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Biodeterioration kinetics and microbial community organization on surface of cementitious materials exposed to anaerobic digestion conditions

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Biofilm
Biodeterioration
Anaerobic digestion
Cementitious materials
Biofouling

ABSTRACT

Anaerobic digestion is a process that can produce renewable energy through the fermentation of biodegradable biomass. Industrial anaerobic digestion tanks are usually made of concrete but the production of various aggressive compounds (CO_2, NH_4^+ and volatile fatty acids) during the microbial fermentation leads to deterioration of the concrete structure. In addition, the formation of a microbial biofilm on the cementitious material surface could generate even more intense biodeterioration. The objective of this study is to gain a better understanding of the involvement of biofilm in the biodeterioration of cementitious materials during an anaerobic digestion process. More specifically, the study focuses on the heterogeneity of microbial populations within the biofilm and the reactive medium in anaerobic digestion. Laboratory scale anaerobic bioreactors mimicking the industrial anaerobic digestion medium were constructed and CEM I cement pastes were immersed in this medium for 2, 3, 4, 5, 10 and 15 weeks. The biodeterioration of the cement pastes was evaluated by determining the deteriorated thickness. The aggressive compounds in the medium were quantified. The biofilm attached to the surface of the cement pastes was analyzed using 16 s rRNA gene sequencing. To evaluate the heterogeneity of the biofilm, the growth of biofilm layers was successively caused to stall by using two distinct biofilm removal techniques. Three microbial fractions were defined: planktonic microorganisms, and the microorganisms within the biofilm that were loosely and strongly attached. The results showed that the planktonic lifestyle was more associated with microorganisms producing methane and consuming volatile fatty acids, while the biofilm was more associated with bacteria producing acids, mainly members of the *Clostridium* genus. A microbial community shift due to a reversible propionic acid accumulation during the first 5 weeks was also observed. In addition, no major differences were spotted between the loosely and strongly attached biomass, indicating homogeneity in the two layers of the biofilm. These results suggest that the biofilm could increase the biodeterioration of concrete since volatile fatty acids could be produced in massive quantities near the surface of the cement samples by the acidogenic microbial population more present within the biofilm.

1. Introduction

Anaerobic digestion is a renewable energy production process based on the fermentation of biomass. It consists of the transformation of biomass into two products of major interest: biogas and digestate. The biogas is mainly composed of CH_4 (50–70%) and CO_2 (30–50%) and can be used to produce energy in various forms [1,5]. It can be: burned to generate heat and/or electricity, depending on the type of process used; purified or enriched in CH_4 and injected into the natural gas network; or transformed into biofuel [4]. The digestate, i.e. the residue in the tank, can be used as fertilizer for agronomic purposes [31]. Anaerobic digestion is a succession of four steps involving different microbial populations, as presented in Fig. 1. The first step is the hydrolysis of macromolecules into monomers. The next step, acidogenesis, is the conversion of these monomers into volatile fatty acids, mainly acetic, butyric and propionic acids, and other compounds such as alcohols (ethanol). The third step, acetogenesis, is the conversion of the products of the previous step into acetate, H_2 and CO_2. The last stage, methanogenesis, results in the production of methane and carbon dioxide through two distinct pathways: the acetoclastic pathway, which uses acetate, and the hydrogenotrophic pathway, which produces methane from H_2 and CO_2 [16]. The acetoclastic and hydrogenotrophic pathways

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respectively account for 70% and 30% of the total CH_4 produced [15]. This sequence of biochemical reactions results from the successive action of different microbial communities belonging to the *Bacteria* domain for the first three steps and to the *Archaea* domain for the methanogenesis. In the *Bacteria* domain, the phyla *Bacteroidetes*, *Firmicutes*, *Chloroflexi* and *Proteobacteria* make up the majority of the digesters (anaerobic digestion tanks) [3]. It is mainly the first two phyla, that are involved in hydrolysis. The class *Clostridia*, of the phylum firmicutes, involved in the first two steps is usually the most strongly represented genus, followed by the class *Bacilli*. Acetogenesis results from the action of acetogens, mostly strict anaerobic, syntrophic acetogens of the genera *Pelotomaculum*, *Smithllela*, *Syntrophobacter* and *Syntrophus* [38,39]. In the case of archaea, about 70% of the methane is usually produced by acetoclastic archaea of the genera *Methanosaeta* or *Methanosarcina*. Nearly all of the remaining methane produced comes from the hydrogenotrophic pathway, which involves the genera *Methanobacterium*, *Methanobrevibacter*, *Methanoculleus*, *Methanospirillum*, and *Methanothermobacter* [33]. It should be noted that the genus *Methanosarcina* is also known to group together archaeal species that can use both pathways for methane production, while the genus *Methanosaeta* is limited to the acetoclastic pathway [19]. In the current period of energy transition to non-fossil renewable energies, the anaerobic digestion process is receiving increasing interest, particularly in the aspects of improved production performance and CH_4 enrichment [26]. In this regard, the focus is on improving the performance of the anaerobic digestion process and the sustainability of anaerobic digestion devices. In this context, one of the major challenges is to improve the durability of the digesters since this directly impacts their lifespan and operating costs, i.e. the maintenance costs included in the OpEx (Operational Expenditures). Large and medium capacity digesters are generally built in concrete as it is an inexpensive material that is easy to use and suitable for the construction of tanks of several m^3. However, anaerobic digestion media are highly aggressive towards cementitious materials as they generate biodeterioration phenomena [14,29] in which microbial metabolisms produce aggressive compounds (CO_2, NH_4, volatile fatty acids) leading to leaching of the cement phases and carbonation of the cement matrix in the case of Portland cements. In addition, the formation of microbial biofilms on the surface of concrete is likely to increase the kinetics of these biodeterioration phenomena [35,36]. A biofilm is an aggregate of microorganisms growing at an interface. The microorganisms are usually embedded in a matrix composed of extracellular polymeric substances (EPS) synthesized by the microorganisms present in the biofilm [8]. A biofilm can be heterogeneous, with micrometer scale concentration gradients and variations in microbial populations within its structure. With this in mind, it is reasonable to hypothesize that a biofilm, by locally increasing the concentration of aggressive compounds, is able to have a deleterious catalytic effect on cement matrices [2,20]. This is even more conceivable since this type of effect has already been demonstrated with acidogenic sulfur-oxidizing biofilms in sewer pipes or in a study on a model acidogenic biofilm to establish the its specific effect [12].

Beyond the established cases, the present work aims to better understand the deleterious involvement of biofilm in the biodeterioration process of concrete in the particular context of digesters (anaerobic digestion tanks) built in concrete. More specifically, the work presented here aims to carry out dynamic monitoring of the production of aggressive compounds (CO_2, NH_4^+ and acetic, butyric and propionic acids) in relation to the measured biodeterioration, particularly the total deteriorated depth in a context of anaerobic digestion over several cycles with replacement of the bio-waste. To this is added a comparison, in this same context, of the dynamics over time of the microbial communities developed on the surfaces of cementitious materials. Additionally this comparison was done according to the location (reaction medium and strongly or weakly attached biofilm) of the microbial community. Finally, the chemical and structural characterization of biodeterioration will help us to position ourselves in relation to the work already carried out on the subject.

2. Materials and methods

2.1. Manufacturing of cement paste samples

Portland CEM I cement pastes were prepared with a water/cement ratio of 0.30. Cylindrical molds with a diameter of 2.8 cm and a height of 6.5 cm were used. The cement pastes were then cured at 20 °C in their sealed molds for 28 days. After hardening, cement paste samples (pellets) with a thickness of about 2.5 mm were obtained by cutting the cylindrical cement paste under water with a circular saw (Prezi,

