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# Biodegradation of polyvinyl chloride plastic films by enriched anaerobic marine consortia

**Lucia Giacomucci, Noura Raddadi*, Michelina Soccio, Nadia Lotti and Fabio Fava**

Department of Civil, Chemical, Environmental and Materials Engineering (DICAM), University of Bologna, Italy.

* For correspondence: Noura Raddadi, Department of Civil, Chemical, Environmental and Materials Engineering (DICAM), University of Bologna, Italy, via Terracini 28, 40131 Bologna, Italy. Phone: +390512090358, Fax: +390512090322 Email: noura.raddadi@unibo.it.

**Abstract**

Plastics remarkably contribute to marine litter, which is raising serious concerns. Currently, little is known about the fate of most plastics entering the marine environment and their potential biodegradation rate and extent under anoxic conditions.

In this work, biodegradation of polyvinyl chloride (PVC) films by consortia enriched from marine samples (litter and water) was evaluated in anaerobic microcosms. After 7 months, three microcosms showed dense biofilms on plastic surfaces, gravimetric weight losses up to 11.7±0.6%, marked decreases in thermal stability and average molecular weight of the polymer, suggesting microbial attack towards polymer chains. After 24 months, further three consortia showed the same abilities. Microbial communities analyzed at month 24 included taxa closely related to those previously reported as halogenated organic compounds degraders. The study is the first report on PVC biodegradation by marine anaerobic microbes and provides insights on potential biodegradation of the plastic film introduced into the sea by native microbes.

**Keywords**: polyvinyl chloride, plastics colonization, anaerobic marine environment, biodegradation, microbial consortia, microcosms, community composition, dehalogenation.

## 1. Introduction

In 2018, global plastic production was 359 million tons, 61.8 of which were produced in Europe (PlasticsEurope, 2019). Over one third of plastics is used to produce disposable products, such as packaging, disposable bags, etc., with a lifespan of 3 years (Gewert *et al.*, 2015; PlasticsEurope, 2019; Shah *et al.*, 2008; Wang *et al.*, 2016). From 2006, the amount of plastics wastes that is disposed of in landfill decreased of about 44%, and in 2016 for the first time, recycling overcome landfilling in the EU countries (PlasticsEurope, 2019). Plastic wastes are also widespread in the marine environment where they were found to be the largest debris contaminating marine habitats, with an estimation of more than 250,000 tons (Avio *et al.*, 2017; Oberbeckmann *et al.*, 2016; Wang *et al.*, 2016; Zettler *et al.*, 2013); and between 1.000 and 3.000 tons of floating plastics have been reported in Mediterranean Sea (Avio *et al.*, 2017). This plastic contamination is generated by direct plastic (macro and micro) transfer into aquatic systems or derived from physical/mechanical degradation of macroplastics in landfill that produces microplastics, which are transferred through leachate to rivers and finally to seas and oceans (Avio *et al.*, 2017; Galloway *et al.*, 2017). Further, additives present in plastics formulations are normally not covalently bonded to the polymer chains and could therefore leach out from plastics and enter the marine environment (Avio *et al.*, 2017; Gewert *et al.*, 2015; Harrison *et al.*, 2014). Plastics entering the marine environment could also absorb persistent organic pollutants (POPs) due to the hydrophobic characteristics of these compounds or be a vector agent for the spreading of harmful organisms inhabiting plastic surfaces (Avio *et al.*, 2017; Harrison *et al.*, 2014; Oberbeckmann *et al.*, 2016; Wang *et al.*, 2016; Zettler *et al.*, 2013).

Among petroleum-based plastics, the PVC is the third one in terms of European plastic demand (5 million tons in 2018) (PlasticsEurope, 2019). PVC plastics have a wide range of applications, such as food packaging, electronics, coatings, medical devices etc., due to low cost, long term stability and mechanical properties depending on quantity and quality of plasticizers added (Bueno-

Ferrer *et al.*, 2010; Glas *et al.*, 2014; Reddy *et al.*, 2010). The environmental concern about PVC plastics is due to their high contents of chlorine and additives required for their processing. Additives and stabilizers, e.g. heavy metals or phthalates, provide the necessary physical/chemical and stability properties to the final products but have hazardous characteristics against humans (Glas *et al.*, 2014). Chlorine atoms could produce environmentally harmful chlorinated compounds, HCl or chlorinated dioxins, when disposed of in landfills or incinerated for energy recovery (Glas *et al.*, 2014; Reddy *et al.*, 2010).

In the last years, there has been an increasing interest in the evaluation of biodegradation of petroleum-based plastics (Ali *et al.*, 2014; Shah *et al.*, 2014; Yang *et al.*, 2015; Tribedi and Dey, 2017; Peixoto *et al.*, 2017; Giacomucci *et al.*, 2019; Raddadi and Fava, 2019) with the perspective to develop innovative strategies for mitigating their environmental impact. Considering their hydrophobic nature and the very low bioavailability, the formation of biofilm on the polymer/plastic surface is a critical step necessary for the biodegradation (Das *et al.*, 2012; Reisser *et al.*, 2014). To date, few PVC polymer and plastic biodegradation studies have been reported and were performed mainly under aerobic conditions and in terrestrial systems (Ali *et al.*, 2014; Anwar *et al.*, 2016; Giacomucci *et al.*, 2019; Shah *et al.* 2008; Webb *et al.*, 2000). Marine anaerobic microorganisms have not been studied yet in depth as plastic biodegraders even if they should be the first actors involved in mitigating the marine litter effects associated with the plastics already in the marine ecosystems.

The Mediterranean Sea has been identified as a plastic accumulating ecosystem, especially for floating polymeric items (Ruiz-Orejòn *et al.*, 2017; Suaria and Aliani, 2014). Oxygen availability in marine water is decreasing due to climate change with the result of modifying by the expansion of low-oxygen zones, especially in coastal systems (Breitburg *et al.* 2018; Dang and Lovell, 2016). Although most waters in the ocean are oxic and contain aerobic microbes, biofilms on submerged surfaces and marine particles in oxic waters may additionally harbor hypoxic and anoxic microenvironments suitable for microbial anaerobic metabolisms. Hence, anaerobic microbes living

in sessile form could be found even in aerobic condition as biofilm structure preserves them from oxygen present in the surrounding environment and thus some bacteria associated to marine fouling need suboxic or anoxic microniches within particles to support microaerophilic or anaerobic metabolism (Celikkol-Aydin *et al*., 2016; Dang and Lovell, 2016).

The aim of the present study was to evaluate the biodegradation of a virgin PVC plastic film under marine anaerobic conditions. For this purpose, 16 anaerobic consortia were enriched from marine samples (water with litter fraction) in the presence of PVC films in laboratory microcosms and their biodegradation abilities were assessed after 7 and 24 months incubation. At the end of the experiment, the PVC film-associated microbial communities were characterized.

