de conditions physico-chimiques très variables et d'une forte densité de bactéries, notamment de protéobactéries, la famille de bactéries générant les résistances aux médicaments les plus problématiques chez l'homme à l'heure actuelle.

La découverte récente de nombreux vers bien adaptés dans plusieurs environnements marins extrêmes (polaires, hydrothermaux, abyssaux, pollués, etc.), en co-occurrence avec un grand nombre et une grande variété de bactéries, a fourni l'occasion d'étudier une source intéressante de molécules inconnues à fort potentiel antimicrobien.

Dans ce contexte, l'objectif de cette thèse était d'élargir les connaissances actuelles sur les PAMs chez les vers marins provenant d'environnements extrêmes, en analysant comment les facteurs externes de l'habitat des vers affectent la structure et la bioactivité des peptides.

Le manuscrit commence par une introduction générale qui explique le concept d'éco-immunologie et pourquoi les AMPs (en particulier les AMPs produits par les vers extrêmophiles) ont été utilisés comme modèle pour étudier l'éco-immunologie. Il s'en suit une description des principales caractéristiques des AMPs (leur source, leur structure, leur composition, leur mode d'action, leurs activités biologiques, etc.). Les généralités se terminent

par une revue (publiée dans Marine Drugs) qui dresse un panorama de l'état de l'art sur les différentes structures et fonctions des PAMs chez les vers (annélides et nématodes), afin de souligner la grande diversité et l'originalité de leurs structures primaires, qui imitent les styles de vie et l'écologie très variés des vers.

Le premier chapitre décrit la recherche de nouveaux groupes de PAMs provenant de trois espèces de nématodes méiobenthiques marins vivant dans sédiments anoxiques. La purification biochimique et l'identification de nouvelles PAMs produites par ces minuscules espèces ont été étudiées, fournissant des preuves que deux des trois nématodes constituent des sources intéressantes d'antibiotiques. Les limites de l'application de la méthode biochimique à des animaux aussi petits, non élevés en laboratoire avec une répartition géographique imprévue, ont également été discutées.

Le deuxième chapitre examine le cas unique de trois membres de la famille BRICHOS-PAM, la polaricine, l'arénicine et l'alvinellacine, issus de polychètes vivant dans des habitats très distincts (respectivement polaire, tempérée et les cheminées chaudes hydrothermales). Nous avons étudié leur adaptation à divers facteurs abiotiques et biotiques, ce qui a permis de mettre en évidence l'adaptation des activités biologiques aux bactéries environnementales et l'influence des températures et du pH sur la sélection naturelle des PAMs. Comme le nombre de ponts disulfure augmente avec la sévérité de l'habitat du ver, nous avons réalisé la même étude sur les analogues PAMs sans ponts disulfure, montrant leur implication dans la stabilité des peptides.

Dans le troisième chapitre, les rôles du domaine BRICHOS du précurseur de l'alvinellacine ont été étudiés. Nous l’avons produit par recombinaison et avons mis en évidence une fonction de type chaperon dans l'immunité externe des vers, les aidant à affronter des habitats extrêmes.

Cette thèse nous permet de conclure que les nématodes et annélides marins extrêmes constituent des sources précieuses de substances bioactives prometteuses, possédant des caractéristiques particulières (telles que structure peu commune, pH et thermotolérance). De plus, ils représentent un modèle remarquablement intéressant pour étudier l'évolution des AMPs, en tant qu'acteurs de la défense immunitaire des vers dans des conditions environnementales extrêmes et fluctuantes.

GENERAL INTRODUCTION

1. Preface: ecoimmunology and AMPs from worms

Ecological immunology (Figure 1A), known as ecoimmunology, is a relatively new interdisciplinary research field that examines causes and consequences of natural variation in immune system through its interactions with the environmental factors and the relations host-microbes [1].

The field of immunology classically examines the physiological and molecular processes underlying host defense against pathogens, usually on laboratory models and under optimal conditions (in absence of environmental constraints). The role and the regulation of immune effector systems under the constraints imposed by life history and ecology came into focus ten years ago [2]. Ultimately, understanding how the immune system works and that its function is context-dependent led researchers from the laboratory to the natural world to understand the influence of the biotic and abiotic factors of the environments on the host immune system (genotype and phenotype; Figure 1B) [3]. To do so, ecoimmunology uses techniques from traditionally laboratory-based disciplines (such as immunology, genomics,

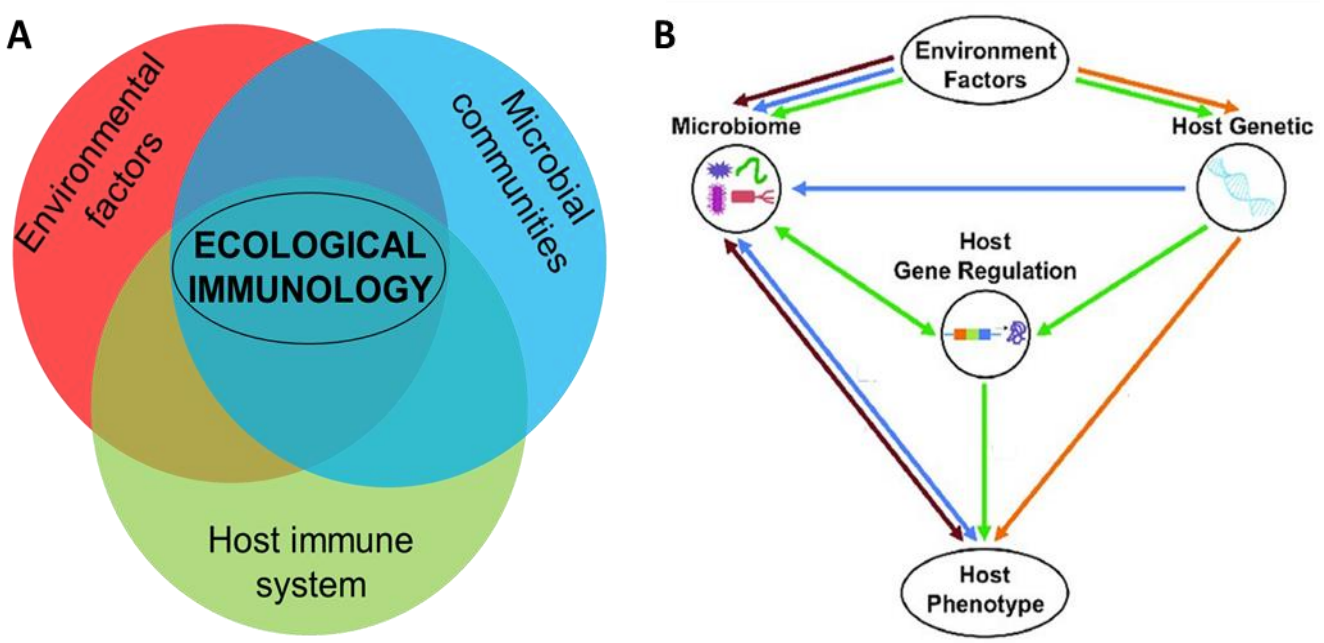


Figure 1: (A) Definition of ecoimmunology as an emerging science studying how the immune system of organisms varies according to the external environment and its relationship with microbial communities; (B) Possible direction of host–microbiome–environment interactions in the context of host phenotypes (Awany et al., 2019).

proteomics and cell biology) to reveal how the immune system of wild organisms shape and/or respond to ecological and evolutionary pressures.

AMPs are in the first line of innate immune defence of all organisms, providing 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 [4–10]. They appear to be essential anti-infectious factors, been conserved during evolution in strategic location at the interfaces between the organisms and its environment, such as phagocytes, body fluids and at epithelial level [11–15]. Therefore, AMPs appear as attractive subjects to study ecoimmunology: in particular, those produced by invertebrates because of the simplicity of their innate immune system mechanisms and for the ease of their physiological manipulation in the context of life history and ecology.

More specifically, the abundance (representing the dominant benthic fauna in terms of biomass and species richness) and wide distribution of worms (annelids and nematodes) in terrestrial, marine, and freshwater habitats (including extreme ones), in coexistence and coevolution with a large number and variety of microbes, make them perfect models for studying their immune systems through ecoimmunology [16,17]. To date, several studies demonstrated that many worms have evolved a variety of physical and chemical defence mechanisms, for instance antimicrobial metabolites [11,12,18–22]. Mostly, they present no shell or a proper exoskeleton (their body is directly exposed to the environmental constraints), hence their antimicrobial secretions represent an extended arm of the immune system (external immune defence) [23]. Moreover, they occupy a key position in the trophic network, as a major food source for fishes, birds and terrestrial fauna.

Nematoda is an ancient animal phylum of unsegmented microscopic roundworms, the only group of multicellular animals (metazoans) that are pervasive in sediments and soils, where they often outnumber other animals [24,25]. This phylum, belonging to the ecdysozoan group, contains to date more than 27,000 described species that most likely represent only a small portion of the total [26]. Nematodes exist in marine, freshwater and terrestrial ecosystems, as well as in plants and animals.

Free-living nematodes have successfully adapted to nearly every ecosystem on Earth, being the most abundant animals in sediments and soils. The ecological success of nematodes is strongly linked to their ability to feed on various food sources that are present in both sediments and soils, and to proliferate rapidly and survive in contrasting environmental conditions [26].

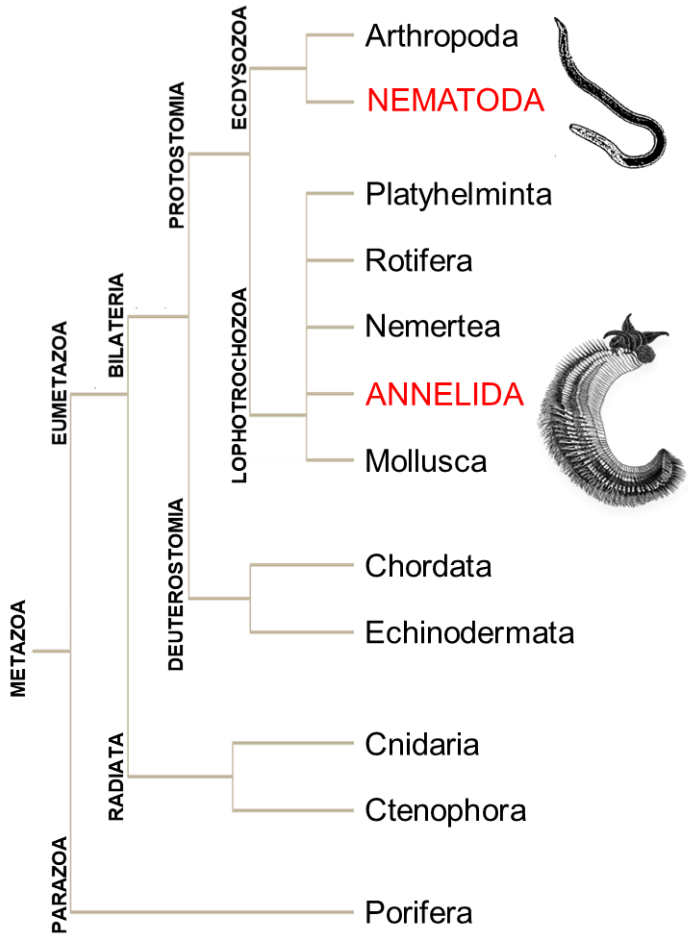


Figure 2: Phylogenetic three of metazoan. In red the phyla (Annelida and Nematoda) object of this thesis.

Annelids (also called segmented or ringed worms) constitute one of the major protostome phyla, within the lophotrochozoan group: they are primitive coelomates that have developed both cellular and humoral immunity against pathogens (ingested during feeding and/or introduced into the body after injury) [27]. This phylum is ecologically and morphologically diverse, comprising over 21,000 described species, whose members occupy a wide range of environments and show high diversity in life modes, feeding and reproductive strategies, body forms and developmental patterns [28].

Formerly, annelids are classified into three main groups: Polychaeta (lugworms), Oligochaeta (earthworms) and Hirudinea (leeches): the large majority of polychaetes is restricted to the marine domain, whereas oligochaetes and leeches can be terrestrial, semi or fully aquatic in freshwater or more rarely in seawater [28].

The combination of these features makes annelids and nematodes interesting subjects to study the evolution of immune genes (such as AMPs) in conjunction with the abiotic and biotic variations of the environments [29,30]. Currently, a growing interest in research is devoted to worms as promising sources for the discovery of novel and unique compounds having a plethora of activities (antimicrobial, antiviral, antifungal, etc.) and applications (reviewed in [31]).

After the discovery in 1989 of cecropin P1 [32], the first nematode AMPs (from the parasite *Ascaris suum*), efforts were mostly focused on the terrestrial genetic model *Caenorhabditis elegans* [33–35]. Later, several groups of AMPs were identified in nematodes: defensin-like antibacterial factors (ABFs), caenopores, caenacins (CNCs) and neuropeptide-like (NLPs) [36–43] (reviewed in [19]).

By contrast to nematodes, most annelid AMPs were biochemically isolated from diverse wild species from different taxa. The first annelid AMP was lumbricin-1 isolated from the earthworm *Lumbricus rubellus* in 1998 and later in leeches [44]. In 2004, the first member of the macin family (theromacin) was characterized in leeches [22]. Another family of AMPs characterized in annelids is the cysteine-rich BRICHOS family [31]; the first member was arenicin isolated from the body fluid of *Arenicola marina*, followed by other marine polychaetes [11,12,45].

Therefore, it seems reasonable to assume that marine worms (nematodes and annelids) are interesting and potential source of still undiscovered bioactive substances, such as AMPs (subject approached in the Chapter 1 and Chapter 2 of this thesis).

2. History of AMPs

The discovery of the first antibiotic can be attributed to Fleming in 1922, finding out the activity of lysozyme in bacterial inhibition [46].

Before the 1980s, other research led to the discovery of several non-gene encoded antibiotics: in 1928, Rogers and Whittier noticed the antibacterial activity of nisin, from fermenting milk cultures [47]; in 1939, Dubos identified gramicidin from a soil bacterium *Bacillus brevis* [48]; and in the 1960s, Zeya and Spitznagel discovered that basic proteins and peptides in polymorphonuclear (PMN) leukocytes display antimicrobial properties [49].

At the beginning of 1980s, Boman and his group showed an active molecule (cecropin) responsible for the strong antibacterial activity in almost all investigated invertebrates, thus paving the way of gene-encoded AMPs as potential antimicrobials [50]. The discovery of the first vertebrate peptides followed, with the identifications of human α-defensins by Lehrer (in 1985) and frog magainins by Zasloff (in 1987) [51,52].

The discovery and the interest in new AMPs have increased exponentially over the last decades: the following graph shows the number of AMPs publications and patents for year (Figure 1) [53].

Persistent use and /or misuse of antibiotics (in humans, agriculture, animal farming, and industry), self-medication, and exposure to infections in hospitals has provoked the emergence of multidrug resistant (MDR) organisms [54].

It is estimated that globally approximately 700,000 deaths are attributed annually to

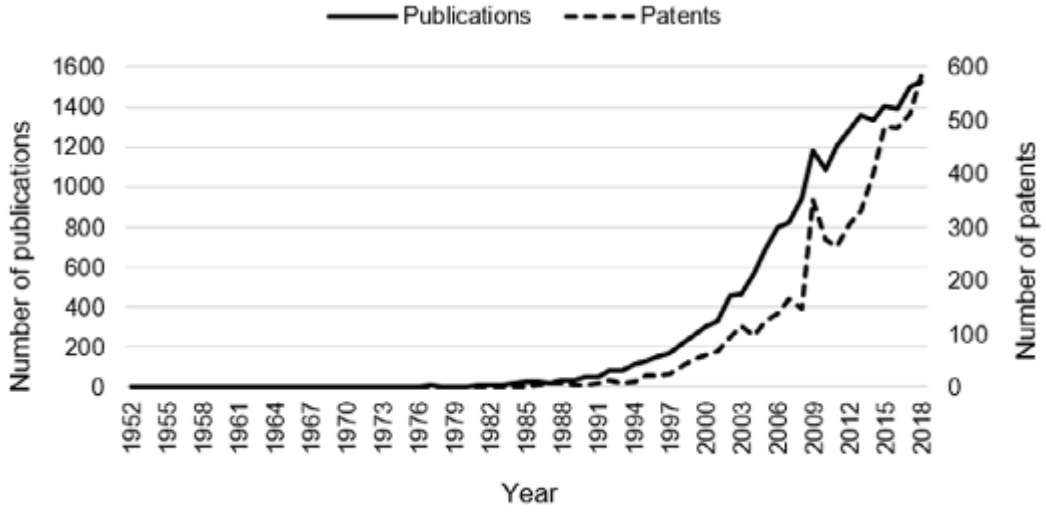


Figure 3 : The number of publications and patents per year for AMPs (Koo and Seo, 2019).

antimicrobial resistance and this could rise to 10 million deaths per year by 2050 [55]. This

emergence in the last decade, also declared by the World Health Organization (WHO), has led to major US government incentives for antimicrobials and an acceleration to bring them into clinical use by the US Food and Drug Administration (FDA) [30,56–59].

The Antimicrobial Peptide Database (APD), originally created by Zhe Wang and Guangshun Wang, was open to the public in August 2003. It originally contained 525 peptide entries. Currently, the APD contains more than 3200 AMPs (last access on 01/10/2020), isolated from bacteria, invertebrates, vertebrates and plants, diversified by rapid evolution between species [60]. Approximately 75% of them come from animal sources, followed by plants and bacteria (Figure 2). Only two percent of AMPs from animals has been characterized and identified from marine organisms (fish, sponge, annelid, echinoderm, *etc.*), mainly because the marine fauna has been dramatically less sampled [11,12,61,62].

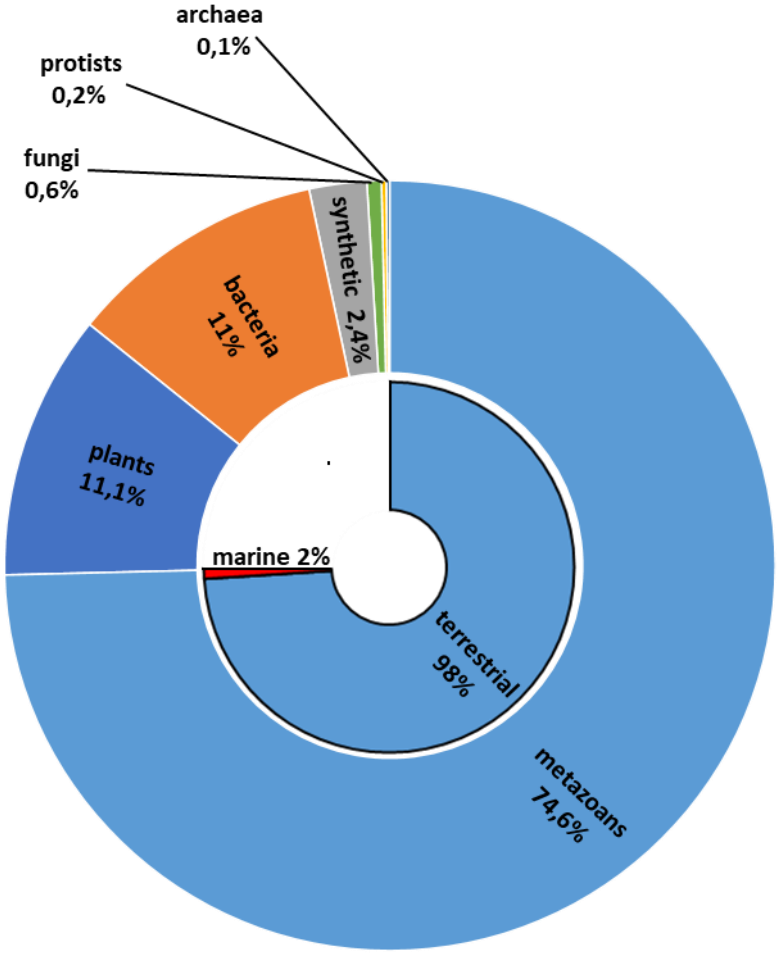


Figure 4: Sources of AMPs (total 3248). Numbers obtained from the APD (Wang 2016), last access on 01/10/2020.

3. Main common features of AMPs

AMPs are small sized molecules, ranging from 5 to one hundred amino acids (less than 10 kDa in mass), with potent antibacterial, antiviral (such as cecropin, indolicin, tachyplesins, targeting important human viruses like influenza, HIV, HCV and SARS) and antifungal activity (such as histatins, penaeidins and gomesin) [4–10,63]. AMPs are ubiquitous, being part of the innate immune defense system of multicellular organisms, microorganisms, plants and animals to help them fight against external invading pathogens and to maintain the commensal population stable [64,65].

In Figure 3A is plotted the number of AMPs as a function of peptide length: the majority of AMPs (~90%) consist of less than 50 amino acids and the peak is at 30; the shortest AMP contains only 5 amino acid and the longest contains 100 amino acids (arbitrary definition of peptides) [60].

Mostly, AMPs have the common characteristics of being short, cationic, hydrophobic, with amphipathic and/or membranolytic properties enabling them to interact, penetrate and/or

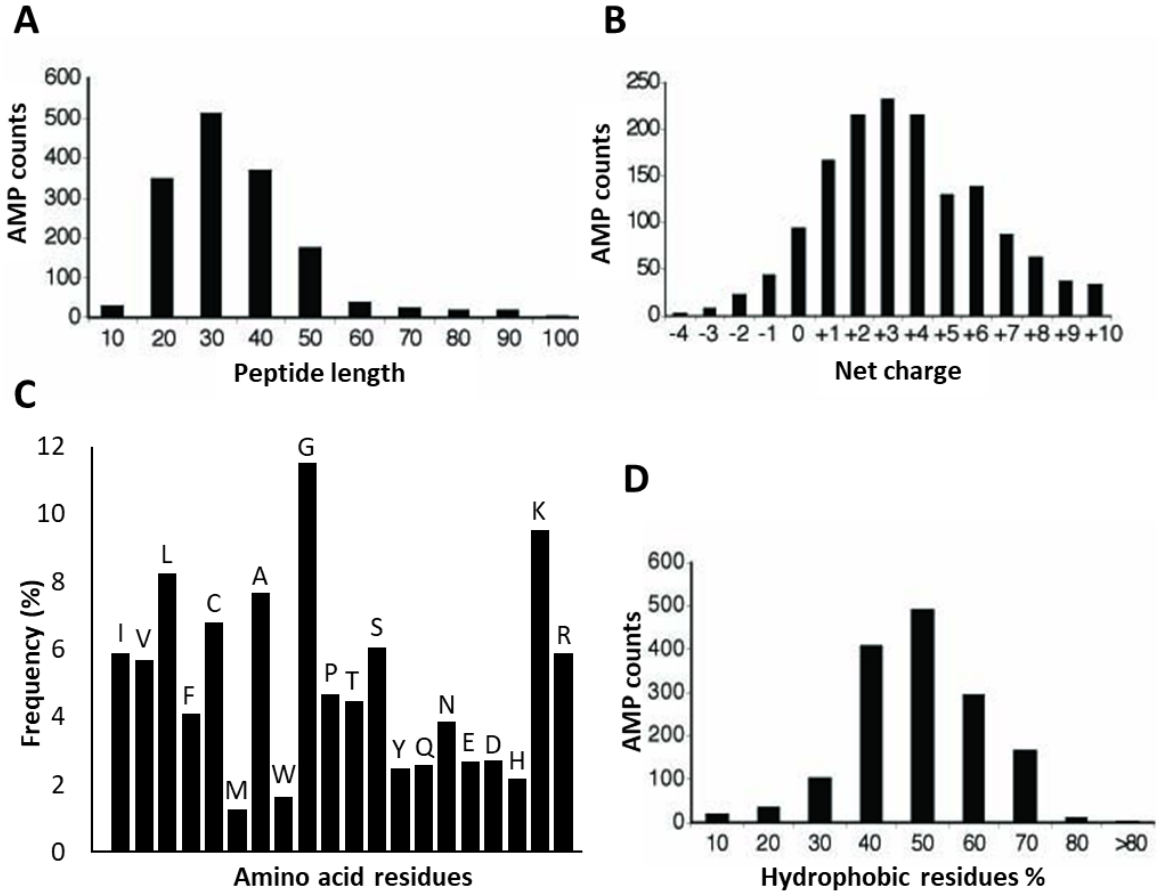


Figure 5: Distribution of AMPs versus (A) peptide length, (B) net charge, (C) percentage of amino acid residues frequency in AMPs sequences and (D) percentage of hydrophobic residues. A total of 3248 peptides are included in the analysis (data by APD, (last access on 01/10/2020), Wang 2016).

disrupt the bacterial membranes [66]. In particular, the initial electrostatic attraction occurs between peptides cationic charge and the negatively charged phospholipids or peptidoglycans, in the outer membrane of respectively Gram-negative and Gram-positive bacteria [67]. Figure 3B shows the number of AMPs as a function of net charge: they are distributed around the peak at +3; 83% are positively charged, 7% are neutral and 6% are anionic (such as theromyzin, isolated from the coelomic liquid of the leech *Thermyzon tessulatum*) [22,68]. In terms of amino acid residues, the hydrophobicity is defined as the proportion of hydrophobic residues Isoleucine (I), Valine (V), Leucine (L), Phenylalanine (F), Cysteine (C), Methionine (M), Alanine (A) and Tryptophan (W) within a peptide, is typically around 50% (Figure 3D). The most frequent residues (with a percentage of approximately 10% or greater) are leucine (L), glycine (G), alanine (A), lysine (K), abundant in AMPs with α-helical structures [69]; in contrast, arginine (R) and cysteine (C) residues are typical in AMPs with known β-hairpin structures (Figure 3C) [11,12,70].

The hydrophobic component of the peptide is required for subsequent peptide anchoring to the bacterial membrane surface: the combination of positive charge and hydrophobicity explains the amphipathic nature of the majority of AMPs (Figure 6).

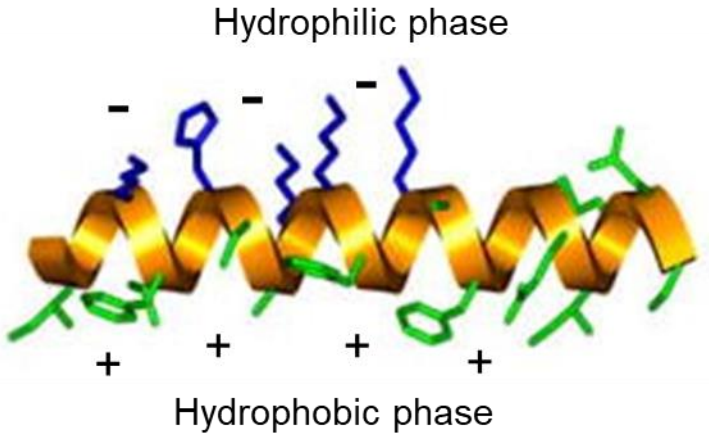


Figure 6: Schematic representation of an amphipathic α-helical peptide with its typical opposite disposition of hydrophobic and hydrophilic phases (respectively in green and blue).

It is proposed that the positively charged, hydrophilic face drives the initial electrostatic attraction to the negatively charged components of the microbial membrane, and the hydrophobic face then inserts into the membrane through van der Waals interactions, leading to loss of the membrane function and increased permeability. Disrupting the amphipathic structure of AMPs can influence their mechanism of action and pore formation [71].

Hydrophobicity play also a role in peptides antimicrobial activity: in α-helical peptides, higher hydrophobicity is correlated with stronger peptide antimicrobial and cytotoxicity activities; a further increase in hydrophobicity will result in a decrease in antimicrobial activity, probably due to increased dimerization, which prevents access to the membrane in prokaryotic cells [72,73]. Recently it was showed that the increase in amphipathicity has led to different effects on β-sheet peptides (arenicin-3, tachyplesin-1, gomesin, polyphemusin-1, protegrin-1 and thanatin), improving the antimicrobial activities of some of them against specific and various bacterial strains [74].

In conclusion, the strategy for a "good AMP" (maximize antimicrobial activity and minimize toxicity) is not determined by a single factor but by a subtle combination of factors such as the sequence, net charge, hydrophobicity and position of cationic residues (Figure 7). In AMP design, all the biochemical determinants discussed above need to be considered together since there is an interdependent relationship between them. Changing one of these parameters to achieve a desired modification of an AMP may alter other parameters which may be essential for the activity of that AMP and its range of target cells [71].

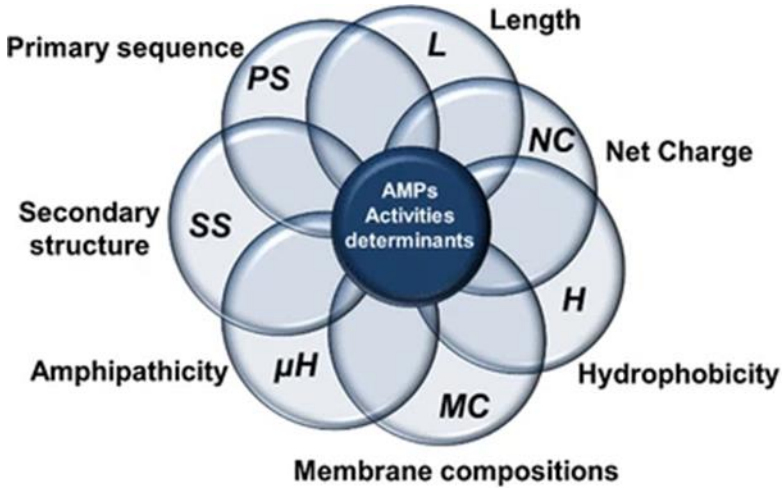


Figure 7: Interdependent molecular determinants of biological activities of AMPs. All parameters collectively determine the efficiency, spectrum of activity, mechanism of action and cell selectivity of AMPs (Shagaghi et al. 2018).

Consequently, there is no strict rule regarding the optimal number of charged and hydrophobic residues for maximum antimicrobial activity and minimum cytotoxicity. Predicting the effects of an AMP modification and/or the function of a synthetic AMP, are still among the challenges in this field.

- RiPPs and NRPs

Natural peptides are biosynthesized by one of two pathways, ribosomally or non-ribosomally, generating ribosomally (RiPPs) and non-ribosomally (NRPs) synthesized peptides. All living organisms contain ribosomes, molecular machines that perform translation of messenger RNA to protein, within their cells. More recently a non-ribosomally machine have been discovered in some bacterial and fungal species.

RiPPs are a diverse group of biologically active molecules, produced by many different organisms and particularly by bacteria (bacteriocins); they are characterized as important defense against microorganisms [75]. RiPPs are ribosomally synthesized as pro-peptides, consisting of an N-terminal signal peptide sequence, a pro-region, and a C-terminal sequence with antimicrobial activity (the peptide itself) once it is cleaved from the rest of the pro-peptide via proteolytic enzymes [76].

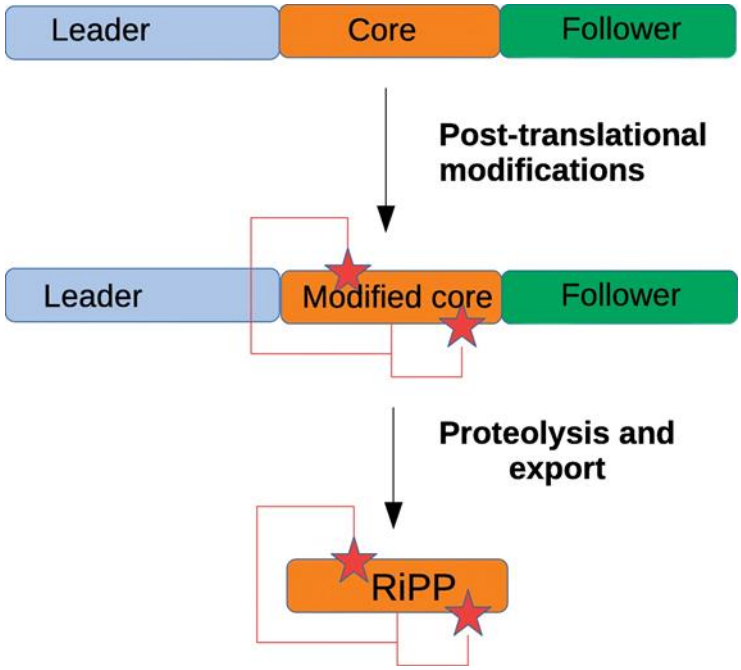


Figure 8: General RiPP biosynthetic pathway. The leader and follower peptide direct the modifications (e.g. addition of functional groups, indicated by stars, or formation of additional bonds, indicated by the connective lines) on the core peptide. After removal of the leader and follower sequence the mature RiPP is released (figure by Vignolle et al. 2020).

NRPs are biosynthesized by a chain of modules (the non-ribosomal peptide synthetases, NRPS), each module contributing a specific amino acid and modifying the growing peptide as it is passed along the chain. Every module is generally composed of an adenylation domain (A), a peptidyl carrier protein (PCP), and condensation domain (C) [75]. The number of

modules is usually consistent with the number of amino acids comprising the final product, except for the case of dimerization or polymerization of the peptide, produced by a single assembly line [77]. NPRs are often quite complex in structure and difficult to synthesize: unlike them, the RiPPs cannot explore amino acids beyond the canonical 20 proteinogenic amino acids, limiting their structural diversity to some degree [78].

4. Diversity of AMPs in animals

Despite their similar general physical properties, AMPs have very low sequences homology and a wide range of secondary structures, with at least four major groups (Figure 4): α-helical peptides, β-sheet peptides, αβ and non-αβ families [79].

The α-helix peptides contain one or more helices, with spatially disjunct hydrophobic and hydrophilic surfaces (Figure 4A) [80]. Most of these peptides are unstructured in aqueous solution but become structured when in contact with trifluoroethanol, detergents/surfactants above critical micellar concentration. Upon association with bacterial membranes, usually the helix-bundle structure may form pores in the membrane thanks to multiple exposed hydrophobic side chains. This is the case of cecropins and saposin-like protein (SAPLIP) family (such as caenopore-5), from *Caenorhabditis elegans* [42,81]. As for hedistin, from the marine annelid *Hediste diversicolor*, it was hypothesised bacterial membrane disruption through a carpet model (Figure 5), due to peptide 3D structure (forming a helix–bend–helix conformation) [21,82].

The AMPs of β-sheet family (deeply described below, see paragraph 7) possess the characteristic structure consisting in two anti-parallel β-sheets linked by a small turn of 3 to 7 amino acids, forming a hairpin shape (Figure 4B). They are stabilized by intra-backbone hydrogen bonds and one or more disulfide bonds between cysteine residues [83]. β-sheet AMPs are more structured in solution and do not undergo major structural changes when going from an aqueous environment to a membrane environment.

The family of αβ peptides contains all AMPs that have mixed structure, with both β-sheets and α-helices (Figure 4C) [84]. Macins, described in leeches (*Theromyzon tessulatum* and *Hirudo medicinalis*), and ABFs (antibacterial factors, characterized only in nematodes) belong to this

family [15,22,36,39,85]. For macins family has been demonstrated the barnacle model, and pore formation for neuromacin and ABFs (such as As-ABF-alpha and Ce-ABF2) [39,86,87].

The members of non-αβ family form neither α-helix or β-sheet structures (such as extended coil peptides), containing a high proportion of one or two amino acids (usually proline, glycine, tryptophan, etc.) often essential for their antimicrobial activity (Figure 4D) [88]. Neuropeptide-like (npls) and caenacins from nematodes and lumbricins, perinerin and ms-hemerycin from annelids belong to this family [20,37,38,44,85,89].

Out of all the AMPs reported, only about 40% of them have had their secondary structure characterized: amongst them, around 70% adopt α-helical, 12% β-hairpin, 17% αβ and less than 1% non-αβ secondary structures [60].

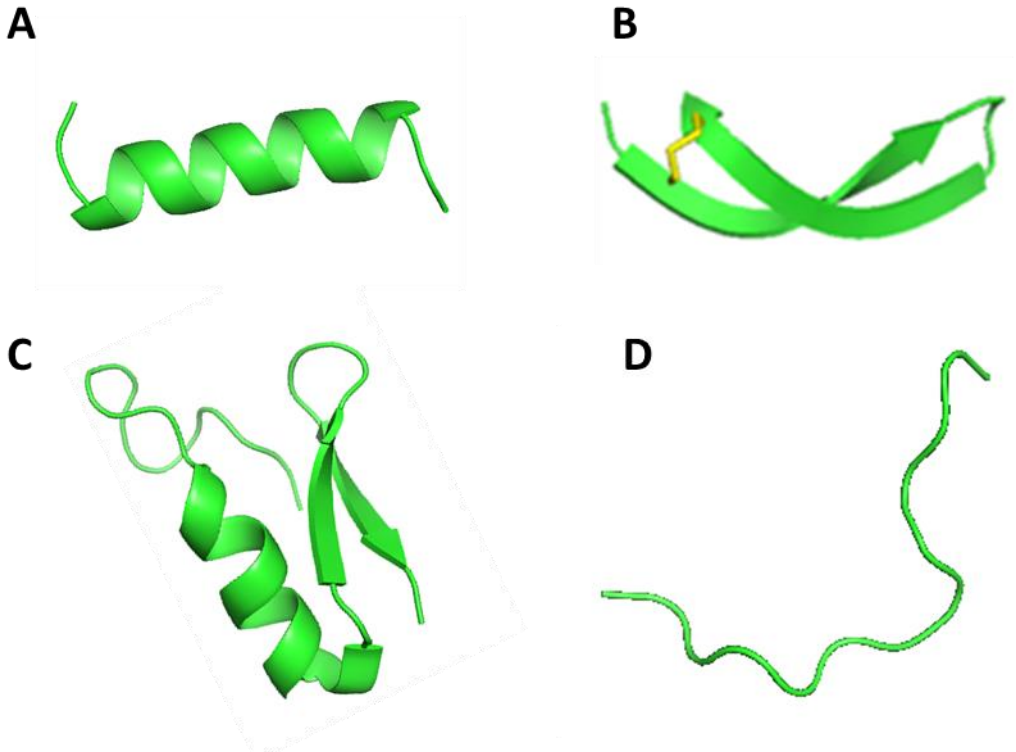


Figure 9: Structural diversity of antimicrobial peptides (AMPs). (A) α-helical peptide (frog magainin, PDB: 2MAG); (B) β-sheet peptide (arenicin-1 from *Arenicola marina*, PDB: 2JSB), with disulfide bridge in yellow; (C) αβ-peptide (sapecin-A from *Sarcophaga peregrina*, PDB: 1L4V); (D) non-αβ extended peptide (bovine indolicidin, PDB: 1G89). The figures were generated using PyMOL (TM) 2.3.2 software: BioLuminate, Schrödinger, LLC, New York, NY, 2019 (www.pymol.org).

5. Mode of actions (MOA)

Broadly, the direct killing AMPs mechanism of action can be divided into membrane targeting and non-membrane targeting.

AMPs target a fundamental difference in design between the bacterial membrane and the membrane of multicellular animals. The outer surface of Gram-positive and Gram-negative bacteria contains mostly lipids with negatively charged head groups, allowing the initial electrostatic attraction with cationic AMPs [90,91]. Whereas the outer leaflet of the animal membranes are made up of zwitterionic phospholipids such as phosphatidylcholine, sphingomyelin and other neutral components such as cholesterol [92].

Moreover, some AMPs (like protegrins-1, magainin-2, melittin, LL37, etc.) are even sensitive to other properties of the lipids (not just the charge); membrane curvature, for instance, may also play an important role [93].

It is assumed that membrane-targeting AMPs disrupt bacterial membranes via pore formation (barrel stave or toroidal models), or by non-pore mechanisms, such as a carpet-like mechanism (Figure 5). They usually accumulate at the membrane surface of the bacteria (negatively charged); then, above a certain concentration threshold, they disrupt or penetrate the membrane [94]. In pore models, the peptides begin to orientate perpendicular to the membrane and insert into the bilayer: in the toroidal model, the peptides are always associated with the lipid head groups; in the barrel-stave model, they form a bundle in the membrane with a central lumen (the peptides represent the staves of the barrel). Alternatively, in the carpet model (Figure 5), the peptides cover the membrane surface orientated in parallel and at high concentrations they disrupt the bilayer in a detergent-like manner, leading to the formation of micelles [95]. Some amphipathic helical cationic antimicrobial peptides (i.e. analogs of magainin 2), polarize the membrane forming anionic lipid clusters [96].

A minority of AMPs, however, do not cause membrane disruption (non-membrane targeting peptides): after crossing the bacterial cell membrane, they interfere with pathogen microbial function or survival by binding to intracellular targets such as ribosomes and RNA polymerases [97,98].

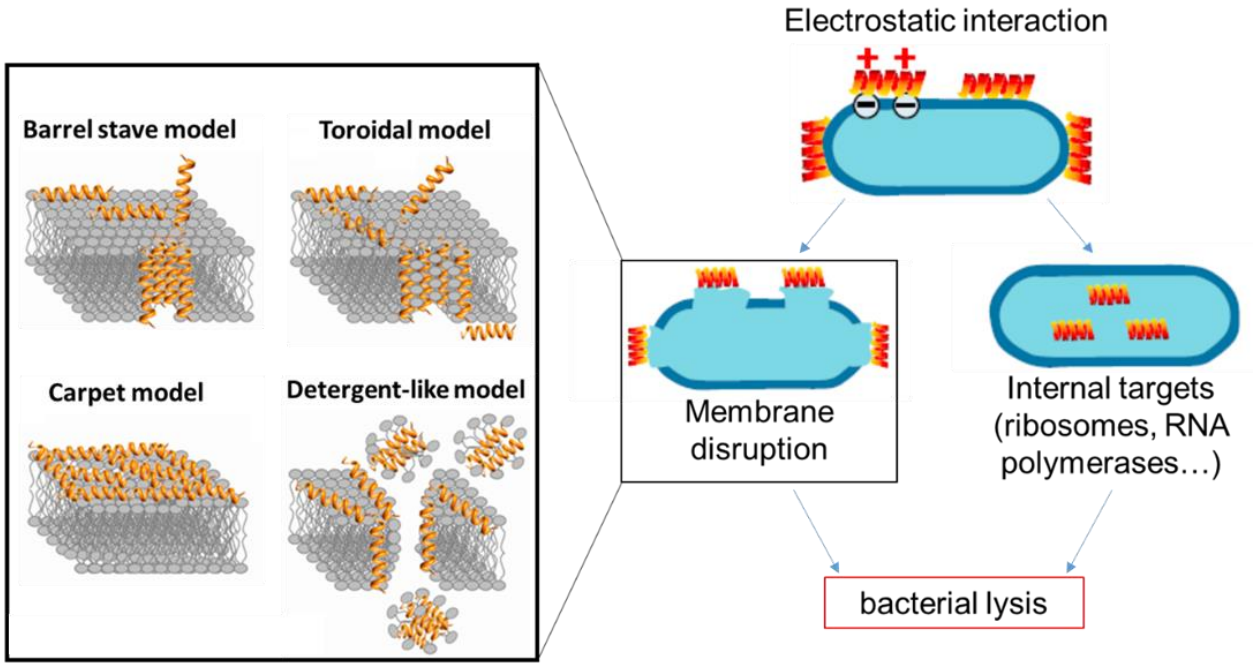


Figure 5: Schematic representation of peptide (in red) interaction with bacterial membrane (in blue). In the black box on the left, representation of the most common AMPs mechanisms of action (by pore formation), Kumar et al., 2018.

- Bacteriostatic *versus* bactericidal activity

Two main classes of AMPs can be distinguished (i) bacteriostatic, restricting the growth and reproduction of the bacteria, such as theromyzin and theromacin against *Micrococcus luteus*, and (ii) bactericidal, causing bacterial cell death (more than 99.9% in 18-24 hours), like hedistin against *M. luteus* and *M. nishinomiyaensis* [21].

The in vitro microbiological determination of bactericidal or bacteriostatic activity depends on the bacterial strain and may be influenced by growth conditions (medium, temperature, pH, salinity, etc.) bacterial density, test duration, and extent of reduction in bacterial numbers [99]. Minimum bactericidal concentration (MBC) assay determines (under strict laboratory conditions) if the peptides activity is bactericidal (or bacteriostatic), expressed as the lowest peptide concentration, causing an at least 99.9% reduction in the number of microorganisms (evaluated as colony forming units) [100].

- Cytotoxicity

Most of the AMPs are selective activity against bacteria: their cationic nature enables the peptide to target anionic pathogens rather than host cells, which are rich in zwitterionic lipids and cholesterol in the membranes. However, some AMPs appear to be poisonous to human

cells and/or to red blood cells (such as LL-37, DP1, AMPs from spiders and scorpions venom, arenicins and derivates, etc.), as they are highly haemolytic especially at high concentration [101–105]. Therefore, a thorough evaluation of the potential cytotoxicity of AMPs is necessary.

6. Application of AMPs in medicine

In this era of MDR's rapid spread, AMPs are one of the most promising classes of potential drug candidates [106]. In the past several decades, some AMPs have already been introduced into the market and many are in the process of clinical trials [59]. The overall drug

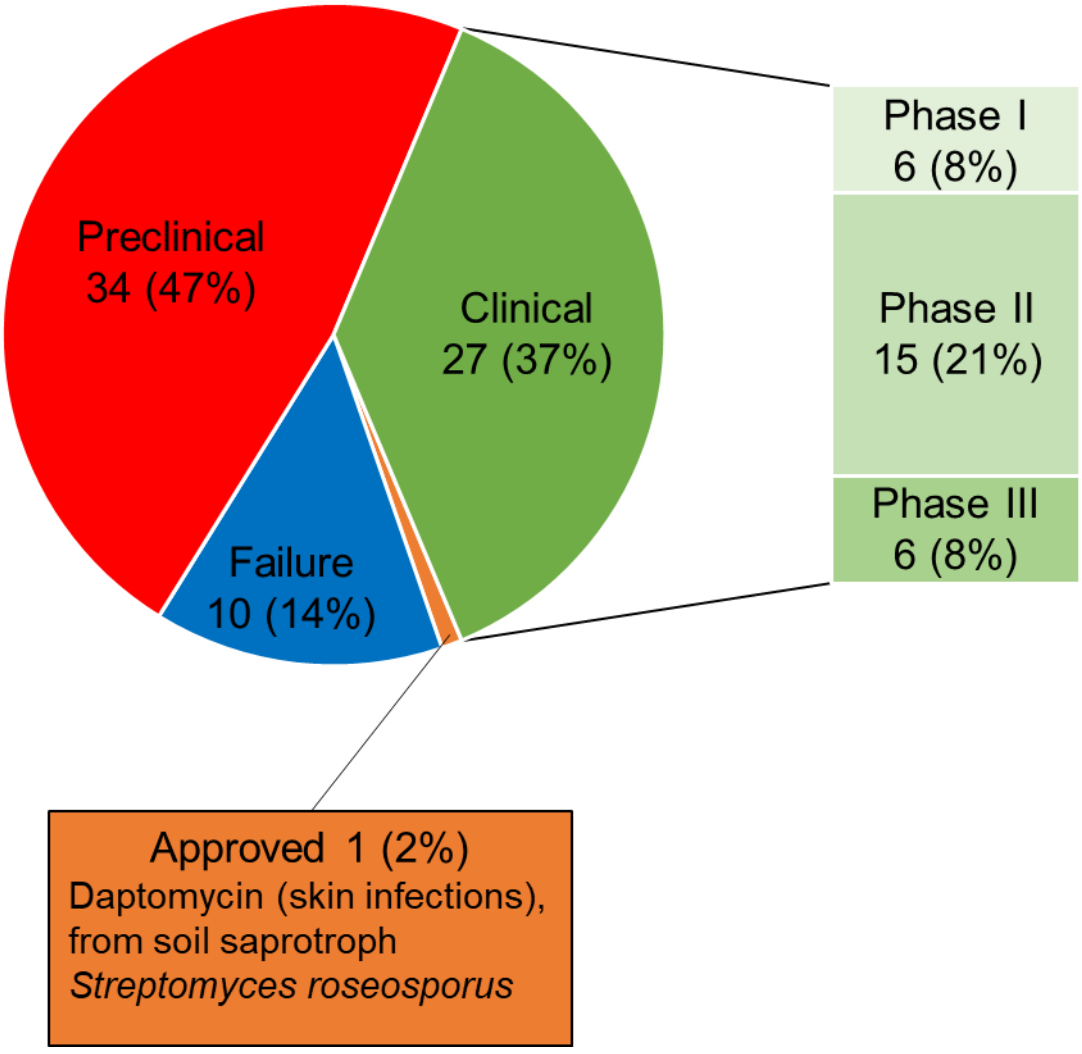


Figure 10: Chart pie representing the number (and percentage) of AMPs under different phases of FDA (U.S. Food and Drug Administration) protocols (Koo and Seo, 2019).

development is a complex process and it takes several years (between 10 and 15) before marketing approval, as the requirements of FDA guidelines (Figure 10) [107].

During preclinical research, the drugs undergo to laboratory and animal testing to answer basic questions about safety (detailed information on dosing and toxicity levels). The clinical phases study the interactions between the drug and the human body: starting from a small group of people (Phase I) to several thousands of volunteers, the researchers can confirm drug effectiveness, monitor side effects, compare it to commonly used treatments, and collect information that will allow the experimental drug or treatment to be used safely [53,108].

Table 1, adapted from a recent review of Patrulea et al. 2020, lists the AMPs at present time approved by FDA and commercialized for topical medications, including those in preclinical and in clinical phases [106]. The source of these AMPs was added to the list. All FDA-approved AMPs were discovered in Gram-positive soil bacteria (already source of many conventional antibiotics); arenicin, from *Arenicola marina* (a marine polychaete) is in preclinical phase [12]. These peptides are small (molecular weight between 1145 and 1882) have mostly been utilized to treat bacterial skin infections, pink eye, or wounds: Neosporin® (gramicidin), Cubicin® (daptomycin), Vancocin®HCl (vancomycin), Orbactiv® (oritavancin), DalvanceTM (dalbavancin), and Vibativ® (telavancin) [53,106–108]. Most of these lipopeptide antibiotics (except colistin) are used for treating Gram-positive bacterial infections, and only a few of them have been administered as oral solutions or tablets because of their poor penetration of the intestinal mucosa (such as oral vancomycin, limited to the treatment of *Clostridium difficile* diarrhea and staphylococcal enteritis) [108]. AMPs to treat infections caused by Gram-negative bacteria are clearly needed.

Despite the strengths of AMPs (broad-spectrum of activity, high effectivity against Gram-negative bacteria, less resistance to their activities, etc.) [95], their production is much more expensive than conventional antibiotics, limiting their rapid development [53].

Table 1: Selected AMPs for topical application under different phases of preclinical and clinical trials, including AMPs FDA-approved based on their reported activities (Patrulea et al., 2020) [106]. 1Oritavancin and telavancin may also act by membrane-pore, channel formation, or lysis of the cell membrane; 2VISA: vancomycin-intermediate S. aureus; 3VRSA: vancomycin-resistant S. aureus; 4MSSA: methicillin-susceptible Staphylococcus aureus; 5LPS: lipopolysaccharides; Gram (+): Gram-positive bacteria; Gram (−): Gram-negative bacteria. MIC: minimal inhibitory concentration.

AMPs	Mechanism of Action	Activity Against	Side Effects	Application and Administration Route	Source
FDA Approved					
Anidulafungin (Eraxis™) in 2006	Inhibition of (1,3)-β-D-glucan synthase	Fungi; *C. albicans* and *K. crusei* (MIC: 0.06 μg/mL)	Hypersensitivity; hepatic effects	Treatment of *Candida* infections, especially esophageal candidiasis; IV infusion	Semisynthetic from *Aspergillus nidulans* (fung.)
Caspofungin (Cancidas) in 2001	Inhibition of β (1,3)-D-glucan production	Fungi; *C. albicans*; (MIC: 0.06 μg/mL); *K. crusei* (MIC: 0.25 μg/mL)	Hypersensitivity; hepatic effects	Treatment of esophageal candidiasis; IV	Semisynthetic from *Glarea lozoyensis* (fung.)
Dalbavancin (Dalvance™) in 2014	Inhibition of bacterial cell wall synthesis	Gram (+); *S. aureus* (MIC: ≤0.008–0.5 μg/mL)	May cause nausea, headache, and diarrhea	Treatment of complicated skin and skin structure infections (cSSSI); IV injection	Semisynthetic from *Streptomyces orientalis* and *Actinoplanes teichomyceticus* (bact.)
Daptomycin (Cubicin®) in 2003	Membrane lytic	Gram (+); MRSA (MIC: 0.25–1 μg/mL); VISA (MIC: 1–8 μg/mL); VRSA (MIC: 0.12–1 μg/mL)	Not approved for pediatric patients	Treatment of cSSSI; IV injection	*Streptomyces roseosporus* (bact.)
Gramicidin (Neosporin®) in 1955	Pore-forming; aggregation; membrane disruption	Gram (+), (−); *E. faecalis* (MIC: 8–16 μg/mL)	Hemolytic activity	Treatment of bacterial conjunctivitis; ointment	*Bacillus brevis* (bact.)
Polymyxins (Polymyxin E = colistin) in 1964	Membrane disruption	Gram (−); *P. aeruginosa* (MIC: 8 μg/mL); *E. coli* (0.5 μg/mL)	Used only as "last-resort" due to neuro- and nephrotoxic effects and neuromuscular blockage	wounds after operations, in superficial eye infections, and to protect minor wounds against infections; cream, ear and eye drops	*Bacillius polymyxa* (bact.)
Oritavancin (Orbactiv®) in 2014	Inhibition of bacterial cell wall synthesis and disruption of bacterial membrane [1]	Gram (+); MRSA and MSSA (MIC: ≤0.008–0.25 μg/mL)	Long-term treatment is ambiguous	Treatment of (cSSSI); IV	Semisynthetic from *Amycolatopsis orientalis* (bact.)
Telavancin (Vibativ™ and Vibativ®) in 2009	Inhibition of bacterial cell wall synthesis and disruption of bacterial membrane [1]	Gram (+); VRSA (MIC: 2–4 μg/mL); VISA (MIC: 0.25–1 μg/mL)	May induce acute kidney injury	Treatment of cSSSI; IV	Semisynthetic from *Amycolatopsis orientalis* (bact.)
Vancomycin (Vancocin® HCl) in 2016	Inhibition of bacterial cell wall synthesis	Gram (+); *Candida* spp. (MIC: >256 μg/mL); MRSA (MIC: 0.5–2 μg/mL); VISA (MIC: 4–8 μg/mL); VRSA (MIC: 32–>64 μg/mL)	May cause nephrotoxicity	Treatment of severe MRSA infections; IV and oral	Semisynthetic from *Amycolatopsis orientalis* (bact.)
Preclinical Phase					
Arenicin	Membrane pore formation	Gram (+), (−); MRSA infection; *E. coli* (MIC: 1 μg/mL); *K. pneumoniae* (2 μg/mL)	Significant toxicity to mammalian cells	Urinary tract infections; hospital-acquired infections	*Arenicola marina* (marine annelid)
Avidocin and purocin	Membrane disruption	Gram (+) and (−)	Safety reported	Treatment of *C. difficile* infections (colitis); topical	Modified bacteriocin
Buforin II	Inhibition of DNA/RNA synthesis	Gram (+), (−); fungi; *E. coli* (MIC: 1 μg/mL); *K. pneumoniae* (MIC: 2 μg/mL)	Safety reported	Used as bacteriostatic; bactericide; anti-sepsis	*Bufo gargarizans* (frog)
Lactocin 160 (Bacteriocin)	Membrane disruption	*G. vaginalis*; *P. bivia*; *Lactobacillus* spp. (MIC: > 200 mg/mL)	Safety reported	Urogenital tract infections; Bacterial vaginosis	*Lactobacillus rhamnosus* (bact.)
LTX-109 (Lytixar)	Membrane disruption and cell lysis	Gram (+); MRSA; [2] VISA; [3] VRSA (MIC: 2–4 μg/mL)	Itching, pain and burning effects	Treatment of diabetic foot ulcers; topical	Synthetic
Mersacidin	Inhibition of cell wall	Gram (+), MRSA (MIC: 2–16 μg/mL); *Clostridium* spp. (MIC: 1–16 μg/mL)	Safety reported	Treatment of hospital-acquired infections	*Bacillus sp.* (bact.)
Planosporicin (Bacteriocin)	Inhibition of cell wall	Gram (+), (−); *Planomonospora* sp., MDR strains; *S. aureus* (MIC: 2–>128 μg/mL); *S. epidermidis*; *E. faecalis* (MIC: 32 μg/mL)	Not reported	Hospital-acquired infections	*Planomonospora alba* (bact.)
Plectasin (NZ2114)	Inhibition of cell wall synthesis	Gram (+), (−); MRSA (MIC: 16–32 μg/mL); *P. aeruginosa* (MIC >128 μg/mL)	Not reported	Pneumococcal peritonitis and pneumonia infections	*Pseudoplectania nigrella* (fung.)

AMPs	Mechanism of Action	Activity Against	Side Effects	Application and Administration Route	Source
		Clinical Phase			
CZEN-002/(CKPV)2 (phase IIb)	Immunomodulation	*C. albicans*	Not reported	Vulvo-vaginal candidiasis; topical	Synthetic
D2A21 (phase III)	Membrane disruption	Gram (+), (−); fungi; Gram (+), (−); *S. aureus*; *E. coli*; *P. aeruginosa* (MIC: 4 µg/mL)	No side effects reported	Burn wound infections; topical	Synthetic
DPK-060 (phase II)	Membrane disruption and immunomodulation	Gram (+), (−); fungi; *S. aureus* (MIC: 4.6 µg/mL)	No side effects reported	Acute external otitis; ear drops	Kininogen derivate (human)
EA-230 (phase IIb)	Immunomodulation	Gram (−)	Safety reported	Sepsis and renal failure protection; IV	hCG derivative (human)
Histatin (phase I)	Membrane disruption	Gram (−); *C. albicans* (MIC: 4–16 µg/mL)	No side effects reported	Treatment of *P. aeruginosa* infections and oral candidiasis	Saliva (human)
hLF1-11 (phase I/II)	Membrane disruption	Gram(+), (−); fungi; *Staphylococcus* spp. (including MRSA), *Streptococcus mitis* (MIC: 1.6–6.3 µg/mL); *A. baumannii*, *Pseudomonas* spp., *Klebsiella* spp., *E. coli* (MIC: 6.3–12.5 µg/mL); *Candida* spp. (MIC >12.5 µg/mL)	Little discomfort at the injection site	LPS-related fungal infections; IV	Lactoferricin derivative
IDR-1 (phase I)	Immunomodulation	MRSA; VRSA	No side effects reported	Infection prevention in immunocompromised patients	Synthetic
IMX942/SGX942 (IDR-1 derivative; phase III)	Immunomodulation	Gram (+), (−)	Safety reported	Treatment of nosocomial infections, neutropenia	Synthetic
LL-37 (phase IIb)	Barrel-stave mechanism of membrane disruption; inhibit [5] LPS binding	Bacteria, fungi and viral pathogens; *P. aeruginosa* (MIC: 64 µg/mL)	Cytotoxic	Diabetic foot ulcers; chronic middle ear infection	Human cathelicidin
LTX-109 (Lytixar, phase II)	Membrane disruption and cell lysis	Gram (+); MRSA; VISA; VRSA (MIC: 2–4 µg/mL)	Itching, pain and burning effects	Treatment of nasal MRSA infections; nasal and topical	Synthetic
Mel4 (phase II/III)	Membrane disruption	Gram (+), (−); *P. aeruginosa* (MIC: 62.5–250 µg/mL)	No cytotoxicity and no resistance reported; no staining of human cornea	Contact lenses	Synthetic
Melimine (phase I/II)	Membrane disruption	Gram (+), (−); *P. aeruginosa* (MIC: 250–500 µg/mL)	No cytotoxicity and no resistance reported; staining of human cornea	Contact lenses	Synthetic
MX-226 (Omiganan®; phase III)	Cell disruption	Gram (+), (−); MRSA (MIC: 2–8 µg/mL); *E. coli* (MIC: 8–16 µg/mL); *C. albicans* (MIC: 64 µg/mL)	Not reported	Prevention of device-related infections; topical	Indolicidin derivative
Novexatin (NP213; phase IIb)	Membrane disruption	Fungi	Not reported	Treatment of dermatophyte fungal infections	Synthetic
Opebacan (rBPI21, neuprex; phase II)	Membrane disruption	Gram (+), (−)	Fever reported	Meningococcal, wound, and burn infections; IV	Synthetic
OP-145 (LL-37 derived; phase II)	Membrane disruption	Gram (+)	Lytic to human cells at high concentrations	Treatment of chronic bacterial middle ear infection; ear drops	LL-37 derivative
PAC113 (P113; histatin 5 analog; phase IIb)	Membrane disruption and immunomodulation	Gram (+), (−); *Candida* spp.; *A. baumannii* (MIC: 38 µM); *P. aeruginosa* (MIC: 47 µM); *E. cloacae* (MIC: 90 µM)	No cytotoxicity reported	Oral candidiasis in HIV patients and prevention of bacterial periodontal disease; topical	Histatin 5 analog
p2TA (AB103; phase III)	Immunomodulation	Gram (−)	No adverse effects reported	Necrotizing soft tissue infections; IV	Synthetic
Ramoplanin (NTI-851; phase II)	Membrane disruption and cell wall synthesis	Gram (+); *C. difficile*; [4] MSSA (MIC: 2–4 µg/mL)	Low local tolerability when injected IV	Treatment of *C. difficile*-associated infections; oral	*Actinoplanes spp (bact)*

7. AMPs functions

AMPs represent a universal feature of defense systems existing in all living forms, protecting the host organisms against pathogens and managing healthy relations with the commensal microbiota. Whereas not all AMPs have been thoroughly evaluated to their full potential, extensive researches on human (cathelicidin LL-37, α- and β-defensins) and *Drosophila melanogaster* have led to discover many functions of natural AMPs (Figure 11) [68,109–112].

