In vitro and *in situ* tests to evaluate the bacterial colonization of cementitious materials in the marine environment

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Highlights:

- The pre-carbonation accelerates the bacterial colonization of cementitious surface in marine environment
- CEM III mortars are more bioreceptive than the CEM I mortars
- Laboratory tests could be quickly identified the bioreceptivity of a cementitious material

Abstract

Civil engineers have a responsibility to take measures to protect marine biodiversity by selecting more bioreceptive construction materials in the design of marine infrastructure, for better biodiversity conservation. In this study, it was shown that pre-carbonation of cementitious materials accelerates their bacterial colorization by lowering the pH of their surface. It has been shown both in the laboratory and *in-situ* tests that the bacterial colonization of cementitious materials is influenced by the pH and the type of cement. By comparing the bacterial colonization of Portland cement mortars, CEM I, and slag cement, CEM III, mortars, it was found that the CEM III mortars are more bioreceptive than the CEM I mortars. This study presented and verified a novel experimental laboratory approach which can be used to evaluate the bacterial colonization (bioreceptivity) of cementitious materials in marine environment. The approach could be taken up in future recommendations to enable engineers to eco-design more eco-friendly marine infrastructure and develop green-engineering projects.

Keywords: Cementitious materials, bacterial colonization, marine environment *in-vitro*/*in-situ* tests, ecological engineering

1. Introduction

There is a world concern to develop a new project based on ecological reconciliation, through a "win-win" approach between human and nature [1]. Today, cementitious materials such as concrete are essential materials for the construction of marine structures such as marine ports and coastal structures [2–4]. There is an increasing research effort into ways that coastal infrastructure can be built to meet engineering requirements, while also increasing its value as habitat for marine life to the benefit of both engineering and nature [5–10]. Structures are aimed to be constructed with a minimal impact on the existing environment and with a maximal possible development of new ecological habitat to encourage ecosystems that replace those that may be lost [11,12]. Combining engineering techniques and ecological understanding can provide cost-effective ways of maintaining or enhancing biodiversity [7,13,14].

The engineers who design civil engineering structures must carry out intensive investigations to ensure a minimum service life of the structures while respecting the economic constraints [15]. In the marine environment, cementitious materials are exposed to a multitude of actions of different natures (physical, chemical and biological) which can be aggressive towards the material and act synergistically, leading to the deterioration of the structure [15–18]. Although actions of a mechanical and physicochemical nature are generally well understood and are subject to standards and recommendations, the actions of a biological nature are much less considered and often neglected [19]. However, it should be noted that the durability of the material and biological interactions between the material and the environment are interconnected, as degradation of concrete structures has been observed to vary in intensity and rate of appearance and propagation due to the presence of microorganisms [20–23]. The biological interactions between concrete and the marine environment can lead to biodegradation or bioprotection of marine structure [23–25]. Any undesirable change in the properties caused by the activities of living organisms is considered as biodeterioration of concrete [26]. Microorganisms affect the stability of concrete by contributing to surface erosion, which increases the porosity of the surface and thus reduces the protection of the concrete cover. Increased porosity of the concrete cover leads to more efficient transport of aggressive ions (Cl^-, Mg^{2+}, OH^-) which can accelerate reinforcement corrosion, cracking, flaking and other damage [20,21,27]. In contrast, microorganisms can also protect the colonized concrete by forming a physical barrier that reduces surface permeability, leading to better durability of the cementitious materials [24,28–32].

In seawater, concrete and any natural or artificial substrata quickly become fouled [33–35]. The term "fouling" is defined as the colonization of any solid surface, living or dead, natural or artificial by living organisms in a marine or wet environment [36–39]. This colonization can be divided into two main stages, micro-fouling and macro-fouling, which are characterized respectively by the formation of bacterial biofilm on the surface and the adhesion of macro-organisms such as algae, barnacles and larvae (macro-fouling) (Figure 1) [14,34,40].

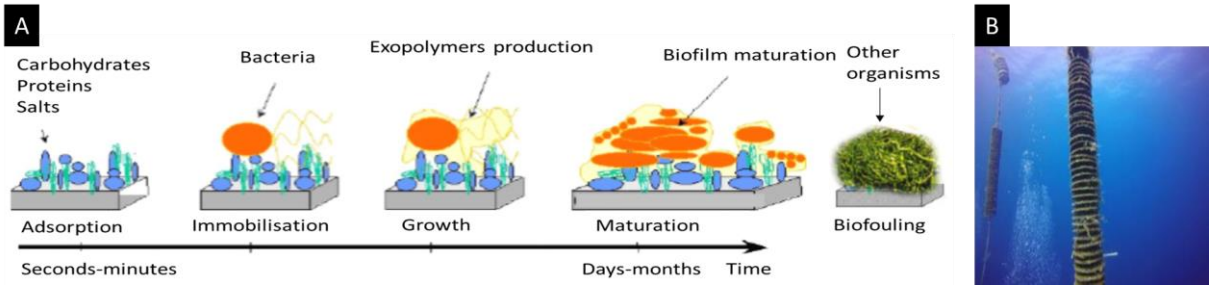

Figure 1. Biofouling in the marine environment. (A) Schematic representation of marine biofouling formation [40]. (B) Photo of marine biofouling [14].

Within minutes of immersing, organic molecules and particles are adsorbed onto the surface of cementitious materials, which is later colonized by bacteria that form a biofilm. Bacterial biofilms are composed of one or multiple bacteria species attached to the substratum (and to each other) and encased within a matrix of extracellular polymeric substances (EPS) [33]. The formation of bacterial biofilm involves i) the reversible and irreversible adhesion of microbial cells to the surface of the cementitious material, ii) the growth and maturation of the biofilm with the secretion of EPS, iii) the partial detachment and dispersion of microbial cells. Mature biofilms have complex, three-dimensional structures, which depend on the species composition of the biofilm, bacterial activity and environmental conditions [41–43]. However, bacteria (bacterial biofilm) are the first colonizers that facilitate the adhesion of other organisms such as fungi, microalgae, macroalgae and invertebrates [44–47]. Fouled structure is characterized by the thickness, density, structure, composition, bioadhesive strength and weight of fouling organisms (Figure 1).