## 2. Materials and Methods

### 2.1. Sampling and pre-enrichment of marine anaerobic consortia

Marine samples were collected from four sampling stations (S1 to S4) in Elefsis Bay (Greece) at a depth of 0 to 1.2 m of the water column. The samples, named S1 to S4 and consisting of marine water and a litter/debris fraction, were used as as source of microbial communitites. To increase the anaerobic microbial biomass to be used as inoculum in biodegradation assays, a pre enrichment step of the microbes adhered to the collected marine litter was performed. Specifically, four anaerobic microcosms from each sampling station were set up, using four different growth media, in 100 ml serum bottles with working volume of 40 ml. For this purpose 4 ml of litter fraction, 16 ml of marine water and further 20 ml of the anaerobic specific medium containing 24 g/L of sodium chloride were mixed. The four culture media (M1, M2, M3 and M4; composition reported in Supplementary material S1) were used in order to try to favour the growth of different anaerobic consortia, i.e. acidogenic/methanogenic (M1 and M2), nitrate reducing (M3) and sulfate reducing (M4) microorganisms. The sixteen different microcosms were flushed with pure sterile nitrogen (in the case of M1, M3 and M4 media) or a mix of nitrogen and carbon dioxide ($N_2$:$CO_2$=80:20, for the M2 medium) and incubated at 20°C. Microbial growth was evaluated after 3 months incubation

based on gas composition analysis of the headspace of each microcosm ($H_2$, $CO_2$, $CH_4$) by gas chromatography (Agilent 3000 MicroGC, Agilent technologies, Santa Clara, CA, USA) equipped with thermal conductivity detector (TCD). The analytical conditions were as follows: injector temperature 90 °C; column temperature 60 °C; sampling time 20 s; injection time 50 ms; column pressure 25 psi; run time 44 s; carrier gas $N_2$. When microbial growth was detected, fresh medium was added to a final volume of 70 ml and microcosms were incubated under the same conditions until a significant growth was detected, before using as inocula in the biodegradation experiment.

A set of four negative controls with the corresponding non inoculated media were also prepared.

### 2.2. PVC film

PVC film containing a total of 30%$_{w/w}$ of additives was provided by an Italian plastic producing company. In order to better monitor the gravimetric weight loss, thick PVC plastic films (300 μm), obtained after pressing several PVC thin layers (20 μm each) at 17.44 bar and 160°C for 4-5 min, were used. Thick films were then cut into small pieces (1.5 cm × 1.5 cm and 1.5 cm × 3.2 cm), degreased and sterilized by 30 min immersion in 70%$_{v/v}$ ethanol solution and washed with sterile distilled water before use. The films used for the determination of gravimetric weight loss were dried under vacuum to constant weight before being used as substrate for microbial growth.

### 2.3. PVC biodegradation assays

The sixteen microbial communities from the pre-enrichment step were used as inocula for PVC biodegradation experiments (Figure S1). Specifically, sixteen microcosms were setup in 100 ml serum bottles with 50 ml of the corresponding medium containing 10 g/l of PVC films as major carbon and energy source and inoculated with 20%$_{v/v}$ of pre-enriched cultures. Inoculated microcosms and abiotic controls were incubated under static conditions at 20°C. Samples of plastic

144 films and culture broth were withdrown after 7 and 24 months incubation and subjected to the
145 following analyses in order to detect putative microbial growth and PVC films biodegradation.

146 *2.3.1. Evaluation of the microbial growth and colonization of the PVC film surface*

147 Viability and growth of anaerobic consortia were evaluated by measuring headspace gas
148 production and composition ($H_2$, $CO_2$, $CH_4$) using MicroGC-TCD.

149 Biofilm formation was assessed by quantifying the proteins available onto the PVC film
150 surfaces. For this purpose, sampled PVC films were incubated overnight (4°C, 150 rpm) in a 6 M
151 urea solution. Protein quantification according to Lowry method (Lowry *et al.*, 1951) was then
152 performed on urea solutions, and urea 6 M was used as blank.

153 *2.3.2. Evaluation of PVC films biodegradation*

154 *Gravimetric weight loss.* Measurement of the dry weight of PVC film was determined after biofilm
155 removal by incubation with a 6 M urea solution overnight at 4°C and further washing with distilled
156 water. The washed PVC films were dried under vacuum at room temperature to constant weight (at
157 least 24 h) before weighing using a balance with a 6 digit accuracy. The weight loss percentage was
158 calculated as follows:

159 Weight loss (%) = (Initial weight-Final weight)/Initial weight*100.

160 *Thermogravimetric analysis (TGA).* Dried plastic samples of 5 to 10 mg were subjected to TGA
161 using a Perkin Elmer TGA7 thermal analyzer under nitrogen atmosphere (gas flow: 40 ml/min).
162 The thermograms were recorded from 40°C to 800°C at a heating rate of 10°C/min. The onset
163 degradation temperature ($T_{onset}$) and the maximum degradation temperature ($T_{max}$) were noted. Non
164 incubated PVC film and PVC resin were used as reference.

165 *Gel permeation chromatography (GPC).* The molecular weight of the PVC films were determined
166 using GPC. Measurements were carried out on a HPLC Lab Flow 2000 equipped with Phenomenex
167 Phenogel Mixed 5µ MXM/MXL columns and a Linear Instrument UVIS-200 detector operating at
168 254 nm. Tetrahydrofuran was used as mobile phase (1 ml/min) after calibration with polystyrene

standards of known molecular mass. A sample concentration of 3 mg/ml was applied. Non incubated PVC film and PVC films incubated under abiotic conditions were used as references.

#### *2.3.3. Statistical analyses*

Results of gravimetric weight losses and GPC analyses were statistically evaluated using one-way ANOVA. Post-hoc Tukey test was applied to determine whether gravimetric or molecular weight changes of PVC films showed significantly different extents. Statistically significant results were depicted by *p-values* < 0.05. All the analyses were performed using GraphPad Prism version 8.0.0 for Windows (GraphPad Software, San Diego, California USA, www.graphpad.com).

### 2.4. Composition of planktonic and biofilm communities

After 24 months incubation, total DNA was extracted using a Powersoil® DNA isolation kit (MOBIO, Carlsbad, CA, USA) from planktonic (from 2 ml of culture broth) and biofilm (PVC adhered microbial cells) microbial communities. Bacterial partial 16S rRNA gene was amplified using 357F-GC and 907R primers and PCR conditions reported by Polo *et al.* (2010) except for $MgCl_2$ and dNTP mix concentration of 1.8-2 mM and 0.2 µM, respectively and annealing temperature of 55°C. Archaeal PCRs were performed using Arch344F-GC and Arch918R (Giovannoni *et al.*, 1988; Stahl and Amann, 1991) primer pair. The archaeal thermal protocol was a modification of the bacterial protocol by elongating each step of 15 sec. DGGE was according to Polo *et al.* (2010), using denaturant gradients of 40–60% and 30–70% for bacterial and archaeal communities, respectively. Principal bands were excised, eluted in 50 µl milliQ water (30°C for 3 h), amplified and identified by sequencing (Macrogen Inc., Republic of Korea). The sequences were checked for chimeras using DECIPHER software (Wright *et al.*, 2012), analyzed using the BLASTN software and submitted to the Genbank database (accession numbers MH520751 to MH520766).

## 3. Results and discussion

### 3.1. PVC films biodegradability after 7 months

#### *3.1.1. Microbial growth monitoring*

Before performing the biodegradation assays, a pre-enrichment step (growth in the absence of PVC film) was performed (please refer to the scheme of the experimental design in supplementary Figure S1) and microbial growth was confirmed by headspace gas analysis. The pre-enriched consortia were used as inocula in the biodegradation assays. Microbial growth in the presence of PVC films as major carbon and energy source was monitored by measuring the volume and composition of the gas produced in the headspace of each consortium. After 7 months incubation, significant gas volumes were measured in 10 microcosms (Figure 1A) reaching a maximum of 19.0±1.0 ml, while no gas production was detected in the four abiotic controls. Headspace gas analysis showed that $CO_2$ was produced in all the microcosms while hydrogen was recorded only in the case of S2-M1 consortium (5.8±0.0 ml) (Figure 1A). Methane (max volume = 5.7±0.1 ml) was produced by the four consortia enriched in M2 medium, while very low/no methane production was recorded in microcosms enriched in M1, M3 and M4 media. These results showed that growth of the native marine microbial communities occurred in the presence of PVC plastic film as major C and energy source. Plasticizers probably sustained the observed growth, in line with previous studies carried out with PVC plastic under sulfate reducing conditions with bacteria enriched from landfill (Tsuchida *et al.*, 2011).