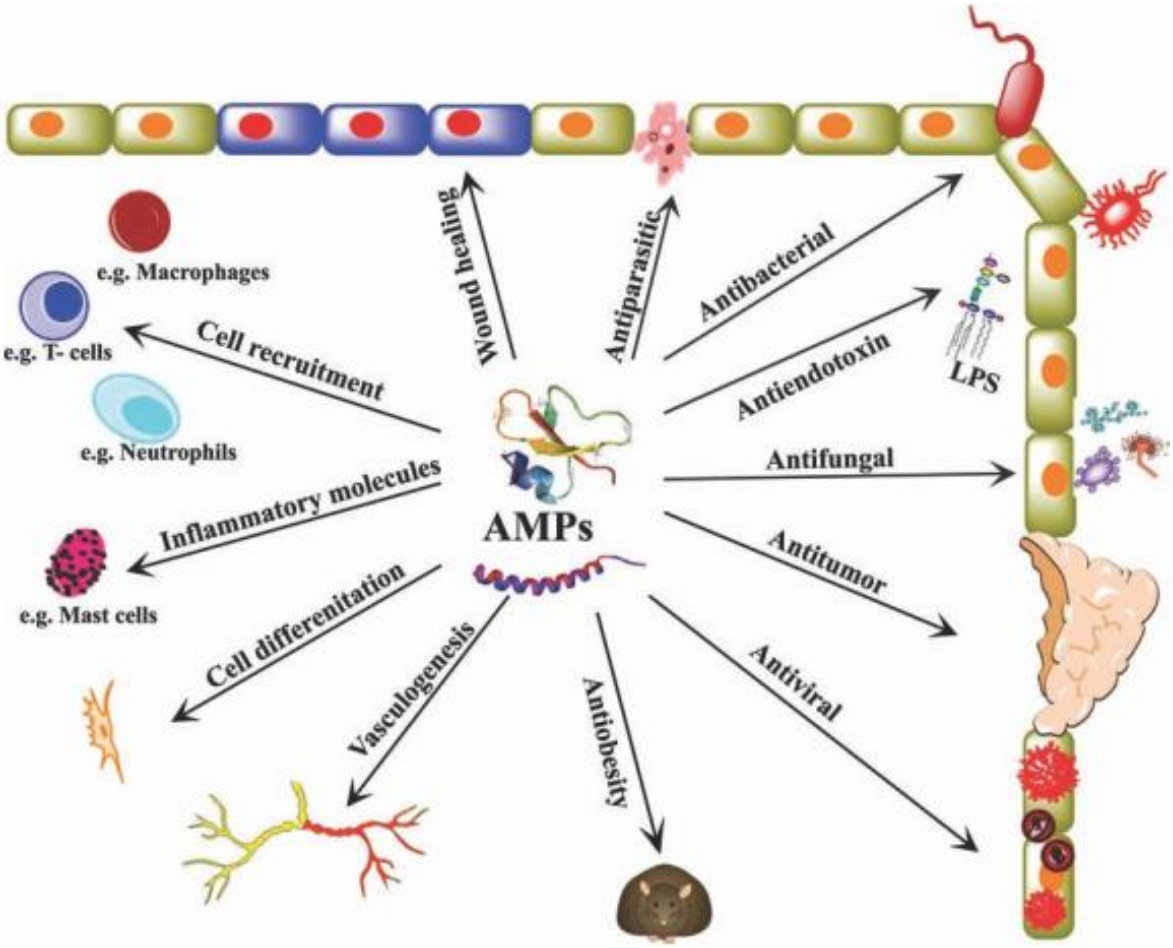


Figure 11: Main functions of AMPs in host immune protection. In addition to antimicrobial activity, some AMPs possess antiobesity and antitumor properties, wound healing mechanism, genesis of vascular system, promote leukocyte recruitment at the site of infections, induction of cell differentiation process, binding to LPS, and preventing pro-inflammatory responses (figure by Pasupuleti *et al*., 2011).

- Immune function (role in bactericidal clearance)

In many cases, AMPs primary role is in the killing of invading pathogenic organisms, bacteria, fungi, and some parasites and viruses, although the importance of these activity in the host

defense may vary between different sites within a particular organism and also between different types of organisms [68,113,114].

They can be expressed constitutively, for example in the hemocytes of marine organisms such as shrimp, oyster, and horseshoe crab [115,116], or induced in response to pathogen challenge, such as antifungal peptides in *Drosophila melanogaster* [117]. Moreover, in multicellular organisms, they can be distributed systemically (like cecropins in the insect hemolymph) and/or localized to specific cell or tissue more often in contact with pathogens, such as mucosal epithelia and the skin [81,118,119].

Direct microbicidal activity is associated with AMPs under physiological conditions, especially where they are found in very high concentrations (mg *per* ml), creating a highly toxic environment for invading bacteria, whereas such concentration are sufficiently diluted in proximity of commensal microorganisms [68,109,113]. However, AMPs biological concentration is generally much lower than the minimal inhibitory concentration (ranging from ng *per* ml to µg *per* ml), and their antimicrobial activity is inhibited by the presence of physiological concentrations of salt, serum proteins and/or lipoproteins and glycosaminoglycans [112,118]. It is now well established that AMPs, also known as host defence peptides (HDP), perform critical immunomodulatory functions on various cell types throughout the body (skin, lungs, intestine and circulatory system) (Figure 12) [68,109,118–120].

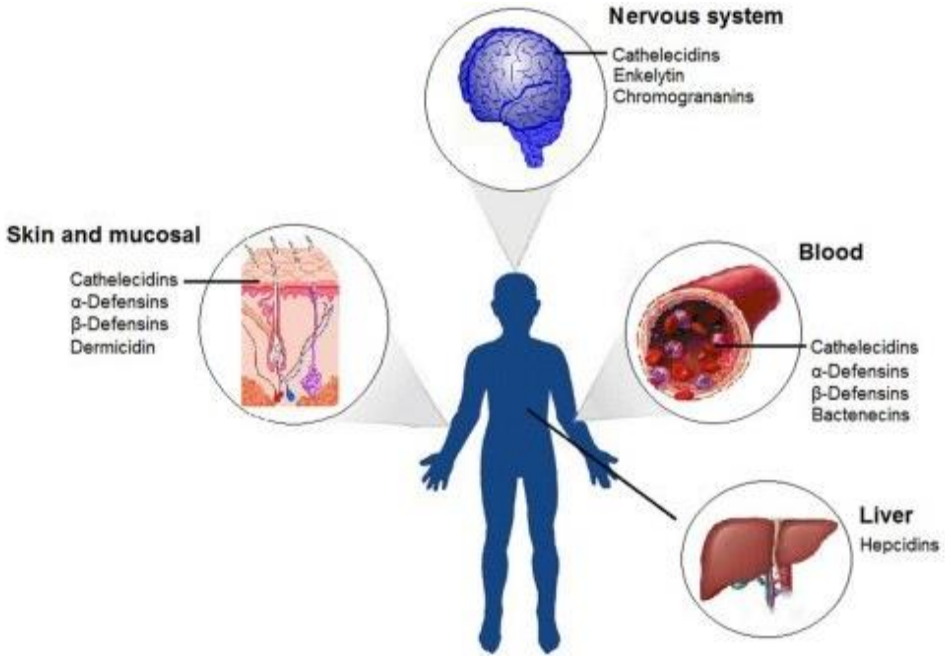


Figure 12: Wide distribution of host-defense peptides described in human body, being found mainly in skin, mucous membranes, blood, nervous system, and liver (figure by Silva et al., 2011).

A primary function associated with certain HDPs is the promotion of immune cell recruitment to the site of infection, directly involved in the clearance of infections. Human cathelicidin LL-37 and human defensins peptide hBD-2 can either directly or indirectly promote recruitment of different immune cells (such as neutrophils, monocytes, immature dendritic cells, T lymphocytes, eosinophils and neutrophils) in the site of infection (reviewed in Choi *et al.*, 2012 and Hancock *et al.*, 2016 [109,112]). Moreover, at low to modest physiological concentrations, they can promote chemotaxis of immune cells indirectly by inducing the production of chemokines. It has also been demonstrated LL-37 and human defensin hBD-3 suppress neutrophil apoptosis (by induction of anti-apoptotic protein BcL-XL) [121].

Some mammalian HDPs, like LL-37, hBD-3 and HNP-1 to -3, influence the differentiation and subsequent change in dendritic cells phenotype (by activation of T and B lymphocytes) to indirectly promote an adaptive immune response [112]. Certain HDPs were shown to have also direct effects on lymphocytes: murine cathelicidin CRAMP and human defensins (HNP-1 to -3) can alter T and B cell responses, playing a role in adaptive immune responses regulation [122].

Several in vivo models of infections and sepsis have shown that cathelicidins LL-37 and BMAP-28 and defensins hBD-2 and hBD-3, can modulate host immune responses for the resolution of pathogen-induced inflammation [123,124]. Their anti-inflammatory activity appears to be targeted and selective, controlling the inflammatory responses and enhanced pathogen clearance. For example, many defensins and cathelicidins have been shown to suppress specific pro-inflammatory responses (such as induction of tumour necrosis factors), while maintaining or enhancing critical immune responses such as cell recruitment and movement and crucial anti-inflammatory mechanisms [123–125].

Broadly, HDPs are known to promote several other immune-related functions, which include promotion of wound healing, angiogenesis (capillary growth) and arteriogenesis (growth of pre-existing vessels), induction of mast cell degranulation and release of histamine and prostaglandin D2. Unfortunately, the relationship between the structures of the various HDPs and how this relates to the diverse immunity-related functions mediated by these endogenous peptides is not yet resolved.

Overall, direct effects of HDPs on immune functions contribute to a wide range of biological effects from infection control to wound healing and maintaining homeostasis.

- Role in symbiostasis

Although not all AMPs possess wide spectrum activity, increasing new evidences indicate that the role of AMPs in host defense goes well beyond direct killing of microorganisms.
More recently, they have also been evidenced to shape, control and confine the symbiotic microflora into specific anatomic compartments (gut, bacteriomes, skin...), thus contributing to the symbiostasis of both invertebrates and vertebrates [14,23,109,114,126,127]. Although their mode of action does not rely on the recognition of specific molecules at the cell surface of microorganisms, AMPs control symbiosis by selectively killing specific bacterial taxa, while being inoffensive for other ones. The AMPs in *Alvinella pompejana* and *Hirudo verbana* were found controlling respectively the epibiotic microflora and the gut microbiota of the host [11,126]. Similarly, in humans, α-defensins and skin AMPs govern intestinal and epidermal microflora [127,128].

- Other functions

Whereas not all AMPs have been thoroughly evaluated to their full potential, extensive researches in multicellular organisms have led to discover many other functions of natural AMPs, such as nerve repair, antiobesity proprieties and spermicidal effect [68,86,109,129].
The activity of AMPs in the process of nerve cord reparation, as a “secondary” activity, was shown by the macins, theromacin and neuromacin from the medicinal leech, *Hirudo medicinalis* [86,130].
Hemopressin, a short AMPs isolated from rat and mice brain, have been shown to modulate the activity of appetite pathways in the brain [131].
A dozen AMPs (such as human cathelicidin LL-37, magainin 2 from *Xenopus laevis* and nisin from *Lactococcus lactis*) are known to possess spermicidal effects and microbicidal properties; being selective to sperm and not to the female reproductive tract, they are eventually useful to avoid pregnancy [132].
Recent findings described the specificity of a group of *Drosophila* AMPs (attacins, cecropins, diptericins, drosocin, drosomycin, metchnikowin and defensin) against specific pathogens, acting as the arbiters of life/death upon certain infection; moreover, an additive and synergistic action of AMPs was shown to suppress *Providencia burhodogranariea* growth in vivo, probably due to their complementary mechanisms of action [133].

8. β-sheet family

β-sheet AMPs are small size (less than 30 residues) peptides with common characteristic: they possess two anti-parallel β-sheets linked by a small turn of 3 to 7 amino acids, forming a hairpin shape and constrained by intramolecular disulfide bridges. Twelve families of β-hairpin AMPs have been discovered and reported so far, from mammals and invertebrates [68,84]. Figure 7 (from, Panteleev et al., 2015 [134]) lists the most representative members of the family, varying from the number of disulfide bond (one to four), including arenicins from coelomocytes of marine annelids, thanatin from the spined soldier bug, protegrin-1 (PG-1) from porcine leucocytes, tachyplesins and polyphemusin I from hemocytes of the horseshoe crab, and gomesin from hemocytes of the tarantula spider [10,12,70,135–137]. The disulfide bonds distant to the β-turn region are more important for the stability of the structure than the one close to the turn [134].

β-hairpin AMPs share close sequence homology and organization: cationic Arg or Lys residues are in position C- and N- termini and adjacent to the turn; they are flanked by hydrophobic and membrane-insertive, Val, Leu, Ile, Tyr or Trp residues [91,138–140]. Together these features describe an amphipathic structure with one hydrophobic face and a basic face, conferring an excellent binding to the lipid bilayer of bacterial membranes [74,141].

The binding to the membranes leads to conformational changes of the peptide molecule (such as arenicins and PG-1): two N-terminal β-strands of peptides form a dimer by parallel association which leads to formation of ion-conducting pores in the target membrane according to the toroidal pore mechanism [138,142,143]. However, other modes of action have been suggested: the model barrel-stave was proposed for tachyplesins [144]. In yeast, arenicin-1 may act indirectly, inducing apoptosis via intracellular accumulation of reactive oxygen species, and directly damages mitochondria and DNA in nuclei [145].

β-hairpin AMPs showed potent antimicrobial activity against a broad-spectrum of fungal, Gram-positive and Gram-negative pathogens, including multidrug resistant Gram-negative strains such as *Klebsiella pneumoniae*, *Escherichia coli*, *Acinetobacter baumannii* and *Pseudomonas aeruginosa* [10,12,70,74,135–137]. However, most members of the class also possess adverse cytotoxicity and hemolytic activity precluding their development as candidate drugs [74,146]. Many efforts were invested in their sequence modification: artificial modified

analogs were designed based on their structure, in order to decrease their adverse effects and to enhance the antimicrobial properties [74,102,147–149].

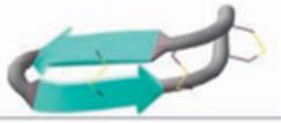
Name	Source	Activity	Amino acid sequence	Spatial structure
Tigerinin-1	*Rana tigerina* (frog skin secretion)	B, M	FCTMIPIPRCY*	-
Bactenecin	*Bos taurus* (bovine neutrophils)	B, V	RLCRIVVIRVCR	-
Thanatin	*Podisus maculiventris* (spined soldier bug hemolymph)	B, F	GSKKPVPIIYCNRRTGKCQRM	
Arenicin-2	*Arenicola marina* (lugworm coelomocytes)	B, F, H	RWCVYAYVRIRGVLVRYRRCW	
Lactoferricin B	Bos taurus taurus (bovine milk)	B, F, V, C, E, I	FKCRRWQWRMKKLGAPSITCVRRAF	
Tachyplesin-1	*Tachypleus tridentatus* (horseshoe crab hemocytes)	B, F, V, C, H, E, I	KWCFRVCYRGICYRRCR*	
Gomesin	*Acanthoscurria gomesiana* (spider hemocytes)	B, F, P, C, H	ZCRRLCYKQRCVTYCRGR*	
Androctonin	*Androctonus australis* (scorpion hemolymph)	B, F, T	RSVCRQIKICRRRGGCYYKCTNRPY	
Protegrin-1	*Sus scrofa* (porcine leukocytes)	B, F, V, C, H	RGGRLCYCRRRFCVCVGR*	
θ-defensin-1	*Macaca mulattav* (rhesus monkey leukocytes)	B, F, V, E, I	GFCRCLCRRGVCRCICTR	
Hepcidin	*Homo sapiens* (human hepatocytes)	B, M	DTHFPICIFCCGCCHRSKCGMCCKT	

Figure 7: Structure and biological activities of β-hairpin AMPs. The disulfide bonds are marked with thin lines. The bold line denotes the peptide bond that forms a θ-defensin cycle. (*) – C-terminal amidation, Z – N-terminal pyroglutamic acid. The biological activities are indicated as follows: B – antibacterial, F – antifungal, V – antiviral, P – antiparasitic, C – anticancer, H – cytotoxic and hemolytic, E – exo- and endotoxin binding, I – immunomodulatory, T – neurotoxic, M – metabolic ones. (figure by Panteleev et al., 2015)

9. Discovery of new AMPs

The most used strategy for the discovery of AMP is the genetic approach, using the homologies between cDNA transcripts or EST-clones and motif from already known peptide (Figure 9B). It is a rapid and easy method, with high rate of success which does not encode any new AMP motif [150].

Similarly, the in silico approach (Figure 9C) is based on known structural characteristics of the new AMP (such as size, net charge, hydrophobicity, etc.) scanned in a complex database of known peptides with the same features. In both methods, after sequence identification, the putative peptide must be produced/synthesized and tested for antimicrobial activity (to be certain that the sequence codes for an AMP) [68,150].

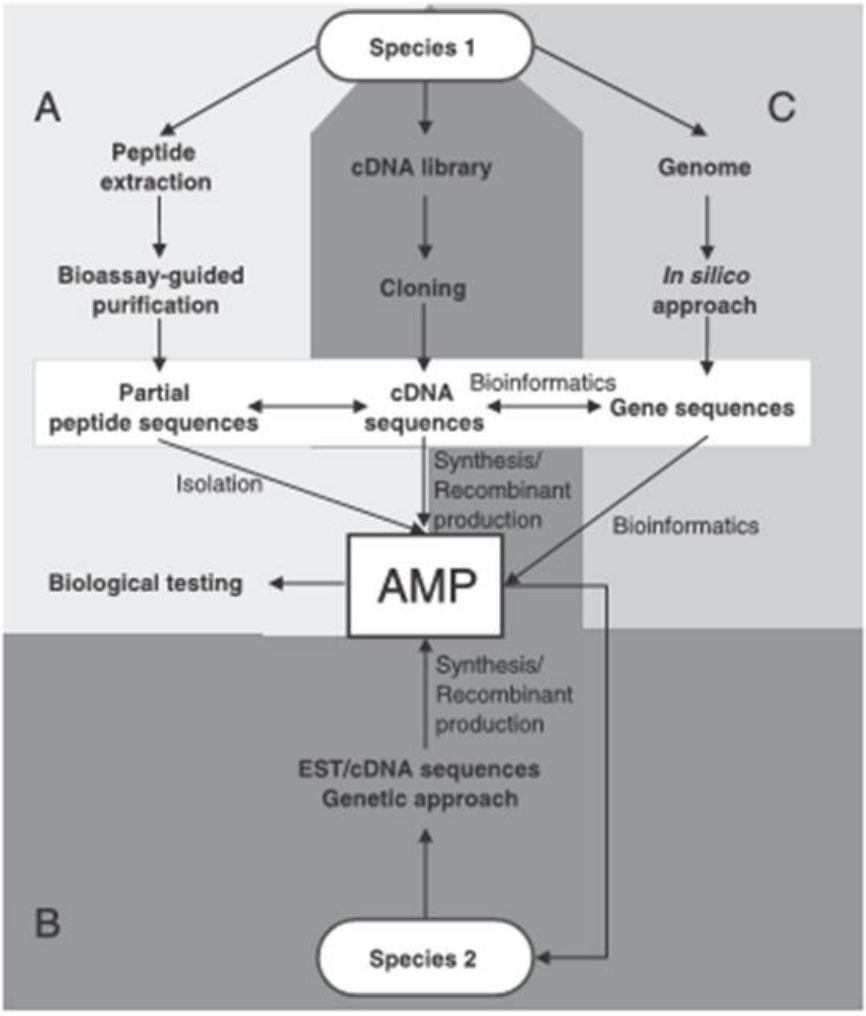


Figure 9: Framework of the methods for isolating new antimicrobial peptides: the bioassay-guided purification approach (A), the genetic approach (B), and the in silico approach (C); figure by Sperstad et al., 2011.

The classic method for discovering natural AMPs is to isolate them from natural sources chromatographically. This approach, bioassay-guided purification (Figure 9A), is the only method for identification of unknown AMPs with novel motifs. It consists in peptide extraction from the sample, followed by its purification, Edman degradation (for amino acid sequencing), mass spectrometry analysis (for mass and purity study) and NMR (Nuclear Magnetic Resonance, for 3D structure definition). These procedures are time-consuming, require intensive work and depend on the activity and the quantity of the extract [150].

However, the three approaches demand a direct selection method for antimicrobial activity, a screening using living microbes. The “broth dilution assay” tests the ability of a peptide to inhibit the growth of microbes in a nutrient broth and in the “agar diffusion assay”, peptides are spotted on a thin layer of nutrient agarose seeded with bacteria.

In the last years, there is a strong desire to produce new AMPs by bioengineering approaches, managing to design more potent and less toxic molecules on the base of already known antimicrobial peptides [68,148]. This approach was successfully used to produce ALP1 and ALP2, starting from the structure of arenicin-1, with the introduction of a second disulfide bridge (by analogy with tachyplesins/arenicin-3 structures) and the shortening of the polypeptide chain length (to diminish hemolytic activity) [148].

Despite the fact that the field of AMP research has been intensively investigated over the last decades, in which many peptide have been discovered, designed and/or engineered, evident sequence-structure-function relationship is rarely successful, creating a bottleneck in the discovery of novel AMPs [68].

Recent works suggests that antimicrobial activity is not depending on specific amino acid sequences or on specific three-dimensional peptide structures [151,152]. The appropriate balance of interactions between and among peptides, water and membrane lipids, called “interfacial activity”, depends more on peptide amino acid composition and its physico-chemical properties than on its exact sequence or structure organization [68]. Design based on the principle of interfacial activity is not yet possible because the physicochemical basis of interfacial activity has not been parameterized, especially with respect to target selectivity [152].

10. AMPs evolution

Throughout evolution, the ability of an organism to cope with microbial or other species invasion has been a key factor for survival [129]. For this purpose, all species from bacteria to humans, have developed and evolved an immune defence system, involving AMPs [128].

Hosts and pathogens live in a strong relationship with each other, fighting for existence and co-evolving by mutations under selective pressures [153]. Pathogens evolve continuously to escape from the immune response of host, which consequently evolves to improve barriers against pathogens (the Red Queen hypothesis), both developing diversity and polymorphism of molecules involved [154]. This arms race between hosts and pathogens led a rapid evolution of immune defense genes, while genes encoding AMPs evolved slower, exhibiting high rates of non-synonymous polymorphisms [155,156].

The generation of different variants in AMP families may be indicative of the functional divergence of isoforms to extend the antimicrobial spectra or acquire novel immune functions [157]. Different patterns of diversification were found among AMP families: some AMP families widely conserved throughout evolution, such as defensins, are found in an extensive spectrum of phyla; other families are restricted to only few species belonging to close phylogenetic groups, such as Anti-lipopolysaccharide factors (ALFs) [157].

In diverse species, the evolution of AMPs has been shown to be driven by recurrent duplications (i.e. creation of paralogs) and balancing/positive selection to face and kill new and/or altered bacterial pathogens that can be encountered in a novel habitat and/or that have rapidly evolved to escape the immune response [29,155,158]. Studies performed in both invertebrates and vertebrates revealed that these non-synonymous mutations strongly affect the antibacterial activity of AMPs and thus resistance to bacterial infection [155,158–160]. As AMPs have a role in the control of gut microbiota, variation in AMPs could thus contribute importantly to the ability of animal hosts to adapt to changing environments through adaptive changes of their symbiotic communities [161–163].

However, most of these studies were performed in the laboratory under specific controlled conditions and/or focusing on the well-protected inner part of the multicellular host (inside the body *sensu lato*). AMPs can also be secreted into the environment surrounding an organism (such as alvinellacin), participating in their external immunity [23]. Considering the body of the organism as a wall buffering external abiotic and biotic variations, selection

processes driven by environmental constraints on innate immunity can be considered to fluctuate more outside the organism than inside [23,164].

Until now, no study has been devoted to the impact of the environmental factors on the evolution/adaptation of AMPs, and alvinellacin represents an interesting model to investigate on it [164].

- Annelids AMPs: the case of alvinellacin

The Pompeii worm (*Alvinella pompejana*) is a polychaete that lives in the extreme and fluctuating environment of hydrothermal vents, one of the harshest on the Earth [165]. The data from a study of our group [11], showed the production of an original AMP (alvinellacin) from a deep-sea animal that endorses a durable relationship with Epsilonproteobacteria and possibly archaea in the face of the hostile vent habitat (Figure 11).

Alvinellacin appears to act as a first line of defence against microbial invasion. Once secreted by the epidermal cells of the worm, alvinellacin (the AMP) is at the direct contact with the environmental abiotic and biotic constraints [11].

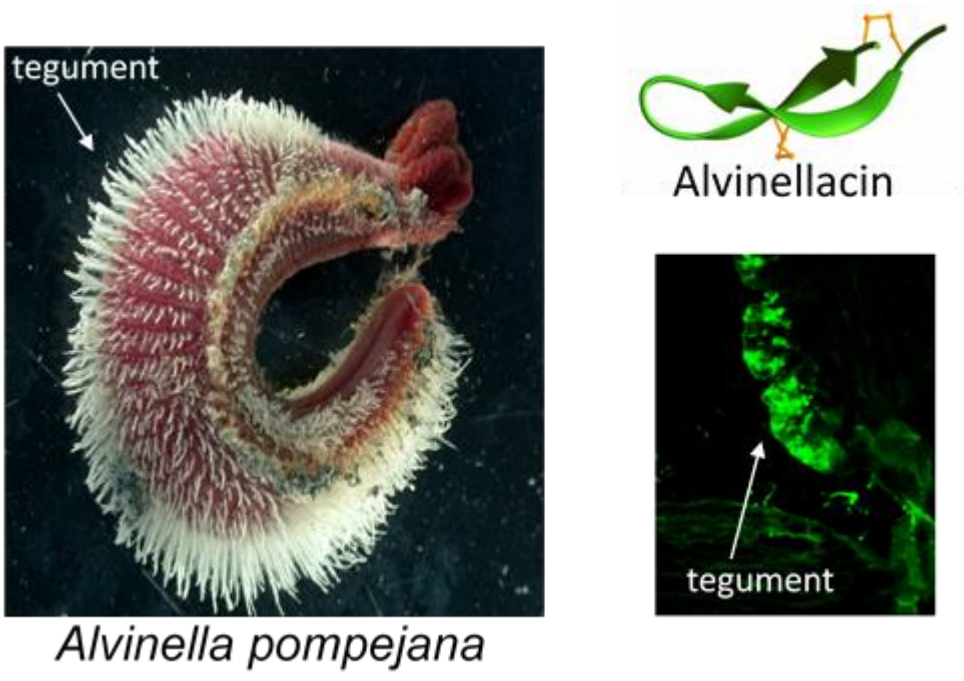


Figure 11: The extremophile worm *Alvinella pompejana*, endemic of the deep hydrothermal vents environment (East Pacific rise, characterised by extreme and extremely changing temperatures and pH and high concentrations of sulphide and heavy metals), lives in association with Gram negative bacteria (ectosymbiont, in white) attached to its tegument to survive in such hostile habitat. It was shown that alvinellacin (the AMP produced by the worm), is secreted by the animal's tegument (external immunity) and control the ectosymbiosis of the worm, vital to its survival in a hydrothermal environment (Tasiemski et al., 2014).

Moreover, it was showed, that alvinellacins, being part of part of the external immunity of *A. pompejana* and *A. caudata*, participates in the control and selection of the symbiotic bacteria that covers the dorsal tegument of the worms (Figure 11) [164].

Alvinellacins were listed in the family of BRICHOS-AMPs because of their main features: the presence of a BRICHOS domain in their precursors, a short amino-acid sequence, a cationic net charge, a hydrophobic region, an amphipathic nature, mainly a β-sheet fold and the formation of disulfide bonds between cysteine residues (Table 2 and Figure 12) [31].

Table 2: Amino acidic sequences, hydrophobicity and net charge of BRICHOS-AMPs. In bold type, cysteine residues involved in disulfide bridges. The Innovagen Pepcalc.com server (https://pepcalc.com/) was used to calculate the net charge at neutral pH and the Peptide2.0 server (https://peptide2.com/) for the hydrophobicity.

AMP Name	Amino Acid Sequence	Hydrophobicity	Net charge at pH 7
Arenicin-1	RW**C**VYAYVRVRGVLVRYRR**C**W	42%	+6
Arenicin-2	RW**C**VYAYVRIRGVLVRYRR**C**W	42%	+6
Arenicin-3	GF**C**WYV**C**VYRNGVRV**C**YRR**C**N	28%	+4
Alvinellacin (*A. pompejana*)	RG**C**YTR**C**WKVGRNGRV**C**MRV**C**T	22%	+6
Alvinellacin (*A. caudata*)	RG**C**YTR**C**WKVGSNGRV**C**MRV**C**T	23%	+5
Capitellacin	RSPRV**C**IRV**C**RNGV**C**YRR**C**WG	29%	+6
Nicomicin-1	GFWSSVWDGAKNVGTAIIKNAKV**C**VYAV**C**VSHK	45%	+3
Nicomicin-2	GFWSSVWDGAKNVGTAIIRNAKV**C**VYAV**C**VSHK	45%	+3

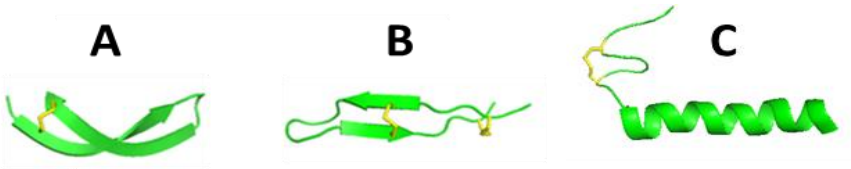


Figure 12: Three-dimensional structures of BRICHOS-AMPs: arenicins (A), alvinellacins (B) and nicomicins (C); in yellow the disulfide bonds between cysteine residues.

Surprisingly, these original peptides were found in annelids and more precisely only in marine polychaetes coming from different habitats (polar, deep-sea hydrothermal vent and temperate-coastal) [11,12,45]. Mature BRICHOS-AMPs are cleaved from a larger protein precursor, that contains a signal hydrophobic region and a pro-region containing the BRICHOS domain (Figure 13) [166]. This structure is generally found in all BRICHOS-containing proteins, with a β-hairpin protein in position C-terminal (Figure 13) [167].

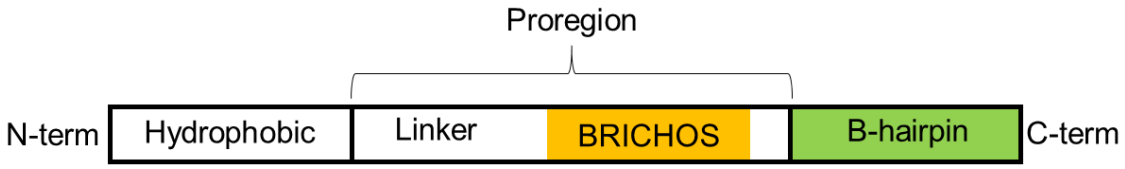


Figure 13: General structure of BRICHOS-containing proteins characterised by a hydrophobic region in N-terminal position, a proregion containing the BRICHOS domain and a β-hairpin in C-terminal position.

BRICHOS (so called from Bri2, CHOndromodulin, and proSurfactant protein C) is a well-preserved domain, characterised by a hundred amino acids and found in very distant protein families [168]. It generally acts as a molecular chaperone and has been shown to prevent the formation of amyloids in humans by stabilising β-hairpin [168,169]. Recently it has been demonstrated that mutation of the domain are associated to major diseases, such as Alzheimer, cancer and respiratory distress syndrome [170,171].

Interestingly, our group performed genetic analyses of the preproalvinellacin gene, carried out on 2000 worms from populations of *A. pompejana* and *A. caudata*, living at 4000 km from each other (Figure 14A) [164]. The evolution of the gene coding for the protein precursor (consisting of 6 exons and 5 introns) was investigated in the sister alvinellid species (Figure 14B) [164].

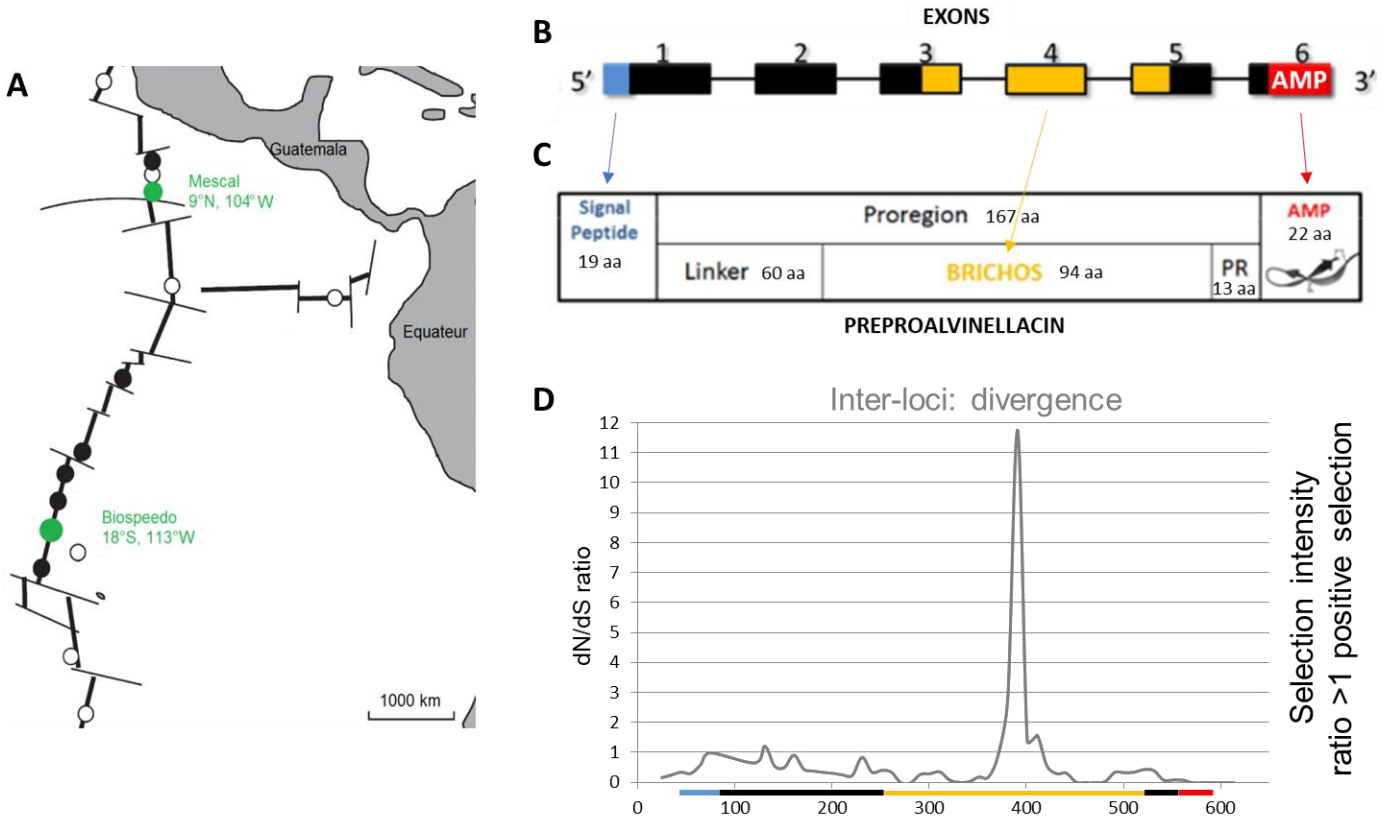


Figure 14: (A) Geographic location of the Pompeii worm collection sites (East Pacific Rise); structures of (B) the gene encoding preproalvinellacin and (C) the protein precursor: signal peptide is in blue, proregion in black, BRICHOS domain in yellow and AMP in red (figure by Papot, 2017); (D) peak of mutations in BRICHOS region (17 variants).

As showed by Figure 14, no variation of alvinellacin gene was reported between individuals from geographically-disjoint populations of *A. pompejana* physically separated since at least two million years (the last exon was monomorphic). Moreover, AMP sequences of the two *Alvinella* species diverge only by one amino-acid replacement, despite speciation having

occurred a long time ago. On the contrary, the BRICHOS region displayed a high number of non-synonymous variants [164]. In contrast to the Red Queen theory (observed in other metazoan), the vital and highly conserved ecto-symbiosis in a fluctuating environment has led to an adaptive diversification of the molecular chaperone of the AMP, but not of the AMP itself [164]. These discoveries led us to hypothesized the possible role of BRICHOS (as molecular chaperone and/or anti-amyloid agent) to preserve the biological activity of the peptides in the different external conditions encountered by the worms (subject approached in the Chapter 3). Because of the uniqueness of its chaperone, the preproalvinellacin gene family represents an interesting model to better understand the evolution of external immunity in *natura* (subject approached in the Chapter 2).

- Nematodes and crustaceans AMPs

Nematodes is an ancient animal phylum, forming the group of Ecdysozoa with arthropods (see above Figure 2). They are both the most successful animals for adapting to almost all environmental conditions over the planet (terrestrial, marine, and freshwater habitats, including extreme ones), with a very old but common ancestry.

To date, in crustaceans (the most abundant marine arthropods), 15 distinct AMP families have been identified. For economic reasons, the most characterized peptides come from farmed species, such as shrimp, crab, crayfish, prawn and lobster [172,173]. Although the great majority (14 families) comes from members of the order Decapoda, some of them were found in all crustaceans studied (such as the ALFs). Other crustacean AMPs, like the penaeidins (restricted to penaeid shrimp), are specific to certain lineages. Besides marine shrimp, AMPs have been also identified and characterized in a number of freshwater (crayfishes and prawns) and terrestrial species (such as isopods). Based on the structure and amino acid composition, crustacean AMP families can be clustered into four main groups: (i) single-domain linear α-helical AMPs and peptides enriched in specific amino acids, (ii) single-domain peptides containing cysteine residues engaged in disulfide bonds such as ALF, (iii) multi-domain or chimeric AMP-like crustins, which are shared by several crustacean species, and (iv) unconventional AMPs including multifunctional proteins or protein-derived fragments that exhibit antimicrobial functions [172,173]. In particular, a remarkable diversity was revealed by marine peptides, in their structural and genetic composition compared to their terrestrial counterparts [172,174].

As for nematodes, while the production of AMPs and their contribution to worm immunity have been well demonstrated in terrestrial species, to our knowledge no AMP has yet been identified in marine species. Nematodes, as crustaceans, over their long evolutionary history, have been facing a wide variety of integrity challenges because their natural habitat is generally overloaded with infectious microorganisms (viruses, bacteria, fungi and other parasites). Their evolutionary success confirms the effective strategies they use to fight against any kind of disease-causing agents and parasites present in their environment.
Due to the rapid molecular evolution and high diversity of AMPs, one can assume that not all families of AMPs are characterized yet in nematodes. In particular, we might expect that marine nematodes display a diversity of bioactive substances, as high as that found for crustaceans [175]. Thus, the exploration and study of novel and unconventional nematodes species appear as a promising source of new AMPs and of different modes of immune defense in link with the ecology/habitat of the species of interest.

11. AMPs from worms

The interest and originality of worm-produced AMPs relies on worm (nematodes and annelids) ability to have colonized all habitats on Earth including very extreme ones (polar, hydrothermal, abyssal, polluted, etc.), co-occurring with a large number and variety of bacteria. As a consequence of hundred millions years of worms' evolution and diversification and natural selection occurring at the interspecific level according to peculiar lifestyles and habitats, they produce a wide diversity of primary and tertiary AMPs structures.
Currently, several groups of AMPs have been identified in nematodes: cecropins, defensin-like antibacterial factors (ABFs), neuropeptide-like (Nlps), caenopores and caenacins. These peptides were mostly found in terrestrial genetic model (*Caenorhabditis* and *Ascarididae* species), by using inverse genetic and/or by screening omic databases.
About annelids (ringed worms), they represent the only worm clade for which the research of AMPs has not been targeted on laboratory models but is rather the result of species exploration over a variety of environments (marine, terrestrial, freshwater, etc.).

This part has been the subject of a review published in *Marine Drugs* (29/08/2019), surveying the current knowledge about the antimicrobial peptides from worms (nematodes and annelids), their sequences, structures, biochemical characteristics and biological functions.

marine drugs

MDPI

Review

Worms' Antimicrobial Peptides

Renato Bruno [1,2,*], Marc Maresca [3], Stéphane Canaan [4], Jean-François Cavalier [4], Kamel Mabrouk [5], Céline Boidin-Wichlacz [1,2], Hamza Olleik [3], Daniela Zeppilli [6], Priscille Brodin [1], François Massol [1,2], Didier Jollivet [7], Sascha Jung [8] and Aurélie Tasiemski [1,2,*]

1 Univ. Lille, CNRS, Inserm, CHU Lille, Institut Pasteur de Lille, U1019 – UMR 8204 - CIIL - Center for Infection and Immunity of Lille, Lille F-59000, France
2 Univ. Lille, CNRS, UMR 8198 - Evo-Eco-Paleo, Lille F-59000, France
3 Aix-Marseille Univ, CNRS, Centrale Marseille, iSm2, Marseille F-13013, France
4 Aix-Marseille Univ, CNRS, LISM, IMM FR3479, Marseille F-13009, France
5 Aix-Marseille Univ, CNRS, UMR7273, ICR, Marseille F-13013, France
6 IFREMER Centre Brest REM/EEP/LEP, ZI de la Pointe du Diable, CS10070, Plouzané F-29280, France
7 Sorbonne Université, CNRS, UMR 7144 AD2M, Station Biologique de Roscoff, Place Georges Teissier CS90074, Roscoff F-29688, France
8 Department of Applied and Molecular Microbiology, Institute of Biotechnology, Technische Universität Berlin, 13355 Berlin, Germany
* Correspondence: renato.bruno@univ-lille.fr (R.B.); aurelie.tasiemski@univ-lille.fr (A.T.); Tel.: +33(0)3 20 33 59 57 (A.T.); +33(0)3 20 33 65 79 (R.B.)

Received: 19 July 2019; Accepted: 27 August 2019; Published: date

Abstract: Antimicrobial peptides (AMPs) are natural antibiotics produced by all living organisms. In metazoans, they act as host defense factors by eliminating microbial pathogens. But they also help to select the colonizing bacterial symbionts while coping with specific environmental challenges. Although many AMPs share common structural characteristics, for example having an overall size between 10–100 amino acids, a net positive charge, a γ-core motif, or a high content of cysteines, they greatly differ in coding sequences as a consequence of multiple parallel evolution in the face of pathogens. The majority of AMPs is specific of certain taxa or even typifying species. This is especially the case of annelids (ringed worms). Even in regions with extreme environmental conditions (polar, hydrothermal, abyssal, polluted, etc.), worms have colonized all habitats on Earth and dominated in biomass most of them while co-occurring with a large number and variety of bacteria. This review surveys the different structures and functions of AMPs that have been so far encountered in annelids and nematodes. It highlights the wide diversity of AMP primary structures and their originality that presumably mimics the highly diverse life styles and ecology of worms. From the unique system that represents marine annelids, we have studied the effect of abiotic pressures on the selection of AMPs and demonstrated the promising sources of antibiotics that they could constitute.

Keywords: Antibiotics; annelids; nematodes; AMP; extremophiles

1. Introduction

Antimicrobial peptides (AMPs) are natural antibiotics produced by all living organisms, from archaea to mammals [1–3]. In pluricellular organisms, they act as key actors of immunity by operating in the first line of defense towards microbes [4–7] such as bacteria, fungi, and protozoa or viruses that attempt to invade and to proliferate into the host [8–12]. AMPs also contribute to symbiostasis (i.e., the regulation of mutualistic and commensal symbionts to avoid proliferation) in vertebrates and invertebrates by controlling, shaping, and confining the symbiotic microflora in specific anatomical compartments (gut, bacteriomes, skin) [13–15]. Because symbionts have been shown to represent a rapid source of innovation for the host to adapt to changing habitats, AMPs are also indirectly involved in the ability of animals and plants to cope with environmental changes [16–19]. In metazoans, active AMPs

are generally matured from a larger inactive protein precursor containing a signal peptide, a proregion, and the AMP itself. The ribosomal synthesis and/or the secretion of AMPs by epithelial and circulating cells are well documented to be regulated by microbial challenges, while few data also evidence an influence of abiotic factors. However, there are increasing examples of an endogenous role of AMPs, i.e., they are active towards the expressing host and work as cannibal toxins [20].

Regarding their application as type of therapeutic drugs, after their first discovery in the early 1980s, AMPs appeared as a promise of novel antibiotics to address issues about the multi-drug resistance (MDR) of pathogenic bacteria. Animals are the most important producers of AMPs (2298 versus 349 from plants or 342 from bacteria), although very poorly described in worms, with only 20 AMPs discovered out of seven species [21]. The definition of an AMP is only based on physico-chemical criteria (<100 amino acids in length, amphipathic, cationic) and on their properties to kill microbes. Recently, a unifying structural signature present in cysteine-stabilized AMPs was discovered: The γ-core motif [22]. Virtually all peptides sharing the γ-core motif interact with the negatively-charged lipid membranes causing ion-channel dysfunction or membrane pore formation in bacteria. One important point is that the multi-target interaction and mechanism of action (MOA) of AMPs with the bacterial membrane makes the appearance of resistance to AMPs more difficult compared to conventional antibiotics. AMPs and AMP-resistance mechanisms have presumably co-evolved through a transitory host–pathogen balance that has characterized the existing AMP collection [23]. Additional bioactivity features of AMPs such as their natural antibacterial biofilm activities, their chemotaxis of immune cells, immunomodulation, endotoxin neutralization, their mediation of nerve-repair activities [23] also add value/benefit to AMPs compared to conventional antibiotics [3,11,24]. However, most of the existing sequences of AMPs have never been exploited so far. Thirty years after their discovery, a better understanding of their MOA, modifications (structural and/or residues substitution), and synthesis is reigniting the commercial development of AMPs, which "stage a comeback" [25].

The production of AMPs and their contribution to host immunity have been well demonstrated in worms (Table 1) [2,12,26–30]. Their involvement in resistance to microbial infection and in symbiostasis is sustained by their strategic location in immune cells (phagocytes), in body fluids (pseudo-coelom, coelom, and blood) and at the interfaces between organisms and their environment, *i.e.*, at epithelial cell levels such as intestinal cells and epidermis cells. The first worm AMP (namely cecropin P1) was isolated and identified in 1989 [31] by the team of H. Boman, who just discovered the existence of AMPs in the butterfly *Hyalophora cecropia* [32]. The cecropin P1 was originally thought to be a porcine cecropin until the workers who isolated it provided evidence in 2003 that, in fact, this AMP originated from the pig intestinal parasitic nematode *Ascaris suum,* and not from its mammalian host [19]. This underlines the non-negligible difficulty and importance of separating host DNA, RNA, or peptides from those of potential parasites and symbionts when searching for a new component. To date, cecropins have been identified mainly in ecdysozoans (insects and nematodes), in one marine tunicate, and in bacteria [18,33,34], but neither in lophotrochozoans (molluscs, annelids, etc.) nor in vertebrates.

In 1996, ABF-type peptides (also called nematode defensins) were discovered in nematodes by Kato *et al*. [35]. Like mollusc and insect defensins, they contain eight cysteine residues and harbor a cysteine-stabilized alpha helix and beta sheet (CSαβ) structure. These common features may suggest an evolution from a common ancestor [36]. However, the lack of a significant sequence similarity or a conserved genomic organization (exon–intron structure) suggests that these groups of AMPs have rather emerged through convergent evolution [37]. In 1998, Banyai and Patthy demonstrated the antibacterial activities of saposin-like proteins (SPP) (called caenopores) from *Caenorhabditis elegans,* a family of AMPs similar to the amoebapores of the unicellular *Entamoeba histolytica* and the granulysin from human cytotoxic T lymphocytes [38]. Amoebapore-like SPPs might have been the first AMPs since this family emerged in protists, *i.e.*, before the advent of multi-celled organisms [39]. In 2002, Mallo *et al.* observed, in *C. elegans* again, the induced expression of a neuropeptide-like peptide (nlp) upon bacterial infection. Later, in 2004, Ewbank's group indirectly demonstrated an antifungal activity for nlp-31 [40]. Until now, nlps have not been identified in non-nematode species, and their MOA and 3D structures remain to be solved. As detailed below, other AMPs were identified in nematodes, but, to

our knowledge, except for cecropins, none of these were purified from crude extracts of worms; their predicted "in silico" sequences are issued from homology-based searches in genomes or transcriptomes starting from already described AMP sequences in other invertebrates [41]. Due to the rapid molecular evolution and high diversity of AMPs, one can assume that not all families of AMPs are characterized yet in nematodes. Efforts were also mainly focused on *C. elegans* and should be extended to wild species and enlarged to different taxa of nematodes. However, one major problem when searching for new AMPs from nematodes, as we have tried with the marine *Metoncholaimus* and *Oncholaimus* spp, is their tiny size (0.2 mm diameter) combined with their highly variable and patchy distribution in their natural habitat, making it complicated and not reproducible the collect of a sufficient number of individuals. Although promising at first, the too low quantity of material was a clear limitation to the use of the bioassay-guided purification, which remains the best and only strategy to discover new AMPs (unpublished data).

By contrast to nematodes, most annelid AMPs were biochemically isolated from diverse wild species from different taxa. The first annelid AMP was lumbricin-1 isolated from the earthworm *Lumbricus rubellus* in 1998 [42] and later in leeches [43]. Its MOA, as well as its 3D structure, have yet to be described. The relatively low antimicrobial activities of lumbricin-like AMPs suggest that the microbial clearance is not the main biological function of this molecule. In 2004, the first member of the macin family (theromacin) was characterized in leeches [44]. Despite their different disulfide arrays, macins and invertebrate defensins share the CSαβ motif also characteristic of the members of the scorpion toxin-like superfamily [6]. By contrast with defensins, macins have been shown to exert neurotrophic and proliferation effects, in addition to their bactericidal activities [6,43]. Based on their functions, their expression sites, their occurrence, and their evolutionary relationship in the animal kingdom, the possibility to consider macins as defensins could be discussed. Another family of cysteine-rich AMPs was characterized in annelids: The BRICHOS (so called from Bri2, CHOndromodulin, and proSurfactant protein C) AMP family; the first member was arenicin isolated from the body fluid of *Arenicola marina* in 2004 [14]. At this time, the presence of a BRICHOS domain in the proregion of the arenicin precursor was not noticed by the authors and was first mentioned later in 2013 in a review written by Knight *et al*. who discovered the BRICHOS domain in 2002 [45,46]. The evidence of other members and the study of their gene evolution confirm the existence of the BRICHOS-AMP family, which seems to be restricted to marine worms [47]. Even if AMPs from this family do not share any sequence similarity, they harbor a beta hairpin structure stabilized by one or two disulphide bridges [48].

Table 1. Dates of antimicrobial peptides' (AMPs) discovery in nematodes and in annelids.

Worm Phylum	Dates	AMP Families	References
Nematodes	1989	Cecropins	[31]
	1996	ABFs	[35]
	1998	Coenopores	[38]
	2002	Caenacins	[39]
	2004	Nlps	[40]
Annelids	1998	Lumbricins	[42]
	2004	Macins	[44]
	2004 and 2013	BRICHOS-AMPs	[14,46]
	2004	Perinerin	[49]
	2006	Hedistin	[50]
	2016	Ms-Hemerycin	[51]

This review surveys the wide diversity of primary and tertiary structures of worm-produced AMPs as a consequence of a hundred millions years of worms' evolution and diversification and natural selection occurring at the interspecific level according to peculiar lifestyles and habitats. We focus on

annelids, which represent the worm clade for which the research of AMPs has not been targeted on genetic/laboratory models as performed in nematodes, but is rather the result of species exploration over a variety of environments (marine, terrestrial, freshwater, etc.). This review highlights that none of the AMP families are universally expressed and that none of the studied worm species seem to produce all types of AMPs, even if the lack of genomes does not allow to firmly confirm this observation. Thus, the exploration and study of novel and unconventional worm species appear as a promising source of new AMPs and of different modes of immune defense in link with the ecology/habitat of the species of interest.

2. AMPs Diversity in Annelids and Nematodes

AMPs' capacity to kill microorganisms lies in their ability to disrupt and/or permeate the target cell membranes. Being generally cationic, they usually accumulate at the membrane surface (negatively charged) of the bacteria. Then, above a certain concentration threshold, they disrupt the cell membrane through very diverse and complex mechanisms [9]. Most of the MOAs studied act via pore formation (barrel-stave or toroidal models) or by non-pore mechanisms, such as a carpet-like mechanism. In both the pore models, at increasing concentrations, peptides begin to orientate perpendicular to the membrane and insert into the bilayer: In the toroidal model, the peptides are always associated with the lipid head groups; in the barrel-stave model, they form a bundle in the membrane with a central lumen (the peptides represent the staves of the barrel) [9,52].

Alternatively, in the carpet model, the peptides cover the membrane surface in a carpet-like manner (orientated in parallel to the membrane) and at high concentrations, they disrupt the bilayer in a detergent-like manner, leading to the formation of micelles [53]. Some AMPs polarize the membrane, forming anionic lipid clusters [54]. A minority of AMPs, however, do not cause membrane disruption: After crossing the bacterial cell membrane, they act on intracellular targets (such as nucleic acids and functional proteins) to activate cell death [55].

AMPs can be classified into several subgroups according to their secondary structure and biochemical characteristics: (i) α-helix peptides, containing one or more helices with spatially disjunct hydrophobic and hydrophilic surfaces [56]; (ii) β-sheet peptides, with β-hairpin-like structure, rich in cysteine and containing disulfide bonds; (iii) α-helix/β-sheets peptides with mixed α-helical and β-sheet organization [4,57]; (iv) extended peptides, which do not adopt regular secondary structures, containing a high proportion of one or two amino acids (such as proline, glycine, tryptophan, etc.) often essential for their antimicrobial activity [57,58]; and (v) peptides derived from larger molecules, exerting multiple functions [59]. Interestingly, representatives from all of these structural groups have been identified in worms (summarized in Table 2). They represent the main subject of this article and are subsequently described below.

Table 2. Repartition of the different groups of identified AMPs according to the phylum and the respective habitats of the worms.

Structure (Group)	AMPs	Worm Phylum	Worm Habitat
Linear α-helix (i)	Cecropins	Nematode	Terrestrial
	Caenopores	Nematode	Terrestrial
	Hedistin	Annelid	Marine
β-sheet (ii)	BRICHOS-AMPs	Annelid	Marine
Mixed α-helix/β-sheet (iii)	ABFs	Nematode	Terrestrial
	Macins	Annelid	Freshwater
Enriched with specific amino acids (iv)	Neuropeptide-like	Nematode	Terrestrial
	Caenacins	Nematode	Terrestrial

	Lumbricins	Annelid	Marine and Freshwater
Derived from larger molecules (v)	Perinerin	Annelid	Marine
	Ms-Hemerycin	Annelid	Marine

2.1. α-helix Peptides

2.1.1. α-helix Peptides in Nematodes

Cecropin and Caenopore Families

Cecropins and cecropin-like peptides have been identified and characterized in insects [60,61], nematodes [19,29], tunicates [18], and bacteria [34]. In worms, cecropins have only been detected in the nematode *Ascaris suum* (cecropin-P1, -P2, -P3 and -P4), a pig intestinal parasite, and other species of *Ascarididae* (at least in *A. lumbricoides* and *Toxocara canis*) [19,62]. These AMPs are short in length, rich in serine, not stabilized by disulfide bonds, and display a linear and amphipathic α-helical structure (Figure 1) [29,63].

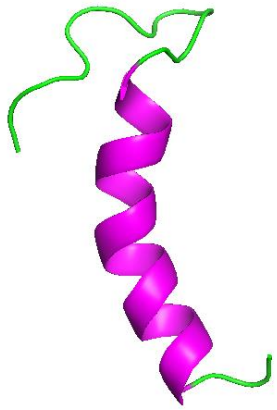


Figure 1. Three-dimensional structure of cecropin-P1, representative of cecropin family (PDB ID: 2N92) [31]. Picture generated using PyMOL (TM) 2.3.2 software: BioLuminate, Schrödinger, LLC, New York, NY, 2019 (www.pymol.org).

Cecropins are derived from precursor molecules, with a common structure, i.e., having a signal peptide, a mature peptide, and a pro-region (Figure 2) [64]. As for α-defensins (mammalian AMPs), the acidic pro-region may inhibit the antimicrobial/cytotoxic activity of the basic mature region, protecting the cells of AMP production sites [65]. The primary structures of the mature cecropins are highly conserved and consist of 31 residues [62].

```
            SIGNAL PEPTIDE      MATURE PEPTIDE                 PROREGION
Cecropin-P1 MFLIYLFVQTAES    SWLSKTAKKLENSAKKRISEGIAIAIQGGPR  RRRFVAEQDAIHSRVSREVPTLSDSV----
Cecropin-P2 MILIYLLVQTAES    SWLSKTYKKLENSAKKRISEGIAIAIQGGPR  RRRFVVQQDTISPRLEVDERFLPNSVQEQI
Cecropin-P3 MFLIYLFVQTAES    SWLSKTAKKLENSAKKRISEGIAIAIKGGSR  RRRSVGEEDAIPSHIEVNKFFLRKPAKEHI
Cecropin-P4 MFLMYLFVQTTES    SWLSKTYKKLENSAKKRISEGVAIAILGGPR  HRRSVAHQEEASLHVKTDELPSPDTVREQL
            * * ** *** **    ****** ************** **** ** *    ** *
```

Figure 2. Sequence alignment of cecropin-family from *A. suum*; * conserved amino acids.

Ascaris cecropins exhibit potent antimicrobial activity. They are upregulated upon bacterial challenge and are active against Gram-positive bacteria (*Staphylococcus aureus*, *Bacillus subtilis*,

Micrococcus luteus), Gram-negative bacteria (*Pseudomonas aeruginosa*, *Salmonella typhimurium*, *Escherichia coli*), and also fungi (*Saccharomyces cervisae*, *Candida albicans)* (Table 3) [62,66,67].

Table 3. Antimicrobial activity spectrum of worm AMPs. The values are expressed in μM: MIC (Minimal Inhibitory Concentration) in black, MBC (Minimal Bactericidal Concentration) in red, and BC50 (50% Bactericidal Concentration) in green.

	Microorganisms	CECROPINS				CAENOPORES			hedistin	BRICHOS FAMILY				ABFS		MACINS			LUMBRICINS		perinerin
		P1	P2	P3	P4	SSP1	SSP5	SSP12		arenicin1	arenicin2	alvinellacin	nicomicin1	AS-α	CE-2	Hm-N	Tt-T	Hm-T	PG	1	
GRAM NEGATIVE	*Escherichia coli*	0,3-0,5	30	9	20		0,1		0,8-1,6	4	4	0,012-0,024	2-16	50		25	25		20	12	12,5-25
	Pseudomonas aeruginosa	0,4-0,5	20	20	20					2			32								3,1-9,2
	Pseudomonas sp.											0,001-0,003									
	Salmonella enterica									0,6											
	Salmonella typhimurium	0,4-0,5	20	8	8																
	Proteus mirabilis									0,6											
	Proteus vulgaris													10							
	Klebsiella pneumoniae	0,5								2-4				70	0,9						
	Vibrio alginolyticus									0,4											
	Vibrio diabolicus											0,048-0,096									
	Vibrio MPV19											0,012-0,024									
	Listonella anguillarum									3,1											
	Bdellovibrio bacteriovorus													0,5	0,06						
	Agrobacterium tumefaciens										5			10	0,05						
	Serratia sp.																		2,5	16	
GRAM POSITIVE	*Micrococcus luteus*	8	30	8	8				0,4-0,8		2,6		0,125	0,8			0,165-0,33				25-50
	Micrococcus nishinomiyaensis								0,4-0,8									1,95-3,8			
	Staphylococcus aureus	22,2	8	3	3				3-6	2-8		0,048-0,096	2	0,6		6,25	100		5	16	
	Staphylococcus epidermidis									4-8											
	Streptococcus mutans																			30	
	Bacillus megaterium					0,1	0,05	0,275			2,6	0,012-0,024				0,20	0,39				2,5-5
	Bacillus subtilis	2	20	10	20					0,31			0,062	1,2						12	
	Bacillus thuringiensis							10													
	Kocuria varians													0,5	0,008						
	Enterococcus faecium	3,4-4								12,5											
	Enterococcus faecalis	9,4																			
	Planococcus citreus									0,03											
	Listeria monocytogenes	4,1								0,6	0,6-0,8										
YEASTS	*Candida albicans*	200	200	200	200					4,5-9	4,5-9								10	16	
	Candida krusei													10	0,3						
	Candida parapsilosis									4,5											
	Trichosporon beigelii									4,5											
	Trichophyton rubrum									9											
	Malassezia furfur									9											
	Fusarium solani										50										
	Saccharomyces cerevisiae	300	300	300	300															12	
	Pichia anomala													30	0,08						
	Paecilomyces heliothis																				12,5-25
	Kluyveromyces thermotolerans													3	0,3						
	REFERENCES	[62,67]	[62]	[62]	[62]	[72]	[72]	[75]	[50]	[14,87,98-100]	[88]	[48]	[110]	[35,117]	[113]	[6]	[6,44]	[43]	[130]	[42]	[49]

The interaction between cecropin and the bacterial membrane is initiated by the C-terminal α-helical structure that plays a crucial role in lipopolysaccharide recognition. Cecropins exert pore formation as a bacterial-killing mechanism [33].Recently, disease-resistant fish and shellfish strains were produced by transgenesis of cecropins-P1 gene, exhibiting elevated resistance to infection by different pathogens [68,69]; cecropin-P4 was used against chicken and pig pathogens as a food supplement to livestock production [70].

Caenopores (from *Caenorhabditis elegans*) belong to the saposin-like protein (SAPLIP) superfamily, a group of small proteins of different sizes and various cellular functions [71]. They are cationic peptides, characterized by the conserved positions of six cysteine residues involved in the formation of three disulfide bonds (Figure 3) [29]. Twenty-three different caenopore-coding genes have been evidenced in *C. elegans*, but antimicrobial activities have only been described for caenopore-1 (SPP-1), caenopore-5 (SPP-5), and caenopore-12 (SPP-12) [72–74].

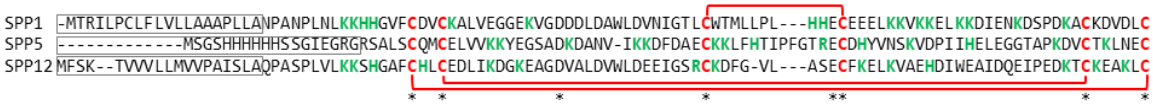


Figure 3. Sequence alignment of caenopores (or saposins); signal peptide in the frame; in red bold type, cysteine residues involved in disulfide bonds; in green bold type, cationic residue; * conserved amino acids.

These three molecules are active against *Bacillus megaterium*; moreover SPP-5 shows significant activity against *E. coli* and SPP-12 is active against *B. thuringiensis* (Table 3) [72,75]. As reported by several authors, natural variants in this AMP family (33 AMPs encoded by 28 different genes) are inducible by different microbes and have a different target spectrum against bacteria and fungi [72,73,76]. Under acidic conditions (pH 5.2), these AMPs are able to form pores, leading to the permeabilization of the bacterial membranes [72]. SSP-5 and SSP-1 are exclusively expressed in the intestine, probably to kill ingested bacteria, and SPP-12 is exclusively expressed in the two pharyngeal neurons [73,75]. In general, it seems that they contribute to both the digestion and the immune defense of the host [73]. To date, only the 3D structure of SSP-5 has been solved at 0.6 Å of resolution, revealing the existence of two conformers (Figure 4).

