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Biofilm monitoring as a tool to assess the efficiency of artificial reefs as substrates: Toward 3D printed reefs

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ABSTRACT

Habitat destruction is one of the main causes of the decline of biodiversity and of fishery resources in the marine environment. An artificial reef (AR) could be a tool for protecting or restoring these habitats and their declining biodiversity, and also help to enhance sustainable fisheries. The goal is to design non-polluting structures that best mimic the complexity of natural habitats in order to improve their service to the community. To date, the assessment of reef performance has been mostly focused on fish assemblages and species of ecological and/or socio-economic interest, and has disregarded the biofilm communities that determine the first level of an AR's trophic network. In this work, we used biofilm formation to compare the quality of substrates used as building parts for an AR, in order to optimize an eco-friendly material that will be used to design a new generation of ARs produced by giant 3D printers. The structure of the photosynthetic communities has been identified using pigment biomarkers and their production of exudates has been analysed. These polymeric substances were quantified in terms of total sugar and protein concentrations. They were further analysed in terms of amino acid content. We found no significant differences between the micro-algae communities developed on the different substrates. These photosynthetic communities were mainly composed of diatoms, prasinophytes, haptophytes, and dinoflagellates. However, we showed that the material for ARs is crucial for biofilm development, especially with regard to its secretions of sugar. The choice of an appropriate substrate for AR construction is thus of particular importance since biofilm secretions determine the organic substrate on which sessile macro-organisms will settle.

1. Introduction

Artificial reefs (ARs) have been defined as "submerged structures placed on the seabed deliberately to mimic some characteristics of natural reefs" (Pickering et al., 1998). The main goal is to protect, regenerate and increase marine resource production, to help measure conservation in the protection and restoration of habitat and to enhance fisheries (UNEP, 2009). The first ARs were made with recycled waste materials, then since the mid-1970 s, innovative trends shifted towards purpose-designed reef structures (Pickering et al., 1998; Barnabe, et al., 2000; Tessier et al., 2015). In the USA, various depolluted waste materials were used, such as wrecks of oil and gas production platforms or charter vessels (Pickering et al., 1998), while in Europe and Japan since the 1980s, concrete blocks have been virtually exclusively used (Pickering et al., 1998; Barnabe et al., 2000; Tessier et al., 2015). In comparison to wrecks, AR design using concrete blocks limits the spatial heterogeneity and cannot mimic the high three dimensional complexity of natural rocky habitats.

To date, the assessment of reef performance has focused mostly on fish assemblages and species of commercial value, and has underestimated the development of epibiosis (or biofouling) (Svane & Petersen, 2001; Tessier et al., 2015). Moreover, material characteristics that affect larval settlement and recruitment have seldom been taken into account (Svane & Petersen, 2001). ARs are colonized as a consequence of the immersion of vacant hard substratum (Wahl, 1989; Svane & Petersen, 2001; Salta et al., 2013); holes and structures provide shelter for motile organisms and the surface is colonized ultimately by epibionts. The establishment of epibiosis is a complex process. It follows the same basic pattern of an initial biochemical conditioning film formed by the ambient water chemistry, followed by early colonisers such as bacteria and unicellular eukaryotes which form a matrix biofilm that composes the living substratum necessary for the successive

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colonisation by multicellular eukaryotes (Wahl, 1989; Svane & Petersen, 2001; Callow & Callow, 2006; Salta et al., 2013). The biofilm is, therefore, a key element in the subsequent colonisation of ARs and the development of their trophic network.

The biofilm is described as a matrix of microbial assemblages bonding with a biological or non-biological immersed surface (Costerton et al., 1994; Hall-stoodley et al., 2004). In marine environment, biofilm is composed of microbes such as bacteria, archaea, microalgae; microscopic fungus, heterotrophic flagellates, and ciliates that previously lived as plankton. The relative abundance of these taxa varies according to environmental conditions, but diatoms and cyanobacteria are those mainly represented (Wahl, 1989; Callow & Callow, 2006; Salta et al., 2013). Absorption and adherence of bacteria to the surface involves physical and chemical interactions and secretion that form the primary film, then unicellular eukaryotes settle several days later, dominated by diatoms that are attached to the surface by mucus secretion (Wahl, 1989). The secretions of bacteria and unicellular eukaryotes, called extracellular polymeric substances (EPS), are composed of macromolecules such as polysaccharides, proteins, nucleic acids, lipids and other polymeric substances (Flemming & Wingender, 2010). Those EPS constitute a matrix giving a tri-dimensional structure to the biofilm and express a particular phenotype of the microorganisms, giving them better access to nutrients, stronger colonisation capability and greater resistance to the environmental pressure (Salta et al., 2013). The EPS are heterogeneous and vary spatially, chemically and physically within the matrix according to environmental gradients (pH, temperature, oxygen, light, etc.; Costerton et al., 1995). To date, only two studies have focused on characterizing the biofilm community of the ARs (Salamone et al., 2016; Liu et al., 2017), but no investigation dealing with the biochemical characterisation of the biofilm has been undertaken to evaluate the nutritional quality of biofilm exudates that may have a function in ARs' trophic network.

