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## The diversification of the antimicrobial peptides from marine worms is driven by environmental conditions

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1 **The diversification of the antimicrobial peptides from marine worms**  
2 **is driven by environmental conditions**

3  
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44

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56 transcriptomic analyses, conceptualization, critical review. Aurélie Tasiemski: Data  
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59

60 **Abstract:** Antimicrobial peptides (AMPs) play a key role in the external immunity of  
61 animals, offering an interesting model for studying the influence of the environment on  
62 the diversification and evolution of immune effectors. Arenicin (ARE), alvinellacin (ALV)  
63 and polaricin (POL, a novel AMP identified here), characterized from three marine worms  
64 inhabiting contrasted habitats (temperate, 'hot' vents and polar respectively), possess a  
65 well conserved BRICHOS domain in their precursor molecule despite a profound amino  
66 acid and structural diversification of the C-terminal part containing the core peptide. Data  
67 not only showed that ARE, ALV and POL display an optimal bactericidal activity against  
68 the bacteria typical of the habitat where each worm species is living but also that this  
69 killing efficacy is optimal under the thermochemical conditions encountered by their  
70 producers *in situ*. Moreover, the correlation between species habitat and the cysteine  
71 content of POL, ARE and ALV led us to investigate the importance of disulfide bridges in  
72 their biological efficacy as a function of abiotic pressures (pH and temperature). The  
73 construction of variants using non-proteinogenic residues instead of cysteines ( $\alpha$ -  
74 aminobutyrate chemical variants) leading to AMPs devoid of disulfide bridges, provided  
75 evidence that the disulfide pattern of the three AMPs allows for a better bactericidal  
76 activity and constitutes an adaptive way to sustain the fluctuations of the worm's  
77 environment. This work shows that the external immune effectors exemplified here by  
78 BRICHOS AMPs are evolving under strong diversifying environmental pressures to be  
79 structurally shaped and more efficient/specific under the ecological niche of their  
80 producer.

81

82

## 83 **1. INTRODUCTION**

84

85 External immunity constitutes the first defense of metazoans to face pathogens by  
86 handling the surrounding microbial communities to both avoid infection and establish  
87 symbiosis [1]. As such, it can be viewed as an extended arm of the immune system to  
88 prevent infection [1]. Many organisms including plants, invertebrates, vertebrates and,  
89 even bacteria, secrete antimicrobial peptides (AMPs) as an extrinsic protective shield  
90 against the surrounding microbiome that is usually rich in potentially pathogenic agents  
91 [2–4]. AMPs are predominantly short cationic peptides (up to 100 amino acids), with a  
92 broad-spectrum of activities against diverse microbes (bacteria, virus or fungi) with a  
93 rapid and selective killing processing mode [4]. Lacking antibodies, invertebrates  
94 nevertheless benefit from a powerful innate immune system, characterized, among other  
95 processes, by a great variety of AMPs [5].

96 Marine organisms constitute almost half of the biodiversity of the Earth, and after a long-  
97 term diversification (Cambrian explosion: 541 to 485 mya ago) and co-evolution with  
98 fungi, bacteria and archaea, acquired the capacity of producing many unique  
99 antimicrobial substances [6–8]. Marine AMPs were found to be structurally different from  
100 their counterparts produced by terrestrial species, usually displaying novel structures,  
101 taxa-specific or even species-specific [6,8,9]. A long evolutionary story of marine  
102 organisms (including bacteria) under diversifying environmental pressures, such as the  
103 microbial community composition and/or highly fluctuating abiotic factors (temperature,  
104 pH, salinity, or hydrostatic pressure, which can vary to extreme values from polar regions  
105 to the Equator and the intertidal to deep sea) have probably driven the evolution of the  
106 physiological adaptation of most marine invertebrates, including on their immune  
107 functions [10].

108 In particular, worms have colonized and dominated in biomass most of marine and  
109 freshwater habitats, coping with a wide number of environmental conditions and  
110 bacterial assemblages, especially in extreme environments such as polar regions, deep-  
111 sea hydrothermal vents, or highly anthropized shallow areas [11,12]. In the case of  
112 annelids (ringed worms), the majority of AMPs is species-specific, probably as the result  
113 of a long parallel evolution of their defense arsenal to a wide variety of habitats on Earth  
114 (aquatic and terrestrial), mimicking their highly diverse life styles and ecology [13]. In  
115 this context, the BRICHOS-domain AMP family of marine annelids living under extreme

116 conditions, which is specifically involved in the worms' external immune defense  
117 constitutes a unique and attractive model to study the adaptive evolution of the immune  
118 system to environmental changes.

119 BRICHOS (initially found in Bri2, chondromodulin, and prosurfactant protein C in human)  
120 is a 100 amino acids domain, present in several protein precursors, acting as an  
121 intramolecular chaperone [17–21]. Despite their very old phylogenetic separation (at the  
122 end of the Cambrian diversification), BRICHOS precursors all display the same structure  
123 consisting of: (i) a highly hydrophobic N-terminal signal sequence, (ii) a pro-region  
124 containing a BRICHOS domain, and (iii) a C-terminal region with  $\beta$ -sheet propensities. In  
125 the unique case of marine worms, this latter corresponds to an AMP which exerts its  
126 antimicrobial activities once cleaved from the precursor [6,14–16]. Among the BRICHOS-  
127 domain AMPs, arenicin (ARE) was the first identified from *Arenicola marina*, an annelid  
128 inhabiting the intertidal zone of the European temperate shore [14]. Later, the AMP  
129 alvinellacin (ALV) was purified from the deep-sea hydrothermal vent annelid *Alvinella*  
130 *pompejana*, which inhabits the hottest part of this peculiar environment [15]. Finally,  
131 another annelid BRICHOS-domain AMP, the nicomicin, was identified from *Nicomache*  
132 *minor*, a polychaete colonizing both temperate and arctic habitats [16].

133 Interestingly, precursors of BRICHOS-AMPs show a well-conserved BRICHOS domain but  
134 highly divergent primary AMP sequences, suggesting either a strong diversifying  
135 selection at the interspecific level of the antibiotic peptides to face the diverse microbial  
136 communities, and/or abiotic conditions, or some exon shuffling between the well-  
137 conserved BRICHOS domain and AMPs of different genomic origins [22]. In order to  
138 enlarge our panel of BRICHOS-AMPs from extreme habitats, we report here a novel AMP,  
139 named polaricin (POL), from an undescribed terebellid polychaete belonging to the genus  
140 *Amphitritides* endemic to subtidal polar waters of the Antarctic habitat. POL together with  
141 ALV and ARE, represent relevant molecules to study AMP adaptation to environmental  
142 stressors as they have evolved under highly contrasted (polar, hot vents and temperate  
143 respectively) environmental conditions from a shared but old ancestor [14,15] (Fig. 1).

144 In this work, we focused our attention on how selective pressures associated with biotic  
145 and abiotic factors have affected the structure and bioactivity of the three POL, ALV and  
146 ARE peptides. Through combined analyses of their bioactivities and structural  
147 characteristics under various set of environmental and bacterial conditions, we

148 deciphered whether these immune peptides better suited to the environmental  
149 conditions typifying the worms' habitats.

150

## 151 **2. MATERIAL AND METHODS**

### 152 **2.1. AMPs and habitat specificities of the worms from which they derived from**

153 Arenicin (ARE) was purified from the lugworm *Arenicola marina* (Annelid, Polychaeta,  
154 Scolecida), a polychaete that inhabits temperate near-shore (intertidal) sediments,  
155 digging tunnels in the sand [60,61]. The burrows of these worms are continuously  
156 exposed to periodic tidal emersion, which imposes severe thermal variations (-5 °C to 25  
157 °C) and temporal hypoxia with mild acidic conditions (pH from 5.6 to 8.2) [24–32].

158 Alvinellacin (ALV) was isolated from *Alvinella pompejana* (Annelid, Polychaeta,  
159 Terebellida), a polychaete that inhabits parchment-like tubes along the outer walls of  
160 active hydrothermal vents chimneys of East Pacific Rise [62]. Its habitat is characterized  
161 by frequent and unpredictable mixing of hot and anoxic fluids with cold seawater (2-3 °C),  
162 which expose the worm to extreme temperatures (from 2 °C to upper 100 °C) that greatly  
163 vary inside the tube (14 °C to 80 °C with a thermal optimum around 42 °C) and acidic  
164 gradients (pH from 5.33 to 6.4) due to the mixing of bottom seawater with the acidic vent  
165 fluid [33–38].