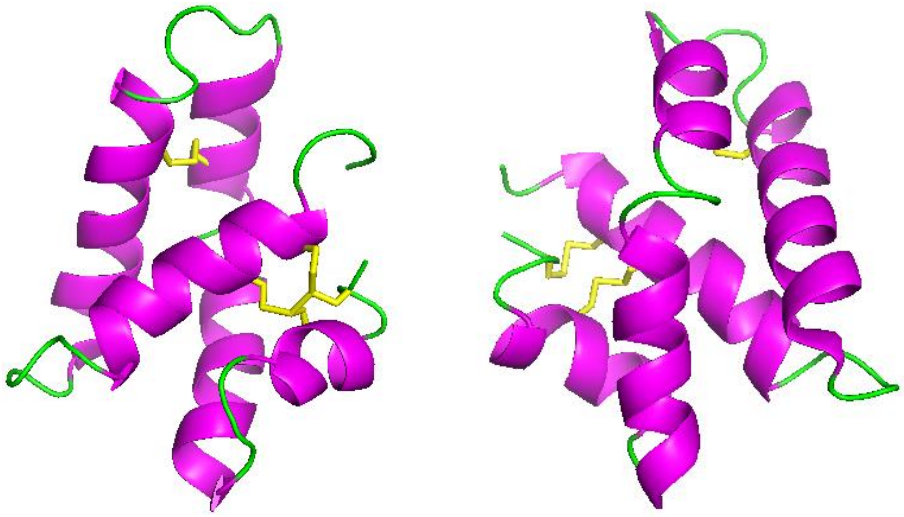
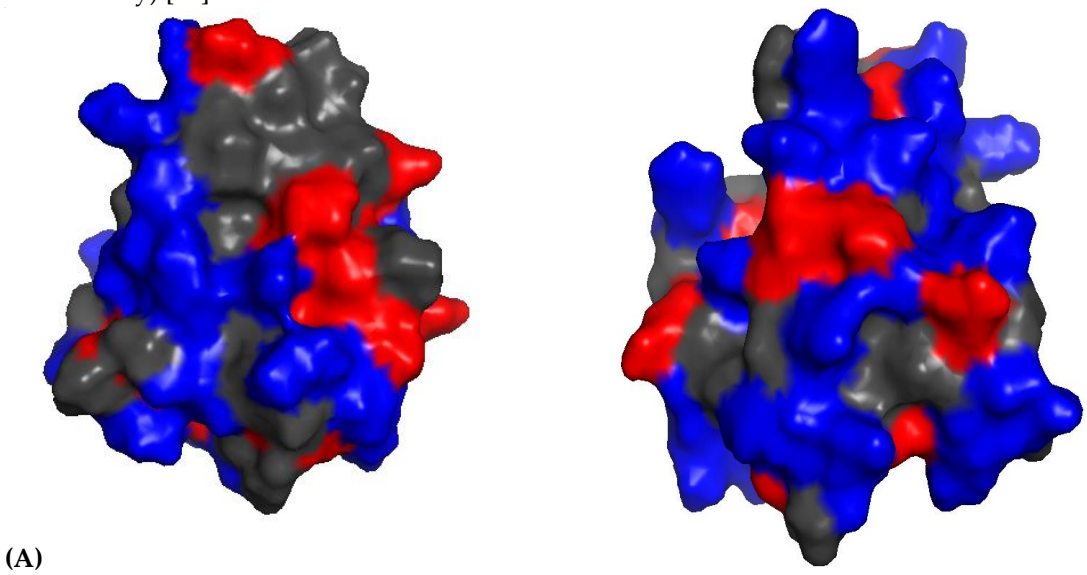


Figure 4. Three-dimensional structure of the SSP-5 conformers: (**A**) *Cis* isomer (PDB ID: 2JS9) [77]; (**B**) *Trans* isomer (PDB ID: 2JSA) [77]. Helices in purple and disulfide bridges in yellow. Pictures generated

using PyMOL (TM) 2.3.2 software: BioLuminate, Schrödinger, LLC, New York, NY, 2019 (www.pymol.org).

The *cis* and *trans* conformers (differing in the isomerization of the peptide bond between Cys80 and Pro81) consist of a bundle of five amphipathic helices which are arranged in a folded leaf with two halves [77]. The 3D structures of both conformers display a large hydrophobic region and an uniformly distributed charged residue covering the surface (Figure 5). SSP-5 was found to exert its antibacterial activity by pore formation (as already shown for amoebapore-like peptides which also belong to the SAPLIP family) [77].



(A) **(B)**

Figure 5. SSP-5 *cis* (**A**) and *trans* (**B**) 3D structures of the surface. Hydrophobic, charged, and polar residues are represented in grey, blue, and red, respectively. Pictures generated using PyMOL (TM) 2.3.2 software: BioLuminate, Schrödinger, LLC, New York, NY, 2019 (www.pymol.org).

2.1.2. α-helix Peptides in Annellids

Hedistin

Hedistin is a linear peptide, identified from the marine annelid *Hediste diversicolor* [50]. To date, no hedistin-like sequences have been found in other species. This ragworm is an euryhaline marine polychaete (order of *Phyllodocida*) able to withstand great variations in salinity. Hedistin (primary structure: LGAW$_{Br}$LAGKVAGTVATYAW$_{Br}$NRYV) is the only annelid peptide containing bromotryptophan residues. As shown for cathelicidin peptides, this modification might be the result of an adaptation that makes the AMP less vulnerable to proteolysis for steric reasons [50,78]. It also carries a C-terminal amidation that increases the cationic charge, and thus its attraction for negatively charged bacterial membranes [50,79]. Hedistin is active against Gram-positive bacteria (especially *Micrococcus luteus* and *Micrococcus nishinomiyaensis*) and the Gram-negative bacterium *Vibrio alginolyticus* (Table 3) [50]. The 3D structure presents three segments, forming a helix–bend–helix conformation that suggests bacterial membrane disruption through a carpet model [50,80]. Hedistin is constitutively and strongly produced by NK-like cells circulating in the body cavity of annelids [50].

2.2. β-sheet Peptides in Annelids

BRICHOS-AMPs Family

Surprisingly, members of this AMP family have been identified in polychaetes only. These AMPs are processed from a larger precursor containing a BRICHOS domain (Figure 6) [14,48,81]. This domain

consists of 100 amino acids and the different BRICHOS family members always show the following structure (Figure 6): (i) A hydrophobic region (a signal peptide or a transmembrane region), (ii) a proregion with a linker and a BRICHOS domain, and (iii) a C-terminal region whose amino-acid residues fold into a double stranded β-sheet (a cysteine rich AMP). While present in a wide range of organisms, the functional properties of the BRICHOS domain has only been explored in mammals [71].

In humans, BRICHOS is a constituent of protein families associated with amyloid formation, found in several major human diseases (Alzheimer's, Parkinson's, diabetes mellitus, dementia, respiratory distress, and cancer) [48,82]. The BRICHOS family member proSP-C (prosurfactant protein C), although the most studied, has no antimicrobial activity due to the absence of the C-terminal extension, i.e., the AMP part. However, in case of proSP-C, BRICHOS binds to the amyloidogenic transmembrane region, preventing it from self-aggregating. The second well studied protein, Bri2, possesses the general structure of BRICHOS family proteins. Current data show that the Bri2 domain interacts as a molecular chaperone on its C-terminal extension (Bri23) to maintain a β-hairpin structure, which has no antimicrobial activity either [82].

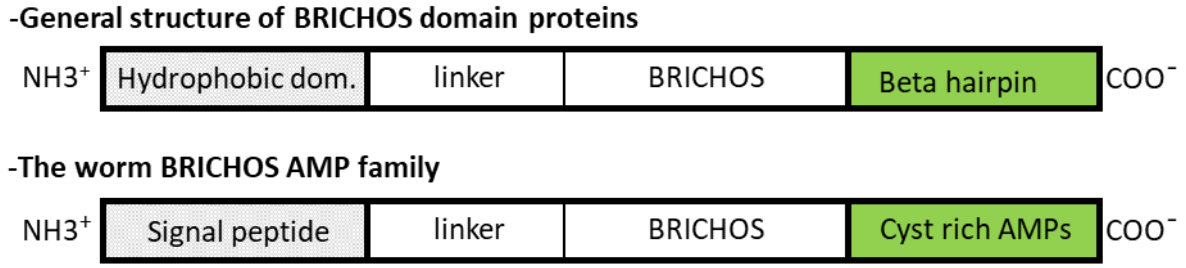


Figure 6. Structural organization of the precursor of a BRICHOS-AMP.

In marine annelids, by contrast with the relatively well conserved BRICHOS domain, the AMP part of the precursor shows a high diversity with sequences that do not share any homologies, suggesting that a strong selection at the interspecific level has probably occurred probably in link with the habitat of the worms [47]. The first discovered members of this family were arenicin-1 and arenicin-2 [14], isolated from the coelomocytes of *Arenicola marina*, a coastal polychaete. This lugworm inhabits sand flats, characterized by high fluctuations of temperature, salinity, oxygen, and sulphide concentrations [83]. The primary structures of the two cyclic isoforms differ only by one amino acid substitution (Val10Ile). They are characterized by 21 residues with a single disulfide bond that connects the N- and C-terminus (Cys3 – Cys20). Later, a third isoform, termed arenicin-3, showing significant differences in the sequence from the first two arenicins and containing one additional disulfide bond (Cys7 – Cys16) was isolated and characterized [84]. Another member of this AMP family named alvinellacin was isolated later and identified from *Alvinella pompejana* the emblematic Pompeii worm that inhabits the hottest part of the black chimneys of the deep eastern Pacific ocean [81]. This animal is considered as the most thermotolerant and eurythermal animal in the world, facing bursts of elevated temperatures as high as 80 °C but also harsh acidic conditions and high pressures (up to 300 bars) [85]. In such a fluctuating and extreme environment, genetic analysis of alvinellacin has given evidence of an adaptive diversification of the molecular chaperone of the AMP, but not of the AMP itself, as the result of the gain of a vital and highly conserved epsilon proteobacteria ectosymbiosis in the face of the joint thermal and sulfide fluctuations of the vent habitat [47]. Biochemical characterization of alvinellacin has revealed that its primary structure is composed of 22 amino acid residues and stabilized by two disulfide bonds [48,86]. However, it is worth noting that BRICHOS-AMP homologs have been also described in other alvinellid and terebellid worms that do not always exhibit bacterial epibioses, and thus represent a very 'old' family of AMPs in annelids.

As mentioned above, annelid AMPs with BRICHOS are characterized by a short amino-acid sequence, a cationic net charge, a hydrophobic region, a β-sheet fold, and the formation of disulfide bonds between cysteine residues, increasing the rigidity of their open-ended cyclic structures (Table 4)

[87–89]. Different specific software can easily determine all these structural characteristics. The Innovagen Pepcalc.com server (Innovagen AB, SE-22370 Lund, SWEDEN; https://pepcalc.com/) was used to calculate the net charge at neutral pH, and Peptide2.0 server (Peptide 2.0 Inc., Chantilly, VA; https://peptide2.com/) to evaluate the peptide hydrophobicity. The positive charge (due to arginine residues) and the hydrophobicity (from valine, leucine, alanine, tryptophan, isoleucine, phenylalanine, and tyrosine) contribute to the amphipathic nature of the peptide. In aqueous solution, they adopt a β-hairpin conformation, formed by two twisted antiparallel β-strands, stabilized by intra-backbone hydrogen bonds and one or two disulfide bonds between cysteine residues (Figure 7) [48,88–90]. This motif was found in other AMPs, like protegrins, gomesin, and tachyplesins, but not in combination with a large residue ring structure (showed in Figure 7) [91–93].

Table 4. Amino acidic sequences hydrophobicity and net charge of BRICHOS-AMPs. In bold type, cysteine residues involved in disulfide bridges.

AMP Name	Amino Acid Sequence	Hydrophobicity	Net Charge At pH 7
Arenicin-1	RW**C**VYAYVRVRGVLVRYRR**C**W	42%	+6
Arenicin-2	RW**C**VYAYVRIRGVLVRYRR**C**W	42%	+6
Arenicin-3	GF**C**WYV**C**VYRNGVRV**C**YRR**C**N	28%	+4
Alvinellacin	RG**C**YTR**C**WKVGRNGRV**C**MRV**C**T	22%	+6
Nicomicin-1	GFWSSVWDGAKNVGTAIIKNAKV**C**VYAV**C**VSHK	45%	+3
Nicomicin-2	GFWSSVWDGAKNVGTAIIRNAKV**C**VYAV**C**VSHK	45%	+3

(A) **(B)** **(C)**

Figure 7. Three-dimensional structure of (**A**) alvinellacin (PDB ID: 2LLR) [48], (**B**) arenicin-1 (PDB ID: 2JSB) [89], and (**C**) arenicin-2 (PDB ID: 2JNI) [88]. Disulfide bridges in yellow. Pictures generated using PyMOL (TM) 2.3.2 software: BioLuminate, Schrödinger, LLC, New York, NY, 2019 (www.pymol.org).

Notably, the structural properties of BRICHOS-AMPs are linked to their membranolytic activity, exhibiting a broad spectrum of activities against Gram-positive, Gram-negative bacterial, and fungal pathogens (Table 3) [94]. Arenicin isoforms display potent antibacterial activity against Gram-positive bacteria (*Listeria monocytogenes*, *Staphylococcus aureus, Staphylococcus epidermidis, Planococcus citreus, Bacillus subtilis, Bacillus megaterium, Micrococcus luteus*), Gram-negative bacteria (*E. coli, Klebsiella pneumoniae, Salmonella enterica, Salmonella typhimurium, Pseudomonas aeruginosa, Proteus mirabilis*, *Vibrio alginolyticus*, *Listonella anguillarum, Agrobacterium tumefaciens*), and also antifungal activity (*Candida albicans, Fusarium solani*) [14,28,88–90,95–100]. Alvinellacin is active against Gram-positive bacteria (*B. megaterium* and *S. aureus*) and Gram-negative bacteria (*E. coli*, *V. diabolicus*, *Pseudomonas* sp., *V. MPV19*). Interestingly, in contrast to the majority of known AMPs, the antimicrobial activity of arenicin-family members is preserved in the presence of salt [14,48,89]. Similarly, low temperature conditions (+4 °C) do not impede arenicin-1 antimicrobial inhibition on *E. coli* and *P. mirabilis* [89].

The peptides kill a number of bacterial strains within minutes by membrane permeabilization, membrane detachment, and release of cytoplasm [14,89]. The mechanism of action of arenicins is still under investigation, and recent studies propose a "toroidal-pore" model, including monomeric or dimeric peptide organization [98,101,102]. The AMP interaction with the anionic phospholipidic bilayer of bacterial membranes is promoted by the high abundance of hydrophobic and positively-charged residues [98,102,103]. The binding to the membranes leads to conformational changes of the peptide molecule [28,104]. Two N-terminal β-strands of peptides associate to form a dimer mediating pore

formation [28,101,104]. In yeast, arenicin-1 may act indirectly, inducing apoptosis via intracellular accumulation of reactive oxygen species, and directly damages mitochondria and DNA in nuclei [105].

Except for alvinellacin, which is not hemolytic or cytotoxic to mammalian cells, arenicins are cytotoxic to human cell lines and cause hemolysis of human red blood cells. Although this precludes their development as candidate antimicrobials, artificial modified analogs were designed based on their structure, in order to decrease their adverse effects and to enhance the antimicrobial properties. Novel derivatives named NZ17074, N2, and N6 were designed and synthesized as linear or with more disulfide bonds by amino acid substitution [90,97,106,107]. By showing a higher antimicrobial activity and a lower cytotoxicity, these latter derivatives were more powerful than the parent molecule. Therefore, these positive results suggest these AMPs as potential candidates for antibacterial drug development [81,107,108].

Arenicin-1 and 2 and alvinellacin transcripts are expressed constitutively in coelomocytes, in the body wall, the foregut, and midgut, suggesting a peptide's involvement in both systemic and epithelial branches of immunity [14,83,109]. These AMPs are also present in a major part of the nervous system, which suggests a possible involvement in the defense and the regeneration of the nerve cord as demonstrated for the cysteine rich AMPs of the leeches (see below) [43,89,109]. Data given also evidences that alvinellacin shapes and controls the specific epibiotic microflora that allows it to thrive in the hydrothermal habitat [48].

Recently, nicomicin-1 and -2 were identified in the artic polychaeta *Nicomache minor* [110]. This worm lives in the cold water, inhabiting hard tubes attached to stones [111]. Nicomicins consist of 33 residues (Table 1), containing BRICHOS domain in the sequences of their prepropeptide. They are characterized by many hydrophobic amino acids (51%) and a disulfide bond (Cys24 – Cys29) [110]. While Nicomicin-2 has no effect on bacteria, Nicomicin-1 exerts strong antimicrobial activity towards Gram-positive bacteria by damaging their membranes; the presence of salt impedes its activity [110]. Conversely, the AMP 3D structure is different from alvinellacin and arenicin and is organized into two independent regions with an α-helix at the N-terminal moiety and a six-residue loop stabilized by the disulfide bridge at the C-terminus [110].

2.3. Mixed α-helix/β-sheet Peptides

2.3.1. Mixed α-helix/β-sheet Peptides in Nematodes

The ABF Family

ABFs (antibacterial factors) are defensin-like AMPs characterized in nematodes only, first in *Ascaris suum* (seven As-ABFs) and then in *Caenorhabditis elegans* (five Ce-ABFs), in *Ancylostoma duodenale* (six Ad-ABFs), and one Cbr-ABF in *C. briggsae* [35,112,113]. This family of peptides appears to be widely distributed in nematodes (86 peptides from 25 species) with different lifestyles and habitats. *A. suum* and *A. duodenale* are hematophageous parasitic, living in the small intestine of mammalian hosts; *C. elegans* and *C. briggsae* are not parasitic and inhabit compost and garden soil. Despite their similarities with macins, they have not been found in annelids. Nematode defensins are cationic and cysteine rich peptides, with formation of disulfide bonds (Figure 8) [114–116].

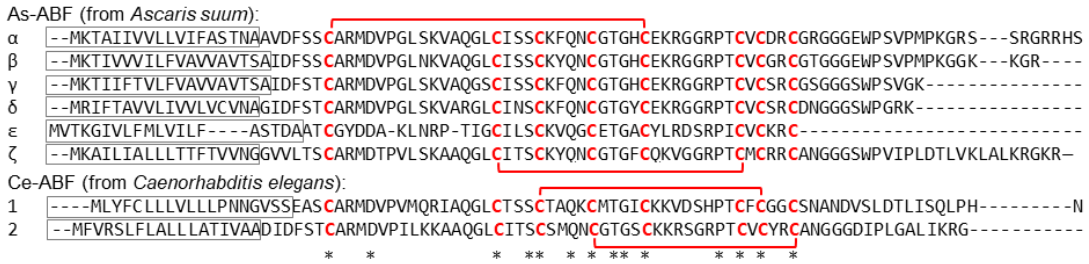


Figure 8. ABF family members' sequence alignment: Signal peptide in the frame; in red bold type, cysteine residues involved in disulfide bridges; * conserved amino acids.

Although the structure for As-ABF-α is the only one having been experimentally determined (Figure 9), the ABFs' structural motif is characterized by an α-helix and two β-sheets stabilized by three disulfide bonds (CS-$\alpha\beta$), the fourth bond contributes to the firmness of the open ended cyclic molecule [4,64].

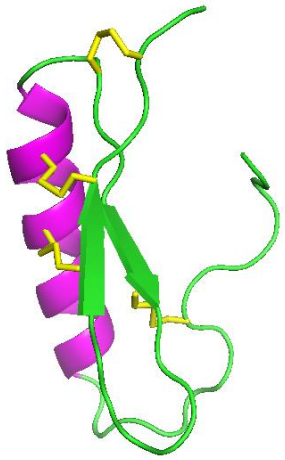


Figure 9. Three-dimensional structure of the As-ABF-alpha (PDB ID: 2D56): In green, antiparallel β-sheets; in purple, α-helix; and in yellow, disulfide bridges [35]. Picture generated using PyMOL (TM) 2.3.2 software: BioLuminate, Schrödinger, LLC, New York, NY, 2019 (www.pymol.org).

The antibacterial activity has been screened for As-ABF-alpha and Ce-ABF2 only, and both exhibit higher antimicrobial activity against Gram-positive bacteria (through pore formation) than against Gram-negative bacteria and yeast (Table 3); the presence of salt inhibits their bactericidal activity [35,112–114,117]. Their expression increases upon bacterial challenge [73,116]. As-ABFs have been detected mainly in the body wall and in other tissues, probably with diversified physiological roles [116]. Conversely, Ce-ABF1 and Ce-ABF2 are mainly produced in the pharynx of *C. elegans*, i.e., the site where live bacteria accumulate after their ingestion [113].

2.3.2. Mixed α-helix/β-sheet Peptides in Annelids

Macin Family

Macins are cationic cysteine-rich AMPs. Members of this family of peptides have been first described in leeches (*Theromyzon tessulatum* and *Hirudo medicinalis*) [43,44], and later in *Hydra vulgaris* [43,118] and in the mollusks *Hyriopsis cumingii* [80] and *Mytilus galloprovincialis* [119]. Both leeches belong to the "Clitellata" class: *T. tessulatum* is a shallow water rhynchobdellid leech, ectoparasite of aquatic birds [120]; *H. medicinalis*, a gnathobdellid leech, is an ectoparasite of mammals which lives in stagnant freshwater and streams [121]. Tt-theromacin (Tt-T) in *T. tessulatum* [44], Hm-neuromacin (Hm-N) and Hm-theromacin (Hm-T) in *H. medicinalis* [43], have several functions that includes bacterial killing, symbiostasis in the gut, immune defense, and regeneration of the damaged nerve cord. Their primary structure is highly conserved with the presence of a signal peptide (except for Hm-Theromacin), four disulfide bridges [122], and a fifth intramolecular disulfide bond (C31:C73) in theromacins (Figure 10) [118].

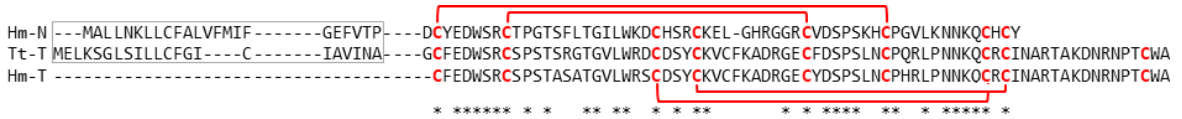


Figure 10. Sequence alignment of Macin family members. Signal peptide in the frame; in red bold type, cysteine residues involved in disulfide bonds; * conserved amino acids.

Macin peptides represent rather long and complex peptides of more than 60 residues. The tertiary structure of macin family members is organized in a knottin-fold according to the arrangement of cysteine bonds, and the peptides' molecular surfaces are divided into two hydrophobic hemispheres (due to the band-like distribution of the positive charges) [118,122]. Figure 11 shows the open-ended cyclic structure of theromacin. The conserved structural features in the macin family are an additional α-helix in N-terminal position and two long flexible loops, distinguishing them from all other peptides of the scorpion-toxin like superfamily in which the macin family belongs [118].

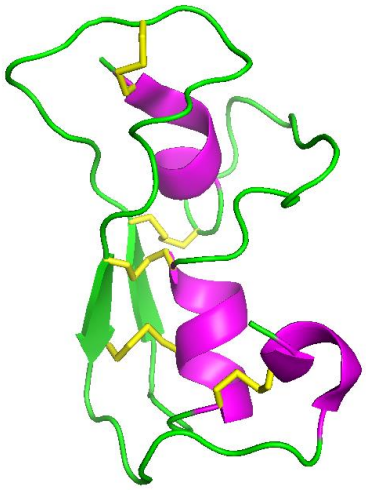


Figure 11. Three-dimensional structure of Tt-theromacin (PDB ID: 2LN8): In green, antiparallel β-sheets; in purple, α-helix; and in yellow, disulfide bridges [6]. Picture generated using PyMOL (TM) 2.3.2 software: BioLuminate, Schrödinger, LLC, New York, NY, 2019 (www.pymol.org).

Theromacin and neuromacin have been evidenced to display antimicrobial activity against Gram-positive bacteria (*B. megaterium* and *M. luteus*) [6] and low antibacterial activity against Gram-negative proteobacteria (*E. coli*) [44]; neuromacin being also active against *Micrococcus nishinomiyaensis* (Table 3) [43]. The activities are impeded with increasing salt concentrations [6]. The MOA of the family (barnacle model) includes the permeabilization of the membrane of Gram-positive bacteria, but also the pore formation as observed for neuromacin [6]. Thanks to their structural double-amphipathic character (two hydrophobic hemispheres sandwiched by a belt of positive charges), initially macins promote aggregation of bacteria, and after, they permeabilize the bacterial membrane [6,118].

In addition to antibacterial activity, both neuromacin and theromacin exert nerve-cord regeneration activity [6,43]. In *H. medicinalis*, theromacin is released in the blood surrounding the nervous system, and neuromacin is produced by nerve cells and accumulates at the wounded site of the central nervous system [123], whereas Tt-theromacin is expressed in large fat cells and released immediately into the coelomic fluid following infections or damages of the central nervous system [6,44].

2.4. Peptides Enriched with One or Two Specific Amino Acids

2.4.1. Peptides Enriched with One or Two Specific Amino Acids in Nematodes

Neuropeptide-Like Peptides and Caenacins

Neuropeptide-like peptides (nlps) and caenacins (CNCs) are basic peptides which are enriched in glycine and aromatic amino acids residues [40,124,125]. They are induced in the hypodermis by infection (i.e., *Drechmeria coniospora*) or wounding in *C. elegans* and other nematodes species, playing diverse roles in nervous system functioning [125]. These two AMP groups represent 111 genes already known. In Figures 12 and 13, some examples of nlp and CNC families are listed, showing YGGWG and YGGYG motifs which are likely to typify this group of AMPs [126].

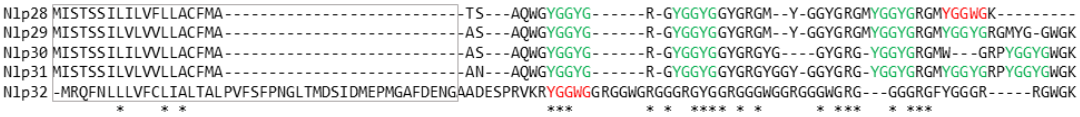


Figure 12. Nlp family members' sequence alignment: Signal peptide in the frame; YGGYG and YGGWG motifs in green and red, respectively; * conserved amino acids.

Interestingly, the expression of nlp and CNC genes in *C. elegans* is downregulated upon challenge with the majority of tested bacteria and upregulated in the case of fungal infections [73]. To date, there have been no direct tests of the antimicrobial/antifungal activity for these peptides and the MOA has not yet been described. Recently, nlp-31 exhibited activity towards *Burkholderia pseudomallei* (a Gram-negative bacterium, resistant to a wide range of antimicrobials), thereby binding to DNA and interfering with bacterial viability without any membrane disruption activity [127].

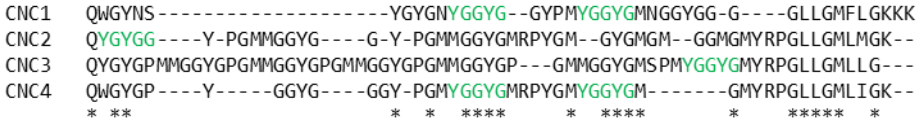


Figure 13. Caenacin members' sequence alignment: YGGYG motifs in green; * conserved amino acids.

2.4.2. Peptides enriched with one or two specific amino acids in annelids

Lumbricin Family

Lumbricins are proline-rich AMPs characterized in oligochaete earthworms *Lumbricus rubellus* (lumbricin-1), *Pheretima tschiliensis* (PP-1), *P. guillelmi* (lumbricin-PG), and *Eiseinia andrei* (Lumbr and LuRP), and in the leech *H. medicinalis* (Hm-lumbricin) [42,43,128–130], but also found in polychaetes such as the Pompeii worm *A. pompejana* (AT & DJ, pers. obs.) and leeches [88].

Their amino acid sequences include numerous prolines and aromatic amino-acid residues (phenylalanine, tyrosine or tryptophan); only lumbricin-1 and lumbricin-PG exhibit a signal peptide sequence (Figure 14).

Figure 14. Lumbricin family members' sequence alignment: Signal peptide in the frame; * conserved amino acids.

Lumbricin-1 exhibits antibacterial activity towards a broad spectrum of Gram-positive and Gram-negative bacteria and fungi (Table 3) [42,43,128–130], but their MOA is still unknown. PP-1 is synthesized in the mucus of the epidermis; the two lumbricins from *E. andrei* have been detected in the

intestine and in other tissues (body wall, gut, ovary, etc.) [43,128,131,132]. Interestingly, Hm-lumbricin gene expression is rapidly enhanced by bacterial challenge [43], whereas Lumbr and LuRP are slowly induced (after 48 h) following the infection [129]. By contrast, lumbricin-1 (present only in adult worms) is not inducible when the animal is subjected to a bacterial challenge [42]. Hm-lumbricin exerts neuroregenerative properties in leeches, as observed for neuromacin [43]. Nowadays, the tertiary structures of lumbricins, nlps, and CNCs have not been solved [114].

2.5. Peptides Derived from Larger Molecules in Annelids

2.5.1. Perinerin

Perinerin is a cationic, hydrophobic, and linear peptide, isolated and characterized from the Asian marine clamworm *Perinereis aibuhitensis* (Grube, 1878) [49,133]. This annelid is a marine polychaete, living in the sediment of estuaries [134]. Perinerin consists of 51 amino-acid residues (primary structure: FNKLKQGSSKRTCAKCFRKIMPSVHELDERRRGANRWAAGFRKCVSSICRY), with a high proportion of arginine and four cysteine residues possibly involved in the formation of two disulfide bonds [49]. Despite the presence of cysteine residues and disulfide bonds, the Perinerin sequence does not show any similarities with the previously described AMPs in annelids, and its average sequence identity to other cysteine-rich AMPs is less than 30% [135]. It exhibits a broad range of antimicrobial activities (antifungal, bactericidal against Gram-negative and Gram-positive bacteria) without any observed microbial resistance (Table 3) [49]. The proposed MOA is pore-forming activity and the bactericidal action against the Gram-positive bacteria *B. megaterium* is very fast (less than 3 minutes) [79]. Perinerin purification is obtained from unchallenged individuals, and suggests that the peptide is constitutively expressed [49]. Until now, no studies describing the three-dimensional structure of Perinerin have been performed.

2.5.2. Ms-Hemerycin

Ms-Hemerycin is an AMP from the polychaete *Marphysa sanguinea*, a marine lugworm that inhabits mudflats [51]. Its amino-acid sequence consists of 14 amino acids (Ac-SVEIPKPFKWNDSF) blocked by a N-terminal acetylation for its stability. Ms-Hemerycin is derived from the split of the N-terminus of the well-known respiratory pigment hemerythrin found in several marine invertebrates. This peptide exhibits potent activity against Gram-negative and Gram-positive bacteria (Table 3). Ms-Hemerycin has been detected constitutively in all examined tissues, with higher concentration in brain and muscle. The secondary structure might be unordered, containing a partial α-helical region. From such an unordered structure, it can be predicted that the MOA should be very different from the other AMPs [30,51].

3. Conclusions and Perspectives

Among biological models, marine worms are particularly attractive for searching and studying the adaptation/evolution of AMPs to environmental conditions despite their high level of divergence. Compared to the terrestrial environment, the sea has remained virtually unexplored for its ability to yield pharmacological metabolites. In the last decades, research has expanded from lands to oceans in order to find new drug candidates. Because the oceans occupy almost 70% of Earth's surface, they offer a vast potential for biological and chemical diversities. Even more interesting are marine worms living in extreme habitats. The peculiar thermochemical and biotic pressures (and notably, the abundance of Gram-negative bacteria where most actual MDR bacteria belong to) that marine worms have to face in hostile environments represent a natural laboratory to select AMPs able to be more acid-resistant, thermostable, salt-tolerant, and active against most bacterial strains. Extremophile worms constitute interesting models to search and study novel drugs [136].

Moreover, the study of AMPs produced by extremophile annelids offers the perspective to add an initial piece in the complex relationship between the external immunity of the host and its ectosymbionts recruitment and growth control [48,137,138].

Funding: This research was funded by the Université de Lille (BQR), the GDR CNRS ECCHIS, the Fondation pour la Recherche sur la Biodiversité (FRB), the Région Hauts de France (VERMER project), and the Total Foundation-Ifremer (PIONEER and DYVA projects).
Acknowledgments： We thank the captain of the crew of the R/V Atalante, the DSV Nautile and ROV group (IFREMER), along with the chief scientists of the MESCAL 2010, 2012 (LE BRIS Nadine, LALLIER François MESCAL, https://doi.org/10.18142/261) and CHUBACARC 2019 cruises.
Conflicts of Interest： The authors declare no conflicts of interest.

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12. Objectives of this thesis

The discovery of marine antibacterial macromolecules plays a significant part in the field of drug discovery and biomedical research, being a promising source of antibacterial agents against several drug resistant strains of pathogenic microorganisms [65]. Despite more than 30,000 natural macromolecules have been identified and reported from marine organisms, however only few macromolecules are being explored and validated [176,177]. The marine environment is relatively poorly explored in terms of potential pharmaceuticals (especially from invertebrates), and it contains an impressive species diversity which evolved in close proximity to microorganisms [150,176]. 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 [178]. 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 [29,30]. 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 [176,179].

In this context, after an updated overview of the current knowledge about the antimicrobial peptides from worms (previously integrated as review “Worms’ antimicrobial peptides”), the goals of my PhD were:

- to identify new AMPs from marine nematodes, as described in Chapter 1 (Screening for antimicrobial molecules in meiobenthic nematodes belonging to the Oncholaimidae family);
- to study the adaptation of AMPs to varying environmental conditions by using as a model the BRICHOS-AMPs family present in annelids, as described in Chapter 2 (Local adaptation of BRICHOS-AMPs to biotic and abiotic environmental constraints);
- to study the roles of the BRICHOS domain in invertebrates AMPs precursor, as described in Chapter 3 (Role of BRICHOS domain).

In conclusion, we present a last chapter containing general discussions and perspectives for future investigations.

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CHAPTER 1. Searching for novel antibiotic substances produced by marine nematodes inhabiting extreme habitats

To date, several group of AMPs have been identified in terrestrial species of nematodes, none of these was purified from marine nematodes.

In this second chapter, even if the approach was unfruitful, I describe our attempts to purify and identify AMPs from marine nematodes by using bioassay-guided purification assays.

We investigated on three Oncholaimidae nematodes species, the most abundant inhabiting the anoxic sediments of two coastal extreme environments: the shallow hydrothermal vent field “Secca delle Fumose” (Naples, Italy) and Roscoff harbour (Roscoff, France). Promising results provided evidences that two of the three Oncholaimidae nematodes species are potential sources of small sized antibiotics.

The limitation of the method was clearly the too low quantity of material, due to nematodes tiny size, combined with the difficulties in raising them in the lab and their unexpected random geographical distribution.

My contribution to this work consisted in: sediment samplings (underwater in Naples, November 2016 and November 2017 and at low tide in Roscoff, July 2017), sorting and identification of sampled nematodes, peptidic extraction, antimicrobial assays and purification by RP-HPLC of active substances.

The paper was accepted in *Cahier de Biologie Marine* (03/02/2021, vol. 62-2).

Additionally, I contributed to a study on the particular environmental conditions and the species composition of macrofauna at the Secca delle Fumose shallow hydrothermal system, evaluating the biological responses in an extreme habitat (such as high temperature, sulfide concentration and low pH condition). The data have been subject of a paper published in *Frontiers in Marine Science* “Environmental and Benthic Community Patterns of the Shallow Hydrothermal Area of Secca Delle Fumose (Baia, Naples, Italy)”, (see Annexe 1).

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

Renato BRUNO[1,2]*, Daniela ZEPPILLI[3], Céline BOIDIN-WICHLACZ[1,2], Roberto SANDULLI[4,5] and Aurélie TASIEMSKI[1,2]*

[1] Univ. Lille, CNRS, Inserm, CHU Lille, Institut Pasteur de Lille, U1019 – UMR 8204 - CIIL - Center for Infection and Immunity of Lille, Lille F-59000, France

[2] Univ. Lille, CNRS, UMR 8198 - Evo-Eco-Paleo, Lille F-59000, France

[3] IFREMER Centre Brest REM/EEP/LEP, ZI de la Pointe du Diable, CS10070, Plouzané F-29280, France

[4] Department of Science and Technology (DiST), Parthenope University of Naples, Naples 80143, Italy

[5] CoNISMa, Piazzale Flaminio n.9, 00196 - Rome, Italy

* Corresponding authors: Renato BRUNO, Aurélie TASIEMSKI; E-mail: renato.bruno@univ-lille.fr - aurelie.tasiemski@univ-lille.fr

Running title: marine nematodes antimicrobials

Keywords: antimicrobial peptides - free-living nematodes - marine worms

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 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.

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; Tortorella 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 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; Donnarumma et al., 2019; Bellec et al., 2020; Appolloni et al., 2020).

In the old harbour of Roscoff (48°43'34.20''N and 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 and 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 and 1.097 g/mL, 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 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 and 1.097 g/mL, 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 and 1.097 g/mL, 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/µL. 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. 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. 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 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 (Figure S1).

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. 4A-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 and 1.097 g/mL, 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 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 4). 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. 5A-B), challenged extract presents two additional peaks (red rectangle in Fig. 5A). 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 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. 5C).

MALDI-TOF mass spectrometry analysis detected that the two active fraction samples (Fig. 5D-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. 5F). 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*., 2020, 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.

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)).

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) and (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).

Figure 3: *Oncholaimus* morpho1 (Roscoff), *Oncholaimus* morpho2 (Naples) and *Metoncholaimus albidus*.

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

Figure 4: Antimicrobial activity of *Metoncholaimus albidus* and *Oncholaimus* morpho1.

Antibacterial solid plate assay of (A) *M. albidus* and *O.* morpho1 crude extracts against *M. luteus* and *A. hydrophila* and (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 5: 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).

Figure S1: Survival curve of *Metoncholaimus albidus* and *Oncholaimus* morpho2 maintained in the laboratory.

Table1: 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 1

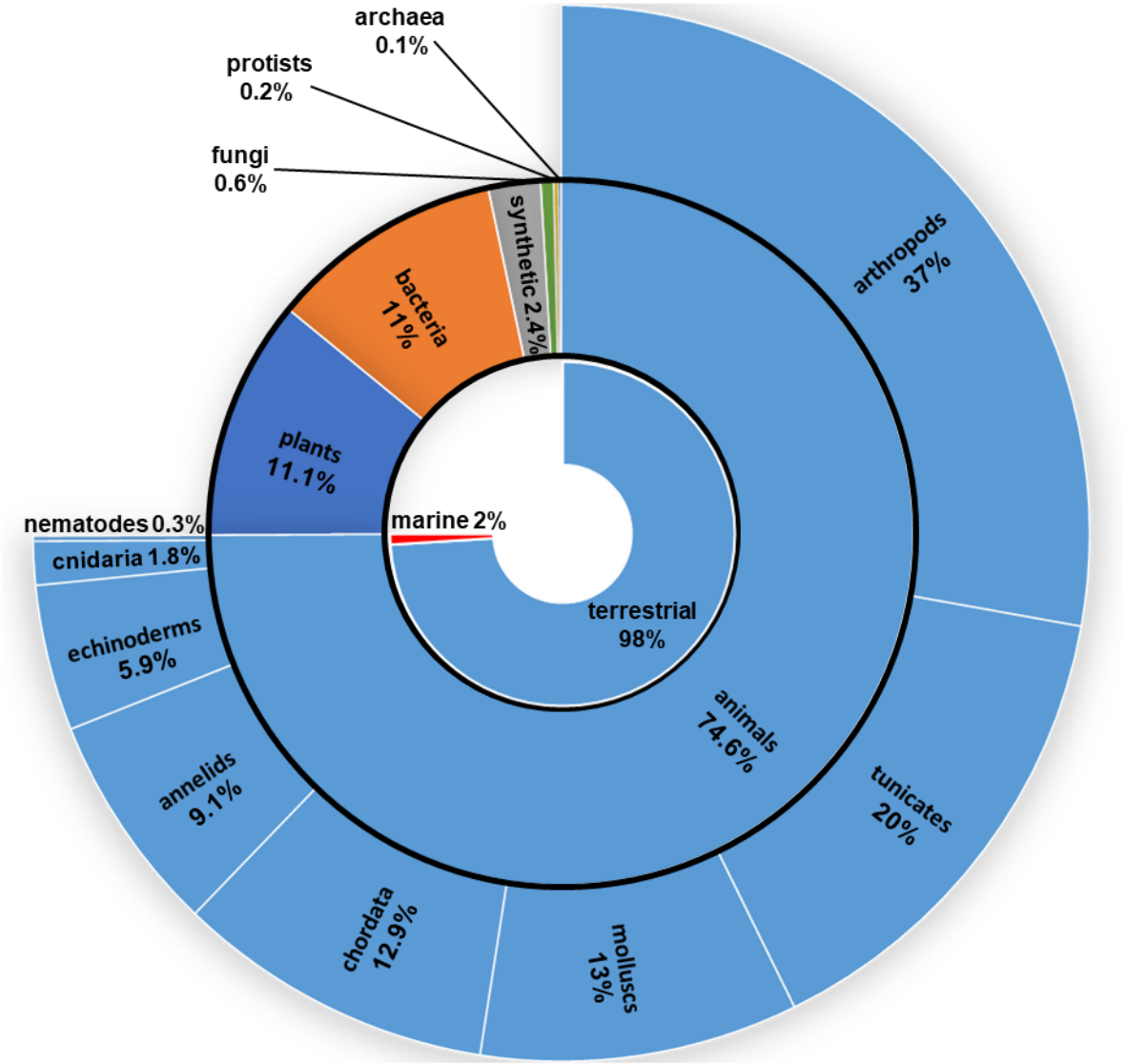
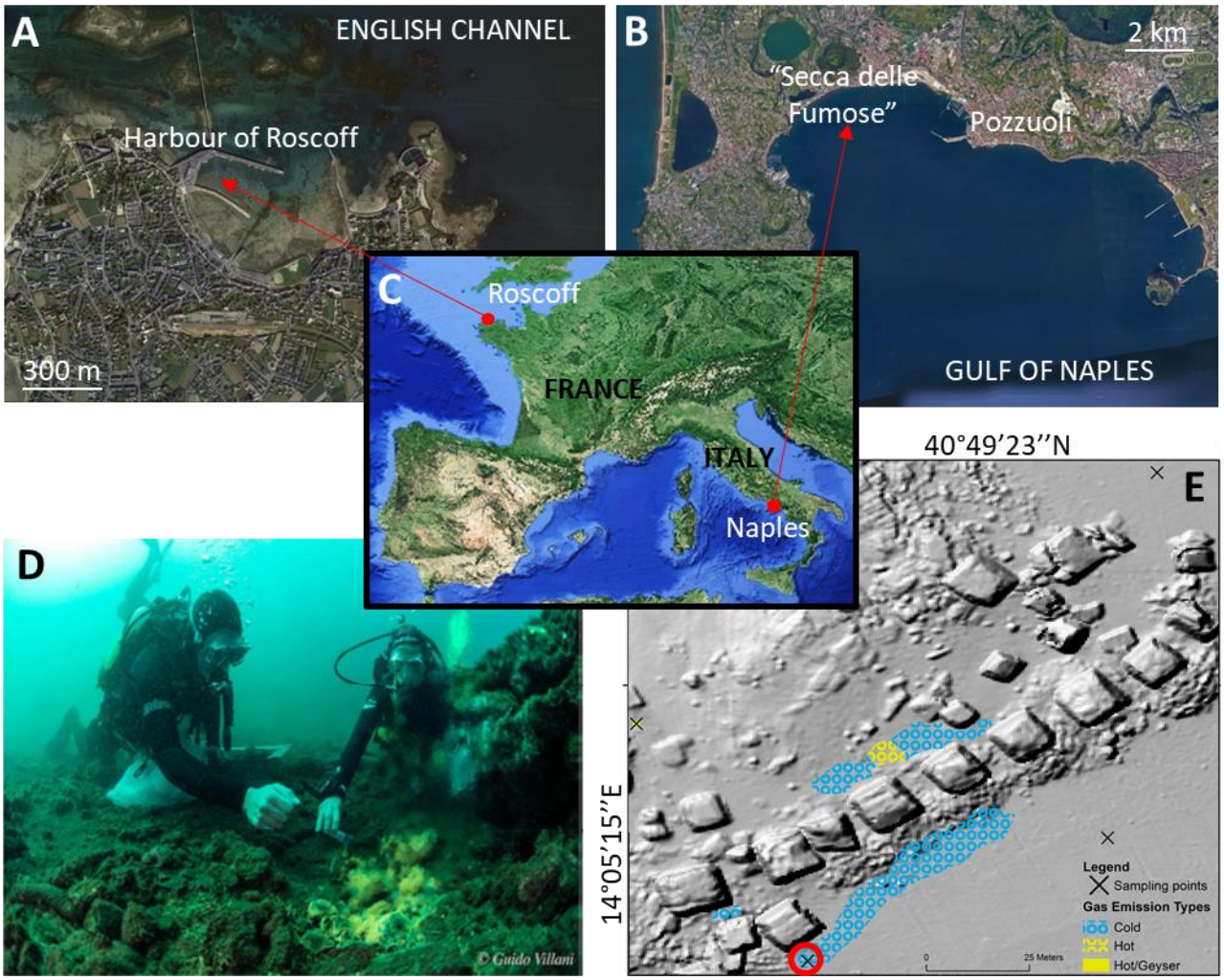


Figure 2



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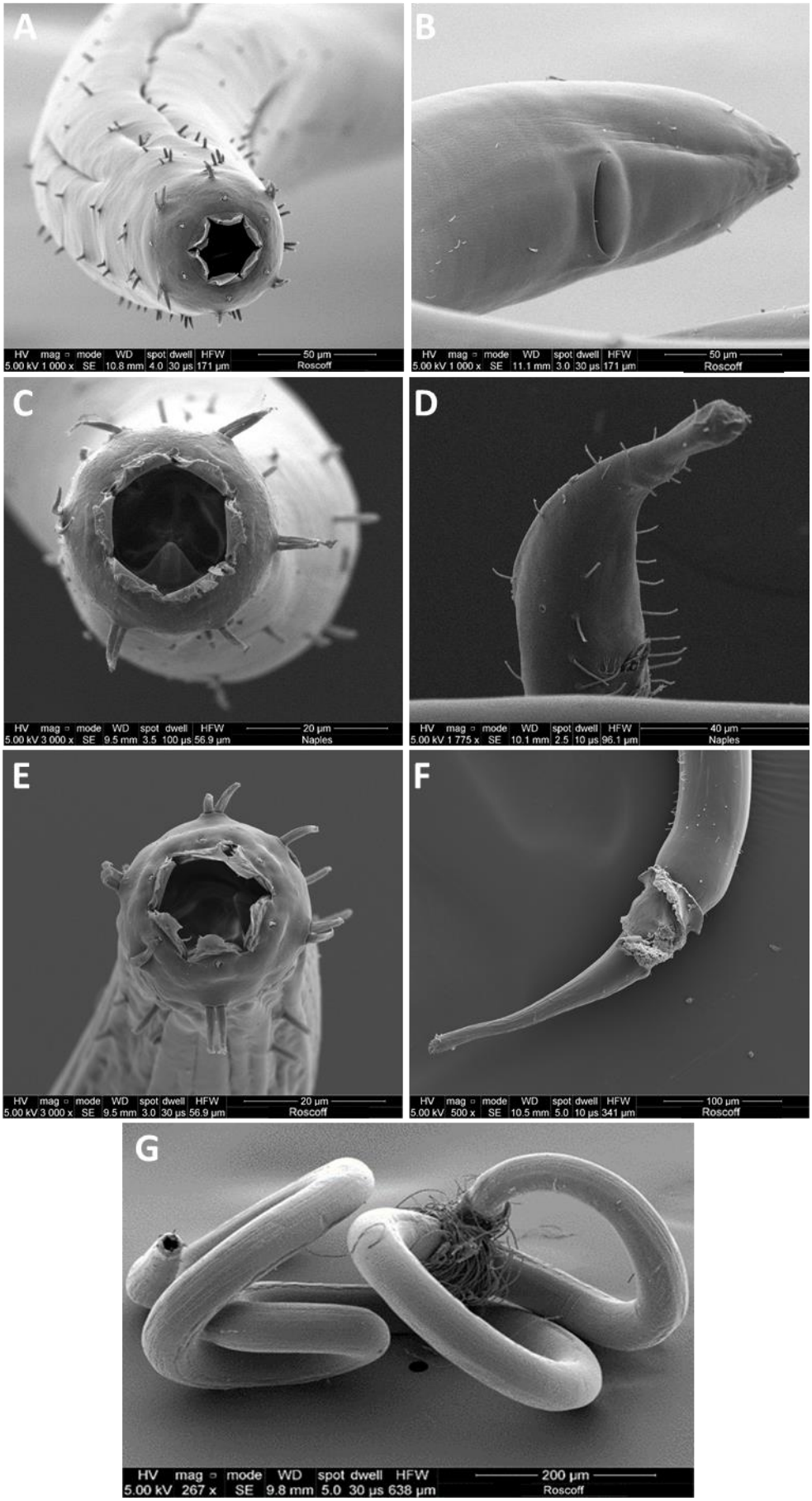

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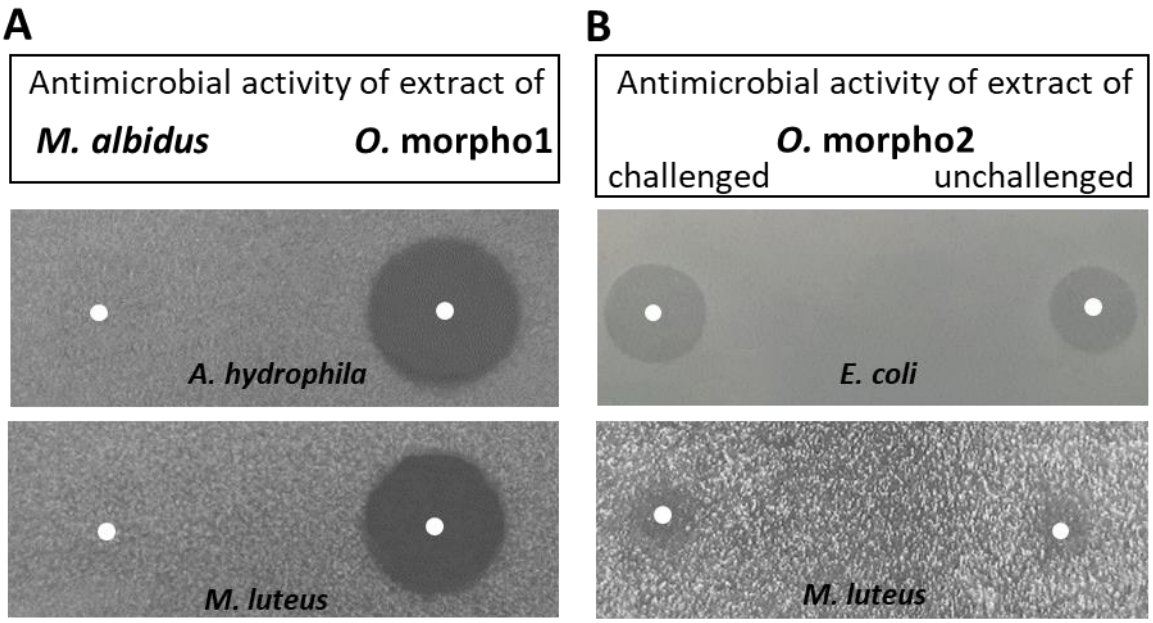


Figure 5

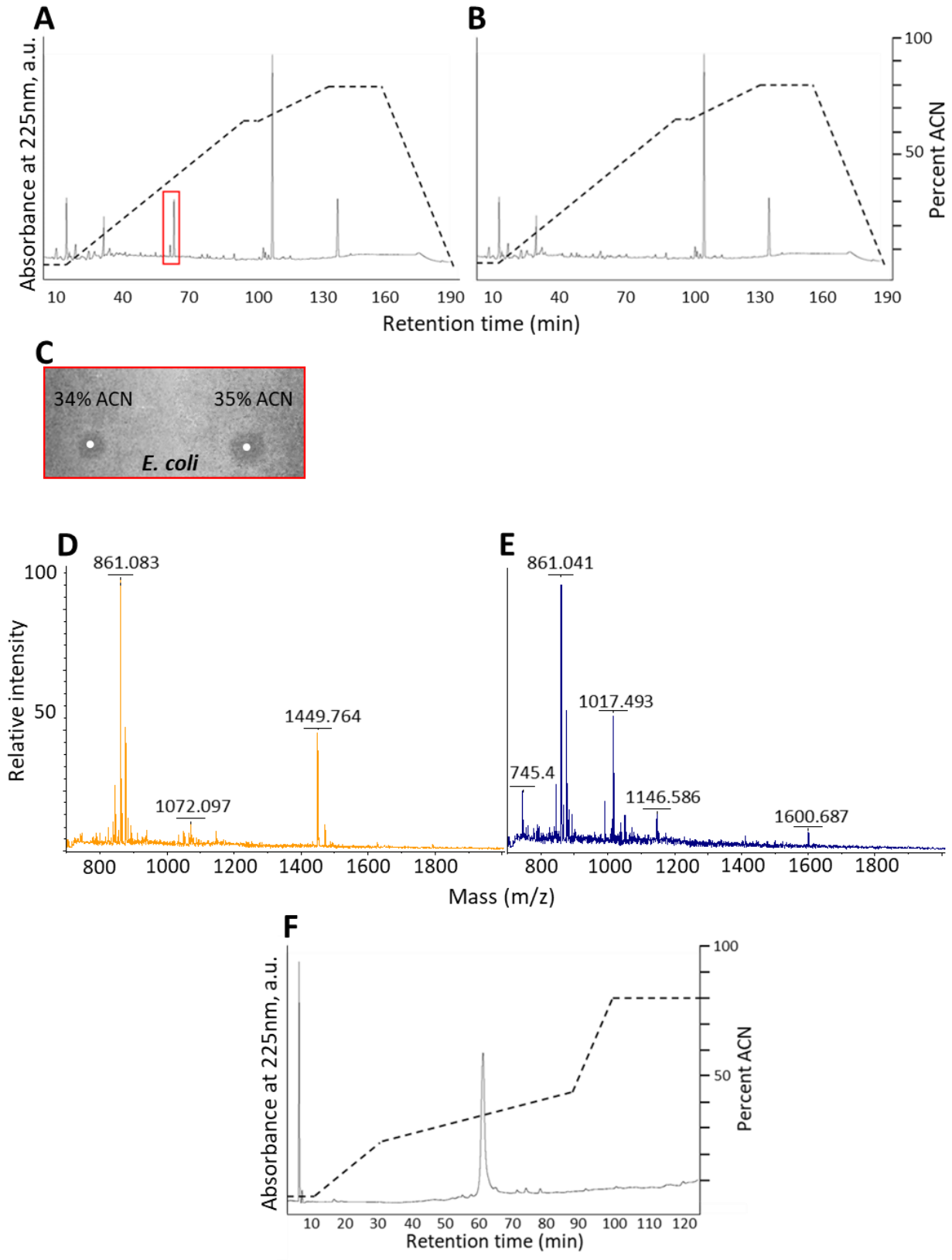
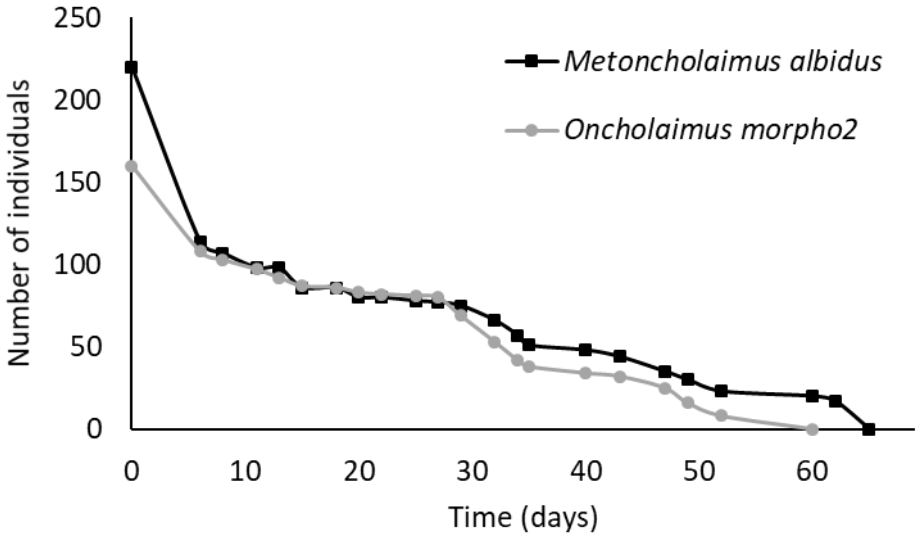


Figure S1



Acknowledgements

We thank Sandra Fuchs, Laure Bellec, Elisa Baldrighi and Chloe Gonzalez for their technical help during sampling and nematode sorting, and peptide purification. The soprintendenza of the Underwater Archeological Park of Baia (prot. 5667, 24/10/2016) is acknowledged for the authorization to sampling. We are in debt to the diving center Centro SUB Pozzuoli (Guglielmo Fragale) for support during diving activities. We thank Luca Appolloni and Luigia Donnarumma for helping in subaquatic sampling activities. Authors are indebted to Nicolas Gayet for his help and support during SEM analyses. Financial support was provided by the project “Prokaryote-nematode Interaction in marine extreme envirONments: a uniquE source for ExploRation of innovative biomedical applications” (PIONEER) funded by the Total Foundation and IFREMER (2016–2019). The funding body played no part in the design, collection, analysis or interpretation of the experiment or in the production of the manuscript.

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CHAPTER 2. Local adaptation of BRICHOS-AMPs to biotic and abiotic environmental constraints

Polaricin (a novel AMP from *Amphitritides* sp.), arenicin and alvinellacin are AMPs isolated from three marine polychaetes, living in various extreme habitats (respectively polar, coastal and deep-sea hydrothermal vents). Playing a key role in the external immunity of marine annelids, they offer an interesting model for studying the influence of the habitat on the selection of AMPs. They share common biochemical features (cationic charge, amphipathicity) and are processed from a larger precursor containing a BRICHOS domain. Surprisingly, members of this AMP family have been identified in polychaetes only. Despite the similarities and relatively well conserved BRICHOS domain, in marine annelids, the AMP part of the precursor shows a high diversity, suggesting that a strong selection at the interspecific level has occurred probably in relation to the peculiar ecology of these organisms. AMPs role in the external immunity makes them directly exposed to the biological and physico-chemical variations of the habitat of the worm.

We studied the adaptation of BRICHOS-AMPs to varying abiotic (thermal and pH variations) and biotic factors (environmental bacterial communities), providing a clear evidence of:

1. the adaptation of the biological activities to the environmental bacteria;
2. the influence of the temperatures and the pH on the natural selection of AMPs;
3. the disulfide bridges involvement in AMPs stability (in terms of biological activities), in the cases of thermal and pH stressors.

This chapter hinges together the main work of my PhD. Here is presented as the Chapter 2, the draft of a paper not yet ready for submission.

Local adaptation of AMPs to environmental factors

Authors: Renato Bruno[1,2], Celine Wichlacz[2], Didier Jollivet[3], François Massol[2], Daniela Zeppilli[4], Kamel Mabrouk[5], Marc Maresca[6] and Aurélie Tasiemski[1,2].

[1] Univ. Lille, CNRS, Inserm, CHU Lille, Institut Pasteur de Lille, U1019 – UMR 8204 - CIIL - Center for Infection and Immunity of Lille, Lille F-59000, France
[2] Univ. Lille, CNRS, UMR 8198 - Evo-Eco-Paleo, Lille F-59000, France
[3] Sorbonne Université, CNRS, UMR 7144 AD2M, Station Biologique de Roscoff, Place Georges Teissier CS90074, Roscoff F-29688, France
[4] IFREMER Centre Brest REM/EEP/LEP, ZI de la Pointe du Diable, CS10070, Plouzané F-29280, France
[5] Aix-Marseille Univ, CNRS, UMR7273, ICR, Marseille F-13013, France
[6] Aix-Marseille Univ, CNRS, Centrale Marseille, iSm2, Marseille F-13013, France

Abstract: Antimicrobial peptides (AMPs) play a key role in the external immunity of animals, offering an interesting model for studying the influence of the environment on the structural diversification and evolution of AMPs. Arenicin, alvinellacin and polaricin (a newly identified AMP), characterized from three marine polychaetes inhabiting in contrasted marine habitats (temperate intertidal, still 'hot' deep sea vents and polar subtidal), possess a well conserved BRICHOS domain in their precursor molecule despite a profound diversification of both the primary and 3D structures of the antibacterial peptide. Here, we showed that the AMP part has an adaptive role to fit both extreme abiotic (thermal and pH variations) and biotic (bacterial communities) factors. Linearized variants (devoid of cysteine residues) were used to demonstrate disulfide bridges functional involvement in peptides structure stability and gene evolution to diverse selective pressures. Our data clearly provided evidences that biological activities of the mature AMPs are markedly adapted to the bacterial communities encountered in each environment and, for the first time, indicated that the external immune is evolving to adapt to abiotic variations of the habitat (temperature and pH) by increasing or reducing the number of disulfide bridges. Altogether, these data highlight that AMPs are the most efficient in the microbial but also in the abiotic environment they have been selected for. These observations also support the importance of considering the optimal/natural environment of AMPs when investigating and optimizing their use in therapeutic treatment of microbial diseases known to modify the cellular environment.

Keywords: BRICHOS-AMPs, abiotic constraint, disulfide bridges, marine annelids, peptide adaptation, extreme environment, bacterial communities, external immunity

2.1. Introduction

External immunity is the first defence response of metazoans to face pathogens by manipulating the surrounding microbial communities in order to avoid infection and also to establish symbiosis [1]. As such, it can be viewed as an extended arm of the immune system [1]. Many organisms including plants, invertebrates, vertebrates and, even bacteria, secrete antimicrobial peptides (AMPs) as an extrinsic protective shield against the surrounding biota that is usually rich in potentially pathogenic agents [2–4]. AMPs are small molecules (up to 100 amino acids), with a large broad-spectrum of activity against many microbes (bacteria, virus or fungi) and a rapid and selective killing activity [4]. Lacking antibodies, invertebrates evolved by developing a potent and complex innate immune system, characterized among other factors by a wide range of AMPs [5]. In the case of annelids (ringed worms), the majority of AMPs is species-specific, probably as a long parallel evolution of their defence arsenal to a wide variety of habitats on Earth (aquatic and terrestrial), mimicking their highly diverse life styles and ecology. Worms have colonized and dominated in biomass most of marine and freshwater habitats, copying with a wide number of environmental conditions and bacterial assemblages, especially in extreme environments such as polar regions, deep-sea hydrothermal vents, or highly anthropized areas [6,7]. Marine organisms constitute almost half of the biodiversity of the Earth, and after a long-term diversification (Cambrian explosion) and co-evolution with bacteria and archaea, they are capable of metabolizing many unique antimicrobial substances [8,9]. Thus, marine annelids living under extreme conditions and more particularly their specific BRICHOS-domain AMP family involved in the worms' external immune defence constitute a model of choice to study immune adaptive evolution to environmental changes.