Recently, D-shape (3D giant inkjet printing company for building construction) and Royal Boskalis Westminster N.V (Netherlands-based Company that provides services relating to the construction and maintenance of maritime infrastructure on an international basis) proposed a new way to build ARs using a giant 3D printer and an eco-friendly material, dolomite Sorel cement (84% dolomite sand and 16% magnesium oxide). 3D printing enables the building of more complex artificial structures, closer to the complexity of a natural rocky habitat. As part of the evaluation of the quality of this new generation of ARs, and to assess the quality of various kinds of substrate used for ARs, we developed a method to monitor the first stage of biofilm colonisation that constitutes the living substrate for epibiont settlement. A comparative study has been performed on different substrate types: grey concrete (which is commonly immersed in Europe), white concrete (that contains less metallic oxide than grey concrete, Telford, 1999; BETOCIB, 2000), and dolomite Sorel cement (used to build the 3D printed ARs). Samples of these substrates were immersed in the Larvotto marine reserve (Principality of Monaco) over a 35-day period in order to determine the colonisation of biofilm from the early phase to maturation and to identify potential biological and biochemical variations of biofilm composition between the substrates. We hypothesised that the substrate type is likely to induce differences (i) in microbial diversity during the colonisation process and (ii) in the composition of biofilm secretion.

2. Materials and methods

2.1. Sampling and site of monitoring

Biofilm communities were monitored on different substrates for 35 days in the coastal waters of the Larvotto reserve (Monaco: 43.743950°N, 7.434700°E). The reserve is a protected marine area managed by the AMPN (*Association Monégasque pour la Protection de la Nature*). Samples were retrieved one day after immersion to analyse the initiation phase, and then every week (during 4 weeks) to follow the maturation of communities. However, a storm occurred the third week of monitoring which delayed the sampling by one week. The five sampling dates are thus unevenly distributed, and sampling finally took place 1, 7, 14, 28 and 35 days after immersion. The samples were immersed at 18 m depth, 2 m above a sandy bottom. They were fixed on 5 different 1 m^2 plastic frames (1 frame for each sampling date), suspended from a 10 L buoy and anchored by concrete blocks. The depth was selected in order to facilitate monitoring by scuba divers and corresponds to the depth at which ARs are commonly immersed in the French Mediterranean Sea (Tessier et al., 2015).

Cobblestones of 5 * 2 * 2 cm were cut within large slabs of dolomite Sorel cement (Ds), standard grey concrete (Cg) and white concrete (Cw). Three replicates of each substrate were dedicated for 3 different analyses (Pigment, EPS, and 16S ribosomal DNA analyses) at each sampling time (i.e. 3 replicates × 3 substrates × 3 analyses × 5 times = 135 samples). Unfortunately, after extraction, the quantity of DNA compared to the PCR inhibitory products was not sufficient to follow the analysis by amplicon sequencing.

Immersion, installation and sampling were done by scuba divers. The retrieval of the plastic frames with the attached cobblestone samples lasts for ca. 2 min from the bottom to the boat. We therefore consider that new irreversible settlement of planktonic microorganisms during the retrieval was very unlikely. Samples were then packed in falcon tubes and immediately frozen with liquid nitrogen. Samples were stored at −80 °C before analysis.

2.2. Pigment analysis

The photosynthetic communities have been analysed by the quantification of pigments by High Performance Liquid Chromatography (HPLC) according to Brotas & Plante-Cuny (2003). Pigments were extracted by scraping the biofilm from the substrate using a scalpel and fiber glass filter (GF/F) with 6 mL of 95% cold buffered MeOH (2% ammonium acetate) for 4 h at 4 °C, in the dark. Extracts were then filtered (0.2 µm) immediately before HPLC analysis. Pigment extracts were analysed using an Agilent 1260 Infinity HPLC composed of a quaternary pump (VL 400 bar), a UV–VIS photodiode array detector (DAD 1260 VL, 190–950 nm), and a 100 µl sample manual injection loop (overfilled with 250 µl). Chromatographic separation was carried out using a C18 column for reverse phase chromatography (Supelcosil, 25 cm long, 4.6 mm inner diameter). The solvents used were A: 0.5 M ammonium acetate in methanol and water (85:15, v:v), B: acetonitrile and water (90:10, v:v), and C: 100% ethyl acetate. The solvent gradient followed the Brotas & Plante-Cuny method (2003), with a flow rate of 0.5 mL·min^{-1}. Identification and calibration of the HPLC peaks were performed with chlorophyll *a*, ββ-carotene, chlorophyll *c2*, diatoxanthin, diadinoxanthin and fucoxanthin standards. All peaks detected were identified by their absorption spectra and relative retention times using the Open Lab CDS software (ChemStation Edition for LC/MS Systems, Agilent Technologies). Quantification was performed by repeated injections of standards over a range of dilutions to establish a standard curve of concentrations. The relative abundance of each pigment (%) was calculated from their respective concentrations (µg·cm^{-2}).

2.3. Extraction of polymeric substances

Since our samples were frozen before extraction, this could have broken the cell wall, and some cell content would have been released and mixed with extracellular polymeric substances (EPS). Thus, although this input of intracellular material is probably negligible, our chemical analyses may reflect the total polymeric substances (PS).