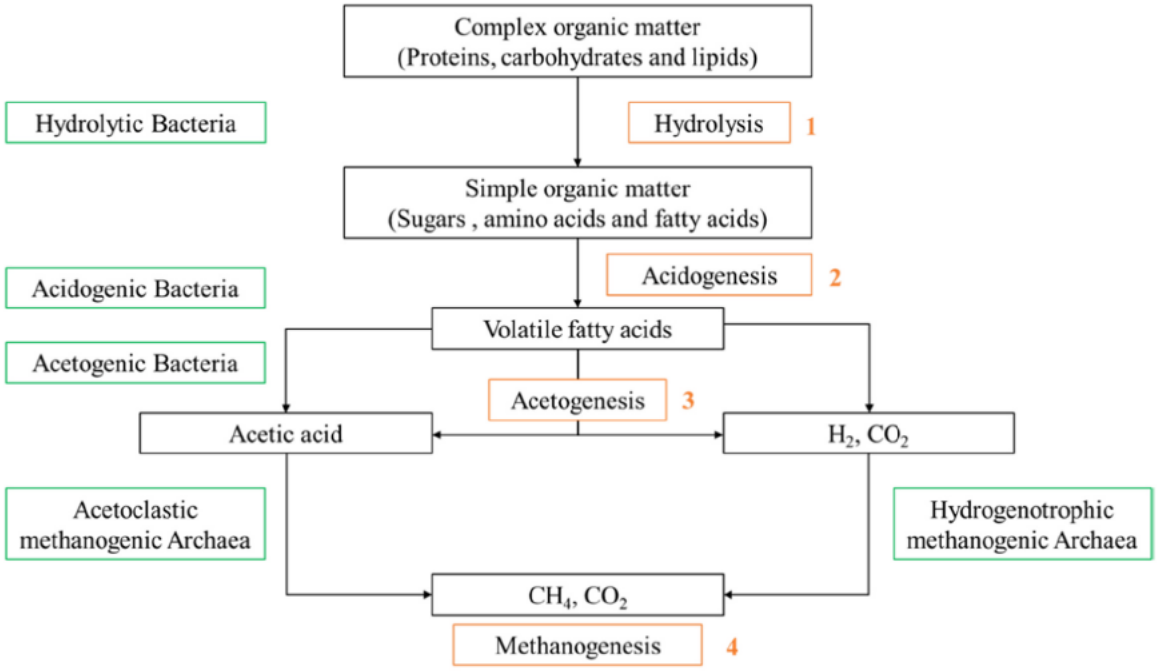

Fig. 1. Synthesis of the 4 successive reaction steps of the anaerobic digestion of complex substrates leading to the production of biogas.

Mecatom 180). The cement paste pellets were then polished with a silicon carbide disc (ESCIL, P800–22 µm).

2.2. Microbial inoculum and reaction medium

The microbial inoculum and the reaction medium were chosen to reproduce the conditions of industrial anaerobic digestion, in accordance with the protocol and methods already described by Voegel et al. [36]. The medium used was a synthetic "reconstituted bio-waste" composed of different organic fractions, which were blended for 5 min at 20 °C (Table 1). The inoculum used was activated sludge taken from a municipal wastewater treatment plant in Castanet-Tolosan (France). The inoculum/culture medium volume ratio was 2.5/1.

2.3. Experimental laboratory device simulating anaerobic digestion conditions

The protocol for immersing the cement paste in the reaction medium was designed to mimic the environmental conditions found in an anaerobic digestion tank [36]. Each reactor, a 100 mL sealed airtight Schott flask, contained a pellet of cement paste immersed in 66 mL of inoculated reaction medium (Fig. 2). Thus, the ratio between the surface area of the solid cement paste and the volume of the liquid phase was 220 cm^2/L. These reactors were incubated under the same conditions at 37 °C with magnetic agitation for times of 2, 3, 4, 5, 10 or 15 weeks. Six replicates runs were performed for each duration of the biodeterioration assay. As one cycle corresponded to 5 weeks, the biodeterioration assay consisted of 1, 2 and 3 cycles with several timing events during the first cycle. A renewal of the medium was carried out every 5 weeks, the theoretical average duration of an anaerobic digestion cycle. For the 10 and 15 week timeframes, the culture medium was therefore renewed, respectively once and twice, by removing 19 mL of reaction medium and replacing it with new reconstituted bio-waste. To avoid the accumulation of biogas in the gas phase, the biogas produced in the reactors was daily evacuated using a needle and a sealed gas collection bag. For each timeframe, 6 replicates were used for chemical, microbiological and physical analyses. A summary of the procedure with the analyses performed is shown in Fig. 3.

2.4. Chemical analysis

Two mL of the reaction medium were collected after 2, 3, 4, 5, 10 and 15 weeks and filtered at 0.2 µm. The ammonium ions concentration was measured using the Hach LCK 304 kit according to the manufacturer's instructions. Dissolved inorganic carbon was determined using a TOC analyzer (TOC-SHIMADZU Combustion). The concentrations of acetic, butyric and propionic acids were determined by high performance liquid chromatography (Thermo Scientific, Accela system, Rezex ROA H^+ 8% column, H_2SO_4 eluent 10 mM, flow rate 170 µL.min ^{1}) [6]. The pH was measured with a Sentix Sur (WTX) surface pH electrode, due to low sample volumes.

2.5. Analysis of microbial populations

The microbial biofilm on the surface of the cementitious paste pellets was sampled in order to gain access to two layers in the thickness of the biofilm. The microbial biomass of the "loosely attached" biofilm was removed from a colonized cement paste pellet by an immersion in Phosphate Buffer (PBS, 0.1 M, pH 7.4) for 15 min. The microbial biomass of the "strongly attached" microbial biomass was removed by 3 min of sonication treatment immersed in PBS (material used: FB 15061 Fisher Scientific, ultrasonic frequency, 37 Hz). For these two types of samples, the entire phosphate buffer containing the detached biomass was then recovered and pelleted for 1 min at 13,000 g. The biofilm removal treatments were not carried out successively. Therefore, the "strongly attached" biomass must also contain the "loosely attached" biomass. The use of the 6 replicates for each sampling period made it possible to carry out each stalling treatment in triplicate. For each sampling period, 2 mL of the reaction medium was also taken to access the biomass suspended in the reaction medium and pelleted for 1 min at 13,000 g. DNA extraction was performed on all three types of samples (biomass suspended in the reaction medium, "strongly attached" biomass and "weakly attached" biomass) using the Qiagen DNeasy DNA extraction kit, according to the protocol described by the manufacturer. Sequencing of the 16S RNA was performed using the 515F and 806R primers targeting both bacteria and archaea. Sequencing was carried out by RTlab (USA). Statistical processing of these data was performed with the R software (R Core Team, (2017)).

An abundance map was produced with the "Marray" package using Ward's classification method and a calculation of distance based on the correlation between the standardized abundance scores of the OTUs (Operational Taxonomic Units). Principal component analyses (PCA) were carried out with the "Factominer" and "Factoextra" packages. Three diversity indices were calculated from the sequencing data: Simpson's index, Shannon-Wiener's index (H') and Pielou's equitability index.

Table 1
Synthetic bio-waste composition.

Organic fractions	Mass %
Water	75.6
Potatoes	8.1
Tomatoes	3.4
Ground meat (beef)	8.1
Shortbread cookies	4.1
Milk powder	0.7

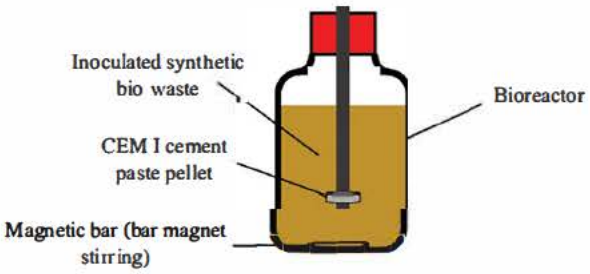

Fig. 2. Experimental laboratory anaerobic bioreactor device reproducing the anaerobic digestion conditions of biogas-producing digesters.

2.6. Physical analysis

Part of the cementitious paste pellet was impregnated, under vacuum, with epoxy resin (Araldite 2020) in circular molds (diameter 2.6 cm). After curing of the epoxy resin, the surface was polished under water using several polishing discs with decreasing particle sizes of: 200, 68, 27 and 15 µm. Finer polishing was then done using diamond polishing discs with decreasing particle sizes (6, 3 and 1 µm). The samples were coated with carbon before being observed with a scanning electron microscope (Hitachi S-4300 SE/N) coupled with an EDS (Thermo Scientific Ultradry) detector operating at 15 kV. One sample was analyzed for each duration tested.

Mineralogical analyses were also carried out in X-ray diffraction using a D8 advance system (Bruker), with a cobalt anticathode, a voltage of 35 kV and an electrical current of 40 mA. Flat rectangular areas of about 1 cm^2 were analyzed, taking care to avoid the edges of the sample, which was also subject to alteration in the radial direction. The first analysis was performed on the surface of the sample and the following ones after abrasion of the sample to the desired depth using 500 and 1200 µm abrasive discs. The abraded depth was measured using a micrometer (KETOTEK). A sample was then analyzed for each maturity.