To date, AMPs from the BRICHOS-domain AMP family have been identified in marine annelids only [10–12]. Arenicin (ARE) of *Arenicola marina*, an annelid inhabiting the intertidal zone of the temperate shore, was the first identified [10]. Later, alvinellacin (ALV) was purified from the hydrothermal hot vent annelid *Alvinella pompejana* [11] and recently nicomicin (NIC) was identified from *Nicomache minor* a polychaete colonizing both temperate and arctic habitats [12]. They all share in common the same precursor structure consisting of: i/an highly hydrophobic N-terminal signal sequence, ii/ a pro-region containing a BRICHOS domain, and ii/ the C-terminal AMP which exerts its biological activities once cleaved from the

precursor[10–13]. BRICHOS (initially found in Bri2, chondromodulin, and prosurfactant protein C in human) is a 100 amino acids domain, present in several protein precursors associated with several major human diseases, acting as an intramolecular chaperone and/or preventing the amyloid formation [14–18]. Even if all the identified BRICHOS precursor present the structure mentioned before, the presence of an AMP at the C-terminal part is unique of marine annelids [13].

Interestingly, BRICHOS-AMPs sequences of annelids show a well-conserved BRICHOS domain but highly divergent primary sequences of the AMP, suggesting either a strong diversifying selection at the interspecific level of AMPs in face of diverse microbial communities and/or abiotic conditions or some exon shuffling between the well-conserved BRICHOS domain and AMPs of different origins [19].

In order to enlarge our panel of BRICHOS-domain AMPs from extreme habitats, we first report here a novel AMP, named polaricin (POL), from an undescribed terebellid polychaete belonging to the genus *Amphitritides* inhabiting polar habitat (Antarctica). POL together with ALV and ARE, thus represent a perfect model system to study AMP adaptation to environmental stressors as they have evolved under highly contrasted environmental conditions and likely share a common ancestor [10,11].

We then focused our study on local adaptation of this specific annelid BRICHOS-domain AMP family involved in the worms' external immune defence under highly different ecological niches (polar/hot vent/temperate), to observe how the pressures of biotic and abiotic factors have affected the structures and bioactivities of the peptides. By combining both biochemical and structural characteristics of these three AMPs with their bioactivities under different conditions, we determine whether these immune peptides fit to the biotic and abiotic factors typifying the worms' habitats. The role of the disulphide bridges in such adaptation of the AMPs was then investigated by comparing the biological activities of our three BRICHOS-AMPs with and without pairs of cysteines, under each set of environmental conditions.

2.2. Results

2.2.1. The newly identified Polaricin (POL) supports the interspecific diversity of AMPs from the BRICHOS-AMP family

The molecular identification of prepropolaricin was obtained by tblastx on the transcriptome assembly of *Amphitritides* sp. using the sequence of the preproalvinellacin as query (Figure 1, Figure S1).

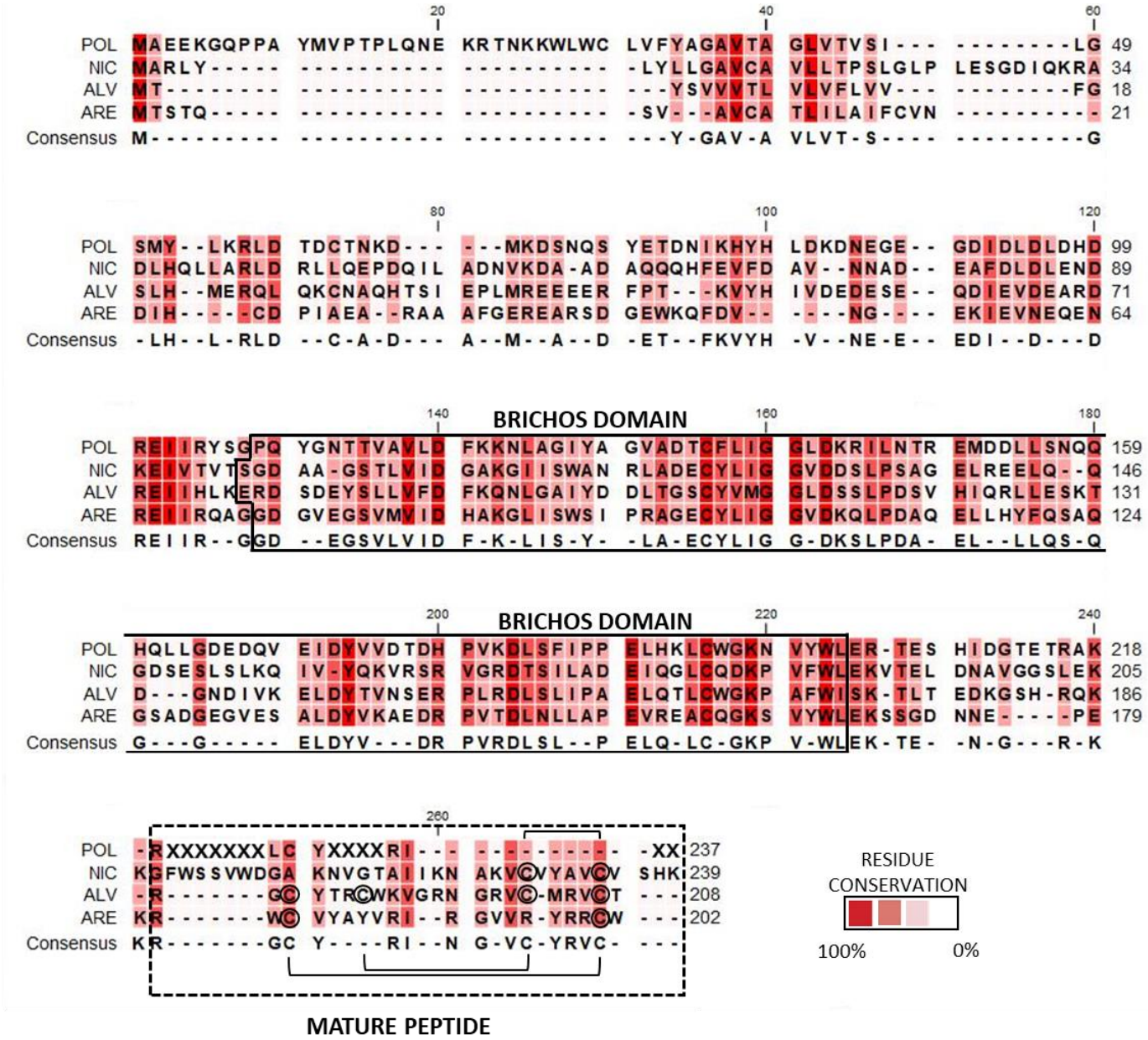


Figure 1: (A) Alignment of the precursors of the BRICHOS-AMP family members: polaricin (POL), nicomicin (NIC), alvinellacin (ALV) and arenicin-1 (ARE). BRICHOS and AMP regions are respectively in black and dotted black rectangles. Cysteine residues involved in disulfide bridges are in black circles. The amino acids background colour underline the higher consensus in the BRICHOS part compared to the AMP part itself. The complete sequence of polaricin has been protected by using "X" instead of some amino acids. The alignment was generated using CLC Sequence Viewer software (version 8.0).

Pfam analysis revealed the presence of a conserved BRICHOS domain, in line with the previously described members of BRICHOS-AMP family, ALV, ARE (used in this study) and with nicomicin (NIC) identified from another marine annelid species distributed both in temperate and polar habitats [10–12]. Data highlighted a high percentage of identity of the BRICHOS domain compared to the low identity rate of the AMPs part of the precursor (table S1).

POL is processed from the C-terminal part of prepropolaricin (Figure 1). It is a cationic 19 amino acids (table S2) which presents an unique cysteine residue by contrast with ARE and ALV which respectively possess 2 or 4 cysteine residues involved in the formation of disulfide bonds that stabilize their β-hairpin conformation (Figure 2).

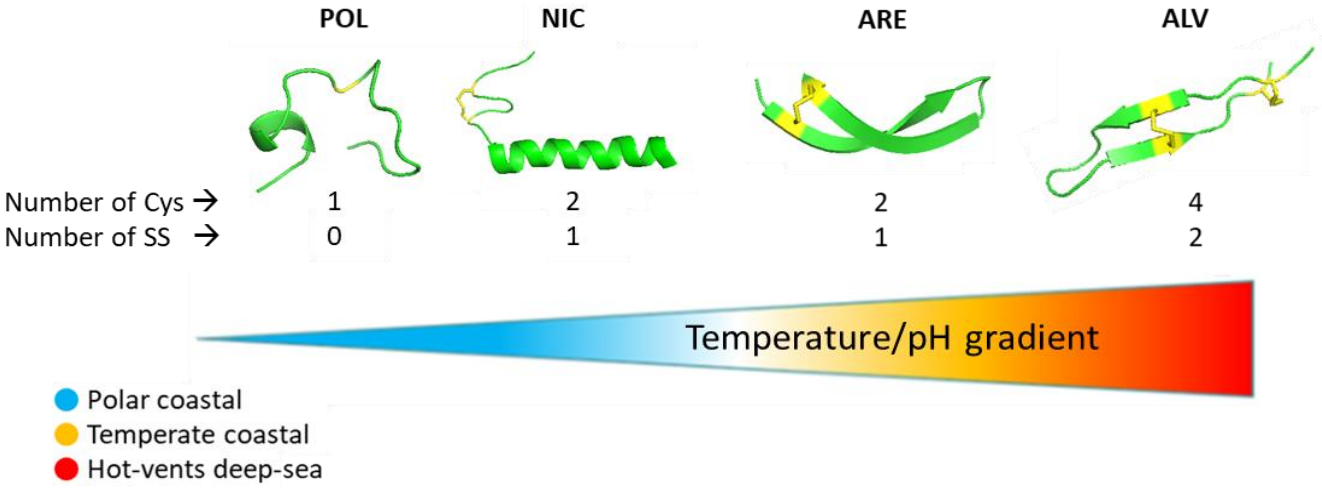


Figure 2: Three-dimensional structures of ALV, ARE, NIC and the predicted one of POL (generated by PEP-FOLD software). The images of the structures were all created using PyMOL 2.3.2 software.
The disulfide bridges and the cysteine residues are depicted in yellow in the 3D representations.
The colour chart indicates the gradient (in term of temperature and pH) of worm environments: from low harshness in light blue (polar), to high harshness in red (hot-vent).

As already evidenced by NMR [10,11], despite the differences in primary structure, ALV, and ARE share the same structural organization (two twisted antiparallel β-strands, forming a β-hairpin conformation stabilized by two or one disulphide bond(s)) while NIC [12] presents an amphipathic α-helix combined with an extended part (Figure 2). Structural analysis of POL, based on a de novo approach to predict 3D peptide structures (see Mat and methods), showed that the polar AMPS adopts an extended or a combination of extended and α-helix conformation: the best predicted model is being represented in Figure 2.

MALDI-TOF mass spectrometry analyses of POL were performed and they revealed that the polar AMP dimerizes presumably through an intermolecular bond engaging each unique cysteine residue of two POL (Figure S2).

2.2.2. AMPs are fitted to the microbial community typifying the worms' habitats

Crossed antimicrobial assays between environmental bacterial strains and ALV, ARE and POL were performed. The Table 1 illustrates the values of MIC (Minimum Inhibitory Concentration) and MBC (Minimal Bactericidal Concentration) of the three AMPs towards marine bacteria (cultivable under the conditions of a MIC assay) that are typical from the temperate-coastal (*Vibrio alginolyticus, Vibrio fluvialis, Shewanella algae and Oceanisphaere spp*.) and from hot-vent deep-sea habitats (*Vibrio diabolicus* and *Pseudomonas sp.*).

Table 1: MIC (Minimal Inhibitory Concentration) and MBC (Minimal Bactericidal Concentration) values of BRICHOS-AMPs towards environmental bacteria.

		MIC (μM)			MBC (μM)		
HABITATS	BACTERIA	**ALV**	**ARE**	**POL**	**ALV**	**ARE**	**POL**
temperate, coastal	*Vibrio alginolyticus*	**0.15 - 0.31**	**0.07 - 0.15**	**0.31 - 0.625**	**0.625**	**0.31**	**5**
	Vibrio fluvialis	**0.31 - 0.625**	**0.156 - 0.31**	**40**	**10**	**1.25**	**>40**
	Shewanella algae	**>40**	**20 - 40**	**>40**	**>40**	**>40**	**>40**
	Oceanisphaere sp.	**0.31 - 0.625**	**0.07 - 0.15**	**2.5 - 5**	**1.25**	**0.625**	**20**
hot-vent, deep-sea	*Vibrio diabolicus*	**1.25 - 2.5**	**2.5 - 5**	**>40**	**20**	**20**	**>20**
	Pseudomonas sp.	**0.035 - 0.07**	**0.07 - 0.15**	**0.15 - 0.31**	**0.31**	**1.25**	**5**

Data showed that ALV displayed antimicrobial activity against all tested bacteria, except for *Shewanella algae*. Among the three AMPs, it is the most efficient against hydrothermal vent bacteria. ARE is active against all tested strains, showing the highest activity against temperate-coastal bacteria than against hot vent bacteria. It is the only of the three AMPs to inhibit the growth of *S. algae*. POL is the less active of the three AMPs against all the bacteria tested none of them being typical of the polar habitat. The local adaptation of the AMPs to the microbial community typifying the worms' habitats was then analysed via a "home vs away" diagram, a method commonly used in ecology to identify a local adaptation (see methods) [20].

To do so, the MIC values of the three AMPs against the different strains were used to build the diagram (Figure 3).

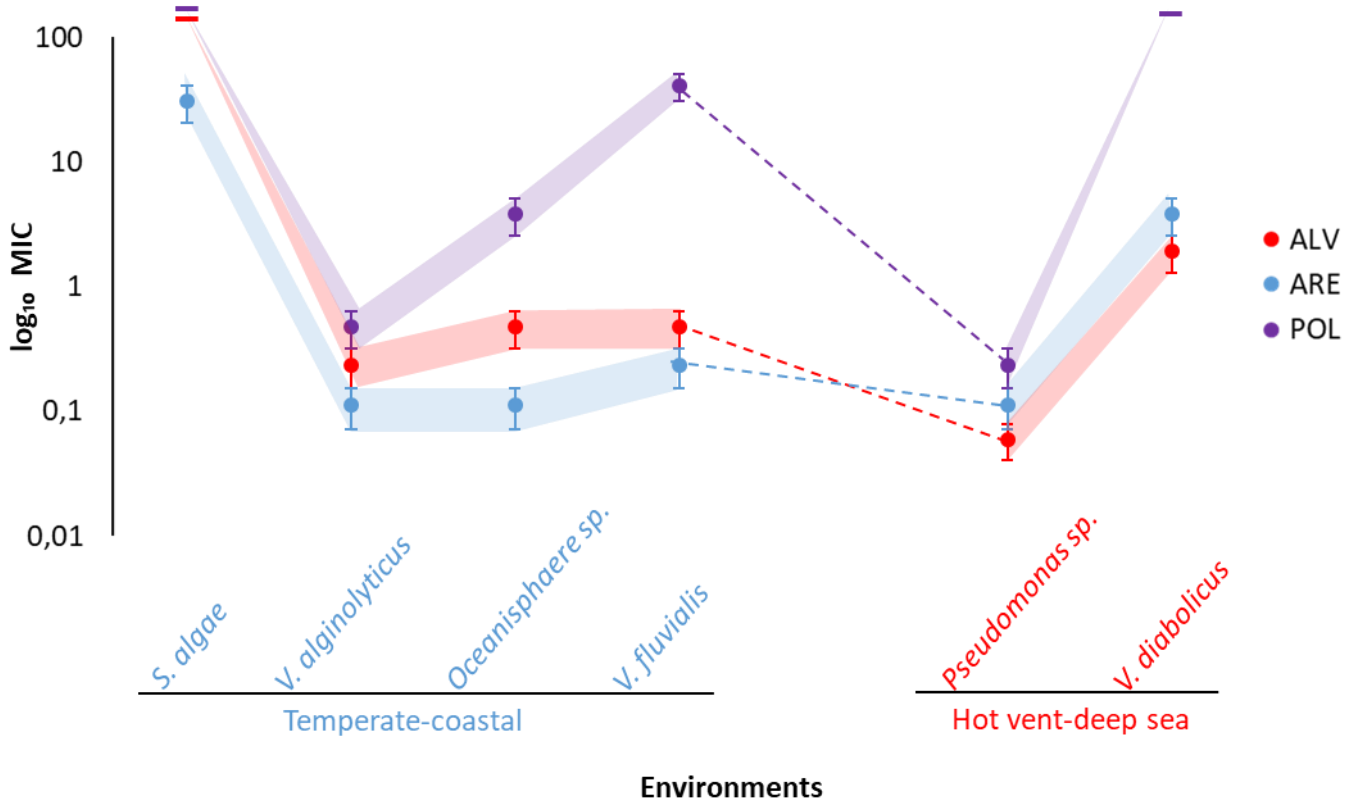


Figure 3: A representation of BRICHOS-AMPs local adaptation (home vs. away diagram) to temperate-coastal and hydrothermal vents-deep sea habitats, depending on their MIC values against environmental bacterial strains. Y-axis, representative of fitness factor, stands for peptides MIC log-transformed data: higher MIC values correspond to lower fitness levels. The coloured areas gather the values belonging to the same peptide. Dashed lines, connecting the areas across the two habitats, show the adaptation of ARE and ALV to their respective habitats, displaying lower MIC values against "home strains" and *vice versa*. Missing polar bacterial strains, POL displays the highest MIC values against the investigated "away strains". Peptides and bacteria sharing a common environment are displayed by the same colour: red for hot vent, blue for temperate and purple for polar. The cases of not-active peptide are represented with – on the top of the diagram, as leaning to infinity.

A pattern showing local bacterial communities as specific targets of the worm's AMPs clearly emerges from this analysis. Assuming that well-adapted peptides display lower value of MIC (higher fitness level) and vice versa, the populations (represented here by the peptides) are locally adapted in the home sites of their "producers". ARE and ALV displayed higher fitness levels (lower MICs) at their native habitats than any other peptides; POL denoted highest MIC values for all tested strains.

2.2.3. AMPs are fitted to the temperature of the worms' habitats

The bactericidal activity which reflects the time required for an AMP to kill bacteria (Figure 4) was investigated through time-kill kinetics assays against Vibrio strains typical of the hot hydrothermal vent versus the temperate environment (*V. diabolicus* and *V. alginolyticus* respectively).

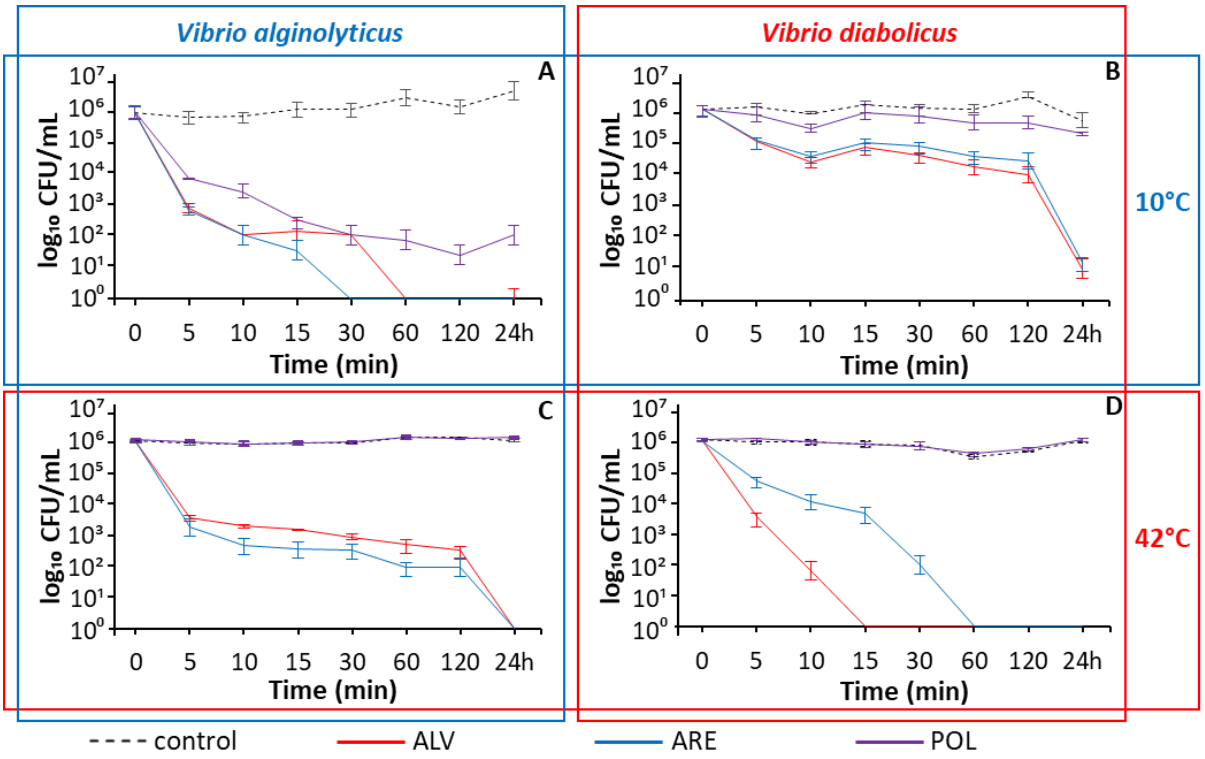


Figure 4: Kinetics profiles of the bactericidal activities of ALV, ARE and POL against *Vibrio alginolyticus* and *V. diabolicus*, at 10°C and 42°C. The results are shown as time-plot of Vibrios viability (log_{10} of CFU/ml), in absence (control) and in their presence of peptide. Temperature, peptide and strain belonging to temperate environment are in blue, hydrothermal vent ones are in red, and the polar peptide is in purple.

This antimicrobial efficacy testing was performed either at 10°C which corresponds to the optimal temperature of the temperate worm [21–29] or at 42°C which corresponds to the temperature optimal for the hydrothermal worm) [30–35]. As also evidenced in table 1, data of the Figure 4 showed that i/ POL is slightly active against *V. alginolyticus* and is inactive against *V. diabolicus* and ii/ ARE and ALV are bactericidal against both strains with a better efficiency against their respective surrounding bacterial communities. When mimicking the local temperature, the local adaptation of the AMP is even more pronounced (15min to kill

100% of *V. diabolicus* at 42°C versus >24h at 10°C for ALV; time killing of ARE of 30min against *V. alginolyticus* at 10°C versus 24h at 42°C). The assays which require to be at the mid-exponential phase of bacterial growth were first adjusted according to the growth curve of the bacteria previously determined at the different temperatures (Figure S3). The observed effects are consequently not the reflect of a direct impact of the temperatures on the bacterial growth but rather the differential activities of the AMPs on the bacteria.

The thermal stabilities of the three AMPs were then tested and their bactericidal properties were compared after having incubated them for 30 min-1h at 4°C, 10°C, 42°C and even 90°C (Figure 5A).

The "polar" AMP, POL kept its antimicrobial activity against V. alginolyticus at 4°C and 10°C only and fully lost it at 42°C and 90°C. As observed before, no activities were observed against V. diabolicus. By contrast, the "hot" AMP, ALV remained active against both bacteria at 42°C and at 90°C with a 50% decrease of its activity after 1h of incubation at 90°C. Data confirmed the higher efficiency of ALV toward V. diabolicus than against V. alginolyticus. The "temperate" ARE presented an intermediate pattern with an activity starting to decrease by 80% at 42°C and almost fully disappearing (20%) after 1h at 90°C.

2.2.4. AMPs are fitted to the pH of the worms' habitats

The effect of the environmental pH on the three AMPs was measured by comparing their antimicrobial activities at pH 4, 6, 7.4, 8 and 10. Because POL is not active against V. diabolicus, only V. alginolyticus was used for the assays (Figure 6A). Data significantly showed that the biological activities of ARE and ALV were not affected by the pH although POL exhibited a reduced efficiency (4-fold in the most extreme conditions pH 4 and 10) as soon as the pH is not neutral.

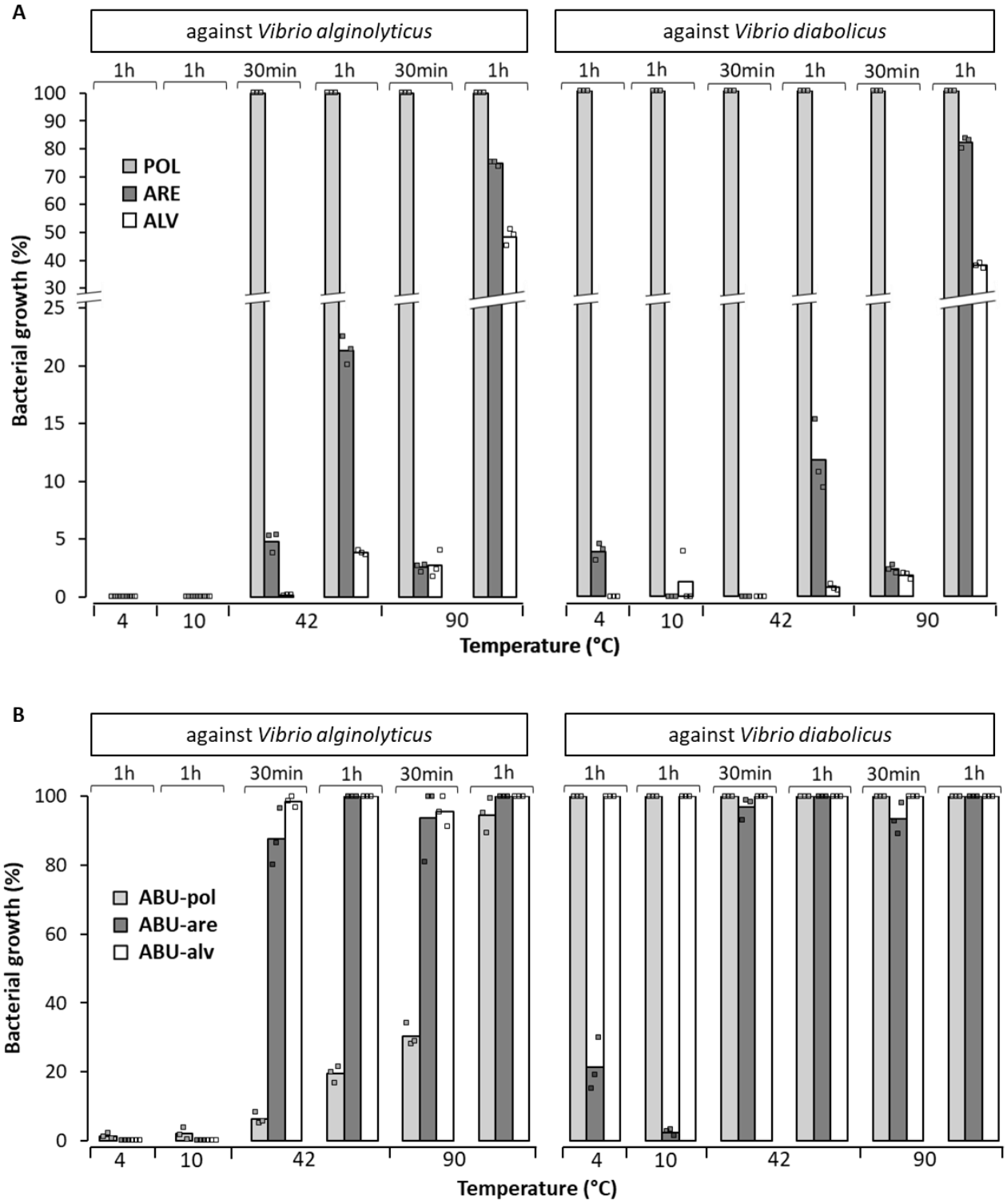


Figure 5: The effect of temperature on biological activity of (A) native peptides and (B) ABU-peptides, against *V. alginolyticus* and *V. diabolicus*, after 30 / 60 minutes of peptides incubation at different temperatures. Each bar represents the average value of the three independent replicates reported in the chart. The Y-axis (in A) presents a break in the range of values to improve its readability.

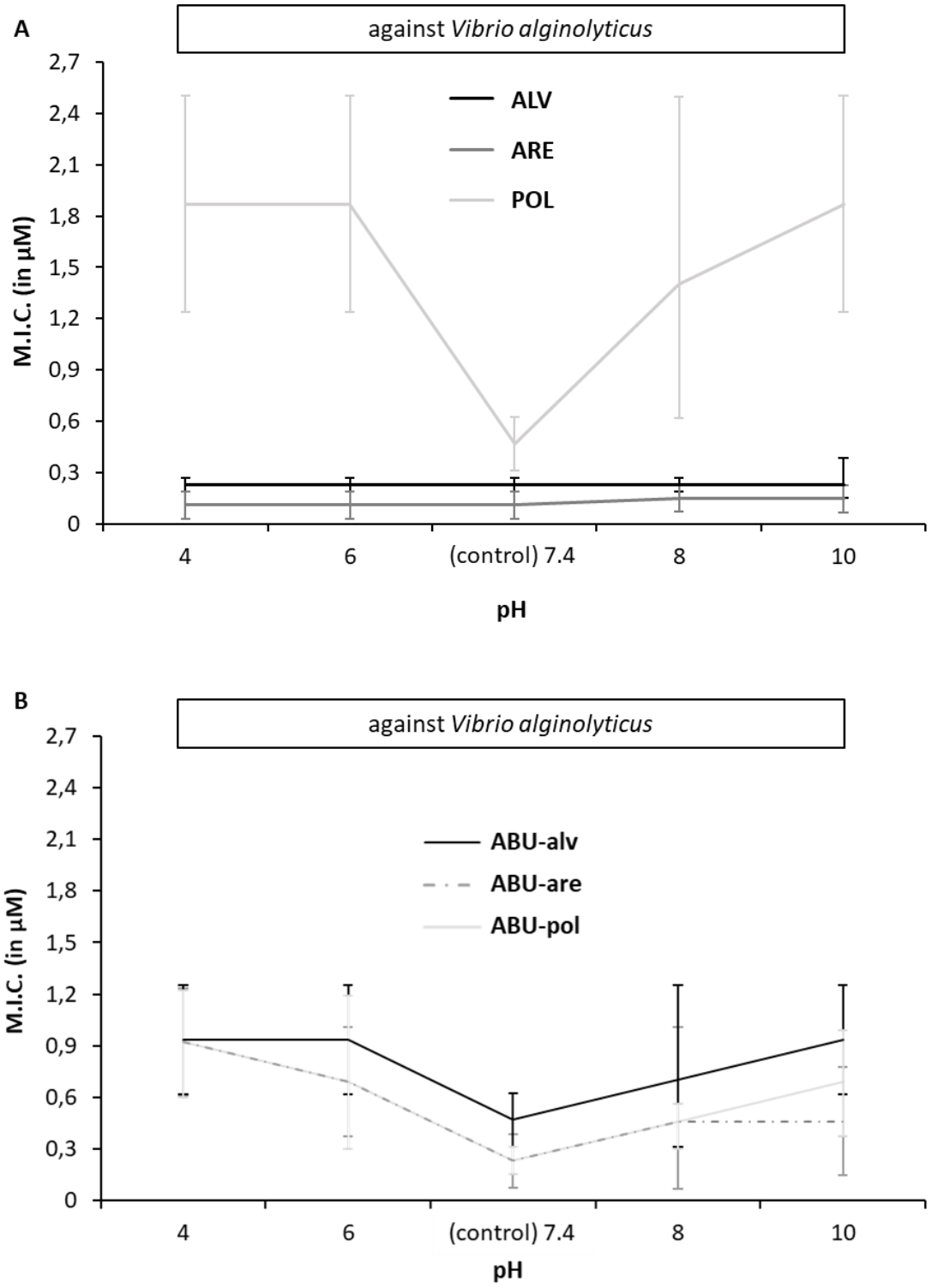


Figure 6: The effect of pH on biological activity of (A) native peptides and (B) ABU-peptides activities, against *V. alginolyticus*. The tests were performed after peptides incubation (3h) in acid/basic conditions (at pH 4, 6, 8 and 10). The control is represented by peptide MIC values in MHB medium (pH 7.4).

2.2.5. Role of the disulphide bridges in the thermal and pH adaptation

The more the marine worms are exposed to high temperatures and high pH variation, the more their BRICHOS-AMPs exhibit a higher number of cysteine residues involved in disulfide bridges (Figure 2). POL, ARE and ALV possess respectively 0, 1 and 2 intramolecular disulfide bridge. Antimicrobial assays were performed with the variants in which the cysteine residues were replaced by α-aminobutyrate (ABU) residues (ABU-POL, ABU-ARE and ABU-ALV). Since the ABU residues are not engaged in disulfide bridges, this approach allowed the removal of the intermolecular (dimerization of POL) and intramolecular disulfide bonds. These amino acid substitutions did no modify the main biochemical features of the AMPs (resumed in table S3), with unvaried net charges and isoelectric points (except for ABU-POL, for which the Pi is slightly increased).

The Table 2 illustrates the values of MIC (Minimum Inhibitory Concentration) and MBC (Minimal Bactericidal Concentration) of the three ABU-peptides towards marine bacteria *Vibrio alginolyticus* and *Vibrio diabolicus*).

Table 2: MIC (Minimal Inhibitory Concentration) and MBC (Minimal Bactericidal Concentration) values of abu-peptides against *Vibrio alginolyticus* and *V. diabolicus*. BRICHOS-AMPs towards environmental bacteria.

	MIC (µM)			MBC (µM)		
	ABU-alv	**ABU-are**	**ABU-pol**	**ABU-alv**	**ABU-are**	**ABU-pol**
V. alginolyticus	0.31 – 0.625	0.15 – 0.31	0.15 – 0.31	1.25	1.25	1.25
V. diabolicus	>40	5 – 10	>40	>40	>40	>40

Figure 5B showed that at 4°C and at 10°C, the three ABU AMPs were active against V. alginolyticus while only ABU-ARE was active against V. diabolicus. The disulfide bridges seem to play a role in the mode of action of ABU-ALV against V. diabolicus. Compared to the folded peptides, the ABU-variants were less effective (or even inactive in the case of ABU-ALV) against the two Vibrios, except for ABU-POL which appeared 2-fold more effective against V. alginolyticus than the native AMP.

Moreover, ABU-alv and ABU-are, exposed to high temperatures (at 42°C and 90°C), completely lost their biological activity (Figure 5B). Conversely, ABU-pol was thermostable at

high temperature, keeping mostly its antimicrobial activity, against V. alginolyticus, after 30 min. at 90°C.

Upon acid/basic conditions, ABU-variants kept their antibacterial activity against V. alginolyticus (Figure 6B), displaying a slightly reduction of activity (2-4-fold) especially at pH4 and pH10.

2.3. Material and methods

Worms habitats and AMPs

Arenicin (ARE) was purified from the lugworm *Arenicola marina* (Arenicolidae), a polychaete that inhabits temperate near-shore (intertidal) sediments, digging tunnels in the sand [9,10]. The burrows of these worms are continuously exposed to periodic drying (tidal emersion), which imposes severe thermal variations (-5°C to 25°C, pH 5.6-8.2) [11–19].

Alvinellacin (ALV) was isolated from *Alvinella pompejana* (Alvinellidae, Terebellida), a polychaete that inhabits parchment-like tubes along the outer walls of active hydrothermal vents chimneys of East Pacific Rise [20]. These areas are characterized by frequent and unpredictable emissions of hot fluid, causing extreme temperature and pH gradients (from 2°C to upper 100°C, with a high temporal variability of these conditions inside the tube (14°C-80°C and thermal optimum around 42°C; pH between 5.33 and 6.4, due to the mixing of bottom seawater with the acidic vent fluid) [21–26].

Polaricin (POL) from *Amphitrides* sp. a marine annelid inhabiting a polar environment (Antarctica) at depth of 100-150 m. This spaghetti worm is sedentary and lives inside a slightly consolidated tube made with sand and coral debris on the Antarctic continental plateau where it faces extremely cold but stable coastal waters (-1.8°C), with very small fluctuations of temperature and pH [27–30]. As indicated by its name, the worm uses its buccal tentacles as a spider net to collect marine particles as a suspensivorous feeder.

Molecular identification of POL/prepropolaricin

The complete cDNA sequence of prepropolaricin was obtained by homology with the preproalvinellacin gene (Tasiemski et al. 2014, accession N° KJ489380) from a tblastx query on the transcriptome assembly previously obtained for the Antarctic Amphitritides sp. under scrutiny (blast database) using the software Geneious (blast cut_off p-value = 10-15). The

Amphitritides RNAseq dataset used for the assembly was obtained from an Illumina 150 bp paired-ends sequencing of a stranded mRNA library on a HiSeq 2500 machine at Genome Québec (half a line: 150 M PE reads). Transcripts (Ngenes=99970, N50=1440 bp, median contig length=488 bp, GC%=36.2) were then obtained with the Trinity 2.4.0 software from R1 and R2 pairs after trimming adapters and regions of low PHRED scores (Trimmomatic 0.36: ILLUMINACLIP: illumina.fa:2:30:10, LEADING:5, TRAILING:5, SLIDINGWINDOW:4:15 MINLEN:36) and a subsequent normalization of the reads.

Prepropolaricin sequence analysis

BRICHOS domain sequence was identified by using MyHits Motif Scan (https://myhits.isb-sib.ch/cgi-bin/motif_scan). SignalP 5.0 software (https://www.cbs.dtu.dk/services/SignalP/) was used to detect the presence of signal peptide.

The analysis of peptide Hydrophobicity/Hydrophilicity was performed by Peptide2 software (Peptide 2.0 Inc., https://www.peptide2.com/).

Amino acid alignment (precursors and peptides)

The sequence alignments of peptides precursors of alvinellacin (GenBank accession number KJ489380), arenicin (GenBank accession number AY684856), nicomicin (GenBank accession number MH898866), capitellacin (GenBank accession number KB309561) and polaricin, was performed by using CLC Sequence Viewer software (version 8.0).

The computation of homology/identity percentage was performed at the SIB (ExPASy software, https://www.expasy.org/) using the BLAST network service (BLASTP, version 2.2.31+).

Peptides physicochemical properties

The physicochemical properties of the peptides (molecular weight, isoelectric point, net charge at different pH) were calculated by Innovagen Pepcalc.com server (Innovagen AB, SE-22370 Lund, SWEDEN; https://pepcalc.com/).

3D design

All 3D peptide models of polaricin were predicted using the PEP-FOLD software (online server http://bioserv.rpbs.univ-paris-diderot.fr/services/PEP-FOLD/). This method, based on structural alphabet (SA) letters describing the possible conformations of groups of four consecutive residues, couples the predicted series of SA letters to a greedy algorithm and a coarse-grained force field [36]. We kept the representative structure of the most populated cluster on 200 run simulations. The software was previously tested, giving as input the amino

acid sequences of ALV, ARE and CAP: strong correlation was observed between predicted and already known 3D structures.

The representations of peptides 3D images were generated using PyMOL (TM) 2.3.2 molecular modelling system (BioLuminate, Schrödinger, LLC, New York, NY, 2019 http://www.pymol.org/).

The three-dimensional structures are assumed for neutral pH.

Mass spectrometry

A sample of POL (about 2 µL, at concentration of 2 mg/mL) was characterized by analytical UPLC–MS using a System Ultimate 3000 UPLC (Thermofisher) equipped with an Acquity peptide BEH300 C18 column (1.7 µm, 2.1 × 100 mm, 300 Å), a diode array detector and a mass spectrometer (Ion trap LCQfleet). Analyse was performed at 70°C using a linear gradient of 0-70% of eluent B in eluent A over 20 min at a flow rate of 0.4 mL min^{-1} (eluent A = 0.1% TFA in H_2O; eluent B = 0.1% TFA in CH_3CN). The column eluate was monitored by UV at 215 nm. The peptide masses were measured by on-line UPLC–MS (LCQ Fleet Ion Trap Mass Spectrometer, ThermoFisherScientific): heat temperature 450 °C, spray voltage 2.8 kV, capillary temperature 400 °C, capillary voltage 10 V, tube lens voltage 75 V.

Synthesis of the peptides

Alvinellacin (ALV), arenicin (ARE) and polaricin (POL) were synthesized by BIOSYNTAN GMBH (Berlin, Deutschland).

Microorganisms

The bacterial strains used in this study are Gram-negative bacteria from marine environment: *Shewanella algae*, *Oceanisphaera sp.*, *Vibrio fluvialis* and *Vibrio alginolyticus* are worldwide distributed, especially in the coastal waters of temperate areas [37,38]; *Pseudomonas sp. AT1238* and *Vibrio diabolicus* HE800 (isolated from *A. pompejana*), are related to a deep-sea hydrothermal vent environment (East Pacific Rise) [39].

V. alginolyticus, *V. diabolicus HE800* and *Pseudomonas sp. AT1238* were provided by IFREMER.

V. fluvialis, *S. algae and Oceanisphaera sp.* ere isolated from sediment of coastal environment: mud collected next to the shaped burrow of the lugworm *Arenicola marina* was spread onto an agar plate and incubated at 30°C overnight. Well-growing colonies were isolated by multiple pricking out. One of the pure colonies isolated was grown in fresh liquid Zobell medium (4g Bacto Proteose Peptone (BD Biosciences), 1g Bacto Yeast Extract (BD Biosciences), 23.4g NaCl, 1.5g KCl, 1.2g MgSO4 7H2O, 0.2g CaCl 2H2O, in 1L of pure water) for 15h.

Fragments of the 16S rRNA gene from bacterial colonies were PCR amplified with the bacterial primer set 16S-F and 16S-R (forward primer 5'-GGACTACCAGGGTATCTAATCCTGTT -3'; reverse primer 5'-TCCTACGGGAGGCAGCAGT -3'), amplification of 900 pb fragments, using GoTaq® G2 Flexi DNA Polymerase (PROMEGA, France). PCRs, performed in a Mastercycler Pro S thermal cycler (Eppendorf, France), included 39 cycles, each consisting of annealing for 1 min at 57°C, elongation for 1,20 min at 72°C, and denaturation for 1 min at 95°C. The PCR product, loaded on an agarose gel, revealed a single band of the expected size, which was purified with the Nucleospin Extract kit (Macherey-Nagel) and cloned in TA Cloning kit (Promega), according to the protocol provided by the manufacturer. DNA plasmids were Sanger-sequenced with FM13/RM13 universal primers using BigDye Terminator (Applied Biosystems, Foster City, CA). The sequences were matched against nucleotide sequences from Eztaxon using BLASTn (http://www.ezbiocloud.net/eztaxon/database).

The marine strains were cultivated at 28°C in MHB (Mueller Hinton Broth, ROTH), under shaking at 140 rpm and maintained on Zobell medium agar at room temperature.

Effect of temperature on *V. alginolyticus* and *V. diabolicus* growth

One bacterial colony (of *V. alginolyticus* or *V. diabolicus*) was grown in MHB medium at 28 °C overnight on a rotary shaker (140 rpm). The freshly grown culture was then diluted (1/100 in 10mL of MHB), and incubated under the same conditions until the mid-log phase (optical density at 600 nm) was reached. Bacteria were diluted to 1×10^6 CFU/ml and incubated at different temperatures (10°C, 28°C and 42°C) on a rotary shaker (at 140 rpm). Optical density measurement at 600 nm was determined by using an Ultrospec 10 cell density meter (Amersham Biosciences, UK), at several time intervals up to 48 hours. The results are the means from at least two sets of independent experiments.

Antimicrobial assay

Peptides minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC), were determined by the broth microdilution method against bacterial growth in microtiter plates as previously describe by Bulet *et al*., 2007 [40].

One bacterial colony was grown in MHB medium, overnight at 28°C (140 rpm). The freshly grown culture was then diluted (1/100 in 10 mL of MHB), and incubated under the same conditions until the mid-log phase (optical density at 600 nm) was reached. Bacteria were diluted to 1×10^6 CFU/mL and added (100µL per well) into sterile 96-well flat bottom plates (CELLSTAR, Greiner bio-one) containing serial dilutions (from 40 to 0.00195 µg/mL) of

peptides (10µL per well). The plates were incubated overnight at 28°C on a rotary shaker (140 rpm). The MIC was taken as the lowest peptide concentration at which observable microbial growth was inhibited, the measurements were realised by a microtitre plate reader (Tecan Sunrise Microplate Reader) at A600. MBC was measured by streaking on proper agar (MHB) petri dishes, the entire volume (110µL) of wells (from previously MIC plates) with no bacterial growth. After incubation for about 24 hours at 28°C, the MBC was defined as the peptide concentration where no colony growth was observed. All tests were conducted in triplicate.

Home versus away

The diagram was constructed on the bases of peptides MIC values (obtained by antimicrobial assays and resumed in Table 2) against environmental marine bacteria. The MIC data were log-transformed and organized in two groups, depending on bacteria typical environment (temperate-coastal and hot vent-deep sea). The coloured areas gather the MIC values of the same peptide to highlight their patterns in their native environment and when transplanted to other habitat.

Kinetics of bactericidal activities (Time course of bacterial killing by AMPs)

The kinetics of bactericidal activity of the native AMPs were assessed at a concentration five times the MIC for each peptide, using *V. alginolyticus* and *V. diabolicus*. The Vibrio bacteria were grown (on a rotary shaker, at 140 rpm and 28°C) to exponential phase and diluted (in MHB medium) to obtain a 1 x 10^6 CFU/mL final concentration. Then they were incubated with peptides (5-fold MICs) for 5, 10, 15, 30, 60, 120 minutes and 24 h, in parallel at 10°C and 42°C, to reproduce a natural-like environment. Viabilities of Vibrio strains were assessed by plating on MH agar plates (sterile petri dish, 90 mm) the bacterial suspensions at various time; the plates were incubated overnight at 28°C and the resulting bacterial colonies were counted (CFU/mL). The results are the mean of three independent experiments; they are expressed by plotting the log-transformed data (log CFU/mL, as they are more sensitive to any differences that may exist in bacterial killing) at different times.

Thermal-stability assay

The biological activities of the AMPs were evaluated after their exposition to thermal stress (high and low temperatures). Two Vibrios were chosen for this assay, *V. alginolyticus* or *V. diabolicus*, as representatives of the two environments. One colony *of Vibrios* was grown in MHB medium overnight at 28°C (140 rpm). The freshly grown culture was then diluted (1/100 in MHB), and incubated under the same conditions until the mid-log phase (optical density at

600 nm) was reached. Bacteria were diluted to 1×10^6 CFU/mL and added (100µL per well) into sterile 96-well flat bottom plates (CELLSTAR, Greiner bio-one) containing a volume of 10µL/well of peptides (at concentration 5-fold MIC values) previously thermally stressed (incubation for 30 minutes and 1 hour at 4, 10°C, 42 and 90°C). The plates were then incubated overnight at 28°C at 140 rpm. The antimicrobial activity was evaluated by the measurements of bacteria growth by a microtitre plate reader (Tecan Sunrise Microplate Reader) at A600. The results were expressed as percentage of bacterial growth, in comparison with the control samples (MHB medium and bacterial strain). The tests were conducted in triplicate.

PH-stability assay

The biological activities of the AMPs were estimated after their exposition to acid/basic stress according to Yang *et al.* 2017 [41]. *V. alginolyticus* was used for these tests, due to the lower MIC values displayed by all peptides. One colony *of V. alginolyticus* was grown in MHB medium overnight at 28°C (140 rpm). The freshly grown culture was then diluted (1/100 in MHB), and incubated under the same conditions until the mid-log phase (optical density at 600 nm) was reached. Bacteria were diluted to 1×10^6 CFU/mL and added (100µL per well) into sterile 96-well flat bottom plates (CELLSTAR, Greiner bio-one) containing serial dilutions of peptides (from 20 to 0.00195 µg/mL), previously incubated for 3 hours at 10°C, in 100mM sodium acetate buffer (pH 4.0), sodium phosphate buffer (pH 6.0), Tris-HCL buffer (pH 8.0), or glycine-NaOH buffer (pH 10.0). The control samples were incubated in MHB medium (pH 7.4) at 10°C. The plates were then incubated overnight at 28°C at 140 rpm. The antimicrobial activity of peptides was determined by the broth microdilution method [42]. All tests were conducted in triplicate and the results were expressed as MIC (in µM) variations.

Abu-peptides

Linearized abu-peptides (ABU-alv, ABU-are and ABU-pol) were designed as variant of ALV, ARE and POL, based on the removal of the disulfide bonds through the replacement of cysteine by α-aminobutyrate (ABU) residues (hydrophobic non-proteinogenic alpha amino acid, also known as homoalanine) which are unable to associate covalently [43,44].

Statistical analysis

All data are presented as the mean ± SEM (standard error of the mean).

2.4. Discussion

Marine AMPs were found to be structurally different from their counterparts produced by terrestrial species, usually displaying novel structures, taxa-specific or even species-specific [45]. Aggressive environmental pressures, as abundance of pathogen microorganisms and hostile factors (extreme and/or varying temperature, pH and salinity values, pressure, etc.) have probably driven the evolution of the physiological adaptation of all marine organisms, including on their immune functions [46].

Amongst all methods to discover new antimicrobial peptides from marine invertebrate, recently our group focused on extreme marine nematodes (see Chapter 1) by using bioassay guided purification protocol, as the only method to discover novel AMP motif [47].

Here, using "in silico approach" and blasting the sequence of preproalvinellacin [11] to *Amphitritides sp.* genome, we identified a novel putative AMP from the polar marine polychaetes, named polaricin. Such as already known AMPs from marine polychaetes (alvinellacins, arenicins, capitellacin and nicomicins), polaricin is processed from a larger precursor molecule containing the BRICHOS domain, prepropolaricin, being part of BRICHOS-AMP family [10–12]. The members of this family (come from annelids living varying and distant habitats) are a remarkably attractive model to study their evolution, as actors of worm's immune defence in extreme and fluctuating environmental conditions.

Recently, our group showed that alvinellacins from two sister species *A. pompejana* and *A. caudata* present no difference at intraspecific level (over about 6000 km of East Pacific Rise hot chimneys walls), only one amino acid replacement between the AMPs and a strong selection of alvinellacin chaperones [19]. Share the same biotic and abiotic environmental conditions, might force parallel evolution of defence mechanisms against common microbial communities [48,49].

Here, we showed that BRICHOS-AMPs markedly differ in primary structures and exhibit highly conserved precursor sequence (especially the BRICHOS region). These data, suggest a common origin of the precursor molecule with a divergent evolution of the AMP part: adaptive changes (nucleotide and amino acid substitutions) in genes involved in the immune defences occur at higher rate than non-immunity related genes [64].

To date, the BRICHOS domain, found in many protein families with a wide range of functions and disease associations, is the only region of the BRICHOS-containing proteins that is

conserved across the superfamily [50]. Surprisingly, this molecular chaperone seems to be linked to AMPs in the unique case of polychaetes precursors. The potential roles of this evolutionarily successful domain linked with such potent antimicrobial peptides deserve further investigations.

From a structural point of view, in contrast to the other member of BRICHOS-AMP family, prepropolaricin lacks a typical signal sequence (likely some members of interleukin family), although it presents many hydrophobic residues in N-terminal position that could allow peptide sorting across the membrane of the endoplasmic reticulum. The AMP part, polaricin, as the other BRICHOS-AMP members, consists in a cationic (+5, net charge at neutral pH) short peptide (19 amino acid residues), containing hydrophobic residues and only one cysteine residue (Cys10). As showed by MALDI-TOF analysis, the presence of a unique cysteine residue in the polaricin sequence, do not allow the formation of intramolecular disulfide bridge (like showed by the other BRICHOS-AMPs). This finding would also result in a different three-dimensional organisation, resulting in a different structure from the more recurrent β-sheet, as evidenced before only by nicomicins [12,13]. Therefore, the putative estimated three-dimensional structures of polaricin displayed a combination of extended and α-helix organization, excluding the β-hairpin conformation. Until now, only nicomicins (another polar peptide) showed a similar and novel scaffold (combining an α-helix and an extended part) among this family of AMPs.

Moreover, from our investigations, it seems apparent that two molecules of polaricin arrange to form dimers.

In this study, polaricin and other two members of the BRICHOS-AMPs family (alvinellacin and arenicin) were tested against many marine strains belonging to different habitats (temperate-coastal and hydrothermal hot-vent environments). They all displayed important antibacterial activity. The increasing number of new bioactive substances (such as AMPs) in extreme worms, living under harsh external pressures (abiotic and biotic), suggest a putative role of AMPs in their adaptation to environmental changes through the selection of the surrounding bacterial communities. For example, our group highlighted the key role of alvinellacin, in its host immune defence shaping the microbiota and preventing colonization and establishment of pathogens in the face of the hostile vent habitat [11]. More recently, we have also showed the presence of antibacterial molecules (most likely AMPs) in meiobenthic nematodes living extreme marine environments in coexistence with a plethora of bacteria (see Chapter 1).

According with common-garden approach, the manipulation in turn of environmental factors (bacterial community, temperature and pH) will show up the need for the evolution of external immune defences [1]. Here, using the home versus away definition, we showed that the AMPs are locally adapted to the bacterial communities of their environment. The MIC data revealed that the tested AMPs killed faster and more effectively than other peptides uniquely the typical strains of their own habitat. Unfortunately, polar strains were not available to complete the assays, as they are hard to trace, isolate and cultivate.

Surprisingly, *Pseudomonas sp.*, isolated from the hydrothermal vent Pompeii worm (*A. pompejana*), was easily eliminated by all peptides regardless their habitat origin. We assumed that this is related to strain belonging to the family of *P. aeruginosa*, an ubiquitous bacterium found in all marine habitats (temperate, polar, hydrothermal vents, etc.).

It was previously showed that marine invertebrates (such as oyster) are well adapted to the biotic and abiotic stressors of their environment compared to transplanted organisms and vice versa [51].

In this study, the effect of temperature and pH (as external abiotic factors) on the biological activity of BRICHOS-AMPs has been investigated. In the last decades, due to the global warming, variation in temperature and pH are affecting coastal and open ocean ecosystems (included deep-sea) throughout the world, causing unpredictable reaction and variation on marine (especially sessile and semi-sessile) organisms [52]. For the first time, we show here a case of external immune adaptation to the abiotic constraints of the habitat. The AMPs retained their antimicrobial activity after exposition to the external stresses typical of their habitats. Previously, the effect of the temperature and pH on many peptides has been studied using a biochemical or biomedical approach (variation of antimicrobial and cytotoxic activity, net charge, secondary structure, binding properties, mode of action) [53–55].

Interestingly, our results showed that external pressures less affected peptides possessing disulfide bridges (ALV and ARE) than POL (without disulfide bridges). These preliminary data were confirmed by using the abu-variants, devoid of disulfide bridges, providing a clear evidence of disulfide bridges involvement in the stability of BRICHOS-AMPs exposed to thermal and pH stresses. The only exception was the case of ABU-pol (abu-variant of polaricin): more effective and displaying more stability at high temperatures and extreme pH values than its native variant. We hypothesised that it could be explained by their different molecular organization and then mode of action. Lacking of cysteine residue, ABU-pol should

be unable to form dimers (probably varying peptides capability to penetrate target membranes). To date, the studies of dimeric forms of bioactive peptide (like magainins and histatin) sequences have shown advantages in enhanced antimicrobial potency and resistance to proteases (reviewed in Lorenzon et al., 2019 [56]). However, other works have shown that the dimerization decreases the antimicrobial activity of cationic AMPs such as aureins and melittin, changing peptides mechanism of action [57]. Otherwise, it has been shown that dimerized arenicin exhibited significantly reduced cytotoxicity but similar antibacterial activities compared to its native AMP [58].

More generally, in agreement with our pH- and thermo-stability results, a recent study on a group of variants of NZ17074 (variant of arenicin-3, another AMP isolated from *A. marina*) showed the importance of disulfide bridges for the stability of the peptides. The variants containing disulfide bridges were thermostable (range 20-80 °C) and not affected by pH variations (4.0 to 10.0); on the contrary, the linear variants exhibited the lowest antibacterial activity [41]. Moreover, many other studies on arenicin and its linear variants have proven the key role of disulfide bridges for maintenance of high stability and biological activity of the peptides, ensuring the β-hairpin structure and their mechanism of action [59–63].

Finally, from our data, it looks that the biological activities of the tested peptides are more affected by high temperatures compared to extreme pH variations.

2.5. Conclusion and perspectives

Our results provides a novel AMPs sequence, polaricin, from an extremophile polar polychaetes *Amphitritides sp*. We have included it among the members of BRICHOS-AMPs family, because it is a short cationic peptide and its precursor, prepropolaricin, contains the BRICHOS domain. Its predicted three-dimensional structure is similar to the other polar AMPs of the family, nicomicins (combining α-helix with an extended part).

Polaricin possesses an important activity against Gram-negative marine bacteria, as the other members of the family. The analysis of their (polaricin, alvinellacin and arenicin) antimicrobial activity against the bacterial strains of various marine environments denotes peptides specificity towards the peculiar bacterial communities of the habitats in which the polychaetes live. For the first time, here, we showed BRICHOS-AMPs local adaptation to not only biotic constraints, but also abiotic stressors of the external environment. Temperature and pH of

worms habitats seem have selected and shaped potent AMPs structurally adapted to exert their activities in specific and often extreme conditions.

Moreover, we have provided evidences that the strategic presence of disulfide bridges in AMPs stabilized their structures, allowing them to retain the biological activities in face of harsh stressors. Therefore, BRICHOS-AMPs containing disulfide bridges, displayed rare and attractive properties such as high thermal stability and tolerance to acid/basic conditions, confirming the potential of molecule from marine invertebrates (notably extremophile ones) in various applications such as biomedicine, food industry, agriculture, aquaculture fields.

The exclusive presence of the evolutionary successful BRICHOS domain linked to potent AMPs, raise the matter of fully investigating on their connection and the role of the domain, since especially in invertebrates it has not been widely discussed. Because of the extreme nature of the environments, we have hypothesised that the BRICHOS may be secreted and serve as molecular chaperone and/or as anti-amyloid agent to preserve the biological activity of the peptides in the different external biotic and abiotic conditions encountered by the worms.

Finally, considering immunity beyond the simplified laboratory conditions and taking in account the multiple interacting and changing variables (biotic and abiotic) in nature, these surveys might provide guidelines of organisms evolutionary response to future scenarios, like ocean warming and acidification [64–66].

Table S1: Percentage of identity from AMP and BRICHOS parts alignment. The computation of identity percentage was performed at the SIB (ExPASy software, https://www.expasy.org/), using the BLAST network service (BLASTP, version 2.2.31+).

AMP	ALV	ARE	POL	NIC
ALV	/	27	20	26
ARE	27	/	15	15
POL	20	15	/	9
NIC	26	15	9	/

BRICHOS	ALV	ARE	POL	NIC
ALV	/	34	40	32
ARE	34	/	37	46
POL	40	37	/	28
NIC	32	46	28	/

Table S2: Amino acid sequences of BRICHOS-AMPs and their main biochemical properties: number and position of disulfide bridges, molecular weight (M.W.), isoelectric point (P.I., the pH at which the net charge becomes zero) and net charge at acid and neutral pH (calculated by Innovagen Pepcalc.com server, Innovagen AB, SE-22370 Lund, SWEDEN; https://pepcalc.com/). Cysteine residues are in black bold and numerated (amino acid position). The complete sequence of polaricin has been protected by using "X" instead of some amino acids.

AMPs	GenBank accession number	Amino acid sequences	Disulfide bridges	M.W. (Da)	P.I.	Charge
ALV	KJ489380	RG**C**$_3$YTR**C**$_7$WKVGRNGRV**C**$_{17}$MRV**C**$_{21}$T	2 (3-21; 7-17)	2601.11	pH 12.29	+6
ARE	AY684856	RW**C**$_3$VYAYVRVRGVLVRYRR**C**$_{20}$W	1 (3-20)	2758.29	pH 11.82	+6
NIC	MH898866	GFWSSVWDGAKNVGTAIIKNAKV**C**$_{24}$VYAV**C**$_{29}$VSHK	1 (24-29)	3537.08	pH 10.42	+3.1
POL	-	RXXXXXXXL**C**$_{10}$YXXXXRIXX	0	2368.9	pH 11.93	+5

Table S3: Amino acid sequences of linear abu-variants and their main biochemical properties: disulfide bridges (SS), molecular weight (M.W.), isoelectric point (P.I., the pH at which the net charge becomes zero) and net charge at neutral pH (calculated with Innovagen Pepcalc.com server, Innovagen AB, SE-22370 Lund, SWEDEN; https://pepcalc.com/). The complete sequence of polaricin has been protected by using "X" instead of some amino acids.

