Reduction of bacterial biofilm formation using marine natural antimicrobial peptides

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Abstract: There is an important need for the development of new "environmentally-friendly" antifouling molecules to replace toxic chemicals actually used to fight against marine biofouling. Marine biomass is a promising source of non-toxic antifouling products such as natural antimicrobial peptides produced by marine organisms. The aim of this study was to demonstrate the efficiency of antimicrobial peptides extracted from snow crab (SCAMPs) to reduce the formation of marine biofilms on immerged mild steel surfaces. Five antimicrobial peptides were found in the snow crab hydrolysate fraction used in this study. SCAMPs were demonstrated to interact with natural organic matter (NOM) during the formation of the conditioning film and to limit the marine biofilm development in terms of viability and bacterial structure. Natural SCAMPs could be considered as a potential alternative and non-toxic product to reduce biofouling, and as a consequence microbial induced corrosion on immerged surfaces.

Highlights

1- Snow crab peptides are promising source of non-toxic antifouling products
2- Peptides interact with natural organic matter for a new conditioning film
3- Snow crab peptides modify the bacterial richness of the marine biofilm
4- Snow crab peptides reduce the bacterial viability of the marine biofilm

Keywords

Antimicrobial peptides; Antifouling; Bacterial diversity; Conditioning film; Marine biofilm

1. Introduction

Biofouling occurs worldwide in various industries, from fishing equipment, offshore oil and gas industries, cooling systems to canalizations using water. Unprotected submerged marine metallic surfaces are inevitably subjected to biofouling and corrosion, leading to considerable economic and environmental consequences for marine industries. The cost of biofouling for marine industries is evaluated at several billions US dollar per year [1] and the development of environmentally friendly antifouling strategies is a great challenge today [2]. Up to recently, synthetic chemicals agents, such as tributyltin (TBT) and 2-méthylthio-4-tert-butylamino cyclopylamino-6-(1,3,5-triazine) (Irgarol 1051) were used in paint formulation to prevent and protect metallic structures from biofouling. However, due to their toxicity for non-target marine organisms, their use was restricted or prohibited (as for TBT in 2008) following the recommendations of the Marine Environment Protection Committee (MPEC) and the International Maritime Organization [3-5]. Although the use of synthetic biocide are still on-going (e.g. 4,5-dichloro-2-n-octyl-4-isothiazolin-3-one (Sea-Nine 211)), "green" alternatives are now the focus of many researchers worldwide.

The first step of the biofouling is initiated by different bacterial species and the compound of seawater as natural organic matter organized into a microenvironment called a biofilm. When the biofilm settles on submerged or periodically submerged metallic surfaces, such as mild steel, it not only modifies physically the surface (and then changes the hydrodynamicity of the installation, e.g. boat hull or pipe) but it may in addition accelerate the corrosion through the microbiologically influenced corrosion process [6]. Christensen et al. [7] and Nielsen et al. [8] have demonstrated that the biofilm is a dynamic microenvironment, where intra and interspecific interactions directly influence its

morphology and bacterial species survival. As a consequence, reducing bacterial adhesion and limiting the expansion of the biofilm are essential to limit the impacts of biofouling on submerged steel structures. Steinberg et al. [9], Holmström and Kjelleberg [10] and Callow and Callow [11] were pioneers in the development of alternative antifouling products from marine biomass, essentially from seaweeds. Since their discovery, over 100 marine natural products were identified as antifouling and several other products are studied for these properties [12, 13].

One of the main advantages of using marine biomass as "green" antifouling strategies is the valorisation of marine by-products, which are considered as under-exploited wastes for marine industries. Many substances have been extracted from these products and several applications in antifouling paints have been developed [14, 15]. Lactones, alkaloids, polysaccharides and fatty acids are among extractable products originating from marine biomass [13, 14, 16]. Another class of biomolecules with promising applications are the antimicrobial peptides [17] principally with the presence of D-amino acids which could inhibit the biofilm formation [18]. These AMP are widely available and derived from a variety of organisms such as animals, plants, bacteria, fungi and viruses [19] but also from marine by-products.

Incorporating a bio-sourced antifouling agent in paint is not the sole way to take advantage of its capacity to limit the development of marine biofilms on immerged metallic surfaces. By designing the antifouling as a free water soluble additive, it can be include in a mitigation strategy of fouling growth on inert parts of confined metallic structures (e.g. seawater cooling system, pipes, ship ballast tanks, seawater storage reservoirs), otherwise difficult to reach during maintenance work.