166 Polaricin (POL) was identified by analogy from the transcriptome of *Amphitritides* sp.  
167 (Annelid, Polychaeta, Terebellida), a marine annelid inhabiting marine sediments at the  
168 base of sponges or bryozoans of the Antarctic plateau at depth of 100-150 m. This  
169 “spaghetti worm” is sedentary and lives inside a slightly consolidated tube made with  
170 sand and coral debris on the Antarctic continental plateau where it faces extremely cold  
171 but stable coastal waters (about -1.8 °C), with very small fluctuations of temperature and  
172 pH [63–66].

### 173 **2.2. Molecular identification of the AMP precursors**

174 The molecular characterization of the preproarenicin and preproalvinellacin has been  
175 described in previous studies done by Ovchinnikova, T. V. *et al* (2004) [14] and by  
176 Tasiemski A. and colleagues [15].

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

181 10-15). The *Amphitritides* RNAseq dataset used for the assembly was obtained from an  
182 Illumina 150 bp paired-ends sequencing of a stranded mRNA library on a HiSeq 2500  
183 machine at Genome Québec (half a line: 150 M PE reads). Transcripts (Ngenes=99970,  
184 N50=1440 bp, median contig length=488 bp, GC%=36.2) were then obtained with the  
185 Trinity 2.4.0 software from R1 and R2 pairs after trimming adapters and regions of low  
186 PHRED scores (Trimmomatic 0.36: ILLUMINACLIP: illumina. fa: 2:30:10, LEADING:5,  
187 TRAILING:5, SLIDINGWINDOW:4:15 MINLEN:36) and a subsequent normalization of the  
188 reads.

### 189 **2.3. Alignments of the precursor amino acid sequences and identification of the** 190 **BRICHOS and AMP domains**

191 BRICHOS domain sequence was identified by using MyHits Motif Scan  
192 ([https://myhits.isb-sib.ch/cgi-bin/motif\\_scan](https://myhits.isb-sib.ch/cgi-bin/motif_scan)) and the SignalP 5.0 software  
193 (<https://www.cbs.dtu.dk/services/SignalP/>) was used to detect the presence of signal  
194 peptide.

195 The amino acid sequence alignments of peptides precursors of ALV (GenBank accession  
196 number KJ489380), ARE (GenBank accession number AY684856), nicomicin (GenBank  
197 accession number MH898866) and POL, was performed by using CLC Sequence Viewer  
198 software (version 8.0). The computation of homology/identity percentage was performed  
199 at the SIB (ExpASY software, <https://www.expasy.org/>) using the BLAST network service  
200 (BLASTP, version 2.2.31+). The amino acid sequences of the three AMPs presented in this  
201 alignment were used for the chemical synthesis of native and ABU peptides as described  
202 further on.

### 203 **2.4. Chemical synthesis of AMPs with (ALV, ARE, POL) and without (ABU ALV, ABU** 204 **ARE, ABU POL) disulfide bridges**

205 The amino acid sequences used for the peptide synthesis are presented in tables S1 and  
206 S2. The chemically synthesized peptides (purity of 98%), *i.e.*, ALV, ARE and POL, were  
207 produced by GenScript (Leiden, Netherlands) with the cysteine (Cys) pattern involved in  
208 disulfide bridges accordingly to their native forms:

209 -ALV: Four cysteine residues engaged in two intramolecular disulfide bonds (Cys3-Cys21;  
210 Cys7-Cys7);

211 -ARE: Two cysteine residues engaged in one intramolecular disulfide bond (Cys3-Cys20);

212 -POL: One cysteine (Cys10) engaged in one intermolecular disulfide bond (Cys10-Cys10).



213 The  $\alpha$ -aminobutyrate (ABU) modified peptides were chemically produced using the solid-  
214 phase method with an automated peptide synthesizer (Model 433A, Applied Biosystems  
215 Inc.) in the Radical Chemistry Institute by the Radical Organic Chemistry and Specialty  
216 Polymers Team (CROPS) laboratory (France, Marseilles) [67,68]. The disulfide bonds  
217 were removed by replacing all the cysteine residues by ABU residues (hydrophobic non-  
218 proteinogenic alpha amino acid, also known as homoalanine) which do not associate  
219 covalently and thus do not form disulfide bridges [69,70]. The identity and degree of  
220 homogeneity of the peptides were verified by: (1) analytical C18 reversed- phase  
221 HPLC(NUCLEODUR 100-5 C18, 250  $\times$  10 mm, MACHERY NAGEL) using a 60-min linear  
222 gradient from 0 to 60% of buffer B [0.08% (v/v) TFA/acetonitrile] in buffer A [0.1% (v/v)  
223 TFA/H<sub>2</sub>O], at a flow rate of 1 mL/min; (2) amino acid analysis after acidolysis [6N HCl/2%  
224 (w/v) phenol, 20 h, 118°C, N<sub>2</sub> atmosphere] and (3) molecular mass analysis by MALDI-  
225 TOF mass spectrometry. The physicochemical properties of all the synthesized peptides  
226 (molecular weight, isoelectric point, net charge at neutral pH) were calculated by using  
227 Innovagen Pepcalc.com server (Innovagen AB, SE-22370 Lund, SWEDEN).

## 228 **2.5. Comparison of the three-dimensional (3D) structures of ALV, ARE and POL**

229 The 3D structure of ARE and ALV was gained from previous studies [14,15]. 3D peptide  
230 models of the homooligomer 1:1 of POL and of the sequence monomer were predicted  
231 using the Alpha Fold software. The confidence in the accuracy of the predicted structures  
232 was measured by the predicted local distance difference test (pLDDT): score 0 to 100. The  
233 peptides 3D images were generated using ChimeraX.

## 234 **2.6. Mass spectrometry of POL**

235 A sample of POL (about 2  $\mu$ L, at concentration of 2 mg/mL) was characterized by  
236 analytical UPLC–MS using a System Ultimate 3000 UPLC (ThermoFisher) equipped with  
237 an Acquity peptide BEH300 C18 column (1.7  $\mu$ m, 2.1  $\times$  100 mm, 300 Å), a diode array  
238 detector and a mass spectrometer (Ion trap LCQfleet). Analysis was performed at 70 °C  
239 using a linear gradient of 0-70% of eluent B in eluent A over 20 min at a flow rate of 0.4  
240 mL min<sup>-1</sup> (eluent A = 0.1% TFA in H<sub>2</sub>O; eluent B = 0.1% TFA in CH<sub>3</sub>CN). The column eluate  
241 was monitored by UV at 215 nm. The peptide masses were measured by on-line UPLC–  
242 MS (LCQ Fleet Ion Trap Mass Spectrometer, ThermoFisherScientific): heat temperature  
243 450 °C, spray voltage 2.8 kV, capillary temperature 400 °C, capillary voltage 10 V, tube  
244 lens voltage 75 V.

## 245 **2.7. Bacterial strains used for the antimicrobial assays**

246 A series of bacterial strains was used to estimate the killing efficiency of the different  
247 AMPs and test whether they are more efficient against bacteria living in the same  
248 environment of the worms. The bacterial strains used in this study are all Gram-negative  
249 from different marine environments:

250 --*Pseudomonas sp.* AT1238 and *Vibrio diabolicus* HE800 provided by Ifremer LMEE culture  
251 collection (Mission HERO'1991) were isolated from a mix of tubes of *A. pompejana* and  
252 small pieces of the surrounding polymetallic sulfides of deep-sea hydrothermal chimneys  
253 (East Pacific Rise) [72].

254 --*Shewanella algae*, *Oceanisphaera donghaensis*, and *Vibrio fluvialis* were isolated from  
255 intertidal mud sediment near to the burrow of *Arenicola marina*. Raw sediment samples  
256 were incubated overnight in a liquid Marine Broth media at 28 °C to stimulate bacterial  
257 growth. Well-growing colonies were isolated by multiple pricking out onto agar plates  
258 containing marine seawater to identify different bacterial strains. Pure colonies were  
259 identified with the Universally applicable bacterial primer set 16S-F and 16S-R (forward  
260 primer: 5'-TCCTACGGGAGGCAGCAGT -3'; reverse primer: 5'-  
261 GGACTACCAGGGTATCTAATCCTGTT -3') as described in *Cuvillier et al* [73] . The  
262 sequences were compared against nucleotide sequences from the Eztaxon database using  
263 BLASTn UPGMA trees were constructed using the Mega-X software package  
264 (<http://www.megasoftware.net>) (Fig. S4).

265 --*Vibrio alginolyticus* provided by the Ifremer is worldwide distributed in coastal waters  
266 of temperate and also polar areas [74,75]. *V. alginolyticus* and *V. diabolicus* are genetically  
267 closely related (Fig. S4).