AMPs	Amino acid sequences	SS	M.W. (Da)	P.I.	Charge
ABU-alv	RG***abu***YTR***abu***WKVGRNGRV***abu***MRV***abu***T	0	2532.97	pH 12.29	+6
ABU-are	RW***abu***VYAYVRVRGVLVRYRR***abu***W	0	2724.22	pH 11.82	+6
ABU-pol	RXXXXXXXL***abu***YXXXXRIXX	0	2350.86	pH 12.28	+5

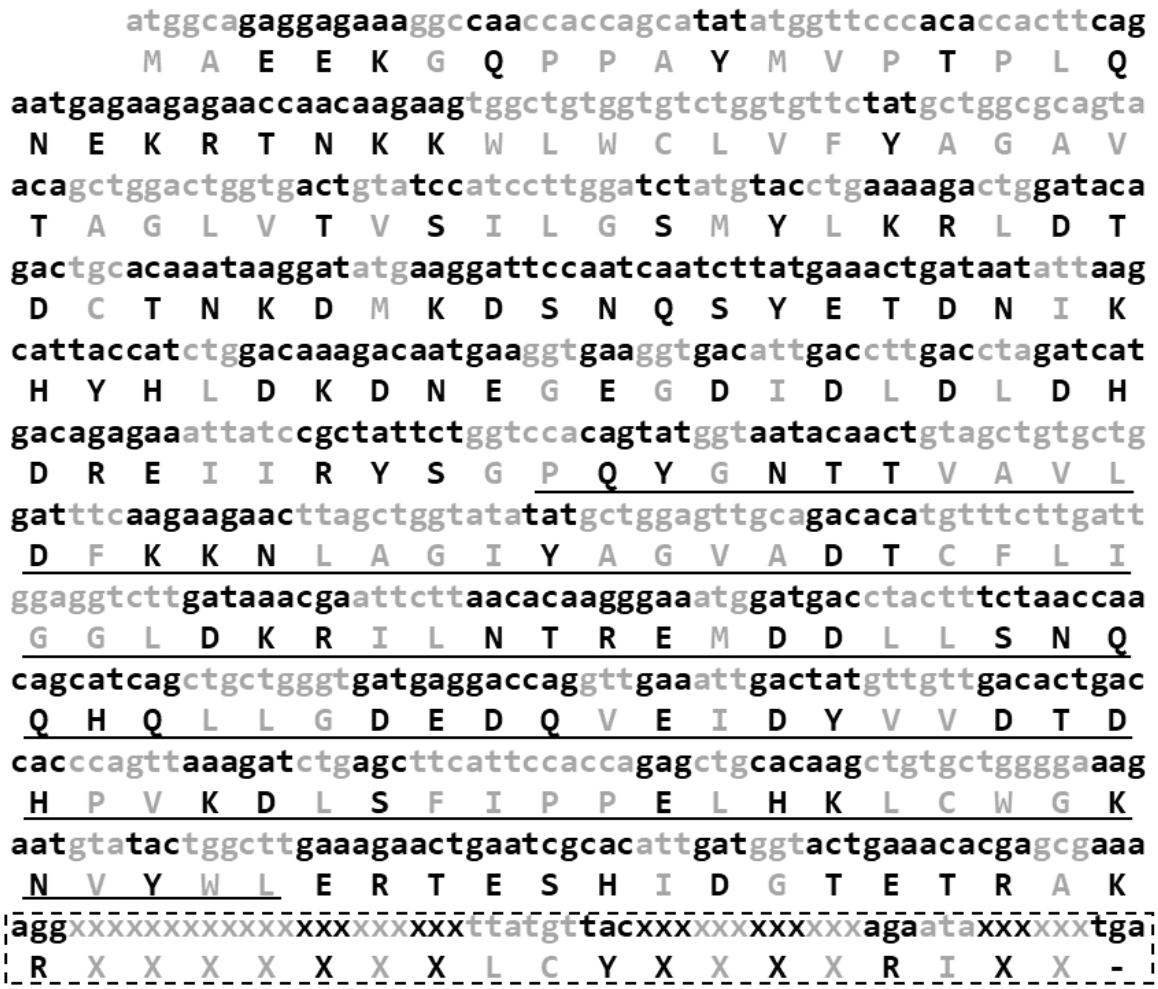
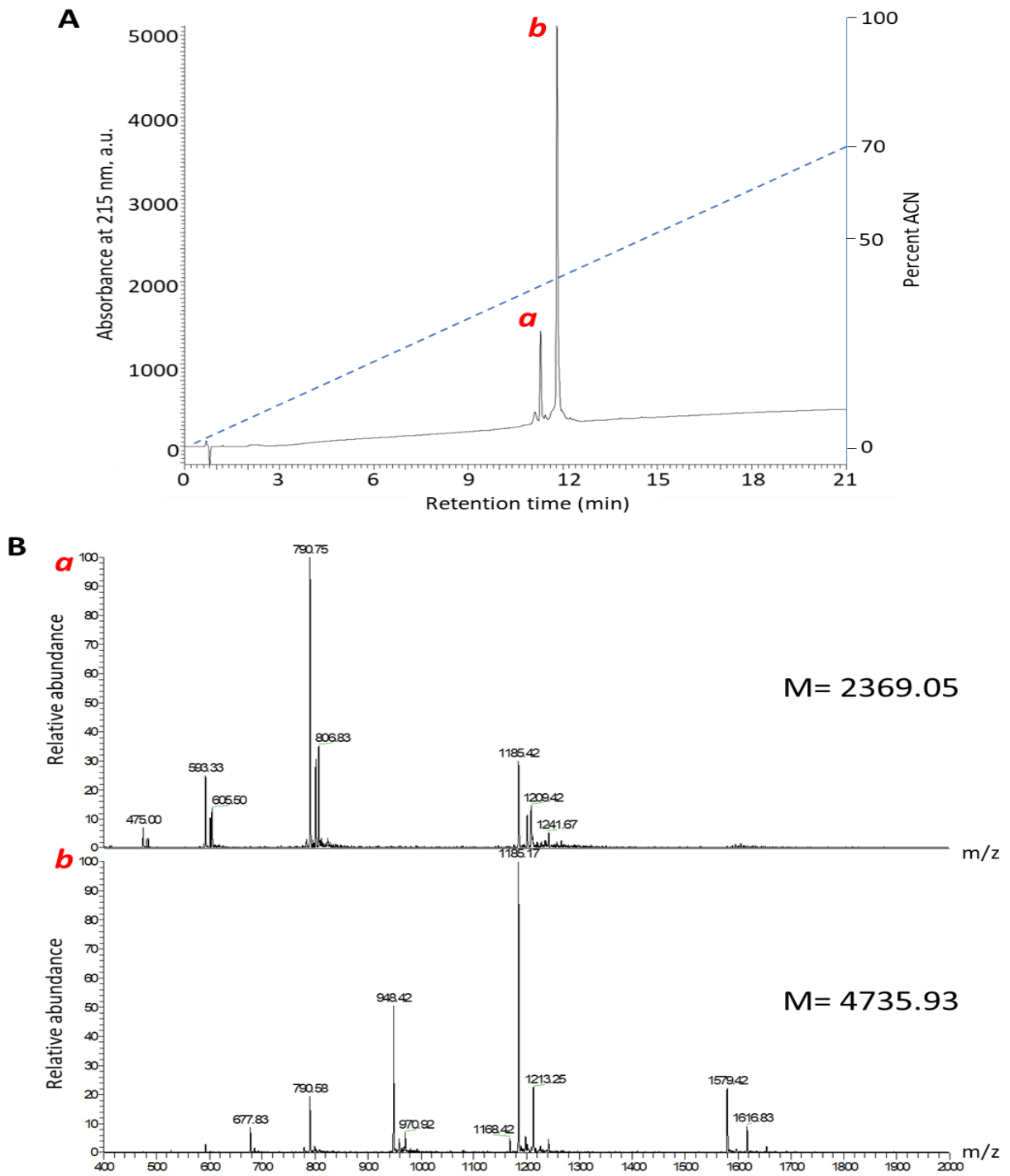


Figure S1: (A) Schematic organisation of the precursor's structure of BRICHOS-AMP family. (B) Translation of POL nucleotide sequence to a protein (amino acidic) sequences using Translate tool (ExPASy, https://web.expasy.org/translate/). In the black dotted square is the AMP part “polaricin”, in grey are the hydrophobic residues, and underlined is the BRICHOS region. The complete sequence of polaricin has been protected by using "X" instead of some amino acids.



C

POL	$(M+2H)^{2+}$	$(M+3H)^{3+}$	$(M+4H)^{4+}$	$(M+5H)^{5+}$	$(M+6H)^{6+}$	$(M+7H)^{7+}$
Monomer (a)	1185.42	790.75	593.33	475.00	/	/
Dimer (b)	/	1579.42	1185.17	984.42	790.58	677.83

Figure S2: UPLC-MS spectra of POL: (A) chromatogram of POL reveals the presence of two peaks, at (***a***) RT= 11.30 min and (***b***) RT= 11.82 min; elution was performed with a gradient of acetonitrile in acidified water (dotted line) and absorbance was monitored at 215 nm.(B) Mass spectrum was acquired on the two peaks (***a*** and ***b***), from 0 to 2000 m/z. The molecular weights were calculated from multi-charged fragments, giving molecular weight of 2369.05, equal with the theoretical value of monomeric POL (B1) and 4735.93, value of dimeric (POL). All values are summarized in the table C.

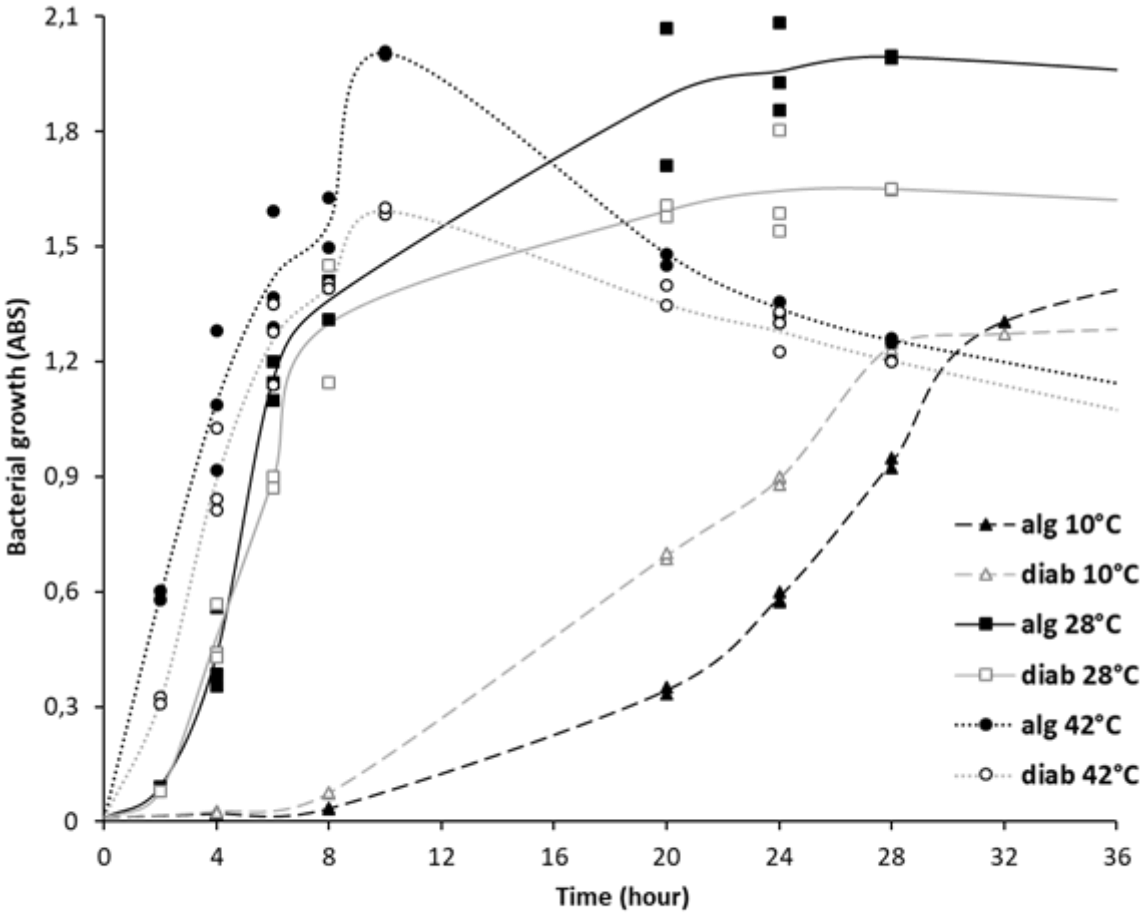


Figure S3: Temperature influence on *Vibrio alginolyticus and V. diabolicus* growth: aerobically incubated in MHB medium at 10°C, 28°C and 42°C on a rotary shaker (at 140 rpm).

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CHAPTER 3. Role of BRICHOS domain

Proteins denaturation can be caused by environmental stresses, fluctuations in physiological and environmental conditions. The resulting accumulation, self-assembling and misfolding of these denatured proteins into large aggregates has harmful consequences for the exposed organism [1]. To date, various maior diseases were discovered in humans and associated to these aggregates (such as Alzheimer and Parkinson diseases, cancer, diabetes, etc.) [2,3].
Molecular chaperones are essential for cellular homeostasis to prevent misfolded and aggregated proteins. There are many different types and mechanisms of molecular chaperones promoting the correct folding of proteins, BRICHOS has been proposed to assist its respective precursor protein to fold correctly during biosynthesis, preventing them from auto-aggregation (having a high propensity to form β-sheet structures) [4,5]. The BRICHOS domain is present in more than 300 proteins of 12 distantly related families, mainly linked to major diseases, such as British and Danish dementia, cancer and respiratory distress syndrome [5,6]. Three functions of BRICHOS domain-containing proteins have been proposed and proven in mammals: intramolecular chaperone-like function, promotion of targeting and secretion and assistance with specialized intracellular protease activity. The functional properties of the domain has not been fully explored in invertebrates.
BRICHOS domain has been identified in many marine polychaeta (considered as the primitive annelids), but to date not functionally described [7–9]. It is the only case of a BRICHOS domain in an AMP precursor. Because BRICHOS-AMPs, especially alvinellacin, are produced and in contact with extreme habitats, they are an interesting model to study the role of the domain in connection with external environmental factors.

This chapter contains preliminary results and it is here presented as the Chapter 3, the first draft of a paper not yet ready for submission.

The BRICHOS domain of marine annelids stabilizes the activities of antimicrobial peptides (AMPs) to face environmental conditions

Abstract:

The family of AMP from marine annelids exhibits the unique and coexisting presence of the evolutionary successful BRICHOS domain and potent antimicrobial substances in their precursor molecules. Amongst them, alvinellacin, from the eurythermal and thermotolerant Pompeii worm, is secreted in an environment extremely changing and harsh in terms of temperature, pH, pressure, etc. Since BRICHOS domain function has not been widely discussed, especially in invertebrates, we examined its chaperone function and the relationship with the antimicrobial peptides.

We demonstrate the presence of alvinellacin and its BRICHOS domain in worms plasma, in strict contact with the external environment of the worm, suggesting a role of the domain in assisting the peptide in the case of harsh conditions (in particular high temperature). Three variants of BRICHOS from preproalvinellacin were produced and used to analyze their potential role through anti-amyloid assay and thermal-stability test. The variants of *Alvinella* BRICHOS did not prevent the auto-aggregation of B-hairpin molecules into beta amyloid structures. They acted as molecular chaperone by increasing the thermostability of the peptide, maintaining their bioactivity in the case of high temperatures.

Keywords:

Molecular chaperone, preproalvinellacin, abiotic constraint, peptides thermostability.

3.1 Introduction

Environmental stresses, fluctuations in physiological and environmental conditions can cause proteins denaturation of the exposed organism. The resulting accumulation, self-assembling and misfolding of these denatured proteins into large aggregates has harmful consequences for organisms [1]. In humans, these aggregates are associated with about 40 human diseases, including Alzheimer and Parkinson diseases, cancer, diabetes, etc [2,3].

Molecular chaperones are essential for cellular homeostasis to prevent misfolded and aggregated proteins. There are many different types and mechanisms of molecular chaperones promoting the correct folding of proteins, BRICHOS has been proposed to assist its respective precursor protein to fold correctly during biosynthesis, preventing them from auto-aggregation (having a high propensity to form β-sheet structures) [4,5].

BRICHOS is a domain of approximately 100 amino acids, initially described in 2002 and identified in Bri protein (Bri2), Chon-dromodulin-1 (ChM-1) and Surfactant-associated protein C (proSP-C) sequences [6]. Later it has been found in more than 300 proteins of 12 unrelated families, associated with major diseases such as dementia syndrome (Alzheimer‘s disease), respiratory distress syndrome and cancer [4]. BRICHOS domain is characterized by low amino acid sequence identity (about 20%) with three residues strictly conserved in all representatives of the BRICHOS superfamily, one aspartic acid and two cysteine residues (forming an intramolecular disulfide bond) [6,10]. However, they displayed similar predicted secondary structures [11]. All BRICHOS containing preproproteins have an N-terminal cytosolic part, a hydrophobic signal/transmembrane (TM) region in N-terminal position, a linker region followed by a BRICHOS domain, and a C-terminal part (usually a β-sheet) [11]. The only exception is proSP-C, which has no additional C-terminal region following the BRICHOS domain. All of these preproproteins have a segment with high β-sheet propensity i.e. the C-terminal region, except in proSP-C, where instead the TM region has high β- sheet propensity [5,6].

While it was found in a wide range of organisms, the functional properties of the BRICHOS domain has not been fully explored. To the best of our knowledge, three functions of BRICHOS domain-containing proteins have currently been showed (mostly in mammals): (i) aid the promotion of targeting and secretion; (ii) assistance to the specialized intracellular protease processing system; and (iii) intramolecular chaperone-like function, protecting and avoiding

proteins aggregation, capable of conformational change (α helix to β sheet aggregates) producing amyloid fibrils [6,10,11].

The BRICHOS domain-containing proteins are present within all living organisms, and to date its mechanisms have been poorly investigated and described in invertebrates. Two BRICHOS domain-containing proteins have been identified in the most studied nematode, *Caenorhabditis elegans*: C25F6.7 homologue of human ITM2B that inhibit amyloid formation, and C09F5.1 a nematode-specific gene without a human homologue [1]. Recently, it has been showed that C09F5.1 was expressed in the sites constantly exposed to external environment and induced by heat-shock, playing a putative role in a temperature stress responses [1]. Unlike mostly BRICHOS domain-containing protein, C09F5.1 had no apparent chaperone function: in particular, it did not decrease auto-aggregation of amyloid proteins (Aβ42) [1].

As for annelids, the BRICHOS domain has been described in many marine polychaeta (considered as the primitive annelids) [7–9]. Moreover, it is the only case of BRICHOS domain presence in precursor protein of antimicrobial peptides (AMPs), participating in the biosynthesis of different structural types of polychaeta AMPs.

Here we used *Alvinella pompejana* as model organism, constantly exposed to extreme and fluctuating environmental conditions and producing a potent AMPs, containing BRICHOS domain in its precursor (preproalvinellacin) [8]. A recent study of our group on preproalvinellacin gene showed that the vital and highly conserved ectosymbiosis and the highly fluctuating physico-chemical conditions have not promoted diversifying selection on the AMP part alvinellacin [12]. On the contrary, a peculiar selective trend promoting the adaptive diversification of the BRICHOS part of the AMP precursor has occurred (exhibiting 17 variants) [12]. In this study, three of these variants of BRICHOS domain (presenting more amino acid substitutions) were produced as recombinant and tested to investigate on their function in invertebrate's model and in association with AMPs.

Moreover, we detected the presence of BRICHOS domain (and alvinellacin) in the extracellular plasma of the worm, strictly in contact with the extreme harsh environment. This finding, led us to assume it might play a role in regulating the integrity of the peptide under stress or changing environmental conditions.

The similarity with the other homologues at both the genomic DNA and protein structure levels suggested that the BRICHOS variants plays similar roles to those previously proposed for this protein family, such as (mostly) chaperone-like functions, assistance in the secretion

pathway and cellular protease activity. We investigated here on its chaperone-like function, potential synergic activity in alvinellacin biological activities and role in AMPs thermotolerance.

3.2. Results

BRICHOS from preproalvinellacin

The amino acid sequence alignment of the BRICHOS domain from preproalvinellacin and other BRICHOS-related proteins (database UniProtKB/Swiss-Prot and UniProtKB/TrEMBL), in Figure 1, clearly underlines the residues strictly conserved in all members of the BRICHOS superfamily, especially one aspartic acid and two cysteine residues.

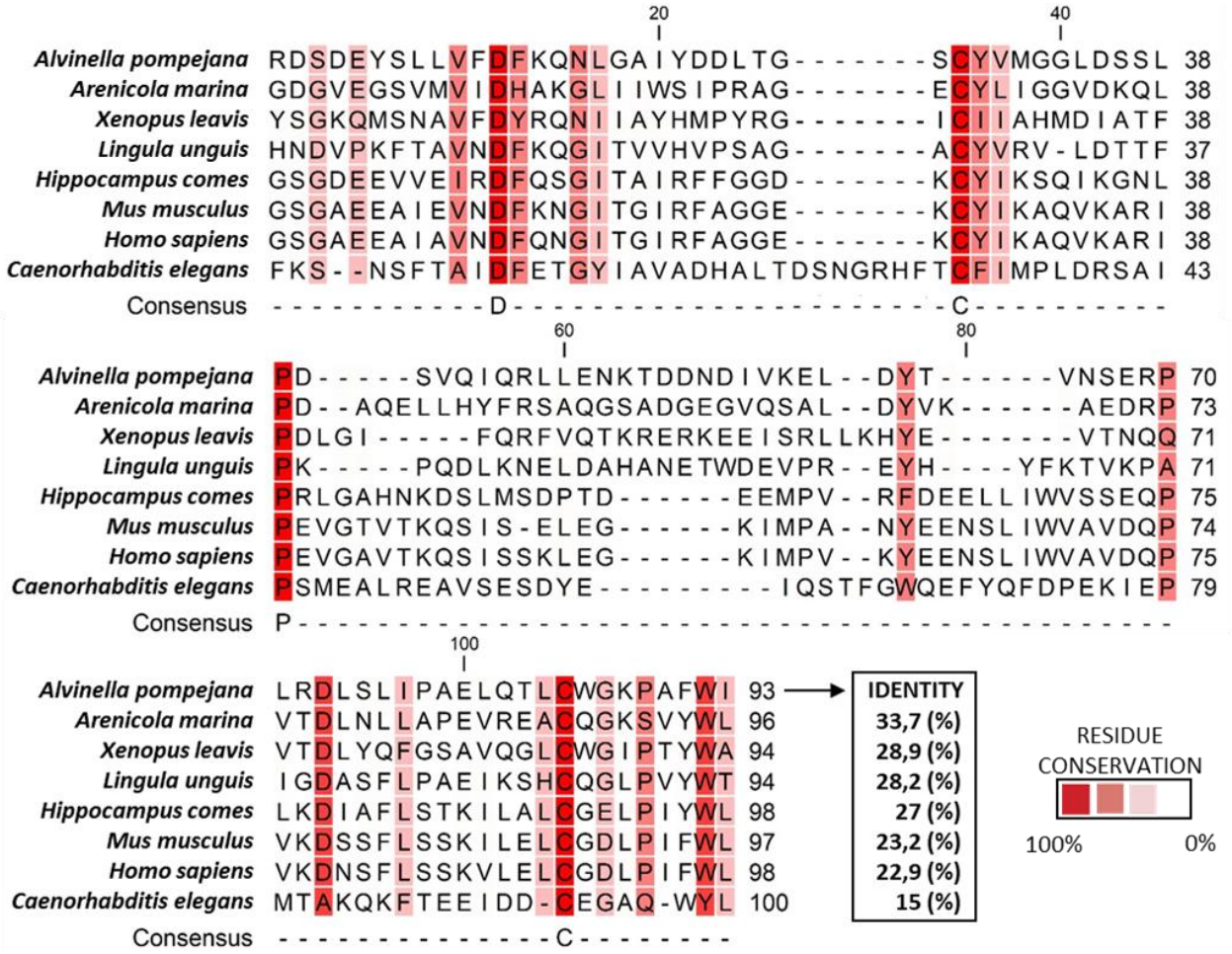

Figure 1: Multiple sequence alignment of BRICHOS region from various animal and their identity (express in percentage) with alvinellacin BRICHOS domain (in the black box), generated by CLC Sequence Viewer software (version 8.0). The background colour (intensity of red) of the amino acid represents their conservation. The computation of identity percentage was performed at the SIB (ExPASy software, https://www.expasy.org/) using the BLAST network service (BLASTP, version 2.2.31+).

To date, the roles of BRICHOS domain-containing proteins have been poorly investigated and described in invertebrates: the only one recently examined, C09F5.1 from *C. elegans*, displays merely 15% of identity with preproalvinellacin BRICHOS [1].

BRICHOS secretion

Samples of plasma extracted from many individuals of *Alvinella pompejana* (from MESCAL cruise, 2012) were used to detect the extracellular presence of BRICHOS domain [13]. A total of 35 samples (previously exposed to different thermal and pressure stresses) were tested by Dot-immunobinding assay (DIA), in Figure 2, using a panel of specific antisera (polyclonal anti-BRICHOS guinea pig antibody and anti-guinea pig as secondary antibody). The presence of BRICHOS in the extracellular fluid of Pompeii worms, as it has been earlier proved for alvinellacin, was detected in all tested sample, not depending on inductions of external stressors (such as pressure or thermal conditions).

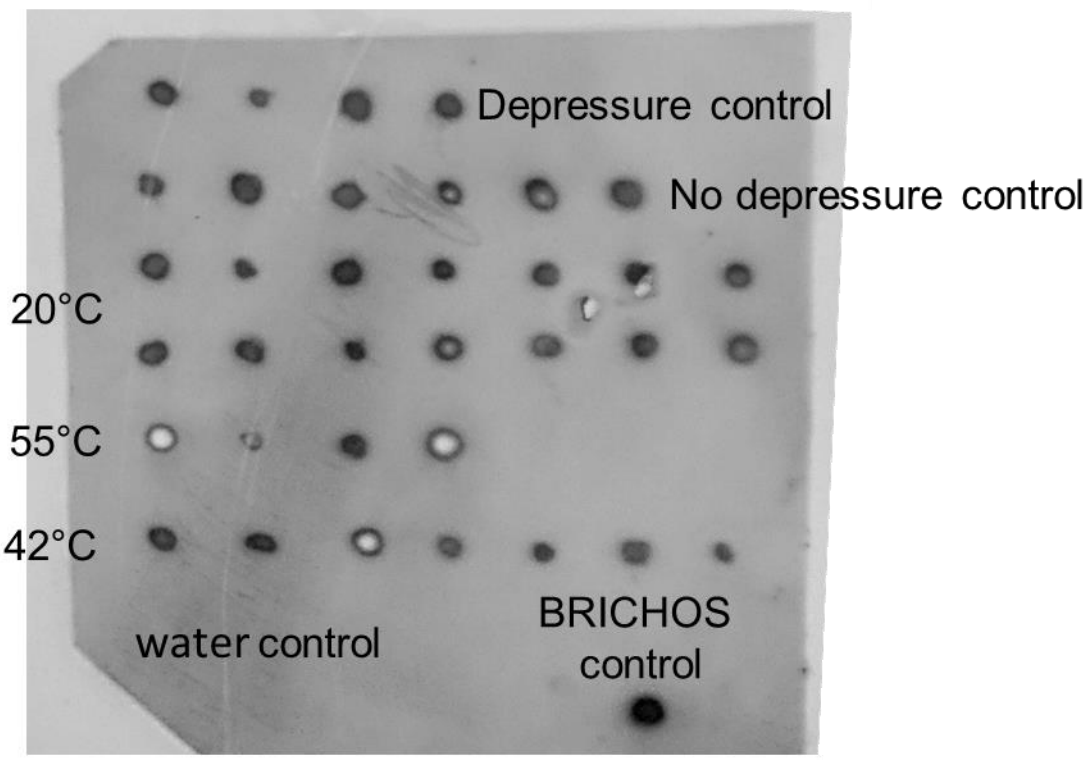

Figure 2: Immunodetection of BRICHOS in cell free plasma of different individuals of *Alvinella pompejana,* exposed to different temperatures and pressures.

BRICHOS variants

As mentioned above, a recent study from our group on alvinellacin precursor gene, showed that the extreme harsh environmental conditions have not promoted diversifying selection on alvinellacin; on the contrary, a positive selection on the proregion was evident with a hot spot mutation on the BRICHOS domain [12]. Among the 17 variants of BRICHOS sequences described by Papot *et al.* 2017, we selected the three variants (named SNC1, SNC4 and SNC10) from different clades and showing more mutations (Figure 3).

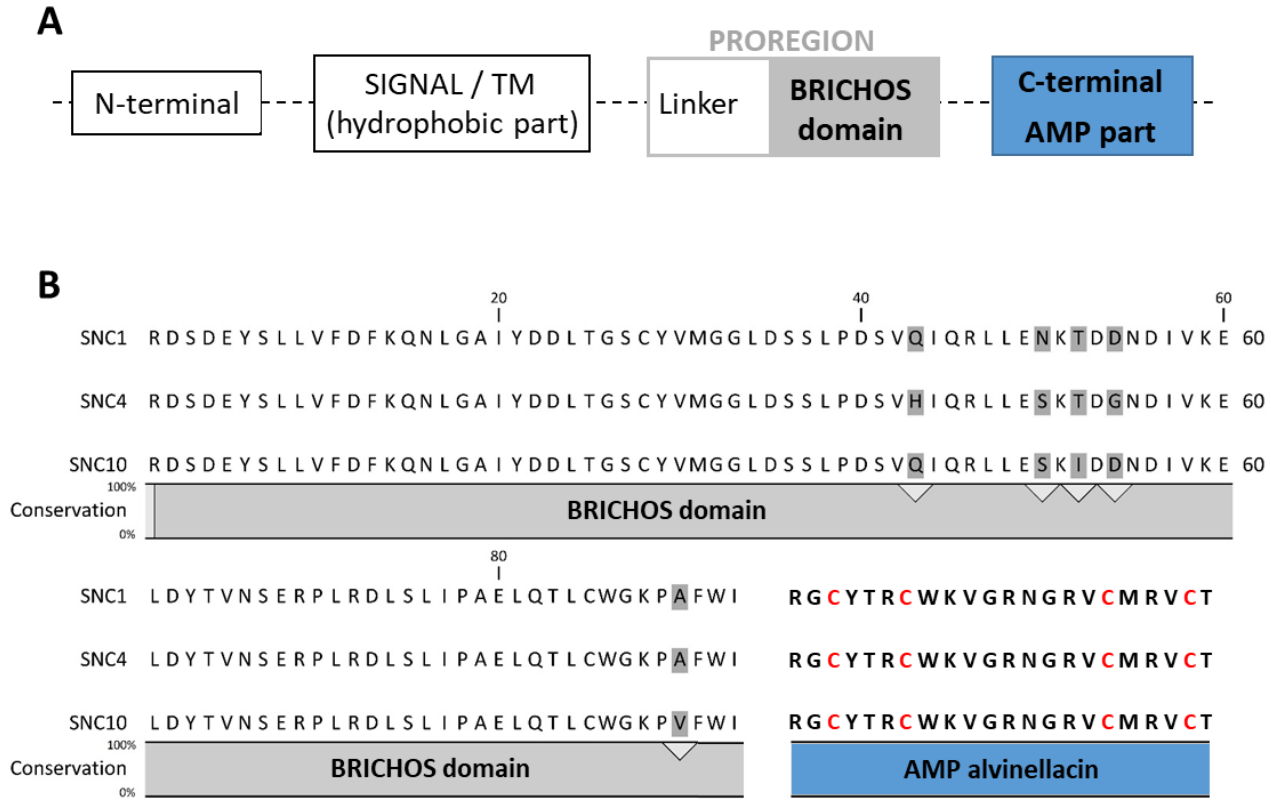

Figure 3: The domain architecture in representative members of the BRICHOS domain-containing families: (A) typical structure of BRICHOS-AMP precursors, with BRICHOS domain in grey and mature AMP part in blue. (B) Amino acid sequence alignment of alvinellacin precursor variants (BRICHOS domain and mature AMP), performed by using CLC Sequence Viewer software (version 8.0).

The three recombinant of BRICHOS sequence (SNC1, SNC4 and SNC10) from precursor of alvinellacin, have been massively produced in *Escherichia coli Origami* (DE3) pLysS (Novagen). The proteins were detected by electrophoresis on gel and Western Blot (in Figure 4) to then be purified by reversed-phase high-performance liquid chromatography (RP-HPLC), in Figures 5/1, 5/2 and 5/3. Mass spectrometry analysis associated to Dot immunoblotting assays, confirmed the presence and the dimension of the proteins (Figures 5/1, 5/2 and 5/3).

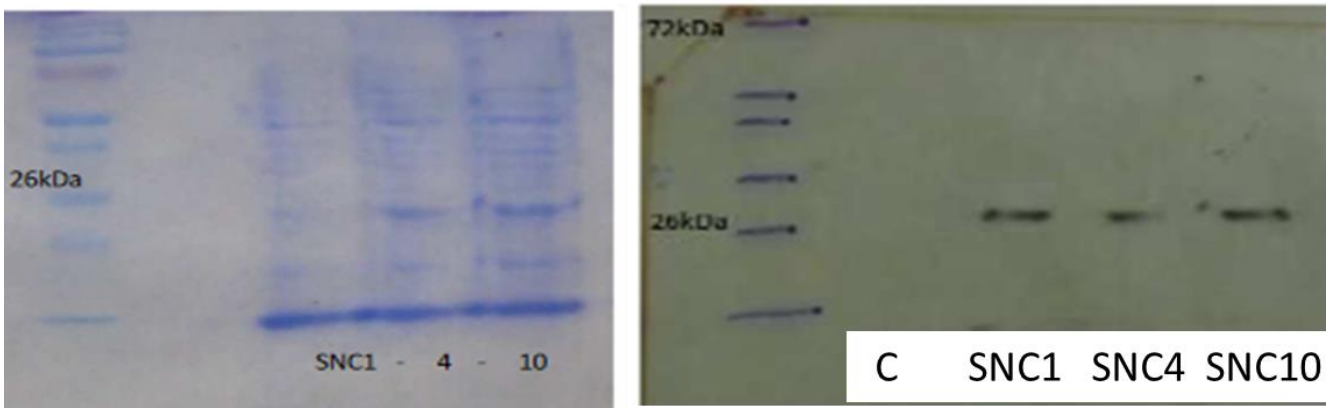

Figure 4: SDS-PAGE (12% acrylamide) and Western Blot, performed after 6h induction and using the anti-BRICHOS antibody for the three variants SNC1-SNC4-SNC10. The band at the expected size (25kDa) is visible, revealing the presence of the BRICHOS protein (10kDa) + expression vector (15kDa).

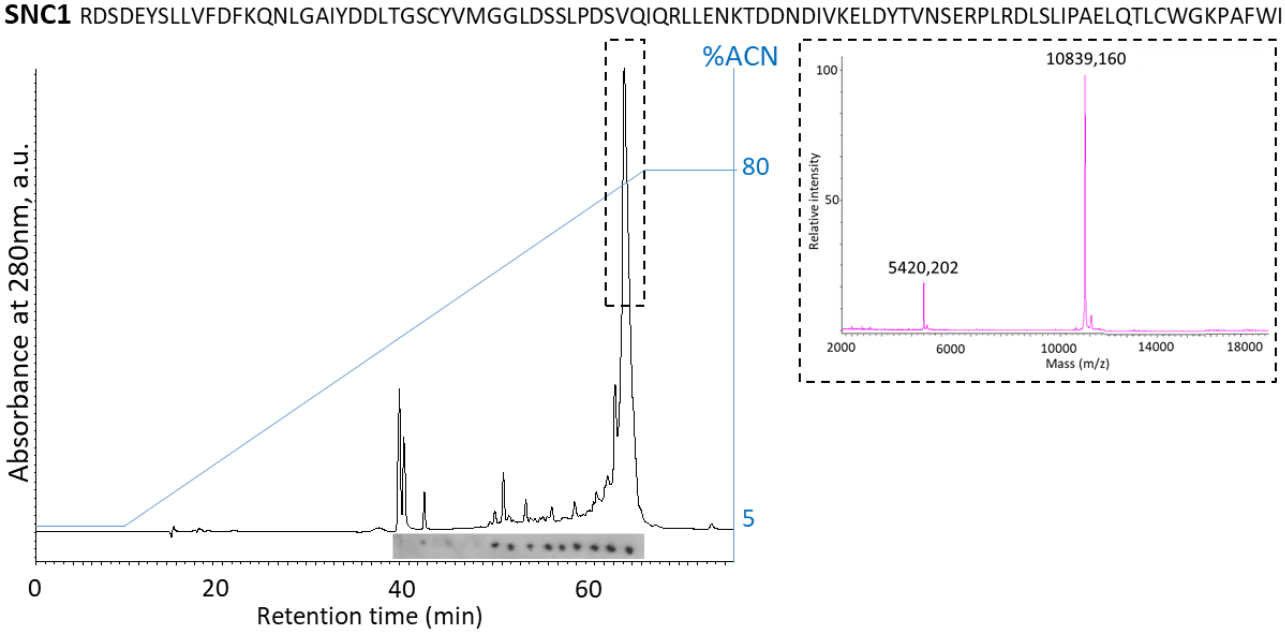

Figure 5/1: Chromatogram of SNC1: the sample was loaded onto a C18 column (250x10mm, Sephasyl). Elution was performed with a monophasic gradient of acetonitrile in acidified water and absorbance was monitored at 280 nm. Under the graphs, the DIA results of the fractions corresponding to the peaks. MALDI mass spectrum of the purified variant, in the dotted rectangle, acquired in the range 2000-18000 m/z, shows the major peaks at m/z 10839.160 [M+H]+ and at m/z 5420.202 [M+2H]2+.

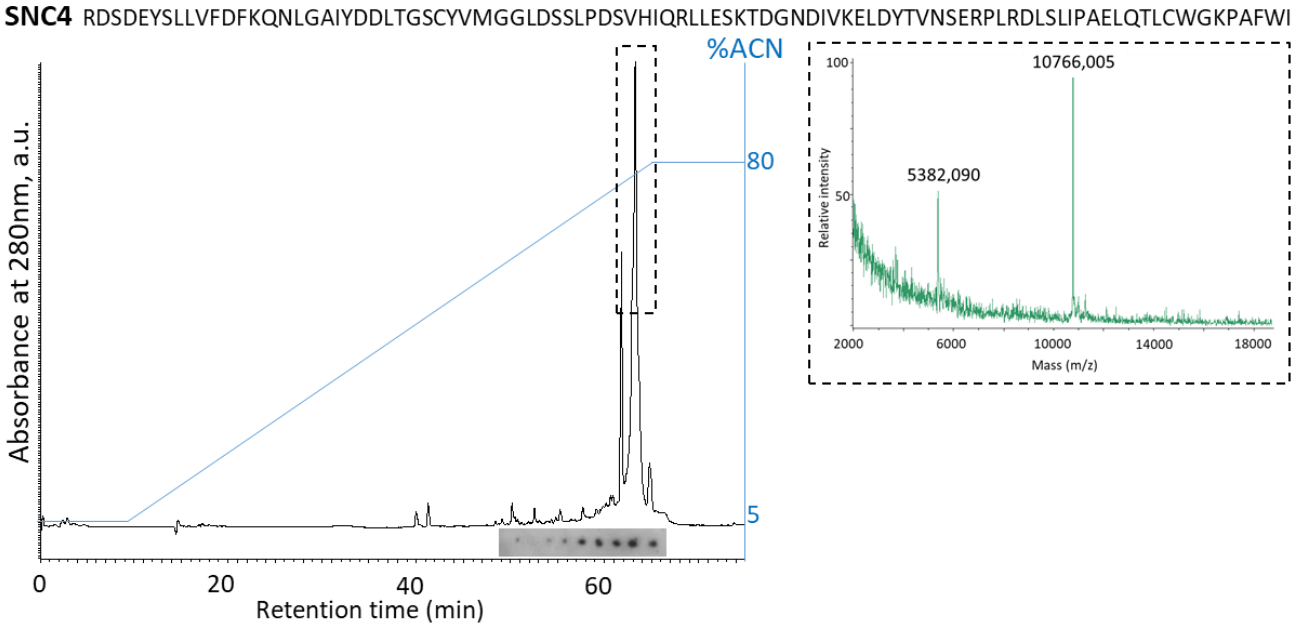

Figure 5/2: Chromatogram of SNC4: the sample was loaded onto a C18 column (250x10mm, Sephasyl). Elution was performed with a monophasic gradient of acetonitrile in acidified water and absorbance was monitored at 280 nm. Under the graphs, the DIA results of the fractions corresponding to the peaks. MALDI mass spectrum of the purified variant, in the dotted rectangle, acquired in the range 2000-18000 m/z, shows the major peaks at m/z 10766.005 [M+H]+ and at m/z 5382.090 [M+2H]2+.

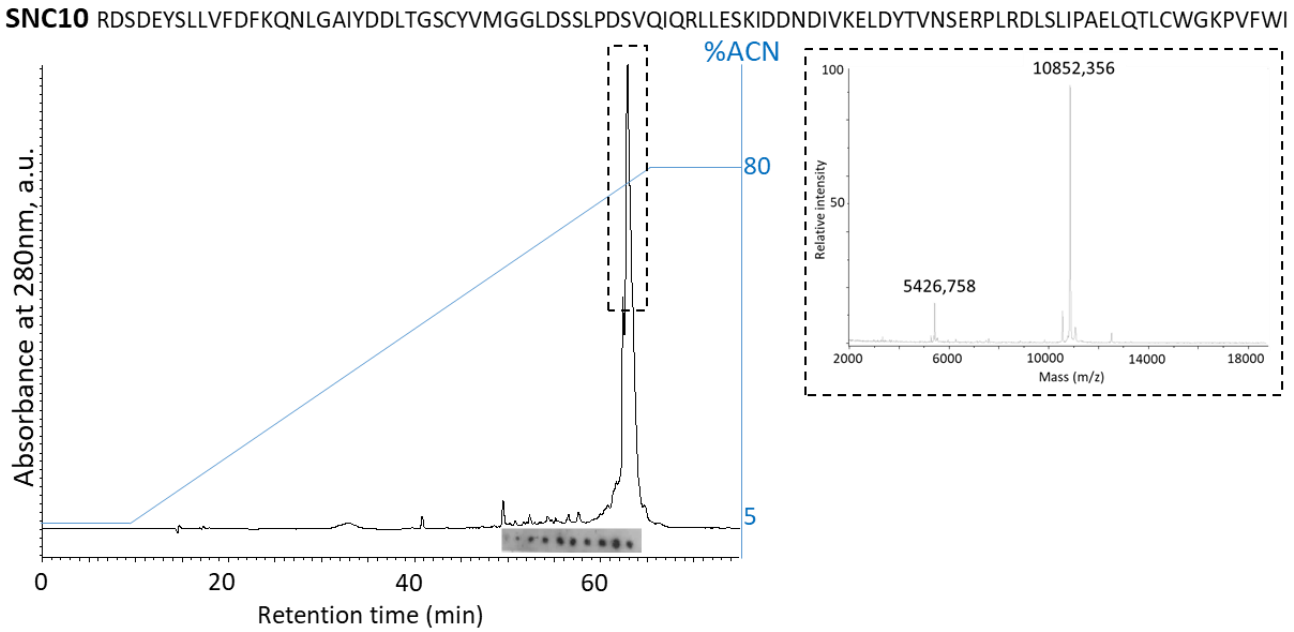

Figure 5/3: Chromatogram of SNC10: the sample was loaded onto a C18 column (250x10mm, Sephasyl). Elution was performed with a monophasic gradient of acetonitrile in acidified water and absorbance was monitored at 280 nm. Under the graphs, the DIA results of the fractions corresponding to the peaks. MALDI mass spectrum of the purified variant, in the dotted rectangle, acquired in the range 2000-18000 m/z, shows the major peaks at m/z 10852.356 [M+H]+ and at m/z 5426.758 [M+2H]2+.

Antimicrobial activities

The three variants so produced in large quantities, were firstly tested as possible co-adjuvant in peptides biological activities. Table 1 shows MIC (Minimal Inhibitory Concentration) and MBC (Minimal Bactericidal Concentration) values of alvinellacin, BRICHOS variants and alvinellacin *plus* variants against *Vibrio alginolyticus* and *V. diabolicus*, to evaluate potential synergic effects between peptide and its BRICHOS domain. The results clearly demonstrate that the three variants have no effects on peptides bacteriostatic and bactericidal activities.

Table 1: MIC (Minimal Inhibitory Concentration) and MBC (Minimal Bactericidal Concentration) values of alvinellacin (ALV), BRICHOS variants (SNC1, SNC4 and SNC10) and alvinellacin+variant, against Vibrios.

BACTERIA	MIC (µM)							MBC (µM)						
	Peptide	BRICHOS			Peptide+BRICHOS			Peptide	BRICHOS			Peptide+BRICHOS		
	ALV	**SNC1**	**SNC4**	**SNC10**	**ALV+ SNC1**	**ALV+ SNC4**	**ALV+ SNC10**	**ALV**	**SNC1**	**SNC4**	**SNC10**	**ALV+ SNC1**	**ALV+ SNC4**	**ALV+ SNC10**
V. alginolyticus	0.15 - 0.31	> 1.25	> 1.25	> 1.25	n.t.	n.t.	n.t.	0.625	>1.25	>1.25	>1.25	n.t.	n.t.	n.t.
V. diabolicus	1.25 - 2.5	>10	>10	>10	1.25 - 2.5	1.25 - 2.5	1.25 - 2.5	10	>10	>10	>10	>10	>10	>10

Anti-amyloid assay

Since the most common function of BRICHOS domain is chaperone activity, preventing the auto-aggregation of β-hairpin molecules into beta amyloid fibrils toxic for the cells, we performed an anti-amyloid assay in a dose-dependent manner, using Aβ42 protein and Thioflavin T (ThT). The test measures changes of fluorescence intensity of ThT upon binding to amyloid fibrils (protein Aβ42) [5,14–17]. The auto-aggregation of the protein Aβ42, with formation of beta amyloid structure, was followed for 48 hours (Figure 6). The charts show that the three variants of BRICHOS domain from preproalvinellacin (SNC1, SNC4 and SNC10) do not prevent auto-aggregation of amyloid proteins. The fibril formation of the amyloid protein was not affected by the presence of the three alvinellacin BRICHOS, even at high concentrations (such as 150% of protein concentration).

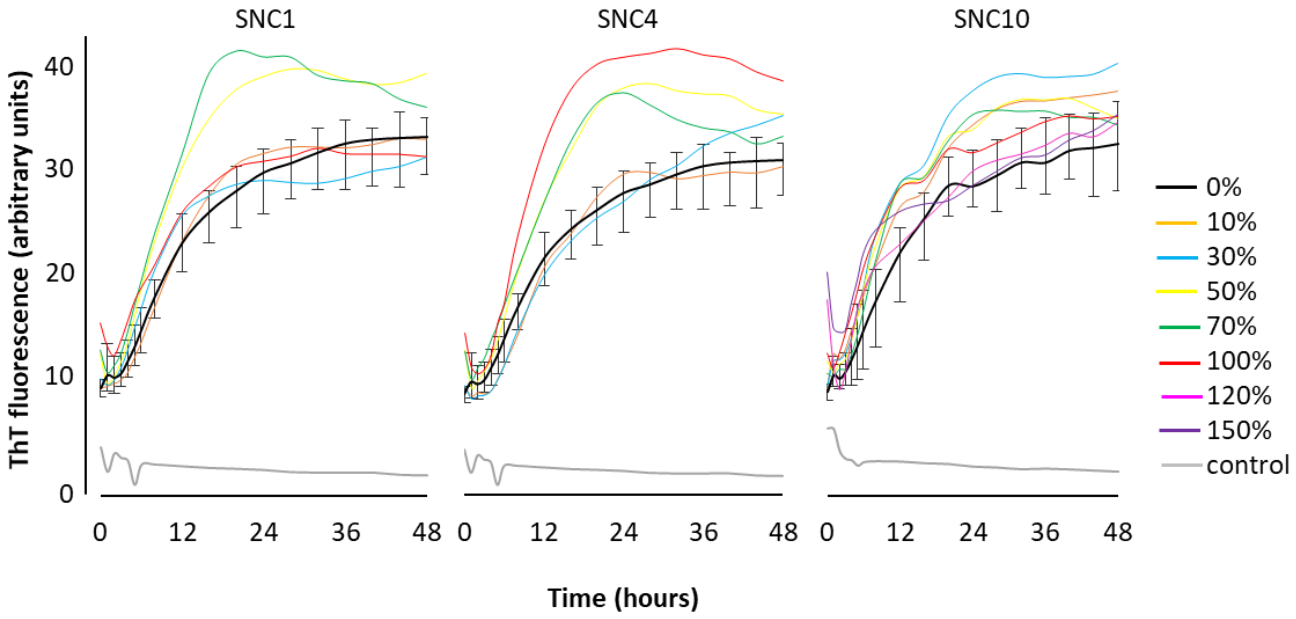

Figure 6: Kinetics analysis of BRICHOS variants (SNC1, SNC4 and SNC10) effects on Aβ42 fibril formation: aggregation of 3 µM of protein Aβ42 in the presence of 0 (black, positive control), 10 (orange), 30 (cyan), 50 (yellow), 70 (green), 100 (red), 120 (fuchsia) and 150% (violet) of BRICHOS variants (molar percentage referred to protein Aβ42 concentration). Fluorescence and aggregation are directly proportional. In light grey are the negative controls, not containing the amyloid-β protein (Aβ42).

BRICHOS chaperone activity

A*lvinella pompejana* is one of the most eurythermal and thermotolerant known species. The finding of BRICHOS and alvinellacin presence in extracellular plasma of the Pompeii worm (above demonstrated), in contact with the harsh hydrothermal vent habitat, led us to investigate on the role of these molecules in correlation with thermal stress.

Based on our recent results about alvinellacin adaptation to extreme temperatures (see Chapter 2 of this thesis), we evaluate here the involvement of BRICHOS domain in the stability of the peptide in the more extreme conditions encountered by the worm.

Here, in Figure 7, we show the results of alvinellacin thermostability test, with and without BRICHOS variants. It is evident that BRICHOS variants significantly contribute to alvinellacin stability in case of heat shock (1 hour at 90°C): *V. diabolicus* growth was drastically reduced (~40%) compared to the samples not assisted by the domain. In addition, we performed the tests with alvinellacin analogs devoid of cysteine residues and disulfide bridges (ABU, in Figure 8) and polaricin (POL, in Figure 9). The results obtained with ABU and POL confirmed that the domain play a role in peptide thermal-stability: only in presence of BRICHOS variants, both peptides inhibited *V. alginolyticus* growth despite their incubation at 42°C.

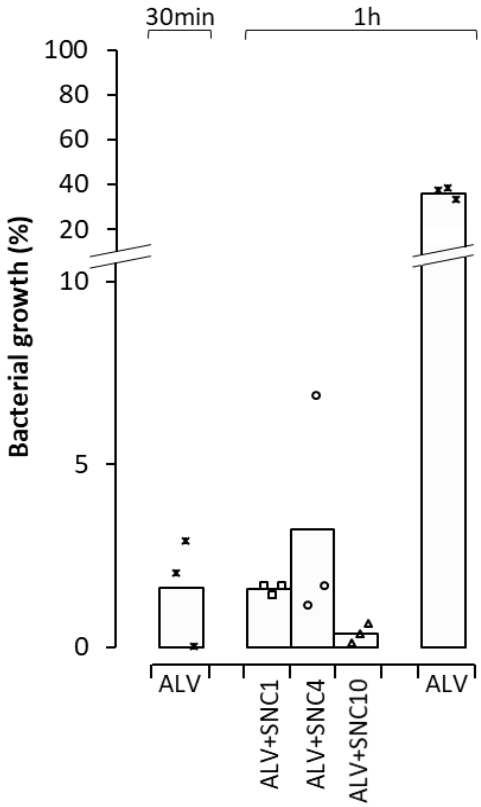

Figure 7: Thermostability assay. Antimicrobial activity of alvinellacin (ALV), evaluated after its incubation at 90°C (30min or 1hour) with and without BRICHOS variants (SNC1, SNC4 and SNC10), against *V. diabolicus*.

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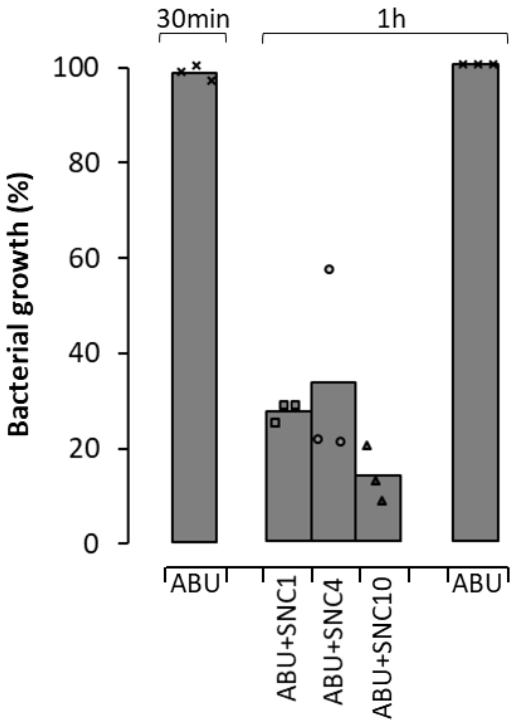

Figure 8: Thermostability assay. Antimicrobial activity of ABU-alvinellacin (ABU), evaluated after its incubation at 42°C (30min or 1hour), with and without BRICHOS variants (SNC1, SNC4 and SNC10) against *V. alginolyticus*.

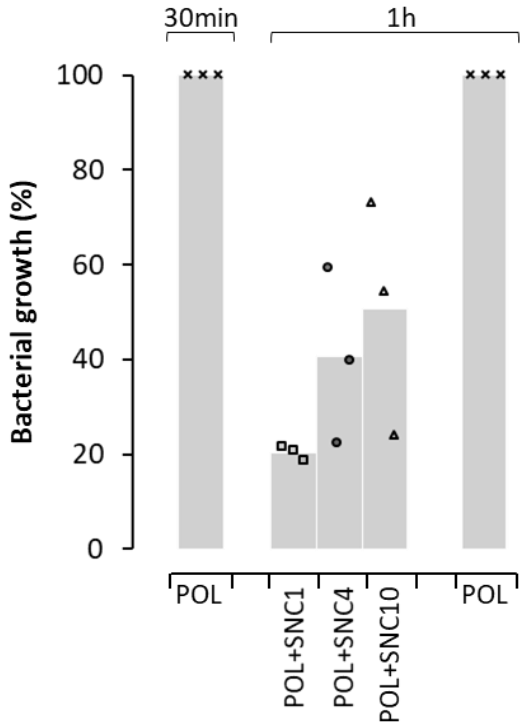

Figure 9: Thermostability assay. Antimicrobial activity of polaricin (POL), evaluated after its incubation at 42°C (30min or 1hour), with and without BRICHOS variants (SNC1, SNC4 and SNC10) against *V. alginolyticus*.

3.3. Material and methods

Sequences alignments

The multiple sequence alignment of BRICHOS region from various animal (selected by using BLAST database, UniProtKB/Swiss-Prot + UniProtKB/TrEMBL), was generated by CLC Sequence Viewer software (version 8.0). The computation of identity percentage, between BRICHOS domains, was performed at the SIB (ExPASy software, https://www.expasy.org/) using the BLAST network service (BLASTP, version 2.2.31+).

Among the 17 variants of preproalvinellacin previously described by Papot *et al.* 2017 [12], we chose to select the three variants (named SNC1, SNC4, SNC10) showing the mutation of each clade (with more amino acid substitutions). The sequence alignments of BRICHOS variants was performed by using CLC Sequence Viewer software (version 8.0).

Plasma samples

Alvinella pompejana specimens were collected using the DSV Nautile during the oceanographic cruise MESCAL (2012) [13]. The samples were subjected to two pressure regimes (varying pressure and constant pressure thanks to Balist aquarium) and three thermal regimes, a constant mild 20°C exposure, and two heat-exposures followed by a 3 hour-recovery period at 20°C. The heat-exposures lasted about 2 hours, the first one ramped from 30°C to 42°C, and the second one from 50°C to 55°C (thereafter referred to as '42°C' and '55°C' experiments respectively). The coelomic fluid was collected from individual worms and stored at -80°C pending analyses.

Dot immunobinding assays (BRICHOS and ALV)

The presence of alvinellacin and BRICHOS in worms plasma was detected by Dot immunobinding assay, using a panel of specific antisera. 1 µl from each sample was spotted onto a nitrocellulose membranes (BIO-RAD, Hercules, CA, USA). Membranes were baked (1 hour; 100 °C), incubated in blocking buffer (1 hour; TBS, 0.1 M; Tween 20, 0.05%; non-fat dry milk, 2%) and probed with the rabbit polyclonal anti-alvinellacin antibody (2 hours; 1/400; TBS, 0.1 M; Tween20, 0.05%). The membranes were washed (3x5 minutes; TBS 0.1 M; Tween20, 0.05%) and incubated for 1 hr with anti-rabbit secondary antibody IgG (1/1000; TBS, 0.1 M; Tween20, 0.05%). The same procedure was performed for BRICHOS detection, using anti-BRICHOS cobay antibody (1/400) and anti-cobay secondary antibody (1/1000). Both membranes were washed again (10 minutes; TBS 0.1 M; Tween20, 0.05%; and 10 minutes in

TBS 0.1 M). ClarityTM Western ECL Substrate (BIO-RAD) was used for the chemoluminescence visualization of the immunolabeling with a Kodak Bio Max light film.

Recombinants of BRICHOS variants

The three recombinant of BRICHOS sequence (SNC1, SNC4 and SNC10) from precursor of alvinellacin, were produced in *Escherichia coli Origami* (DE3) pLysS (Novagen), chosen because it allows the formation of disulfide bridges in the produced molecule, according to a standard protocol (50 µl of bacteria transformed with 20 nanograms of expression vector pet32c (Novagen, Madison, WI) by thermal shock of 40seconds at 42°C). The constructions were already available at my arrival to the laboratory [18].

For a massive production of the variants, a preculture was prepared by adding 50µL of the stored constructions to 5mL of Luria-Bertani (LB Broth Lennox, Athena ES) medium containing 100µg/mL of ampicillin and incubated overnight (at 37°C, 180rpm). The expression was induced by the addition of preculture in 1L of LB medium containing 100mg/ml of ampicillin and 0.25mM of isopropyl β-D-1-thiogalactopyranoside, at 25°C and 180rpm, until the O.D. reach 0.5 at 600nm.

The samples were centrifuged at 1000g for 20 minutes at 4°C, and the pellets were incubated with lysozyme (1mg/ml) for 1hour on ice and then through a French press (at about 1200 psi). The supernatants was collected after 30 minutes centrifugation at 4°C at 14000rpm and eluted on 1mL HisTrap HP nickel resin (GE HealthCare Life Science) containing 5ml of fixing buffer (20mM sodium phosphate, 0.5M NaCl, pH7.4) with gradient of imidazole concentration (50mM, 250mM and 500mM). The proteins were detected (25kDa) in the fractions eluted with 250mM imidazole, by using SDS-PAGE (in denatured conditions) on 4-12% gels, with a marker protein Ladder (10-170 kDa, Euromedex) and visualized by Coomassie Brilliant Blue staining.

The protein concentration was determined by using centrifugal filter devices (Amicon Ultra-15, 10K, Millipore, Germany) for 15 minutes at 4000g. After protein quantification with Qubit Fluorometric (Qubit 4 Fluorometer, Invitrogen, Thermo Fisher Scientific, Waltham, USA), enterokinase light-chain (New England Biolabs) was used (0.00016µg for 25µg f proteins) for cleavage, at 25°C for 16 hours.

The samples were subjected to reversed-phase high-performance liquid chromatography (RP-HPLC), carried out on a Perkin Elmer series 200 HPLC system with a variable wavelength detector. The column effluent was monitored by absorbance at 280 nm. The elution was performed on an Uptisphere C4 column (250 × 10.0 mm, model UP5WT4-250/100, Interchim),

with a biphasic gradient of 5 - 80% ACN in acidified water for 60 min, at a flow rate of 1 ml/min. The fractions corresponding to absorbance peaks were collected in polypropylene tubes, pulled, dried, reconstituted in HPLC grade water, and tested for its immunoreactivity to the BRICHOS Ab by Dot immunobinding Assay (see above).

The samples were finally characterized by Mass spectrometry analysis (sinapinic acid 20mg/ml in TFA/ACN (7:3, v/v), sample/matrix ratio 1:2, FlexAnalysis 3.4 - Bruker Daltonics.

Synthesis of the peptides

Alvinellacin (ALV) and polaricin (POL) were synthesized by BIOSYNTAN GMBH (Berlin, Deutschland). ABU-alv was designed as variant of ALV based on the removal of the disulfide bonds through the replacement of cysteine by α-aminobutyrate residues (see Chapter 2).

Microorganisms

The bacterial strains used in this study are Gram-negative bacteria from marine environment, *V. alginolyticus* and *V. diabolicus HE800*, provided by IFREMER. The marine strains were cultivated at 28°C in MHB (Mueller Hinton Broth, ROTH), under shaking at 140 rpm and maintained on MH agar at room temperature.

Antimicrobial assays

The synergic activity of BRICHOS and AMPs was determined by evaluating peptides biological activities when incubated in presence of BRICHOS variants: the antimicrobial activity was determined by the broth microdilution method against *V. alginolyticus* and *V. diabolicus*, as described above [19]. BRICHOS variants and peptides were mixed and added at equal concentration by serial dilution (10 to 0.0195 µg/mL of pure water). Antimicrobial activity of BRICHOS variants was screened against Vibrios strains, as control.

One colony of Vibrios strain was grown in MHB overnight at 28°C (140 rpm). The freshly grown culture was then diluted (1/100 in 10 mL of MHB), and incubated under the same conditions until the mid-log phase (optical density at 600 nm) was reached. Bacteria were diluted to 1×10^6 CFU/mL and added (100µL per well) into sterile 96-well flat bottom plates (CELLSTAR, Greiner bio-one) containing serial dilutions (10 to 0.0195 µg/mL of pure water) of BRICHOS variant and peptide mixed at equal concentration. The plates were incubated overnight at 28°C at 140 rpm. The MIC (Minimal Inhibitory Concentration) was taken as the lowest peptide concentration at which observable microbial growth was inhibited, the measurements were realized by a microtitre plate reader (Tecan Sunrise Microplate Reader) at A600. MBC was measured by streaking on proper agar (MHB) petri dishes, the entire volume (110µL) of wells

(from previously MIC plates) with no bacterial growth. After incubation for about 24 hours at 28°C, the MBC was defined as the peptide concentration where no colony growth was observed. All tests were conducted in triplicate.

Anti-amyloid assays

The analysis of the kinetics of amyloid fibril formation was performed by ThT assay [17]: 80µL solution containing 3µM of beta-amyloid 1-42 (Aβ42, Enzo Life Sciences), 10µM of Thioflavin T (ThT, Sigma-Aldrich) and different concentrations of BRICHOS variants (10-30-50-70-100-120-150% of Aβ42 concentration) were added to each well of 96-well half area black polystyrene microplates with clear bottom and non-binding surface (Greiner Bio-One, Germany), incubated at 37°C. The fluorescence was recorded (for 48h) using a 400±50 nm excitation filter and a 480±20 nm emission filter (POLARstar Omega from BMG Labtech, Ortenberg, Germany). The tests were conducted in triplicate.

Thermal-stability assays

The chaperone activity of BRICHOS was determined by evaluating peptide biological activities, after their exposure to thermal stress. One colony of Vibrios strain was grown in MHB overnight at 28°C (140 rpm). The freshly grown culture was then diluted (1/100 in MHB), and incubated under the same conditions until the mid-log phase (optical density at 600 nm) was reached. Bacteria were diluted to 1×10^6 CFU/mL and added (100µL per well) into sterile 96-well flat bottom plates (CELLSTAR, Greiner bio-one) containing ALV and BRICHOS, mixed at equal concentration (5-fold MIC) and previously incubated for 1 hour at 42°C or 90°C (on a digital dry block heather, Grant QDB2). The plates were incubated overnight at 28°C at 140 rpm. ALV antimicrobial activity (with BRICHOS assistance) was evaluated by the measurements of *V. diabolicus* growth by a microtitre plate reader (Tecan Sunrise Microplate Reader) at A600. The results were expressed as percentage of bacterial growth, in comparison with the control samples (MHB medium and *V. diabolicus*). MBC was measured by streaking on proper agar (MHB) petri dishes, the entire volume (110µL) of wells (from previously MIC plates) with no bacterial growth. After incubation for about 24 hours at 28°C, the MBC was defined as the peptide concentration where no colony growth was observed. All tests were conducted in triplicate.

Statistical analysis

All data are presented as the mean ± SEM (standard error of the mean).