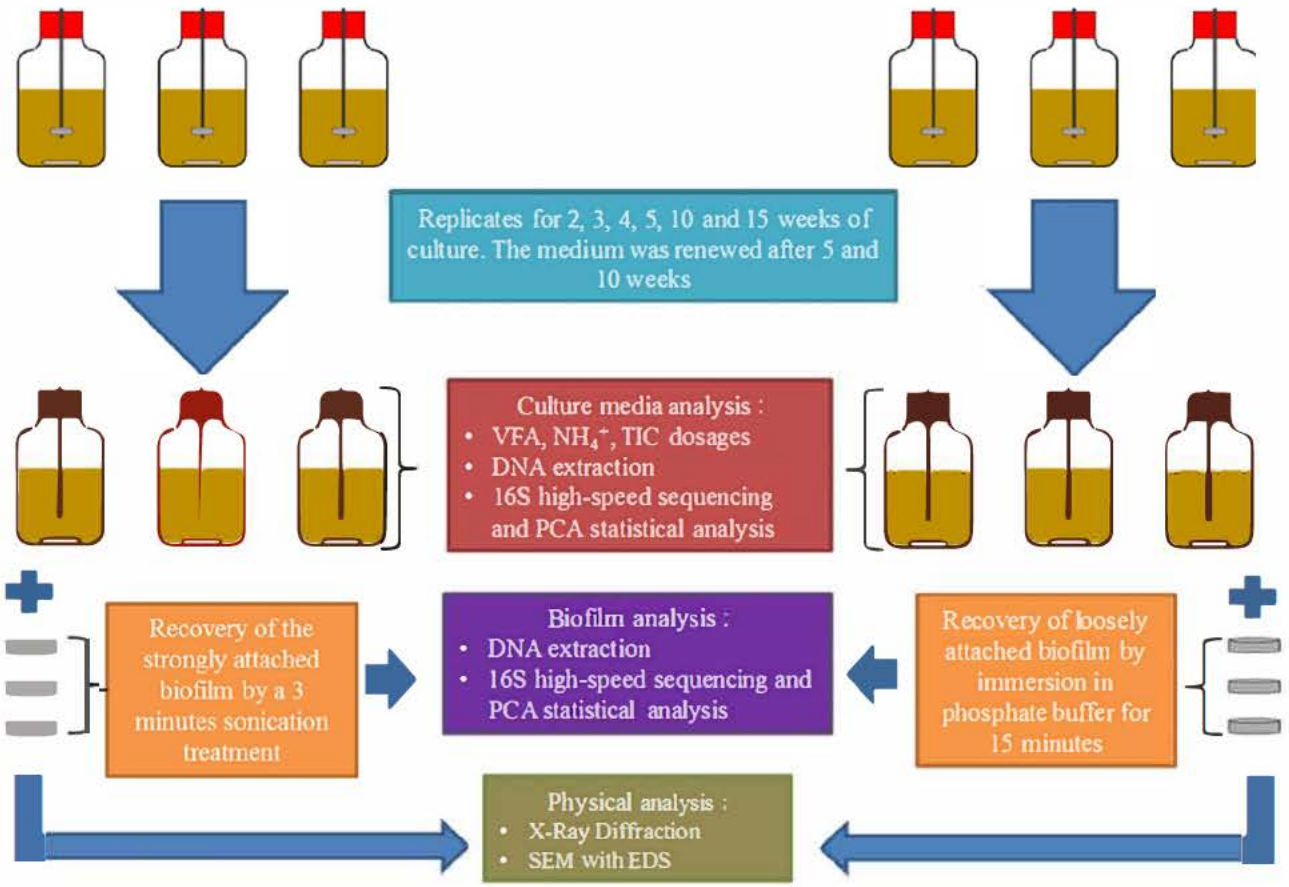

Fig. 3. Synthesis of the operating procedure and the analyses carried out.

Dehydrated biofilm samples were observed under SEM according to the protocol of Marchand et al. [21] and Voegel et al. [35] after gold coating (Field Emission Gun, JEOL 7100F TTLS).

3. Results

CEM I pastes were exposed for increasing periods of time (2, 3, 4, 5,

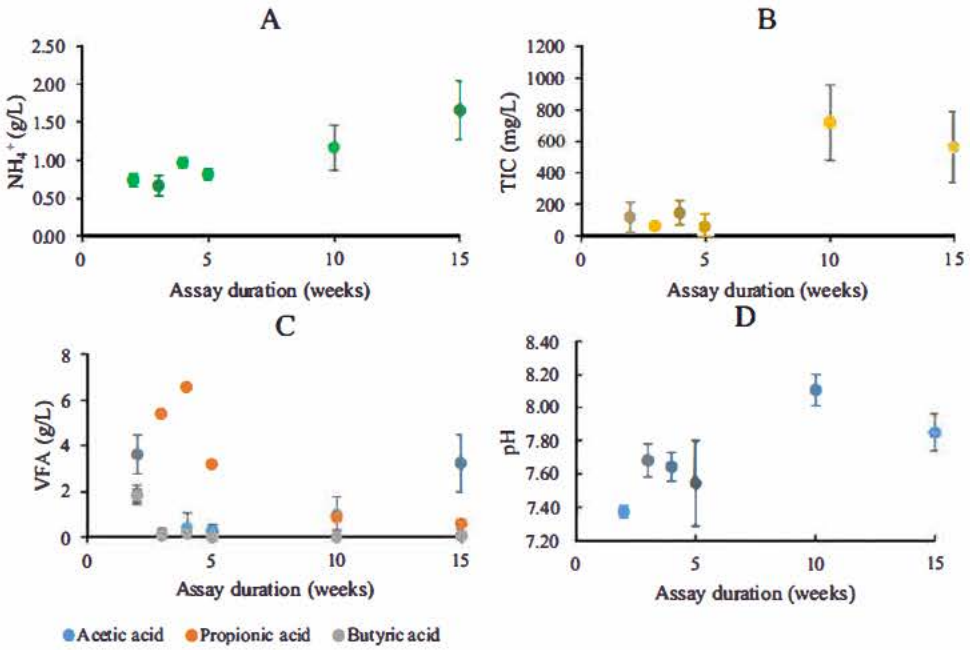

Fig. 4. Evolution of the concentrations of aggressive compounds (ammonium ions (A), total inorganic carbon (B), VFA (C) and pH (D) during 2–15 weeks of anaerobic digestion tests. The values shown are the averages of the 6 samples collected from the liquid medium, the error bars correspond to the standard deviations associated with these values.

10 and 15 weeks) and the biodeterioration in an anaerobic digestion environment was followed by a method developed previously by Voegel et al. [36]. The evolution of the concentrations of aggressive agents (VFA, NH_4^+ and CO_2) was measured in the liquid phase. Then, the biodeterioration of the cementitious materials was qualitatively and quantitatively evaluated by electron microscopy analysis of the polished surfaces of pellet cross sections and also by X-ray diffraction. Finally, the biofilms established on the surface of the cementitious materials were removed using two protocols developed to recover, on the one hand, the part of the biofilm loosely attached to the cementitious surface and, on the other, the part of both the biofilm strongly and loosely anchored on the surface, i.e. the basal layers of the biofilm. The DNA of these samples, and of samples taken from the reaction medium, was extracted and sequenced. For each sampling time, among the 6 replicates, 3 replicates were used to recover the weakly attached biofilm and the 3 others for the strongly attached one. At the end of each test period, there were therefore 6 samples of reaction medium and 3 of each biofilm layer.

3.1. Evolution of aggressive compounds during the biodeterioration test

Fig. 4 shows the evolution of ammonium ions, total inorganic carbon, pH and organic acid (acetic, butyric and propionic acids) concentrations in the reaction medium during the entire duration of the biodeterioration assay. Ammonium ions were produced throughout the biodeterioration test (Fig. 4A). After slight production during the first two weeks of the first cycle, resulting in a concentration of about 0.7 g/L, an increase of about 0.1–0.2 g/L was observed during the second half of the first cycle, with the amount of ammonium ions produced increasing in subsequent cycles. During the second cycle, the average concentration increased by 0.35 g/L and, for the third cycle, the increase was even more significant, with a production of 0.5 g/L of ammonium ions resulting in an ammonium ions concentration in the medium of 1.67 g/L. Since the production of ammonium ions comes from the fermentation of amino acids during acidogenesis [24], the relatively low production observed in the second half of the first cycle can be explained by the fact that the majority of ammonium ions were probably produced during the first two weeks of the cycle. The same phenomenon is found for the production of VFA, the concentrations of which are shown in Fig. 4C. During the first cycle, the production of acetic and butyric acids took place during the first two weeks before their almost complete consumption by the third week. For propionic acid, production took place during the first 4 weeks, up to a value of 6.6 g/L before its partial consumption during the 5th week, resulting in a concentration of 3.2 g/L. For the following cycles, propionic and butyric acids were completely consumed and the acetic acid produced was only partially consumed, its concentration at the end of the second cycle being a little less than 1 g/L, and 3.2 g/L at the end of the third cycle. For the first cycle, the evolution of butyric and acetic acids concentrations corresponds to what could be expected from an anaerobic digestion process, i.e., hydrolysis followed by acidogenesis in the first two steps of the anaerobic digestion process, during the first ten days. At the end of these steps, acetogenesis began and acetate accumulated in the system.