## 268 **2.8. Determination of the spectrum of antimicrobial activities of ALV, ARE and** 269 **POL**

270 Peptides Minimal Inhibitory Concentration (MIC) and Minimal Bactericidal Concentration  
271 (MBC), were determined by the broth microdilution method against bacterial growth in  
272 microtiter plates as previously described by Bulet [76]. The AMPs were resuspended in  
273 sterile pure water. Briefly, 100 µL of bacterial dilutions (10<sup>6</sup> Colony Forming Unit/mL)  
274 were added to each well into sterile 96-well flat bottom plates (CELLSTAR, Greiner bio-  
275 one) containing serial dilutions (from 40 to 0.00195 µg/mL) of peptides (10 µL per well).  
276 The plates were incubated overnight at 28 °C on a rotary shaker (140 rpm). The MIC was  
277 taken as the lowest peptide concentration at which observable microbial growth was  
278 inhibited, the measurements were realized using a microtiter plate reader (Tecan Sunrise

279 Microplate Reader) at A600nm. MBC was measured by streaking on proper agar (MHB  
280 with 15 g/L bacteriological agar, Euromedex) petri dishes, the entire volume of each well  
281 (110 µL) with no bacterial growth in the previously obtained MIC plates. After incubation  
282 for about 24 hours at 28°C, the MBC was defined as the peptide concentration where no  
283 colony growth was observed. All tests were conducted in triplicate.

284 A 'home versus away' graph was constructed from antimicrobial assays against the whole  
285 set of environmental marine bacteria with log-transformed MIC data organized in two  
286 groups, depending on the habitat (polar/temperate intertidal vs deep-sea hot vents) to  
287 highlight patterns of AMP efficiency in both native and transplanted habitats.

### 288 **2.9. Kinetics of bactericidal activities of ALV, ARE and POL against *Vibrio* strains** 289 **depending on the thermal range**

290 Kinetics of bactericidal activity of the three AMPs were assessed on two bacteria belonging  
291 to the same genus but colonizing two contrasted habitats: *V. alginolyticus* (polar and  
292 temperate) and *V. diabolicus* ("hot" hydrothermal vent) according to the protocol  
293 described in Tasiemski et al [77].

294 Cultures were incubated with peptides (at a concentration of 5-fold MICs) for 5, 10, 15,  
295 30, 60, 120 minutes and 24 h at two temperatures (10 °C and 42 °C) to mimic the worms'  
296 thermal environment. Viabilities of *Vibrio* strains were assessed by plating the bacterial  
297 suspensions on MH agar plates (sterile petri dish, 90 mm). The plates were then incubated  
298 overnight at 28 °C and the resulting bacterial colonies were counted (CFU/mL). Three  
299 independent experiments were run in parallel and data were log-transformed (log  
300 CFU/mL).

### 301 **2.10. Thermostability assays on ALV, ARE, POL and their ABU forms against** 302 ***Vibrio* strains**

303 The biological activities of AMPs were evaluated after their exposition to a thermal stress  
304 (high and low temperatures). Two *Vibrio* species genetically closely related, *V.*  
305 *alginolyticus* and *V. diabolicus* were chosen for this assay, as representatives of the two  
306 marine environments. The 10<sup>6</sup> CFU/mL diluted culture was added (100 µL per well) into  
307 sterile 96-well flat bottom plates (CELLSTAR, Greiner bio-one) containing a volume of 10  
308 µL/well of peptides (at a concentration of 5-fold MIC values) previously incubated for 30  
309 minutes and 1 hour at 4, 10, 42 and 90°C. The plates were then incubated overnight at  
310 28°C at 140 rpm. The antimicrobial activity was evaluated by measuring bacteria growth  
311 on a microtiter plate reader (Tecan Sunrise Microplate Reader) at 600 nm. The results

312 were expressed as percentage of bacterial growth, in comparison with the control (no  
313 AMP added to the MHB medium). The tests were conducted in triplicate.

314 The same experiment was also conducted in triplicate with the ABU forms to determine  
315 the role of the disulfide bonds in the thermal stability of the three native AMPs

### 316 **2.11. pH-stability assay of ALV, ARE, POL and their ABU forms against *V.*** 317 ***alginolyticus***

318 The biological activities of the three AMPs were estimated after their exposition to an  
319 acid/basic stress following the method developed by Yang *et al.* 2017 [46]. For  
320 comparative purposes, only a strain sensitive to the three AMPs was used: *V. diabolicus*  
321 was excluded and *V. alginolyticus* was chosen because of its lowest MICs to all three AMPs.  
322 One colony of *V. alginolyticus* was grown in MHB medium overnight at 28°C (140 rpm).  
323 Diluted bacterial cultures ( $1 \times 10^6$  CFU/mL) were added (100  $\mu$ L per well) into sterile 96-  
324 well flat bottom plates (CELLSTAR, Greiner bio-one) containing serial dilutions of  
325 peptides (from 20 to 0.00195  $\mu$ g/mL), previously incubated for 3 hours at 10°C in a pH  
326 buffered solution. Four acid-to-basic 100 mM solutions (sodium acetate buffer (pH 4.0),  
327 sodium phosphate buffer (pH 6.0), Tris-HCL buffer (pH 8.0), and glycine-NaOH buffer (pH  
328 10.0)) were tested against control samples incubated in MHB medium (pH 7.4) at 10°C.  
329 The plates were then incubated overnight at 28°C and 140 rpm. The antimicrobial activity  
330 of peptides was determined by the broth microdilution method [78]. All tests were  
331 conducted in triplicate and the results were expressed as MIC values (in  $\mu$ M).

332 The same experiment was then conducted in triplicate with the ABU forms to determine  
333 the role of the disulfide bonds in the pH stability of the three native AMPs.

334

## 335 **3. RESULTS**

336

### 337 **3.1. The newly identified Polaricin (POL) supports a wider sequence and** 338 **structural diversification of AMPs from the BRICHOS-AMP family**

339 The molecular identification of prepropolaricin was performed following a tblastx on the  
340 transcriptome assembly of the Antarctic terebellid worm *Amphitritides* sp. using the  
341 sequence of the preproalvinellacin as query (Fig. 2 and Fig. S1). Pfam analysis revealed  
342 the presence of a conserved BRICHOS domain, in line with the previously described  
343 members of BRICHOS-AMP family [14–16]. Data highlighted a higher percentage of  
344 sequence identity of the BRICHOS domain compared to the AMP itself, which seems to

345 have evolved at a much faster rate (Table S1). As opposed to the AMP itself, the BRICHOS  
346 domain keeps a clear phylogenetic signal in our set of polychaetes with *Alvinella* and  
347 *Amphitritides* being closer one to each other when compared to other annelid species even  
348 if they inhabit opposite habitat.

349 POL is processed from the C-terminal part of prepropolaricin (Fig. 2). It is a cationic 19  
350 amino acids peptide (Table S2) containing an unique cysteine residue by contrast with  
351 ARE and ALV, which respectively possess two and four cysteine residues involved in the  
352 formation of disulfide bonds that stabilize a  $\beta$ -hairpin conformation (Fig. 3).

353 As already evidenced by Nuclear Magnetic Resonance (NMR) [14,15], ALV and ARE share  
354 the same monomeric organization despite great differences in primary structure: two  
355 twisted antiparallel  $\beta$ -strands, forming a  $\beta$ -hairpin conformation stabilized by two or one  
356 disulfide bond(s) respectively. By contrast with ARE and ALV, MALDI-TOF mass  
357 spectrometry analyses of POL revealed that 3/4 of the polar AMP molecules dimerize  
358 presumably through an intermolecular bond engaging the cysteine residue of POL (Fig.  
359 S2). Depending on the dimerization state, the predicted 3D structure for POL, based on an  
360 *in silico* approach (see Methods) is different: The monomer adopts an alpha-helix  
361 conformation while the homodimer forms two parallel  $\beta$ -strands exposing face to face  
362 each cysteine residue. The best predicted models are presented in Figure 3.