Based on these results, the 10 microcosms displaying clear microbial growth on PVC films (S2-M1, S2-M2, S3-M2, S4-M2, S1-M3, S3-M3, S4-M3, S2-M4, S3-M4 and S4-M4) were selected for further analysis.

#### *3.1.2. Evaluation of microbial colonization of PVC film surface.*

Among PVC films incubated in the 10 microcosms, four showed significantly higher (>10 times) amount of adhered proteins compared to their corresponding abiotic controls (Figure 1B),

suggesting an extensive microbial colonization of plastic surface. These include S2-M2, S3-M2, S4-M3 and S2-M4, with a protein content (mean±SD) of 57.3±2.9, 45.6±2.3, 88.9±4.4 and 76.3±3.8 µg of BSA equivalent/mg of PVC film, respectively. Microbial colonization of PVC films was reported under aerobic conditions. Specifically, unpretreated plasticized PVC films exposed to the atmosphere (Blackley, Manchester, United Kingdom) for 95 weeks were found to be colonized by fungi while no bacterial colonization was recorded (Webb *et al.*, 2000). Dang *et al.* (2008) evaluated biofilm formation on different polymer surfaces including PVC plates, submerged in seawater (1 m below the water surface) near the Qingdao coast for 72 h. Biofilm formation was shown to occur on the different surfaces based on detection of 126 operational taxonomic units with prokaryotes being predominant.

To the best of our knowledge, to date there is only one report on microbial colonization of PVC plastic surface under anaerobic conditions. The Archaeal strain *Methanosarcina barkei* was found to be able to adhere on PVC films after 2 h of exposure and to putatively produce exopolymeric substances (EPS), as revealed by epifluorescence and scanning electron microscope observations (Nguyen *et al.*, 2016).

Biofilm formation is considered as the first step of biodegradation activity against water insoluble polymeric materials (Das *et al.*, 2012; Reisser *et al.*, 2014; Wang *et al.*, 2016; Zettler *et al.*, 2013). Hence, only microbial consortia that showed significantly high adhered protein contents on PVC films, i.e. S2-M2, S3-M2, S4-M3 and S2-M4 (Figure 1B), were selected for further analyses. Indeed, for the PVC films incubated in these microcosms, a 10-fold protein content was recorded compared to the corresponding abiotic controls. This threshold was fixed in order to overcome the limitations of the Lowry assay related to the interference of molecules other than proteins (like compounds that derive from the additives components of the plastic film), in the colorimetric measurements (Everette *et al.*, 2010).

### *3.1.3. Evaluation of PVC biodegradation*

Biodegradation activity was evaluated on film samples withdrawn from the four (S2-M2, S3-M2, S4-M3 and S2-M4) selected microcosms. Gravimetric measurements revealed a statistically higher decrease in their weights ($p < 0.05$; Figure 2 A-D) compared to those recovered from the corresponding abiotic controls. Specifically, weight reductions (mean±SD%) of 11.67±0.58%, 10.71±0.54%, 6.26±0.31% and 5.77±0.29% were recorded for the films incubated in S4-M3, S2-M4, S2-M2 and S3-M2 microcosms, respectively. Among the biological effect of the four selected consortia, the highest gravimetric weight decrease was recorded by films incubated with S4-M3 and S2-M4 consortia, which showed the same statistical film weight reduction followed by the action of S2-M2 and S3-M2 consortia ($p < 0.05$; Figure 2 D). The corresponding abiotic controls incubated in the growth media M3, M4 and M2 showed statistically equal weight losses of 3.71±0.28%, 3.91±0.2% and 3.51±0.18% respectively ($p < 0.05$; Figure 2 A-D) due to additives leaching from the PVC films (Kastner *et al.*, 2012; Suhrhoff and Scholz-Bottcher, 2016; Wang *et al.*, 2016). These evidences are the first proof of plastic biodegradation; indeed, the reduction of gravimetric weight, which is a direct evidence of the loss of material, is widely used in biodegradation tests (Shah *et al.* 2008; Wang *et al.*, 2016). Thus some components of the PVC films were degraded by the enriched microbial communities. Since the films used in this study had about 30%$_{w/w}$ of additives, further analyses were performed in order to elucidate if the biodegradation regarded the sole additives or also the PVC polymer.

TGA showed a lower thermal stability of the films recovered from the four active microcosms (Figure 2A-C) compared to those incubated under abiotic conditions. Specifically, a $T_{max}$ ranging from 287°C to 277°C was recorded for the former compared to a $T_{max}$ of 292°C for those of abiotic controls or 294°C for the non incubated PVC film. Considering that the presence of additives (citrates, polyadipates, epoxidized soybean oil, Zn etc; data supplied by the production company) is lowering the thermal stability of PVC resins once in PVC plastic film, the (bio)degradation of the sole additives would lead to an increase of the film thermal stability i.e. a shift of the TGA curve

toward that of the resin. On the opposite, here a reduction of such parameter was observed and would suggest that a microbial attack towards PVC polymer chains occurred.

Also GPC analyses exhibited a decrease of the average molecular weight ($M_n$%; mean±SD) from 100% to 91.1±3.2%, 94.3±3.3%, 95.5±3.3%, and 98.1±3.4% for PVC films incubated with S4-M3, S3-M2, S2-M2, and S2-M4 consortia, respectively (Figure 2E). According to statistical analysis, PVC film incubated with S4-M3 exhibited a statistically lower $M_n$(%) compared to the film incubated with its abiotic control ($p < 0.05$, Figure 2E), suggesting that this consortium was able to statistically reduce PVC molecular weight. On the other hand, no statistically significant differences were recorded between molecular weights of films incubated with all consortia and pristine PVC film ($p > 0.05$; Figure 2E). However, the fact that the differences between biotically treated and pristine PVC films are not statistically significant does not imply that no biological action has occurred and the interpretation of the results should focus on the scientific importance rather than on the statistical significance. Indeed, considering the nonbiodegradable nature of the polymer even small changes that are not statistically significant are important from a biodegdataion view point. Hence, the GPC results are in line with those of TGA and suggest that some polymer chains were biodegraded with the production of low $M_w$ PVC chains in the case of S4-M3, S3-M2 and S2-M2 microcosms (Figure 2E). On the other hand, GPC analyses revealed an increase of $M_n$ of abiotic controls up to 105.5±3.7% (mean±SD; Figure 2E) although no appreciable changes in their thermal stability were recorded. Such results could be explained by a loss of water-soluble low molecular weight additives other than thermal stabilizers, which has led to $M_n$ increase without change in the thermal stability.

### 3.2. PVC films biodegradation after 24 months

Figure 3 shows the gravimetric weight reductions of the PVC films incubated in the 16 biologically active microcosms or in the corresponding abiotic controls after 24 months incubation. According to statistical analysis, eleven consortia (three in M2, 4 in M3 and 4 in M4 medium) were

shown to significantly reduce film weight compared to their abiotic controls ($p < 0.05$; Figure 3). Four and three consortia enriched in M4 and M3 media reduced the gravimetric weight (mean±SD) of the PVC films by up to 12.97±0.65% ($p< 0.05$; showed by d letter in Figure 3). A statistically lower weight loss of up to 9.23±0.46% ($p < 0.05$; showed by c and e letters in Figure 3) was recorded for three microcosms set up in M2 and for S2-M3 consortium, while a maximum weight loss of 4.21±0.21%, statistically comparable to that recorded under abiotic conditions (4.32±0.22%), was observed for the four consortia enriched in M1 and in S4-M2 consortium ($p < 0.05$; Figure 3).

Based on these results, only 11 consortia (S1-M2, S2-M2, S3-M2, S1-M3, S2-M3, S3-M3, S4-M3, S1-M4, S2-M4, S3-M4 and S4-M4), exhibiting statistically significant gravimetric weight losses ($p < 0.05$), were selected for further analyses.