All samples (i.e. Ds, Cg and Cw cobblestones) were mixed with 2 mL of artificial seawater and 2 g of cation exchange resin (Dowex® Marathon™ C sodium form previously activated in PBS) for 1.5 h with a

Table 1
Composition of the solutions used in the modified LOWRY assay protocol.

Solutions	Composition
1	143 mM NaOH with 270 mM Na_2CO_3
2	57 mM $CuSO_4$
3	124 mM Na-tartrate
4	mix of solution 1, 2 and 3 solutions with fraction 100:1:1 (v:v:v).
5	Folin with distilled water 5:6 (v:v)

tube roller (Denley Instruments). Samples were then centrifuged (6030 *g*, 10 min) and the supernatants containing the polymers (exo- and intra-) were retrieved (PS fraction: intracellular material with colloid and bond substances from extracellular material). Previous studies showed that the cation exchange resin method is more efficient than other extraction methods (e.g. with EDTA or NaOH), leading to high EPS yields with no apparent cell lysis and no impact on subsequent biochemical analysis (Jachlewski et al., 2015).

2.4. Quantification of carbohydrate concentration by colorimetric assay

Carbohydrate analyses were performed following the phenol assay protocol (Dubois et al., 1956). Briefly, 200 µl of the PS fraction were mixed with 200 µl phenol (5%) and 1 mL sulphuric acid (98%). Mixtures were then incubated for 35 min at 30 °C and the carbohydrate concentration was measured with a spectrophotometer at 488 nm (Milton Roy Spectronic Genesys 2). A calibration curve was prepared using glucose as standard.

Protein analyses were performed following the modified LOWRY assay protocol (Raunkjær et al., 1994; Frølund et al., 1996), using five reagents as described in Table 1.

250 µl of the PS fraction were mixed with 250 µl of SDS (2%) and 700 µl of solution 4, and mixtures were then incubated for 15 min at 30 °C. 100 µl of solution 5 were added to each tube and vortexed immediately. Mixtures were then incubated for 30 min at 30 °C. The protein concentration was measured with a spectrophotometer at 750 nm (Milton Roy Spectronic Genesys 2). A calibration curve was prepared using bovine serum albumine (BSA) as standard.

2.5. Amino acid composition of polymers by HPLC

Amino acids of the PS fraction were identified and quantified by HPLC. PS fractions were dialysed against distilled water (cut-off 12–14 kDa) and freeze-dried. 10 mg were then mixed with 200 µl of HCl (6 N). The acid mixture was carefully degassed to reduce the level of oxidative destruction and proteins were then hydrolysed (24 h at 110 °C) in vacuum using a sealed glass ampule. Ampules were then dried using a speedvac after hydrolysis. The resulting amino acids were then reconditioned in Pickering diluent prior to injection in the HPLC system. Amino acids were separated by ion-exchange HPLC using a high-efficiency sodium column (4 × 150 mm; Pickering Lab, LCTech, Dorfen, Germany) with a Waters 2695 separation module (Waters). The elution buffers and gradient conditions were those recommended by the manufacturer (Table 2).

Separating amino acids were first subjected to post-column derivatization with Ninhydrin (Pickering Lab.) by using a PCX 5200 derivatizer (Pickering Lab.) and later detected on a Waters 2996 Photodiode as a UV module detector at 570 nm for all the amino acids containing a primary amine, and at 440 nm for the Proline which holds a secondary amine. Quantification was performed by repeated injections of standards over a range of dilutions to determine the relationship between peak area and standard concentrations. The relative abundance of each amino acid (%) was calculated from their respective concentration (µg·cm^{-2}), and protein concentration was calculated from the total amino acid concentration.

Table 2
HPLC solvents for amino acid detection.

Buffer	Composition
Pickering diluent NA220	Water (97.9%), sodium citrate (2%), Pro Clean 400 (< 0.1%)
A: Buffer Na pH 3.14 Sodium eluant 1700-0112	Water (93%), Sulfolane (5%), Hydrogen Chloride (0.6%), Sodium acetate (1.8%), Phenol (< 0.1%, pH 3.15)
B: Buffer Na pH 7 Sodium eluant N740	Water (94%), Sodium chloride (5%), sodium acetate (1.4%), phenol (< 0.1%, pH 7.40)
C: NaOH Sodium regenerant RG011	Water (99%), Sodium hydroxyde (0.6%), Sodium chloride (0.4%, pH 13)

2.6. Data analysis

All statistical tests have been done with the open source software R (3.4.1), using “FactoMineR” (Husson et al., 2016), “vegan” (Oksanen et al., 2017) and “agricolae” (De Mendiburu, 2016) packages. Differences in time and between substrates of the total concentration of pigment, sugar and proteins were tested using the univariate non-parametric Van-der-Warden test. Permutational Multivariate Analysis of variance (PERMANOVA) was performed using a Bray-Curtis dissimilarity index calculated with pigment percentages. The multivariate homogeneity of group dispersion (PERMDISP2 procedure; Anderson, 2001) was verified before applying a PERMANOVA. Post-hoc pairwise PERMANOVA tests were performed to identify significant differences between modalities of factors (Anderson, 2001). A Principal Component Analysis (PCA) was applied on pigment concentrations and supplementary variables (sugar and protein concentrations).