Understanding the interactions between microorganisms and cementitious materials is crucial and constitutes a fundamental step towards more durable, safer and higher quality structures in many contexts [30,48–50]. However, as mentioned, the material's bioreceptivity (ability to be colonized by living organisms) is determined by the nature and the physico-chemical properties of the surface [48,51]: the chemical composition [52–55], roughness [56,57], porosity [57–59], hydrophobicity [53,60–62], and pH [57,63]. In the marine environment, additional studies are necessary to determine the different factors that can influence the biocolonization of cementitious materials. The main factors seem to be the pH, the chemical composition and the surface roughness [62,64–66].

In order to study the influence of the type of cement (chemical compositions) and surface pH on the bacterial colonization of cementitious materials immersed in seawater, this paper presents laboratory and field experiments allowing quantification of bacterial biofilm formed on cementitious materials with different cement and surface pH. The longer-term objective of this study is to develop an experimental approach that could help civil engineers to design green-marine structures by specifying the type and physicochemical characteristics of cementitious material to be used.

2. Materials and methods

2.1. Preparation of cementitious materials specimens

Three types of cementitious materials were produced: concrete, cement paste and mortar. Table 5 gives an overview over the investigated samples, the pre-treatment, the exposure and the techniques applied.

2.1.1. Preparation of cement paste specimens

In order to test our laboratory experimental approach, a Portland cement paste was prepared by mixing (mixer, 500 rpm for 60 seconds and 1000 rpm for 30 seconds) in a ratio w/c of 0.5, Portland cement (Portland cement CEM I 52,5 N PM ES) and water. After mixing, the cement pastes were cast in cylindrical molds with 2.2 cm diameter and 2 cm height and were kept 7 days at 20 °C in a laboratory room. Then, the cement pastes were demolded and placed for 7 days in the laboratory room at 20 °C.

2.1.2. Preparation of mortar specimens

In order to study the influence of the type of cement and surface pH on the natural bacterial colonization of mortars, four types of mortar specimens were prepared; two with CEM I Portland cement (Portland cement CEM I 52.5 N PM ES), and two with CEM III (composed of 60% of ground granulated blast-furnace slag NF EN 15167-1, provided by Ecocem, N° CAS: 65996-69-2). Table 1 shows the major constituents of this type of slag.

Table 1. Chemical composition of the blast furnace slag (traces of TiO_2, Na_2O and K_2O are also detected in the slag [67])

Component	Percentage (%)
CaO	35-48
SiO_2	32-41
Al_2O_3	9-18
MgO	1-9
MnO_2	0.4-0.7
Fe_2O_3	0.2-3
SO_3	0.4-1

The mortar had a water-cement-ratio (w/c) of 0.5 and was composed of 450 g cement and 1350 g sand (see Table 2). After mixing, the mortars were cast in cylindrical molds with 5.5 cm diameter and 6 cm height and were kept sealed with a lid at 20 °C. After 7 days of hardening, mortar samples with a diameter of 2.8 cm and a height of 3 cm were cut out from mortar cylinders. In order to reduce excessive leaching of $Ca(OH)_2$ during the test, the cut mortar specimens were first immersed in distilled water for at least 14 days with weekly water replacement. This pre-leaching has the role of lowering the pH of the surface and then makes easier material colonization.

After the pre-leaching procedure, half of the mortar samples were placed at 20 °C in an aerated chamber for 7 days to obtain carbonated mortar samples (Table 2). The surface pH was evaluated using pH paper (described below) and the phenolphthalein solution [68]. When applied, phenolphthalein indicators give a pink color to the non-carbonated surface while the surface becomes colorless if it is carbonated.

Table 2 gives an overview of the four types of mortar specimens: 1- carbonated CEM I, 2- Non-carbonated CEM I, 3- carbonated CEM III, 4- Non-carbonated CEM III. The abbreviation, cement type used, composition and pre-treatment for the four specimens are specified.

157 Table 2. Types and compositions of mortar specimens investigated in this study.

Mortar specimen	Cement	w/c	CEM I (g)	Ecocem (g)	Water (g)	Sand (g)	pre-treatment
1C	CEM I	0.5	450	0	225	1350	14 days in distilled water 7 days in aerated chamber
1NC							14 days in distilled water
3C	CEM III	0.5	180	270	225	1350	14 days in distilled water 7 days in aerated chamber
3NC							14 days in distilled water

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159 **2.1.3. Preparation of concrete specimens**

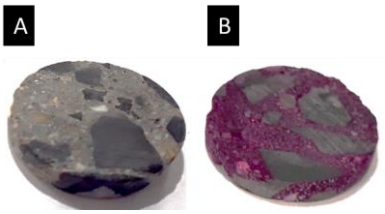
160 Concrete specimens used in this study (Table 5) were extracted from concrete discs (diameter
161 11 cm and height 7 cm) already prepared in 2016 by Souche *et al* [14,56]. Table 3 shows
162 information regarding the composition of the concrete. The concrete was prepared with water
163 to cement ratio of 0.6, a Portland cement CEM I 52.5 N PM ES (the same type of cement is
164 used in mortar and cement paste samples), sand (0/4, Languedoc Roussillon Matériaux, LRM),
165 a natural silica-limestone gravel (5.6/11.2, LRM), and a superplasticizer with a high water
166 reducing properties.

167 Table 3. Composition of concrete prepared in 2016 by Souche *et al* [56].

Compound	Density (kg/m^3)	Quantity (kg/ m^3) of concrete
CEM I 52.5 N PM ES	3.19	333.3
sand (0/4)	2.62	827.0
Gravel (5.6/11.2)	2.56	961.0
Superplasticizer	1.06	2.4
Water	1.00	220.3

168

169 The concrete samples used in this study (diameter 2 cm and height 0.3 cm) were obtained by
170 coring (with a crown of 2.2 cm) and sawing the concrete discs. Each concrete sample obtained
171 after sawing was inspected and selected to be representative (presence of paste and aggregates).
172 The samples obtained from the end of the discs were considered as carbonated samples and the
173 rest of the cylinder was considered as non-carbonated. The samples obtained were hermetically
174 stored in sealed tubes for 1 day and then emerged in seawater. As was the case for mortars, the
175 pH of the concrete surface was evaluated using pH paper and the pH indicator phenolphthalein.
176 The phenolphthalein test revealed that the sawn samples at the end of the discs are carbonated,
177 which is not the case with the sawn samples from the rest of the discs (Figure 2).