363

### 364 **3.2. BRICHOS-AMPs co-evolved with the worms' microbial community from which** 365 **they are derived from**

366 Crossed antimicrobial assays (Table 1) were performed between bacterial strains  
367 (cultivable under MIC assay conditions) typical of the polar/temperate shallow water or  
368 hot vents, and the three BRICHOS-AMPs (*i.e.*, POL, ARE and ALV). AMPs specificities for  
369 microbial communities characterizing thermal habitats encountered by the worms were  
370 then analyzed *via* a "home *versus* away" diagram, a method commonly used in ecology to  
371 identify a local adaptation (see Material and methods) [23]. To do so, MIC values of the  
372 three AMPs against the different strains were plotted together to build the diagram (Fig.  
373 4). The peptides which are the best locally adapted to the home site of their producers  
374 display the lowest values of MIC (higher fitness level) and *vice versa*. Analysis showed that  
375 ALV displayed a strong antimicrobial activity against all the bacteria tested with a better  
376 fitness against hydrothermal vent bacteria than against temperate/polar bacteria. ARE  
377 was also active against all the strains tested including *S. algae*, showing unlike ALV, a

378 better fitness against temperate-coastal bacteria than against hydrothermal vent bacteria.  
379 Among the three peptides, POL was not active against the hot vent *V. diabolicus* and has  
380 the lowest efficacy against all the other bacterial strains tested. However, its fitness was  
381 the best against *V. alginolyticus* a strain that can also be found in polar habitat.  
382 Consequently, BRICHOS-AMPs are locally adapted to (*i.e.* specifically target) the microbial  
383 community associated with the worm habitat from which they derive from.

384

### 385 **3.3. BRICHOS-AMPs are fitted to handle the thermal and pH ranges sustained by the** 386 **worms**

387 In a first step, the bactericidal activity, which reflects the requested time for an AMP to  
388 kill bacteria, was investigated through killing-time kinetics assays against the two *Vibrio*  
389 strains typical of the deep-sea 'hot' vents (*V. diabolicus*) and of the intertidal  
390 temperate/polar environment (*V. alginolyticus*) (Fig. 5). The antimicrobial efficacy was  
391 tested either at 10°C, which corresponds to the optimal temperature of *Arenicola marina*  
392 [24–32] or at 42°C, which corresponds to the optimal temperature of *A. pompejana* [33–  
393 38]. The assay was first adjusted according to the growth curve of the bacteria previously  
394 determined at the different temperatures (Fig. S3). The observed effects are consequently  
395 not reflecting a direct impact of these temperatures on the growth of these bacterial  
396 strains but rather differential AMP activities on the bacteria. Figure 5 shows that POL was  
397 slightly active against *V. alginolyticus* at 10°C and inactive against *V. diabolicus* at both  
398 10°C and 42°C. Figure 5 shows also that ARE and ALV are bactericidal against both strains  
399 with a better efficacy against their respective surrounding *Vibrio* communities. When  
400 mimicking local temperatures, the efficiency of ARE and ALV is even more pronounced  
401 against the local strain (*i.e.*, 15 min to kill 100% of *V. diabolicus* at 42°C versus >24 h at  
402 10°C for ALV and 30 min to kill 100% of *V. alginolyticus* at 10°C versus 24 h at 42°C for  
403 ARE).

404 In a second step, the thermal stabilities of the three AMPs were tested by comparing their  
405 bactericidal properties after being incubated for 30 min to 1 hour at 4 °C, 10 °C, 42 °C and  
406 even 90 °C (Fig. 6A). The “polar” AMP (POL) exerted its antimicrobial activity against *V.*  
407 *alginolyticus* at 4 °C and 10 °C only and lost all its activity following incubations at 42 °C  
408 and 90 °C. By contrast, the “vent” AMP, ALV remained active against both bacteria  
409 following incubations at 42 °C and 90 °C with a 50% decrease of its activity after 1 h of  
410 incubation for the highest temperature tested. Data confirmed the higher efficiency of ALV

411 toward *V. diabolicus*. The “temperate” ARE displayed an intermediate pattern in which the  
412 activity started to decrease by 80% at 42 °C and almost disappeared after 1 h at 90 °C with  
413 only 20% of residual activity.

414 The effect of the environmental pH on the three AMPs was evaluated by comparing their  
415 antimicrobial efficacies at various pH (4, 6, 7.4, 8 and 10) corresponding to the pH range  
416 of the habitat of each worm (Fig. 3B), the narrowest range being the polar environment  
417 (7.8-8.6 versus 3.9-8.5 for the hot vent). Because POL is not active against *V. diabolicus*,  
418 only *V. alginolyticus* was used for the assays (Fig. 7A). The peptides remained soluble  
419 whenever the pH. Antibacterial activities of ARE and ALV were not affected by their  
420 incubation under acid-to-basic pH conditions although POL exhibited a reduced efficiency  
421 (4-fold in the most extreme conditions pH 4 and 10) as soon as the pH is not neutral.

422

#### 423 **3.4. Importance of disulphide bridges for the antibacterial activities of BRICHOS-** 424 **AMPs under different thermal and pH conditions**

425 The more the marine worms are exposed to high temperatures and extreme pH values,  
426 the more their BRICHOS-AMP exhibits a higher content of cysteine residues engaged in  
427 disulfide bridges (Fig. 3). Cysteine replacement by alpha amino butyric acid (ABU) in the  
428 three AMPs enabled removing the intermolecular (ABU-POL) or intramolecular disulfide  
429 bonds (ABU-ALV, ABU-ARE) without modifying their main biochemical features  
430 (summarized in Table S3).

431 When comparing both the MIC and MBC values (Table 2) of the three AMPs and their ABU  
432 forms, ABU ALV and ABU ARE were slightly less efficient against *V. alginolyticus* while  
433 ABU POL has become slightly more active against this bacterium. Against *V. diabolicus*, the  
434 replacement of cysteines had a strong negative effect on the killing efficiency of the three  
435 AMPs. The disulfide bridges are likely to play a crucial role in the mode of action against  
436 *V. diabolicus*.

437 The importance of the cysteines in the thermostability of the BRICHOS-AMPs was also  
438 investigated. The ABU forms of the three BRICHOS-AMPs were exposed for 30 min to 1  
439 hour to the three temperatures (4 °C, 10 °C and 42 °C) considered as optimal for their  
440 respective worms (Fig. 6B) before being used in antibacterial assays against *V.*  
441 *alginolyticus* and *V. diabolicus*. ABU-POL kept the same activity as POL (Fig. 6A) against *V.*  
442 *alginolyticus* at 4 °C and 10 °C. However, when incubated at higher temperatures (42 °C  
443 and 90 °C), ABU-POL still inhibited partly the growth of *V. alginolyticus* unlike the native

444 peptide POL, which is inactive in such conditions. ABU-POL even showed a reduced but  
445 still existing activity against this bacterium after being heated 30 min at 90 °C. Both POL  
446 and its ABU form were inactive against *V. diabolicus* whatever the AMP thermal  
447 incubations tested. ABU-ARE was as active as ARE against *V. alginolyticus* and *V. diabolicus*  
448 at 4 °C and 10 °C. At 42 °C and above, ABU-ARE became almost inactive against the two  
449 tested strains. Without disulfide bridges, ALV was only active against *V. alginolyticus* at 4  
450 °C and 10 °C. No activity was observed at 42 °C and above.

451 Because POL, ABU-POL and ABU-ALV were not active against *V. diabolicus*, the importance  
452 of the disulfide bridges in the pH stability of the ABU-AMPs versus the AMPs was only  
453 investigated against *V. alginolyticus* (Fig. 7). Upon acid/basic conditions, ABU-variants  
454 kept their antibacterial activity against *V. alginolyticus* (Fig. 7B), displaying a slight  
455 decrease of activity (2-4-fold) especially under the most extreme pH conditions (4 and  
456 10). ABU-POL was more resistant to pH changes than the native form, with an  
457 antimicrobial activity that slightly increased compared to POL.

458

#### 459 **4. DISCUSSION**

460 The members of the BRICHOS AMP family constitute a remarkably attractive model to  
461 study the evolution of AMPs, as actors of worm's immune defense in extreme and  
462 fluctuating environmental conditions. In a previous study, genetic analyses of the *ALV*  
463 *precursor* encoding gene in two sister and syntopic species of alvinellid worms (*A.*  
464 *pompejana* and *A. caudata*) sharing the same hydrothermal vent conditions, showed no  
465 differences of the AMP sequence over a geographic range spanning the 6000 km of East  
466 Pacific Rise [39][22]. Despite multiple genetic duplications the *ALV precursor* gene in both  
467 sister species [22], sharing the same biotic and abiotic environmental conditions  
468 constitutes a strong genetic purifying way of maintaining exactly the same AMP to interact  
469 with common microbiome during the evolution race [40,41].

470 Here, an “*in silico* approach” consisting in blasting the sequence of preproalvinellacin on  
471 the Antarctic *Amphitritides sp.* transcriptome [15] allowed us to identify a novel member  
472 of the BRICHOS-AMP family, named polaricin (POL). POL does not show any similarities  
473 with other known molecules. Focusing on BRICHOS-AMP precursors (ARE, POL and ALV)  
474 from annelids living in opposite habitats (temperate, polar, hot vent), we provided  
475 evidence for almost no similarity in AMP primary sequences and conformations, while  
476 exhibiting a nearly conserved BRICHOS domain.



