Report:
Collaboration with Expedition Med
(Sampling 2019, draft)

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1. Introduction

The Expedition Med has planned to carry out a plastic collection from Mediterranean Sea, in ports and rivers in 2019. Thanks to the collaboration between Expedition Med and the proteomics and microbiology laboratory of Professor Ruddy Wattiez from the University of Mons, various analyzes will be realized on these plastics. The aim is to understand the effects of plastics as well as their role in ecosystem. Indeed, the plastic presence into oceans provides a very large number of new substrates on which microorganisms develop. It constitutes a major vector for the dispersal of species such as pathogenic species across the environment. These plastics are self-sufficient ecosystems that are mandatory to study and better understand. For this, different analyzes will be carried performed such as the microbial community study of biofilms by sequencing and by proteomic. In addition, some bacteria in these communities could constitutes potential candidates for plastic degradation. In order to highlight these, enrichment cultures will be realized. The ultimate goal of these enrichment will be to find a new sustainable alternative for waste management and recycling. Finally, plastic samples will be counted in order to have an idea of plastic penetration into the environment.

More than 110 plastics have been sent to us by expedition med. With this macro-, meso- and microplastics, bacterial community will be study by 16S rRNA amplicon sequencing to better understand the plastisphere. For that, the bacterial composition will be studied in comparison of different parameters: (1) plastic natures (Polypropylene, polyethylene, polystyrene, …); (2) plastic size (micro-, meso-, microplastic), (3) plastic colors; (4) sampling places (inshore, offshore, harbor, river); (5) water temperature; (6) sampling geography. Enrichment culture were realized with samples containing a lot of biofilm. These cultures were realized in medium with low concentration of carbon and in presence of plastic as the main carbon source (PVC, LDPE, LMWPE, PS and PET). Moreover, to better characterize plastic waste find into the Mediterranean, the nature of each plastic will be determined by ATR-FTIR. Finally, metaproteomic and metagenomic will be performed on in-shore plastic in comparison with river.

2. Materials and methods

2.1. Sampling

Expedition med sampled plastics across the Mediterranean Sea from July 21th, 2019 to August 9th, 2019. Sampling were carried out at different place: in-shore, off-shore sampling performed using a manta net towed by the boat during 30 min, and several sampling in harbor and rivers were realized (Fig.1). Once collected, the plastics were immerged in sea water in sterile 50 ml falcon and store at 4 °C during the transportation. A table summarize all the plastics sampled by expedition med as well as the physico-chemical parameters of the water (Table S1).