In Canada, the harvesting of snow crab is one of the most successful fisheries, with a landing volume around of 103,000 metric tons per year. As a consequence, year to year, over than 30,000 tons/yr of snow crab by-products (cephalothorax shells, digestive systems, including hepatopancreas and hemolymph) are buried in landfill sites in the province of Québec (Canada) [20]. However, upcoming environmental regulations will forbid landfilling of marine wastes in 2020. This challenges the Canadian fishing industry to diversify their activities on by-products valorisation and on biotechnology to ensure aquatic biomass enhancement [21]. Untapped residues of snow crab transformation could constitute a valuable source of components for antifouling strategies. Whereas several AMP were identified or cited in the literature, no AMP from snow crab (*Chionoecetes opilio*) (SCAMPs) were listed among the 2000 AMP present in the Antimicrobial Peptides Database (http://aps.unmc.edu/AP/main.php). One of our previous studies have shown that SCAMPs inhibit the growth of specific bacteria in pure cultures [22] but, to our knowledge, there is still no information concerning their potential as antifouling agents. The aim of our study was to demonstrate the efficiency of SCAMPs as antifouling agents by limiting the formation of marine biofilms on mild steel plates immerged in seawater.

2. Materials and Methods

2.1. Enzymatic hydrolyzed fractions of snow carb by-products

Snow crab hydrolysate fractions were produced at Merinov, the Quebec Fisheries and Aquaculture Innovation Centre (Gaspé, QC, Canada) according to a procedure by Beaulieu

et al. [20]. Briefly, 100 kg of grinded snow crab by-products were added to equal amount of demineralized water (w/w), the total volume was heated to 45°C. Then, 100 g Protamex (Novozymes, Bagsvaerd, Denmark) were added to start the hydrolysis. After 120 minutes hydrolysis at 45°C, the tank temperature was increased to 90°C, to inactivate proteases. The liquid fraction was decanted using a clarifying decanter and then centrifuged at 11,000 g to separate suspended insoluble matter and lipids from the hydrolysate. The hydrolysate was then ultrafiltered (spiral membrane with cut off of 10 kDa) to separate proteins and peptides according to the molecular mass. Permeate from the 10 kDa membrane at 200 Da was nano-filtered (Model R, GEA filtration, Hudson, WI, USA) to obtain a 10 kDa – 200 Da retentate (SCAMPs). Nano-filtration retentate was spray-dried and kept at 4°C until analyses.

2.2. Amino acid identification

Amino acid determination of fractions was performed according to the method described by Beaulieu et al. [23] using the AccQ-Tag amino acid analysis procedure (Waters, Canada). Briefly, the AccQ-Tag method is a pre-column derivatization technique for amino acids in peptide and protein hydrolysates. The amino acids were separated by reversed-phase high performance liquid chromatography (RP-HPLC) and quantified by fluorescence detection. The HPLC system used was equipped with a Waters Alliance e2695 Separations Module (Waters, Mississauga, ON, Canada) and a Waters 2475 Multi λ Fluorescence Detector. Analyses were performed in duplicate and averages are shown.

2.3. Peptide identification by tandem mass spectrometry

Analyses by mass spectrometry were performed using the proteomics platform from Quebec Genomics Centre (Québec, QC, Canada) following the procedure described by

Beaulieu et al. [24]. Briefly, 10 µg of proteins were washed 3 times with 50 mM ammonium bicarbonate buffer and 1 µg of trypsin was added before analysed by electrospray mass spectrometry (ES-MS/MS) (Agilent 1200, AB Sciex, Framingham, MA, USA). All MS/MS peak lists were analysed by Scaffold software (version Scaffold_4.2.0, Proteome Software Inc., Portland, OR, USA). Peptide identifications were accepted if they could established at greater than 85% probability by the Peptide prophet algorithm [25] with Scaffold delta-mass correction.

2.4. Growth conditions and biofilm formation assays

The experiments were designed as part of a larger project on the potential of SCAMPs as inhibitor of corrosion of mild steel [26].The biofilm development on metallic surface, with and without bioactive peptides, was monitored during 10 days on 36 mild steel coupons (2.5 cm x 4 cm) in natural seawater collected from the St. Lawrence Estuary (Rimouski, QC, Canada). For each treatment the coupons were immerged in a 10 L seawater tank, and kept at a temperature of 20°C ± 0.01 (Digital temperature controller 1196D, VWR) throughout the experiment. This temperature, close to room temperature, was chosen according to previous results on microbial induced corrosion performed in the laboratory that demonstrated no significant difference between corrosion inhibition at 15°C and 20°C [26]. The seawater (containing around $1.8 \times 10^6 \pm 0.6 \times 10^6$ bacteria.mL^{-1}) and the first tank containing this seawater was used as control whereas the second was SCAMP-treated (300 mg.L^{-1}). In seawater, bacteria were enumerated using an EPICS ALTRATM cell sorting flow cytometer (Beckman-Coulter Inc., Mississauga, Canada) equipped with a laser emitting at 488 nm according to Doiron et al. [27]. The biofilm formation was followed by collecting six plates at 3, 24, 48, 96, 168 and 240 hours. At each sampling