3.4. Discussion and conclusion

The BRICHOS domain was found in many proteins from a wide range of species, exerting chaperone-like functions, assistance in the secretion pathway and cellular protease activity [5,6,20]. While it has various disease associations, the functional properties of the BRICHOS domain has not been fully explored, especially in invertebrates.

This study extends the knowledge of BRICHOS domain functions by using as model the precursor of alvinellacin from the Pompeii worm. Preproalvinellacin contains a BRICHOS domain and alvinellacin (as AMPs part), belonging to the family of BRICHOS-AMPs. To date, the presence of BRICHOS domain in association with AMPs is restricted to the family of marine polychaetes peptides [7–9].

From our findings, the BRICHOS region and the AMP part of preproalvinellacin were both detected in Pompeii worm extracellular plasma, exposed to the harsh hydrothermal vent conditions. We hypothesized a function of BRICHOS in stabilization of alvinellacin in the context of variable abiotic (especially thermal) factors. Recently, in a similar way, it had been shown the localization of C09F5.1 BRICHOS domain-containing protein identified in *Caenorhabditis elegans* and displaying a role in thermotolerance [1].

Overall, we demonstrate the key role of alvinellacin BRICHOS domain in the assistance to the biological functions of AMPs in the case of heat stress.

In our previous work (Chapter 2 of this thesis), we showed that the biological activities of alvinellacin samples, against two Vibrio bacteria, were reduced (about 50% of bacterial growth) by their incubation at 90°C for 1 hour. In presence of the BRICHOS variants, presumably assisting alvinellacin (since they have not exhibited antimicrobial activity nor synergistic effect), we registered less than 5% of bacterial growth.

Moreover, our results showed that the three BRICHOS variants are not alvinellacin-specific, supporting various AMPs with different structures. In Chapter 2, we showed that the exposition of polaricin and ABU-alvinellacin to thermal stress (at 42°C), caused the complete loss of their biological activities against *V. alginolyticus*. Surprisingly, both peptides displayed high rates of bacterial killing, when incubated (at 42°C) in presence of BRICHOS variants.

From our preliminary results, it appears that the variants possess a peptides specificity: while SNC1 was the most performant for the stability of polaricin, we reported less significant rates of bacterial inhibition for alvinellacin and ABU-alvinellacin. Conversely, SNC10 was extremely

efficient in association with alvinellacin and its analogs, as if the mechanism relied on amino acid sequence patterns. The presence of various variants and their variable effectiveness, might also suggest their potential contribution in the case of different external stresses (pH, salinity, redox and hypoxic conditions, etc.).

A variety of proteins have been shown to possess the ability to assemble into fibrils with amyloid characteristics, and at least 40 different proteins can form amyloid in humans, each protein associated with a specific disease [2,3]. BRICHOS domain (especially in humans) is known to prevent the auto-aggregation of β-hairpin molecules into toxic amyloid structures [4]. The structural similarity of preproalvinellacin BRICHOS domain with other BRICHOS containing proteins suggested that it might possess a chaperone-like function that decreases amyloid cytotoxicity by inhibiting fibril formation [10,20]. Apparently, the BRICHOS domain from the Pompeii worm has no chaperone function: specifically, the three tested variants did not prevent neither reduce auto-aggregation of Aβ42 protein, as showed by C09F5.1 BRICHOS domain from *C. elegans* [1]. Despite the low consensus between the two worms BRICHOS sequences, they seem to exert similar function.

These interesting findings suggest deeper investigations on functions and mechanisms of invertebrates BRICHOS domains. In particular, because the peptides belonging to BRICHOS-AMP family come from distinct habitats and present different structures, they can be used as model to study the evolution of the domains in changing environments, not excluding potential different properties.

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GENERAL DISCUSSION

External immunity, an extended arm of the immune system, is the first defence response of metazoans to face pathogens by manipulating the surrounding microbial communities in order to avoid infection and also to establish symbiosis [1]. Many organisms including plants, invertebrates, vertebrates and, even bacteria, secrete antimicrobial peptides (AMPs) as an extrinsic protective shield against the surrounding biota that is usually rich in potentially pathogenic agents [2–4]. 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 [5,6]. 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) [7,8]. 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 [9]. 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 [10]. 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 [11,12]. 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 [7,13].

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 [14].

In this context, the principal aim of this thesis was to increase the current knowledge on AMPs from extreme marine worms, focusing on Nematodes and Annelids.

More specifically, the abundance (representing the dominant benthic fauna in terms of biomass and species richness) and wide distribution of these worms in terrestrial, freshwater, and marine habitats (including extreme ones), in coexistence and coevolution with a large number and variety of microbes, make them perfect models for studying their immune systems through ecoimmunology [15,16]. To date, several studies demonstrated that many worms have evolved a variety of physical and chemical defence mechanisms, for instance antimicrobial metabolites [17–23]. Mostly, they present no shell or a proper exoskeleton (their body is directly exposed to the environmental constraints), hence their antimicrobial secretions represent an extended arm of the immune system (external immune defence) [1]. Moreover, they occupy a key position in the trophic network, as a major food source for fishes, birds and terrestrial fauna.

The combination of these features makes annelids and nematodes an interesting subject to study the evolution of immune system genes (such as AMPs) in conjunction with the abiotic and biotic variations of the environments [11,12].

To date, about 75% of the AMPs investigated come from the animal kingdom, only two percent have been characterized and identified from marine organisms (except nematodes), suggesting that we may be facing the sheer tip of the iceberg of potential new compounds (Antimicrobial Peptides Database APD3, [24]). After the discovery in 1989 of cecropin P1 [25], the first nematode AMPs (from the parasite *Ascaris suum*), efforts were mostly focused on the terrestrial genetic model *Caenorhabditis elegans* [26–28]. Later, several groups of AMPs were identified in nematodes: defensin-like antibacterial factors (ABFs), caenopores, caenacins (CNCs) and neuropeptide-like (NLPs) [29–36] (reviewed in [18]).

By contrast to nematodes, most annelid AMPs were biochemically isolated from diverse wild species from different taxa. The first annelid AMP was lumbricin-1 isolated from the earthworm *Lumbricus rubellus* in 1998 and later in leeches [37]. In 2004, the first member of the macin family (theromacin) was characterized in leeches [23]. Another family of AMPs characterized in annelids is the cysteine-rich BRICHOS family [38]; the first member was arenicin isolated from the body fluid of *Arenicola marina*, followed by other marine polychaetes [19,22,39]. Therefore, it seems reasonable to assume that both marine nematodes and annelids are interesting and potential source of still undiscovered bioactive substances, such as AMPs.

In this thesis, we firstly focused on searching for new active molecules from extreme marine worms, subsequently we analysed the adaptation of the members of BRICHOS-AMP family to biotic and abiotic environmental factors and finally we investigated on the roles of BRICHOS domain from preproalvinellacin.

Our first attempt was to fill the lack of data about AMPs from marine nematodes. Numerous families of AMPs were identified in terrestrial nematodes species (mostly *Caenorhabditis* genus); furthermore, many unique and potent AMPs were recently characterized from marine annelids (such as arenicins, alvinellacin, hedistin, perinerin, nicomicins, etc.) [18,19,22,39]. Using the protocol already applied for the identification of new AMPs from invertebrates and selecting extreme environments (known for the abundant presence of nematodes), we found two novel species (belonging to the *Oncholaimus* genus) producing uncharacterized compounds (probably AMPs) with promising bioactivities. We encountered many problems related to the amount of material, which did not lead us to the final identification of the active molecules.

Concerning AMPs from marine annelids, we found a novel antimicrobial peptide from an undescribed terebellid polychaete belonging to the genus *Amphitritides* inhabiting polar habitat (Antarctica). Expecting high similarities with already known AMPs from marine polychaetes (alvinellacins, arenicins, capitellacin and nicomicins), we used the "in silico approach", blasting the sequence of preproalvinellacin [19] to *Amphitritides sp.* genome. The new AMP, polaricin is processed from a larger precursor molecule containing the BRICHOS domain, prepropolaricin, being part of BRICHOS-AMP family [19,22,39]. BRICHOS-AMPs markedly differ in primary structures while exhibit highly conserved precursor sequence (especially the BRICHOS region). Therefore, they (coming from annelids living varying and distant habitats) are a remarkably attractive model to study their evolution, as actors of worm's immune defence in extreme and fluctuating environmental conditions.

We investigated on the influence of the external factors of the environment on three members of this family, living in highly distinct habitats (polar, temperate and hot chimneys of hydrothermal vents) [19,22,39]. By combining analyses of structure, antimicrobial and time-killing assays, as well as thermo- and pH-stability assays, we demonstrated the biochemical adaptation of AMPs not only to the typical bacterial communities but also to abiotic constrains of the environments. In addition, we showed the key role of disulfide bridges in peptides stability, in the case of thermal and acid/basic conditions.

Finally, we examined the functions of three BRICHOS domain variants from preproalvinellacin: we focused especially on its common role of molecular chaperone, already widely shown in mammals and on the unique link with AMPs [40]. Our results clearly highlighted that the variants had no chaperone function in decreasing protein Aβ42 aggregation (unlike those of other proteins containing this domain), nor synergy in the antimicrobial activity of the AMPs, but they acted by assisting bioactivities of AMPs in case of thermal stress.

1. New AMPs from marine nematodes

A growing interest in research is currently 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 [19,41,42].

In the first chapter, we showed a preliminary investigation on crude extracts from extreme marine nematodes, showing clearly the presence of small molecules with antibacterial properties. These 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 [18]. 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) [41]. In sulfide-rich black mud, marine organisms are permanently in close contact with very high densities of microbes [16]: 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 [43,44].

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.

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 [19,21,45]. The AMP from the extreme Pompeii worm was patented for its potential use

in human antibiotherapy. The same procedure of AMP purification than the one used for annelids was then applied to the three species of Oncholaimidae presented here. The research of new AMPs, with unique and novel motifs requires the bioassay-guided approach [9], not existing genetic database or transcriptome of these new species for the “in silico” approach.

Because they inhabit hostile habitat (sulfide rich, reduced and anoxic 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 [19]. Oncholaimidae being described as major constituent of the biomass of the meiofauna at hydrothermal vent sites [46], 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).

Unfortunately, we did not reach the identification and characterization of the compounds, due to insufficient amount of worms. 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 [47–49] while other species such as the marine annelid *Capitella* sp. known to be an opportunistic species [50], inferred to habitats enriched in sulfides was observed at 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., 2021, 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. Besides antimicrobials produced by marine organisms, it has been 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 [43]. To date, the biological role of immune molecules in marine host-symbiont association is a burgeoning field [51,52]. 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* [19,53]. Therefore, we hypothesised the unexpected lack of antimicrobial activity in *M. albidus* as a result of a too low amount of biological material available.

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 research 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 too 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 species that was relatively abundant in 2016 was then planned the following year without any success. To date, there is no description of AMPs of this molecular weight in nematodes and in crustaceans, the other mayor group of Ecdysozoa inhabiting the sea. Among the invertebrates including marine organisms, small sized AMPs (around 10 amino acids) have been characterized in Molluscs, Annelids and in 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*) [54–56].

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 molecules by bioassay-guided purification.

The work carried out in the first chapter, provides for the first time the evidence of marine nematodes as source of natural antimicrobial agents, probably different from their terrestrial counterparts (based on their size) and potentially possessing novel patterns. The diversity of the marine environments has provided an enormous genetic and biological diversity in nematodes, and although the difficulties faced in detection and identification of AMPs, the research for new drugs candidates should continue along all possible strategies, especially in periods of medical crisis.

2. Polaricin, a new member of BRICHOS-AMP family

Marine AMPs were found to be structurally different from their counterparts produced by terrestrial species, usually displaying novel structures, taxa-specific or even species-specific [57]. Aggressive environmental pressures, as abundance of pathogen microorganisms and hostile factors (extreme and/or varying temperature, pH and salinity values, pressure, etc.) have probably driven the evolution of the physiological adaptation of all marine organisms, including on their immune functions [7].

Using “in silico approach” and blasting the sequence of preproalvinellacin [19] to *Amphitritides sp.* genome, we identified a novel putative AMP from the polar marine polychaetes, named polaricin.

The precursor of polaricin includes the BRICHOS domain, such as other AMPs from annelids (alvinellacin, arenicins, capitellacin and nicomicins) [19,22,39]. From a structural point of view, in contrast to the other member of BRICHOS-AMP family, prepropolaricin lacks a typical signal sequence (likely some members of interleukin family), although it presents many hydrophobic residues in N-terminal position that could allow peptide sorting across the membrane of the endoplasmic reticulum.

Polaricin shares the following main characteristics with the other member of BRICHOS-AMP family: short amino acid sequence (19 residues) containing cysteine residue (Cys10), positive net charge (+5) and amphipathic nature. Alignment studies between the member of this family, showed low consensus (less than 20% of identity) between AMP parts and higher identity between the domain parts (30-40% of identity). These data, suggest a common origin of the precursor molecule with a divergent evolution of the AMP part.

Performing a UPLC-MS (Ultra Performance Liquid Chromatography tandem Mass Spectrometry) analysis, we showed that the presence of a unique cysteine residue in the polaricin sequence, do not allow the formation of intramolecular disulfide bridge (like showed by the other BRICHOS-AMPs).). This finding would also result in a different three-dimensional organisation, resulting in a different structure from the more recurrent β-sheet, as evidenced before only by nicomicins [38,39]. Therefore, the putative estimated three-dimensional structures of polaricin displayed a combination of extended and α-helix organization, excluding the β-hairpin conformation. Until now, only nicomicins (another polar peptide)

showed a similar and novel scaffold (combining an α-helix and an extended part) among this family of AMPs.

Moreover, from our investigations, it seems apparent that two molecules of polaricin arrange to form dimers.

Although less effective than other family members against the bacteria tested, polaricin displayed antimicrobial activity against many Gram-negative marine bacteria (*Vibrio alginolyticus*, *V. fluvialis*, *Pseudomonas sp.* and *Oceanisphaere sp.*) [41].

From an ecologic point of view, the member of BRICHOS-AMPs family represent an unique model to study peptide (and BRICHOS) evolution in face of different extreme environments, as actor of worm's immune defence.

3. Local adaptation of BRICHOS-AMP family

- Biotic constraint

The discovery of a new member of BRICHOS-AMP family could help the understanding of the evolutionary mechanisms responsible for their structural and functional diversities. We hypothesized that biotic and abiotic pressures of various environments participate in the shaping of AMPs (as component of worms external immunity) [1]. Arenicin, alvinellacin and polaricin, come from worms inhabiting distant and diverse habitat: temperate-coastal, hot-vent deep-sea and polar-coastal environments respectively. Their biological activities were tested using many marine bacterial strains, typical of temperate-coastal (*Vibrio alginolyticus*, *V. fluvialis*, *Shewanella algae*, *Oceanisphaera sp.*) and hot-vent deep-sea environments (*V. diabolicus* and *Pseudomonas sp.*). As expected, the peptides were more effective in killing their local bacterial communities and vice versa with exogenous strains.

Until now, two popular models of co-evolutionary dynamics between host and pathogen genes have been proposed: the "arms race" and the "Red Queen" models [58]. These two types of coevolution have radically different consequences on hosts' immune defense genes (such as AMPs). Arms races lead to a rapid evolution of the genes involved, with a larger amount of amino acid replacements between the species but low intra-species polymorphism in the regions tightly linked to the selected sites [58,59]. Differently, the Red Queen model results in balanced polymorphisms with deep coalescence times, promoting recombination

and sexual reproduction [58,59]. From our findings, BRICHOS-AMPs seem to fit the arms race model: we found a high rate of polymorphism between the peptides of the different species (low identity percentages between their amino acid sequences), probably caused by the different pathogens that the hosts have to face (to which they are well adapted).

- Abiotic constraint

Assuming that the effectivity of external immunity should include the correct functioning of AMPs under the stressors of the external environment, we investigate the biological activities of AMPs in the case of thermal and pH variations. For the first time, we showed that abiotic constraints of a habitat selected AMPs perfectly adapted to exert their biological functions in the range (thermal and pH) of their external environment.

Alvinella pompejana inhabits active deep-sea hydrothermal edifices, characterised by hot and acid fluid emission, that suddenly comes out of the chimneys [60–64]. In our study, alvinellacin displayed its antimicrobial activity in the tested thermal and pH-range (4°C to 90°C; pH 4 to pH 10), typical of its own environment.

Similarly, arenicin showed high stability to temperature and pH variations (for a shorter period of exposure than alvinellacin), being *Arenicola marina* continuously exposed to physico-chemical variation, typical of the temperate near-shore (intertidal) sediments (such as -5°C to 25°C and pH 5.6-8.2) [65–72].

As for polaricin, the Antarctic coastal waters are extremely cold, with very small fluctuation of temperature and pH (about 0°C; pH 8) [73–76]: the effect of high temperature and acid/basic conditions on peptide caused respectively the loss and/or decrease of its activities.

Structurally the three AMPs differ for the number of disulfide bridges, which increases with the harshness of the environment to which the worm species are exposed to, and we demonstrated here that this structure is indispensable for peptides stability under harsh conditions (especially in the case of high temperature). It can be assumed that the evolution, through the forcing of the external environment, has selected structural solutions (such as disulfide bridges) that guarantee the AMPs to exercise their biological functions in the conditions to which they can be subjected by their habitat itself.

Recent studies on β-sheet peptides, such as a group of variants of NZ17074 (variant of arenicin-3, isolated from *A. marina*), showed similar results about the involvement of disulfide bridges in the thermal- and pH-stability of the peptides (in the ranges 20-80 °C and pH 4.0 to

10.0) [77–82]. Also AcAMP, a 51-aa cysteine-rich antimicrobial peptide from *Aspergillus clavatus* ES1, was stable between pH 5.0 and 10.0, and heat resistant (15 min at 100°C) [83,84]. The heat and pH stability and the wide spectrum of activity are very rare and useful characteristics for the potential application of BRICHOS-AMPs (such as food preservative, biological control of plant diseases, etc.).

About polaricin and ABU-polaricin, we assumed that the ABU-variant benefited in terms of thermostability and antimicrobial activity, as a result of the lack of the cysteine residue which avoids the formation of dimers. To date, the studies of dimeric forms of bioactive peptide (like magainins and histatin) sequences have shown advantages in enhanced antimicrobial potency and resistance to proteases (reviewed in Lorenzon et al., 2019 [85]). However, other works have shown that the dimerization decreases the antimicrobial activity of cationic AMPs such as aureins and melittin, changing peptides mechanism of action [86]. Otherwise, it has been shown that dimerized arenicin exhibited significantly reduced cytotoxicity but similar antibacterial activities compared to its native AMP [87].

4. Preproalvinellacin as model system for studying BRICHOS domain roles and its interactions with the AMP

Environmental stresses, fluctuations in physiological and environmental conditions can cause proteins denaturation of the exposed organism. The resulting accumulation, self-assembling and misfolding of these denatured proteins into large aggregates has harmful consequences for organisms [88]. In humans, these aggregates are associated with about 40 human diseases, including Alzheimer and Parkinson diseases, cancer, diabetes, etc [89,90].

Molecular chaperones are essential for cellular homeostasis to prevent misfolded and aggregated proteins. There are many different types and mechanisms of molecular chaperones promoting the correct folding of proteins, BRICHOS has been proposed to assist its respective precursor protein to fold correctly during biosynthesis, preventing them from auto-aggregation (having a high propensity to form β-sheet structures) [91,92].

The BRICHOS domain (approximately 100 amino acids) is present in more than 300 proteins of 12 distantly related families, mainly linked to major diseases, such as British and Danish dementia, cancer and respiratory distress syndrome [40,92]. While it was found in a wide range of organisms, the functional properties of the BRICHOS domain has not been fully

explored (especially in invertebrate). Three functions of BRICHOS domain-containing proteins have been proposed and proven in mammals: intramolecular chaperone-like function, promotion of targeting and secretion and assistance with specialized intracellular protease activity. While it has various disease associations, the functional properties of the BRICHOS domain has not been fully explored, especially in invertebrates.

Two BRICHOS domain-containing proteins have been identified in *C. elegans*: C09F5.1, a nematode-specific gene without a human homologue and C25F6.7, a homologue of human ITM2B that inhibits amyloid formation [93]. C09F5.1, unlike most BRICHOS domain-containing protein, did not exhibit a chaperone function and its expression was induced by heat shock with likely involvement in temperature stress response [88].

As for annelids, the BRICHOS domain has been identified in many marine polychaeta (considered as the primitive annelids), but not functionally described [19,22,39]. It is the only case of a BRICHOS domain in an AMP precursor. We used preproalvinellacin, the precursor of alvinellacin from the Pompeii worm containing the BRICHOS domain, as model to study the roles of the domain.

From our findings, the BRICHOS region and the AMP part of preproalvinellacin were both detected in Pompeii worm extracellular plasma, exposed to the harsh hydrothermal vent conditions. We hypothesized a function of BRICHOS in stabilization of alvinellacin in the context of variable abiotic (especially thermal) factors. Recently, in a similar way, it had been shown the localization of C09F5.1 BRICHOS domain-containing protein identified in *Caenorhabditis elegans* and displaying a role in thermotolerance [88].

Overall, we demonstrate the key role of alvinellacin BRICHOS domain in the assistance to the biological functions of AMPs in the case of heat stress.

From our preliminary results, it appears that the variants possess a peptides specificity, as if the mechanism relied on amino acid sequence patterns. The presence of various variants and their variable effectiveness, might also suggest their potential contribution in the case of different external stresses (pH, salinity, redox and hypoxic conditions, etc.). The structural similarity of preproalvinellacin BRICHOS domain with other BRICHOS containing proteins suggested that it might possess a chaperone-like function that decreases amyloid cytotoxicity by inhibiting fibril formation [91,94]. Apparently, the BRICHOS domain from the Pompeii worm has no chaperone function: specifically, the three tested variants did not prevent neither

reduce auto-aggregation of Aβ42 protein, as showed by C09F5.1 BRICHOS domain from *C. elegans* [88].

These interesting findings suggest deeper investigations on functions and mechanisms of invertebrates BRICHOS domains. In particular, because the peptides belonging to BRICHOS-AMP family come from distinct habitats and present different structures, they can be used as model to study the evolution of the domains in changing environments, not excluding potential different properties.

CONCLUSIONS AND PERSPECTIVES

Marine invertebrates are recognized as a rich source of promising antibiotic substances, in particular AMPs. Living in very exigent, competitive, and aggressive environments (very different from the terrestrial), these organisms produce a wide range of specific and potent active molecules. Some of them and/or their derivatives are already in different phases of the clinical and preclinical process as potential new drugs [12,95].

In this thesis, we focused on marine worms (as nematodes and annelids) living extreme environments. Their success in colonizing the harshest habitats of the sea, with a wide species diversity and in coevolution with several pathogens, suggest that they possess the credentials to produce highly effective antimicrobial substances.

To date, no AMPs have been identified by marine nematodes whereas many potent AMPs from marine annelids have displayed novel and original structures (such as arenicins, alvinellacin, hedistin, nicomicins and perinerin) [19–22,39].

The work of Chapter 1 is the first to provide an evidence that marine nematodes are an abundant source of antibacterials. Two out of three species investigated showed important activity against gram-positive and gram-negative bacterial strains. Although the difficulties encountered did not lead us the identification of the molecules, knowing their size (thanks to the mass spectrometry results) we can definitely affirm that such small substances have never been found until now in nematodes. Future works should attempt to identify and characterize these molecules, possibly thanks to other massive sampling, not excluding more efforts in animals breeding.

More generally, following the encouraging results of this work, further investigations on antibiotic substances from marine nematodes are needed.

The importance of identifying AMPs from marine nematodes also lies in the addition of an important piece to the question of the invertebrates peptides evolution in marine environment and their adaptation to the extreme constraints of the habitat, remembering their putative unique features in terms of future applications.

These subjects were mainly the objects of Chapter 2, where the effects of biotic and abiotic varying conditions of extreme environments were evaluated on three annelid BRICHOS-AMPs. More precisely, we demonstrated that the biological activities of these three peptides are specific against the bacterial strains (biotic constraint) they typically have contact with, living

in the same worm environment. A rapid and effortless elimination (represented respectively by AMPs fast killing and low MIC values) of bacteria within their own habitat, means that the peptides are adapted to their local bacterial communities. In reciprocal transplant experiments the “home vs away” pattern was confirmed: each locally adapted AMP in its native site has higher fitness than any other population in the same site and vice versa. The diagram presented must be completed by using endemic polar bacteria, despite the difficulties in using these strains in terms of their maintenance.

Regarding the abiotic constraints, we took in consideration temperature and pH, demonstrating for the first time that the AMPs are adapted to the external constraints of their own environment. More specifically, worm habitat selected AMPs perfectly adapted to exert their biological functions in the range (thermal and pH) of the external environment.

These findings suggest that the peptides are structurally adapted to these constraints: we observed that the presence and the number of disulfide bridges could play a key role in AMPs stability. Using AMPs analogues, devoid of disulfide bridges, we demonstrated our hypothesis. It would be interesting to evaluate the mode of action of native peptides and their variants, under the different conditions imposed by the external environments biotic (bacterial communities) and abiotic (temperature, pH, salinity, etc.), with and without BRICHOS domain. Polaricin predicted three-dimensional structure (combining α-helix with an extended part) should be confirmed by NMR spectroscopy. In parallel, we suggest the determination of AMP structures by NMR spectroscopy under the various conditions listed above to understand the impact, the role and mechanisms of external constraints on peptides molecular organization. Deeper investigation on BRICHOS domain involvements in AMPs stability are needed.

Potential of BRICHOS domains from annelids AMP precursors

The encouraging results obtained using the BRICHOS variants from preproalvinellacin, in assisting the biological activities of the peptides under thermal stress, drive further investigation in that direction (external constraints). The massive production of these three variants would allow a wider analysis of the conditions under which the domain protects the molecules, not considering only the thermal factor: it has been shown that changes in pH, salinity, redox, etc., have an effect on the antimicrobial activity of the peptides. Being the

Pompeii worm in close contact with this kind of environmental stressors, we propose to extend the investigations, starting from the tolerance ranges of the worms.

Moreover, we observed slight differences in performance between the three tested variants. Remembering that our group identified 17 variants of BRICHOS from preproalvinellacin, it would be interesting to know whether they are specific to different environmental factors [96]. The method consisting in the evaluation of AMP biological activity under different stress conditions, with and without the chaperone domain, could be easily used (as it has already been performed for thermostability and pH-stability assays).

Additionally, we noticed that the variants have a different response by varying the peptides: they should be tested extending the survey to various molecules (for example with agro-alimentary, pharmaceutical applications) to improve their resistance to similar stressors (temperature, pH, salinity, etc.).

Our investigations, did not include the mechanism(s) of action of the domain in the peptides protection.

The finding of BRICHOS domain secretion and its like-chaperone function with AMPs, does not exclude its putative role within the cell. The intracellular functions of the domain could be examined, expressing it with GFP (green fluorescent protein) inside eukaryotic cells and following it inside the cells, eventually evaluating the possibility to expose the used cells to various stress (temperature, pH, etc.).

Because BRICHOS domain is typical of this family of AMPs, our data encourage producing and looking into the counterparts from arenicins and polaricin. Coming from distinct environments and acting with structurally different peptides, we expect they possess potential different properties.

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ANNEXES

ANNEXE 1. *Environmental and Benthic Community Patterns of the Shallow Hydrothermal Area of Secca Delle Fumose (Baia, Naples, Italy)*

Authors: Luigia Donnarumma, Luca Appolloni, Elena Chianese, Renato Bruno, Elisa Baldrighi, Rosanna Guglielmo, Giovanni F. Russo, Daniela Zeppilli and Roberto Sandulli.

Published: 08 November 2019, in *Frontiers in Marine Science.*

Keywords: shallow hydrothermal vents; interstitial water; sediments; biodiversity; Mediterranean Sea

Abstract: The occurrence of hydrothermal vent ecosystems at Secca delle Fumose, Pozzuoli Bay (Gulf of Naples), represented an opportunity to study the benthic assemblages under the thermal stress of hydrothermal emissions in a very shallow environment (9–14 m water depth). In autumn 2016, the macrobenthic community was sampled by scuba divers at four sites located in the Baia Underwater Archeological Park. Two sites were characterized by vent emissions (one with white bacterial mat scattered on the bottom and one with a yellow substrate around a geyser opening) and two at about 100 m away, used as control. Sediment and interstitial water environmental variables were measured to determine their influence on the structure of macrobenthic assemblages. A total of 1,954 macrofaunal individuals was found, characterized by great differences in abundance and species richness among sites. This pattern was correlated to the dominance of a particular set of variables that drastically change in a very small spatial scale, from one site to another. The control sites, characterized by the highest percentage of gravel in the sediments (19.67 ± 2.6%) and normal level of major ions such as Ca^{2+}, K^{+}, and Mg^{2+} in the interstitial waters, showed the highest values of sinecological indices. The “white” hydrothermal site exhibited the lowest species richness, abundance and species diversity, influenced by low pH values (∼7.6), high temperatures (∼37.53°C) and by the highest total organic carbon content (TOC 34.78%) in the sediment. The “yellow” hydrothermal site, with sediment TOC equal to 30.03% and interstitial sulfide ions measuring 130.58 *ppm*, showed higher values of sinecological indices than those recorded at the “white” site. Therefore, taxonomic analysis revealed a high turnover between control and vents sites. This highlights the preference for hydrothermal vents by a few resistant species, such as the gastropod *Tritia cuvierii* and the polychaete *Capitella capitata*, confirming the role of the latter species as opportunistic in extreme environments like Secca delle Fumose.

INTRODUCTION

The relationship between environmental factors and benthic communities is of primary importance in determining the structure of biocenoses and their functioning (e.g., Feder et al., 1994; Ellingsen, 2002; Lloret and Marín, 2009; Arribas et al., 2014). A huge variety of marine species and bioconstructions is widely distributed along the bathymetrical gradient, from very shallow to deep-water. Among extreme marine systems, hydrothermal vents have wide global distribution, occurring in all oceanic bottoms, at different latitudes and depths and harboring rich and peculiar biological communities (Parson et al., 1995; Dando et al., 2000). Several studies revealed that the occurrence of benthic organisms in the hydrothermal systems is strongly related to the volcanic fluids that outflow from the bottom, characterized by high concentrations of iron, zinc sulfides and gases, such as CH4, H2S, H2, and CO2 (e.g., Van Dover and Fry, 1989; Micheli et al., 2002; Hall-Spencer et al., 2008; Martin et al., 2008; Yao et al., 2010). A great biological difference occurs between deep and shallow vents. The former are mainly characterized by chemolithotrophic bacteria using H2S as energy source, representing the basis for a complex heterotrophic ecosystem, while the latter are composed both by chemolithotrophic bacteria and by communities energetically driven by photosynthetic organisms, such as diatoms and algal-bacterial mats absent in deep-sea vents communities (Vismann, 1991; Lutz and Kennish, 1993; Tarasov et al., 2005; Raghukumar et al., 2008).

In the Mediterranean Sea, very shallow hydrothermal vents were reported for the Tyrrhenian and Aegean Sea, ranging from few meters to about 30 m depth (Dando et al., 1999). They are related to tectonically active coastal zones, where the volcanic fluids are characterized by high temperatures and mainly composed by sulfide and/or CO2 (Dando et al., 2000). Even though shallow benthic communities include tolerant species to natural or anthropogenic stressors, many studies have suggested that the increasing temperature and sulfides negatively affect a wide variety of benthic assemblages, reducing the water oxygen concentration and producing toxicity to the majority of aerobic species (Caldwell, 1975; Wang and Chapman, 1999; Vaquer-Sunyer and Duarte, 2010, 2011). Similarly, natural CO2 emissions in seawater produce a change in carbonate chemistry, resulting in a local seawater acidification, which, in turn, impacts on calcification and growth processes of many planktic and benthic species (Fabry et al., 2008; Doney et al., 2009; Wicks and Roberts, 2012).

Volcanic emissions, rich in CO2, were previously reported by Hall-Spencer et al. (2008) for the cold vents of Ischia Island, in the Gulf of Naples (Italy). Here, sulfides are absent while the high percentage of CO2 (~90–95%) considerably reduces the seawater pH which negatively affects calcifying organisms (Cigliano et al., 2010; Donnarumma et al., 2014; Lucey et al., 2016; Teixidó et al., 2018).

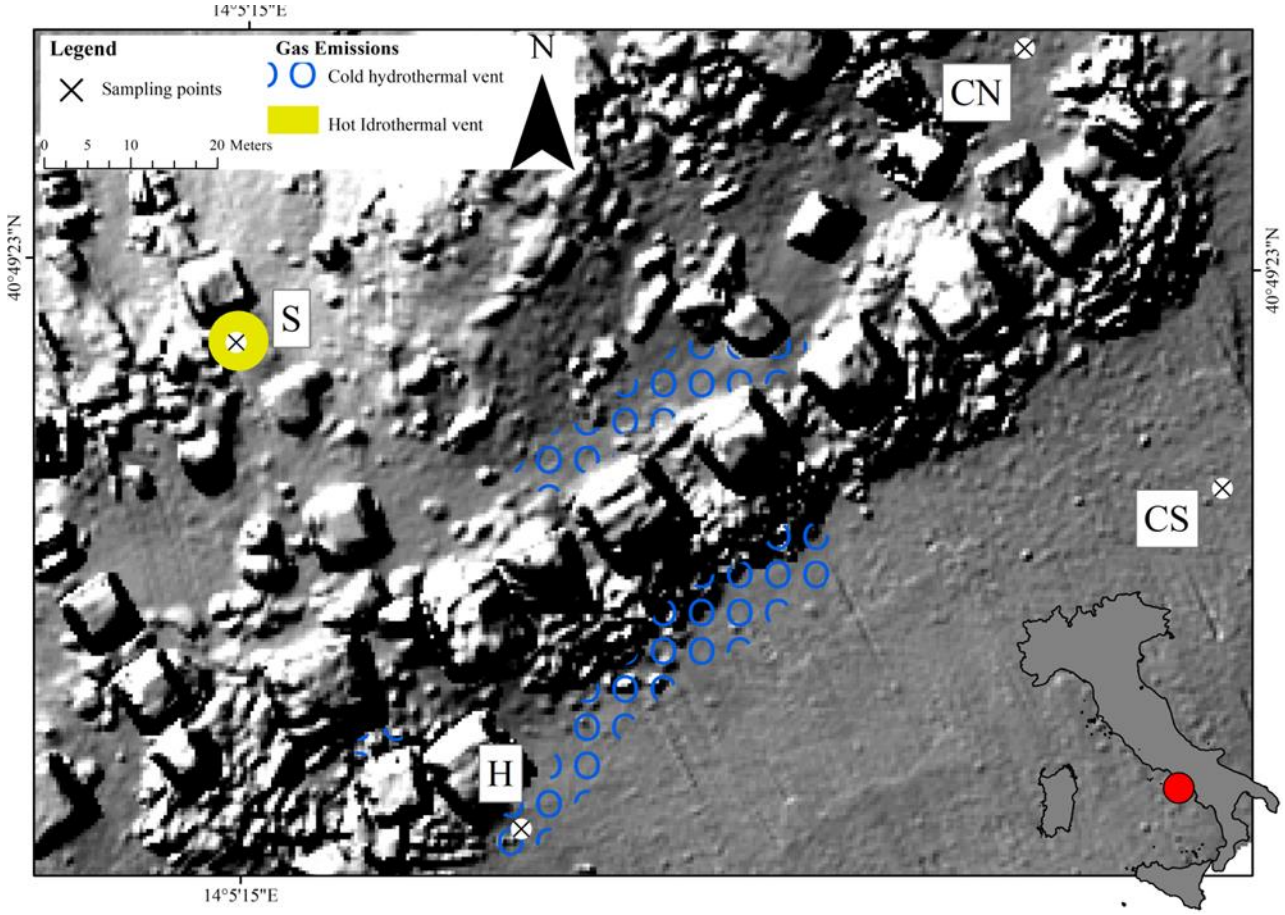

Figure 1: Study area showing the sampling sites (H – White hydrothermal vent; G – Geyser; CN - Control North; CS - Control South) around the Roman pillars at Secca delle Fumose.

A few kilometers away from Ischia Island, in the Campi Flegrei caldera, Di Napoli et al. (2016) reported a remarkable variation in seawater pH (~7.3–8.3) due to the gas-rich hydrothermal fluids occurring in a shallow submarine relief, namely Secca delle Fumose. This area was only recently investigated from a geological (e.g., Tedesco et al., 1990; Passaro et al., 2013) and microbiological (Maugeri et al., 2010), point of view. High-resolution morpho-bathymetric data and archeological surveys indicate that the relief is largely anthropogenic, consisting of a dense aggregation of pillars of the Roman age (first century BC), with a perimeter of 9 m × 9 m and a height of 7 m, mostly standing on a seafloor at 12 m depth where hydrothermal vents occur. Since there is a lack of information concerning the other biological and ecological components, the aims of this paper are: (i) to evaluate the spatial variation of macrobenthic

community at the Secca delle Fumose, (ii) to assess the most important abiotic parameters affecting soft-bottom assemblage structure and (iii) to estimate the taxonomic diversity among hydrothermal vents and non-vent sites. To our knowledge, this study represents the first investigation of macrobenthic assemblages inhabiting the shallow hydrothermal systems of Campi Flegrei.

MATERIALS AND METHODS

Site Description

"Secca delle Fumose" (SdF) belongs to the largest degassing structure offshore of the Campi Flegrei caldera. With an extension of approximately 0.14 km2, is located about 800 m off the coastline in the north-western part of Pozzuoli Bay (Gulf of Naples, Italy) (40°49'23 ''N 14°05'15''E) (Tedesco et al., 1990; Passaro et al., 2013; Di Napoli et al., 2016). In this area, four sampling sites were selected (Figure 1), two control sites (CN; CS) and two in proximity of very different vents: a first characterized by white bacterial mats (H) and a second by yellow substrate around a solitary geyser opening (G). The control sites were distant about 65 m each other and 100 m away, in the hydrothermal area, the other two sites were sampled at the same distance from each other.

Sampling Collection and Analytical Procedures

In each site (H, G, CN, and CS) in November 2016, environmental parameters were measured (temperature and pH) and samples for interstitial water chemistry, grain size, total organic carbon (TOC) and sediment macrofauna were collected in triplicate.

Sediment temperature was measured in situ by means of an underwater thermometer. Water samples at water/sediment interface were collected for pH evaluation (pH/ORP Meter, HI98171, and probe HI 1230, Hanna instr.). Interstitial water (20 ml) for ions and metals determination was sampled using syringes and kept frozen until analyses; sediment samples were collected for the grain size and TOC analysis, by means of a cylindrical corer (5.5 cm diameter) pushed 10 cm into the sediment.

In the laboratory, interstitial water was filtered with cellulose filters (0.20 µm) and treated with H2O2 (100 µl in 10 ml of sample) for the digestion of organic content; samples were then fractioned in two aliquots for ions and metals determination.

For the analysis of major ions concentration, interstitial water samples were analyzed through ICS1100 ion chromatographic system, equipped with a double column system for simultaneous analyses of both anions and cations (Chianese et al., 2019); anions were detected with an AS22 column working with a cell volume of 100 µl and a solution 3.5 mM of sodium carbonate/bicarbonate as eluent, while cations were determined with a CS12A column working with a cell volume of 25 µl and 20 mM methanesulfonic acid solution as eluent. For both anions and cations, calibration curves were calculated using certified multistandard solutions; anions and cations detectable with this method are respectively: Cl–, F–, Br–, NO−2, NO−3, PO3−4, SO2−4 (as inorganic species), HCOO–, CH3COO–, C2O−24 (as organic species) and Li+, Na+, K+, NH+4, Ca2+, and Mg2+. In addition the S2– ion was estimated using a chromatographic method, converting it in sulfate ion after oxidation with H2O2. Heavy metals (Pb, Cd, Cu, and Zn) were estimated by means of a polarographic method, with a Metrohm 797 VA Computrace; this system uses a multimode working Mercury electrode and an Ag/AgCl electrode as reference. Using this method, metals that are soluble in mercury such as zinc, cadmium, lead, and copper are simultaneously determinable (Chianese et al., 2019). Also in this case, calibration curves were calculated using certified multistandard solutions.

For the grain size analysis (Eleftheriou and McIntyre, 2008), sediment was sieved over a series of 11 sieves with mesh size ranging from 1 cm to 63 µm. Fractions were dried in oven at 60°C for 48 h and weighed; data were expressed as percentages of the total sediment dry weight, differencing it in three size classes: gravel (>2 mm), sand (2 mm <Ø> 0.063 mm), and mud (<0.063 mm). TOC was determined according to Schumacher (2002) and expressed as% of sediment.

As for macrofauna community, samples were collected at each site by scuba-diving operators using an air-lift pump equipped with a 0.5 mm nylon mesh size bag (Benson, 1989; Chemello and Russo, 1997) within a 50 cm × 50 cm frame, reaching a depth of 10 cm in the sediment. In the laboratory, the samples were fixed in 70% ethanol and macrofauna was sorted and analyzed under a stereomicroscope. Macrofauna organisms were analyzed up to the lowest taxonomic level, when possible, and their identification was cross-checked with the World Register of Marine Species (WoRMS Editorial Board, 2018).

Data Analysis

Multivariate ordination by principal component analysis (PCA) was performed on normalized environmental variables in order to determine their distribution patterns among the four sampling sites.

Sinecological indices, such as number of individuals (N) per 25 dm3, species richness (SR), Shannon-Weaver diversity (H': log2) and Pielou's evenness (J) were calculated based on three replicate samples for each site. The quantitative (DI, percentage of individuals of a given species upon total individuals collected in the sample) and qualitative dominances (DQ, percentage of species of a given taxon upon the total of species collected in the sample) were also calculated. Differences of sinecological variable among sites were detected by permutational analysis of variance (PERMANOVA; Anderson, 2001a), based on Euclidean distance (Terlizzi et al., 2007). A one-way experimental design with n = 3 was involved with the fixed factor Site (four levels). PERMANOVA analysis, based on Bray–Curtis similarity, was also performed in order to assess differences in the structure of community assemblages among sites. 4999 permutations were always applied (Anderson, 2001b) and a PERMANOVA pairwise t-test was used in order to evaluate differences between pairs of sites. Prior to analysis, data were log (x + 1) transformed (Clarke and Warwick, 2001) in order to normalize the data. To examine the structural variation of benthic communities among sites, canonical analysis of principal coordinates (CAP; Anderson and Willis, 2003) was used and similarity percentage (SIMPER) was calculated among the replicates for each site and, then, it was applied to identify those species that contributed more to the similarity among sites. Only species that cumulatively contributed to 50% to similarity were considered.

Relationships between macrobenthic community composition and environmental variables were tested by distance-based linear modeling analysis routine (distLM, Anderson, 2004). The aim was to identify which variables were mostly related with assemblages and to better explain the biological pattern among sites. Then, distance based redundancy analysis (dbRDA, Legendre and Anderson, 1999) was used to visualize the influence of variables identified by distLM. For distLM analysis, interstitial water variables, expressed in ppm and ppb, were log10 transformed to better compare different scales (Underwood, 1997). All multivariate analyses were undertaken using the PRIMER-PERMANOVA + v.6 software package (Anderson et al., 2008).

Table 1 – Environmental condition of the study area. Data were expressed as percentages or averaged (±S.D.) among four replicates at each site (H; G; CN; CS).

VARIABLES		H	G	CN	CS
Sediment variables					
Temperatures (°C)		37.53±2.28	29.1±2.81	21.8	21.8
pH		7.56±0.05	8	8.1	8.1
TOC (%)		34.78	30.03	17.05	18.14
Gravel (%)		7.41	13.96	17.84	21.52
Sand (%)		90.12	83.40	79.60	76.67
Mud (%)		2.47	2.64	2.57	1.81
Interstitial water variables					
Ions (*ppm*)	**Na^+**	8668.260±4.5	9973.210±10.5	10776.925±12.5	11120.825±18.7
	Cl^-	19512.965±20.5	23726.385±15.7	26039.500±17.5	25976.560±18.6
	K^+	317.855±7.6	426.340±10.2	407.405±8.5	399.125±6.2
	Mg^{2+}	805.000±7.3	954.705±3.8	1219.475±8.8	1179.500±5.2
	Ca^{2+}	327.500±8.3	472.200±9.5	503.290±9.2	385.725±7.3
	NO_3^-	28.77±0.06	25.50±0.06	26.07±0.03	25.68±0.32
	SO_4^{2-}	3152.300±3.6	2658.500±6.5	3369.880±3.1	3888.500±5.9
	S^{2-}	n.d	130.58	n.d	n.d
Metals (*ppb*)	**Zn**	33.66±0.51	34.56±3.86	39.09 ±0.50	33.65±0.50
	Pb	62.02±0.16	31.29±0.52	18.31±0.60	62.02±0.16
	Cd	4.42±0.19	n.d.	n.d.	4.42±0.18
	Cu	n.d.	8.88±0.21	5.25±0.21	n.d.

n.d. data not detected

RESULTS

Environmental Variables

Environmental characteristics of SdF are summarized in Table 1. A solitary hydrothermal vent (geyser) at G site is present at 10 m depth. The vent opening was about 10 cm in diameter; the hydrothermal fluid temperature reaches ∼80°C at the outlet, while a lower temperature (29.1 ± 2.81°C) and a moderate pH value (8) occurred in the sediment at a distance of 20 cm from the vent center. Rocky substrate surrounding the geyser was covered by yellow sulfur deposits (Figure 2), while soft substrate among the rocks presented a TOC content of 30.03%. Interstitial water had a sulfur ion S2– concentration of 130.58 ppm. Here, the most abundant ions were sulfate (SO4)−2, with a concentration of 2658.500 ± 6.5 ppm, and Mg, with a concentration of 954.705 ± 3.8 ppm, furthermore, relevant values of metals such as Zn (34.56 ± 3.86 ppb) and Pb (31.29 ± 0.52 ppb) were detected. About 1 m from the vent, where

macrofauna was collected, the sediment was composed by sand (83.40%), gravel (13.96%), and mud (2.64%).

The H site was approximately 65 m from the G site, at a depth of 14 m. The sediment temperature was about 37.53 ± 2.28°C and the pH value 7.56 ± 0.05, indicating an acidified condition, where some gas bubbling occurred. This site was characterized by a soft bottom covered by a white microbial mat (Figure 3), with a TOC content of 34.78%. Sediment was mainly composed by sand (90.12%), gravel (7.41%) and mud (2.47%), while interstitial water showed the highest mean value of NO−3 (28.77 ± 0.06 ppm) respect to the other sites, and a high mean value of Pb (62.02 ± 0.16 ppb) among metals.

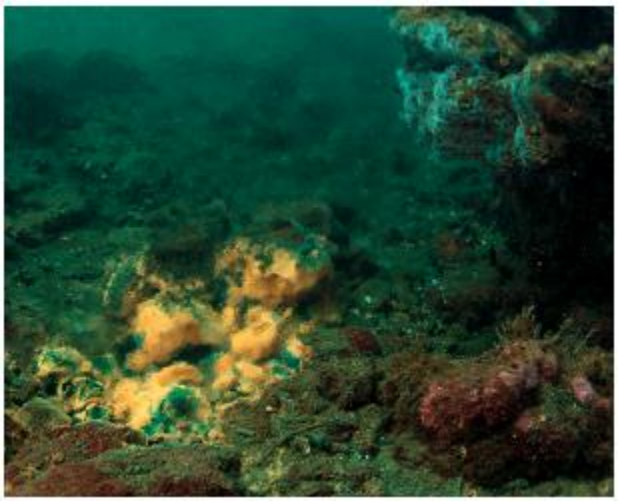

Figure 2: The hydrothermal gyser xith sourrounding rocky substrate covered by yellow sulfur deposit

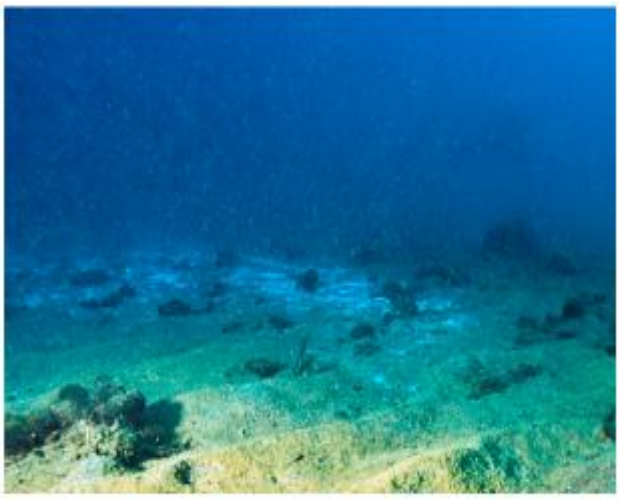

Figure 3: Hydrothermal site with oft bottom covered by white microbial mat

The two control sampling sites (CN and CS) were located respectively to the north and south of Roman pillars and 100 m from G and H at a depth of 9.8 and 12.1 m respectively. In these sites, the gas emissions and the white microbial mat were absent, while the sediment temperature of 21.8°C was comparable to that of sea water column; the pH values (average 8.1) were within the range of normal conditions. The content of the TOC in the sediment varied from 17.05 to 18.14%. The sediment grain size was characterized by a high percentage of sand (CN 79.60%; CS 76.67%) and a lower content of gravel (CN 17.84%; CS 21.52%) and mud (CN 2.57%; CS 1.81%). High concentrations of heavy metals occurred in the interstitial water in both sites: the CN site was mainly characterized by a Zn content of 39.09 ± 0.50 ppb,

while CS by Pb concentrations of 60.02 ± 0.71 ppb. This latter site presented also the highest mean value of (SO2)−4 (3888.500 ± 5.9 ppm).

The sediment quality characteristics from sampling sites were shown by multivariate PCA (Figure 4). In particular, PC1 accounted for 47.5% of variation among sites, and PC1 and PC2 together accounted for 84.5% (Table 2). Along the PC1 axis, hydrothermal vent sites (H and G) were separated from the control sites (CN and CS), according to temperature and ion NO−3 , that were high at the active sites; on the contrary other ions (e.g., Mg2+; Na+) and pH, were high at control sites (Figure 4 – PC1). Along the PC2 axis, the graph showed a clear separation between southern (H and CS) and northern (G and CN) sites; the former sites were displaced on the graph according to heavy metals content such as Cd and Pb and the SO2−4 ion; the latter sites according to Cu, S2– ion and% of mud (Figure 4 – PC2).

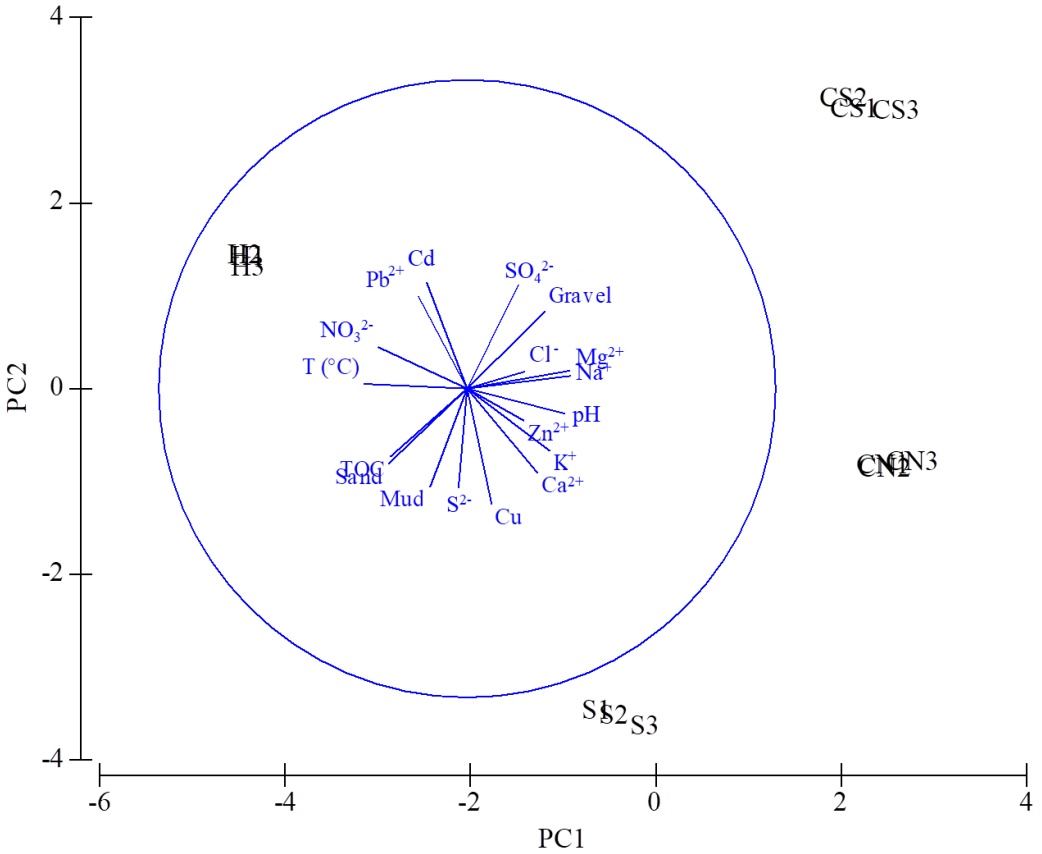

Figure 4: Ordination of environmental variables at the four stations of the Secca delle Fumose using PCA.

Table 2 - Principal component loadings for hydrothermal and control sites from PCA of environmental data from 4 sites sampled at the Secca delle Fumose hydrothermal zone. Bold values were considered high (≥|0.290|).

Environmental variables	PC1	PC2
pH	**0.317**	-0.081
T (°C)	**-0.335**	0.015
TOC (%)	-0.250	-0.221
Na^+	**0.337**	0.041
Cl^-	0.189	0.056
Ca^{2+}	0.229	-0.274
K^+	0.269	-0.202
Mg^{2+}	**0.333**	0.058
NO_3^{2-}	**-0.290**	0.135
SO_4^{2-}	0.167	**0.337**
S^{2-}	-0.028	**-0.322**
Zn	0.185	-0.104
Pb	-0.159	**0.299**
Cd	-0.132	**0.345**
Cu	0.080	**-0.376**
Gravel (%)	0.253	0.251
Sand (%)	-0.256	-0.244
Mud (%)	-0.120	**-0.319**

Table 3 – Taxonomic list of total fauna occurring at Secca delle Fumose, with abundance (N) of each taxon at each site (H – White hydrothermal vent; G – Geyser; CN - Control North; CS - Control South). Dashes indicate absence.

TAXA	H	S	CN	CS
Nemertea	-	-	5	-
Sipuncula	-	71	58	477
Polychaeta				
Amphictene auricoma (O.F. Müller, 1776)	-	-	1	-
Aphelochaeta marioni (Saint-Joseph, 1894)	-	-	7	-
Aphelochaeta multibranchis (Grube, 1863)	-	-	4	-
Aponuphis bilineata (Baird, 1870)	1	3	26	53
Capitella capitata (Fabricius, 1780)	4	-	-	-
Chrysopetalum debile (Grube, 1855)	-	-	1	1
Dialychone acustica Claparède, 1870	-	-	9	-
Diplocirrus glaucus (Malmgren, 1867)	-	-	1	-
Drilonereis filum (Claparède, 1868)	-	-	1	-
Eteone longa (Fabricius, 1780)	-	-	4	-
Euclymene oerstedi (Claparède, 1863)	-	-	5	-
Eulalia sp.	-	-	3	2
Eunice pennata (Müller, 1776)	-	-	-	45
Eunice vittata (Delle Chiaje, 1828)	-	11	81	16
Exogone sp.	-	-	3	-
Glycera unicornis Lamarck, 1818	-	-	12	2
Harmothoe longisetis (Grube, 1863)	-	2	2	3
Hesionidae indet.	-	-	7	-
Hydroides dianthus (Verrill, 1873)	-	1	-	-
Hydroides uncinata (Phillipi, 1844)	-	-	1	-
Laonice cirrata (M. Sars, 1851)	-	-	1	-
Lepidonotus clava (Montagu, 1808)	-	-	-	2
Levinsenia gracilis (Tauber, 1879)	-	-	7	-
Lumbrineris latreilli Audouin & Milne-Edwards, 1834	-	-	7	-
Lysidice unicornis (Grube, 1840)	-	4	4	20
Malmgrenia andreapolis McIntosh, 1874	-	-	3	-
Mysta picta (Quatrefages, 1866)	-	-	7	-
Neanthes kerguelensis (McIntosh, 1885)	-	-	-	5
Nereis rava Ehlers, 1868	-	6	14	-
Notomastus latericeus Sars, 1851	-	1	6	1
Owenia fusiformis Delle Chiaje, 1844	-	-	20	-
Perinereis cultrifera (Grube, 1840)	-	7	-	-
Phyllodoce lineata (Claparède, 1870)	-	-	5	-
Pista cristata (Müller, 1776)	-	-	18	-
Platynereis dumerilii (Audouin & Milne Edwards, 1833)	-	1	9	-
Pontogenia chrysocoma (Baird, 1865)	-	-	-	1
Protocirrineris chrysoderma (Claparède, 1868)	-	-	4	-
Protodorvillea kefersteini (McIntosh, 1869)	-	-	9	-
Pseudoleiocapitella fauveli Harmelin, 1964	-	-	1	-
Sabellidae indet.	-	-	4	-
Serpula vermicularis Linnaeus, 1767	-	3	1	-
Sigambra tentaculata (Treadwell, 1941)	-	-	3	-

Spio filicornis (Müller, 1776)	1	-	-	-
Spiophanes bombyx (Claparède, 1870)	1	-	-	-
Spirobranchus triqueter (Linnaeus, 1758)	-	5	-	-
Sthenelais limicola (Ehlers, 1864)	-	-	-	1
Syllidae indet.	-	-	16	3
Polyplacophora				
Acanthochitona crinita (Pennant, 1777)	-	-	-	1
Acanthochitona fascicularis (Linnaeus, 1767)	-	-	1	1
Callochiton septemvalvis (Montagu, 1803)	-	1	-	-
Leptochiton scabridus (Jeffreys, 1880)	-	-	1	-
Lepidochitona sp.	-	1	10	21
Gastropoda				
Alvania cancellata (da Costa, 1778)	-	1	-	-
Alvania discors (Allan, 1818)	1	3	1	-
Alvania lineata Risso, 1826	-	5	-	-
Alvania pagodula (Bucquoy, Dautzenberg & Dollfus, 1884)	-	-	-	1
Aplysia parvula Mörch, 1863	-	-	-	1
Ascobulla fragilis (Jeffreys, 1856)	-	-	1	6
Bela nebula (Montagu, 1803)	-	-	1	-
Bittium latreillii (Payraudeau, 1826)	-	5	1	9
Bolma rugosa (Linnaeus, 1767)	-	-	-	1
Bulla striata Bruguière, 1792	-	1	-	-
Caecum auriculatum de Folin, 1868	-	1	-	6
Caecum glabrum (Montagu, 1803)	-	-	1	-
Caecum sp.	-	-	-	26
Caecum trachea (Montagu, 1803)	-	-	1	4
Calyptraea chinensis (Linnaeus, 1758)	-	1	3	8
Cerithium vulgatum Bruguière, 1792	-	1	1	1
Chrysallida indistincta (Henn & Brazier, 1894)	-	-	-	1
Eulimella sp.	-	-	1	-
Euspira nitida (Donovan, 1804)	-	-	1	-
Fusinus sp.	-	-	-	3
Gibbula ardens (Salis Marschlins, 1793)	-	1	1	-
Gibbula fanulum (Gmelin, 1791)	-	-	-	1
Gibbula guttadauri (Philippi, 1836)	-	-	3	1
Gibbula sp.	-	-	1	-
Haminoea sp.	1	-	-	10
Hexaplex trunculus (Linnaeus, 1758)	-	-	-	5
Mangelia costulata Risso, 1826	-	-	2	-
Mangelia scabrida Monterosato, 1890	-	1	-	-
Manzonia crassa (Kanmacher, 1798)	-	-	1	-
Odostomia sp.	-	-	-	1
Ondina vitrea (Brusina, 1866)	-	-	2	-
Philine sp.	-	-	1	4
Rissoa splendida Eichwald, 1830	-	-	1	-
Tectura virginea (O. F. Müller, 1776)	-	-	1	-
Tritia cuvierii (Payraudeau, 1826)	8	15	-	-
Tritia incrassata (Strøm, 1768)	-	7	-	-
Pusia savignyi (Payraudeau, 1826)	-	-	-	2
Pusia tricolor (Gmelin, 1791)	-	-	-	1

Vitreolina sp.	-	-	-	1
Weinkauffia turgidula (Forbes, 1844)	-	-	-	1
Williamia gussoni (Costa O. G., 1829)	-	-	-	2
Bivalvia				
Arca noae Linnaeus, 1758	-	1	-	1
Asbjornsenia pygmaea (Lovén, 1846)	-	-	10	-
Cardites antiquatus (Linnaeus, 1758)	-	-	-	3
Centrocardita aculeata (Poli, 1795)	-	-	2	2
Centrocardita sp.	-	1	28	24
Ctena decussata (O. G. Costa, 1829)	-	-	4	9
Dosinia exoleta (Linnaeus, 1758)	-	-	1	-
Flexopecten hyalinus (Poli, 1795)	-	-	1	-
Gari costulata (W. Turton, 1822)	-	-	5	2
Gari tellinella (Lamarck, 1818)	-	-	1	-
Glans trapezia (Linnaeus, 1767)	-	-	1	10
Gouldia minima (Montagu, 1803)	-	1	5	21
Gregariella semigranata (Reeve, 1858)	-	12	1	9
Hiatella arctica (Linnaeus, 1767)	-	40	5	71
Kurtiella bidentata (Montagu, 1803)	-	-	17	8
Laevicardium crassum (Gmelin, 1791)	-	-	1	-
Limaria tuberculata (Olivi, 1792)	-	1	1	-
Loripinus fragilis (Philippi, 1836)	-	-	1	2
Lucinella divaricata (Linnaeus, 1758)	-	-	1	-
Modiolula phaseolina (Philippi, 1844)	-	-	1	-
Moerella donacina (Linnaeus, 1758)	-	-	5	2
Musculus costulatus (Risso, 1826)	-	-	7	-
Musculus subpictus (Cantraine, 1835)	-	-	4	3
Papillicardium papillosum (Poli, 1791)	-	-	1	-
Peronidia albicans (Gmelin, 1791)	-	-	-	1
Polititapes aureus (Gmelin, 1791)	-	-	13	5
Rocellaria dubia (Pennant, 1777)	-	2	1	3
Striarca lactea (Linnaeus, 1758)	-	5	3	11
Thracia villosiuscula (MacGillivray, 1827)	-	-	1	-
Venus verrucosa Linnaeus, 1758	-	8	7	14
Amphipoda				
Ampithoe ramondi Audouin, 1826	-	-	1	-
Apherusa chiereghinii Giordani- Soika, 1949	-	-	1	-
Caprellidae	-	-	-	4
Dexamine spinosa (Montagu, 1813)	-	-	3	7
Gammarus sp.	-	-	2	9
Microdeutopus anomalus (Rathke, 1843)	-	-	1	-
Microdeutopus spp.	-	-	2	1
Pereionotus testudo (Montagu, 1808)	-	-	6	12
Pseudolirius kroyeri (Haller, 1897)	-	-	1	-
Perioculodes sp.	1	-	-	-
Decapoda				
Alpheus glaber (Olivi, 1792)	-	1	-	-
Anapagurus bicorniger (A. Milne-Edwards & Bouvier, 1892)	-	2	13	5
Athanas nitescens (Leach, 1813)	-	2	-	-

Clibanarius erythropus (Latreille, 1818)	-	4	-	-
Ebalia deshayesi Lucas, 1846	-	-	1	5
Eriphia verrucosa (Forskål, 1775)	-	1	-	-
Galathea sp.	-	2	-	3
Lysmata seticaudata (Risso, 1816)	-	-	2	-
Necallianassa truncata (Giard & Bonnier, 1890)	-	-	1	-
Paguristes eremita (Linnaeus, 1767)	-	-	-	1
Pagurus cuaensis Bell, 1844	-	3	-	-
Pagurus sp.	1	-	-	-
Pisa armata (Latreille, 1803)	-	-	-	1
Processa macrophthalma Nouvel & Holthuis, 1957	-	1	-	1
Sirpus zariquieyi Gordon, 1953	-	-	1	2
Synalpheus gambarelloides (Nardo, 1847)	-	-	1	-
Upogebia stellata (Montagu, 1808)	-	2	-	-
Xantho pilipes A. Milne-Edwards, 1867	-	4	-	-
Isopoda				
Anthuridae	-	-	3	16
Cymodoce truncata Leach, 1814	-	1	14	7
Kupellonura mediterranea Barnard, 1925	-	1	2	-
Tanaidaea				
Chondrochelia savignyi (Kroyer, 1842)	-	-	2	10
Cumacea				
ind.	-	-	5	-
Echinoidea				
Echinocyamus pusillus (O.F. Müller, 1776)	-	-	7	7
Holothuroidea				
indet.	-	-	2	2
Ophiuroidea				
Ophiothrix sp.	-	1	-	3
Amphipholis sp.	-	3	4	11
Plathelmynthes	-	-	1	-
Chordata				
Branchiostoma lanceolatum (Pallas, 1774)	-	-	6	1

Table 4 - Macrofauna assemblage. Number of individuals (N) *per* 25 dm^3, species richness (SR), Shannon-Weaver diversity (H') and Pielou's evenness (J) measured for each site (H; G; CN; CS) (mean ± SD), and results of PERMANOVA test on Euclidean distance (F: F-value, *p*(perm): calculated probability value, Unique perms: the number of unique permutations).