3. Results

3.1. Pigment analysis

Pigment concentrations varied between 0.006 µg·cm^{-2} and 6.647 µg·cm^{-2}, taking all substrates and times together (Fig. 1). On all substrate samples, it increased drastically after two weeks of immersion (Fig. 1). At this time, maximum average concentration was measured on grey concrete (Cg: 6.503 ± 0.054 µg·cm^{-2}; Cw: 5.494 ± 0.181 µg·cm^{-2}; Ds: 4.630 ± 0.465 µg·cm^{-2}). It then remained stable until the end of monitoring for white concrete despite a slight decrease after the storm. However, it decreased dramatically on grey concrete and dolomite Sorel cement after the storm event. In addition, dolomite Sorel cement had a lower concentration than grey and white concrete after the storm event (day 28: Cg: 17.801 ± 1.672 µg·mm^{-2}, Ds: 6.333 ± 0.190 µg·mm^{-2}, Cw: 15.537 ± 1.479 µg·mm^{-2}). At the end of the monitoring period, concentrations remained constant on grey and white concrete and increased again on dolomite Sorel cement up to a level comparable to the two other substrates.

On average, chlorophyll *a*, fucoxanthin and chlorophyll *c* were the major pigments detected (Supplementary Table S1). The results of the PERMANOVA revealed significant differences for each factor (time and substrate) and their interaction (2-way PERMANOVA: time: $p = 0.001$, substrate: $p = 0.035$, interaction: $p = 0.001$; Permutation test for homogeneity of multivariate dispersions: $p = 0.067$). However, pairwise post-hoc test revealed significant differences only between sampling times except between d01 and d07 (Post-hoc pairwise PERMANOVA between sampling time with Bonferroni correction: d01 – d07: $p = 1$, d07 – d14, d14 – d28: $p = 0.01$, d28 – d35: $p = 0.02$). During the first week, we only detected fucoxanthin and chlorophyll *a* on all substrates (Supplementary Table S1). After two weeks of immersion, the proportion of chlorophyll *a* and fucoxanthin decreased due to the detection of chlorophyll *c*, pheopigments, prasinoxanthin,

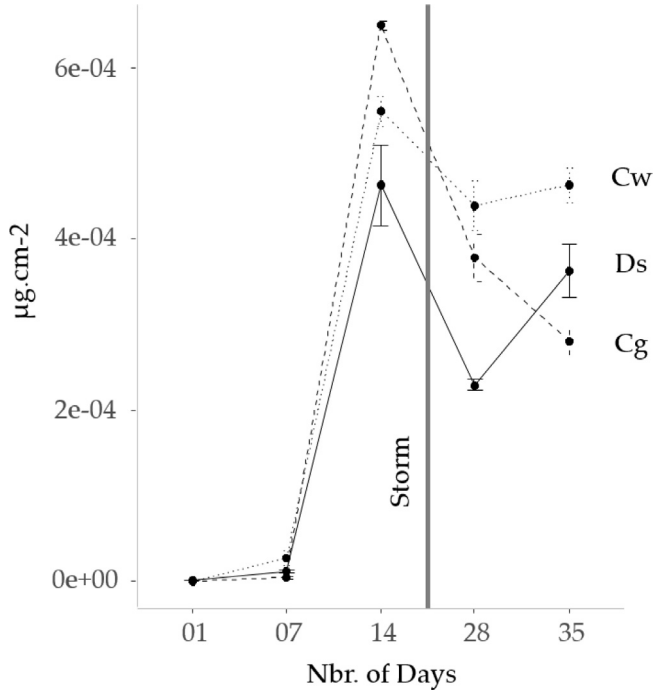

Fig. 1. Variation of pigment concentration over time on the different substrates (dashed line: grey concrete Cg, solid line: dolomite Sorel cement Ds, dotted line: white concrete Cw). (Van-der-Waerden test on substrates: p = 0.867).

diadinoxanthin, and a small proportion (< 1%) of carotene, hex-fucoxanthin, diatoxanthin, antheraxanthin, zeaxanthin and chlorophyll *b*. After the storm event (between day 14 and day 28), carotenes and chlorophyll *b* could not be detected anymore, whereas chlorophyll *c* and prasinoxanthin increased. During the last week of monitoring, chlorophyll *b* and carotenes were detected anew and chlorophyll *c* and prasinoxanthin decreased (Supplementary Table S1).

3.2. Sugar and protein concentration dynamics on the different substrates

Sugar concentrations ranged between 0.674 and 14.628 $\mu g \cdot cm^{-2}$ (Fig. 2) and were significantly different between dolomite Sorel cement and the two types of concrete (Van-der-Waerden: p = 0.0315, post hoc test: Cg-Ds: p = 0.049; Cg-Cw: p = 0.537; Ds-Cw: p = 0.0104). They increased gradually over the monitoring period on grey and white concretes samples to reach on average 7.904 ± 2.472 $\mu g \cdot cm^{-2}$ and 10.546 ± 1.295 $\mu g \cdot cm^{-2}$, respectively, at the end of monitoring. The concentration of sugars on dolomite Sorel cement samples increased slightly, with a maximum mean concentration of 2.733 ± 0.240 $\mu g \cdot cm^{-2}$ at the end of monitoring. Protein concentrations ranged between 0.208 and 1.552 $\mu g \cdot cm^{-2}$ (Fig. 2) and followed approximately the same pattern as the sugar concentration over time. However, the concentrations did not differ between substrates (Van-der-Waerden: p = 0.322).