time, three plates were placed into a 30 mL solution of NaCl 9‰, sonicated three times for 1 minute at 20°C, filtered on polycarbonate membranes (0.2 µm pore size, 25 mm diameter) and the filter was conserved at -80°C until further analyses of bacterial composition by PCR-DGGE (Denaturing gradient gel electrophoresis) (C.B.S. Scientific Company, CA, USA). The remaining three plates were immediately analyzed for biofilm by confocal laser scanning microscopy LSM700 (CLSM) (Carl Zeiss, Germany).

2.5. Fourier transform infrared spectrometry

Fourier transform infrared spectrometry (FTIR) was used to determine the presence of peptides groups on metallic surfaces. Spectral acquisition were realized with a FTIR (Nicolet 6700, Thermo Scientific, USA), in an infrared medium spectral domain (400 cm^{-1} to 4 000 cm^{-1}) with a 40 scans numbers and a 4 cm^{-1} resolution.

2.6. Bacterial cell arrangement, viability and thickness of biofilm

Confocal microscopic observations were performed on a LSM700 (Carl Zeiss, Germany) microscope at 40X magnification. Biofilm was stained directly on mild steel plates with the LIVE/DEAD® Bac Light™ Bacterial Viability Kit (cat. no. L-7012, Molecular Probes Inc, Eugene, Oregon, USA). Briefly, a 10 µM SYTO9 (λ excitation and emission: 480 and 500 nm) and 60 µM propidium iodide (PI) (λ excitation and emission: 490 and 635 nm) mix was added onto the biofilm and each plate was stained during 15 minutes in the dark [28, 29]. After staining, biofilm thickness and viability were evaluated using Zeiss software (Carl Zeiss, Germany).

2.7. Bacterial community composition in biofilm

Total DNA was extracted from biofilm using the MoBio PowerSoil DNA Isolation Kit (cat. no. 12888-05, Mo Bio Laboratories, Carlsbad, CA, USA). Bacterial community

composition was analyzed on each sample according to the PCR-DGGE procedure described in Moreau et al. [30]. Briefly, PCR amplification of the 16S rDNA gene was performed using the universal primers 341F-GC and 907R according to Schäfer and Muyzer [31]. Three PCR amplifications were performed on each DNA sample to overcome the effect of PCR biases [32]. Amplicons were pooled then purified with the MinElute (Qiagen, Mississauga, ON, Canada) and stored at -20°C until DGGE analysis. DGGE analysis was performed using a DGGE-4001-Rev-B (C.B.S. Scientific Company, CA, USA) system according to Schäfer and Muyzer [31]. After migration, gels were stained with a half-diluted solution of SYBR Green I (10,000X, Invitrogen, Inc.) in TAE buffer for 1 hour. Gels images were analyzed using an AlphaImager HP (Alpha-Innotech, San Leandro, CA, USA). The number of bands, corresponding to different operational taxonomic units (OTUs) [33], was determined, and the comparison between DGGE fingerprints was performed using the Phoretix 1D Pro software (Nonlinear Dynamics, Newcastle Upon Tyne, UK) on the basis of a similarity matrix using Jaccard's index [34, 35].

2.8. Statistical analysis

For each treatment, two-way ANOVA was used to test for differences for biofilm thickness and viability. All statistical analyses were done using SYSTAT software version 12.0 (Systat Software Inc., Chicago, USA) with $\alpha = 0.05$. Normality of the data was examined using Kolmogorov-Smirnov test. Homoscedasticity was tested with the Levene test. The Tukey test was chosen for comparative between samples when the probability was significant.

3. Results

3.1. Amino acid, proteins and peptides composition

The amino acid composition of the SCAMPs 10 kDa – 200 Da peptidic fractions from *C. opilio* is presented in Table 1. The SCAMPs fraction was composed of 41.08% of essential amino acids as compared to 55.80% for the non-essential amino acids. Leucine, a non-polar amino acid, and lysine, with charged polar side chain, are the most abundant essential amino acids with 7.85% and 7.36%, respectively. Whereas aspartic acid (10.58%) and glutamic acid (11.30%) are the most abundant non-essential amino acids, both negatively charged amino acid at seawater pH around 8. The peptides fragments were identified by ES-MS/MS using a *Pleocyemata* database. A total of 187 sequences were identified representative of the different categories of the proteins (Fig. 1). Using the Mascot program (confidence of >95% homology) with UniProt as protein sequence database, all peptides were identified (data not show). Figure 1 shows that the main precursor is muscular proteins to 59.0%. Cuticular, ribosomal and antimicrobial proteins represent 14.7%, 8.8% and 8.0%, respectively. Hemocyanins represent 4.8% as well as unidentified proteins. Protein precursors of Scaffold software identified peptides were submitted to BLAST (http://www.camp3.bicnirrh.res.in) for determine their potential antimicrobial properties. A total of five peptide sequences were identified as antimicrobial (Table 2).