477 According to the mass spectrometry analysis, the Antarctic AMP seems to adopt a  
478 dimerized form presumably through a disulfide bridge engaging each unique cysteine  
479 residue of POL, what is unusual for an AMP. The difference between the predicted  
480 structural conformations of the homodimer (two parallel beta strands) *versus* the  
481 monomeric form (alpha helix) of POL underlines puzzling mechanisms that require  
482 further investigations. The best predicted 3D structures of POL do not show any  $\beta$ -hairpin  
483 formation with two antiparallel strands unlike the solved structures of ARE and ALV.  
484 These data suggest a common origin of the precursor molecule with a clear divergent  
485 evolution of the AMP itself, which seems to be driven by strong disruptive ecological  
486 constrains associated with the worm's lifestyle.

487 According to common-garden approaches, the manipulation of environmental factors  
488 (microbial community, temperature and pH) is a powerful method to test for the role of  
489 the species *preferenda* in the evolution of their external immune defenses [1]. Using the  
490 "home *versus* away" approach, we showed that ARE and ALV are locally adapted to the  
491 bacterial communities of the worm's environment they belong to, while POL is  
492 maladapted to interact with both the shallow temperate and the deep hydrothermal-vent  
493 microbiota. According to the MIC data, ALV and ARE indeed kill faster and more efficiently  
494 the bacterial strains encountered in the habitat of their own worm. Interestingly, POL was  
495 inefficient against the hydrothermal vent *V. diabolicus* but displayed the best efficacy  
496 against *V. alginolyticus*, a bacterium that colonizes both polar and cold temperate habitats,  
497 what supports a local adaptation of this AMP. The demonstration would have been even  
498 more convincing by observing a strong activity of POL against a strictly polar marine  
499 bacterium but this requires the use of a strain cultivable under the conditions of a MIC  
500 assay. Such a strain was not available so far. Unlike *Vibrio*, *Pseudomonas sp.* isolated from  
501 hydrothermal vents was easily killed by the three AMPs regardless of their habitat of  
502 origin. The *Pseudomonas* genus is cosmopolitan to marine waters and terrestrial habitats  
503 (e.g., freshwater, soil...) and can be considered as one of the most successful bacterial  
504 groups on Earth. Worms studied here likely have encountered species from the  
505 *Pseudomonas* genus close to the species used here. Such AMP specificities support the role  
506 of AMPs in the invertebrate adaptation to environmental changes through selective  
507 constraints associated with the surrounding bacterial communities as previously  
508 exemplified in studies demonstrating their involvement in the immune defense and in the  
509 control of the vital ectosymbiosis of *A. pompejana* and of the shrimp *Rimicaris exoculata*

510 [15,43]. Data confirmed not only that ARE, ALV and POL display an optimal killing activity  
511 against the *Vibrio* bacterium typical of the habitat where each worm species is living, but  
512 also that this killing efficacy is optimal/kept under the thermo-chemical conditions under  
513 which the worms are living.

514 The correlation between species habitat and the cysteine content of the BRICHOS-AMPs  
515 led us to investigate the importance of disulfide bridges in the biological efficacy of the  
516 three molecules as a function of abiotic pressures (pH and temperature). The use of ABU-  
517 chemical variants, provided evidence of the strong involvement of internal disulfide  
518 bridges in the stability of BRICHOS-AMPs when exposed to thermal and/or pH stresses.  
519 This allowed us to conclude that the disulfide bridge patterns of ALV and ARE are not only  
520 designed for a better bacterial killing but also a way to sustain the highly fluctuations of  
521 the worm's environment (tidal emersion and seasonality for *A. marina* and chaotic mixing  
522 of hot fluid with cold seawater for *A. pompejana*) [24,32,35,44]. These results are  
523 consistent with previous data on chemically synthesized fragments (*i.e.* not naturally  
524 existing) designed from an ARE isoform [45]: the synthetic fragment containing disulfide  
525 bridges were not affected by pH variations (4.0 to 10.0) and more thermostable (range  
526 20-80 °C) than alternative linear fragments (devoid of disulfide bridges) which also  
527 exhibited lowest antibacterial activities [46]. By ensuring the  $\beta$ -hairpin structure, the  
528 disulfide bridges confer both a higher stability and a much more efficient bactericidal  
529 activity to ALV and ARE [47–51].

530 Unexpectedly, ABU-POL was more effective against both mesophilic and thermophilic  
531 bacteria, displaying a more stable structure at higher temperatures and more acid or basic  
532 waters than the native dimeric form. To date, the dimerization of AMPs was mainly  
533 performed with the aim of improving the performance of synthetic peptides for  
534 therapeutic use (reviewed in Lorenzon et al., 2019 [52]). Chemical dimerization *via* the  
535 creation of disulfide bonds of some AMPs such as magainin and histatin conducted to an  
536 enhanced antimicrobial potency and a reinforced resistance to proteases while the  
537 dimerization of others such as the alpha helical aurein, reduced the antimicrobial  
538 activities by changing the mechanism of action of the peptide [53]. In the specific case of  
539 POL, the formation of homodimers might have been selected during the evolution race,  
540 probably as a response to the progressive cooling of the Antarctic continent, about 25 Mya  
541 [54,55]. Although less efficient against temperate to thermophilic bacterial strains,

542 peptide dimerization might be advantageous against polar psychrophilic microbes and  
543 such hypothesis as well as the structural conformation of POL still need to be investigated.

544

### 545 **Conclusion and perspectives**

546 Based on POL, a novel BRICHOS-AMP sequence discovered in a new terebellid polychaete  
547 strictly inhabiting Antarctica, we have performed a comparative analysis between  
548 different BRICHOS-domain AMPs, specific of marine polychaetes inhabiting contrasted  
549 thermal habitats. Due to its unusual dimerization, further investigations are however  
550 required to elucidate both the structure and the mode of action of this novel polar AMP.  
551 The presence of a BRICHOS domain linked to a potent AMP and its successful evolution in  
552 polychaetes raised the matter of fully investigating on their specific molecular interaction  
553 notably by investigating the role of the BRICHOS domain as a molecular chaperone to the  
554 folding/unfolding of the host defense factors of these worms.

555 For the first time, we provided evidence that BRICHOS-domain AMPs evolved under  
556 strong diversifying environmental pressures to be more efficient under the ecological  
557 preferences of the worm they belong to. They are structurally shaped and adapted to exert  
558 their activities under specific conditions and against specific microbial targets.  
559 Considering the multiple interacting and changing nature of the biotic and abiotic  
560 conditions of the worms, these surveys might provide guidelines of the evolutionary  
561 response of organisms to future scenarios, like ocean warming and acidification [56–58].  
562 The present data also support the importance of considering the optimal/natural  
563 environment of active substances when investigating and optimizing their use in  
564 therapeutic treatment of microbial diseases known to modify/alter the cellular  
565 microenvironment [59].

566

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572

573 **Conflicts of Interest:** The authors declare no conflicts of interest.

574

575 **TABLES**

576 **Table 1:** Antibacterial activities against environmental bacteria of the three BRICHOS-  
 577 AMPs: the “cold” POL, “temperate” ARE and “hot” ALV. MIC: Minimal Inhibitory  
 578 Concentration; MBC: Minimal Bactericidal Concentration; > not active at this  
 579 concentration and beyond

HABITATS	BACTERIA	MIC (µM)			MBC (µM)		
		ALV	ARE	POL	ALV	ARE	POL
Temperate, polar Intertidal	<i>Vibrio alginolyticus</i>	0.31	0.15	0.625	0.625	0.31	5
Temperate Intertidal	<i>Vibrio fluvialis</i>	0.625	0.31	40	10	1.25	>40
Temperate Intertidal	<i>Shewanella algae</i>	>40	40	>40	>40	>40	>40
Temperate Intertidal	<i>Oceanisphaera donghaensis</i>	0.625	0.15	5	1.25	0.625	20
Hot vent Deep sea	<i>Vibrio diabolicus</i>	2.5	5	>40	20	20	>20
Hot vent Deep sea	<i>Pseudomonas sp.</i>	0.07	0.15	0.31	0.31	1.25	5

580

581 **Table 2:** Comparison of the antibacterial activities against the hot vent *Vibrio diabolicus*  
 582 and the polar/temperate *Vibrio alginolyticus* of the three BRICHOS-AMPs (the “cold” POL,  
 583 “temperate” ARE and “hot” ALV) versus their respective ABU variants devoid of disulfide  
 584 bridges. MIC: Minimal Inhibitory Concentration; MBC: Minimal Bactericidal  
 585 Concentration; > not active at this concentration and beyond

BACTERIA	MIC (µM)						MBC (µM)					
	ALV	ABU-ALV	ARE	ABU-ARE	POL	ABU-POL	ALV	ABU-ALV	ARE	ABU-ARE	POL	ABU-POL
<i>V. alginolyticus</i>	0.31	0.62	0.15	0.31	0.625	0.31	0.625	1.25	0.31	1.25	5	2.5
<i>V. diabolicus</i>	2.5	>40	5	>40	>40	>40	20	>40	20	>40	>20	>40

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839

840 **FIGURE LEGENDS**

841 **Figure 1:** Geographical distribution of the three polychaetes studied together with the  
842 description of their marine habitats.