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179 Figure 2. Phenolphthalein tests on the surface of the concrete samples obtained after sawing.
180 (A) Sample taken from the end of the concrete disc. (B) Sample taken from the rest of the
181 concrete disc.

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2.2. Biofilm laboratory test

Biofilm laboratory test was performed on both concrete and cement paste samples. In both cases, the medium used was seawater recovered from the IFREMER station at Palavas (Biology Research Unit for exploited marine organisms) in sterile glass bottles (autoclave, 121°C for 15 minutes). The chemical composition of this seawater resembles to that of the Mediterranean Sea (Table 4)

Table 4. Main ionic species present in the Mediterranean seawater [69].

Ionic species	Cl^-	SO_4^{2-}	Br^-	Na^+	Mg^{2+}	Ca^{2+}	K^+
Concentration (g/L)	17.8	2.5	0.2	10.0	1.5	0.4	0.3

In the case of concrete, two types of samples were used, carbonated and non-carbonated concrete, while in the case of cement paste the variable was the medium used; natural and sterile seawater (natural seawater autoclaved by an autoclave at 121°C for 15 minutes). An overview of the samples prepared, how they are exposed, and how they are investigated is proposed in Table 5

To carry out this test, sterile samples of concrete or cement paste (sterilized by autoclave at 121°C for 15 minutes) were deposited on the bottom of the 250 ml sterile Erlenmeyer flasks (Duran, Dutscher 092049) containing 50 ml of seawater (4 samples / Erlenmeyer flask) [29]. The Erlenmeyer flasks, sealed with caps (screw cap GL45 with Polytetra-Fluorethylene filter membranes), were then incubated at 20 °C and 80 rpm to ensure the oxygenation of the medium which is necessary for the growth of microorganisms. After each incubation period (0, 1, 2, 8, 10, 15, 25 and 35 days), the samples were recovered from the Erlenmeyer flasks and were gently rinsed three times with 1 ml of sterile seawater to remove non-adhered microorganisms from their surfaces. Then, the adhering bacteria were detached from the surface using an ultrasonic bath (Bandelin SONOREX™) for 10 minutes at 20 °C (the samples are placed in sterile tubes containing sterile seawater and the tubes are immersed in the ultrasonic bath). The obtained solution was diluted using sterile seawater. Then, 100 µl of diluted solution were spread on plates containing Marine Agar (Dutscher, 490614). These plates were then incubated at 20 °C and colony count was performed at least 72 hours. The results are expressed as colony forming units per cm^3 of cementitious materials (CFU / cm^3). This biofilm quantification method known as "culture-based methods" is widely used in the literature [25,70,71]. In this method, the culture medium has a major impact on microorganism growth. We used in this study the Marine Agar, a medium which is widely used for the culture of marine bacteria [43,72,73].

2.3. pH measurement

pH measurements of the seawater and the surface of the cementitious materials were performed using pH electrode (Hanna instrument, HI1230, accuracy 0.1 pH unit) and pH indicator paper (Whatman, 0.0 to 14.0, accuracy 1 pH unit) respectively. In the case of the pH measurement of seawater, the pH electrode was rinsed thoroughly with distilled water before being dipped in a well-agitated seawater (30 ml). The pH value was noted when the pH reading is stable. In the case of cementitious materials, one ml of ultra-pure water (Milli-Q water) was added to the surface of the sample. After one minute, the pH paper was deposited on the surface and the pH value was evaluated after 30 seconds of contact between the pH paper and the surface [57,58]. Then, this pH was verified using phenolphthalein indicator (goes from colorless to pink at pH $\geq$ 9). This method allows evaluating the pH of the surface and not the pH of the complete

material. It should be noted that this evaluated pH can be influenced by the biofilm present on the surface. However, the use of phenolphthalein and pH indicator paper allows the rather qualitative evaluation of the pH of the surface and does not give an accurate quantification of the pH of the material [68].

2.5. Biofilm *in-situ* test

Biofilm *in-situ* test was carried out using the 4 types of mortars. The *in-situ* exposure site is located at the IFREMER station in Palavas (France). It is a flat basin (polyester, length 6 m height 0.6 m and width 2 m) with a seawater inlet and outlet, which allows for an open water circuit.

To ensure the correct progress of the experiment and to avoid any type of contamination, the basin was first cleaned and disinfected. Mortar samples sterilized in the laboratory by autoclave were then placed in the basin and completely covered with seawater.

After each incubation time (0, 1, 3, 8, 15 and 45 days), three mortar samples of each mortar type were used to quantify the formation of bacterial biofilm. The bacterial colonization are quantified as described above and the results are presented as colony forming units per cm^3 of mortar (CFU / cm^3).

2.6. Statistical analyses

To evaluate the significance of the different results obtained, statistical analysis was done via GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA) using t-test, one-way and two-way ANOVA tests. Statistical significance was accepted by $P_{value} < 0.05$ obtained using Bonferroni or Tukey multiple comparison post-tests.

3. Results and discussion

3.1. Biofilm laboratory test using cement paste samples

Several studies have been dedicated to the development of a laboratory test for the evaluation of the bioreceptivity of cementitious materials [29,61,74–76]. The laboratory test must be reproducible, inexpensive and easy to carry out. It should also discriminate the intrinsic parameters of cementitious materials for biocolonization. The studies carried out are rather focused on building materials with air as an environment, or sewer systems with wastewater as an environment. However, no study to date has been carried out on maritime structures meaning with seawater as environment, as is done in the current study. The bacterial biofilm developed on the surface of paste samples immersed in seawater under laboratory conditions was quantified during 34 days. Since the development of bacterial biofilm is influenced by the alkalinity of the medium and that of the surface to be colonized, the pH of the surface of the cement paste samples and seawater was measured throughout the experiment. The results obtained are shown in figure 3.

3.1.1. pH of cement paste surfaces

The pH of the surface is very basic already from the start of this test, with a value equal to 11.75 (Figure 3 A). The pH was also verified using phenolphthalein indicators which gave a pink coloration after contact with the surface of the cement paste indicating a pH higher than 9. The pH of the pore solution of hydrated cement paste with CEM I ranges generally between 13 and 14 [77]. The measured pH of the surface is therefore lower than expected. This might be due to the pre-treatment of the samples.