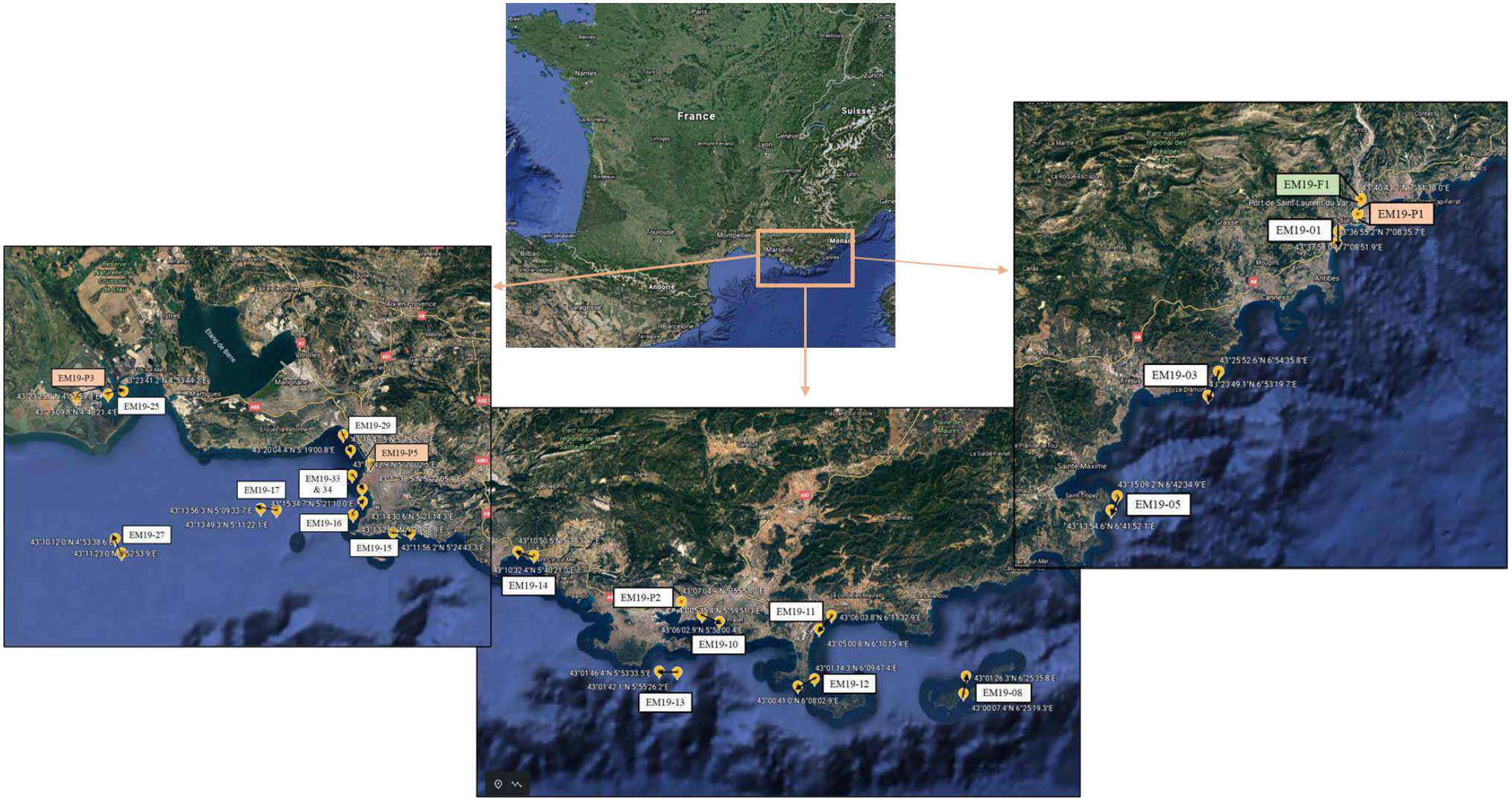

Figure 1: Map of all the sampling that we received in 2019 from the Mediterranean Sea. The localization of the different samplings by manta net are represented by a white rectangle. The first point shows the beginning of the sampling and the second, the end; the arrow show the direction of the sampling. The plastic sampling in harbor are represented in orange rectangle and the river in green.

2.2. Plastic characterization

Biofilms were removed from plastic and used for the bacterial community analysis and the enrichment culture. Before the identification of the plastic nature by ATR-FTIR, plastic films were rinsed with ethanol 70% (V/V) and deionized water to remove organic coatings and dried at 30°C for 1 day. The spectra of the surface films were obtained using Fourier transform infrared spectroscopy (FTIR) using the attenuated total reflectance (ATR) technique (Bruker, Tensor 27) with OPUS 6.5 software. The spectra were acquired over the wavelength range of $4000 - 600\ cm^{-1}$ with 64 spectral scans (Mahoney *et al*., 2013). The size and the color of each plastic sample are collected in order to classify the plastic samples: macroplastics (> 20 mm), mesoplastics (5-20 mm) and microplastics (<5 mm) (Reisser *et al*., 2014; Barnes *et al*., 2009) (Table 3 and S3).

2.3. Enrichment culture

Samples with a lot of biomass were used to perform enrichment culture to try to select bacteria able to degrade plastic (Delacuvellerie *et al.*, 2019) (Table 1). Plastic samples were rinsed in sterile salt water (35 g/L of Sigma Sea Salt) for marine samples and in sterile fresh water for river samples to remove microorganisms that were not attached to the biofilm. Biofilm were scraped with a sterile scalpel blade to recover the maximum amount of bacterial and cultured in glass tubes containing 5 ml of low carbon source marine media for the marine samples (0.2% ammonium sulfate, 0.05% yeast extract, 3.5% salts (Sigma Sea Salt) and 1% trace elements (0.1% $MgSO_4.7H_2O$, 0.1% $FeSO_4.7H_2O$, 0.01% $ZnSO4.7H_2O$, 0.01% $CuSO_4.5H_2O$ and 0.01% $MnSO_4.5H_2O$) in 20 mM (N-morpholino) propane sulfonic acid (MOPS) pH 8; adapted from Yoshida *et al*., 2016) and 2 cm^2 plastic films (all the plastics are in film until the LMWPE that which is in the pellet form). Concerning the river samples, the composition of the medium is the same except that there is no sea salt. Five types of plastic were tested for each sample (Table 2). Plastics were sterilized in 70% ethanol overnight and dried in petri dishes in sterile air. Enrichment cultures were shaken at 140 rpm at 30 °C. After 80 days of culture, biofilm formation was observed in several tubes (Table S3). The bacterial communities from these biofilms were analyzed by DGGE and 16S rRNA sequencing. The weight loss method was used to follow the plastic degradation. After drying, the films were weighed and the percentage of weight loss was determined as follows (Roy *et al.*, 2008):

$$\text{Weight loss}\ (\%) = \frac{(m_i - m_f)}{m_i} \times\ 100$$

where m_i is the weight of the plastic at the initial time and m_f, the weight after the immersion time.