Table 3
means of sugar to protein ratio over time on each substrate (Cg: grey concrete, Ds: dolomite Sorel cement, Cw: white concrete; se: standard error of mean proportion).

	Cg		Ds		Cw	
	mean	*se*	mean	*se*	mean	*se*
Day 1	4.79	*0.15*	4.26	*0.56*	5.46	*0.39*
Day 7	5.80	*1.36*	3.9	*0.37*	4.34	*0.89*
Day 14	7.98	*0.65*	3.99	*0.32*	10.37	*0.79*
Day 28	8.9	*0.36*	4.17	*0.72*	7.18	*0.43*
Day 35	8.26	*0.88*	4.38	*0.32*	7.96	*0.53*

The ratio of sugar to protein was equivalent between substrates for the two first sampling times (mean of the ratio for the 3 substrates at d01: 4.84 ± 0.13, at d07: 4.68 ± 0.29). It remained the same on dolomite Sorel cement till the end of the monitoring, while it increased drastically on the two other substrates from the second week of monitoring and then remained stable at high values till the end of the monitoring (Table 3). Overall, it showed significant differences between dolomite Sorel cement and other substrates (Van-der-Waerden: p = 0.0042, post hoc test: Cg-Ds: p = 0.0022; Cg-Cw: p = 0.9472; Ds-Cw: p = 0.0026).

3.3. Amino acid composition

Proportions of amino acids showed significant differences over time but not between substrates (2-way PERMANOVA: time: p = 0.001, substrate: p = 0.078, interaction: p = 0.918; Permutation test for homogeneity of multivariate dispersions: p = 0.716). Post hoc analyses revealed significant differences before and after the storm event

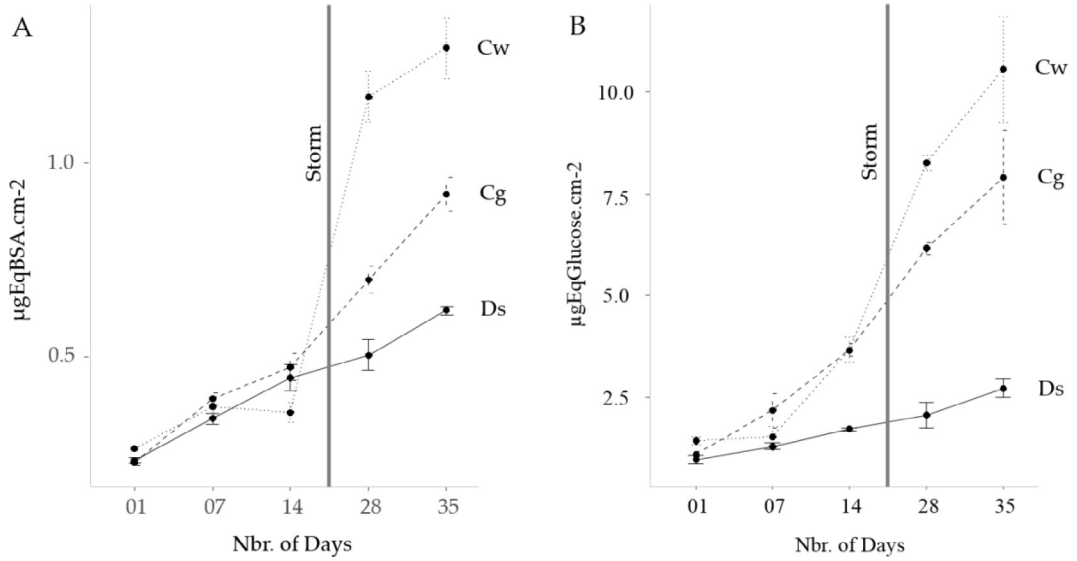

Fig. 2. Variation of (A) sugar concentration (equivalent glucose) and of (B) protein concentration (equivalent BSA) over time on the different substrates (dashed line: grey concrete Cg, solid line: dolomite Sorel cement Ds, dotted line: white concrete Cw). (Van-der-Waerden test on substrates: sugar: p = 0.0315; proteins: p = 0.322).