When cement paste is immersed or in contact with seawater, it will leach due to the high ionic strength of the pore solution of the cement paste compared to the seawater. The pH of a CEM I paste is generally between 13 and 14 whereas the pH of seawater varies between 7.5 and 9.0, with an average of around 8.2 [78,79]. Alkali metals such as potassium, as well as calcium and hydroxide ions will leach out of the cement paste which will lead to a decrease in the pH of the material [80]. However, the pH of the surface of the cement paste remained almost constant throughout our test. There are two potential reasons for this: (1) the cement paste has been pre-leached during the pre-treatment and reached some kind of steady state prior to the experiment. (2) the volume of the exposure solution (seawater) is constant a rather small (50 mL) which can have lead to rapid saturation of the solution with alkali metals, calcium and hydroxide limiting further leaching during the experiment.

3.1.2. pH of seawater

The “carbonic acid – bicarbonate - carbonate” system is the main pH buffer for seawater. The pH of seawater varies between 7.5 and 9.0, with an average of around 8.2 [78,79]. Figure 3 B shows that the pH measured for seawater was 8.2 at the start of the experiment. Due to leaching, the pH of seawater gradually increases to reach a value of 12 after 8 days of immersion. Then, the pH remains almost constant throughout the experiment at a value between 11.5 and 12.

3.1.3 Quantification of bacterial biofilm

In their natural or artificial environment, the majority of microorganisms adhere to biotic or abiotic surfaces. This microorganism-surface duality is conditioned by the properties of the

substrate, properties of the bacterial surface, and environmental conditions [48]. In seawater, the microorganisms live at pH values around 8.2 and colonize all natural or artificial surfaces emerged [81,82]. Figure 3 C shows that the adhesion of bacteria to cement pastes was impossible throughout the experiment. This can be explained by the very basic pH of the seawater and of the cement paste surface (Figure 3 A and B); a very basic or very acidic pH can inhibit the biofilm formation by marine microorganisms [83–85].

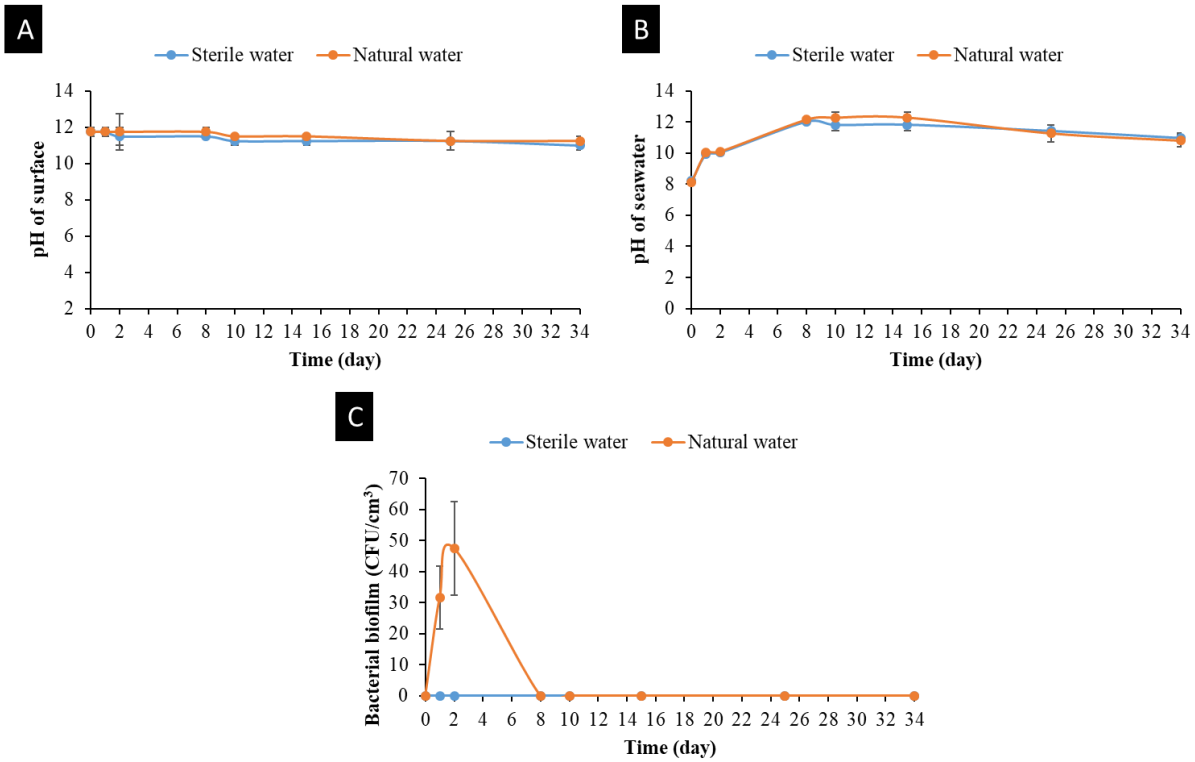

Figure 3. Results obtained with the biofilm laboratory test cement paste samples in seawater. A. pH evaluation of cement surface. B. pH measurement of seawater. C. Quantification of bacterial biofilm. Each experiment was performed in triplicate and the error bars present the standard deviation from the obtained values.

3.2. Biofilm laboratory test using concrete samples

The results obtained with the laboratory test on cement paste show that the pH of the surface and the seawater alkalinity have a crucial role in the bacterial colonization of cementitious materials under laboratory conditions. Under these conditions, it is impossible to work with non-pre-carbonated or non-pre-leached cementitious materials whose high surface pH inhibits the adhesion of marine bacteria on the surface.

In order to avoid this high surface pH, another test was carried out in the laboratory under the same conditions as the previous one. This time, the test was carried out using concrete discs naturally cured for 4 years and stored at 20 °C in a laboratory room [56]. Two types of concrete discs were obtained from these specimens; carbonated (low pH) and non-carbonated (basic pH) (see materials and methods). The advantage of working with these samples is to use concrete with a moderate pH (pH < 10) which does not affect the alkalinity of seawater during immersion.