Finally, methanogenesis began and consumed the acetic acid produced. Its consumption occurred between the second and third weeks of the first cycle, so methanogenesis took place at this time. For propionic acid, on the other hand, consumption started late, from the fourth week, which resulted in the accumulation of propionic acid in the medium before four weeks. Nevertheless, propionic acid was then completely consumed by the end of the next two cycles, indicating that the accumulation could not prevent the smooth running of the anaerobic digestion process. The evolution of the concentration of acetic acid seems to indicate a slight accumulation of acetic acid at the end of the last two cycles, especially at the end of the third cycle, where 5 out of the 6 samples had more than 2.7 g/L of acetic acid, while the last one had 1.5 g/L. The total inorganic carbon concentration varied slightly between 30 and 130 mg/L during the second half of the first cycle and reached about 720 and 560 mg/L at the end of the second and third cycles, respectively (Fig. 4B). The average pH values, shown in Fig. 4d, are between 7.4 and 8.1 for all samples. The lowest values correspond to the first cycle where the VFA concentrations were the highest, whereas at the opposite the highest pH value was measured at the end of the second cycle when the VFA concentration was the lowest. This range of pH is really classical in a normally operating anaerobic digestion tank [22]. These concentrations are below the concentrations usually encountered in anaerobic digestion, with bicarbonate concentrations (about 95% of the total inorganic carbon under anaerobic digestion conditions) that are rather around 2–5 g/L [11]. The concentrations observed during the first cycle were of the same order as those observed by Voegel et al. [37], who worked with the same reaction medium, and those of the second cycle could indicate an evolution of the system towards stabilization around the values usually encountered in anaerobic digestion [17,34].

3.2. Characterization of the microstructure, chemical composition and deteriorated depth of cement paste samples

Table 2 shows the evolution of the total biodeteriorated depth with the duration of the associated biodeterioration assay. This depth varies from about 540 μm at 2 weeks to almost 820 μm after 15 weeks of immersion. After 4 weeks of immersion, we were unable to measure the total deterioration depth accurately, because of a variation of the total deteriorated depth among the samples. Fig. 5 shows the pictures used to determine this value for the 5 weeks immersion time.

Fig. 5A shows the biodeterioration profile (from the surface to the core) of a cement paste sample immersed in the bio-waste for 5 weeks. The combination of the SEM micrograph (Fig. 5A) and the mapping representing the distribution of the elements calcium and silicon (Fig. 5B and C) was used to identify several areas. Four zones can be distinguished: a first zone, darker than the others, of about 100 μm, a second zone, of about 20–30 μm, identified around a crack parallel to the surface of the pellet, a third zone, with a thickness of about 400 μm, and a fourth zone constituting the core of the sample and corresponding to the non altered paste. Fig. 5B and C show the distribution of the elements calcium and silicon in the sample respectively. This mapping allows decalcified zone 1, slightly enriched in silicon, and zone 2, highly enriched in calcium, to be easily identified. The transition from Zone 3 to the healthy paste was also easily identified by a slight increase in the amount of calcium.

Fig. 6 shows the evolution of the relative proportions of calcium (Ca), silicon (Si) and aluminium (Al) from the outer surface to 900 μm in the depth of the biodeteriorated cement paste sample for all exposure times tested. Zone 1, the closest to the cement surface, has a low proportion of Ca, about 10%, a high proportion of Si, about 30%, and a high proportion of Al, about 7%, compared to the other zones. Zone 2 has a particularly specific composition: the proportion of Ca is significantly higher there than in any other zone, up to 70–76% in the two samples immersed for 3 and 4 weeks, and a lower proportion of Al and Si, respectively of the order of 1% and 5%. The presence of phosphorus was also observed in the first two zones (data not shown). Zone 3 is close to that corresponding to the sound paste, only a very slight divergence in composition being detected - between 20% and 30% Ca, about 10% Si and 1.5–2% Al on average. The Ca, Si and Al composition is therefore different for each zone. Zones 1, 2 and 3 are present at each exposure time tested with a relatively comparable overall composition. Fig. S1 (Supplementary data) shows the mineralogical composition of the different zones for a cementitious paste immersed in the reaction

Table 2
Evolution of the total biodeteriorated depth according to the immersion time.

Exposure time (weeks)	2	3	4	5	10	15
Total biodeteriorated depth (μm)	542	569	–	622	756	817

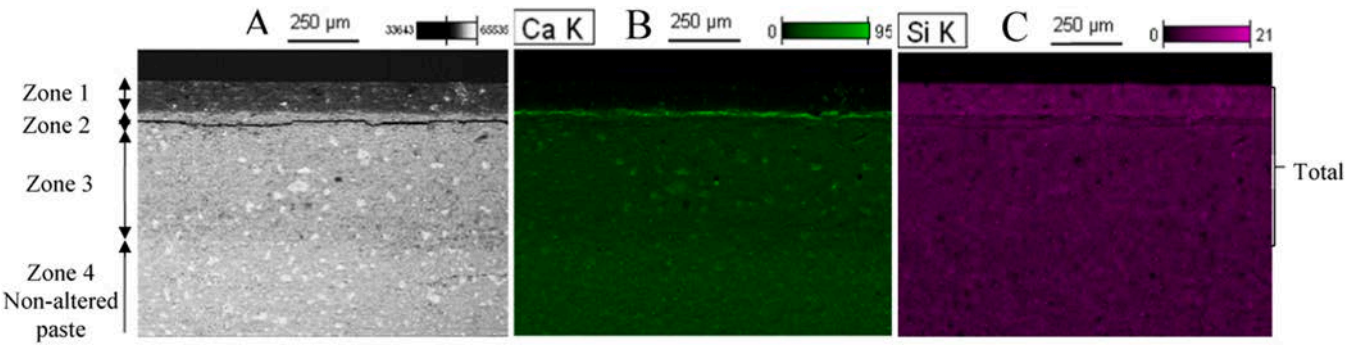

Fig. 5. Distribution of the biodeteriorated zones (A) and mapping of the distribution of the elements calcium (B) and silicon (C) according to the depth of the cement paste sample immersed for 5 weeks in the methanation reaction medium.

medium after 3 weeks. As the surface of the pellet showed calcite precipitates (confirmed by DRX), these were removed. Zone 1 is characterized by an intense signal corresponding to calcite and a lighter signal corresponding to Brownmillerite ($Ca_2(Al,Fe)_2O_5$). Zone 2 has a mineralogical composition close to that of Zone 1 with the appearance of vaterite (a less stable form than calcite). Zone 3 contains mostly ettringite and calcium silicate ($C_3S_)$ with the presence of larnite (C_2S). Finally, the sound paste has these three phases, to which can be added portlandite, a phase mostly present in this zone. The mineralogical composition of the different zones is identical regardless of the duration of exposure to the anaerobic digestion reaction medium.

3.3. *Evolution of microbial populations during the biodeterioration test*

The results of the 16S high throughput sequencing of the DNA extracts from the liquid samples and the biofilm removed from the cement paste pellets after 2, 3, 4, 5, 10 and 15 weeks of incubation are summarized in Fig. 7. The groupings by similarity allow two large groups of samples to be distinguished, A and B, associated with two clusters, of OTUs I and II, respectively. Group A contains all samples associated with the second and third cycle separated into two subgroups according to the cycle. Group B concerns the integrality of the samples associated with the 2-, 3-, 4- and 5-week biodeterioration tests. The two-week-old samples are pooled in a separate subgroup and all other Group B samples are pooled in three other subgroups.