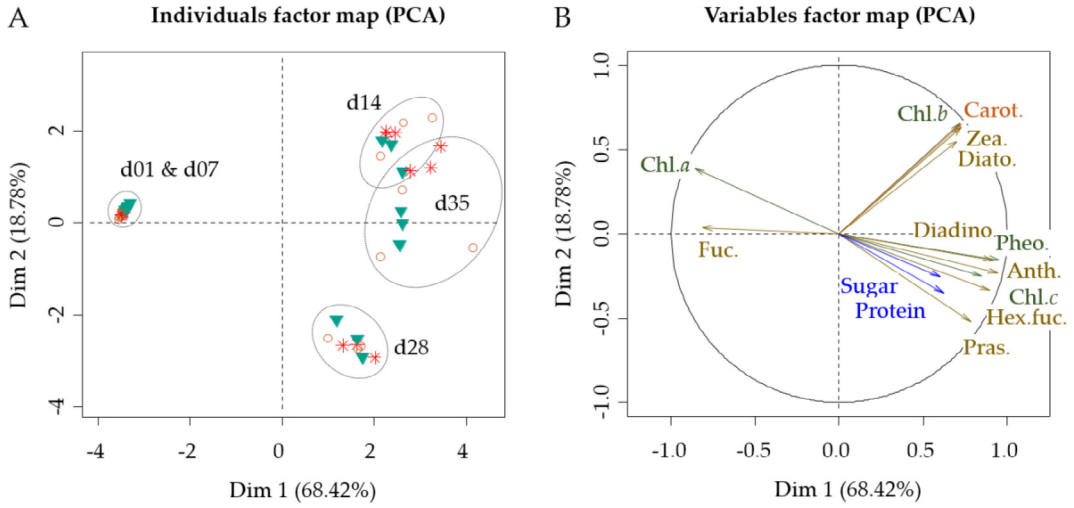

Fig. 3. Principal component analysis: A. Ordination of the samples according to sampling time (d01: first day, d07: 1 week, d14: 2 weeks, d28: 4 weeks, d35: 5 weeks) and substrates (red star: grey concrete; green triangle: dolomite Sorel cement; brown circle: white concrete). B. Correlation circle of the pigments variables (Carotenes (orange): Carot.; Chlorophylls (dark green): Chl.*a*: Chlorophyll *a*, Chl.*b*: Chlorophyll *b*, Chl.*c*: Chlorophyll *c*, Pheo.: Pheopigments; Xanthophylls (brown) Anth.: Antheraxanthin, Diadino.: Diadinoxanthin, Diato.: Diatoxanthin, Fuc.: Fucoxanthin, Hex.fuc.: Hex-fucoxanthin, Pras.: Prasinophyte, Zea.: Zeaxanthin) and supplementary variables (sugar and protein concentrations). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(between d14 and d28, Post-hoc pairwise PERMANOVA, Bonferroni correction: $p = 0.01$). On average, the PS fraction contained amino acids mainly composed of glutamic acid (20.83 ± 0.14%), aspartic acid (10.25 ± 0.08%), glycine (11.80 ± 0.09%), alanine (8.23 ± 0.4%), proline (7.70 ± 0.21%) and serine (9.31 ± 0.12%), 26.18 ± 1.12% were essential amino acids (Supplementary Table S2). Even if no significant differences were detected between substrates, dolomite Sorel Cement showed a lower proportion of essential amino acids than on the two types of concrete at d14 (Cg: 27.78 ± 2.31; Ds: 18.94 ± 0.91; Cw: 28.04 ± 1.77) and d35 (Cg: 32.25 ± 1.57; Ds: 24.78 ± 0.27; 27.70 ± 1.10).

3.4. Principal component analysis

The PCA was performed on pigment variables (12 pigments) and supplementary variables (sugar and protein concentrations) have been computed to verify potential correlations. The samples were collected according to substrates (Ds, Cg and Cw for dolomite Sorel cement, grey concrete and white concrete, respectively) and sampling dates (from d01 to d35; Fig. 3). The first axis, which represented 67.03% of total inertia, mainly structured the samples. The second axis also explained a large part of the total inertia (19.23%), while the third axis represents 5.4% of the total inertia and was not retained in the analysis (< mean threshold 1/12 = 8.33). The first two dimensions showed a good projection of the data, as all variables were close to the correlation circle, except the supplementary variables, and individuals had cumulative squared cosinus above 0.7 on these two dimensions (except the first and third replicates from Cw at d35). Chlorophyll *a*, fucoxanthin, pheopigments, diadinoxanthin, antheraxanthin, hex-fucoxanthin and chlorophyll *c* concentrations from d01 and d7 samples, and the Ds and Cw samples from d35 mainly contributed to the construction of the first dimension. Chlorophyll *b*, diatoxanthin, zeaxanthin, and prasinoxanthin concentrations measured in the d14 and d28 samples and the grey concrete sample at d35 mainly contributed to building the second dimension. On one hand, chlorophyll *a* and fucoxanthin concentrations from samples of the first week were negatively correlated with the first dimension, while pheopigments, diadinoxanthin, antheraxanthin, hex-fucoxanthin and chlorophyll *c* concentrations of the Ds and Cw samples at d35 were positively correlated with the first dimension. On the other hand, chlorophyll *b*, diatoxanthin, zeaxanthin within d14 samples and grey concrete samples at d35 were positively correlated with the second dimension, whereas prasinoxanthin and d28 samples were negatively correlated with this dimension (Fig. 3). Supplementary variables did not show significant correlations with the first two dimensions.