In order to validate our experimental approach and study the influence of carbonation on the biocolonization of concrete surfaces in the marine environment, carbonated (low pH) and non-carbonated (basic pH) concrete samples were incubated in the laboratory (Erlenmeyer flasks) at 20 °C and 80 rpm in natural seawater. The bacterial biofilm formed on the concrete surface was quantified after 0, 1, 3, 7, 10, 15, 24, 29 and 60 days. Similarly, the pH of the concrete surface and the pH of seawater were determined. The results obtained are shown in figure 4.

3.2.1. pH of concrete surfaces

The pH at the surface of the carbonated and non-carbonated samples at T0 is 8 and 10 respectively (Figure 4 A). The surface has also been sprayed with phenolphthalein indicator, resulting in a pink coloration only for the non-carbonated surface. Then, the pH measured at T0 is smaller than the known pH for carbonated and non-carbonated concrete surfaces, 9 and 12 respectively [86,87]. This difference can be explained by the aging of these concrete samples prepared and stored in a laboratory room before 4 years (natural carbonation). In contact with air, concrete is under the action of a carbonation reaction (aging) due to CO_2. CO_2 from the atmosphere diffuses in gaseous form into the pores of concrete and dissolves in the pore solution, and reacts to $CaCO_3$ thereby lowering the pH of the pore solution. This phenomenon gradually changes the chemical composition and the pH of concrete [84,88,89].

Upon immersion, leaching and colonization by bacteria [80,90] lead to a gradually decrease of the pH of the carbonated and non-carbonated surfaces and reaching a value of 6.5 after 15 days of immersion. Upon further immersion the pH remains almost constant until the end of the experiment (Figure 4 A).

3.2.2. pH of seawater

Figure 4 B shows that the pH of the seawater containing non-carbonated samples increases to 10 after 1 day of immersion due to $Ca(OH)_2$ and KOH release (leaching). Hereafter, the pH gradually decreases to 8 after 7 days of incubation and then remains almost constant until T60. However, in the case of carbonate samples the pH remains almost constant throughout the experiment, which indicates that the use of pre-carbonated samples in the laboratory prevents the increase of the pH of the seawater and then allows continuous growth of microorganisms.

3.2.3 Quantification of bacterial biofilm

Figure 4 C shows that the formation of bacterial biofilm on the carbonated and non-carbonated samples started with a latency phase followed by a phase of growth and accumulation of cells on the surface. These kinetics of the colonization process were also observed by Tran *et al* during *in vitro* and *in-situ* colonization tests on mortar samples in air [57,58].

In the case of carbonated samples, the formation of bacterial biofilm was spontaneous with an almost non-existent lag phase. The biofilm accumulation on the surface reaches a maximum after 7 days of incubation and then decreases slightly to reach a plateau phase. However, in the case of non-carbonated samples, an induction phase of 7 days was observed and the biofilm formation reached a maximum after 24 days of incubation with a value equal to that in the case of carbonated samples (Figure 4 C). Then, a plateau phase was observed until T60.

The lag period difference between this two sample cases can be explained by the basic (value equal 10) and lower pH (value equal 8) at T0 of the non-carbonated and carbonated samples

respectively. This influence of surface pH has also been identified in several studies concerning the biocolonization of cementitious materials. These studies showed a higher lag phase in the case of non-carbonated samples [57,58,91]. Similarly, Dooley *et al.* (1999), Guilbeau *et al.* (2003), Prieto *et al.* (2004) showed that carbonation promotes the attachment and growth of microorganisms (algae) during accelerated laboratory tests [92–94].

In addition, a decrease in the surface pH was found to be necessary for the bacterial biofilm development on non-carbonated samples; the biofilm growth started at 5 days when a decrease in the surface pH from 10 (T0) to 9 (T5) was observed. These results indicate that carbonation plays a primary role in concrete biocolonization in seawater. This pH effect of concrete is widely known in the literature: it has been proven that a concrete surface must have a low pH (carbonated surface) in order to be colonized by microorganisms [58,85,95].

In summary, the presented laboratory test is effective and allows a quick and inexpensive way to test the bioreceptivity of cementitious material intended to be immersed in seawater. With this work, it has been shown that the pre-carbonation of concrete accelerates the development of bacteria on their surface by lowering their pH, which shortens the latency phase observed in the case of carbonated samples.