Compared to abiotic control and pristine (non-incubated) PVC films, the films withdrawn from S2 and S3 consortia enriched in M2, the S4 consortium enriched in M3 as well as S1, S2 and S4 consortia enriched in M4 displayed a decrease of TGA-assessed thermal stability (Table 1), confirming the evidences observed after 7 months incubation.

On the other hand, the GPC analyses showed an increase of the $M_n$ (%) of the films incubated with 8/11 consortia (S2-M2, S3-M2, S2-M3, S4-M3, S1-M4, S2-M4, S3-M4 and S4-M4) including those that exhibited $M_n$ (%) lower than 100% at month 7, even if no statistically significant differences were found among $M_n$ (%) after the analysis of variance (ANOVA: $p = 0.19$; $F = 1.44$; $df = 14$) (Figure 2E, Figure 4). Once again, the fact that no statistically significant differences in $M_n$ (%) were observed does not imply no modifications of the Mn (%) has occurred. Indeed, it is important to notice that $M_n$ values for polymeric substances are very high numbers and therefore differences in such number should be very high to be considered statistically different. Consequently, in the case of data obtained from a biodegradation of materials known as “non biodegradable”, small differences in $M_n$ (%) are often observed and should be acknowledged as of scientific importance rather than of statistical significance. Hence, these results suggest that the

three consortia found to be the most active at the 7th month together with consortia S1, S2 and S4 enriched in M4 that displayed activity after longer incubation period, are apparently able to degrade PVC plasticizers and polymer chains. Indeed, considering that biodegradation of low molecular weight compounds affects prevalently the $M_n$ (%) rather than the gravimetric weight, the $M_n$ increase observed after 24 months incubation could be explained by the full biodegradation of the short polymer chains putatively produced within the first months of incubation and/or the release of the broken chains and of the lower molecular weight additives into the growth medium.

### 3.3. Characterization of microbial communities

Characterization of the anaerobic communities enriched in the presence of PVC films was performed on the 11 different consortia exhibiting a mass reduction significantly higher than the corresponding abiotic controls after 24 months incubation.

From the DGGE profiles (Figure 5), 13 (band #1 to #13) and 3 (band #A to #C) bands were cut from the bacterial and archaeal communities, respectively.

The bacterial biofilm communities enriched in M2, M3 and M4 media were characterized by the presence of two bands that were detected in most consortia and which include band #8 and band #10 detected in 9 and 8 out of the 11 consortia, respectively (Figure 5, Table 2). The band #8 has highest sequence similarity with different Clostridia including uncultured *Clostridium* clone reported from an Alaskan mesothermic petroleum reservoir (Pham *et al.*, 2009), Clostridiales bacteria able to degrade the explosive UXO5-23 (Zhao *et al.*, 2007) or *Clostridium* sp. AN-D reported from high pressure prokaryotic enrichments from deep marine gas hydrate sediments (Parkes *et al.*, 2009). The band #10 has 98% similarity with *Acetobacterium* sp. Other two bands (band #9 and band #7) which have, respectively, 100% and 99% of similarity with *Dethiosulfovibrio* sp. and *Sporobacter* sp. were detected in 3/11 consortia (Figure 5, Table 2). Different other bands were detected in some of the communities enriched in specific media and having homologies with: i) *Erysipelothrix* sp. (band #1) mainly in M4 communities; ii) *Fusibacter*

(band #13) and *Psychromonas* (band #4) in some M3 communities and iii) *Desulfovibrio* (band #12), *Cupriavidus* (band #5), *Cohaesibacter* sp. (band #11) and *Pleomorphochaeta* (band #6) detected mainly in M2 consortia (Figure 5, Table 2).

For all the consortia, almost no differences in the composition of the bacterial biofilm and its corresponding planktonic community were observed. Similarly, composition of the planktonic and biofilm archaeal communities for each consortium were very similar. They were composed of bands having high similarities with i) *Methanosaeta* sp. (band A) present in all M4 and M3 communities, ii) *Methanococcoides sp.* (band B) detected in the three M2 communities and iii) *Methanogenium* sp. (band C) found only in some M2 communities (Figure 5, Table 2).

Although the chemical analyses indicate the biodegradation of PVC polymer by the consortia S2-M2, S3-M2, S4-M3, S1-M4, S2-M4 and S4-M4, none common microbial phylotype was observed in all such consortia and this seems to suggest that more than a microbial specie were involved in the biodegradation. The occurrence of high NaCl contents in the microcosm media did not allow to determine changes in chlorine ion concentration in the culture supernatants and therefore to assess if the degradation was accompanied by the dehalogenation of PVC polymer. However, the following observations support PVC dechlorination. Among the microbial components of two communities (S1-M2; S1-M3), bands having a high similarity with the facultative organohalide respiring bacterium *Desulfovibrio dechloracetivorans (band #12,* S1-M2) (Sun *et al.*, 2000) and with *Fusibacter* sp. *(band #13,* S1-M3) were detected. *Fusibacter* sp. was reported as dominant component of tidal flat communities where *Dehalococcoides*-like bacteria were not detected although dechlorination of PCE-to-cis-DCE has occurred (Lee *et al.*, 2011). Further, bands having highest sequence similarity with Clostridia were detected in almost all consortia. The class of Clostridia includes bacterial species (bacteria from the genus *Clostridium)* reported to play an important role in dechlorinating consortia, producing $H_2$ that is used as electron donor by the dechlorinating bacteria (Bowman *et al.*, 2010).

Bacteria of the genus *Acetobacterium* were detected in 8/11 consortia. Among these homoacetogens, some species have been reported to be able to mineralize tetrachloromethane (Egli *et al.*, 1988). *Acetobacterium* spp. was also detected among the components of consortia able to dechlorinate 1,1,2,2-tetrachloroethane (Manchester *et al.*, 2012). Patil *et al.* (2014) reported successful dechlorination of tetrachloroethene by a microbial community that did not include classical dechlorinators due to the synergistic work of the community i.e. *Enterobacter* and *Desulfovibrio* spp. as putative dechlorinators and *Bacteroidetes* and *Firmicutes* as acetate fermenters (Patil *et al.*, 2014).

Other bands having high similarity with Bacteroidales bacterium CF (band#3, 93% of identity), were also detected. This bacterium was found to be the most abundant non dechlorinating bacterium in a community that reductively dechlorinates multichlorine molecules; it is able to ferment lactate, ethanol and glucose in the presence of concentrations of chloroform or trichloroethane that usually inhibit reductive dechlorination and methanogenesis (Tang *et al.*, 2013).

The archaeal communities present in 8/11 consortia enriched in M3 and M4 media were composed mainly by members of the Phylum Euryarchaeota, with a unique member of the Methanosarcinales (Genus *Methanosaeta*, species *pelagica*, Figure 5, Table 2) known for its acetoclastic methanogenic activities. Oh *et al.* (2008) showed that acetoclastic methanogenic Archaea may play an indirect role in dechlorination of halogenated organic compounds such as PCBs by synthetizing the corrinoid cofactor cobalamin required for vitamin B12 synthesis, which is necessary for assembling functional reductive dehalogenase systems (Patil *et al.*, 2014; Rupakula *et al.*, 2014; Yan *et al.*, 2013). Cobalamine and other precursors are typically produced by methanogenic Archaea as cofactors for methyltransferases involved in methane production (Yan *et al.*, 2013).

Specialized dehalorespiring bacteria among the components of the anaerobic consortia studied were, however, not detected. This could be due to PCR bias i.e. the necessity to use more specific

amplification primers in order to reveal the presence of such bacteria (Aulenta *et al.*, 2004; Dowideit *et al.*, 2010; Duhamel *et al.*, 2006; Manchester *et al.*, 2012).