4. Discussion

The assessment of the quality of total polymeric substances (PS) in conjunction with photosynthetic communities of biofilm on different types of substrates enabled a better understanding of the first step of colonisation of AR, and established the first basis for a protocol to assess the ecological quality of different AR materials. We hypothesised that the substrate type is likely to induce differences (i) in microbial diversity during the colonisation process, and (ii) in the composition of biofilm secretions. The influence of substrate on biofilm attachment and diversity has been extensively investigated and most of the studies revealed significant differences (Dexter et al., 1975; Dexter, 1979; Fletchert & Loeb, 1979; Fletcher & Pringle, 1985; Pringle & Fletcher, 1986; Fletcher & Callow, 1992; Cooksey & Wigglesworth-Cooksey, 1995; Finlay et al., 2002; D'Souza et al., 2005; Patil & Anil, 2005; Jones et al., 2007; Sweet et al., 2011; Lakshmi et al., 2012; Ozkan & Berberoglu, 2013; Tan et al., 2015). In this study, diversity, abundance and secretion were significantly different according to sampling time, but only sugar secretion showed significant differences between substrates.

The biofilm on each substrate became mature after two weeks of monitoring, as shown by the high concentration and diversity of pigments, and the sugar to protein ratio that reached a stable value. Previous studies showed that the maturity of biofilm influences the settlement of various larvae of *Mytilus galloprovincialis* (Bao et al., 2007), *Hydroides elegans* (Huang & Hadfield, 2003; Chung et al., 2010), *Enteromorpha* sp. (Dillon et al., 1989), *Balanus amphitrite* (Faimali et al., 2004) and *Bugula neritina* (Dahms et al., 2004) according to diversity, density and secretion of PS.

The overall pattern of changes of the communities is well illustrated by the two first axes of the PCA performed on pigment variables. The first axis represented the diversity of these biofilm communities over time, where communities of the first week (d01 and d07) were related to chlorophyll *a* and fucoxanthin, and the other sampling times to chlorophyll *c* and all the minor pigments and supplementary variables

(sugar and protein concentrations). The second axis represented the split due to the storm event that eroded the biofilm, with samples at d14 characterised by the presence of the less abundant pigments (chlorophyll *b*, diatoxanthin, zeaxanthin and carotene), contrasting with samples at d28 characterised by the loss of rare pigments and the increase of prasinoxanthin, antheraxanthin, chlorophyll *c*, hex-fucoxanthin and diadinoxanthin. At the end of monitoring, the communities are more scattered and recovered slowly to reach the initial state (d14). The loss of zeaxanthin, carotene and chlorophyll *b* might be due to the limitations of HPLC detection. The concentrations of these pigments were very low before the storm and could be undetectable after it. Thus, this loss is not to be considered as a variation in terms of diversity of the pigment composition of communities, but rather as a variation in terms of proportion of the different pigments.

Consistent with the presence of the three types of chlorophyll (*a*, *b* and *c*), the communities were composed of green and brown algae, and perhaps of photosynthetic bacteria (cyanobacteria including prochlorophytes, Jeffrey et al., 2011). According to the proportions of fucoxanthin, chlorophyll *c*, diadinoxanthin and diatoxanthin, brown algae were dominant. Diatoms should be the main taxon represented, since fucoxanthin can represent up to 60% of their pigment content (Strain et al., 1944). It is known that Raphids diatoms generally represent the main taxa of marine biofilm (78%; Wahl, 1989; Callow & Callow, 2006; Salta et al., 2013). The presence of hex-fucoxanthin may reveal the specific presence of benthic haptophytes of the Coccolithophyceae class (Jeffrey et al., 2011), although this was not documented in the biofilm community. The association of antheraxanthin and prasinoxanthin may reveal the presence of prasinophytes among the green algae (Egeland et al., 1995; Jeffrey et al., 2011). Finally, endosymbiotic dinoflagellates with pigments of haptophyte, diatoms and prasinophytes origin, can also occur (Jeffrey et al., 2011). Dinoflagellates and green flagellates (like prasinophytes) are known to each represent 1% of the biofilm communities (Wahl, 1989; Callow & Callow, 2006; Salta et al., 2013). The increase of diadinoxanthin, hex-fucoxanthin and prasinoxanthin after the storm might reveal a shift in the community toward the increase of flagellate organisms (dinoflagellates, coccolithophores and prasinophytes), perhaps coming from the water column after the storm, since those flagellate organisms are planktonic (Jeffrey et al., 2011). Besides, the increase of sugar concentrations on grey concrete and white concrete might also reveal the increasing activity of diatoms. Diatoms, previously identified as the main taxa of the community, secrete a mucilage rich in polysaccharides to adhere to substrates (Hecky et al., 1973; Myklestad et al., 1989; Hoaglang et al., 1993; Underwood & Paterson, 2003; Stal & Défarge, 2005; Bruckner et al., 2008). Pigment concentrations, mainly related to fucoxanthin produced by diatoms, strongly increased on all substrates from d14, thus the dynamic of sugar secretions appeared not to be synchronous with their settlement on these different substrates. Previous culture studies have shown that benthic diatom cells produce higher concentrations and greater proportions of extracellular carbohydrates when cells enter the transition from exponential growth to stationary phase (Sutherland et al., 1998; Underwood & Smith, 1998; Staats et al., 1999; Underwood & Paterson, 2003). It might be possible that diatoms settled on grey concrete and white concrete start their stationary phase after the 14th day of monitoring, whereas they continue to grow on dolomite Sorel cement. These differences in secretion of PS between substrates might also be due to differences in diatom species composition. Culture studies showed that species composition can affect the amount of PS secreted, some species having significantly higher rates of PS production than others under the same conditions (Smith & Underwood, 2000; De Brouwer et al., 2002; Underwood & Paterson, 2003). Although HPLC detection of pigments could not identify differences in terms of diatoms diversity, our results are consistent with previous studies that showed that substratum type can influence the diversity of the diatom community and of their PS secretion (Cooksey & Wigglesworth-Cooksey, 1995; Finlay et al., 2002; Patil & Anil, 2005; Chung et al., 2010; Ozkan & Berberoglu, 2013). Diatoms represent a large part of the feeding resources and they are often associated with the post-larval dietary requirement for suspension feeders (Lam et al., 2003) or grazing juvenile invertebrates, such as gastropods (Slattery, 1992; Bryan & Qian, 1998; Siqueiros-Beltrones and Voltolina, 2000; Siqueiros Beltrones & Valenzuela Romero, 2004; Dahms et al., 2004), sea urchins (Rahim et al., 2004) and sea cucumbers (Ito & Kitamura, 1997). Thereby, the significant differences in sugar concentration on dolomite Sorel Cement compared to the other substrates might have a negative impact on the feeding resources of suspension feeders and grazing invertebrates.