The grouping of the samples according to their similarity to each other corresponds mainly to the duration of their immersion in the biowaste. It also means that the disparity between samples depends more on their immersion time than on the type of sample (liquid, loosely or strongly attached biofilm). It therefore appears that the main factor of disparity within our samples was their duration of contact with synthetic biowaste. Concerning the first two groups, group I contains OTUs that are mostly found in samples from group B cultures, i.e. 5 weeks or less. For group II, even though these OTUs are mostly found in samples from cultures of 2 or 3 cycles, some OTUs of this group are present in samples corresponding to 3, 4 and/or 5 weeks. Among these is the OTU corresponding to *Methanoculleus chikugoensis*, a hydrogenotrophic methanogen, present in all samples except those aged 2 weeks. Similarly, several group II OTUs, corresponding to species of the genus are present in 4- and 5-week old cultures. Both groups contain methanogenic archaea. For group I, these are species of the genera *Methanosarcina*, *Methanosphaera* and *Methanobrevibacter,* i.e. acetoclastic and hydrogenotrophic methanogens. For group II, they are mainly archaea of the genera *Methanoculleus* and *Methanobacterium* hydrogenotrophs. It therefore appears that, over time, the populations encountered in the bio-waste inoculated with activated sludge evolve into two distinct microbial communities. In particular, and even though a *Methanosarcina* species of group I is present in several samples aged 2 cycles (i.e. 10 weeks), acetoclastic methanogens are much less present in cultures of more than 1 cycle and, moreover, the *Methanosarcina* genus is almost absent in samples of 3 cycles.

The evolution of the mean diversity within the sequenced microbial communities according to the duration of the biodeterioration test was calculated using the Simpson diversity index, the Shannon-Wiener index and the Pielou equitability index and is summarized in Table 3. The evolution of these three indices according to the duration of the biodeterioration test is relatively identical for all of them: the three linear correlation coefficients associated with the three indices are all greater than 0.95. This shows a slight increase in diversity between two and three weeks and then a stabilization throughout the rest of the first cycle. All three indices show a decrease in diversity after the second cycle and, to a lesser extent, after the third cycle. We can conclude that the community in Group II is less diverse than that in Group I.

Sequencing data from samples taken from the reaction medium and biofilm surface after 2, 3, 4, 5, 10 and 15 weeks of biodeterioration were statistically analyzed by principal component analysis (PCA), the results of which are presented in Fig. 8. It shows the distribution of the individuals, i.e. the different samples sequenced, according to the first two principal components (PC), defined by the analysis. The first PC, on the X-axis, explains 38% of the variance. The distribution of the individuals according to this PC is done according to the duration of the biodeterioration assay. The samples are ordered from left to right, from the least aged, 2 weeks of immersion in the biodeterioration liquid, to the most aged, 3 cycles (i.e. 15 weeks). This corroborates the fact that the assay duration is the most important factor of variability acting on the samples. The Y-axis explains 12% of the variance. It also appears that the samples from the biofilm, in red, are generally located above the samples from the liquid medium in black. However, there is no noticeable position difference among samples according to the biofilm removal treatment. While the first PC is highly correlated with the duration of the tests, the second PC is related to the type of samples used, depending on whether the biomass is sessile or planktonic.

Fig. 8B presents the variables, i.e. the sequenced OTUs and their best correspondence in the database, related to the first two PCs and well represented ($Cos^2 > 0.6$) by them.

The variables related to the first PC will be considered as related to the duration of the biodeterioration assay. The variables correlated with the second PC will be considered weakly correlated with the sample type, planktonic or sessile. The variables shown to have a high positive correlation with the first PC in Fig. 8B correspond to species of the genera *Clostridium*, *Methanoculeus*, *Symbiobacterium* and *bacteroides*. Among them, the species *Clostridium butyricum*, *Clostridium paratrificum* and *Methanoculleus chikugoensis* could be identified. The OTUs identified as negatively correlated with the first PC belong to the genera *Tannerella*, *Methanosphaera*, *Clostridium, Streptococcus* and *Selenomonas*. For these genera, the species that have been identified are: *Selenomonas bovis*, *Bifidobacterium adolescentis*, *Lactobacillus mucosae*, *Megasphaera elsdenii* and *Methanobrevibacter smithii*. For methanogenic populations,

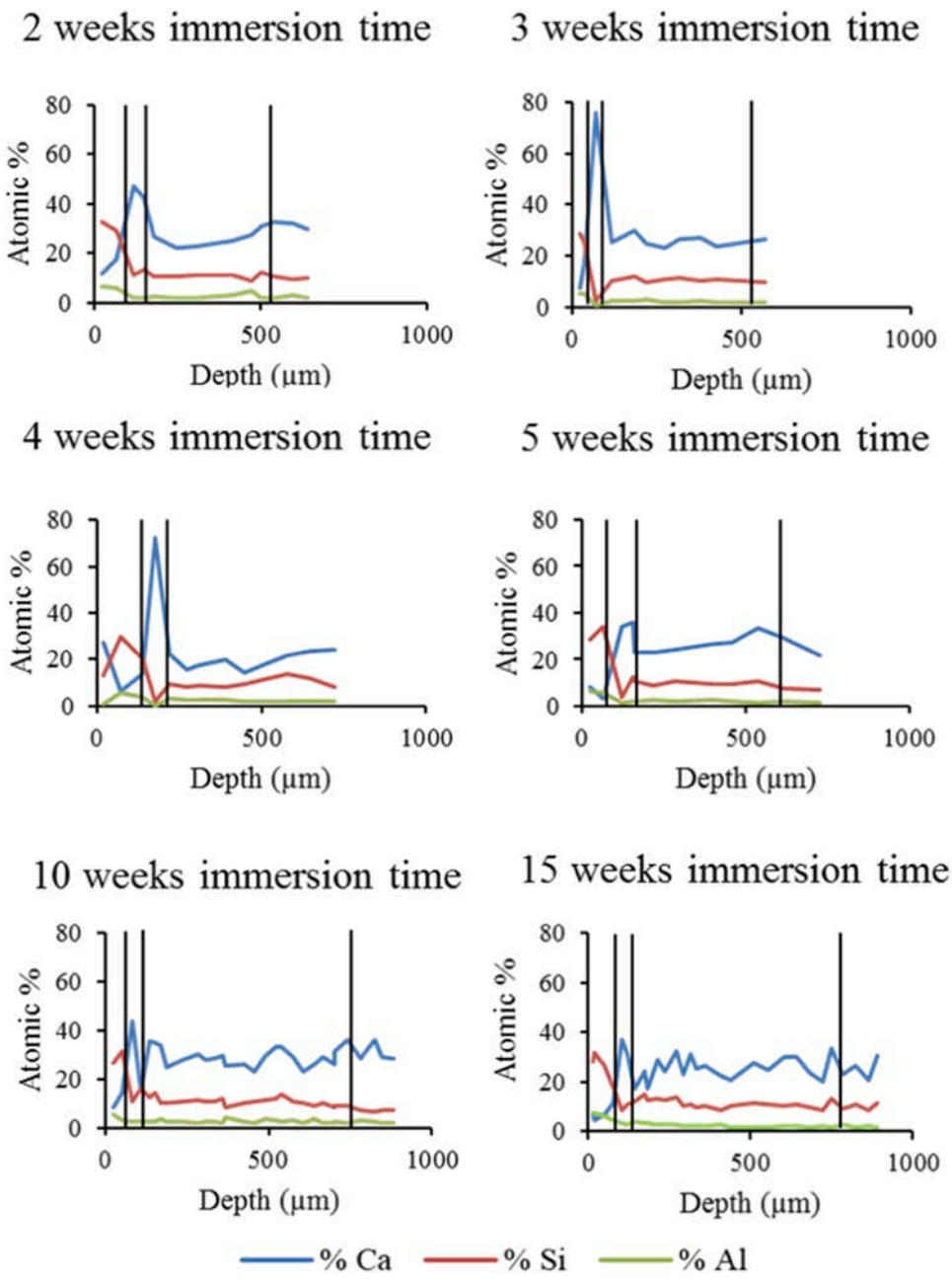

Fig. 6. Evolution of the relative atomic proportions of calcium, silicon and aluminum in the depth of cement pastes immersed in the medium for 2–15 weeks. The black vertical bars correspond to the boundaries between the 4 zones identified. For the sample submerged for 4 weeks, the boundary between the third and fourth zones could not be measured with satisfactory accuracy and is therefore not shown.

the results are in agreement with those presented in Fig. 7: the hydrogenotrophic genera *Methanoculleus* are associated with longer exposition time and the genera *Methanobrevibacter* and *Methanosphaera* also hydrogenotrophic with shorter exposition time. The other genera or species correlated positively or negatively with the first PC correspond to populations involved in the first three stages of anaerobic digestion (hydrolysis, acidogenesis and acetogenesis) and mostly in the first two. For OTUs related to the second PC, they are less numerous. Among the species and genera identified and positively correlated with the second PC are 3 OTUs associated with the genus *Clostridium* and one species of the genus *Tannerella,* which are acidogenic [18,25]. For the OTUs identified as negatively correlated, only the species *Methanoculleus chikugoensis* is present. As the second PC is correlated with the lifestyle of the sample collected, planktonic or sessile, it appears that the planktonic lifestyle contains more OTUs associated with methanogenic bacteria, while the sessile lifestyle contains several acidogenic bacteria.