Site	SR	N	J	H'
H	3.33±2,08	6.33±5.03	0.56±0.48	1.22±1.07
G	24±12.16	86±50.68	0.78±0.05	3.47±0.82
CN	63±5.29	205.66±50.52	0.87±0.01	5.23±0.18
CS	51.33±10.78	353.33±107.77	0.64±0.05	3.63±0.37
F	29.31	16.314	0.958	16.28
***p*(perm)**	0.0006	0.0004	0.473	0.001
Unique perms	2643	4171	4256	4272

Macrofauna Diversity and Community Structure

A total number of 1,954 individuals, belonging to 164 taxa grouped in eight macrobenthic groups, were classified to different taxonomic levels as follows: Mollusca (610 ind.), Sipuncula (606 ind.), Polychaeta (513 ind.), Crustacea (172 ind.), Echinodermata (40 ind.), Chordata (7 ind.), Nemertea (5 ind.), and Platyhelminthes (1 ind.) (Table 3). The whole benthic community drastically increased in abundance and species richness away from the vent sites (H: DI = 0.97% – DQ = 5.49%; G: DI = 13.20% – DQ = 29.88%) to control sites (CN: DI = 31.58% – DQ = 68.29%; CS: DI = 54.25% – DQ = 48.78%).

The main taxa structuring the benthic community were Mollusca, Sipuncula, Polychaeta and Crustacea, reaching a dominance of 97.28%. With the only exception of Sipuncula, three taxa were detected at all sites, differentially contributing to the communities living at each site (Figure 5).

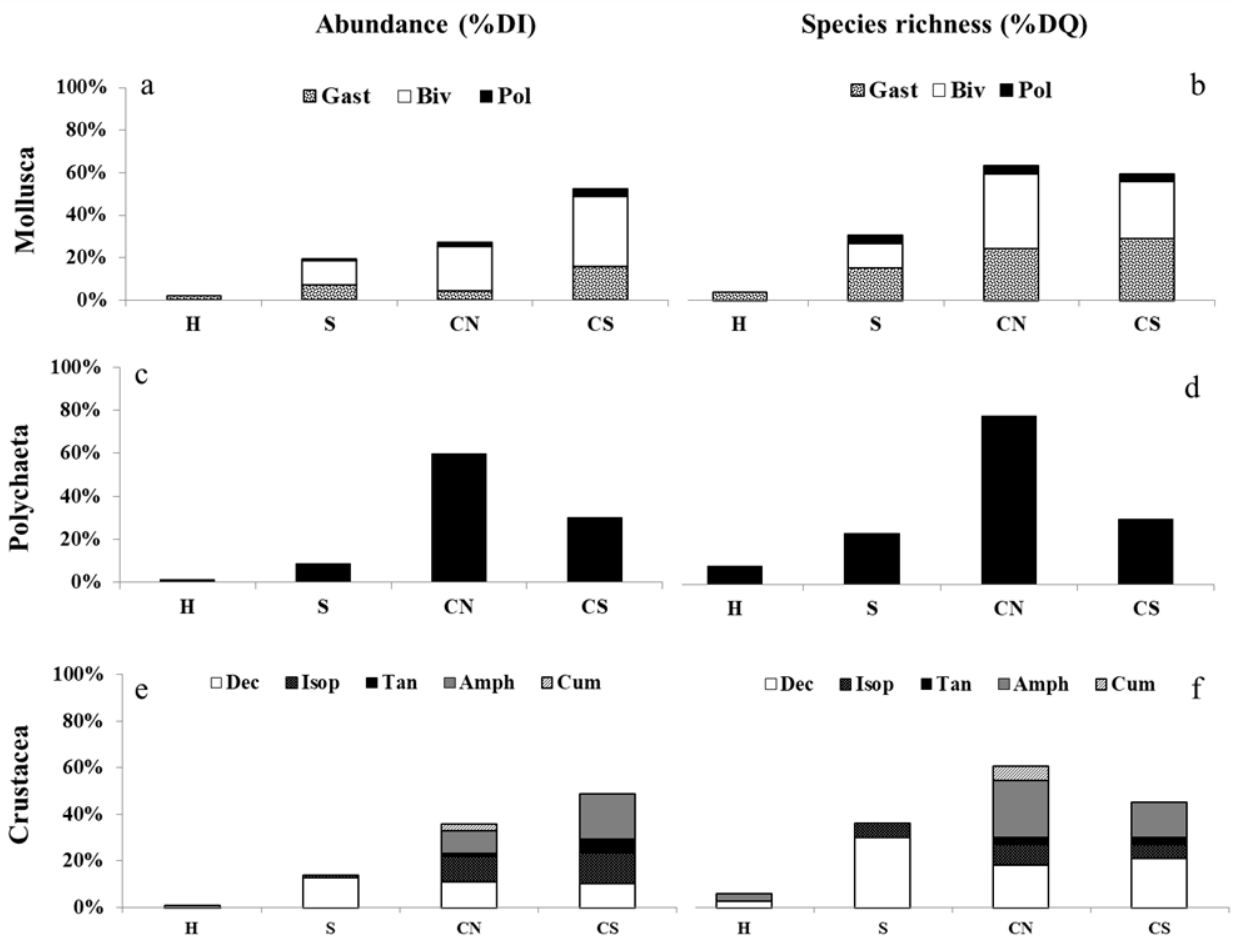

Figure 5: Total contribution to quantitative (Abundance - %DI) and qualitative (Species richness - %DQ) dominances of three major taxonomic groups (Mollusca; Polychaeta; Crustacea) for each habitat (H; G; CN; CS).

Mollusca showed the highest species richness, being represented by 76 species, belonging to the classes Polyplacophora, Gastropoda, and Bivalvia. Bivalvia was the dominant group in term of abundance (Figure 5A), with 400 individuals (65.57%) belonging to 30 species (39.47%). Gastropods were the dominant group in term of species richness (Figure 5B), with 41 species (53.94%) and an abundance of 173 individuals (28.36%). Polyplacophores were poorly represented, both in abundance (37 ind.; 6.06%) and species richness (5 sp.; 6.57%).

The highest percentage of mollusks abundance mainly occurred in the control site CS (52.46%), where bivalves were mainly represented by the species *Hiatella arctica* (76 ind.), gastropods by *Caecum* sp. (26 ind.) and polyplacophores by *Lepidochitona* sp. (31 ind.). On the other hand, the lowest percentage of mollusks occurred in H (DI 1.64%) with only three species: *Tritia cuvierii* (8 ind.), *Haminoea* sp. (1 ind.), and *Alvania discors* (1 ind.).

Polychaeta, with a total of 513 individuals belonging to 47 species, were mainly represented by the species *Eunice vittata* (108 ind., 21.05%) and *Aponuphis bilineata* (83 ind., 16.18%). This group was dominant in the control site CN (Figures 5C,D), both in abundance (307 ind., 59.84%) and in species richness (36 sp., 76%). The opposite occurred in H, where a total of only seven individuals belonging to four species, among which four individuals of the polychaete *Capitella capitata*, were recorded.

Crustacea were represented by 172 individuals belonging to 33 species, grouped in five orders: Decapoda, Isopoda, Tanaidacea, Amphipoda, and Cumacea. Only five species, represented by the decapod *Anapagurus bicorniger*, the amphipods *Dexamine spinosa* and *Pereionotus testudo*, the isopods *Cymodoce truncata* and Anthuridae indet., reached a dominance of 52.35% of the total crustacean assemblage. The highest percentage of crustaceans individuals was detected in CS (84 ind., 48.84%; Figure 5E) and species richness in CN (20 sp., 60.61%; Figure 5F), while only 2 species, the decapod *Pagurus* sp. and the amphipod *Perioculodes* sp., occurred in H with only 1 individual.

Among the sinecological indices (Table 4), the highest density (N) and species richness (SR) values were recorded in CN and CS, while the lowest values in H, which also showed the lowest values of diversity (H') and evenness (J). For each index, PERMANOVA test highlighted significant differences among sites, except for Pielou's evenness (J) (Table 4). A significant difference was also detected analyzing macrofauna composition (PERMANOVA: $F = 3.411$, $p = 0.0004$). In particular, pairwise comparisons showed differences between hydrothermal vents

(G; H) and non-vents (CN; CS) sites, with the highest average similarity among these latter sites (Average similarity 38.30%; Table 5). These differences were also evident in the plot of CAP analysis (Figure 6), where the three replicates formed consistent clusters for each site. Along the CAP1 axis, vent sites (H and G) were separated from control ones (CN and CS), while along the CAP2 axis the graph showed a clear separation between the southern (H and CS) and northern (G and CN) sites. In particular, H and G were strongly polarized respectively in the positive and negative part of CAP2, while CN and CS were aggregated around the zero value of CAP2, respectively in the positive and negative part. The average multivariate similarity of macrofaunal assemblage composition for each site ranged from 11.94 to 59.92% (Table 6), while the similarity of dominant species among sites was 19.62%, attributable to sipunculans, mollusks, and polychaetes (Table 7).

Table 5 - Results of PERMANOVA pairwise comparisons among sites, using 4999 permutations, and average similarity between sites.

Site	t	Unique perms	*p*(MC)	Average Similarity
H, G	1.4325	10	0.1286	7.66
H, CN	1.9607	10	0.0344	0.75
H, CS	2.013	10	0.0378	1.28
G, CN	1.9645	10	0.038	18.83
G, CS	1.8827	10	0.0416	24.18
CN, CS	2.0479	10	0.0236	38.3

Relationship Between Environmental Variables and Macrobenthic Community

The pattern of community structure was significantly correlated with some environmental parameters of sediments and interstitial waters (Table 8) and visualized in the dbRDA (Figure 7), where vectors indicate the direction of increasing influence of each variable on community changes.

Concerning the first group of variables, gravel, sand, pH, temperature, and TOC explained a significant variation in benthic community when tested individually. In particular, only gravel and pH represented the most important driving factors influencing benthic community distribution among sites, explaining 52.76% of community variation.

As for interstitial water variables, all the investigated ions, with the only exception of S2– in the site G, had a significant effect on community variability, even though the greatest influence was due to the Mg2+, K2+, and Ca2+ ions, which explained 56.63% of community variation.

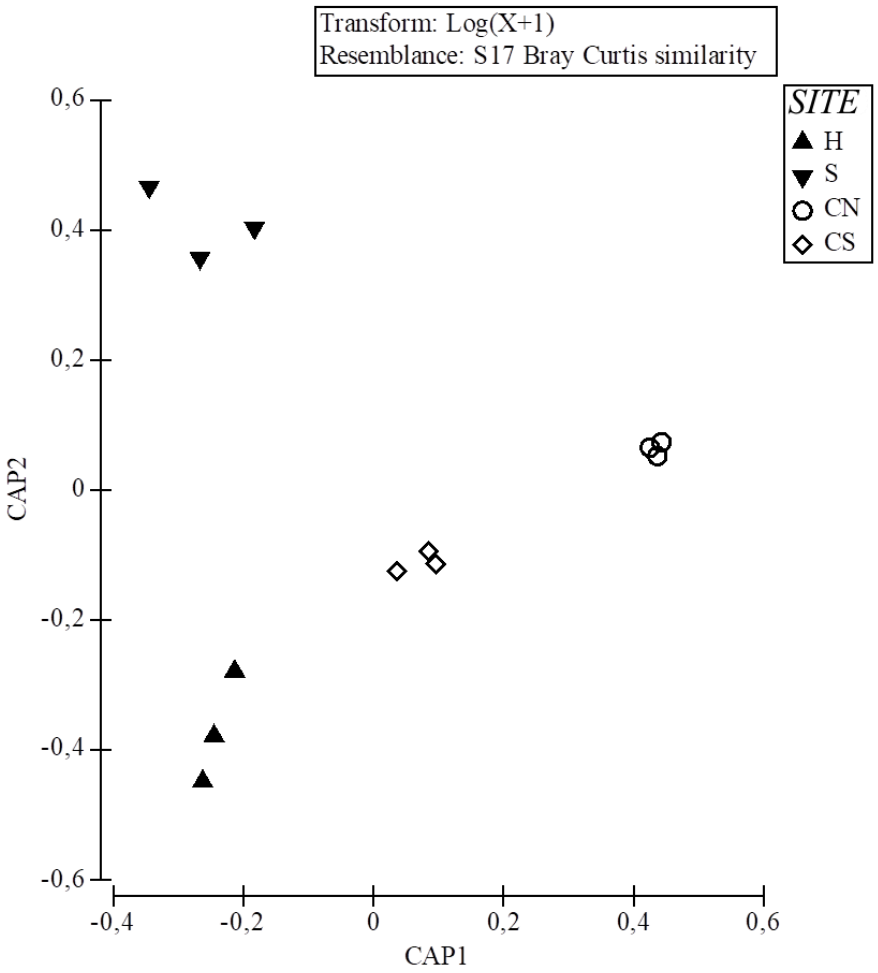

Figure 6: Canonical Analysis of Principal coordinates (CAP) on benthic community dataset classified for each site (H; G; CN; CS).

Table 6 – DistLM results comparing benthic community data and environmental variables (a; b). Bold data are significantly different at $\alpha = 0.05$.

VARIABLES	Marginal tests			Sequential tests			
	Pseudo-F	*p*	Proportion of variation explained	Pseudo-F	*p*	Proportion of variation explained	Cumulative variation
a) Sediment variables							
Gravel (%)	3.3023	**0.0028**	0.24825	3.3023	**0.0024**	0.24825	0.24825
Sand (%)	3.3547	**0.0032**	0.2512	1.6102	0.0954	0.11409	0.36234
Mud (%)	1.5694	0.0956	0.13565	0	1	1.45E-15	0.36234
pH	3.408	**0.0068**	0.25418	2.7984	**0.0042**	0.16525	0.52759
Temperatures (°C)	4.7266	**0.0002**	0.32096	1.9971	0.0622	0.10486	0.63245
TOC (%)	3.1083	**0.0056**	0.23713	0.67269	0.7442	3.71E-02	0.6695
b) Interstitial water variables							
Ca^{2+}	2.3312	**0.0258**	0.18905	2.3312	**0.0244**	0.18905	0.18905
K^{+}	3.2656	**0.0084**	0.24617	2.2943	**0.0274**	0.16474	0.35379
Mg^{2+}	4.5698	**0.0006**	0.31365	3.8457	**0.0014**	0.20979	0.56358
NO_3^{-}	3.3708	**0.009**	0.2521	1.0131	0.398	5.52E-02	0.61876
SO_4^{2-}	2.1855	**0.034**	0.17935	0.89609	0.5072	4.95E-02	0.6683
S^{2-}	1.7078	0.0796	0.14587	0.50382	0.8168	3.04E-02	0.69866
Zn^{2+}	1.6187	0.1072	0.13932	1.3074	0.2978	7.42E-02	0.77289
Pb^{2+}	1.6385	0.1018	0.14078	1.3553	0.302	7.07E-02	0.84356
Cd	1.4394	0.1482	0.12583	1.1787	0.3936	5.80E-02	0.90157
Cu	1.3934	0.1556	0.1223	0.76951	0.5662	4.28E-02	0.94438

Table 7 - Results of similarity of percentages test, showing taxa that mostly contributed to similarity among sites. Average similarity: 19.62 %; Contrib% >3% cut-off.

Taxa	Av.Abund	Av.Sim	Sim/SD	Contrib%	Cum.%
SIPUNCULA	2.74	2.98	1	15.19	15.19
MOLLUSCA					
Tritia cuvierii (Payraudeau, 1826)	0.6	1.44	0.26	7.34	22.53
Hiatella arctica (Linnaeus, 1767)	1.52	1.27	0.67	6.48	29.01
POLYCHAETA					
Aponuphis bilineata (Baird, 1870)	1.45	1.26	0.97	6.43	35.44
Eunice vittata (Delle Chiaje, 1828)	1.45	0.95	0.64	4.84	40.28
MOLLUSCA					
Centrocardita akabana (Sturany, 1899)	1.18	0.78	0.63	3.97	44.25
POLYCHAETA					
Lysidice unicornis (Grube, 1840)	0.87	0.69	0.74	3.52	47.76
ECHINODERMATA					
Amphipholis sp.	0.73	0.6	0.78	3.05	50.81
MOLLUSCA					
Venus verrucosa Linnaeus, 1758	0.88	0.59	0.64	3.01	53.82

DISCUSSION

The hydrothermal vent system at Pozzuoli Bay provides an opportunity to study the macrobenthic assemblages and composition in a shallow extreme environment. Through this work, the macrobenthic community at Secca delle Fumose was investigated for the first time. The results highlighted a strong change in density, species richness and diversity between two non-vent (CN and CS) and two different vent (H and G) sites.

When compared to the hydrothermal sites, the control sites showed higher abundance and species richness, and the environment was characterized by normal pH and ions concentrations (e.g., Ca2+, K2+, and Mg2+), and by a significant percentage of gravelly sediment that markedly affected the macrobenthic composition (DistLM Table 6; dbRDA Figure 7).

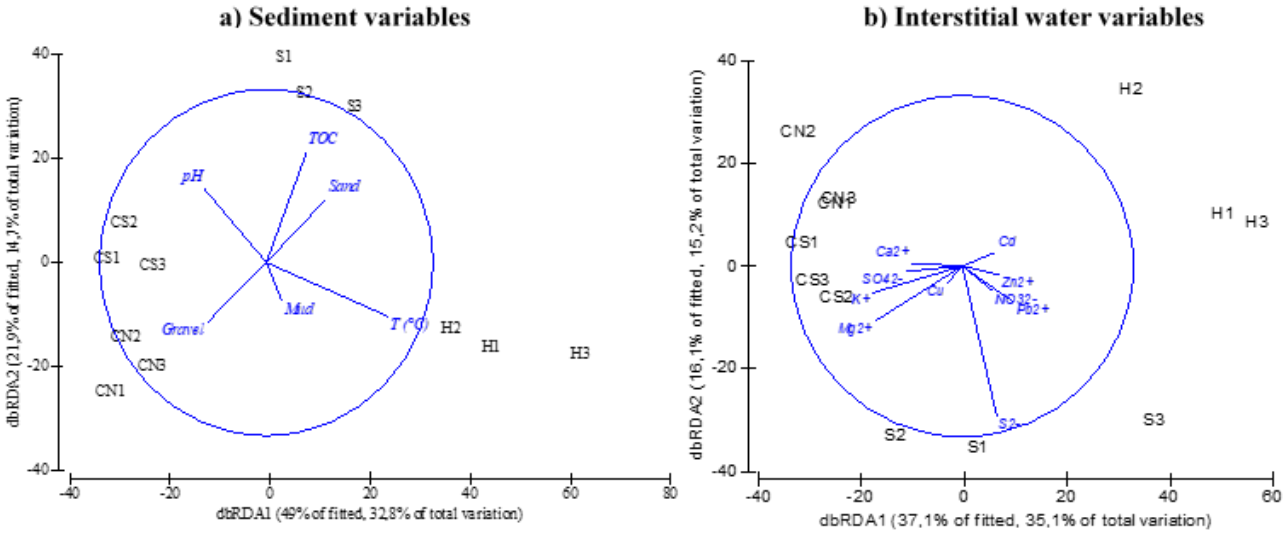

Figure 7: dbRDA plot of benthic community structure fitted to sediment and water variables, showing each site replicated (H; G; CN; CS).

It is well-known that benthic species are functionally and structurally related to the main features of the habitat they reside (e.g., Woodin, 1978; Thrush et al., 1991; Desprez, 2000; Riera et al., 2012; Donnarumma et al., 2018; Casoli et al., 2019). In particular, the taxa composition recorded in the control sites was consistent with a marked occurrence of gravel, which offers microhabitats suitable for settlement and refuge. This is also proven by the high dominance of the byssate bivalve *H. arctica*, which is commonly found on hard substrates, where become strictly aggregated in dense groups (Purchon, 1977). The occurrence of high

abundance of gastropod Caecum spp. is probably due to the presence of sand in the sediment with only tracks of mud (Fretter and Graham, 1978). Species belonging to the genus Eunice (Annelida, Polychaeta) also occurred with a very high abundance in the control area, in agreement with their cosmopolitan nature (George and Hartmann-Schröder, 1985; Dounas and Koukouras, 1989; Gusso et al., 2001; Fauchald et al., 2009), as well as for the crevice-dwelling isopod *C. truncata* and sipunculans, capable of hiding in the narrow cracks of rocks and in empty or fragmented shells (Ferrero-Vicente et al., 2013).

In addition to the relationship between benthic assemblages and structural characteristics of the substrate, the understanding of environment state is also related to the occurrence of sensitive or tolerant species to the environmental changes (Simboura and Zenetos, 2002; Washburn et al., 2016). For instance, the high abundance of C. truncata restricted only in the control area might be related to the suitable habitat conditions, as reported for the normal pH conditions at Ischia Island by Cigliano et al. (2010). Conversely, the absence of this species at the geyser site (G) may be due to the crustaceans sulfide sensitivity (Gray et al., 2002), as well as its absence in the white hydrothermal vent (H) could be also related to the metabolic sensitivity of *C. truncata* to high pCO2 condition (Turner et al., 2016).

Heavy metals (Zn, Cd, Pb, and Cu) occurred in the interstitial waters of sediments at all sampling sites (Table 1). However, their low quantity is compatible with the existence of a well-structured macrobenthic assemblage in the control sites, as reported in previous investigations (Bryan, 1976; Yoshida et al., 2002; Raghukumar et al., 2008). Moreover, the presence only in the control sites of the cephalochordate Branchiostoma lanceolatum attests to the good quality of the sites, since it is a species sensitive to organic enrichment and polluted water (Simboura and Zenetos, 2002; Rota et al., 2009).

At the two hydrothermal sites (G and H), a drastic decrease of benthic biodiversity was observed. This can be mainly attributable to environmental conditions, in particular to presence of sulfide, high temperatures and seawater pH variations generated by volcanic activity. Moreover, as observed by Tarasov et al. (2005), the hydrothermal fluids in these sites produced a slight reduction of water salinity, measured as Na+ and Cl– ions.

Several studies (e.g., Thiermann et al., 1997; Tarasov et al., 1999; Dando, 2010) have reported a decrease of both density and diversity of benthic communities as corresponding to the occurrence of high temperatures and sulfide concentrations, leading to an increase of temperature- and/or sulfide-tolerant species. The same result was also found in the present

study where high sediment temperatures and sulfide ions (S2–) occurred at the geyser site (G), resulting in a 69% reduction of taxonomic richness and a 73% reduction in number of individuals compared to control sites. At this geyser site we observed the absence of obligate vent-associated species, which were previously reported for deep-sea vent systems (Tarasov et al., 2005 and reference therein; Schander et al., 2010; Stevens et al., 2015). Our data are consistent with results from other coastal shallow water hydrothermal vents (Dando, 2010; Bianchi et al., 2011). In particular, the fauna around the geyser at the Secca delle Fumose was composed by the most representative species inhabiting the 'background' area (e.g., the polychaetes *E. vittata* and *A. bilineata*, the bivalves *H. arctica* and *Venus verrucosa*, the ophiuroid *Amphipholis* sp.), but with a very low number of individuals. This benthic assemblage may represent a "simplified" community as suggested by Dando (2010), who defined the living fauna around hydrothermal vent and cold seep sites as a subset of the background biota.

According to Thiermann et al. (1997), the harsh hydrothermal conditions drastically affect the macrobenthic composition, as also observed at the "white" hydrothermal site (H). Here, the lowest species richness (9 sp.) and abundance (19 ind.) are mainly due to the seawater acidification (pH ∼7.6), in agreement with Di Napoli et al. (2016) who recently detected acidic CO2-rich fluids in the SdF area, and to the high sediment temperature (37.53 ± 2.28°C), that was almost the double of that detected in the control sites (21.8°C). These factors produced a reduction of 83.64% in taxonomic richness and 86.29% in number of individuals if compared with those found around the geyser and a reduction of 93.92 and 97.75% respectively if compared with control sites.

The dominant species in H site were the gastropod *T. cuvierii* (8 ind.) and the opportunistic polychaete *C. capitata* (4 ind.), while the other species, each occurring with only one individual, could be considered very rare in the hydrothermal area. The occurrence of the vagile nassariid gastropod *T. cuvierii* and the sediment-dwelling polychaete *C. capitata* clearly underlines the faunal similarity of our study area with other shallow-water hydrothermal vents. Indeed, nassariid species *Tritia neritea* (= *Cyclope neritea* Linnaeus, 1758), was among the dominant organisms in hydrothermal vents off Milos in the Aegean Sea (Dando et al., 1995; Southward et al., 1997; Thiermann et al., 1997) and in the Papua New Guinea (Tarasov et al., 1999). Population density of nassarids species is often influenced by food availability (Zhao et al., 2011), similarly the high abundance of *T. cuvierii* in the white hydrothermal site

(H) may be due to the high food source consisting in the microbial mat (Cardigos et al., 2005), so as also occurs for the congeneric species *T. neritea* in other vent systems. High TOC concentration in the sediment (Table 1) might represent a further food source of this gastropod. The organic enrichment could be responsible for the dominance of the opportunistic polychaete *C. capitata* (Grassle and Grassle, 1976), which is a tolerant species to high temperatures and sulfide concentrations (Gamenick et al., 1998a). This work does not address directly the genetics of *C. capitata* complex (Blake, 2009; Nygren, 2014) that is also reported in hydrothermal vents and sulfidic habitats (Gamenick et al., 1998a, b), nevertheless such complex of sibling species so as for gastropods (e.g., Colognola et al., 1986) will be the focus of future research on its genetic variation under the extreme environmental conditions occurring in the study area.

CONCLUSION

This work represents the first study describing the particular environmental conditions and species composition of macrofauna at the Secca delle Fumose shallow hydrothermal system, an easily accessible coastal area, to evaluate the biological responses in an extreme habitat (i.e., characterized by high temperature, sulfide concentration and low pH condition). The results showed that the studied macrobenthic community appears to be strongly driven by high sediment temperatures, by sulfide concentration around the geyser and by low pH value in the white microbial mat area with the occurrence of some CO2 gas bubbling. These key factors led to a drastic reduction of biodiversity, compared to the surrounding non-vent area, highlighting the great importance of environmental state in structuring benthic systems. Future studies should also take into account other key elements of ecosystem functioning, such as meiofauna and microfauna communities, for a better understanding of the complex characteristics related to this very shallow extreme environment of the Campi Flegrei area.

Author Contributions

DZ and RS conceived the study. LD wrote the manuscript. LD, LA, EB, RB, DZ, and RS provided samples from the field. EB and RB performed the samples pretreatment. EC provided chemical analysis. LD and RG provided taxonomic analysis. LD and LA performed statistical analysis. DZ,

RS, and GR provided the sampling design and improved the manuscript. All the authors read, edited and approved the final manuscript.

Acknowledgments

We are grateful to Soprintendenza of the Underwater Archeological Park of Baia (prot. 5667, 24/10/2016) for the authorization to sampling. We are thankful to Dr. Guido Villani for assistance during the underwater sampling and permission to use photos that enriched the manuscript. Thanks are also due to the diving center Centro SUB Pozzuoli (Guglielmo Fragale) for support field activities and Dr. Aurélie Tasiemski and Dr. Céline Boidin-Wichlacz for helping in sampling activities. We also thank the reviewers for the suggestions and comments. We are indebted to Dr. Jeroen Ingels (Florida State University) for useful discussions.

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ANNEXE 2. *First occurrence of a symbiotic relationship between the opportunistic coastal Capitella spp and environmental microorganisms: a matter of sediment and sulfides?*

Authors: Stéphane Hourdez; Céline Boidin-Wichlacz; François Massol; Didier Jollivet; Maria Claudia-Rayol; Renato Bruno; Daniela Zeppilli; Ludovic Lesven; Gabriel Billon; Sébastien Duperron; Aurelie Tasiemski

Submitted: 07 October 2019, in *Science of the Total Environment*.

Keywords: Marine annelid, ectosymbiosis, epibiosis, *Thiomargarita*, pollution

Abstract: *Capitella* spp. are common species in fine silty sediments in different regions of the world. This coastal polychaete inhabits organic-rich, reduced, and sometimes polluted sediments. In populations from Roscoff (Brittany, France), we observed a facultative epibiotic association covering the tegument of some specimens (20 to 30%) from an anthropized site (the Harbor) while individuals from a reference, unanthropized site (Le Laber) were devoid of any visible epibiosis. By RNAseq and by microscopic analyses, we showed that epibiotic *Capitella* from the Harbor host a greater microbial diversity than non epibiotic specimens from the same location or from the reference site. More particularly, sulfur-oxidizing bacteria amongst which the giant bacterium *Thiomargarita* to date only described in deep sea habitats, are characteristic of this epibiosis. Survey of the associated *Capitella* combined with the geochemical analysis of their sediment revealed that epibiotic specimens are always found in sediments with the highest concentration of sulfides. Given the very low level of intraspecific genetic differences between specimens harboring or not an epibiosis, the facultative acquisition of thiobionts is likely a plastic physiological adaptation that presumably allows the worms to face toxic levels of sulfides that can be temporally reached during the summer.

INTRODUCTION

Despite a multitude of studies on biodiversity erosion over the last decades, understanding the ways by which marine species persist despite anthropic pollution and eutrophication remains at the heart of current concerns, given both the increasing importance of these changes in the functioning of the biosphere and the complexity of biotic and abiotic interactions in marine systems (Harvell et al., 1999; Ruhl and Smith, 2004). Several studies have shown that, when they are not directly lethal, thermal and/or chemical modifications of the environment often induce endocrine and behavioral changes in marine organisms, as well as alterations in their energetic metabolism and immunity (Harvell et al., 1999; Waldichuk, 1979). Recently, Cuvillier-Hot et al. (2018) showed that heavy metals and phthalates, even at concentrations below the toxicity index, alter the immune response of natural populations of the coastal annelid *Hediste diversicolor* and make them less resistant to an experimental infection by the environmental bacterium *Bacillus hwajinpoensis* SW-72 isolated from the burrow of the worm. These observations clearly show the impact of changing environmental conditions on host-bacteria interactions in marine invertebrates (Cuvillier-Hot et al., 2018). The past decades have seen an increasing number of studies with the aim of characterizing the biology of bacterial symbionts in a wide variety of invertebrates and plants, as well as their role on community structure and ecosystem functioning (Brooks et al., 2017; Carrier and Reitzel, 2017; Ferrari and Vavre, 2011; Gilbert et al., 2012; Moran and Wernegreen, 2000). It is now widely admitted that symbiotic associations can be responsible for some of the most noticeable changes in phenotypes, as they constitute a low-cost source of evolutionary innovation for their host (Margulis, 1991). The very short generation time of associated microorganisms could allow a faster adaptation of the host to changing environments than the fixation of favorable alleles in the host genome, and therefore accelerate the acquisition of new phenotypes more adapted to novel ecological conditions. For instance, it is now well established that diagnostic traits of numerous symbiotic species are in fact a response to the microorganisms they are associated with (McFall-Ngai, 2008; McFall-Ngai et al., 2013). Well-known examples include hydrothermal vent metazoans associated with chemolithotroph bacteria, such as the cephalothorax enlargement of the shrimp *Rimicaris exoculata* (Petersen et al., 2010), the modification of the dorsal tegument or the notopodia of either *Alvinella pompejana* or its sister species *A. caudata* to bear dense bacterial filaments (Campbell et al.,

2003; Grzymski et al., 2008; Haddad et al., 1995; Le Bris and Gaill, 2007) or the development of a specific organ, the trophosome dedicated to harbour intracellular bacteria in the giant tubeworm *Riftia pachyptila* (Bright and Lallier, 2010; Cavanaugh et al., 1981). There is also ample empirical evidence of symbioses providing protection against specific natural enemies, e.g. in aphids facing parasitoids and predators (Dion et al., 2011; Oliver et al., 2003; Polin et al., 2014; Vorburger et al., 2010). Such symbioses have also been suggested as a potential means to explain the success of some invasive species in new habitats (Amsellem et al., 2017; Traveset and Richardson, 2014). More generally, symbioses have been shown to affect adaptive traits, from trophic niche (Kohl et al., 2014) to temperature dependence (Morsy et al., 2010), salinity tolerance (Nougué et al., 2015), resistance to oxidative stress (Richier et al., 2005), or resistance against pathogens (Kaltenpoth and Engl, 2014; Tasiemski et al., 2015), that may have an early effect during organism development (Gasnier-Fauchet et al., 1986; Gilbert et al., 2015). While many of the aforementioned symbioses are *endosymbioses*, symbionts found in the gut (i.e. gut microbiota), have also been the focus of much attention. They are now known to influence the life history of the host and to provide an adaptive advantage for feeding on different resources or acting against specific pathogens (Macke et al., 2017; Shapira, 2016). Consequently, understanding the adaptation of marine species to changing environments requires the further exploration of how the environment impacts the host-symbiont associations and their evolution for either endo- or ecto-symbioses (epibiosis). Until now, the symbiotic microflora of marine animals was often considered as a random consortium (McFall-Ngai, 2008). However, multiple lines of evidence show that this microflora corresponds in fact to a highly specialized microbial community forming a specific and stable symbiosis with its host, with dedicated roles. In chemosynthetic environments, such as hydrothermal vents and sulfide-rich sediments, some marine invertebrates are commonly colonized by specific symbiotic bacteria (Dubilier et al., 2008; Bellec et al. 2019). In coastal sediments, some nematodes, amphipods and ciliates also harbor sulfide-oxidizing proteobacteria that aid the host in detoxifying environmental sulfide and, in some cases, contribute to its nutrition (Bulgheresi, 2018; Gillan and Dubilier, 2004). Recently, the nematode *Metoncholaimus albidus*, reported in the same Harbor location as the present study, has been shown to be associated with distinct microbial communities known to be involved in sulfur metabolism (Campylobacterota and Gammaproteobacteriota) (Bellec et al., 2019).

The coastal annelid Capitella spp. is a ubiquitous species that has a propensity to dominate organically enriched sediments. They have long been described as associated with areas particularly rich in sulfide, to the point where relatively high H2S concentration has been considered as a cue for *Capitella* larval settlement (but see (Dubilier, 1988) for a reassessment of this hypothesis). Interestingly, differential tolerance to sulfide was observed between *Capitella* sibling species, leading to the conclusion that these ecophysiological differences were genetically fixed and that sulfidic environments could have been the driving force of such genetic differentiation (Gamenick et al., 1998). Despite the known links between sulfide concentration and the occurrence of symbioses, the existence of microbial communities associated with genus *Capitella* was never properly assessed.

The main purpose of this paper is to explore the microbial diversity associated with *Capitella*, evaluate whether durable host-bacteria interactions occur and assess the influence of the environment on the composition of this microbiota. To that end, animals and sediments were sampled at two locations around Roscoff (Brittany, France) characterized by different anthropic influence: the old eutrophic Harbor (Harbor site), and a nearby site located deep inside a marine inlet, near a fresh water outlet site (Le Laber site). Habitats of the worms were chemically characterized and correlated to the diversity, abundance and specificity of prokaryotic and eukaryotic microorganisms associated with *Capitella* with the help of a RNAseq approach combined with morphological observations. Altogether, this study is the first investigation of *Capitella*-associated microbiota in the context of varying anthropic pressures. Data reveals, in particular, the existence of a specific and facultative tegumental association between the worm and a giant bacterium whose occurrence varies both in space and time depending on the nature of the environment. The influence of animal's size, gender and genotype on the prevalence of epibiotic associations was also investigated in order to determine the intrinsic versus extrinsic factors driving the temporary symbiosis that have been observed.

MATERIALS AND METHODS

Sediment and animals were collected at the same time in the two locations: the old Roscoff Harbor and Le Laber near Roscoff (Brittany, France). A map with the GPS coordinates is presented in supplementary data (Fig. S1).

2.1. Sediments

Sampling and pretreatments – Sediments of the two study sites were characterized in terms of trace metals concentrations (total metals and metals extracted with 1M HCl), reduced sulfur species content (AVS: Acid Volatile Sulfides and CRS: Chromium Reducible Sulfur), dissolved sulfides, granulometry and total carbon and nitrogen contents. Sediments were collected using a 5 cm long (for the top 0–5-cm surface sediment) or a 35 cm long (for sediment profiles) Perspex tubes (internal diameter: 7.5 cm). Cores sampled with the Perspex tubes were put into a glove box, previously flushed with nitrogen, and sliced every 1 cm at both sites. Each sediment sample was then stored under nitrogen untreated in a plastic bag at -18 °C prior to perform AVS, CRS and metal analysis. A slice of each core was also dried for the measurements of the other parameters mentioned previously. Additional sediment cores were sampled for exposure to DGT (Diffusive Gradients in Thin films)-AgI passive samplers used for dissolved sulfide determination.

Total metal concentrations – Sediments from both sampling sites were dried at room temperature under a laminar-flow hood and then sieved to retain the fine fraction (< 63 µm). About 0.2 g of sediment from this fraction was then mixed with 10 mL of concentrated high-purity hydrofluoric acid (Prolabo Normapur) followed by an aqua regia [HCl + HNO3 (ultrapur), 3/1 v/v, 10 mL] attack (Lesven et al., 2008). To achieve the recovery of total amounts of metals, the recovered solutions were subjected to a quantitative evaporation and diluted with ultrapure water (provided by a Milli-Q Plus filter apparatus, Millipore) and quantified by inductively coupled plasma atomic emission spectrometry (ICP-AES; Varian, Vista Pro, axial view). The ICP-AES spectrometer was calibrated using standard solutions, blank corrections were applied when necessary, and certified sediment materials (MESS-3, HISS-1 and PACS-2) were analyzed for quality control (Lourino-Cabana et al., 2014).

AVS, CRS and HCl-extractable metals – Reduced sulfur species (AVS and CRS) were quantified for each core of 30 cm depth. A time series analysis of AVS concentrations was also performed from 28th of July to 8th of December 2015 in the first 5 cm of sediment. AVS was converted into H2S gas (trapped in a NaOH-EDTA 1M solution) before quantification by a sequential extraction procedure previously described by (Cornwell and Morse, 1987). Briefly, AVS were extracted with a 6M HCl solution for 1 h. Afterwards, a hot digestion of the sediment residue for 2 h after addition of a Cr(II) solution was carried out to recover sedimentary pyrite and

elemental sulfur. For both extractions, sulfide concentration in the trapping solution was titrated using potentiometry with a 1000 mg L^{-1} Cd^{+2} standard solution using a sulfide ion-selective electrode (Ag2S, Orion) and a Hg/Hg2SO4 reference electrode. Concentrations of Cd, Co, Cu, Ni, Pb and Zn, called SEM (Simultaneously Extracted Metals) were simultaneously extracted with AVS and measured with an ICP-AES (see description above). Each measurement was run three times for each sample and results are given as means.

Toxicity index calculation – The toxicity index (TI) was calculated as the ratio SEM/AVS to predict metal sediment toxicity towards benthic species (Ankley et al., 1993). Its relevance has been demonstrated via toxicity tests on several benthic organisms (notably the polychaetes Capitella capitata and Neanthes arenaceodentata), in natura or through experimental exposure to contaminants. For each sample, the TI has been calculated, according to the following relation: TI = log ([SEM]/[AVS]) (Ankley et al., 1993). Previous studies have shown that sediments with TI > 0 are toxic for animals whereas sediments with TI ≤ 0 are not (Hansen et al., 2005). For the two study sites and the time series at both sites (from 28 of July to 8 of December 2015), AVS and SEM data of the 5 first cm of the sediment were used to calculate the TI values.

Carbon and nitrogen contents – Total carbon (TC) and nitrogen (TN) contents were measured from dried sediments with a CHN elemental analyzer (FLASH 2000, Thermo Scientific). Total Organic carbon (TOC) was estimated by difference between the total carbon and inorganic carbon contents. This later parameter was measured after heating the sample at 450 °C for 12 h to eliminate the organic carbon fraction from the sediment.

Granulometry – Grain size distribution was determined by laser granulometry (Malvern, Mastersizer 2000) and the fine fraction, including mainly clays and silts, was calculated as the percentage of particles with a diameter smaller than 63 µm (Lesven et al., 2008).

Dissolved Sulfides – Dissolved sulfides were measured using DGT-AgI probes (Gao et al., 2009). Briefly, dissolved sulfides diffuse from pore-water through an acetate cellulose filter (0.45 µm pore size) into a polyacrylamide gel containing a AgI precipitate, which color changes from white to black when forming Ag2S with sulfides. After a known exposure time of the filter in pore-water samples, the precipitate is scanned using a commercial flatbed scanner and color intensity is then digitized and calibrated to calculate the concentrations initially present (Lourino-Cabana et al., 2014; Teasdale et al., 1999). Calibration of the DGT-AgI probes in standard sulfide solutions were performed using the same conditions.

2.2. Worms

Sampling for RNaseq and morphological analyses – *Capitella* spp. were collected at low tide. At both locations, *Capitella* individuals were abundant, representing the most dominant species in the Harbor, and with abundance similar to that of oligochaetes in the nearby site Le Laber. The sediment was sieved on a 500 µm mesh in the field and animals were brought back to the laboratory for sorting under a dissecting microscope. For the RNAseq, animals collected in 2013, were checked for filamentous epibionts under the microscope and were separated into three groups: 1/ non epibiotic animals from the Le Laber 2/ non epibiotic animals from the Harbor and 3/ epibiotic animals from the Harbor (Figs 1B and 2B). For each group, 30 individuals were placed in RNA-laterAt the time of sampling for transcriptome sequencing, *Capitella* covered by epibionts were only found at the Harbor site; No epibiotic individuals were found in Le Laber. For the morphological analyses, five specimens of each group were fixed in glutaraldehyde 2.5% for electron microscopy and five were fixed in paraformaldehyde 4%, for fluorescence in situ hybridization in 2013 and in 2014.

Seasonal survey of *Capitella* association with *Thiomargarita* – From March to December 2015, samples were collected at two-week intervals from both Le Laber and the Harbor sites (19 sampling events per site). Each individual worm was then preserved in 85% ethanol in a microtube. 57 individuals were used for the genetic analysis (see below).The worms were later observed individually under a binocular dissection microscope to check for presence of epibiotic microorganisms and measure the width of the body at the fourth setiger. In total, 5900 worms were sampled (with 150-160 worms collected at each sampling event at each site). To obtain a better estimation of the association prevalence among the worms, the association of large epibiotic microorganisms with *Capitella* was modeled as a Bernoulli random variable through a generalized linear model (GLM) with binomial error and logit link between the explanatory variables and their effect on the association probability. We built 166 different GLM based on the “complete model”, which incorporated the effects of site (Le Laber vs. Roscoff Harbor), worm size and Julian date. The other 165 models were obtained as the sub-models nested within the complete one (i.e. models lacking one or more explanatory variables or interactions thereof). The goodness-of-fit of each model as its corrected Akaike Information Criterion (AICc) were computed and models were ranked from best to worst following increasing values of AICc. To obtain a more robust estimation of model predictions, model averaging procedures were used based on the Akaike weight of each model (Burnham

et al., 2011). For all these statistical analyses, R (v 3.2.3) was used with package 'fields' to make the heatmaps and package 'MuMIn' for automated model goodness-of-fit comparisons and model averaging.

2.3 Morphological observations of associated microorganisms

Optical microscopy - For each sample of Le Laber and the Harbor, worms with and without epibionts were examined alive or fixed (paraformaldehyde 4%) using an optical microscope (Zeiss Axio Imager M2) and a stereomicroscope (Zeiss Stemi 305).

Electron microscopy of the epibiotic microflora - Specimens of the three groups (epibiotic from the Harbor and non-epibiotic from the Harbor or from Le Laber) fixed in 2.5% glutaraldehyde were dehydrated in a series of ethanol solutions of progressively increasing concentrations (75–100%), critical-point-dried with a Balzers SCD 30 (temperature 37°C and pressure 70 kg cm), mounted on stubs, covered with a layer of 10–20 nm of gold, and observed under the SEM using a JEOL JSM-840A Scanning Electron Microscope at 20 kV accelerating voltage.

Fluorescence in situ hybridization (FISH) of epibiotic microflora - FISH experiments were performed using generalist probes targeting Eubacteria (EUB338), Gammaproteobacteria (GAM42), and the probe NON338 (antisense of EUB338) as a negative control (Amann et al., 1990; Manz et al., 1992). All hybridizations were conducted using 30% formamide at 46°C for 3 hours, followed by a 15 minutes rinse in appropriate buffer using the protocol described in (Duperron, 2017). FISH hybridizations were performed on whole specimens of *Capitella* fixed in paraformaldehyde 4% to visualize epibionts, as well as on 8µm-thick cross sections of specimens that were previously embedded in Steedman Wax as described in (Duperron et al., 2008), using DAPI as a background stain. Hybridized samples were visualized under a BX61 epifluorescence microscope (Olympus, Japan).

2.4. Assessing microorganisms biodiversity associated with *Capitella* by RNAseq sequencing

RNA extraction and sequencing – To assess microorganisms co-occurring with *Capitella*, RNAs from the three groups (see worm sampling) were extracted and sequenced to obtain transcriptomes representative of eukaryotes and prokaryotes associated with the worms. The total RNAs of each group were extracted with the TRI-Reagent solution (Sigma), following the manufacturer's protocol. The RNAs were re-suspended in DEPC-treated water and the quality

and quantity were evaluated on a Nanodrop. An Illumina library was prepared for each of the three groups. Each library was sequenced on one lane of HiSeq 2000 (100 million clusters, 2x100 bases paired-end). RNAseq sequencing was performed at Genoscreen (Lille, France).

Assembly and determination of the abundance of assembled contigs –The analyses were all carried out in the Galaxy environment and the computing power was provided by the ABiMS platform (Station Biologique de Roscoff, France). The 100-base sequences for each group were first filtered for quality with Prinseq-lite, and the pairs of sequences of sufficient quality were established (GetPairs) (Schmieder and Edwards, 2011). The ribosomal sequences were separated from the remaining sequences based on similarity with a rRNA database (riboPicker) (Schmieder et al., 2011). These reads (typically about 25 million paired reads per library) were then assembled with Trinity after normalization to reduce the size of the dataset. This was performed for the three libraries and the resulting contigs were concatenated. Redundancy was removed with CAP3 (Huang and Madan, 1999). This final assembly of all rRNA sequences in the libraries was then used as a reference for quantification of the contigs in each library with RSEM (Li and Dewey, 2011). The results were normalized for the size of the contigs, and the sequencing effort, and are expressed in Fragment Per Kilobase of transcript per Million reads of sequencing (FPKM). The closest sequences in GenBank were identified by Blastn and the identifier recovered for all contigs (Altschul et al., 1997).

Full length sequencing of the 16S rRNA from *Thiomargarita* sp. – DNA was extracted individually from four *Capitella* specimens, two with and two without visible epibionts, using the QiaQuick Kit (Qiagen, USA) according to the manufacturer's instructions. A fragment of the gene encoding bacterial 16S rRNA was amplified by PCR using primers 27F and 1492R as described in Muyzer et al. (1995) using 30 PCR cycles (Muyzer et al., 1995). For each gene and specimen, three PCR products were pooled, cloned using a TOPO TA Kit (Invitrogen, CA), and inserts from 96 clones were full-length sequenced (GATC Biotech, Germany). Sequences corresponding to *Thiomargarita* sp. were found in the two specimens displaying epibionts. A 16S rRNA dataset was built by collecting sequences available from *Thiomargarita* and related groups. Sequences were aligned using ClustalX (Larkin et al., 2007). Phylogenetic relationships were estimated based on maximum likelihood using a General Time Reversible (GTR) model and a 5-category discrete Gamma distribution of rates with invariants. Positions with gaps and missing data were not used, resulting in a 1107-bp dataset. Phylogenetic reconstructions were generated using the software MEGA 7 (Kumar et al., 2016).

2.5. Genotyping of epibiotic and non-epibiotic Capitella individuals

DNA extraction and barcoding – 57 *Capitella* collected during our temporal survey (see before) in both Le Laber and the Harbor were used entirely for DNA extraction using a NucleoSpin Tissue XS (Macherey-Nagel) according to manufacturer's protocol. A 569 bp fragment of the mitochondrial gene, cytochrome oxidase subunit 1 (Cox-1) was amplified using newly designed primers CO1F and CO1R: Forward 5'- GTACAGAACTTGCGCGTTCCT- 3' and Reverse 5'- CCACCACCAGTAGGATCAAA -3'. Amplifications were carried out with a GoTaq® G2 DNA Polymerase (Promega). Reaction mixture for PCR amplification contained 10 µM of each primer, 10 µM of each desoxynucleotide triphosphate (dNTP), 1X Go Taq® Flexi buffer (Promega), and 5U of GoTaq G2 Flexi DNA polymerase (Promega). The final volume was adjusted to 25 µl with sterile water. DNA amplification was performed on a Thermocycler (Eppendorf) with the following conditions: (1) an initial denaturation step at 95°C for 15 min without enzyme, followed by (2) a series of 39 cycles of denaturation at 95°C for 30 s, of annealing at 56°C for 30 s, and elongation at 72°C for 1 min with the enzyme, and (3) a final elongation step at 72°C for 5 min. The PCR products were then visualized onto a 1.5% agarose gel with ethidium bromide following electrophoresis at 100 volts for half an hour. PCR products were then purified with nucleofast 96 PCR cleanup kit and then Sanger-sequenced on an ABI 3100 using BigDye (PerkinElmer) terminator chemistry following the manufacturer's protocol. (Applied Biosystems, Foster City, CA).

Sequence analysis – Chromatograms were checked manually using SeqScape V2.5. The sequence data were aligned manually with BioEdit v.7.2.5. Sequences of *Capitella* from other localities were recovered from Genbank to position our sequences within the genus *Capitella* for phylogenetic purposes. Maximum likelihood tree reconstructions were performed using the software Mega7 following the HKY model of substitutions with the pairwise deletion option (Kumar et al., 2016) to check whether *Capitella* sp. populations found at Le Laber and the Roscoff harbor represent admixtures of cryptic species.

3. RESULTS

3.1. Geochemical characterization of sediments in both sites

General parameters - Sediment granulometry was very similar for both sites (Fig. S2): silts (2-63 µm) are the most abundant fraction (40-50%), and their proportions increased toward the sediment-water interface. In the fine fraction, smaller than 63 µm, the amount of Ca, Fe and

Al were higher in the Harbor than in Le Laber, suggesting that sandy particles, less reactive than clays, carbonates and iron oxides, were more frequent in sediments of Le Laber (Table 1). In the Harbor, the layer with the highest proportion of silts extends to a depth of about 3 cm when compared with the site Le Laber (less than 2 cm depth). In this top layer, organic and inorganic carbon contents were greater at the Harbor (Table S1). Total nitrogen contents however, are very similar. At sediment depth greater than 3.5 cm, no significant difference between the two sites was noticeable.

Reduced Sulfur Species – At the time when the worms were collected for NGS sequencing, the two locations greatly differed by the amount of sulfide in the upper layer of the sediment (Fig. 1A). At 1 cm depth, concentrations of reduced sulfur species were 5-6 times higher in the Harbor than in the Le Laber site. Below the depth of 3 cm, concentrations of AVS (the less stable fraction of solid reduced sulfur to oxidation) and CRS (the less reactive fraction of solid reduced sulfur) ranged from 141 to 978 mgS kg^{-1} and from 447 and 712 mgS kg^{-1} for the Harbor and Le Laber sites, respectively. At the water-sediment interface, the concentrations of solid reduced sulfur species increased in the Harbor but not in Le Laber sediment.

A survey of dissolved sulfide concentrations monitored with DGT-AgI probes in August-September 2015 showed that these species were more abundant in the deeper part of the cores (i.e. below 4-5 cm depth). Interestingly, sulfide concentrations were on average higher in the Harbor (from 8.2 to 11.60 mg L^{-1}) than in Le Laber (from 0.58 to 5.52 mg L^{-1}) (Fig. S3), in a way similar to the solid reduced sulfur species (AVS and CRS). More precisely, in the first 3 cm, where the worms live, the inter-site differences are even more marked, with levels ranging between 1.08 and 5.75 mg L^{-1} for the Harbor as opposed to 0 and 0.27 mg L^{-1} for Le Laber (Fig. S3 and Table 2).

Trace metals - Total metal concentrations (Cd, Co, Cr, Cu, Mn, Ni, Pb and Zn) in the sediments clearly indicate that the Harbor is enriched in metals when compared to Le Laber site (Table 1), especially for Cu for which the ratio reaches 6.1 (Harbor/Le Laber). Conversely, the ratio for Cr was close to 1. The toxicity index (TI) was calculated for each sample (averaged over the first 5 cm of sediment) from July to December 2015 (Fig. S4). During this period, all TI values remained below zero, indicating that no significant toxicity has been encountered in sediments (i.e. most of trace metals are efficiently trapped by sulfides forming AVS). The bioavailability of metals should therefore be extremely limited as sulfides are in excess. The TI

values are however consistently greater in the Harbor throughout the sampling period because of higher Zn concentration values.

3.2. Microbes and microeukaryotes associated with Capitella differ between Le Laber and the Harbor

Morphological observations – At the Harbor only during the RNAseq sampling in 2015 or at both locations during the seasonal survey, around 20% of worms exhibited an epibiosis with long white hair-like projection (Figs. 2B, D). Electron microscopy provides evidence of an assemblage of filamentous structures (Fig. 3A), with small microorganisms attached to larger and more visible ones strongly anchored in the tegument (Figs. 3B, C, D, E). During sampling for transcriptome sequencing, none displayed this characteristic feature at Le Laber. FISH hybridizations using the probe EUB338 confirmed that the body of all *Capitella* specimens with epibionts was covered by bacteria with various morphotypes including rods and filaments, and present in large numbers (Figs. 3F, G). The epibiotic microorganisms did not respond to FISH probes (see below), but the filamentous structures sitting on these projections were confirmed to be bacterial morphotypes including slender filaments.

A few worms were also parasitized by nematodes (*Trophomera* sp.) living in the coelomic cavity of the worm (Figs.4D, E), by vorticellid ciliates attached to the tegument (Figs. 4A, B,C) or by gut gregarines (*Ancora saggitata*) (Figs. 4F, G).

Abundance of symbionts lineages based on RNAseq data – The most abundant assembled rRNAs correspond to *Capitella* RNAs and were not considered in the following analyses. The other recovered contigs correspond to organisms associated to *Capitella*, which could be either epibionts (tegument), part of the gut contents, or parasites. In the following analyses, we only considered contigs with abundances greater than 100 FPKM in at least one of the libraries. Some of these may correspond to different fragments of the same organism (e.g. fragment of 28S, another fragment of 28S, fragment of 18S, etc.). The sequence assembly followed by quantification allowed us to identify contigs corresponding to associated organisms that are found in all three groups (1/ non epibiotic *Capitella* from Le Laber 2/ non epibiotic *Capitella* from the Harbor and 3/ epibiotic *Capitella* from the Harbor) but in variable abundance (Table 3), contigs that are more common at Le Laber (Table 4), and contigs that are more abundant in the group corresponding to animals with epibiotic microorganisms (Table 5). Capitella from the three compared groups are host to a variety of eukaryotes at intermediate occurrence (Tables 3-5).

Among the organisms found in all three groups in variable proportions, there is a total of 51 contigs with abundances greater than 100 FPKM in this category (Table 3). The great majority of these organisms are eukaryotes (86.3%), in particular apicomplexan fish parasites (31.8%) for which *Capitella* could be an intermediary host (e.g. *Eimeria leucisci*, *Sphaerospora dicentrarchi*, *Kudoa iwatai*, *Sarcocystis* sp). The contig that ranks second in Table 3 corresponds to the known parasitic gut gregarine *Ancora sagittata* (Apicomplexa, Ancoridae) (Simdyanov et al., 2017) specifically associated to Capitella (Fig. 4). Other abundant types of organisms are ciliates (e.g. *Vorticella* sp.), nematodes, and annelids that could be part of the gut contents *(Paramphinome jeffreysi*, *Tubificoides brownie*; Fig. 3). Bacteria occupy lower ranks in this list (ranks 28, 31, 34, 35, 44, 49, and 51), which could be a reflection of their lesser abundance and also the fact they are single-celled (as opposed to most eukaryotes mentioned earlier). The 15 top-ranking contigs are usually more abundant in the epibiotic animals from the Harbor, with the exception of ranks 1 (a polychaete, possibly from the gut contents), 6 and 11 (a nematode) that are more abundant in the animals from the control site. The animals from the Harbor that do not exhibit an epibiosis tend to have low or very low abundances of these contigs.

The animals from Le Laber site harbor a series of taxa corresponding to the contigs that are found in very low abundances in the Harbor (Table 4). Six of these eight contigs correspond to apicomplexan parasites, the two remaining ones correspond to a bivalve (likely found in the gut contents), and *Corynebacterium*.

The contigs that are found in much greater abundance in epibiotic animals (Table 5) differ greatly from the organisms found in Tables 3 and 4. The *Capitella* specimens from the two other groups (non epibiotics from the Harbor and from le Laber) have very low abundances of these contigs (FPKM ≤ 20). 31 out of the 38 contigs (81.6%) correspond to bacteria, mostly within the Gammaproteobacteria. Eight of these bacterial contigs (21%) correspond to sulfur-oxidizing bacteria (*Thiomargarita*, the most abundant, and *Thioalkalivibrio*). Six of the bacterial contigs (15.8%) correspond to mollicutes, a group usually found in the guts of invertebrates.

The PCR-cloning-sequencing results (Table S4) confirm the main observations made from RNASeq data. Gammaproteobacteria (oceanospirillales, closely related to *Endozoicomonas*) are the most abundant clones (68 out of 93 sequences), and these are found on both animals with and without epibiosis. Six sequences (out of 93) correspond to *Thiomargarita*, and a

minority of sulfide oxidizers (*Thiotrix*). Finally, mollicutes and spirochaetes are also represented.

3.3. Phylogenetic affiliation of the large bacterial epibiont to the genus *Thiomargarita*

As for RNASeq, sequences related to *Thiomargarita* were found only in *Capitella* specimens harboring the epibiotic flora. All these sequences were identical, representing a single 16S rRNA phylotype that shared 98 % of sequence identity with sequences of *Candidatus 'Thiomargarita nelsonii'* recovered from the Costa Rica margin and the Namibian upwelling zone and is well distinct from the *Candidatus 'T. namibiensis'* clade (Salman et al., 2011) (Fig. 5). Under the fluorescence microscope, *Capitella* specimens with epibiotic organisms displayed dense coverage of their surface by microorganisms compared to non-epibiotic specimens (Figs. 2, 3). Morphotypes resembling some *Thiomargarita* recently documented attached to shells of gastropods and byssal threads of mussels *Bathymodiolus* from cold seep were found on epibiotic *Capitella* at lower than in these previously accounted for other invertebrates (Bailey et al., 2011; Salman et al., 2011). *Thiomargarita* are recognizable thanks to their rod shape and very large size (for bacteria) reaching 50 microns from basal to apical ends, the lack of a nucleus based on DAPI staining (Fig 3E), the presence of a large vacuole in the center of the cells, and inclusions resembling sulfur granules within their cytoplasm (Fig 3D, E). The presence of a single second cell apparently budding from the apical end of the animal-attached bacterium is another feature already documented in *Thiomargarita*, although daughter cells were previously described as round-shaped, while they appear as rods here (See (Bailey et al., 2011) and Fig. 2E, 3D). The *Thiomargarita*-like morphotypes did neither respond to the generalist EUB338 and GAM42 probes, nor to a specific probe designed to target the identified 16S rRNA sequence.

3.4. Prevalence of the epibiosis with *Thiomargarita* according to the season, the *Capitella's* size and sex

Observed prevalence of *Thiomargarita* fluctuated between zero and 0.44 at a single sampling date (average over the year: 0.10), with 95% of observations between zero and 0.31 and a median single-sampling date prevalence of 0.08. Worm size (width at the fourth setiger) varied between 0.19 and 1.56 mm (average: 0.54 mm) with a slightly fluctuating average value (between 0.43 and 0.68 mm), without any clear temporal trend. The numbers of males, females and undetermined individuals also do not vary much between sampling times (Fig.

S7). A statistical analysis of time-series was performed using the association occurrences as a quantitative variable and the sampling date, size and gender of the worm as explanatory variables. Overall, the probability of association with *Thiomargarita* increases in summer and increases with the worm's size (Fig. 6). Independently of worm's size, this probability is also higher for males and undetermined than for females (Figs. S5 and S6). As many models have comparable AICc and Akaike values (Table S5), model predictions have been explored using the Akaike-weighted average of all tested models (Figs. 6, S5 and S6). The analysis of evidence ratios of each individual explanatory variable or combination of explanatory variables (Table S6) indicates that all variables have likely effects, except 'site' (*implausible* effect), and 'sex:date', 'sex:date²' and 'sex:size:date²' interactions (only *plausible* effects) using the vocabulary of (Massol et al., 2007).