3.2. Fourier transform infrared spectrometry (FTIR)

Figure 2 shows the differences obtained by FTIR analysis between control and SCAMP-treated after 3 hours of immersion of the mild steel coupons. In SCAMP-treated, the absorption band at 1738, 17575 and 1216 cm^{-1} were attributed to ester, amide II and amide III, respectively (Figure 2b). These bands were not present in control treatment (Figure 2a).

3.3. Bacterial cell arrangement, viability and thickness of biofilm

Figure 3 and table 3 represent the temporal evolution of biofilms with and without addition of peptides related to the cellular arrangement, cellular viability and thickness. At 24 hours, for both treatments, only bacterial adhesion was present with a cellular viability to 72.13% for the control and 75.48% for the SCAMP-treated. Microcolonies appeared at 48 hours with a thickness for control and SCAMP-treated of 31 µm and 28 µm, respectively. For the control, a significant increase of biofilm thickness was observed at 96 hours reaching 43 µm ($p > 0.001$) with an evolution the microcolonies by a bacterial mat that continued to grow for the rest of the experimentation. Moreover, at 96 hours, the presence of the bacterial mat was important comparatively to the control but a higher occurrence of dead cells was evidenced by red coloration with a proportion of the dead cells at 80.54% compared to 32.25% dead cells for the control. At the end of the experimentation, water channel had formed in the control biofilm only and the difference of the percentage of cellular viability is the 59.55% for the control and 57.55% for the SCAMP-treated.

3.4. Bacterial richness in biofilms

Eight OTUs were present in the control after 3 hours, increased to 18 after 96 hours and finally decreased at 14 OTUs after 240 hours. In SCAMP-treated, 3 OTUs were present after 3 hours, increased up to 13 after 24 hours and finally decreased at 6 OTUs after 240 hours (Figure 4). At half-time of exposure, no highest variation was observed in the number of OTUs between control and SCAMP-treated. The DGGE patterns also indicate that the first bacterial species present in the biofilm, after 3 hours, were different between treatments (79% dissimilarity) (Figure 4).

4. Discussion

The economic and ecological impacts of biofouling on marine systems constitute a great challenge for the development of many marine industries worldwide. Metallic structures immerged in marine waters are usually rapidly colonized by a variety of organisms and this biofouling affects the performance of the material and may cause its early deterioration. Even if several anti-biofilm studies have focussed on the formation of monospecific biofilm (e.g. *Pseudomonas aeruginosa* or *Staphylococcus aureus*) on immerged surfaces [4, 36], monitoring natural biofilm formation, involving complex communities, is essential to address environmental perspectives. However, such studies are complex due to the interaction between surfaces, microorganisms and chemical compounds that are naturally present in the seawater.

This study on biofilm inhibition or reduction by SCAMPs was part of a larger project; indeed, Tassel et al. [26] have demonstrated that the addition of water-soluble SCAMPs reduced the corrosion of mild steel in natural seawater by 81%. The present study makes a link between the conditioning films, the cellular arrangement, the cellular viability and the diversity of bacterial species. Our study has demonstrated a potential antifouling-effect of SCAMPs combined with natural organic matter (NOM) in seawater. Thus, SCAMPs are hypothesized to form a conditioning film (SCAMPs-NOM) on mild steel plates that modifies the physicochemical properties of their surfaces. The results obtained in FTIR spectrometry show that SCAMPs were absorbed by mild steel plates compared to control. Indeed, the signals obtained are different between the two treatments, having a strong presence of protein groups for the treated plates. Several authors consider that the conditioning film is the first stage of biofilm formation [37-40]. The nature of the