Table 1: Plastic sampled with a high amount of biofilm used to realized culture enrichment.

Type of sample	Sample name
River	EM19-F1 (replicate n°1, 2, 3 and 4)
Harbor	EM19-P1-1
Harbor	EM19-P4-1
Harbor	EM19-P5-4
Sampling by Manta net	EM19-03 (replicate n°3 and 6)
Sampling by Manta net	EM19-05-06
Sampling by Manta net	EM19-29-07

Table 2: Plastic characteristic used for the enrichment culture.

Plastic type	Provider	Form	Thickness	Density	Crystallinity
LDPE	Goodfellow	Film	0.2 mm	0.95 g/cm^3	40%
PET	Goodfellow	Film	0.2 mm	1.3-1.4 g/cm^3	Amorphous
PVC	Goodfellow	Film	0.2 mm	1.4 g/cm^3	Amorphous
PS	Goodfellow	Film	0.125 mm	1.05 g/cm^3	Amorphous
LMWPE (MW 2,000)	Polysciences	Pellet	/	0.97 g/cm^3	70%

2.4. Denaturing Gel gradient electrophoresis (DGGE)

The bacterial community structure before and after the enrichment culture were studied by DGGE. Touchdown PCR amplification of 200 bp fragments of the 16S rRNA gene was carried out using the following primers: 518R (5'-ATTACCGCGGCTGCTGG-3') and GM5F-C12 (5'-CGCCCGCCGCGCCCCGCGCCCGTCCCGCCGCCCCCGCCCGCCTACGGGAGGCAGC 46AG-3 ') (Gillan *et al*., 2005). The PCR cycle was as follows: 10 min at 95 °C, and then the first cycle was carried out 20 times using a denaturation temperature of 94 °C for 30 seconds, a hybridization temperature of 65 °C for 45 seconds (decreasing by 0.5 °C at each cycle) and an elongation temperature of 72 °C for 30 seconds. The second cycle was performed 10 times using the same denaturation and elongation temperatures and a hybridization temperature of 55 °C for 45 seconds. The PCR was terminated by a heating at 72 °C for 10 minutes (Gillan, 2004). Forty microliters of PCR products were analyzed by DGGE in a gel containing 25-75% (v/v) denaturants (100% denaturants corresponding to 40% (v/v) formamide and 7 M urea) (Gillan

et al., 2005). The gel also contained 0.5% (v/v) TAE buffer (Tris, Acetate, EDTA) and 10% acrylamide (v/v). Finally, 0.2% final (w/v) ammonium persulfate (APS) and 0.1% final (v /v) Temed were used to polymerize the gel. The gradient of the gel was made with a U-tube-type Gradient Former Model 385 Bio-Rad and the ISMATEC ® peristaltic pump at 0.170 ml/min. The gel was placed in the Bio-Rad DcodeTM vat and filled with 40 µl PCR products per well. The migration was carried out in 0.5% (v/v) TAE buffer for 16 hours at 60 °C and 75 volts (Gillan, 2004). The gel was then stained in a solution containing 0.005% Gel Red (v/v). The gel was visualized and photographed by UV Universal Hood II BIO-RAD.

2.5. DNA extraction for 16S rRNA amplicon sequencing

For the DNA extraction, plastic biofilms were scrapped with a sterile scalpel blade to recover a maximum of biomass. The DNA extraction were performed with the biofilm DNA isolation kit (NORGEN BIOTEK CORP. ©) following the manufacturer's instructions.