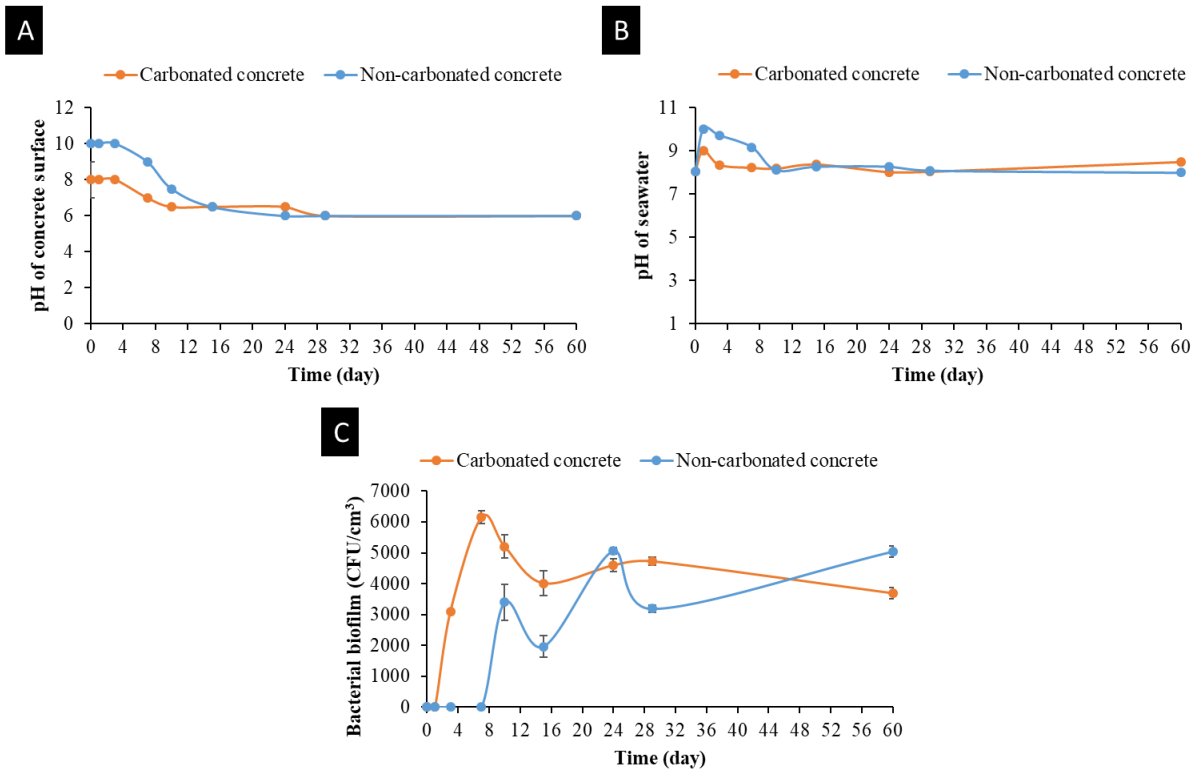

Figure 4. Results obtained with the biofilm laboratory test using concrete samples. A. pH evaluation of the concrete surface. B. pH evaluation of seawater containing the carbonated and non-carbonated samples. C. Quantification of bacterial biofilm. Each experiment was performed in triplicate and the error bars present the standard deviation of the obtained values.

3.3. Biofilm *in-situ* test using mortar samples

In order to (i) compare the results obtained in the laboratory test with the natural bacterial colonization of cementitious materials (ii) study the influence of the type of cement and surface pH on the bioreceptivity of cementitious materials, four types of mortar specimens (non-

carbonated CEM I, carbonated CEM I, non-carbonated CEM III, carbonated CEM III) were immersed in seawater under natural conditions at IFREMER institute (Palavas – France). The bacterial biofilm formed on the mortar surface was quantified after 0, 1, 3, 8, 10, 15 days. At the same time, the pH of the mortar surface and the pH of seawater were determined.

3.3.1. pH of mortar surfaces

Figure 5 A shows that the pH of CEM I mortar samples at T0 is around 9.3 and 7.3 in the case of non-carbonated and carbonated mortars respectively, which indicates that the carbonation and leaching of the samples during their preparation succeeded in lowering the pH of the mortars (see materials and methods). After immersion, in the case of non-carbonated samples, the pH decreases gradually and reaches a value of 7.5 at T15 due to leaching. However, in the case of the carbonated samples, the pH of the mortar surface remains nearly constant throughout the experiment.

The CEM I and CEM III mortar samples were prepared in the same way and they were subjected to the same carbonation and leaching conditions. Similarly to the case of CEM 1 samples, the pH of CEM III mortars at T0 is of the order of 8.6 and 7.3 for non-carbonated and carbonated samples respectively (Figure 5 B). As in the case of CEM I mortars, the pH of non-carbonated CEM III mortars gradually decreases over time and reaches a value of 7.3 at T15 while the pH remains almost constant in the case of carbonated samples.

3.3.2. Temperature and pH of seawater

Figure 5 C shows that the seawater temperature remained almost constant throughout the experiment with an average of 20.8 °C (between 21.4 °C at T0 and 23.5 °C at T15), which is an optimal temperature for the growth of most marine bacteria [43,96–98]. Temperature is an environmental factor which acts on the biocolonization of cementitious materials [99–102]. Maintaining optimal environmental conditions for the growth of microorganisms facilitates the discrimination of the support parameters (intrinsic parameters of cementitious materials) for biocolonization of cementitious materials. In 2014, Tran *et al.* compared laboratory and *in-situ* colonization of carbonated and non-carbonated mortar samples in air. With laboratory tests, they found that carbonation affects colonization, whereas this was not observed in the case of the *in-situ* tests. They explained this observation by climate conditions unfavorable to microorganisms growth during *in-situ* tests [57].

Moreover, the pH of seawater remains constant throughout this test (Figure 5 C). The release of $Ca(OH)_2$ and KOH resulting from the leaching reaction of the mortar samples after immersion did not affect the pH stability of seawater because the test was carried out here in an open seawater circuit.