843

844 **Figure 2:** Alignment of the precursors of the BRICHOS-AMP family members: polaricin  
845 (POL), nicomicin, alvinellacin (ALV) and arenicin (ARE). BRICHOS and AMP regions are  
846 respectively in black and dotted black rectangles. In the AMP part, cysteine residues  
847 involved in disulfide bridges are in black circles. The number of residues relative to the N-  
848 terminal segment of the precursor is shown on the right side of each sequence.

849 The color of amino-acids background underlines the higher level of conservation in the  
850 BRICHOS part as compared to the AMP part itself. The alignment was generated using CLC  
851 Sequence Viewer software (version 8.0).

852

853 **Figure 3:** Three-dimensional structures of ALV, ARE and the predicted ones of POL  
854 generated by Alpha Fold software. AlphaFold produces a per-residue confidence score  
855 (pLDDT) between 0 and 100: Very high confidence (pLDDT > 90); Confident (90 > pLDDT  
856 > 70); Low (70 > pLDDT > 50); Very low (pLDDT < 50).

857 Number of cysteines in the three AMPs as a function of thermal and pH encountered in  
858 the habitat of each worm.

859

860 **Figure 4:** A representation of BRICHOS-AMPs local adaptation (home vs. away diagram)  
861 to temperate-coastal and hydrothermal vents-deep sea habitats, using their MIC values  
862 against environmental bacterial strains. Y-axis, representative of fitness factor, stands for  
863 peptides MIC log-transformed data: higher MIC values correspond to lower fitness levels.  
864 The colored areas gather the values belonging to the same peptide. Dashed lines,  
865 connecting the areas across the two habitats, show the molecular adaptation of ARE and  
866 ALV to their respective habitats, displaying lower MIC values against “home strains” and  
867 *vice versa*. Missing polar bacterial strains, POL displays the highest MIC values against the  
868 investigated “away strains”. Peptides and bacteria sharing a common environment are  
869 displayed by the same color: red for hot vent, blue for temperate and purple for polar. The  
870 cases of not-active peptide are represented with – on the top of the diagram, as leaning to  
871 infinity.

872

873 **Figure 5:** Kinetics profiles of the bactericidal activities of ALV, ARE and POL against *V.*  
874 *alginolyticus* and *V. diabolicus*, at 10 °C and 42 °C. The results are shown as time-plot of  
875 *Vibrio spp.* viability ( $\log_{10}$  of CFU/mL), without (control) and with peptides.  
876 Temperatures, peptides and strains belonging to the temperate, hydrothermal vent and  
877 polar environments are in blue, red and purple respectively. The dotted grey lines are the  
878 negative controls with no peptides.

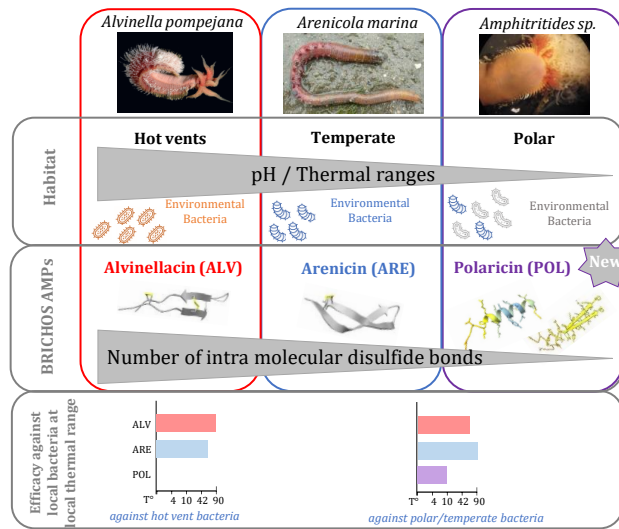
879

880 **Figure 6:** Effect of temperature on biological activity of (A) native peptides and (B) ABU-  
881 peptides, against *V. alginolyticus* and *V. diabolicus*, after 30 minutes to 1 hour of peptide  
882 incubation at different temperatures (4, 10, 42 and 90 °C). Each bar is expressed as the  
883 average with error bars, representing  $\pm$ SD from 3 independent experiments (n = 3). The  
884 Y-axis (in A) displays a break in the range of values to improve its readability.

885

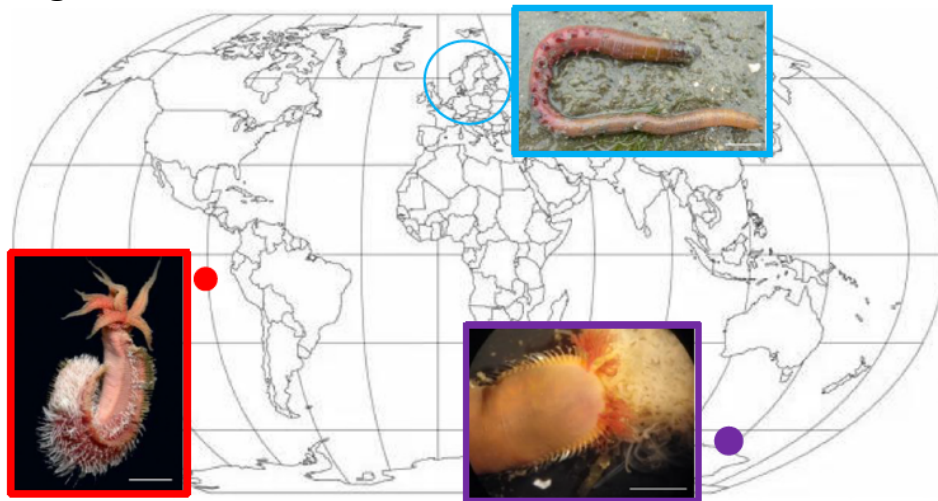
886 **Figure 7:** Effect of pH on the biological activity of (A) native peptides and (B) ABU-  
887 peptides activities, against *V. alginolyticus*. The tests were performed after peptide  
888 incubation (3 hours) under acid/basic conditions (at pH 4, 6, 8 and 10). The control is  
889 represented by peptide MIC values in MHB medium (pH 7.4) for each peptide.