2.6. PCR for the 16S rRNA amplicon sequencing

A 460 bp fragment of the hypervariable V3-V4 region of the 16S rRNA gene of Bacteria and Archaea was amplified by PCR using the following primers: 806R (5'-GGACTACNNGG GTATCTAAT-3') and 341F (5'-CCTAYGGGRBGCASCAG-3') supplemented by overhang (adaptator illumina):

Forward overhang: 5' TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG-[341F]

Reverse overhang: 5' GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG-[806R]

3. Results

3.1. Plastic characterization

In the table 3, the first results of the plastic characterization of the sampling in 2019 are available. All these samples were not analyzed yet. With our first results, we can observe that the plastic nature that is the most represented into the plastic samples is the polyethylene (PE) (Fig.2) and the second plastic nature is the polypropylene (PP). Concerning the sampling near the coast and the harbor, polystyrene plastic (PS) were also detected. Concerning the plastic (Fig.3), most of plastic sample are smaller than 5 mm and so are microplastics. Some macroplastics are found near the harbor, the estuary and the coast. Interestingly, most of plastics are white color (Table 3).

Table 3: first result in the plastic characterization. The nature plastic is identified by ATR-FTIR. Unfortunately, some plastics were disintegrated after the DNA extraction and so the plastic nature was not defined (N.D). Plastics were classified according to the size: microplastic (> 20 mm), mesoplastic (5-20 mm) and microplastic (<5 mm) (Barnes *et al.*, 2009). The DNA concentration after the extraction is given. A part of the sample is being analyzed and the boxes are in blue, the work is in progress.

Sample name	DNA concentration (ng/ µl)	Nature plastic	Plastic color	Size (mm)	Macro-/meso- / micro-plastic
EM19-01-1	0,6	Polystyrene	white	2,5*2,5	microplastic
EM19-01-2	0,278	N.D.	white	1*1	microplastic
EM19-01-3	0,526	Polyethylene	white	3*1,5	microplastic
EM19-01-5	1,39	Polypropylene	transparent	5*5	mesoplastic
EM19-03-01	15,8	Polypropylene	transparent	35*14	macroplastic
EM19-03-02	0,329	Polystyrene	white	4*4	microplastic
EM19-03-03	3,49	Polyethylene	white	30*15	macroplastic
EM19-03-04	2,34	Polyethylene	white	6*6	mesoplastic
EM19-03-05	1,51	Polyethylene	white	11*7	mesoplastic
EM19-03-06	5,45	Polyethylene	white	130*90	macroplastic
EM19-05-01	2,93	Polyethylene	white	8*4,5	mesoplastic
EM19-05-02	12,1	Polyethylene	white	22*7	macroplastic
EM19-05-03	5,34	Polyethylene	transparent	12*8	mesoplastic
EM19-05-04	0,508	N.D.	white	3*3	microplastic
EM19-05-05	0,756	Polyethylene	blue	8*0,3	mesoplastic
EM19-05-06	2,37	Polypropylene	transparent	120*70	macroplastic
EM19-F1-1	3,47	Polyethylene	translucent	30*15	macroplastic
EM19-F1-2	5,86	Polyethylene	white	70*25	macroplastic
EM19-F1-3	10,2	Polystyrene	white	60*40	macroplastic
EM19-F1-4	1,38	Polyethylene	grey	30*25	macroplastic
EM19-P1-UM-1	13,9	Polystyrene	white	5*4; 6*3; 4*3	mesoplastic
EM19-P1-UM-2	2,04	Polyethylene	white	12*7	mesoplastic
EM19-P1-UM-3	1,47	Polyethylene	transparent	19*21	macroplastic
EM19-P1-UM-4	1,76	Polyethylene	translucent	10*5	mesoplastic
EM19-P1-UM-5	0,106	Polyethylene	white	3*3	microplastic
EM19-08-01	0,547	N.D.	translucent	3,5*2	microplastic
EM19-08-02	0,075	N.D.	white	3*2	microplastic
EM19-08-03	0,163	Polyethylene	beige	3*3	microplastic
EM19-08-04	12,7	Polyethylene	brown	20*7	mesoplastic
EM19-08-05	0,096	polypropylene	blue	3*1	microplastic
EM19-10-01	1,07	Polypropylene	pink	19*9	mesoplastic
EM19-10-02	1,8	polypropylene	transparent	27*8	macroplastic
EM19-10-03	6,73	polyethylene	white	12*11	mesoplastic
EM19-10-04	0,521	polyethylene	pink	95*4	macroplastic
EM19-10-05	0,334	polyethylene	mauve	9*4	mesoplastic