4. Discussion

4.1. Kinetics of the cement matrix biodeterioration

Concerning biodeterioration, the phenomena observed here are identical to those highlighted by previous work on the same systems, namely a leaching of calcium and, to a lesser extent, silicon from the dissolution of the hydrated phases of the cement paste [35–37]. This phenomenon is directly related to the diffusion of calcium, according to Fick's law, within the cementitious paste. Consequently, the depth of the deteriorated cementitious paste is assumed to grow according to a linear relationship with the square root of time [7]. The evolution of the total biodeteriorated depth with time is shown in Fig. 9, where it is visible

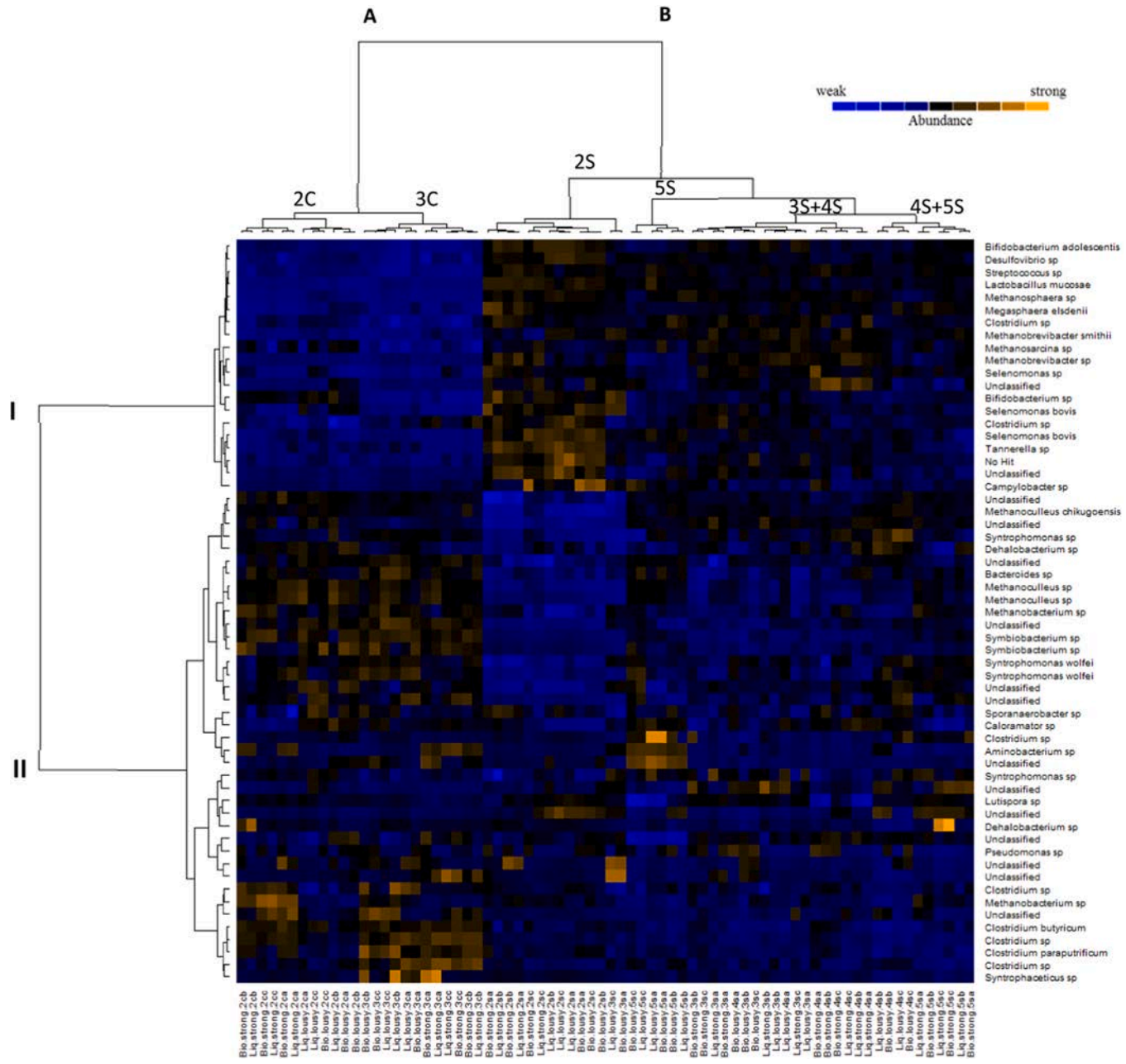

Fig. 7. Heatmap with color scale of the 16S high throughput sequencing of DNA extracts from samples of reaction medium (liquid) and biofilm taken from the surface of cement paste samples after 2–15 weeks of biodeterioration. Y-axis: OTU with an abundance of at least 2% in a sample. X-axis: samples. "bio" and "liq" correspond to the type of associated sample, respectively biofilm and liquid. "lousy" and "strong" correspond to the biofilm removal treatment, respectively immersion in buffer and sonication treatment. 2s, 3s, 4s, 5s, 2c and 3c indicate the duration of the biodeterioration test, i.e. 2,3,4 and 5 weeks of the first cycle and then the 2nd and 3rd cycle cultures, i.e. 10 and 15 weeks. Finally a, b and c correspond to the triplicates.

Table 3
Averages of Simpson diversity index, Shannon-Wiener diversity index and Pielou equitability within all samples by duration of biodeterioration test.

		Duration of the biodeterioration test (weeks)					
		2	3	4	5	10	15
Indices	Simpson	0.87	0.91	0.91	0.91	0.82	0.78
	Shannon	4.79	4.99	5.13	5.01	3.90	3.89
	Pielou	0.54	0.56	0.56	0.56	0.47	0.47

that this evolution has, as expected, a linear relationship with the square root of time, with a squared coefficient of determination of 0.99. In the case of unidirectional diffusion with a constant diffusion coeficient, the relationship between the diffusion front, Δx, the diffusion coefficient, D, and time corresponds to Eq. (1).

$$\Delta x \quad \sqrt{2Dt} \tag{1}$$

The value of the slope observed in Fig. 9 can be used to calculate the diffusion coefficient, which is therefore: $1.13.10^{-14}$ m^2/s. Voegel et al. [37] worked with CEM I Portland cement pastes exposed to the same aggressive conditions and obtained total deteriorated depths of 650 μm and 750 μm after 5 and 10 weeks, respectively. Assuming a type of kinetics similar to that observed for our samples, this would correspond to a diffusion coefficient of $1.92.10^{-14}$ m^2/s, a value very close to the one we obtained. To position our results in another type of aggressive environment, Goni et al. [9] calculated the calcium diffusion coefficient