3.5. Prevalence of the epibiosis with *Thiomargarita* according to *Capitella* genotypes

As species belonging to the genus *Capitella* often represent complexes of cryptic species (see Grassle et al. 1985), a series of individuals with and without epibionts from Le Laber and the Harbor were barcoded using the mitochondrial *Cox-1* gene to test whether the epibiotic association was species-specific. The obtained phylogenetic tree (Fig. 7) revealed the co-occurrence of three different mitochondrial lineages in *Capitella* sp. The lineages are divergent enough to represent distinct cryptic species with a minimum divergence between the two most related taxa of c.a. four fixed substitutions. The two most closely related species dominate the assemblage and correspond to about 90% of the sampling. These OTUs are grouped into specific clades, which clearly differ from *Capitella teleta* and *Capitella capitata* which were described from the East coast of the USA and Canada, respectively, but also collected in the Mediterranean Sea. The epibiosis with the *Thiomargarita*-like epsilon proteobacteria was checked and present on all the mitochondrial lineages examined.

Table 1: Total and HCl 1M-extracted metals concentrations in the first 5 cm depth sediments of Le Laber and the harbor (fraction <63µm). For HCl 1M extraction, an average has been calculated from results obtained between July and December 2015. See table S3 for discrete values.

		Concentration (mg kg^{-1})								Concentration (g kg^{-1})			
		Cd	Co	Cr	Cu	Mn	Ni	Pb	Zn	Ca	Fe	Mg	Al
Laber	Total	0.1	6.5	51	6.0	165	6.9	19.4	42	14	10.2	4.3	38.5
	HCl 1M	0.1	ND	2.2	1.6	15	0.9	4.1	11.1	5.6	2.1	0.8	-
	Lability (%)	76.0	-	4.3	17.9	9.0	13.7	21.3	26.6	39.2	20.9	18.7	-
Harbor	Total	0.4	10.1	54	37.6	264	13.3	30	111	69	21.2	10.7	51.2
	HCl 1M	0.1	0.0	4.8	6.7	32	1.6	8.6	27.8	15	3.8	0.9	-
	Lability (%)	15.1	0.1	8.7	17.9	12.1	11.7	28.7	25.1	21.2	18.1	8.5	-
Ratio of tot Harbor/Laber		4	1.55	1.06	6.27	1.60	1.93	1.55	2.64	4.93	2.08	2.49	1.33

ND: Not detected

Table 2: Dissolved sulfide concentrations (mg L^{-1}). Averaged values for 0-3, 3-15 and 0-15 cm sedimentary horizons from Le Laber and Roscoff Harbor.

		Concentration (mg/L)							
		11/8	12/8	18/8	21/8	1/9	9/9	15/9	24/9
Le Laber	**0-3 cm**		**0.15**	**0.01**		**<0.005**	**-**	**<0.005**	**0.27**
	3-15 cm		0.68	3.1		3.1		5.9	6.8
	0-15 cm		0.58	2.5		2.5		4.8	5.5
Roscoff harbor	**0-3 cm**	**5.8**			**1.1**		**2.8**		
	3-15 cm	13			10		12		
	0-15 cm	12			8.2		9.8		

Table 3: Contigs with intermediate FPKM values (ratios between 30 and 0.03). Le Laber sample (FPKM1), Roscoff harbor sample without (FPKM2) or with (FPKM3) epibiotic microorganisms. Only hits for FPKM values greater than 100 are represented. Contigs ranked in decreasing order of the greatest FPKM value (shaded in grey).

Accession number	E-value	Genbank description	Biology	FPKM1	FPKM2	FPKM3
AY838865.1	0	*Paramphinome jeffreysi* 28S ribosomal RNA gene, partial sequence	Polychaete	2702	658	243
KX982503.1	0	*Ancora sagittata* isolate Ancora2011 external transcribed spacer, partial sequence; 18S rRNA gene, ITS 1, 5.8S rRNA gene, ITS 2, and 28S rRNA gene, complete sequence; and external transcribed spacer, partial sequence	Gregarine of *Capitella*	925	48	1594
DQ779991.1	$6\ 10^{-88}$	*Gymnodinium aureolum* strain GrAr01 18S ribosomal RNA gene, partial sequence; ITS 1, 5.8S ribosomal RNA gene, ITS 2, and large subunit ribosomal RNA gene, complete sequence; external transcribed spacer, partial sequence	Dinoflagellate algae	1042	19	1550
EF100367.1	10^{-114}	Uncultured eukaryote clone D5P10A10 18S ribosomal RNA gene, partial sequence	Apicomplexan?	509	33	1134
EF100398.1	0	Uncultured eukaryote clone D2P03E11 18S ribosomal RNA gene, partial sequence	?	875	31	1086
GU479649.1	0	*Eimeria leucisci* isolate BLI637-#637 18S ribosomal RNA gene, partial sequence	Coccidian apicomplexa fish parasite	936	43	106
AY179976.1	$9\ 10^{-177}$	Uncultured eukaryote clone CCI31 18S small subunit ribosomal RNA gene, partial sequence	Apicomplexan?	530	258	869
KC558064.1	$5\ 10^{-89}$	Uncultured fungus clone NTS_28S_047E_2_f6 28S ribosomal RNA gene, partial sequence	?	121	0	863
JX178933.1	0	*Vorticella* sp. 4 JG-2011 clone 33 18S ribosomal RNA gene, partial sequence; ITS 1, 5.8S ribosomal RNA gene, and ITS 2, complete sequence; 28S ribosomal RNA gene, partial sequence	Peritrich ciliate	150	170	840
GU927604.1	$4\ 10^{-94}$	Uncultured eukaryote clone F5K2Q4C04IDZ7B 28S ribosomal RNA gene, partial sequence	Ciliate	166	16	636
EF990727.1	$5\ 10^{-88}$	*Rhabditoides inermiformis* strain SB328 28S large subunit ribosomal RNA gene, partial sequence	Free-living nematodes	634	25	542
AY256244.1	$2\ 10^{-119}$	Uncultured eukaryote isolate E6 small subunit ribosomal RNA gene, partial sequence	?	369	152	586
GU927618.1	$4\ 10^{-94}$	Uncultured eukaryote clone F5K2Q4C04IVOMC 28S ribosomal RNA gene, partial sequence	?	88	0	578
AY835682.2	0	Uncultured peritrich clone IAFDv27 18S ribosomal RNA gene, partial sequence	Peritrich ciliate	125	23	555
GU927271.1	$4\ 10^{-25}$	Uncultured eukaryote clone F5K2Q4C04H81PH 28S ribosomal RNA gene, partial sequence	?	252	56	430
AB189984.1	$7\ 10^{-83}$	*Contracaecum spiculigerum* gene for 28S ribosomal RNA, partial sequence	Bird parasite	46	446	128
KF147653.1	0	Nematoda environmental sample clone NEMAK34 18S ribosomal RNA gene, partial sequence	?	156	7	414

FJ417074.1	0	*Sphaerospora* sp. M0379 28S large subunit ribosomal RNA gene, partial sequence	Fish parasite	285	8	76
AY641571.1	0	*Kudoa iwatai* isolate J small subunit ribosomal RNA gene, partial sequence	Fish parasite	276	6	102
FQ032815.1	$4\ 10^{-131}$	Uncultured *Sphingobacteria* bacterium, whole genome shotgun sequence	?	265	0	11
JQ723993.1	$5\ 10^{-180}$	*Vorticellides* sp. 2 MD-2012 small subunit ribosomal RNA gene, partial sequence; macronuclear	Peritrich ciliate	58	29	257
AY179976.1	$3\ 10^{-177}$	Uncultured eukaryote clone CCI31 18S small subunit ribosomal RNA gene, partial sequence	?	246	67	76
HM031979.1	0	*Cytophaga* sp. UDC385 16S ribosomal RNA gene, partial sequence	Free-living bacterium	244	2	15
JX391440.1	$5\ 10^{-150}$	Uncultured bacterium clone N0004 16S ribosomal RNA gene, partial sequence	?	233	0	18
EF067920.1	$5\ 10^{-98}$	*Phaeodactylum tricornutum* chloroplast, complete genome	Diatom	222	0	117
DQ377695.1	0	*Sphaerospora* sp. IF-2006 from Mugil curema small subunit ribosomal RNA gene, partial sequence	Fish parasite	218	0	75
EF100398.1	0	Uncultured eukaryote clone D2P03E11 18S ribosomal RNA gene, partial sequence	?	191	15	155
FJ417074.1	$8\ 10^{-178}$	*Sphaerospora dicentrarchi* isolate M0749 28S large subunit ribosomal RNA gene, partial sequence	Fish parasite	170	0	81
GU928472.1	$6\ 10^{-68}$	Uncultured eukaryote clone F5K2Q4C04H5ZBN 28S ribosomal RNA gene, partial sequence	?	33	0	169

KF601317.1	$4\ 10^{-54}$	*Sarcocystis arctica* isolate VI2.2 28S ribosomal RNA gene, partial sequence	Vertebrate parasite	201	0	398
KC869522.1	$9\ 10^{-88}$	*Isodictya grandis* voucher NCI439 28S ribosomal RNA gene, partial sequence	Marine sponge	380	74	394
FJ969135.1	0	*Plectus tenuis* strain ChGaSp5 small subunit ribosomal RNA gene, partial sequence	Free-living nematodes	17	22	371
EF100367.1	$3\ 10^{-105}$	Uncultured eukaryote clone D5P10A10 18S ribosomal RNA gene, partial sequence	?	366	153	60
AB611781.1	$3\ 10^{-64}$	*Fukuia kurodai* ooyagii gene for 28S ribosomal RNA, partial sequence, specimen_voucher: personal:Kameda Y.:5609	Gastropod	343	0	240
EF100367.1	$3\ 10^{-125}$	Uncultured eukaryote clone D5P10A10 18S ribosomal RNA gene, partial sequence	Apicomplexan?	173	0	323
FJ417074.1	0	*Sphaerospora dicentrarchi* isolate M0749 28S large subunit ribosomal RNA gene, partial sequence	Fish parasite	320	3	65
KF147653.1	0	Nematoda environmental sample clone NEMAK34 18S ribosomal RNA gene, partial sequence	?	11	38	319
FJ417074.1	10^{-140}	*Sphaerospora dicentrarchi* isolate M0749 28S large subunit ribosomal RNA gene, partial sequence	Fish parasite	306	0	69
JX391808.1	0	Uncultured bacterium clone NS093 16S ribosomal RNA gene, partial sequence	?	302	0	23
HG315671.1	0	*Formosa agariphila* KMM 3901, complete genome	Algal bacterial associate	287	1	19

AF185190.1	$2\ 10^{-133}$	Eurythoe sp. AMW4444 28S ribosomal RNA gene, partial sequence	Polychaete	154	36	117
FN563149.1	0	*Rhodococcus equi* 103S chromosome	Pathogen causing pneumonia in horses	148	1	7
AB636470.1	10^{-98}	*Kudoa ogawai* gene for 18S ribosomal RNA, partial sequence	Fish parasite	142	0	81
FJ557946.1	0	Uncultured bacterium clone ET_G_4f03 16S ribosomal RNA gene, partial sequence	?	135	0	6
FJ417058.1	10^{-69}	*Kudoa dianae* isolate M0290 28S large subunit ribosomal RNA gene, partial sequence	Fish parasite	133	0	77
GU479649.1	0	*Tubificoides brownae* isolate CE3387 18S ribosomal RNA gene, partial sequence; ITS 1, 5.8S ribosomal RNA gene, and ITS 2, complete sequence; 28S ribosomal RNA gene, partial sequence	Free-living oligochaete	131	0	13
FJ417074.1	$4\ 10^{-103}$	*Sphaerospora dicentrarchi* isolate M0749 28S large subunit ribosomal RNA gene, partial sequence	Fish parasite	128	18	37
JX178767.1	$2\ 10^{-108}$	*Zoothamnium* sp. 1 JG-2011 18S ribosomal RNA gene, partial sequence	Peritrich ciliate	95	49	120
CP004404.1	$7\ 10^{-169}$	*Psychromonas* sp. CNPT3, complete genome	Facultative anaerobic free-living	21	0	113
JQ743689.1	$6\ 10^{-134}$	Uncultured peritrich ciliate clone GDH_F10 18S ribosomal RNA gene, partial sequence	Peritrich ciliate	46	113	33
KF077586.1	0	Uncultured bacterium clone nck74g02c1 16S ribosomal RNA gene, partial sequence		103	0	3

Table 4: Contigs found with greater FPKM values in Le Laber sample (FPKM1) compared to the Roscoff harbor without (FPKM2) or with (FPKM3) epibiotic microorganisms samples. Only hits for FPKM values greater than 100 are represented. Contigs ranked according to decreasing values of FPKM1.

Accession number	**E-value**	**Genbank description**	**Biology**	**FPKM1**	**FPKM2**	**FPKM3**
KC816721.1	$8\ 10^{-113}$	Apicomplexa sp. type N clone N66 clone 2 18S ribosomal RNA gene, partial sequence	Coral parasite	675	0	0
JX044549.1	$7\ 10^{-77}$	*Toxoplasma gondii* strain CASTELLS chromosome Ia region 5 genomic sequence	Animal parasite	651	95	1
JN256118.1	$6\ 10^{-72}$	*Sarcocystis* sp. ex *Corvus monedula* isolate kuos1 28S ribosomal RNA gene, partial sequence	Bird parasite	582	0	1
FJ417076.1	$4\ 10^{-114}$	*Sphaerospora* sp. M0379 28S large subunit ribosomal RNA gene, partial sequence	Fish parasite	478	79	2
X75453.1	$7\ 10^{-99}$	*Toxoplasma gondii* (strain P) rDNA for 17s,5.8s,26s, and 5s ribosomal RNA	Animal parasite	468	5	0
CP001601.1	0	*Corynebacterium aurimucosum* ATCC 700975, complete genome	Mycolic acid-containing actinomycetes	436	0	2
HQ243019.1	$3\ 10^{-35}$	Uncultured *Glomus* clone ZHwq2-227 18S rRNA gene, partial sequence; ITS 1, 5.8S rRNA gene, and ITS 2, complete sequence; and 28S rRNA gene, partial sequence	Bivalve	355	0	0
AF109679.1	$5\ 10^{-58}$	*Sarcocystis mucosa* small subunit ribosomal RNA gene, partial sequence	Mammal parasite	211	16	0

Table 5: Contigs found with FPKM values at 50 times greater in animals with (FPKM3) and without (FPKM2) epibiotic organisms compared with animals from Le Laber (FPKM1). Only hits for FPKM values greater than 100 are represented. Contigs ranked according to decreasing values of FPKM3.

Accession number	E-value	Genbank description	Biology	FPKM1	FPKM2	FPKM3
HF954103.1	0	Uncultured *Thiomargarita* sp. partial 16S rRNA gene, clone NAM094	Giant sulfur bacterium	2	0	2708
JX198551.1	0	Uncultured bacterium clone Tui57 16S ribosomal RNA gene, partial sequence	Oceanospirillales symbiotic with vent snail *Alviniconcha*	19	0	2477
JQ768460.1	$7\ 10^{-130}$	*Spiroplasma* sp. crk 16S ribosomal RNA gene, partial sequence; 16S-23S ribosomal RNA intergenic spacer, 23S ribosomal RNA gene, and 23S-5S ribosomal RNA intergenic spacer, complete sequence; and 5S ribosomal RNA gene, partial sequence	Field cricket gut mollicute	5	0	1897
NR_121985.1	0	Candidatus *Hepatoplasma crinochetorum* 23S ribosomal RNA, complete sequence	Isopod midgut gland mollicute bacterium	4	7	1625
NR_076721.1	0	*Thioalkalivibrio* sp. K90mix strain K90mix 23S ribosomal RNA, complete sequence	Haloalkaliphilic sulfur-oxidizing bacterium	9	1	1359
FR774200.1	0	*Thiomargarita* sp. NAM092 partial 23S rRNA gene and ITS1, clone NAM092	Giant sulfur bacterium	3	0	1257
FJ654610.1	0	Uncultured gamma proteobacterium clone 005_D02_06-017371_low_week_1 16S ribosomal RNA gene, partial sequence	Cnidarian-associated	20	0	1239
JN935865.1	$5\ 10^{-66}$	*Mycoplasma pulmonis* strain Ash (PG34) 16S ribosomal RNA gene, partial sequence; 16S-23S ribosomal RNA intergenic spacer, complete sequence; and 23S ribosomal RNA gene, partial sequence	Animal parasite	0	0	1219
FR690959.1	$3\ 10^{-121}$	Candidatus *Thiomargarita nelsonii* partial 16S rRNA gene and ITS1, isolate NAM071	Giant sulfur bacterium	0	0	1170
FR690946.1	10^{-109}	Candidatus *Thiomargarita nelsonii* partial 16S rRNA gene, isolate NAM057	Giant sulfur bacterium	0	0	1167
EU795103.1	0	Uncultured bacterium ARCTIC45_G_10 genomic sequence	?	7	0	1004
FO203512.1	10^{-74}	*Oleispira antarctica* strain RB-8, complete genome sequence	Hydrocarbonoclastic aerobic bacterium	0	0	957
HQ153940.1	0	Uncultured bacterium clone V1SC07b35 16S ribosomal RNA gene, partial sequence	Hydrothermal vent microbial mats	8	0	819
EU101262.1	0	Uncultured bacterium clone RS06101_B70 16S ribosomal RNA gene, partial sequence	Sulfur-oxidizing	0	0	778
NR_076671.1	0	*Kangiella koreensis* DSM 16069 strain DSM 16069 23S ribosomal RNA, complete sequence	Oceanospirillales free-living bacterium	3	0	589
NR_076212.1	$2\ 10^{-131}$	*Treponema denticola* ATCC 35405 strain ATCC 35405 23S ribosomal RNA, complete sequence	Periodontal disease associate	5	0	546
GU567978.1	$6\ 10^{-93}$	Uncultured gamma proteobacterium HF0200_34B07 genomic sequence	?	0	0	522

GU928698.1	$9\ 10^{-90}$	Uncultured eukaryote clone F5K2Q4C04JA1DB 28S ribosomal RNA gene, partial sequence	?	1	0	521
FJ202296.1	0	Uncultured bacterium clone SGUS1039 16S ribosomal RNA gene, partial sequence	?	1	0	521
HE610322.1	10^{-140}	Uncultured Mycoplasmataceae bacterium partial 16S rRNA gene, clone 3-B9	Mud-crab intestinal mollicute	4	0	476
EF990727.1	$4\ 10^{-116}$	*Rhabditoides inermiformis* strain SB328 28S large subunit ribosomal RNA gene, partial sequence	Nematodes living on vegetation debris	0	0	466
HE610322.1	0	Uncultured Mycoplasmataceae bacterium partial 16S rRNA gene, clone 3-B9	Mud-crab intestinal mollicute	3	0	464
NR_076858.1	10^{-58}	*Marinomonas mediterranea* MMB-1 strain MMB-1 23S ribosomal RNA, complete sequence	Free-living melanogenic bacterium	0	0	460
NR_076770.1	10^{-144}	*Spirochaeta smaragdinae* DSM 11293 strain DSM 11293 23S ribosomal RNA, complete sequence	Free-living thiosulfate and sulfur reducer	3	0	432
EU101262.1	$2\ 10^{-169}$	Uncultured bacterium clone RS06101_B70 16S ribosomal RNA gene, partial sequence	Sulfur-oxidizing	1	0	381
NR_121913.1	0	*Desulfuromonas acetoxidans* strain DSM 684 23S ribosomal RNA, complete sequence	Anaerobic sulfur reducer	3	0	380
EU101262.1	0	Uncultured bacterium clone RS06101_B70 16S ribosomal RNA gene, partial sequence	Sulfur-oxidizing	1	0	314
GU908489.1	0	*Spiroplasma litorale* 16S ribosomal RNA gene, partial sequence; 16S-23S ribosomal RNA intergenic spacer, 23S ribosomal RNA gene, and 23S-5S ribosomal RNA intergenic spacer, complete sequence; and 5S ribosomal RNA gene, partial sequence	Dipteran-associated gut mollicute	4	1	311
HM103460.1	$4\ 10^{-93}$	Uncultured metazoan clone Ma29_1E_24 18S ribosomal RNA gene, partial sequence	?	0	0	290
FO203512.1	0	*Oleispira antarctica* strain RB-8, complete genome sequence	Hydrocarbonoclastic aerobic bacterium	0	0	262
JN018328.1	$3\ 10^{-56}$	*Damon gracilis* voucher MNHN-JAB38 28S ribosomal RNA gene, partial sequence	Whip-spider	0	0	230
NR_102551.1	0	*Thioalkalivibrio nitratireducens* DSM 14787 strain DSM 14787 23S ribosomal RNA, complete sequence	Haloalkaliphilic sulfur-oxidizing bacterium	1	2	208
DQ174761.1	$2\ 10^{-73}$	Uncultured spirochete clone HaTB8 large subunit ribosomal RNA gene, partial sequence	Coral protistan agal symbiont	2	0	205
AJ879862.1	$2\ 10^{-103}$	Uncultured organism 28S rRNA gene, clone ASt-53	?	0	0	176
JN145195.1	$2\ 10^{-81}$	Uncultured eukaryote clone NZAS-293 18S ribosomal RNA gene, partial sequence	?	0	20	167
NR_076865.1	$3\ 10^{-61}$	*Desulfobacca acetoxidans* DSM 11109 strain DSM 11109 23S ribosomal RNA, complete sequence	Deltaproteobacterium Sulfate reducer	0	0	138
GU245692.1	$4\ 10^{-59}$	*Krefftascaris sharpiloi* isolate 2 18S rRNA gene, partial sequence; ITS 1, 5.8S rRNA gene, and ITS 2, complete sequence; and 28S rRNA gene, partial sequence	Turtle parasite	0	0	137
NR_103985.1	$2\ 10^{-77}$	*Spiroplasma chrysopicola* DF-1 strain DF-1 23S ribosomal RNA, complete sequence	Dipteran-associated gut mollicute	1	0	116

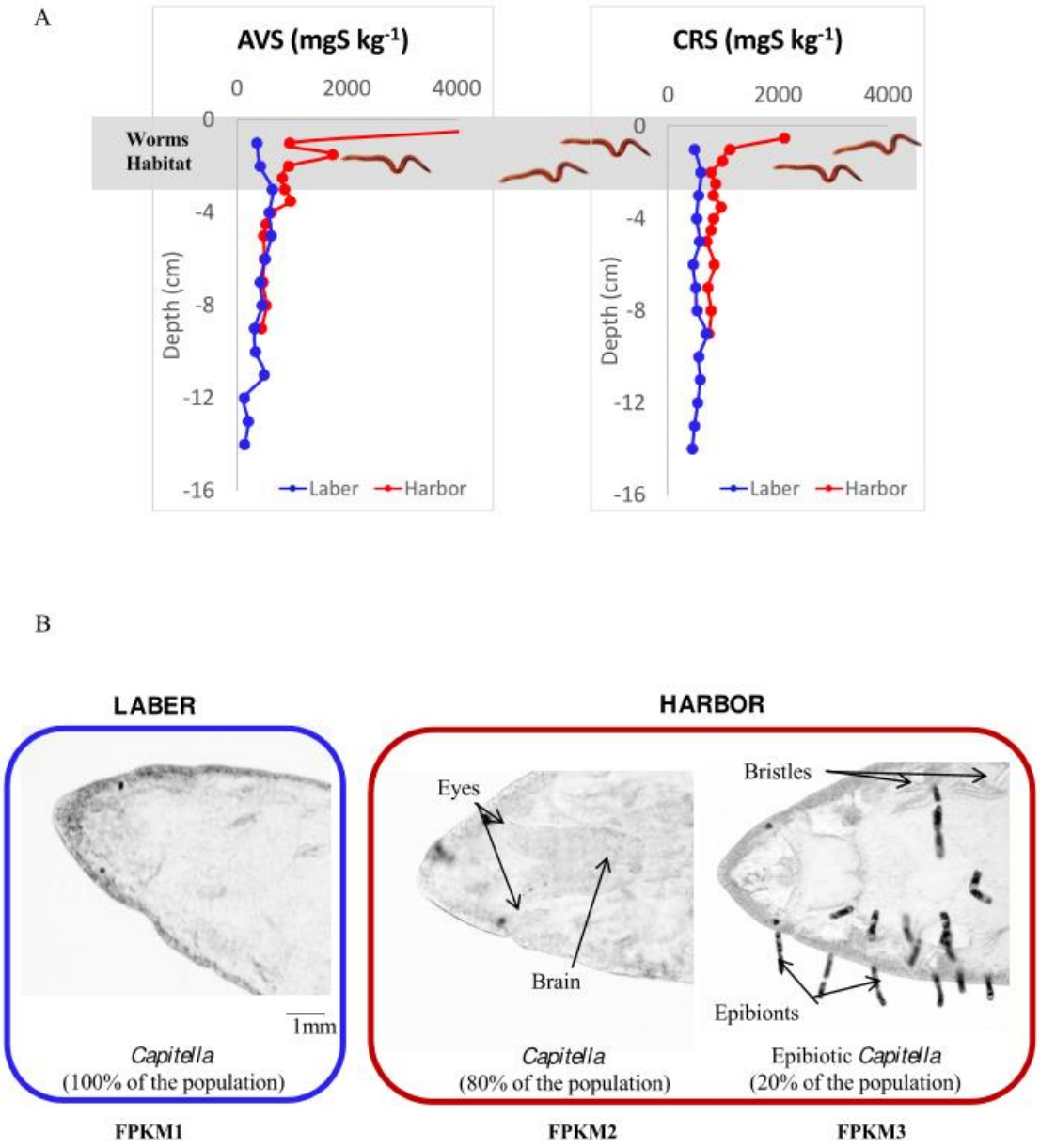

Figure 1: (A) Sedimentary AVS and CRS concentration profiles (mgS kg^{-1} sed) in Roscoff harbor (blue line) and Le Laber (red line) performed in November 2014 (corresponding to the sampling of the animals for NGS sequencing). (B) Semi-thin sections of *Capitella* sampled for the NGS sequencing: not colonized (in Le Laber, FPKM1 and in the Harbor, FPKM2) and colonized by the epibiotic community (in the Harbor only, FPKM3).

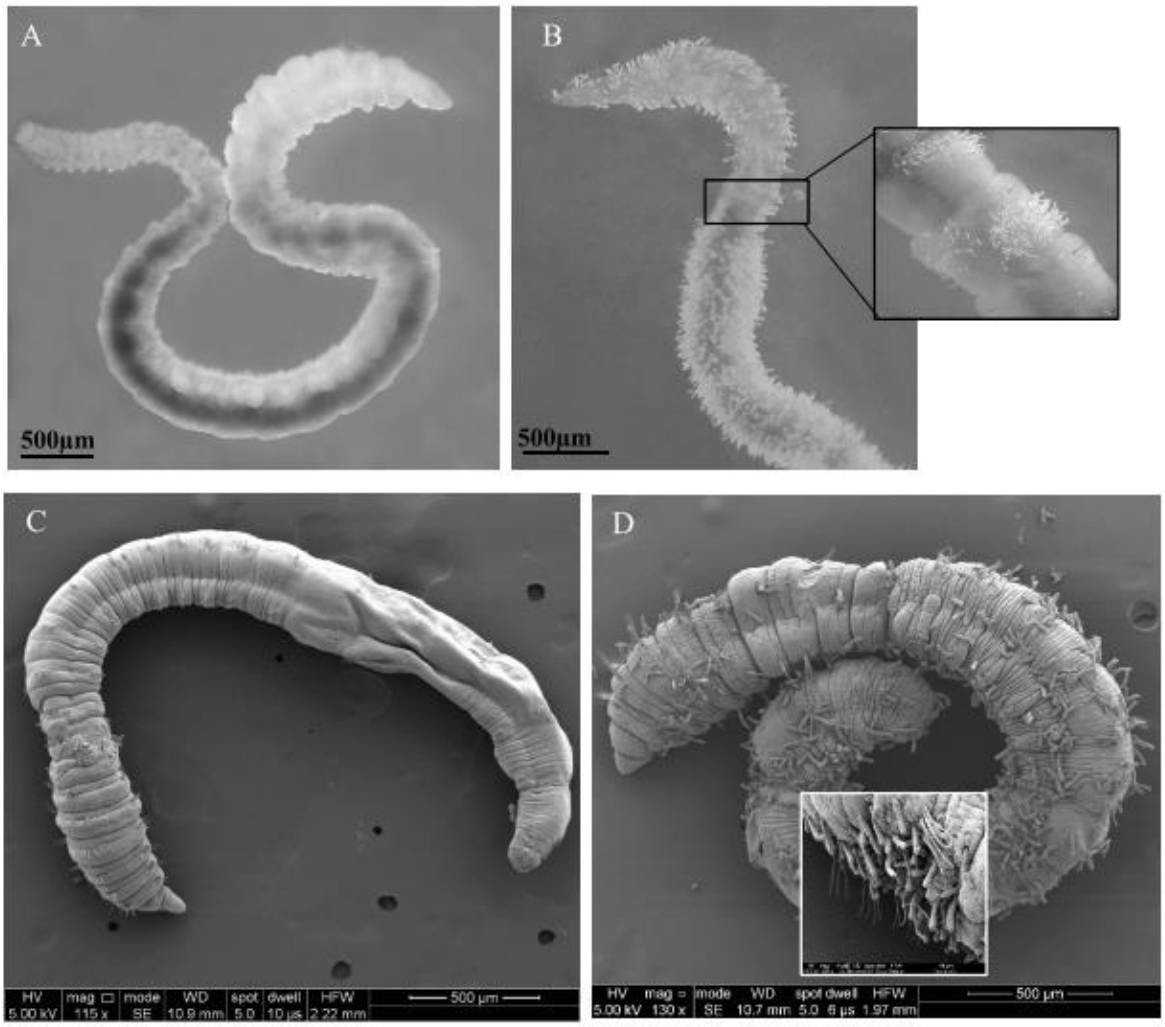

Figure 2: Visible (top) and electron microscopy (bottom) showing non epibiotic (A, C) and epibiotic *Capitella* (B, D). Squares show a zoom on the microbial epibiotic community.

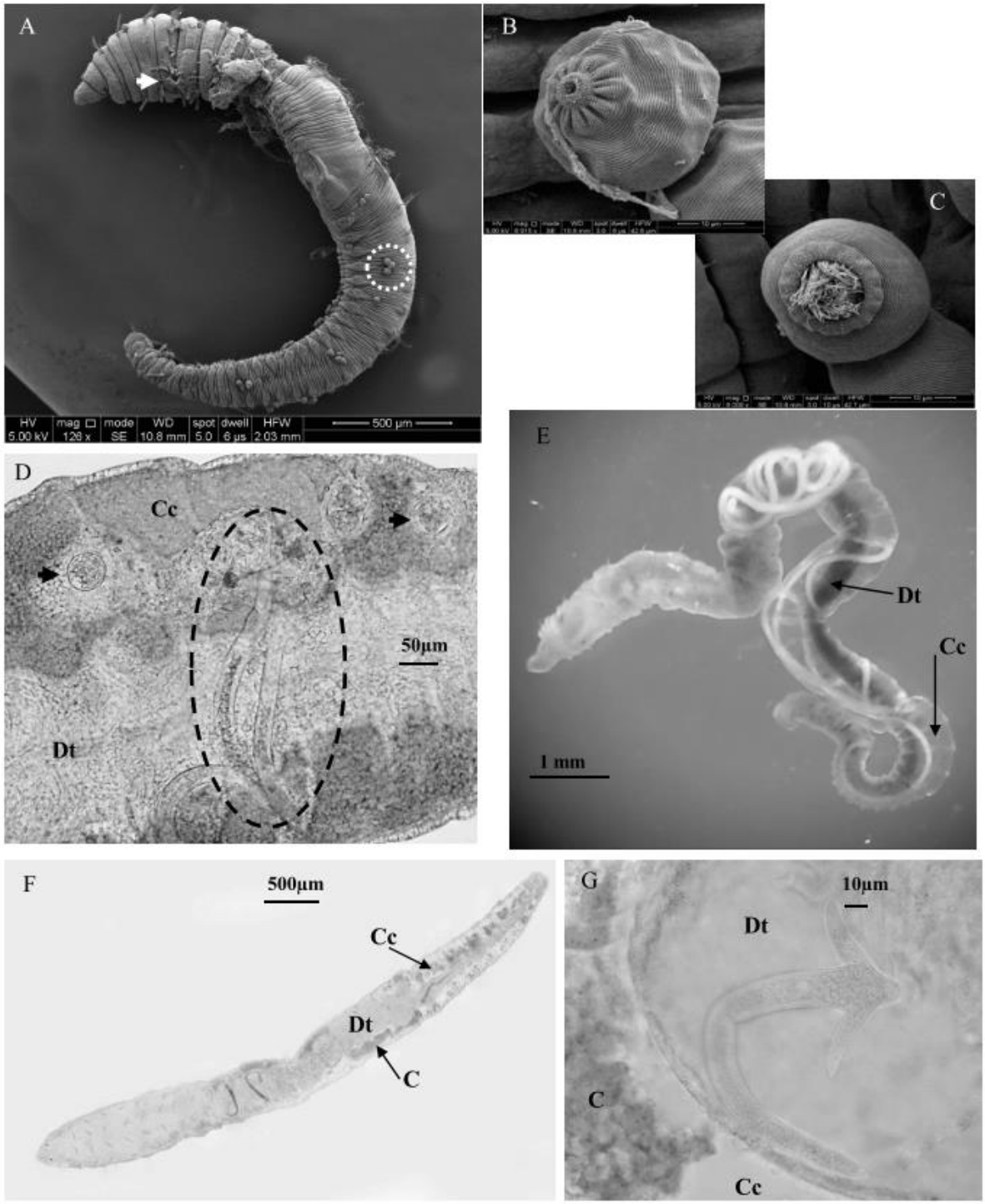

Figure 3: Epibionts of *Capitella* spp. (A) Electron microscopy of the *Thiomargarita*-like bacteria (C) Notice that *Thiomargarita*-like bacteria are strongly anchored on the tegument and (A, B) themselves host epibiotic communities most likely consisting of bacteria some displaying filamentous morphologies. (D) Several *Thiomargarita*-like structures and other microbial morphotypes. (E) DAPI staining of a *Thiomargarita*-like structure (in the center) attached to the tegument of *Capitella*. (F, G) FISH hybridization on the tegument of an epibiotic *Capitella* specimen using the generalist probe EUB338. Notice the abundance and diversity of bacterial morphologies including rods, cocci and filamentous bacteria.

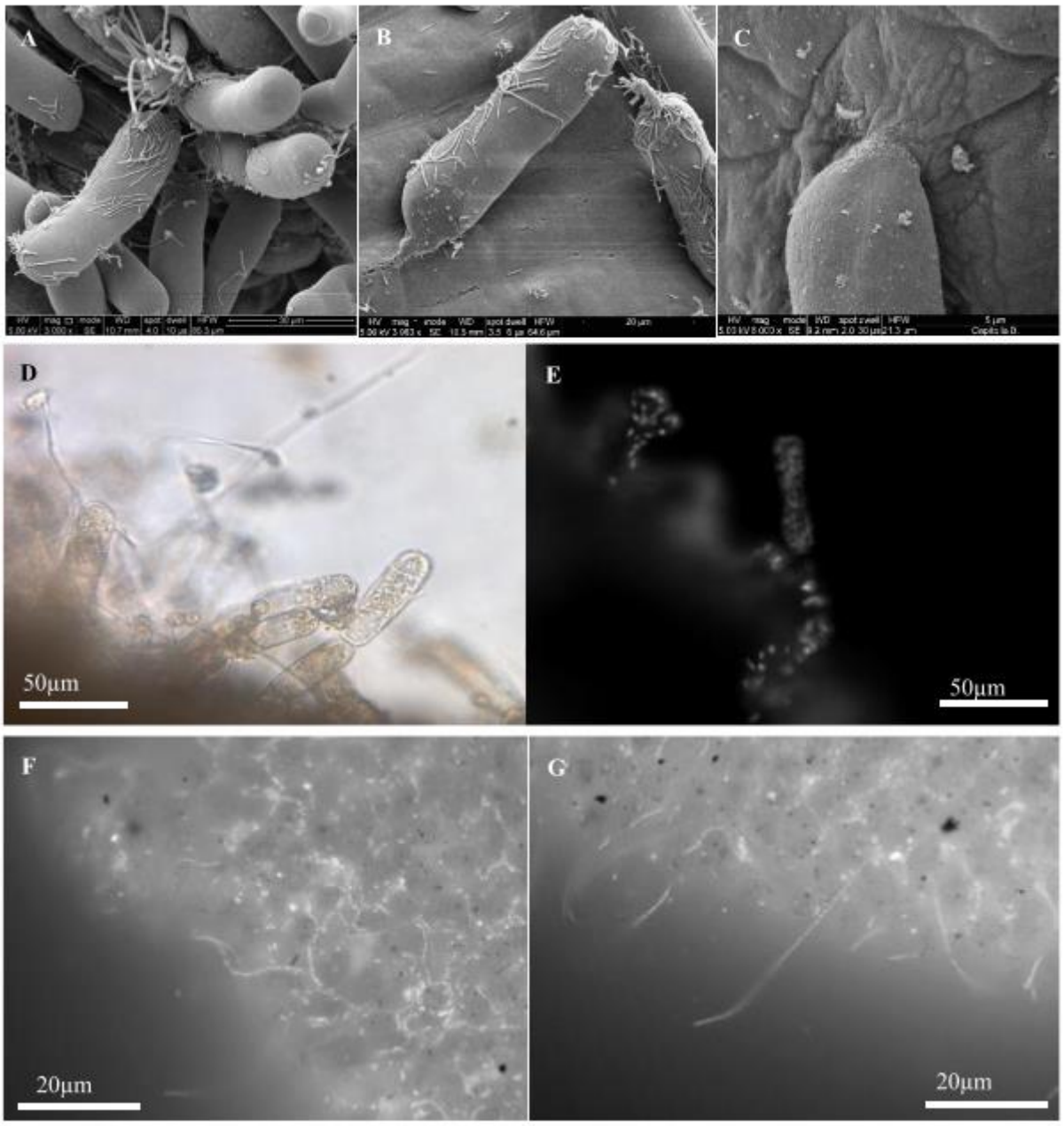

Figure 4: Eukaryotic communities associated with *Capitella* (A) Electron microscopy of an epibiotic Capitella also associated with vorticellid ciliates (white circle). (B, C) Zoom on the ciliates attached to the tegument of *Capitella*. (D, E) Trophomera nematodes (black circle) and their eggs (black arrow) in the body cavity of *Capitella*. (F, G) *Ancora saggitata* in the digestive tract of *Capitella*. Abbreviations: Dt, digestive tract; Cc, coelom; c, coelomocytes.

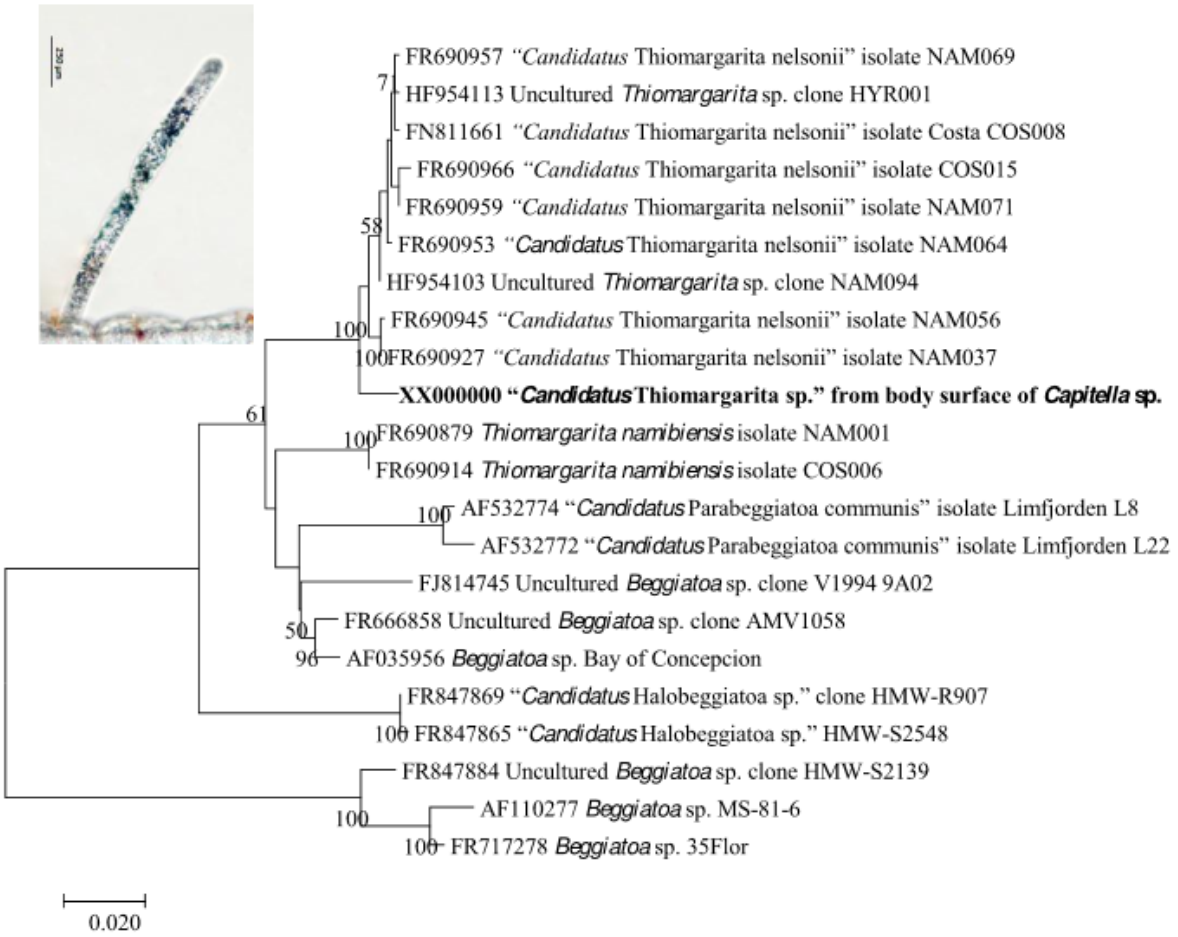

Figure 5: Phylogenetic reconstruction of the position of the *Thiomargarita* sp. sequence obtained from 16rRNA clone libraries obtained from epibiont-covered *Capitella* annelids. See material and methods for detail (FYI: Maximum likelihood using a General Time Reversible Model using MEGA7. Heterogeneity in rates of evolution was accounted by using Gamma distributed rates (5 categories and invariants). 1140 nucleotide positions were analyzed. Scale bar corresponds to 2 % sequence variation. Bootstrap values at nodes were obtained based on 100 ML replications (>50 shown).

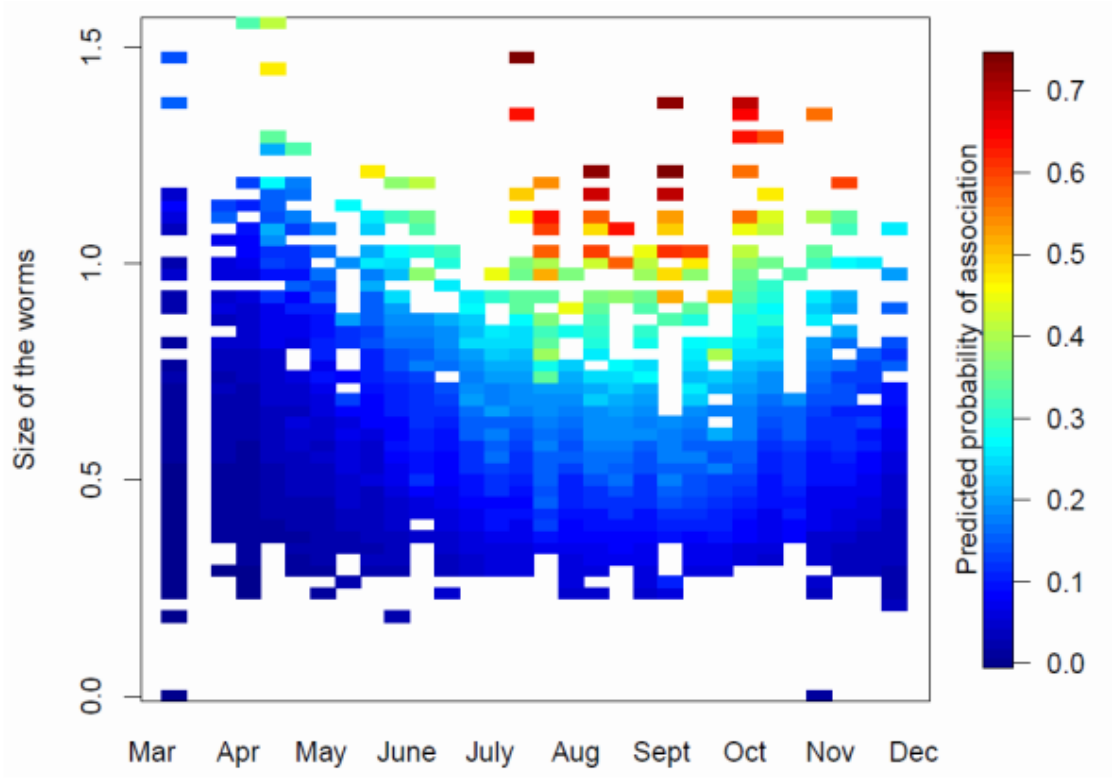

Figure 6: Predicted probability of association with epibiotic microorganisms as a function of the time of the year (month, x-axis) and the size of the worm (in mm, y-axis), obtained from model-averaging 166 GLMs linking site, size, date, date² and sex to association with epibiotic microorganisms. Predictions are made for a uniform sampling of worms among the sexes (undetermined, females and males represent 1/3 of the sample each), the sizes (uniform distribution between 0 and 1.8 mm), the sampling dates and the sampling sites. The color of each square on the heatmap indicates the average predicted probability of association of all worms of that size sampled at that date, following the legend on the right.

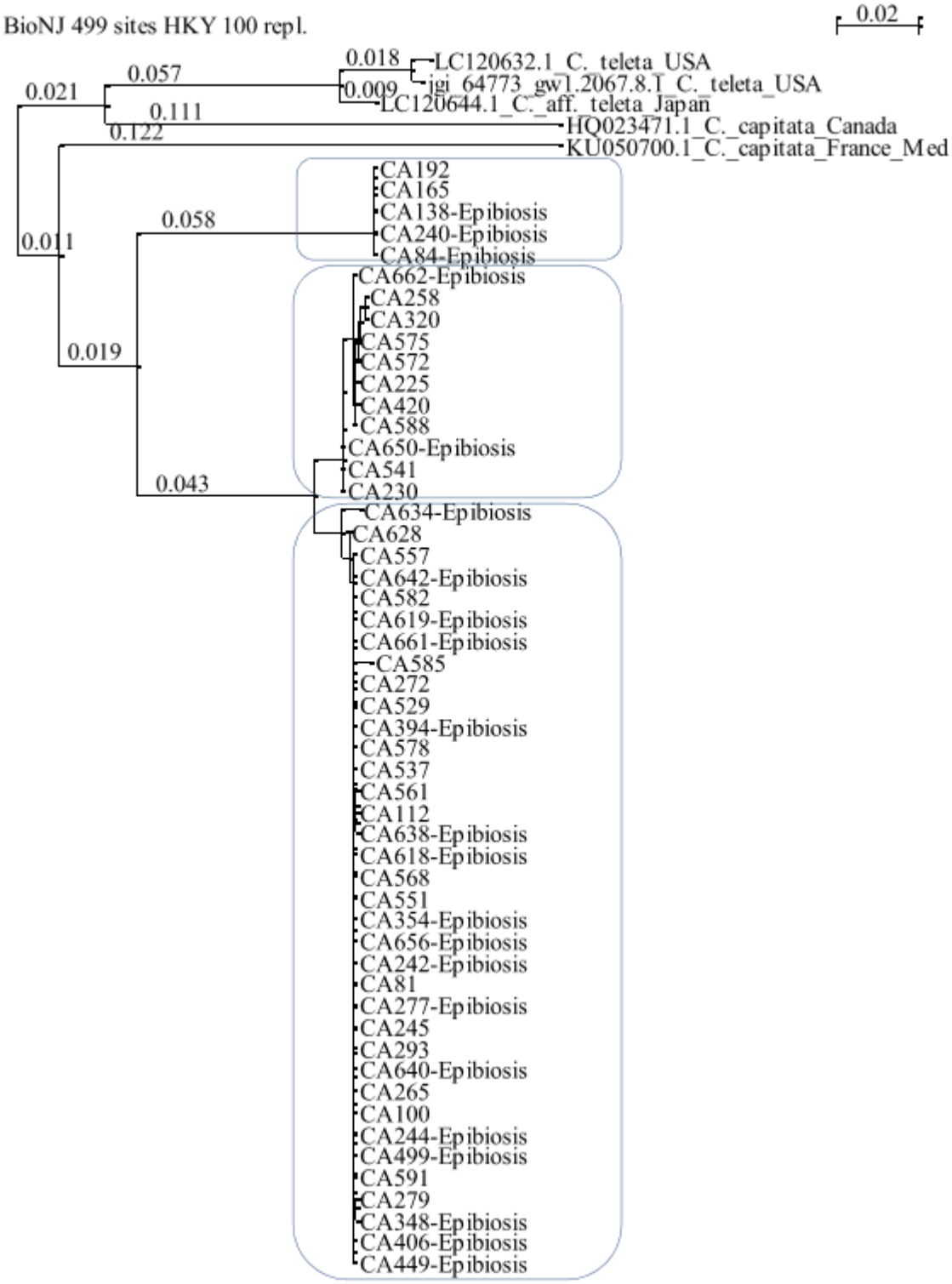

Figure 7: Neighbor-joining tree reconstruction of epibiotic and non-epibiotic *Capitella* spp. individuals barcoded using the mitochondrial marker Cox-1. Distances between individuals were calculated according to the substitution model HKY.

4. DISCUSSION

Differences in the sediment geochemistry lead to different symbiotic associations in *Capitella* spp

Capitella spp. are gonochoristic species with a short indirect lecithotrophic development in the maternal tube, and whose juveniles leave the brooding tube directly after (Wichlacz et al., in prep). This opportunistic species occupies the top 5 cm of sediment, a zone rich in organic matter especially in the muddy sediments of harbors and estuaries. Both surveyed sites are enriched in silts, with a high concentration of organic carbon in the Harbor. The sites exhibited strong differences in AVS concentrations, which could be explained by hydrologic differences and anthropogenic contamination. The seasonal survey shows that sulfide production takes place throughout the year in the Harbor while it mostly occurs in summer at Le Laber. The confinement of the Harbor added to the accumulation of cadavers of crabs due to fishing offloading activities in this zone, promotes a high retention of organic matter (with enrichments in TOC and nitrogen contents), green algal proliferation and, as a consequence, a greater production of sulfide due to microbial degradation over the year when compared with Le Laber. By contrast, Le Laber is open to the ocean and is not affected by off falls. However, it could be also subjected to local eutrophication due intense proliferation of benthic algae at the surface of the sediment in this area during the summer period. These differential compositions of the sediment probably change qualitatively and quantitatively the microorganisms and macro organisms co-inhabiting with *Capitella*.

Concomitantly with the presence or absence of dissolved and particulate (AVS) sulfide concentrations, we observed two distinct phenotypes of *Capitella*: one characterized by a tegument covered by a consortium of filamentous bacteria and another one with an epidermis perfectly clean of any microorganisms as checked by electron microscopy. Epibiosis involving the filamentous bacteria were only observed in the sediments where sulfide concentrations were particularly high. Under these conditions, the prevalence of the epibiotic association is around 20-30% and mostly affects larger individuals.

Trace metals do not seem to affect the epibiosis, as during our survey over the year, only sulfide was shown to increase in the Le Laber site concomitantly with the appearance of animals exhibiting associations with *Thiomargarita*. Total metal concentrations measured in Le Laber sediments are in the same range than those from the Authie estuary located in northern France (Billon), which is considered as a non-polluted site (Natura 2000 site). The

availability of trace metals as chemically estimated through SEM indicates that sediments in the Harbor are able to deliver more important quantities of trace metals to the organisms, although concentrations slightly varied during the monitoring period (Table S2). Concentrations and labilities of metals are however greater in the Harbor than in Le Laber site (excepted for Cr). But the levels of contamination measured in the harbor are not as high as those reported in industrialized harbors of northern France (e.g. Boulogne harbor (Cuvillier-Hot et al., 2018) (Fig. S5)). The sediments never exhibited toxicity index values greater than zero. In addition, TI values were calculated at a macroscopic scale from about 1 g of sediments. In the presence of very local oxygenated areas, which is probably the case near the water/sediment interface, TI values may increase because of AVS re-oxidation and exceed the threshold value of zero. Incidentally, the worms actively pump oxygenated overlaying seawater in the burrows, which could yield to TI values locally above the threshold.

Plastic derivatives (most common phthalates: DEP, DBP, DEHP) were detected at both sites at the same very low concentrations (Data not shown). Unfortunately, the concentration of the pollutant tri-butyl-tin (TBT) was not measured in our study although the Harbor is one of the sites used in on-going studies of this persistent pollutant on local invertebrates. TBT is known to affect the reproduction of mollusks in the harbor by inducing pseudo- hermaphroditism or imposex (Oehlmann et al., 1998; Wirzinger et al., 2007) but it has never been reported to affect any symbiotic interactions (or the immune system).

***Capitella* sp. exhibits association with multiple microbial partners**

We assessed the diversity of microorganisms associated with the worm using a RNAseq approach on animals with and without epibionts in the two distinct nearby habitats. First assignments of contig sequences shown that these small worms are associated with a wide variety of prokaryotes and eukaryotes. The composition of the association varies according to the environmental setting. All animals for the RNAseq study were collected at the same time of the year. Although in all three groups (i.e. Le Laber worms without epibionts, Harbor worms with and without epibionts) the apicomplexan fish parasites are very common, bacterial associates were quite distinct.

Even though *Capitella* with and without epibionts were found in the same sediment sample at the Harbor, associated communities from epibiotic animals were quite distinct from non-epibiotic *Capitella*. Assuming the animals were exposed to the same environmental conditions in the Harbor, this observation suggests that the two groups are characterized either by

physiological or genetic differences. The barcoding effort revealed that up to three lineages are present in Roscoff and three can be host to the large epibiotic filamentous bacteria. Intraspecific genetic differences do not explain the occurrence of epibiosis and physiology seems a good explanation. Pollution, even at sub-lethal levels can affect the physiology of organisms and affect their relationships with other organisms. In polluted fresh water areas in Egypt, a study has showed that the tegument of leeches was more commonly (Gouda, 2006) occupied by vorticellid ciliates. It was however not clear whether these ciliates caused the damage at their attachment point or the damage was pre-existing and allowed the establishment of the ciliates. In our samples, we also found vorticellid ciliates attached to the tegument of *Capitella*. We did not observe any lethal effects of ciliates on *Capitella* maintained in the laboratory (unpublished data).

A new *Thiomargarita*-like association with the intertidal worm *Capitella* spp. exposed to high concentrations of sulfide

The combined analyses of the RNAseq data, the targeted bacterial 16S amplification results and the microscopic observations, allowed the estimation of the abundance and identifying the diversity of the epibiotic bacteria associated with the epibiotic population of *Capitella* in the Harbor. Most abundant bacteria fall into three groups: (i) sulfur-oxidizing bacteria (mostly *Thiomargarita* but also *Thiotrix*, *Thioalkalivibrio*, and *Sulfuromonas*), (ii) mollicutes (including *Spiroplasma*), typically found in invertebrate guts, and (iii) spirochaetes. We identified the largest and most visible epibiont as being a large gammaproteobacterium belonging to genus *Thiomargarita*. This new sequence clustered with all available sequences from *Candidatus 'Thiomargarita nelsonii'* with a 100% bootstrap support in the phylogeny, and is well distinct from the *Candidatus 'T. namibiensis'* clade.

This is the first report of *Thiomargarita* in a coastal ecosystem while this giant bacteria was often encountered with deep sea animals living in sulfidic environments (Bailey, Salman et al. 2011). To date this chemolithotrophic bacterium was described as a free-living species associated with microbial mats. The filamentous bacteria was also found attached to the byssus of a mussel at deep-sea hydrothermal vents (Schulz, 2006), the shell of gastropod *Provanna laevis* at deep-sea methane cold seeps, and on the integument of other seep fauna (Bailey et al., 2011). The ecological behavior of the gastropod *Provanna laevis* was shown to be modified by the presence of *Thiomargarita*, which oriented its shell downward to allow its *Thiomargarita* epibionts to be exposed to sulfide-rich water while the animal had access to

the oxygen-rich overlaying water, leaving its head partially exposed (Bailey et al., 2011). The fluctuating chemosynthetic environment with high sulfide levels appears as an obvious similarity between the *Capitella* and the seep fauna habitats.

Unlike its close relatives *Thioploca* and *Beggiatoa*, the giant bacterium *Thiomargarita* is not mobile. They store elemental sulfur as granules at the periphery of a very large vacuole that occupies 98% of the cell volume where nitrate is stored (Schulz, 2006). Because of their lack of mobility, *Thiomargarita* cells must live in an environment where they will be alternatively exposed to sulfide in the porewater and to nitrate in the overlaying seawater. Compared to previously reported *Thiomargarita* morphologies, the cells attached to *Capitella* are more elongated but the observation of budding structures are similar to those observed in *Provanna laevis* at the Costa Rican seep (Bailey et al., 2011), and suggests that the cells are actively growing. Unlike *Thioploca*, whose populations decline at oxygen concentrations greater than 3 µM, and *Beggiatoa* mats, which thrive with oxygen concentration of 1-2.5 µM, *Thiomargarita* cells can withstand exposure to full atmospheric oxygen concentrations (Schulz, 2006). *Thiomargarita* morphotypes have also been observed attached to various debris while sorting the sediment samples, suggesting their ability to efficiently colonize a wide variety of surfaces, including *Capitella*. The presence of *Thiomargarita* can easily be viewed as a form of biofouling. Their density was, however, highest on the worms, suggesting that these animals offer a more suitable environment. Moreover, we found that *Thiomargarita* was present on the tegument of all three *Capitella* species, but at a higher prevalence on large worms and in summer, irrespectively of sex, and on males and indeterminate individuals than on females. Given the sexual dimorphism on size (in our samples, indeterminate-sex individuals had a mean size of 0.53 mm, while females were on average 0.57 mm-long, and males, 0.56 mm) and the possibility of phenological differences between different *Capitella* species, it still remains to be investigated whether there might be separate cohorts of associated *Capitella* of different species during a single year (with each species associating mostly at the end of its juvenile stage), or whether all *Capitella* species jointly associate with *Thiomargarita* based on their size and the current season.

Is the *Thiomargarita* epibiosis a mutualistic or an opportunistic association?

Animals are exposed to high concentrations of sulfide in the sediment while pumping overlaying oxygenated water by peristalsis in their burrow. Association with the animal could thus be an opportunistic strategy from the bacterial viewpoint, bridging the oxic-anoxic gap

and allowing bacteria access to both electron donors and acceptors. On the other hand, sulfide uptake might contribute to detoxify the environment of *Capitella* and be a positive by-product of the bacterium's activity, although this needs to be tested. The other sulfur bacteria detected could correspond to smaller filamentous bacteria observed at the surface of *Thiomargarita* cells, as already shown in Namibia sediments (Bailey et al., 2011) but also found in association with the hydrothermal vent species *Alvinella pompejana* (Le Bris and Gaill, 2006).

In our survey of epibiosis occurrence over 10 months, we found a greater abundance of animals with epibionts in summer during the high bacterial growth period and in larger animals, at both sites. Summer is the time of the year when temperatures are highest and thus bacterial degradation of organic matter in the sediment, producing sulfide, is likely to be at its highest. The *Thiomargarita* association which oxidizes dissolved sulfide in the pore water, is probably favored under these conditions (Schulz, 2006).

Capitella is a typical member of the *'sulfide system'*. Fenchel & Riedl (1970) coined this term to describe life under these hostile conditions (later called 'thiobiome' or 'thiobios' by Boaden (Boaden, 1975)). Although the thiobiome allows less competitive stress, specific physical and structural adaptations are needed for the survival and thriving of this complex and specific biome. Our observations suggest that at highly "toxic" levels of hydrogen sulfide, physiological adaptations of *Capitella* alone could not be sufficient to detoxify the reduced sulfur compounds and that a facultative epidermal association with *Thiomargarita* and other sulfur oxidizing bacteria available in sediment may constitute a vital additional strategy. Besides detoxication, sulfur-oxidizing epibionts may provide nutrients to the host as suggested for deep sea hydrothermal annelids (Desbruyères et al., 1983). *Capitella* has been shown to feed on free-living autotrophs that use sulfide oxidation to fix CO2 (Hiroaki et al., 2001). A derived question was to know if this *Capitella-Thiomargarita* association was species-specific; to find a specific niche may allow to avoid competition with congeneric species. *Capitella teleta* and *C. capitata* are part of a cryptic species complex (Grassle and Grassle, 1976; Nygren, 2014). Even if the populations of *Capitella* inhabiting Roscoff also constitute an assemblage of cryptic species (manuscript in preparation), barcode analyses performed on the main lineages showed that the epibiotic association is not fixed by the host genetic. The facultative association is likely due to physiological differences between individuals, more or less correlated to their size as evidenced here. The observation could also mean that

Thiomargarita and other epibiotic bacteria correspond to biofouling/parasitic agents capable of colonizing a range of invertebrates, including *Capitella* from different species, when they are under high sulfidic stresses.

Conclusion

Overall, whether the observed epibiosis is beneficial, detrimental or neutral to the host when subjected to sulfide-rich environments remains to be demonstrated. Our data provide clear evidence of the impact of sediment geochemistry on associations between *Capitella* and its surrounding microorganisms with the peculiar development of a thiotrophic epibiosis in worms exposed to high sulfide concentrations. Occurrence and maintenance of an epibiotic community depend on the hosts' ability to control the epibiont's colonization and proliferation through its immune actors. Such defense is probably influenced by variable environmental conditions. Consequently, the next step will investigate how the immune system of *Capitella* can become permissive to the establishment of this facultative ectosymbiosis under challenging conditions. Regardless of the future findings, this emphasizes the importance of investigating associations in their proper environmental context.

Acknowledgements

This project was funded by the CNRS INEE APEGE PolCa program (2012), by the FRB- Nord Pas de Calais VERMER program (2013-2016), the BQR emergent Université de Lille (2013), the Total Foundation PIONEER project (2015-2018). MCR benefitted from a Brazil-France Sandwich fellowship for her fieldwork in Roscoff. The Soil Analyses Laboratory (INRA, Arras) is warmly acknowledged for the analysis of the total organic carbon and nitrogen in the sediments.

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