EM19-10-06	0,898	Polyethylene	translucent	4*4	microplastic
EM19-P2-01	10,8	Polypropylene	white	14*5	mesoplastic
EM19-P2-02	0,73	Polyethylene	translucent	11*5	mesoplastic
EM19-P2-03	1,81	Polypropylene	transparent	12*7	mesoplastic
EM19-P2-04	1,5	Polyethylene	transparent	54*26	macroplastic
EM19-P2-05	0,628	Polyethylene	white	4*4	microplastic
EM19-11-01	0,617	Polyethylene	red	23*3	macroplastic
EM19-11-02	0,079	Polyethylene	blue	5*3	mesoplastic
EM19-11-03	0,114	Polyethylene	white	4*2	microplastic
EM19-11-04	0,144	Polyethylene	white	4*2	microplastic
EM19-11-05	0,081	Polyethylene	transparent	6*3	mesoplastic
Em19-12-01	0,27	Polyethylene	blue	6*3	mesoplastic
Em19-12-02	/	N.D.	transparent	1,5*0,5	microplastic
Em19-12-03	0,124	Polyethylene	white	3*2	microplastic
Em19-12-04	/	Polyethylene	blue	2*1	microplastic
Em19-12-05	0,424	Polyethylene	white	3*2	microplastic
Em19-13-01	/	Polyethylene	mauve	2*2	microplastic
Em19-13-02	0,098	Polyethylene	translucent	5*3	mesoplastic
Em19-13-04	0,071	Polyethylene	translucent	2*2	microplastic
Em19-13-05	0,181	Polyethylene	black	8*2	mesoplastic
Em19-14-01		Polystyrene	translucent	2*1	microplastic
Em19-14-02		N.D.	translucent	2*1	microplastic
Em19-14-03		Polystyrene	white	1,5*1,5	microplastic
Em19-14-04		N.D.	white	1*1	microplastic
EM19-15-01		N.D.	white	1*1	microplastic
EM19-15-02		Polystyrene	white	2*1	microplastic
EM19-15-03		Polyethylene	translucent	2*1	microplastic
EM19-16-01		Polyethylene	white	4*4	microplastic
EM19-16-02		Polystyrene	white	4*2,5	microplastic
EM19-16-03		Polyethylene	white	4*3	microplastic
EM19-16-04		Polyethylene	white	4*4	microplastic
EM19-16-05		Polyethylene	translucent	6*6	mesoplastic
EM19-17-02		Polyethylene	white	2*1	microplastic
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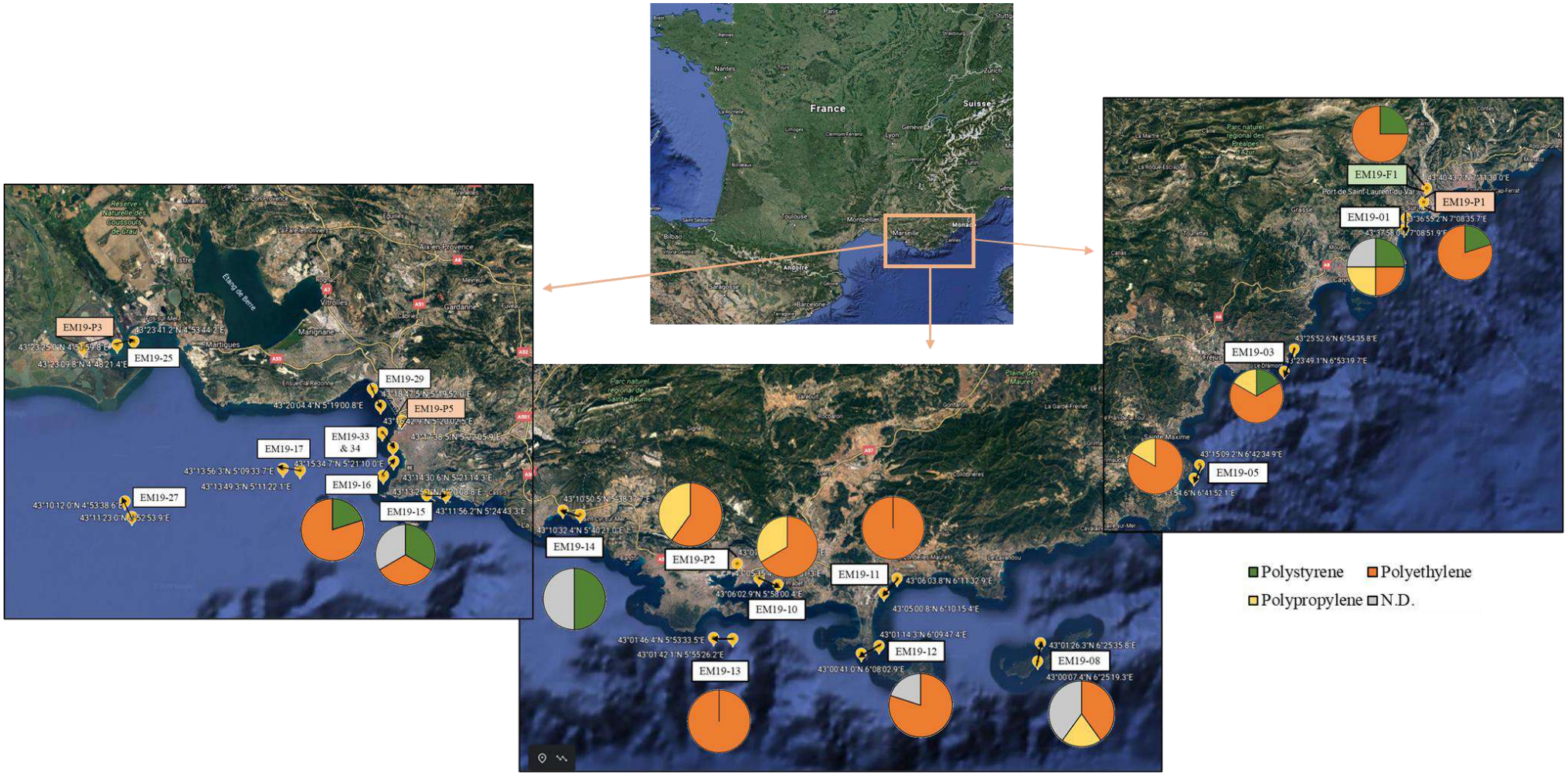