conditioning film is a pre-requisite for cell adhesion that influence the diversity of microbial species present in the biofilm [41, 42]. On immerged surfaces, the natural conditioning film is mainly composed of proteins favouring interactions with microorganisms [43]. These interactions between proteins and bacterial cells are mainly due to van der Waals forces and electrostatic charges [44]. Kolodkin-Gal et al. [18] have shown that the presence of D-amino acids such as D-tyrosine, D-leucine, D-tryptophan and D-methionine could inhibit the biofilm formation in liquid medium as well as on a solid surface. Natural peptides are mostly composed of D-amino acids rather than L-amino acids [19]. Moreover, three of the four amino acids mentioned above were present in the peptide extract used in this study. It is thus possible that the addition of snow crab peptides in natural seawater have altered the nature of the conditioning film on the metallic surface, modifying bacterial species colonizing the surface as well as corrosion [26]. In fact, SCAMPs are composed of molecules already present in NOM but probably shift the distribution of the chemical classes of dissolved organic matter toward those with a limiting effect on biofilm settlement. The lowest concentration of SCAMPs at which inhibition is measurable have not been determined in this study, but it is likely that rapid dilution in the marine environment will preclude any negative effect because of biodegradation of active components of SCAMPs. Despite the large amount of peptides sequences, the presence of the hyastatin, a known antimicrobial, and others AMPs less known, demonstrate the potential of snow crab as antifouling [45]. In addition, some peptide fragments generated from the C-terminal part of crustacean hemocyanin have been shown to possess antimicrobial activities [46, 47]. Di Luca et al. [48] mentioned that the presence of the conditioning film, as well as its composition, is a critical parameter for the subsequent

biofilm formation and that AMPs can interfere with the early adhesion of bacterial cells on this film. This hypothesis is confirmed by our PCR-DGGE results, which demonstrate a difference between the initial bacterial community richness on steel coupons in SCAMP-treated seawater versus untreated seawater. In SCAMP-treated seawater, the low number of the OTUs presents on the metallic surfaces suggests a bacterial selection at the early stage of the biofilm formation.

In natural environment, the diversity of bacterial species present at the early stage of the biofilm formation influence the bacterial species succession for the next stages of the biofilm settlement. The biofilm formation is dependent on the first attachment and the conformation of the mature biofilm is determined by the present species and their proportion within the biofilm [49]. The different bacterial species can modulate the cellular arrangement and the dynamic of subsequent species succession [50]. The addition of SCAMPs might influence the cellular arrangement within the biofilm by interacting with the bacterial membranes and increasing the selective mortality of some bacterial species within the biofilm [51]. Moreover, using natural AMPs avoid the risk of bacterial resistance mechanisms development [52, 53]. The results obtained by confocal microscopy showed that 3D-arrangement and dynamic of the biofilm differ in the presence of the SCAMPs. Indeed, the SCAMPs avoid formation water channels, an indicator of the biofilm maturity, by a higher mortality of the bacterial cells comparatively to the control biofilms. Yala et al. [54] also demonstrated that the peptides modified surfaces and that the mode of actions of the immobilized peptides is bactericidal and not anti-adhesive. These results suggest that the SCAMPs selected bacteria-resistant of SCAMPs and that these bacteria caused the biofilm decrease.

5. Conclusion

The presence of the SCAMPs interacts with the natural organic matter present in seawater to modify the conditioning film on the mild steel. This finding brings a new perspective in the treatment of submerged metal surfaces against biofouling. Further researches on the mode of action of these peptides on biofilm formation and the selection of the colonizing bacteria will allow a better understanding of mechanisms implied in marine biofilms formation. In addition, it could give a better respond in the treatment of biofouling, as SCAMPs could be used as a free water-soluble antifouling agent to protect confined steel structure in contact with seawater, without damaging the coastal environment through release of persistent synthetic chemicals.

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7. References

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510 Tables:

511 Table 1: Total amino acids in 10 kDa – 200 Da hydrolyzed snow crab (*Chionoecetes opilio*)

512 fraction expressed as % (g*100g protein $^{-1}$) on a dry matter basis.

Amino acid	***(%)***
Essential	
Histidine	2.77
Isoleucine	5.04
Leucine	7.85
Lysine	7.36
Methionine	2.51
Phenylalanine	4.37
Threonine	4.47
Tryptophan	N/A.
Valine	6.71
Total (a)	**41.08**
Non-essential	
Alanine	6.47
Arginine	8.98
Aspartic acid	10.58
Cysteine	0.00
Glutamic acid	11.30
Glycine	4.76
Proline	5.81
Serine	3.64
Tyrosine	4.26
Total (b)	**55.80**

513

514 Table 2: Peptide fragments identified using mass spectrometry after a trypsin digestion from the < 10 kDa fraction of *Chionoecetes*

515 *opilio*.