To the best of our knowledge, analyses of global amino acids in *in situ* biofilm development are rare. Bhosle and Wagh (1997) and Bhosle et al. (2005) monitored the amino acid composition of biofilms on aluminium panels in an Indian tropical bay, focusing on the contribution of the major sources of organic matter in the biofilm chemical composition. They observed that the distribution of individual amino acids did not vary in time, and was very similar to that observed in the two main sources of organic matter, and was dominated by aspartic acid, glycine, alanine, serine, leucine, lysine and glutamic acid. In our study, the amino acid composition of the biofilms was dominated by glutamic acid, glycine, aspartic acid, proline, alanine and serine. This composition could stem from the sinking organic matter of marine snow and/or from the microbial community itself that produces protein. But it is generally difficult to distinguish differences between proteins produced by different taxa on the basis of their amino acid composition. The amino acid composition of the proteins is highly conserved among different species, even those performing specific functions. For instance, it has been shown that 16 microalgae species exhibited minor differences in the amino acid composition of their hydrolysates (Brown, 1991). Aspartic acid and glutamic acid are generally found at the highest concentrations, while cysteine, methionine, tryptophan, histidine and proline are found at the lowest concentrations. Therefore, it would be spurious to estimate biofilm diversity on the basis of their amino acid composition. However, the high degree of conservation in amino acid composition among species exudates makes it an interesting biomarker to monitor changes in biofilm functioning. Amino acids can indeed play a key role in the settlement of the macrofouling, Trapido-Rosenthal & Morse (1985) showed that the presence of lysine at a micromolar concentration facilitates the induction of larval settlement and the metamorphosis of *Haliotis rufescens*. In addition, the proportion of essential amino acids in the biofilm is a useful proxy of biofilm nutritional quality for higher trophic levels. The slight differences detected between the dolomite Sorel cement and the other substrates at d14 and d35 could potentially imply a lower nutritional quality on the dolomite Sorel cement.

Taken together, these results suggest that the use of different substrates for ARs may lead to differences in the biofilm composition and secretions, and thus biofilm characterization in this context proves to be a good tool. Through the characterization of the PS composition secreted by the biofilm communities, we have been able to highlight significant differences in sugar concentration of the biofilm between the dolomite Sorel cement and the two types of concrete. This could potentially have an impact on organisms that settle and/or feed on this biofilm. Thus, it is essential to understand these mechanisms in the context of ARs, in order to enhance the colonisation. Otherwise, the two concrete substrates did not show significant differences in the parameters surveyed in this study, while white concrete contains less metallic oxide than grey concrete (Telford, 1999, BETOCIB, 2000). Further analyses are thus needed to determine whether or not those two substrates present equivalent substrate quality for AR communities. Further research is also needed to understand the mechanisms involved in the adaptation of communities to substrates and to determine whether those differences might imply differences in the settlement of sessile macro-organism larvae and propagules at higher trophic levels. We need to determine whether the physical or chemical properties or both

are involved in these mechanisms.

5. Conclusions

We identified a photosynthetic biofilm community mainly composed of diatoms, prasinophytes, coccolithophores and dinoflagellates on each substrate. We also identified differences between communities on the dolomite Sorel cement and those of white and grey concrete. The lower sugar to protein ratio on the dolomite Sorel cement community might be the result of differences in biofilm community composition and activity and is potentially determinant in the recruitment of various larvae and with regard to the diet of grazing invertebrates. We showed that biofilms are useful bio-indicators in that they develop quickly and give a rapid estimation of the quality of AR substrates.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ecoleng.2018.06.005.

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