Figure 2: Map of all the 2019 sampling from the Mediterranean Sea with plastic nature analysed by ATR-FTIR on the pie chart. The green color represented the polystyrene, the orange the polyethylene, the yellow the polypropylene and in grey, is “not determined plastic” (due to its disintegration after the DNA extraction).

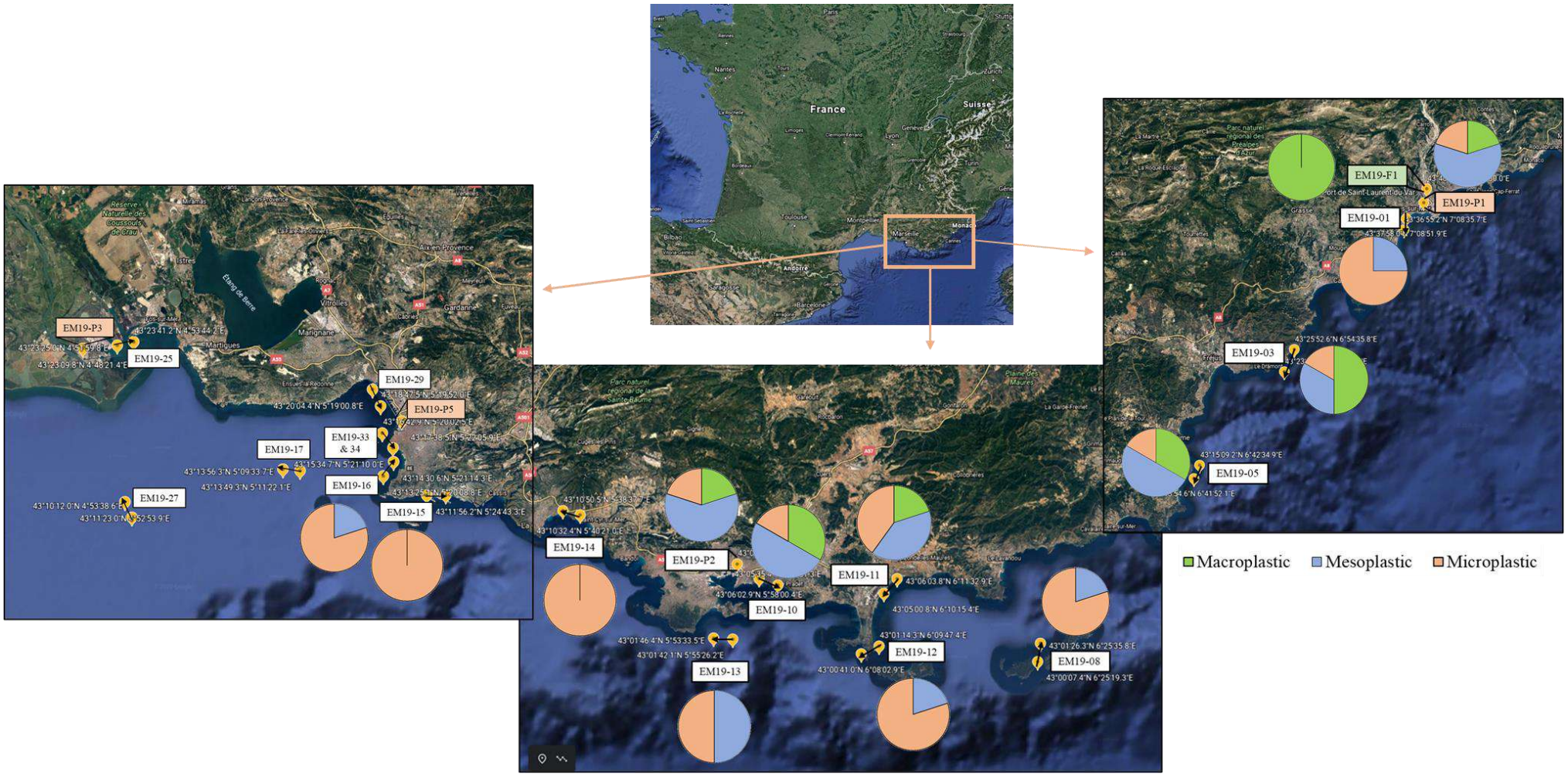

Figure 3: Map of all the 2019 sampling from the Mediterranean Sea with plastic size on the pie chart. The green color represented the macroplastic (> 20 mm), the blue color the mesoplastic (5-20 mm) and the orange the microplastic (<5 mm)

3.2. Enrichment culture

The aim of the enrichment culture is to try to select bacteria able to degrade plastic. Enrichment culture were performed on 5 plastics nature as the main carbon source: LDPE, LMWPE, PET, PS and PVC. After 80 days of culture, biofilm formation was observed in several tubes (Table S3; figure S1). The most developed biofilms were observed on the LDPE and LMWPE plastic. This observation can be explained by the fact that the polyethylene is the most hydrophobic plastic and this plastic floats, so the oxygen accessibility is better. The weight loss method was used to follow the degradation (Table 4). A weight loss was observed in few tubes with, in general, a weight loss of less than 1 percent. The percentage is higher for 3 samples: enrichment culture from EM19-03-06 on LMWPE, EM19-P4-01 on PS and EM19-P5-04 on LMWPE, with 5.6%, 2.11% and 1.58%, respectively. The process of the plastic degradation by the bacteria is very slow and is carried out in optimum conditions that explained the low weight loss. The bacterial communities from these biofilms were analyzed by DGGE (Fig.4-13).

Table 4: Percentage of the weight loss of plastic after the 80 days of culture enrichment. The red boxes represent plastic where there was no biofilm formation. NA is for plastic that some plastic pieces were lost during the experimentation.

	PVC	PET	PS	LDPE	LMWPE
EM19-03-03			0	0	0
EM19-F1-1	0	0,28	0,46	0	0