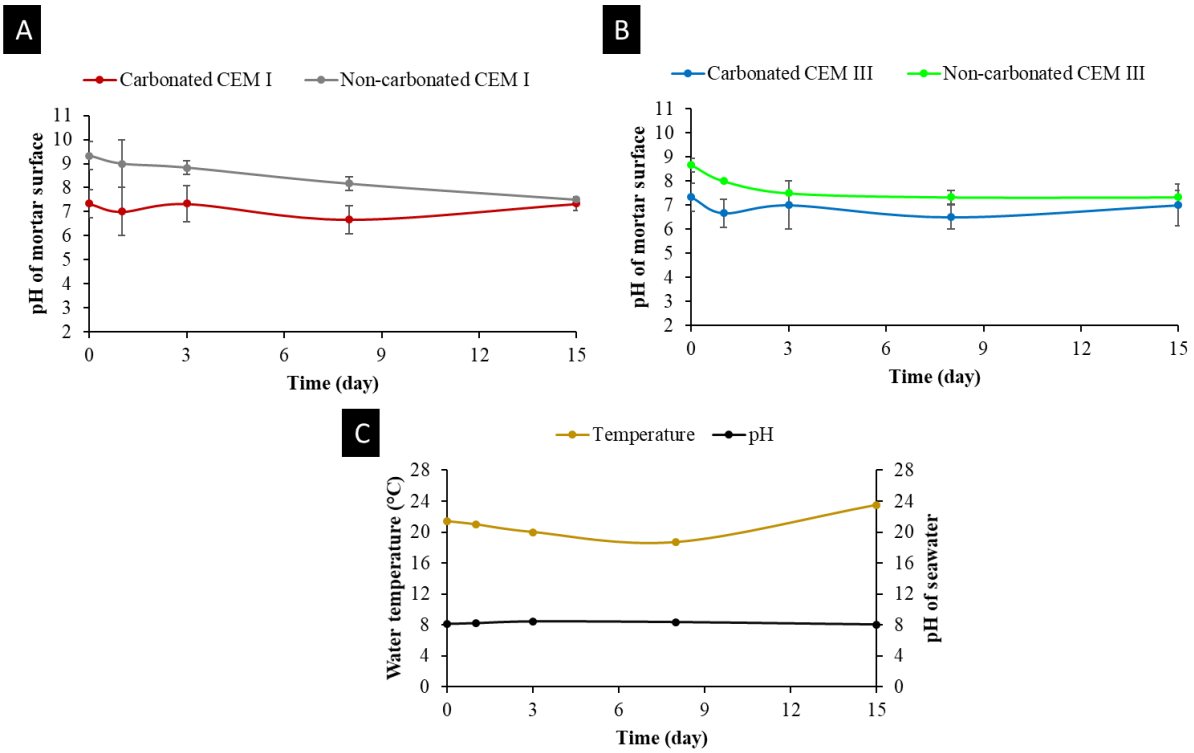

Figure 5. Results of temperature and pH obtained in the biofilm *in-situ* test using mortar samples. A. pH evaluation of the CEM I mortar samples. B. pH evaluation of the CEM III mortar samples. C. temperature and pH evaluation of seawater. Each experiment was performed in triplicate and the error bars present the standard deviation of the obtained values.

3.3.3. Quantification of bacterial biofilm

Figure 6 shows that the bacterial colonization of CEM I and CEM III mortar samples. It starts with a latency phase followed by a phase of growth and accumulation of cells on the surface, as was the case in the laboratory test. The formation of a bacterial biofilm is faster and higher in the case of carbonated samples compared to the non-carbonated both of CEM I and CEM III mortars. These results confirm the conclusion obtained from the laboratory test; carbonation promotes the attachment and growth of bacterial biofilm on cementitious materials in marine environment.

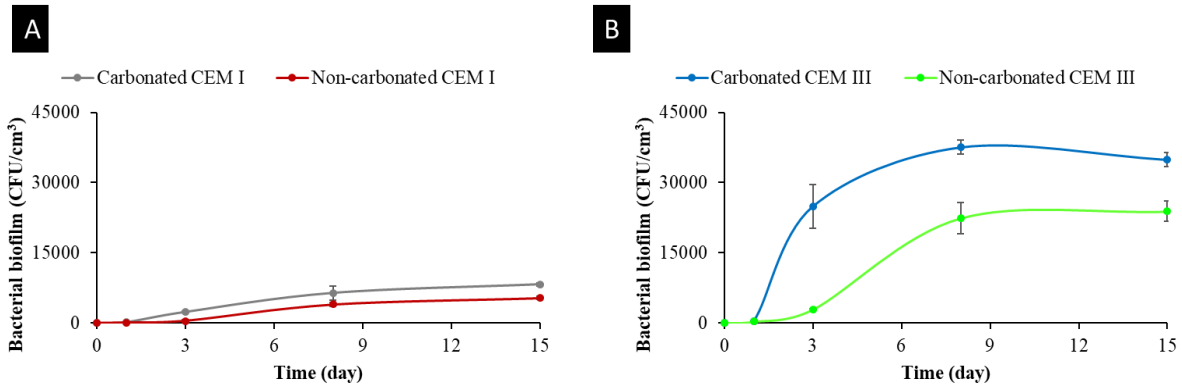

Figure 6. Quantification of bacterial colonization of mortar samples. A. CEM I mortar samples. B. CEM III mortar samples. Each experiment was performed in triplicate and the error bars present the standard deviation of the obtained values.

However, carbonation of cementitious materials leads to a decrease in pH but also to a change in the mineral phases on the surface such as the appearance of calcium carbonate [103,104]. In the marine environment, the formation of bacterial biofilm is influenced by the presence of divalent cations such as Mg^{2+} and Ca^{2+} [105–110]. In general, Ca^{2+} enhanced the biofilm growth in a dose-dependent manner by binding to the EPS components of the biofilm (especially extracellular DNA), whereas Mg^{2+} significantly increased the cell growth in biofilm [105,111]. We propose that the pre-carbonation of the samples before immersion increases the formation of bacterial biofilm not only by the decrease in pH but also by the change in the mineral phases on the surface.

3.3.4. CEM I vs CEM III mortar samples

Figure 7 shows that the type of cement has a large influence on the bacterial colonization of cementitious materials in marine environment. The formation of bacterial biofilm is significantly higher in the case of CEM III mortars regardless of the state of carbonation. For example, at 3 days of incubation, the bacterial colonization of carbonated CEM III mortar is approx. 10 times greater than that of carbonated CEM I mortars (Figure 7 A). At 8 days of incubation, the bacterial colonization of non-carbonated CEM III mortars is about 5 times greater than that of carbonated CEM I mortars (Figure 7 B). These results are in agreement with the literature in which a similar effect of chemical composition on the biocolonization of cementitious materials has been reported [56,60,64,106]. In addition, Ahmed showed that cementitious materials prepared with CEM III are more bioreceptive than those formulated by CEM I using laboratory and field-scale tests in river water [112].

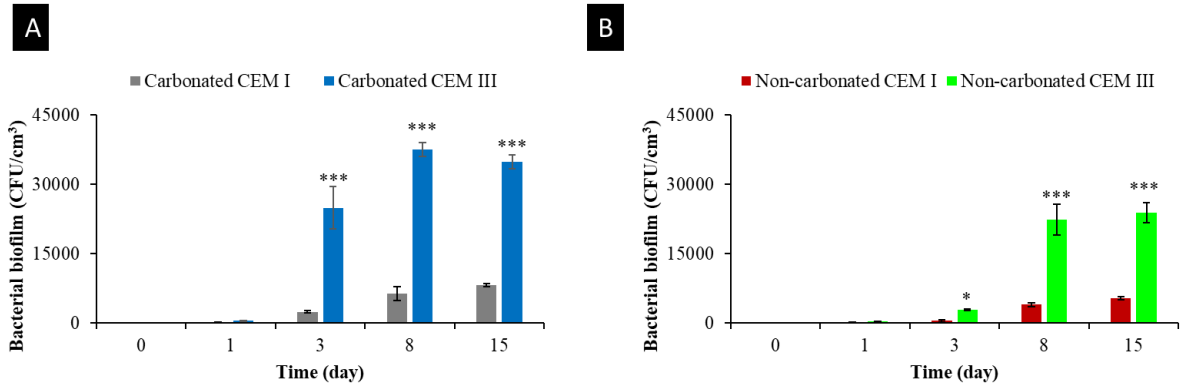

Figure 7. Formation of bacterial biofilm results using mortar samples. A. Quantification of bacterial biofilm on carbonated CEM I and CEM III mortars. B. Quantification of bacterial biofilm on non-carbonated CEM I and CEM III mortars. Each experiment was performed in triplicate and the error bars present the standard deviation of the obtained values. The experiments highlighted by asterisks were significantly different compared to its control (Bonferroni; *: $p < 0.01$, ***: $p < 0,001$) at the indicated time.