Sequence	Protein (accession number)	Protein name	Protein identification probability	Species sharing >95% homology	Peptide molecular mass [43]	Related antimicrobials reported in the literature sharing homology of 67-100% identities with the identified peptide[a]
MKLVVLALAA	Q5XLK1	Arthrodial cuticle protein AMP6.0	100%	*Callinectes sapidus*	1151.51	Beta-defensin 118, *Equus caballus*, CAMPSQ6957, Predicted
YAYAEDSGTYTCRAT	Q95YM2	I-connectin	100%	*Procambarus clarkii*	1346.70	Protein THN-2, *Caenorhabditis elegans*, CAMPSQ6276, Predicted
NLGGGIGSTRP	C4NZN9	Hyastatin	100%	*Hyas araneus*	1027.54	Hyastatin, *Hyas aranus* (Great spider crab), CAMPSQ2582; active against *C. gluamicum*, *E. coli*, *S. aureus*, *P. aeruginosa* [45]
VLLLLALAAAAA	A1X8W2	Vitellogenin	100%	*Callinectes sapidus*	1356.64	Thaumatin-like protein (Fragment) , *Zea mays* subsp. *parviglumis*, CAMPSQ5221, Predicted
QELEEAE	Q6E7L5	Slow-tonic S2 myosin heavy chain	100%	*Homarus americanus*	1330.59	Hepcidin-like, *Takifugu rubripes* (Japanese putterfish), CAMPSQ6968.

[a] CAMP R3 Collection of Anti-Bacterial Peptides. Blast tools matrix PAM30.

516

517 Table 3: Average thickness (μm) and cellular viability (%) by CLSM of each marine
518 biofilm formed on mild steel during 10 days without (Control) and with bioactive peptides
519 (SCAMP-treated).

	Control		**SCAMP-treated**	
Time (hours)	Average thickness (μm)	Cellular viability (%)	Average thickness (μm)	Cellular viability (%)
24	0	72.13 ± 4.17	0	75.48 ± 5.07
48	31 ± 9	59.00 ± 4.72	28 ± 9	64.83 ± 2.84
96	43 ± 0	67.75 ± 6.81	28 ± 5	19.40 ± 5.32
168	28 ± 1	68.32 ± 6.77	22 ± 3	31.10 ± 3.07
240	44 ± 15	59.55 ± 3.80	39 ± 7	42.43 ± 3.41

520

Figures captions

Graphical abstract

Figure 1: Precursors of the identified peptides, expressed in percentages, by electrospray mass spectrometry (ES-MS/MS).

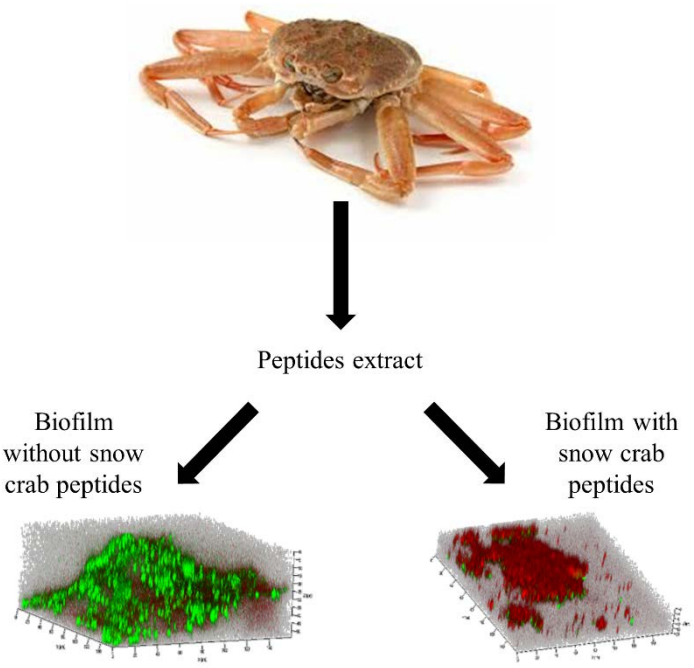
Figure 2: Spectra FT-IR obtained after 3 hours of immersion of the mild steel coupons in natural seawater (A) and SCAMP-treated (B).

Figure 3: Confocal laser scanning microscopy (CLSM) images of the bacterial community in the different marine biofilms formed on mild steel during 10 days without (Control) and with bioactive peptides (SCAMP-treated). The colour green means living cells and the colour red, the dead cells. The letter A represent the location of the water channel.

Figure 4: Dendrogram of the DGGE fingerprint patterns of the bacterial community in the different marine biofilms formed on mild steel during 10 days without (Control) and with bioactive peptides (SCAMP-treated). The cluster analysis was based on Jaccard coefficient similarity indices and constructed with the Phoretix 1D Pro software (Nonlinear Dynamics, Newcastle upon Tyne, UK).