EM19-F1-2	0	0	0	0	NA
EM19-F1-3	0	0	0	0,52	0
EM19-F1-4		0	0	0	NA
EM19-P1-01	0	NA	0	0	0
EM19-03-06	0,18	0	0	0	5,6
EM19-05-06				0,65	0
EM19-P4-01	0	0,59	2,11	0	0
EM19-P5-04	0	0	0	0	1,58
EM19-29-07	0		0	0,25	NA
NC fresh water	0	0	0	0	0
NC sea salt	0	0	0	0,2	0

On the first DGGE gel, we compare the bacterial community composition before the enrichment culture with the microbial composition after the 80 days of culture enrichment on a specific plastic (PET, PVC, LDPE, LMWPE and PS) (Fig.4-8). The first observation is that the richness of the community is high, we obtained a high number of bands on our DGGE gel. Interestingly, the bacterial community composition after the enrichment culture is significantly different from the initial community due to the change of culture conditions (temperature,

medium with low concentration of carbon,…). However, some bands are similar. If we compare the bacterial composition after the enrichment culture developed on the different plastic, interestingly, some bacteria are developed on all the plastic (white rectangle on the figure 4) but some bacteria were enrichment only on one plastic nature, e.g. the blue rectangle one the PS on the fig.4. It is very interesting that some bacteria are enrichment on only one plastic nature. It will be interesting to identify these bacteria to observe of these microorganisms could be a role in the plastic degradation. The LDPE and the LMWPE have a same structure but the molecular weight and the plastic shape are different (film *vs* pellet). The bacterial community able to developed on polyethylene could have been the same on these two plastics. It is not the case, so we can conclude that the plastic nature has a significantly role in the enrichment culture but not only. Indeed, the plastic shape and the molecular weight can also influence the development of the microbial biofilm. On some sample, one bacterium is very enriched and dominate the communty as seen on the figure 6 for the EM19-03-03 on the PS (Fig.6).