Besides of the bacterial and archaeal phyla potentially playing an important role in dechlorinating PVC, the other bacterial and archaeael clades were detected and could be involved in additives biodegradation. These include *Dethiosulfovibrio* sp.; *Erysipelothrix* sp.; *Sporobacter* sp.; *Psychromonas* sp.*; Cupriavidus* sp. and *Cohaesibacter* sp. (Bonin *et al.*, 2002; Canion *et al.*, 2013; Diels *et al.*, 2009; GrechMora *et al.*, 1996; Hwang *et al.*, 2008; Pantazidou *et al.*, 2007; Stackebrandt *et al.*, 2006; Surkov *et al.*, 2001) as well as *Methanococcoides* sp. (99% of identity, Figure 5, Table 2) detected in three M2 consortia or *Methanogenium* sp. detected only in S1-M2 consortium (Figure 5, Table 2) (Miller *et al.*, 2016).

## 4. Conclusions

A significant biodegradation of plasticized unpretreated PVC films by marine anaerobic microbial consortia was demonstrated in this study. After 7 months incubation, three of 16 enriched consortia were able to degrade the films apparently acting against both the additives and the polymer chains. For these consortia, GPC showed a decrease in $M_n$ suggesting that some polymer chain scission took place leading to the formation of smaller fragments, while TGA and gravimetric weight loss (%) revealed a decrease in thermal stability and a mass loss. Prolongation of the incubation to 24 months allowed the selection of other three consortia able to display the same potential.

To our knowledge, this is the first study reporting on biodegradation of plasticized PVC films by marine microbial communities enriched under anoxic conditions and providing information about the composition of such communities potentially responsible for it. The PVC film-associated communities of the biodegrading microcosms encompasses microbial phyla closely related to those

421 previously reported from laboratory/field enrichments for their ability to degrade halogenated
422 organic compounds and hydrocarbons.
423 The enriched consortia originate from marine samples and the activity recorded was obtained
424 under lab conditions mimicking the sulfate reducing, nitrate reducing and methanogenic conditions
425 occurring in marine environment. Thus the present study provides relevant insights on the potential
426 fate of PVC plastic films occurring in anoxic marine impacted ecosystems.
427

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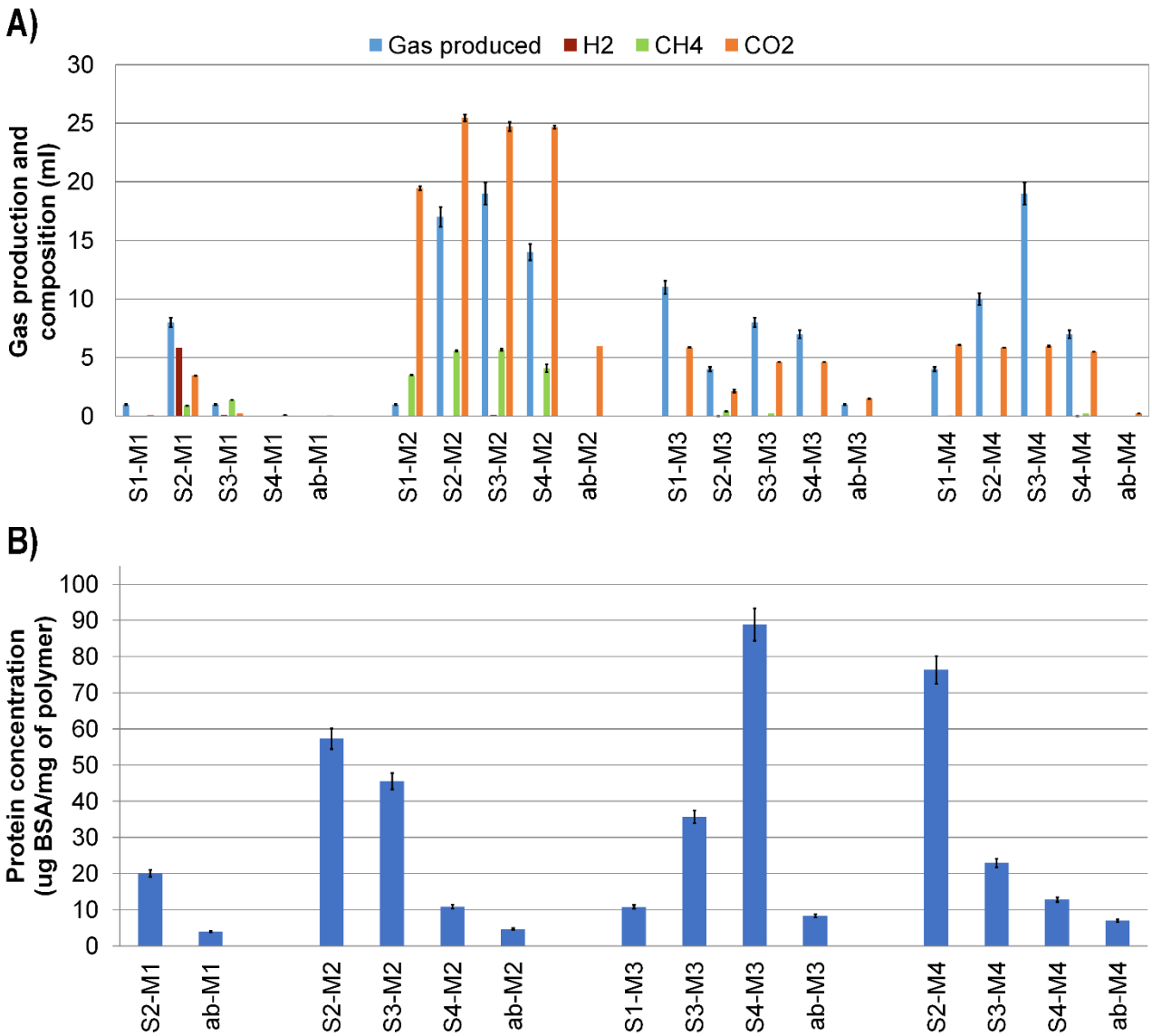



**Figure 1.** Evaluation of microbial growth and biofilm formation after 7 months incubation. Volume and composition of gases produced by the sixteen different enriched marine consortia (the combination of 4 native marine/litter communities, i.e. S1 to S4, grown in the presence of 4 different media, i.e. M1 to M4) (a); Concentration of proteins adhered on surfaces of PVC films incubated under abiotic conditions (ab-M1 to ab-M4) or with the ten different enriched marine consortia showing highest microbial growth by gas analysis (b). Bars indicate standard deviation.