539 Graphical abstract

540



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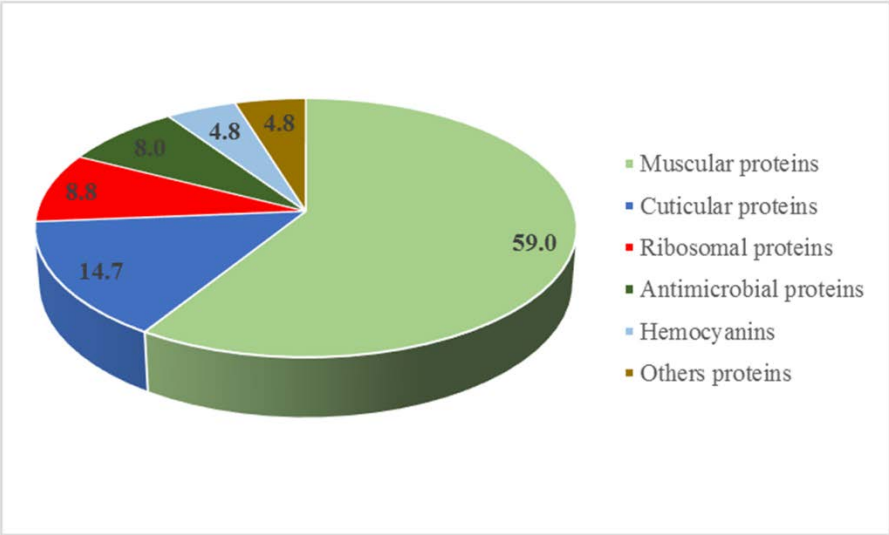
543 Figure 1



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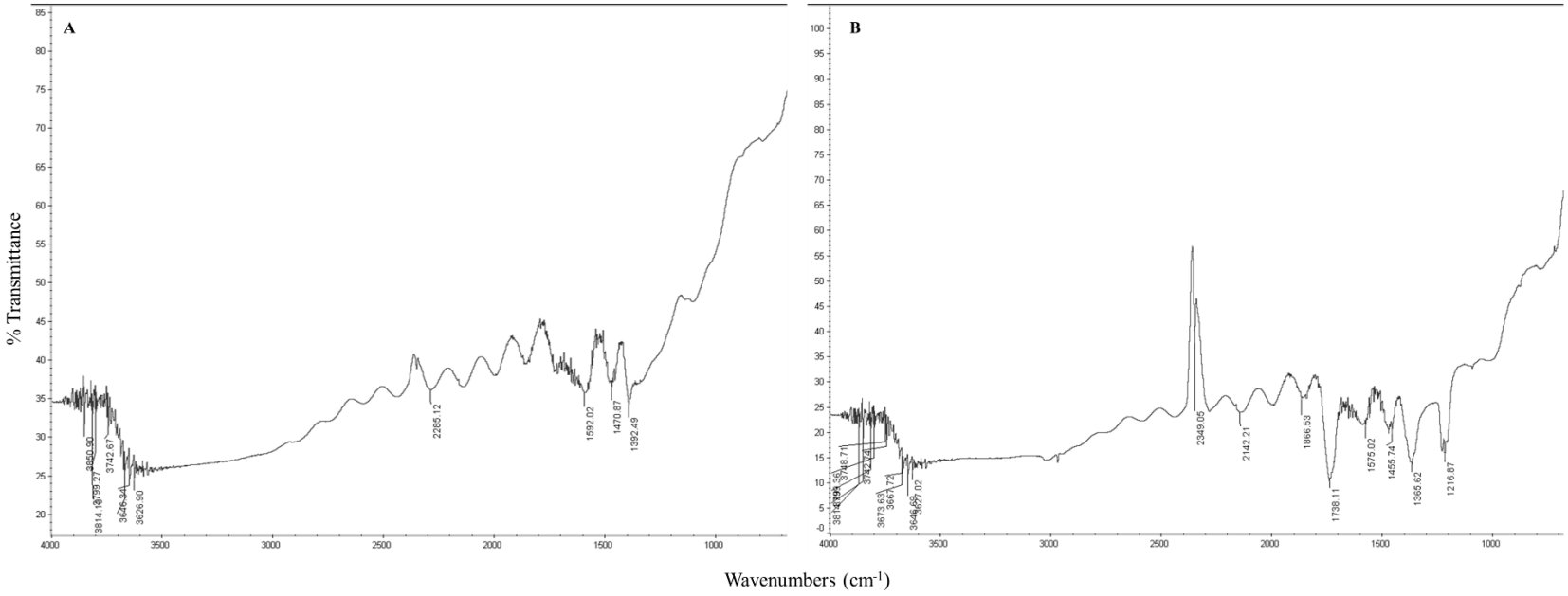
546 Figure 2