## Graphical abstract



- Local adaptation of AMPs to the ecological niche of the producers

Figure 1



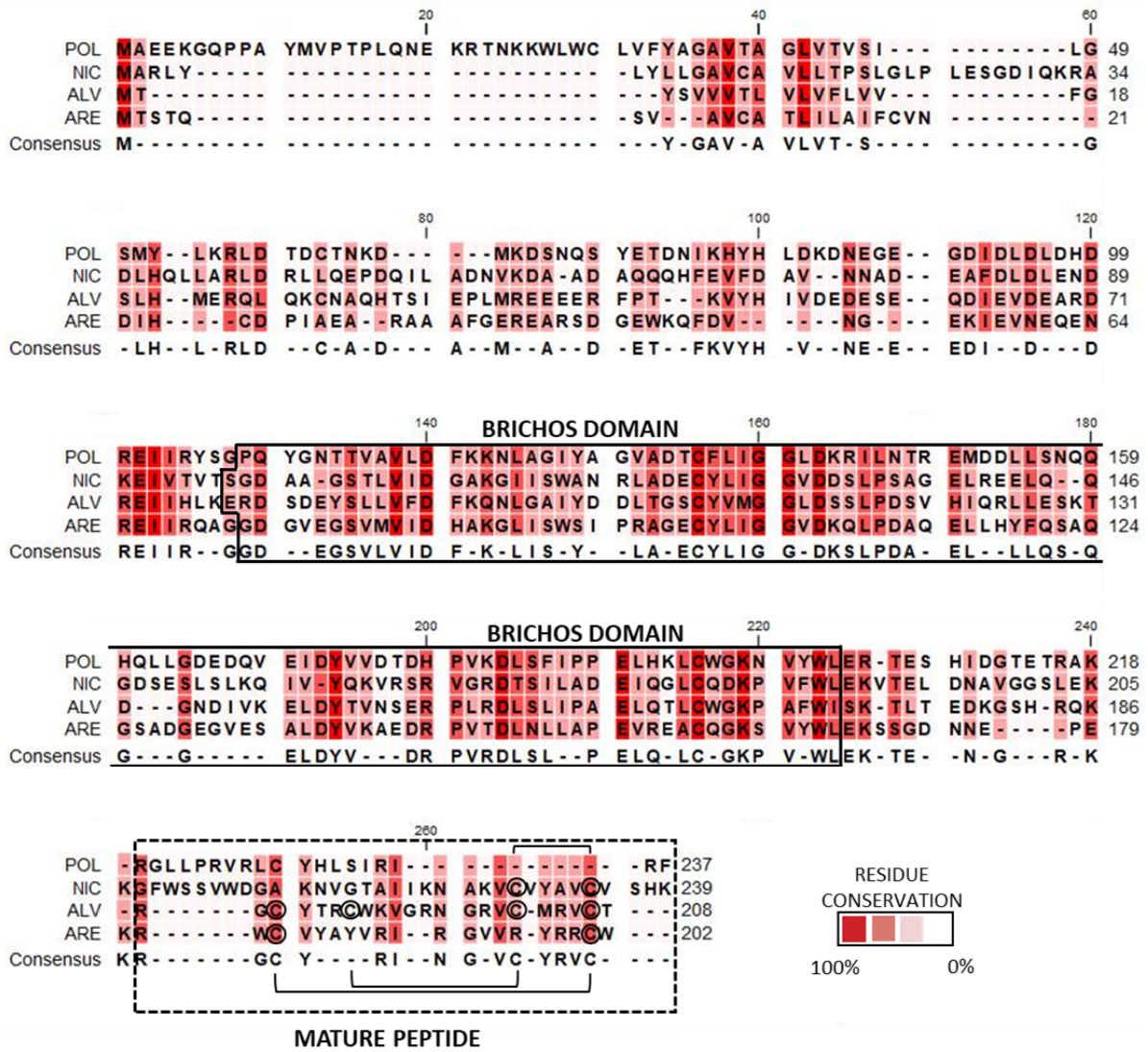
Annelid species	<i>Amphitritides</i> <i>sp.</i>	<i>Arenicola</i> <i>marina</i>	<i>Alvinella</i> <i>pompejana</i>
AMP name	Polaricin (POL)	Arenicin (ARE)	Alvinellacin (ALV)
Environment	Polar, coastal	Temperate, coastal	Hot chimneys of hydrothermal vents, deep sea,
Distribution	Antarctica (Adelie Land)	North Sea	East Pacific Rise 9 North



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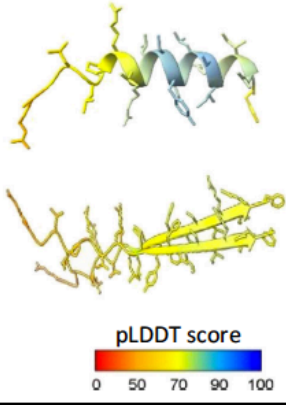

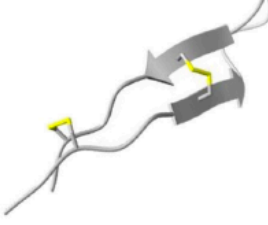
Figure 2



895

Figure 3

896

	POL	ARE	ALV
Structure	 <p>pLDDT score 0 50 70 90 100</p>	 <p>PDB : 2JSB</p>	 <p>PDB : 2LLR</p>
Number of Cys	1	2	4
Number of intramolecular disulfide bridges	0	1	2

Thermal range of the worms' habitat

		<i>Amphitritides sp.</i>	<i>Arenicola marina</i>	<i>Alvinella pompejana</i>	
HABITAT	T°	Range	-1.8 °C – 1 °C	5 °C – 25 °C	2 °C - >100 °C
		Optimum	~0 °C	~10 °C	~42 °C