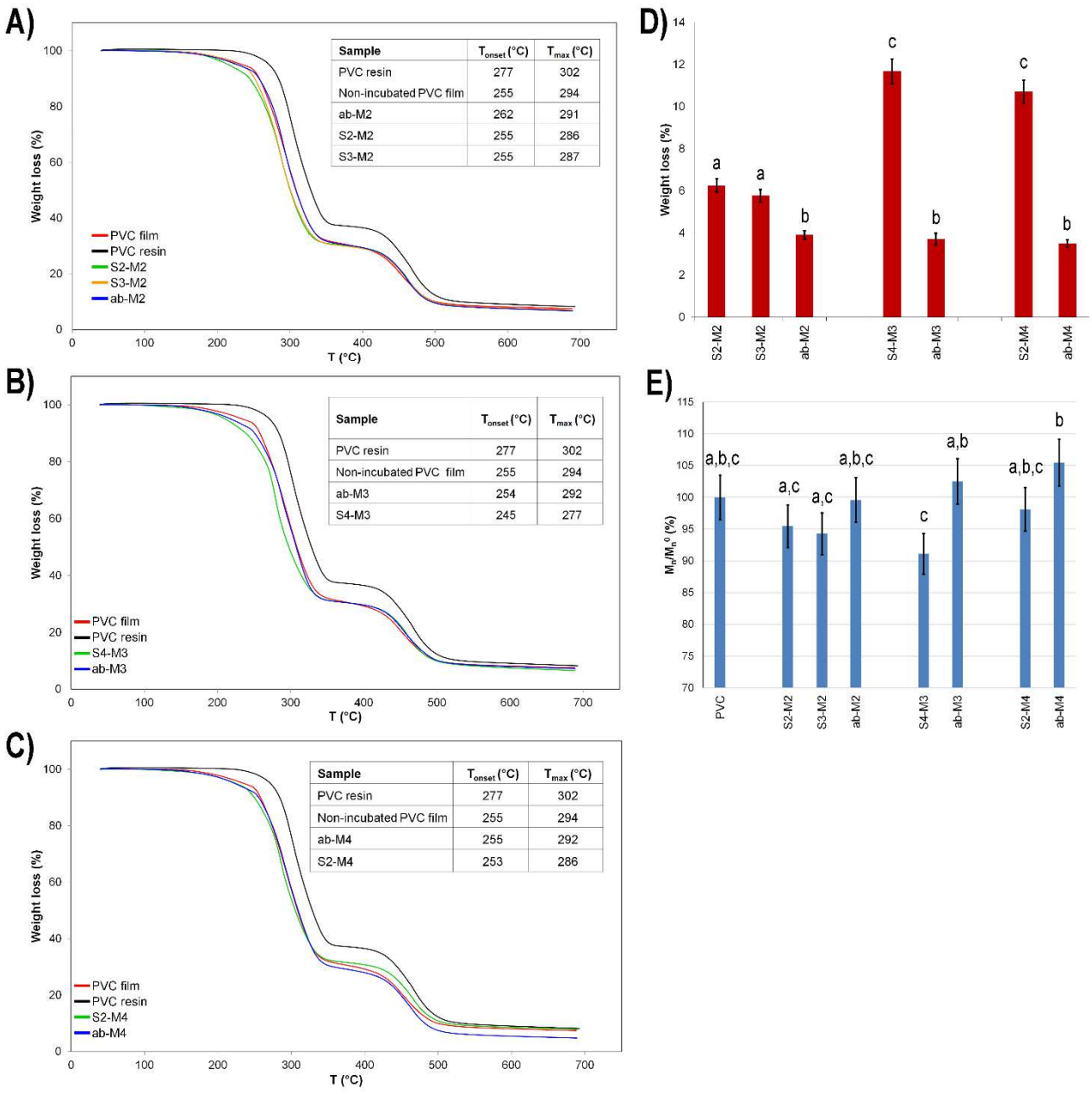



**Figure 2.** Chemical-physical analyses on PVC films withdrawn at month 7. TGA curves of PVC films incubated with the four different consortia enriched in M2 (A), M3 (B) and M4 (C) media or in the four corresponding abiotic controls. Gravimetric weight loss percentage of PVC films incubated with the four different consortia or in the four corresponding abiotic controls (D) (ANOVA: $p < 0.0001$; $F = 249.9$; $df = 6$). Number average molecular weight (Mn) of selected PVC films (ANOVA: $p = 0.0025$; $F = 5.42$; $df = 7$) (E). Mn (%) is defined as (Mn/Mn0)*100, where Mn is the molecular weight at sampling time t and Mn0 is the molecular weight of the non-incubated PVC film. Bars indicate standard deviation. Letters a, b, c show results of post-hoc analyses for film

gravimetric or molecular weight losses ($p < 0.05$), different letters indicate a significant statistical difference in gravimetric or molecular weight reduction values.

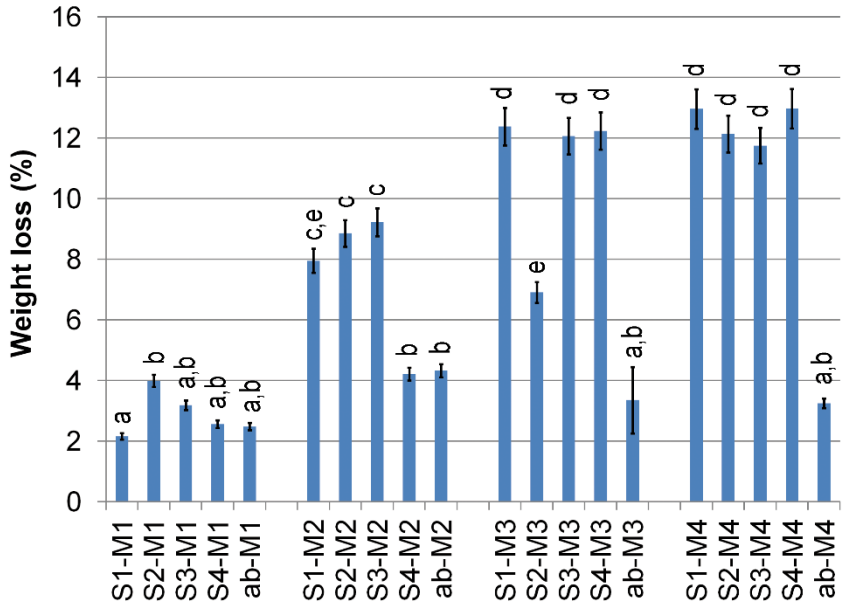
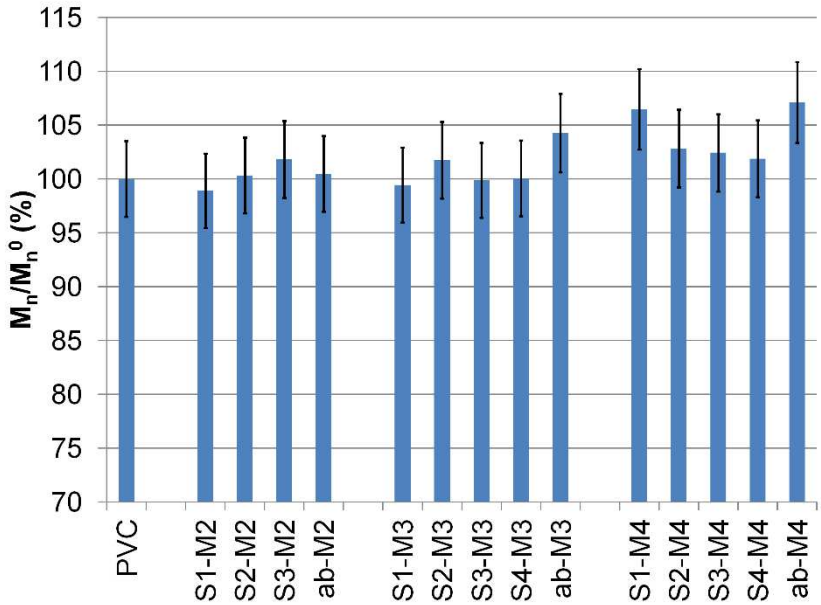



**Figure 3.** Gravimetric measurements at month 24. Weight loss (%) of PVC films incubated with the sixteen enriched consortia and the corresponding four abiotic controls. Bars indicate standard deviation (ANOVA: $p < 0.0001$; $F = 222.3$; $df = 19$). Letters a, b, c, d, e show results of post-hoc analyses for film gravimetric or molecular weight losses ($p < 0.05$), different letters indicate a significant statistical difference in gravimetric weight reduction values.




667

668 **Figure 4.** PVC mean molecular weight at month 24. GPC results for PVC films incubated with

669 eleven different consortia enriched in M2, M3 and M4 media and their corresponding abiotic

670 controls (ab-M1; ab-M2; ab-M3 and ab-M4) (ANOVA: $p = 0.19$; $F = 1.44$; $df = 14$).