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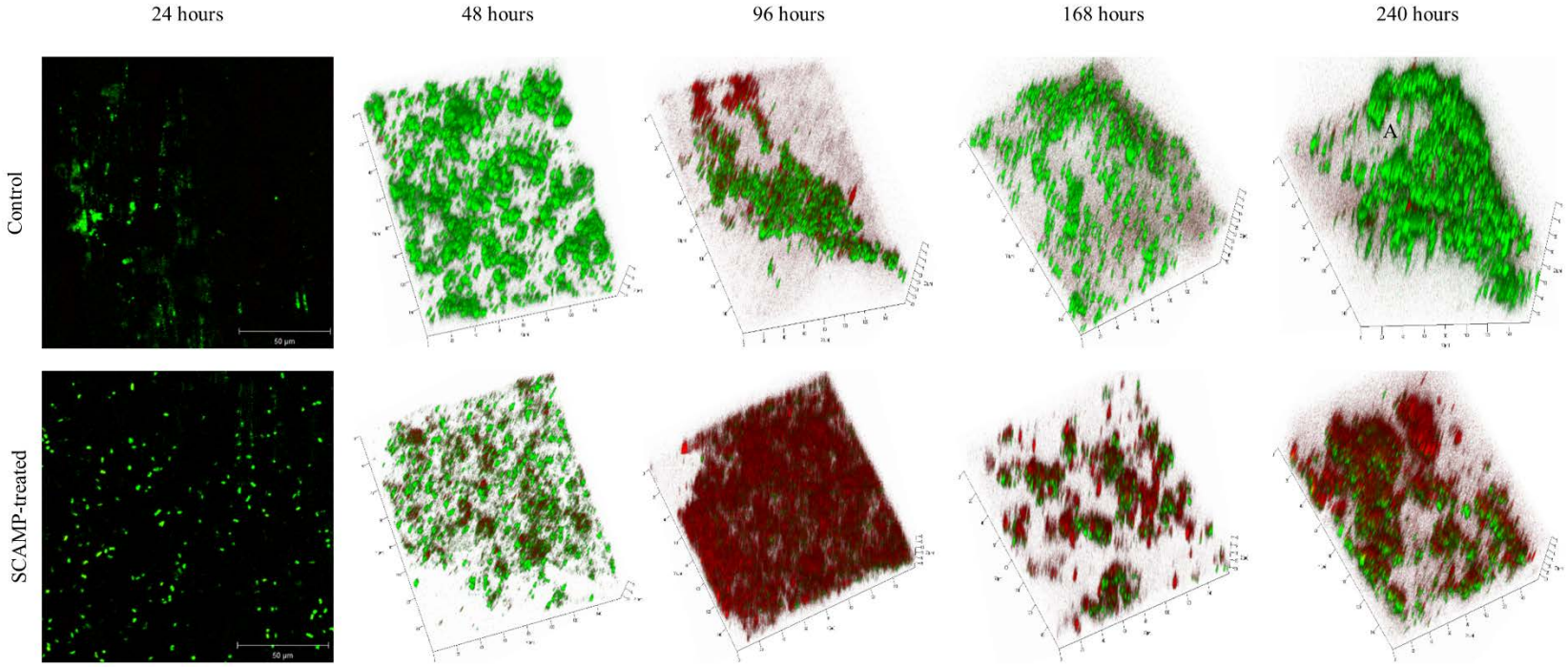

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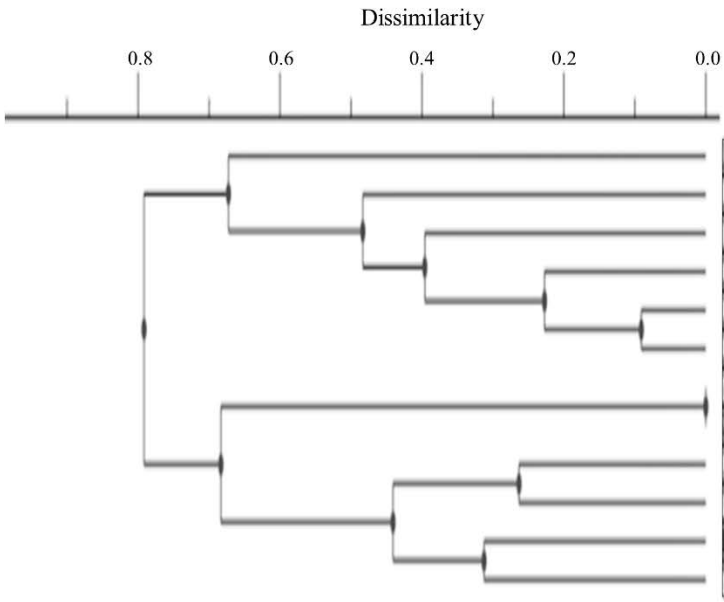

Experiments	Time (hours)	OTUs number
SCAMP-treated	3	3
SCAMP-treated	24	13
SCAMP-treated	240	6
SCAMP-treated	168	9
SCAMP-treated	96	11
SCAMP-treated	48	10
Control	24	8
Control	3	8
Control	96	18
Control	48	15
Control	240	14
Control	168	13

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