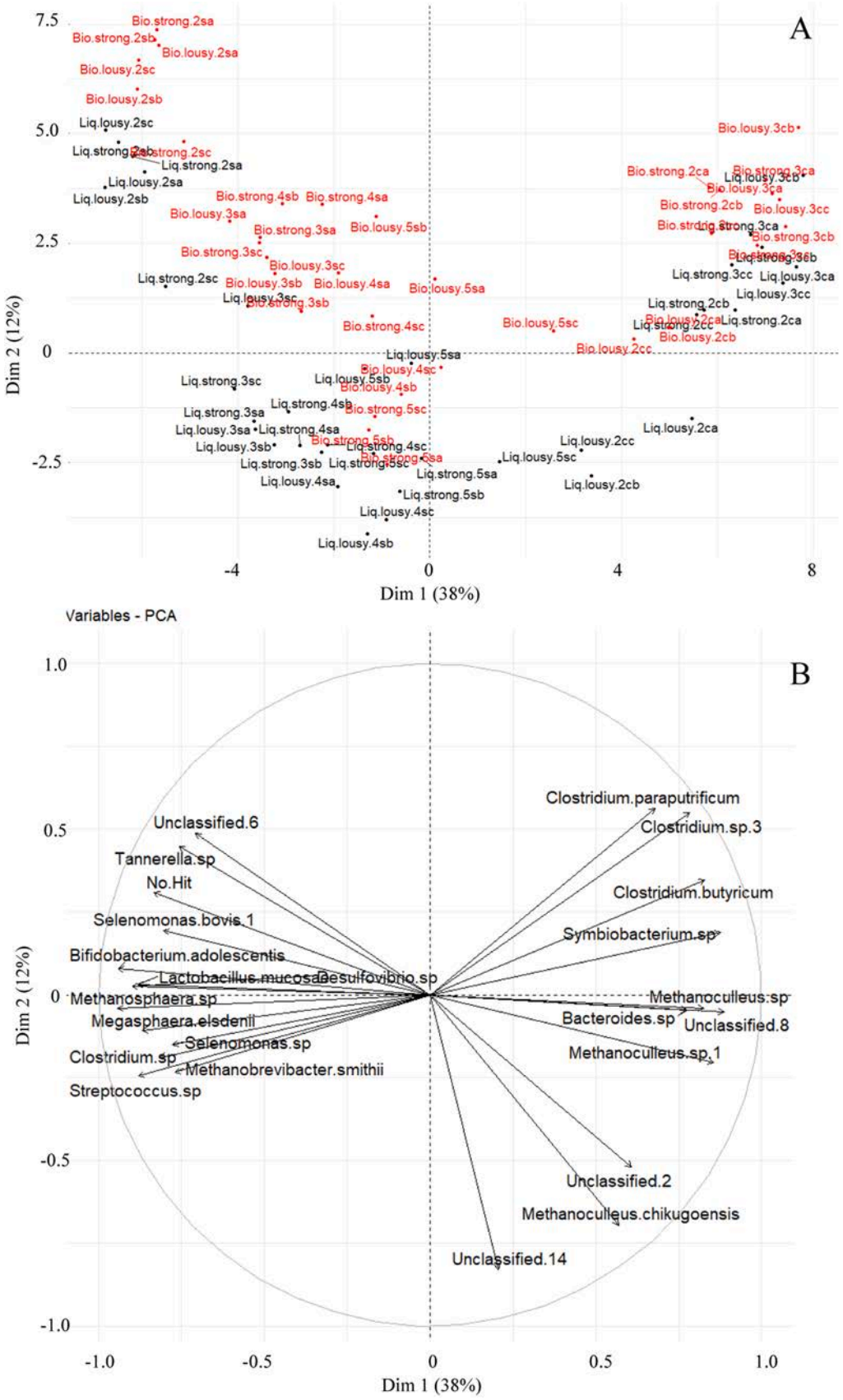

Fig. 8. PCA of microbial communities in anaerobic digestion samples of bio-waste. The PCA was performed with the sequencing data of the samples from the liquid (in black) as individuals. The data from the stalled biofilm samples (in red) were added as illustrative data. Part A represents the individuals according to the first two main components. Part B shows the variables with $\cos^2 > 0.6$. Concerning the names of the individuals, "lousy" and "strong" correspond to the biofilm removal treatment undergone by the cementitious paste pellet, respectively immersion in buffer and sonication treatment. 2s, 3s, 4s, 5s, 2c and 3c indicate the duration of the culture, i.e. the 2, 3, 4 and 5 weeks of the first cycle and then the cultures of 2 and 3 cycles. a, b and c correspond to the triplicates. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

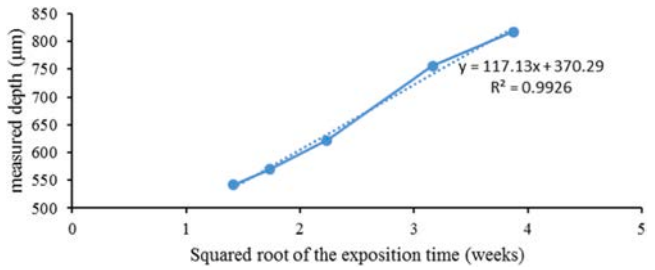

Fig. 9. Evolution of the total biodeteriorated depth with the squared root of the exposure time.

in the same way in Portland cement pastes CEM I exposed to a synthetic reaction medium simulating seawater aggression for 90 days. They obtained diffusion coefficients of the order of 1.10^{-14}~1.10^{-15} m^2/s (two distinct velocities were found according to whether the data corresponded to the first 7 days or the following days). These values are also close to those obtained and are associated with an environment considered aggressive for concrete, confirming the aggressiveness of the anaerobic digestion media towards cement.

Fick's law is governed by differences in the concentration of the compound that diffuses between two points in a solution to compensate for the concentration gradient. Therefore, if this compound, in our case calcium, is consumed by a chemical or biological reaction at one point (i.e. locally in the solution) it will accelerate the diffusion phenomenon and thus the kinetics of biodeterioration. The production of VFA, ammonium ions and CO_2, which react with calcium to form, among other things, calcium salts and calcium carbonates, such as calcite and vaterite, observed on the surface of CEM I pastes and in zones 1 and 2 (Fig. S1) can increase this kinetics. Thus, the presence of bacteria producing VFA, NH_4^+ and/or CO_2 directly in contact with the cementitious paste is likely to amplify the calcium leaching from the cementitious paste and thus its biodeterioration kinetics. We recall that failure to detect the presence of a compound in the reaction medium does not indicate the absence of its production in the system. In anaerobic digestion, VFAs are simultaneously produced (steps 2, 3 and 4 in Fig. 1) and consumed. It is therefore interesting to look at the distribution of these microbial populations within the system.

4.2. Selection of microbial populations according to lifestyle, planktonic or sessile

The microbial communities associated with sessile or planktonic lifestyles, in the same sample, appear to be relatively close. In fact, grouping the samples by similarity does not highlight groups associated with these lifestyles (Fig. 7). Moreover, no significant difference between bacterial populations could be found between the biomass of the loosely attached biofilm and that of the strongly attached biofilm. Statistical analysis of the results also seems to partially confirm this, since the second PC, weakly correlated with the lifestyle of the cells in the sequenced sample, alone explains 12% of the variability, whereas the first PC, very strongly correlated with the duration of the biodeterioration test, explains 38% of the variability (Fig. 8). Nevertheless, statistical analysis revealed some differences between sessile and planktonic microbial communities. These differences seem to be mostly observable in the samples exposed for longer durations since, with the exception of one OTU associated with a species of the genus *Tanerella,* all OTUs correlated with the second PC are on the right side of Fig. 8B, indicating a positive correlation with the first PC and thus with the duration of the biodeterioration test. Therefore, a selection of microbial populations in relation to their sessile (biofilm) or planktonic (suspended in the liquid medium) lifestyle seems to be made progressively during the biodeterioration test. Acidogenic populations of the genus *Clostridium* would then develop preferentially in the biofilm on the CEM I cement paste, whereas methanogenic populations would be more often found in the liquid medium. The low number of OTUs correlated with the second PC (Fig. 8B) may be due to the large difference in variability explained between the two PCs; the first PC could prevail over the variables correlated with the second PC. Therefore, Table 4 groups the OTUs correlated with the second PC with a p value lower than 0.05. The value of the associated correlation coefficient, r, is also shown.

The genus *Clostridium* is clearly more present in the biofilm than in the reaction medium since 6 out of the 10 OTUs detected as having a sessile lifestyle belong to this bacterial genus containing bacteria involved in the hydrolysis and acidogenesis steps (steps 1 and 2, Fig. 1). Among the other 4 OTUs also associated with this lifestyle are another acidogen of the genus *Tanerella*, a syntrophic acetogen of the genus *Syntrophaceticus*, a hydrogenotrophic methanogen of the genus *Methanobacterium* and a bacterium of the genus *Pseudomonas* known for its ability to form pathogenic biofilms under anaerobic conditions. This genus is indeed known to achieve CO_2-producing nitrate respiration [25,28,41]. OTUs associated with the planktonic lifestyle are less numerous; only 6 have been detected. Among them, 2 methanogens have been identified: a hydrogenotrophic *Methanoculleus chikugoensis* and a simultaneously acetoclastic and hydrogenotrophic *Methanosarcina.* Three OTUs of the genus *Syntrophomonas,* known as acetogenic syntrophic bacteria, and a last bacterium of the genus *Dehalobacterium,* usually producing VFAs from dichloromethane, have also been detected [30,32]. Microorganisms responsible for the first two stages of anaerobic digestion were found mainly in the biofilm, while those responsible for the last two stages (stages 3 and 4, Fig. 1) were more often detected in the liquid medium. This selection conditioned by lifestyle seems to be completely independent of the duration of the biodeterioration test, since the two methanogens found in the liquid are of the genera *Methanoculleus* and *Methanosarcina,* which belong to groups I and II (Fig. 7). Thus, we can propose the hypothesis that the first steps of anaerobic digestion preferentially take place in the biofilm, while the last two steps would take place in the liquid medium.