897

Figure 4

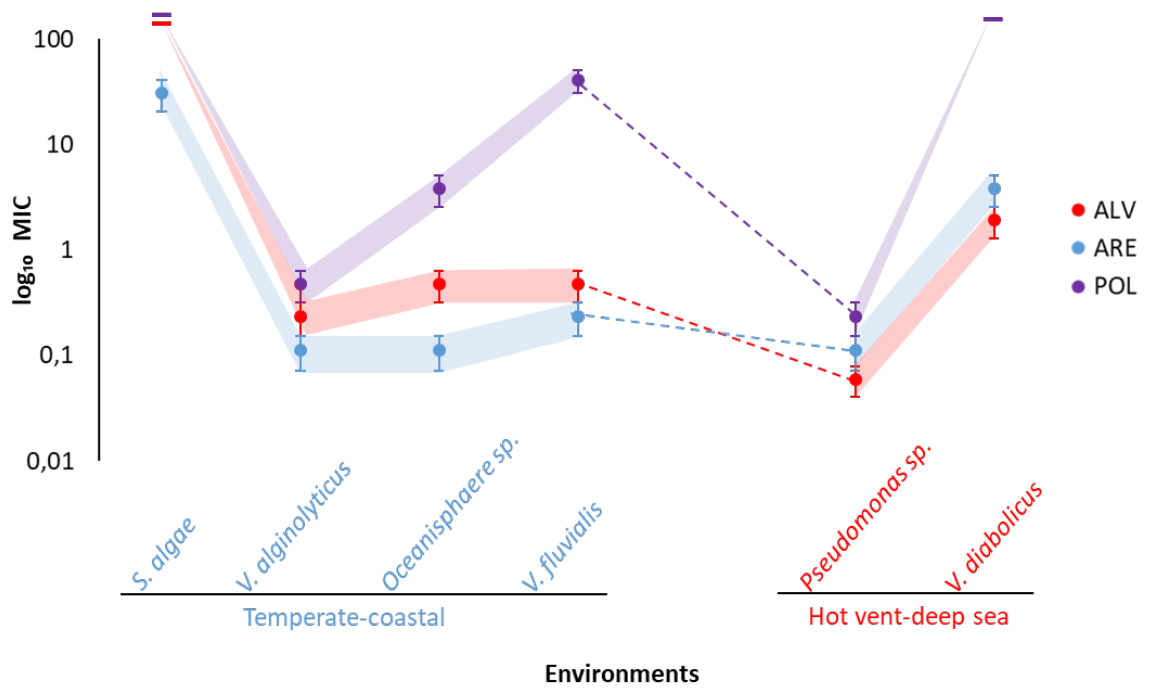




Figure 5

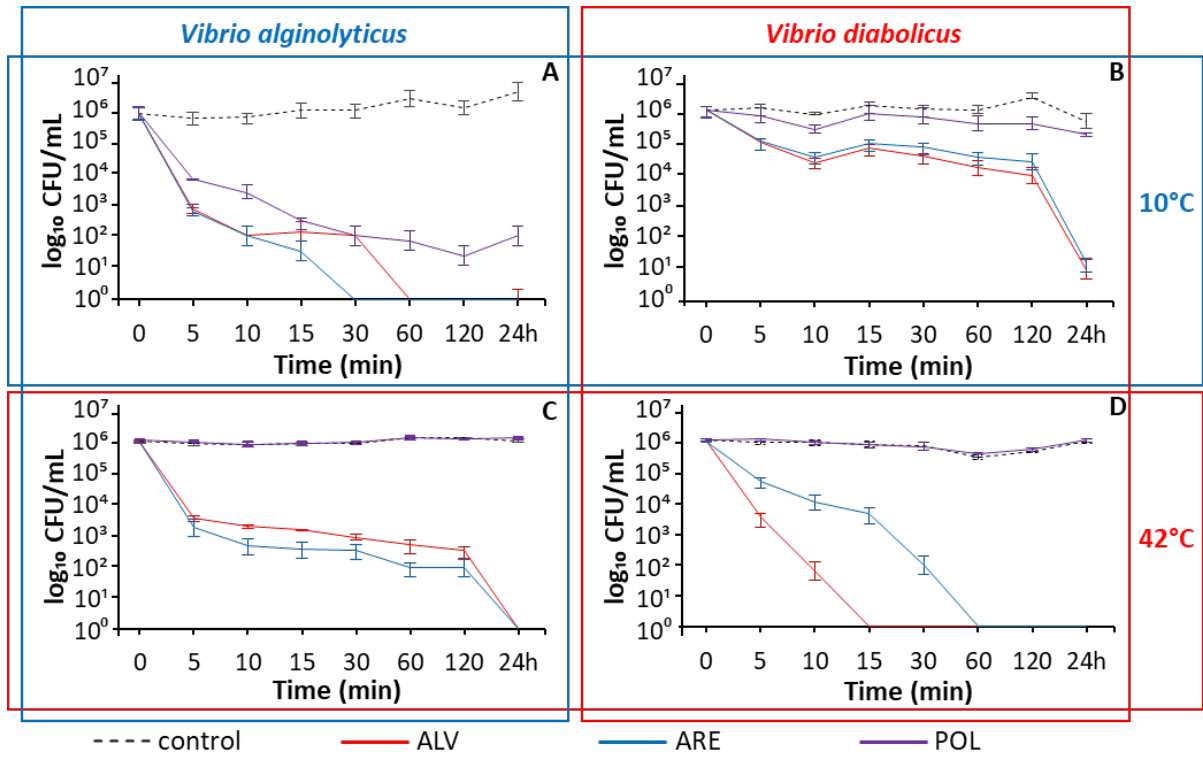
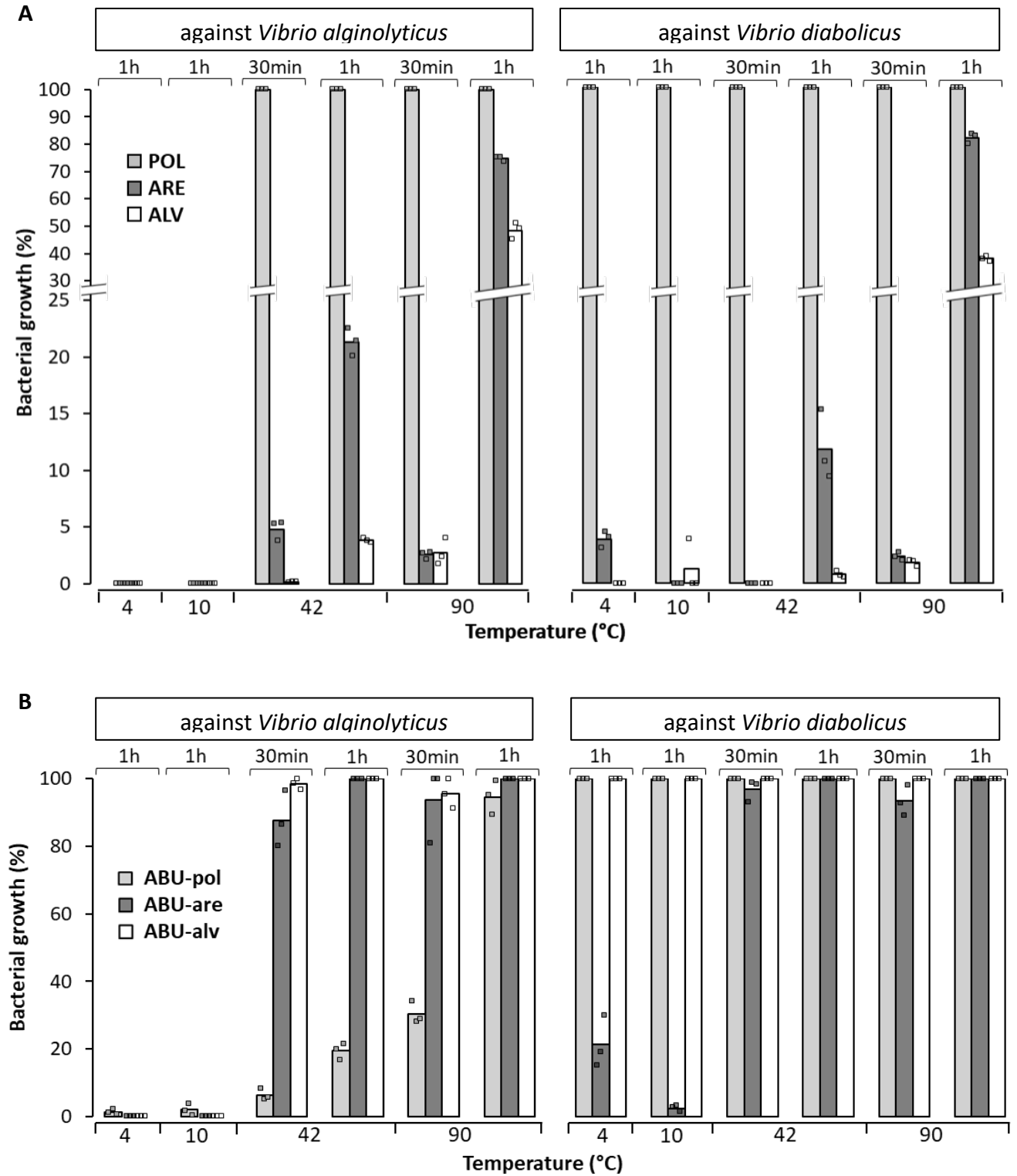


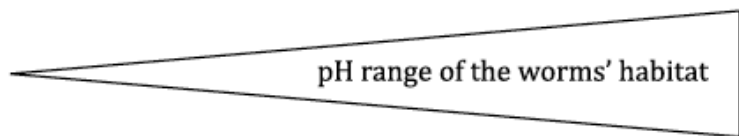
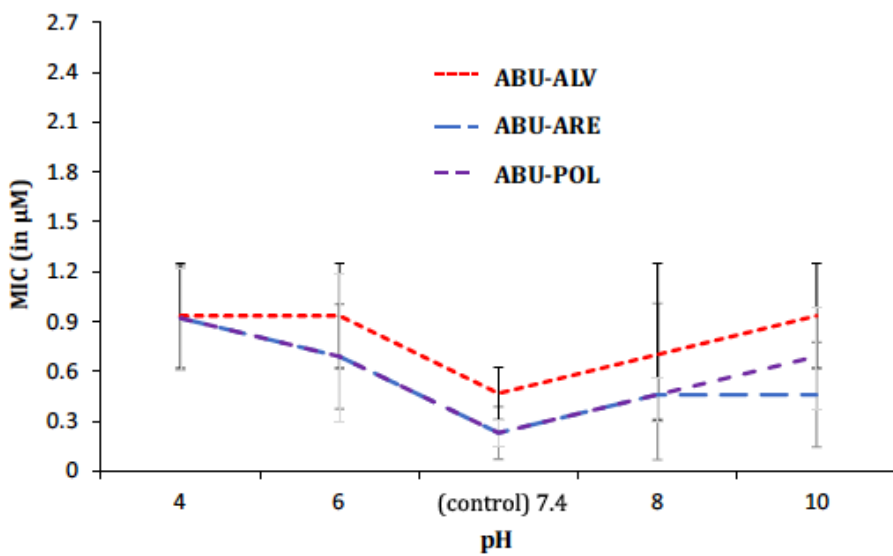
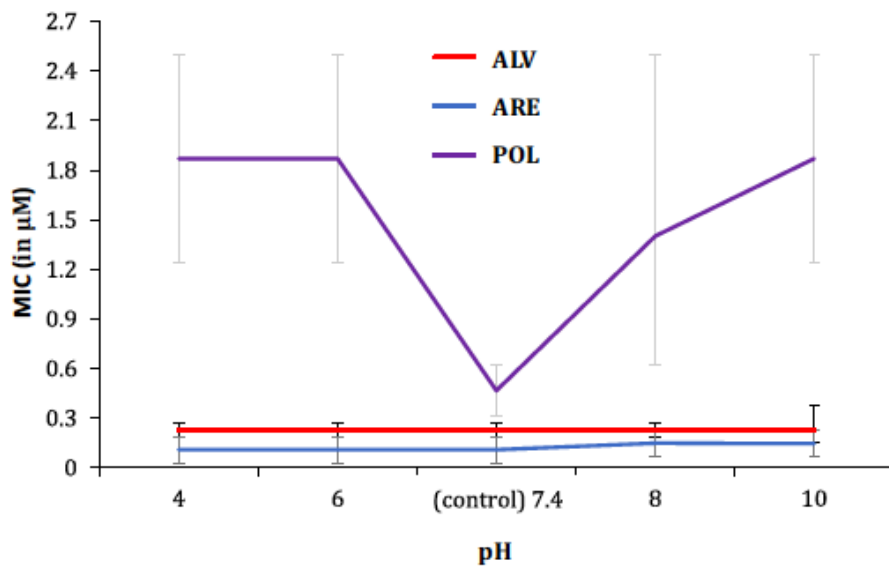
Figure 6



901

902

Figure 7



		<i>Amphitritides sp.</i>	<i>Arenicola marina</i>	<i>Alvinella pompejana</i>	
HABITAT	pH	range	7.8 - 8.6	5.4 - 8.4	3.9 - 8.5
		optimum	7.5 - 8	6.5 - 8	5.7 - 8

903