In the marine environment, bacterial biofilms are known to interact directly with macro-fouling organisms [44,106] and differences in biofilm community structure and quantity may influence their attachment [34,113]. The physical properties of bacterial biofilms, biotic composition of biofilms, and accumulation of chemical compounds, as well as the dynamics of these parameters provide a discriminative mechanism in shaping biofouling communities including algal, larvae and invertebrate colonization [36,47]. For these reasons, we propose that the

chemical composition of submerged cementitious materials can influence not only the quantity of bacterial biofilm, but also macrofouling and subsequently biodiversity in the marine environment. To enhancing marine biodiversity, it is better to manufacture marine structures using CEM III cement.

3.4. Laboratory versus *in-situ* tests

Table 5 summarizes the results of the laboratory and field tests.

The first laboratory immersion test was carried out with non-carbonated cement paste samples. The pH at T0 of these samples was of the order of 12 which increased the pH of the seawater and inhibited the growth of bacteria. This test have shown that biocolonization in laboratory immersion tests only occurs on carbonated or leached samples whose pH is lower than 12.

The second laboratory immersion test was carried out on samples of long-term cured concrete. The pH at T0 of these samples was of the order of 8 and 10 for the carbonated and non-carbonated samples respectively. This low pH of samples kept the average pH of seawater at 8.33. Then, the bacteria were in good growth conditions and they colonized the samples. The results obtained with this laboratory immersion test show that the carbonated samples are colonized more quickly and with a greater amount of bacteria.

Finally, *in-situ* immersion test was carried out with CEM I and CEM III mortar samples. This immersion test confirmed the results obtained with the second laboratory immersion test; surface carbonation promotes the attachment and growth of bacterial biofilm on cementitious materials in marine environment. However, in the case of CEM I mortars, the lag phase is smaller than that observed in the laboratory immersion test for non-carbonated samples (3 days versus 7 days respectively). Furthermore, in the laboratory immersion test the quantity of bacterial biofilm on the surface is higher. These differences can be explained by the different conditions between the laboratory and the field immersion tests; (i) the use of an open seawater circuit for the field immersion test allowed faster leaching of the non-carbonated samples. (ii) The seawater flow allowed a continuous washing of the sample surfaces, which leads to a partial detachment of the bacterial biofilm throughout the experiment [74].

491 Table 5. Results of the laboratory and *in-situ* tests.

Test	Type of samples	Storage before immersion	pH at T0	Duration of lag phase during bacterial colonization (days)	Maximum bacterial colonization (CFU/cm^3)	Time to reach the maximum (day)	pH average of seawater during immersion	Results
Laboratory test	Cement paste	7 days in laboratory room at 20°C	12	ND	ND	ND	11.50	Absence of bacterial colonization; it is impossible do have biocolonization in the laboratory (closed water circuit) using non-carbonated or non-leached samples
Laboratory test	Carbonated concrete samples	4 years in laboratory room at 20°C	8	1	61711	7	8.33	Surface carbonation promotes bacterial colonization with a lower latency phase
	Non-carbonated concrete samples	4 years in laboratory room at 20°C	10	7	50601	24	8.71	
In-situ test	Carbonated CEM I mortar samples	14 days in distilled water 7 days in aerated chamber	7.3	1	8229	15	8.23	Surface carbonation promotes the attachment and growth of bacterial biofilm on cementitious materials in marine environment The type of cement has a large influence on the bacterial colonization of cementitious materials in marine environment; CEM III mortar are more bioreceptive than CEM I mortar
	Non-carbonated CEM I mortar samples	14 days in distilled water	9.3	3	5378	15	8.23	
	Carbonated CEM III mortar samples	14 days in distilled water 7 days in aerated chamber	7.3	1	37538	8	8.23	
	Non-carbonated CEM III mortar samples	14 days in distilled water	8.6	3	22378	8	8.23	

492

4. Conclusions

This study proposes a new fast and reliable laboratory test to control factors that can influence the bacterial colonization of cementitious materials in the marine environment in an easy and inexpensive way. This test allows reproducing, with a simple experiment in Erlenmeyer flasks, the results observed with *in-situ* tests. The study shows that the laboratory tests have made it possible to mimic the natural environment and to lead to similar conclusions: the carbonation (surface pH) and the type of cement play a primary role in cementitious materials colonization by bacteria in the marine environment. Carbonated concrete and mortar are more bioreceptive that the non-carbonated ones in the primary days which is consistent with the literature [54]. The type of cement influences the kinetics and the amount of the development of bacterial biofilm and might have an influence on the biocolonization quality in the marine environment (biodiversity). However, after two weeks, both the materials are colonized in accordance with the study of Jakobsen *et al*., (2016) [110]. Pre-carbonation of the exposed surface seems to have a stronger beneficial effect on biocolonization compared to the chemical composition of the cement.

Eco-design of marine structures is a major focus for many researchers and construction companies working in marine environment, to enhance durability and also since few years to minimize and mitigate human impacts toward “no net loss” on biodiversity policies [14,114]. These companies have long experience in the field of the design of the marine structures and are seeking to improve the construction in marine ecosystems by for example the inspection of the old structures or the use of innovative concrete “eco-blocks” (e.g. https://www.concretelayer.com). We will collaborate with one of these companies to validate the laboratory test proposed in this paper on an industrial scale by carrying out parallel in vitro and *in-situ* test on real marine structures. We will also delve deeper into some issues such as the effect of hydrophobicity material on biocolonization in marine environments and the effect of formwork oil and curing products on the kinetic of biofilm formation.

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