4.3. Inhibition of acetoclastic methanogenic populations in favor of hydrogen methanogens

The success of an anaerobic digestion process is directly linked to the state of the methanogenic populations in the digester, as they are responsible for the last stage of anaerobic digestion, methanogenesis (stage 4, Fig. 1). Moreover, these microbial populations are among the

Table 4
OTUs correlated with the lifestyle of the sequenced sample. Only correlations of the second CP with OTUs identified with a p value <0.05 are presented. The PCA was performed in a manner strictly identical to that observed in Fig. 8.

Sessile biomass (biofilm)			Planktonic biomass (suspended in the liquid medium)		
OTU identified	r	p value	OTU identified	r	p value
Pseudomonas sp	0.67	9.58E-06	*Methanoculleus chikugoensis*	-0.70	2.39E-06
Clostridium sp.2	0.57	2.75E-04	*Syntrophomonas sp*	-0.52	1.25E-03
Clostridium paraputrificum	0.56	3.44E-04	*Syntrophomonas* sp.1	-0.45	0.01
Clostridium sp.3	0.55	5.08E-04	*Syntrophomonas wolfei*	-0.40	0.02
Syntrophaceticus sp	0.53	9.82E-04	*Methanosarcina sp*	-0.40	0.02
Tannerella sp	0.45	6.20E-03	*Dehalobacterium* sp.1	-0.39	0.02
Clostridium sp.5	0.41	0.01			
Clostridium sp.4	0.38	0.02			
Methanobacterium sp.1	0.35	0.03			
Clostridium butyricum	0.35	0.04			

most fragile and sensitive microorganisms present in the digester. Since their growth is very slow (doubling time of about 2 weeks), any inhibition of these populations can temporarily affect the methane production of an anaerobic digestion unit [16]. It should also be recalled that, of the two main metabolic pathways of methanogenesis encountered in anaerobic digestion, the acetoclastic pathway is usually responsible for 70% of methane production [33]. Therefore, the progressive disappearance of acetoclastic methanogenic populations, in our case the genera *Methanosarcina* and *Methanobrevibacter* (Fig. 7), from the end of the first cycle is an abnormal phenomenon whose origin must be discovered. Most often, an inhibition of methanogenic archaea is due to an environmental factor (unsuitable temperature, too high a concentration of an inhibitory compound.). In our case, the NH_4^+ concentrations (Fig. 4A) exceeded 1.2 g/L at the end of the second cycle and 1.5 g/L at the end of the third; which could correspond to values sufficiently high to be the origin of an inhibition. However, when the increase in ammonium ions concentration in a digester is progressive, methanogenic populations are able to adapt to it and can tolerate concentrations above several g/L without proven inhibition [23,40]. In our case, the accumulation of ammonium ions in the tank was relatively slow, of the order of 250 mg/L over 5 weeks, and it is therefore conceivable that inhibition by ammonium ions remained limited. Moreover, high concentrations of ammonium ions usually do not inhibit members of the family *Methanosarcinaceae,* such as the genus *Methanosarcina* [13]. Another cause of this inhibition could lie in the concentrations of VFA, particularly propionic acid, which exceeded 6 g/L during the first cycle. Therefore, propionic acid is known to inhibit methanogenic acetoclastic archaea and it is usually the hydrogen methanogens in syntrophic relationship with propionic acid metabolizing syntrophic bacteria that come to form the majority [10,27]. Among the populations thus selected are the genera *Syntrophomonas*, *Methanobacterium* and *Methanoculleus* found in the microbial community associated with long duration cultures (Figs. 7 and 8). The loss of diversity and the change in the microbial community from the second cycle onwards can therefore be explained as a result of inhibition due to the excessively high concentration of propionic acid. Han et al. [10] suggest that this type of inhibition is usually reversible, which seems to be confirmed by the decrease in propionic acid concentration (Fig. 4C) since it decreases to almost zero at the end of 15 weeks. However, the inhibition of acetoclastic methanogens leads to an accumulation of acetate in the medium (Fig. 4C), which could subsequently, whether the acetoclastic methanogens manage to recolonize the digester or not, be consumed or accumulate and cause further inhibition.

5. Conclusion

Laboratory tests were conducted to better understand the role of biofilm in the biodeterioration of cement materials in the context of anaerobic digestion. The composition of aggressive agents, the sessile microbial populations, with their distribution in the biofilm, and the planktonic populations, as well as the state of the microstructure of CEM I cement paste pellets immersed in a medium reproducing the anaerobic digestion conditions, were monitored over time. The phenomena evidenced are synthesized in Fig. 10. The statistical analysis of the microbial populations showed a progressive adaptation of the microbial community to an inhibition by propionic acid favoring the hydrogenotrophic pathway of methanogenesis. This type of inhibition could be detrimental to an anaerobic digestion process but is theoretically reversible, and assays over longer periods of time would confirm whether acetoclastic methanogens could reappear. It has also been shown that planktonic and sessile lifestyles favor methanogenic and acidogenic populations respectively. These results indicate a greater involvement of biofilm in the hydrolysis and acidogenesis stages, which is one element that may explain the deleterious effect of biofilm on cement matrices.

CRediT authorship contribution statement

Perez Cédric: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing - original draft, Visualization. **Lors Christine:** Conceptualization, Methodology, Validation, Investigation, Writing - original draft, Writing - review & editing, Supervision, Project administration, Funding acquisition. **Floquet Pascal:** Software, Formal analysis, Writing - original draft, Writing - review & editing, Visualization. **Erable Benjamin:** Conceptualization, Methodology, Validation, Writing - original draft, Writing - review & editing, Supervision, Project administration, Funding acquisition.

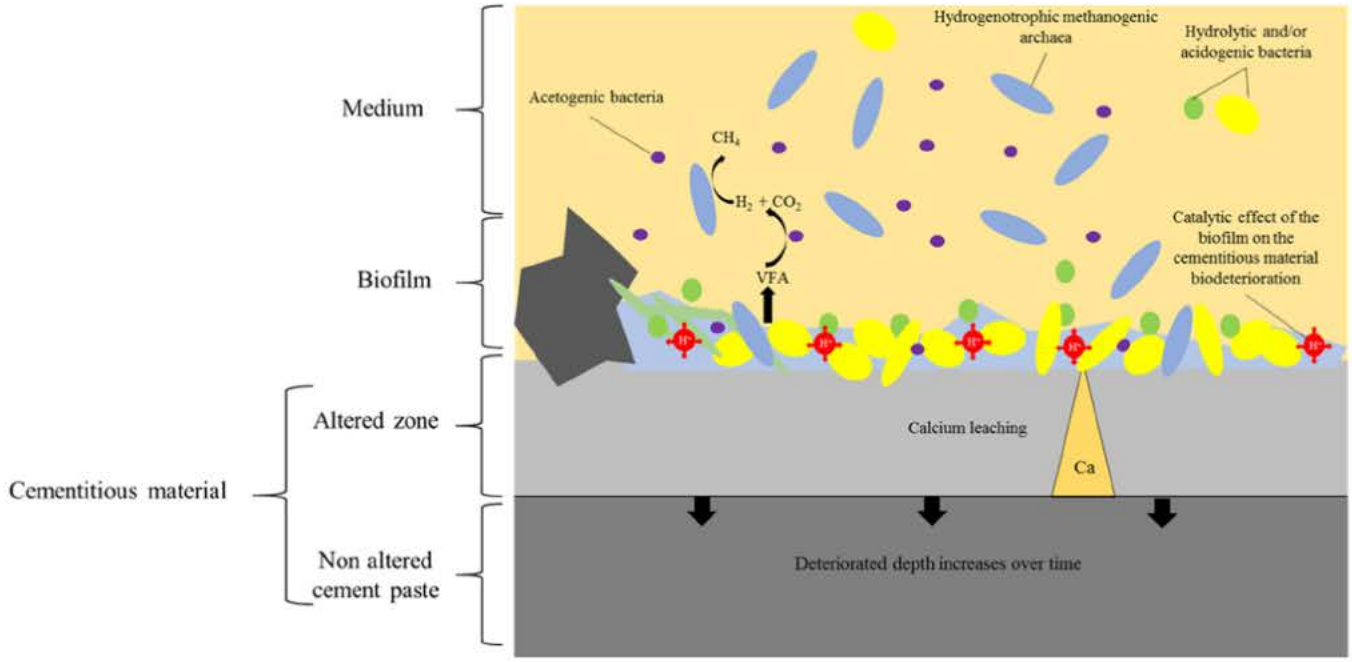

Fig. 10. Synthesis of the phenomena of interactions between the cement matrix and the biofilm under methanization conditions.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jece.2021.105334.

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