671

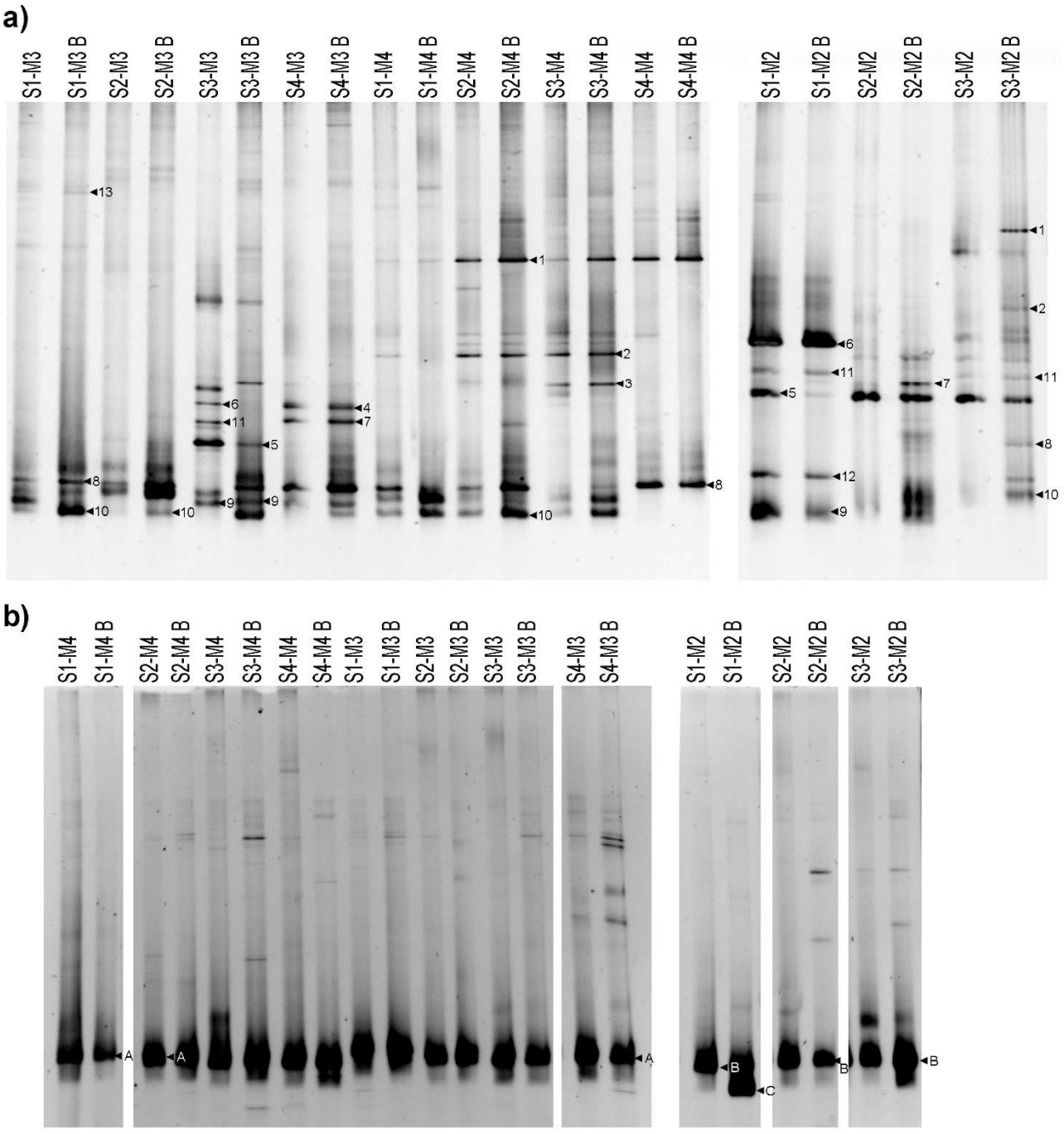



**Figure 5.** Bacterial (a) and archaeal (b) diversities of the 24 months PVC film-enriched anaerobic communities. DGGE profiles of 16S rRNA genes amplified from planktonic and biofilm microbial communities from the eleven different consortia showing high weight losses compared to the corresponding abiotic controls. Names over the lanes refer to the ID of the consortium. The letter B after consortia names stands for “biofilm community”. The identity of sequences of bands marked with arrows is given in Table 2 according to the band ID (1 to 13 and A to C).

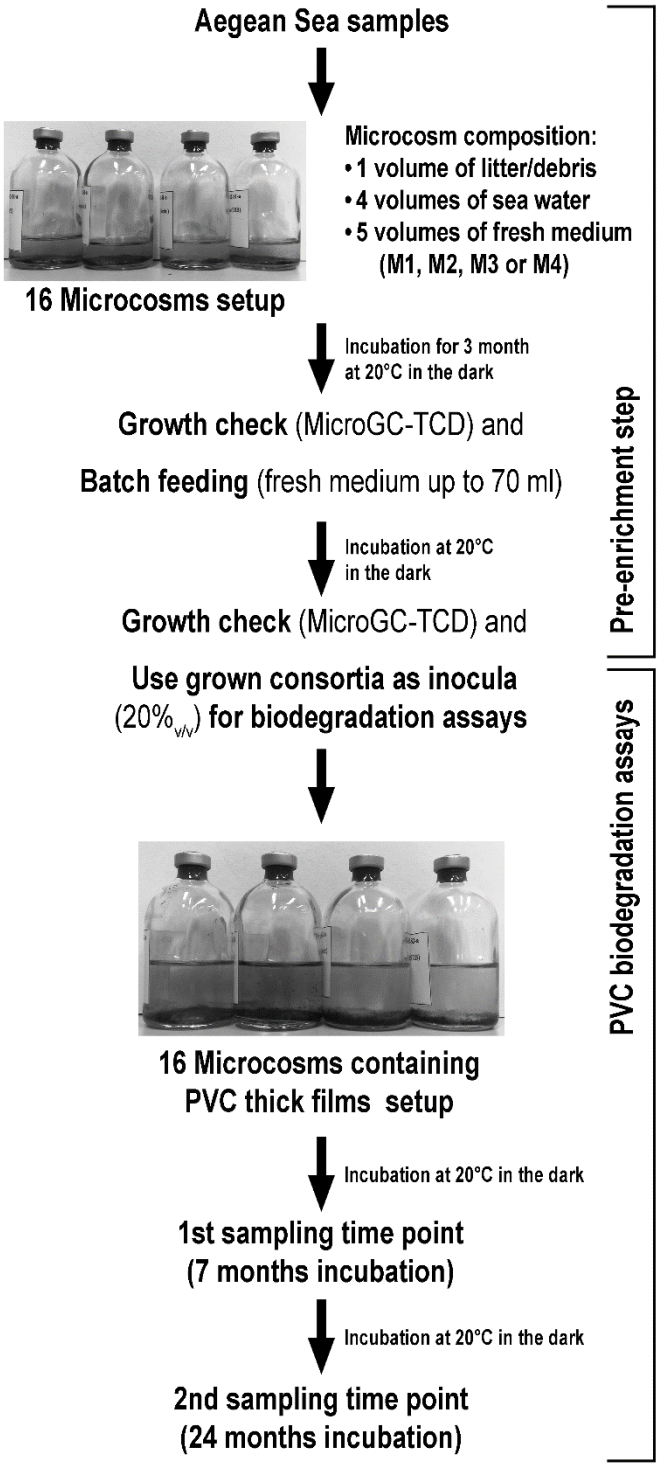



**Figure S1.** Experimental design. Schematic presentation of the experimental sequence for assessment of PVC film biodegradation.

## Supplementary material – S1

Supplementary material S1 contains the composition of the four media used to enrich the different marine anaerobic consortia and used to perform PVC biodegradation study.

*Medium M1* was composed of (g/l): sodium formate, 2; $Na_2CO_3$, 0.1; $(NH_4)_2SO_4$, 0.5; mineral solution (per litre: $KH_2PO_4$, 18 g; NaCl, 18 g; $CaCl_2 \cdot 2H_2O$, 0.53 g; $MgCl_2 \cdot 6H_2O$, 0.4 g; $MnCl_2 \cdot 4H_2O$, 0.2 g; $CoCl_2 \cdot 6H_2O$, 0.02 g), 50 ml; acetic acid, 1.53 ml; vitamin solution (per litre: thiamine-HCl, 20 mg; Ca-D-pantothenate, 20 mg; nicotinamide, 20 mg; riboflavin, 20 mg; pyridoxine-HCl, 20 mg; biotin, 0.5 mg; folic acid, 0.25 mg; vitamin $B_{12}$,0.2 mg), 5 ml; hemin solution (10 mg in 100 ml of 50% ethanolic), 5 ml; cysteine-sulphide solution (L-cysteine-HCl$\cdot H_2O$, 2.5 g/l; $Na_2S \cdot 9H_2O$, 2.5 g/l; pH 11), 5 ml; pH 6.7 (Cacciari et al. 1993).

*Medium M2* was composed of (g/l): glucose, 2; yeast extract, 1 g; tryptone, 1 g; beef extract, 0.5 g; $K_2HPO_4$, 1.5 g; $MgCl_2 \cdot 6H_2O$, 0.21 g; $FeSO_4 \cdot 7H_2O$, 0.1 g; trace element solution (per litre: $MnSO_4 \cdot 7H_2O$, 0.01 g; $ZnSO_4 \cdot 7H_2O$, 0.05 g; $H_3BO_3$, 0.01 g; $CaCl_2 \cdot 2H_2O$, 0.01 g; $Na_2MoO_4$, 0.01 g; $CoCl_2 \cdot 6H_2O$, 0.2 g), 10 ml; vitamin solution (per litre: L-ascorbic acid, 0.025 g; citric acid, 0.02 g; piridoxine-HCl, 0.05 g; para-aminobenzoic acid, 0.01 g; D-biotin, 0.01 g; vitamin $B_1$, 0.02 g; riboflavine, 0.025 g) 1 ml; 5 $\%_{w/v}$ L-cysteine solution, 10 ml; pH 7.2-7.5.

*Medium M3* was composed of (g/l): glucose, 2; $KH_2PO_4$, 0.5; $NH_4C1$, 0.3; $MgSO_4 \cdot 7H_2O$, 0.5; $CaCl_2 \cdot 2H_2O$, 0.1; $NaNO_3$, 3; $NaHCO_3$ solution, 30 ml; vitamins solution, 1 ml; EDTA-chelated mixture of trace elements, 1 ml; selenite-tungstate solution, 1 ml; pH 7.2. All solutions composition were from Widdel and Bak (1992).

*Medium M4* was composed per litre of distilled water: sodium lactate, 4.5 g; $Na_2SO_4$, 6 g; $KH_2PO_4$, 0.2 g; $NH_4Cl$, 0.25 g; $MgCl_2 \cdot 6H_2O$, 0.4 g; KCl, 0.5 g; $CaCl_2 \cdot 2H_2O$, 0.15 g; DSMZ SL-10 trace elements solution, 1 ml; selenite-tungstate solution (Widdel and Bak, 1992), 1 ml; mixed vitamin solution (per 100 ml of 10 mM sodium phosphate pH 7.1: p-aminobenzoic acid, 4 mg; biotin, 1 mg; nicotinic acid, 10 mg; D,L-Ca pantothenate, 5 mg; pyridoxine-HCl, 15 mg; thiamine-HCl, 10 mg), 1 ml; 0.005$\%_{w/v}$ vitamin B12 solution, 1 ml; 84 mg/l $CaHCO_3$ solution, 30 ml; 4$\%_{w/v}$ sulfide solution, 7.5 ml; pH 7.0-7.5.

## Tables

**Table 1. TGA at month 24.** Results of TGA of PVC resin, PVC film (non-incubated) and PVC films incubated under abiotic conditions (ab-M2 to ab-M4) or with the consortia enriched in three media that showed lower thermal stability compared to abiotic controls.

| Sample | $T_{onset}$ (°C) | $T_{max}$ (°C) |
|---|---|---|
| PVC resin | 277 | 302 |
| PVC film | 255 | 294 |
| ab-M2 | 261 | 290 |
| S2-M2 | 260 | 284 |
| S3-M2 | 259 | 283 |
| ab-M3 | 260 | 289 |
| S4-M3 | 263 | 285 |
| ab-M4 | 260 | 289 |
| S1-M4 | 263 | 283 |
| S2-M4 | 262 | 285 |
| S4-M4 | 261 | 284 |

**Table 2. Identification of microorganisms characterizing the different PVC film associated communities according to DGGE profiles.**

| Band ID | Closest cultured relative (BLAST) | Accession No. | Identity (nt%) | Putative classification | No. positives[a] |
|---|---|---|---|---|---|
| *Bacteria* | | | | | |
| 1 | *Erysipelothrix* sp. (KX156777) | MH520751 | 95 | *Erysipelotrichaceae* | 5/11 |
| 2 | Uncultured *CFB group* bacterium (FJ024711) * | MH520752 | 98 | *CFB group* | 3/11 |
| 3 | *Bacteroidales bacterium* CF (CP006772) | MH520753 | 93 | *Bacteroidetes* | 2/11 |
| 4 | *Psychromonas* sp. (NR116830) | MH520754 | 99 | *Psychromonas* | 1/11 |
| 5 | *Cupriavidus* sp. (MG948149) | MH520755 | 100 | *Cupriavidus* | 4/11 |
| 6 | *Pleomorphochaeta* sp. (NR134177) | MH520756 | 98 | *Pleomorphochaeta* | 4/11 |
| 7 | *Sporobacter* sp. (KT183425) | MH520757 | 99 | *Ruminococcaceae* | 3/11 |
| 8 | *Clostridium* sp. a-nd (FN397991) | MH520758 | 98 | *Clostridiaceae* | 9/11 |
| 9 | *Dethiosulfovibrio* sp. (NR029034) | MH520759 | 100 | *Dethiosulfovibrio* | 3/11 |
| 10 | *Acetobacterium* sp. (NR074548) | MH520760 | 98 | *Acetobacterium* | 8/11 |
| 11 | *Cohaesibacter* sp. (KT324976) | MH520761 | 99 | *Cohaesibacter* | 2/11 |
| 12 | *Desulfovibrio* sp. (KU892724) | MH520762 | 99 | *Desulfovibrio* | 1/11 |
| 13 | *Fusibacter* sp. (KJ420408) | MH520763 | 96 | *Fusibacter* | 1/11 |
| *Archaea* | | | | | |
| A | *Methanosaeta pelagica* (NR113571) | MH520764 | 99 | *Methanosaeta* | 8/11 |
| B | *Methanococcoides methylutens* (CP009518) | MH520765 | 99 | *Methanococcoides* | 3/11 |
| C | *Methanogenium* sp. (NR104730) | MH520766 | 99 | *Methanogenium* | 1/11 |

[a] Number of samples positive for the presence of the specific band in DGGE analysis compared to total number of PVC plastic associated biofilm communities analyzed.

* The sequence related to band #2 demonstrated a very low homology with cultivable bacteria, it was analyzed including the uncultured/environmental sample sequences present in nucleotide databases.