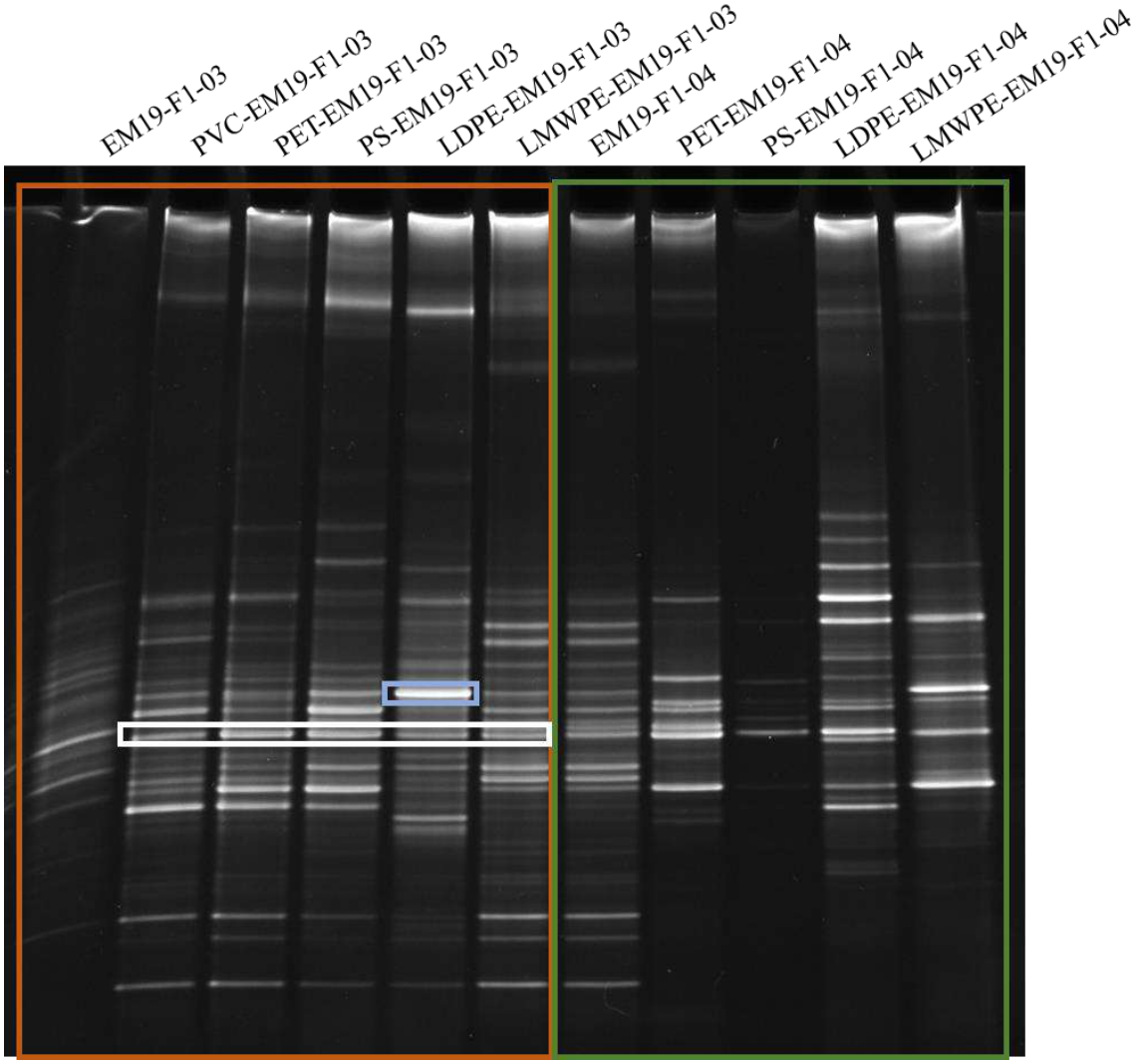

Figure 4: bacterial composition before the enrichment culture (EM19-F1-03 & EM19-F1-04) and after the 80 days of enrichment culture on several plastic (PVC, PET, PS, LDPE and LMWPE.

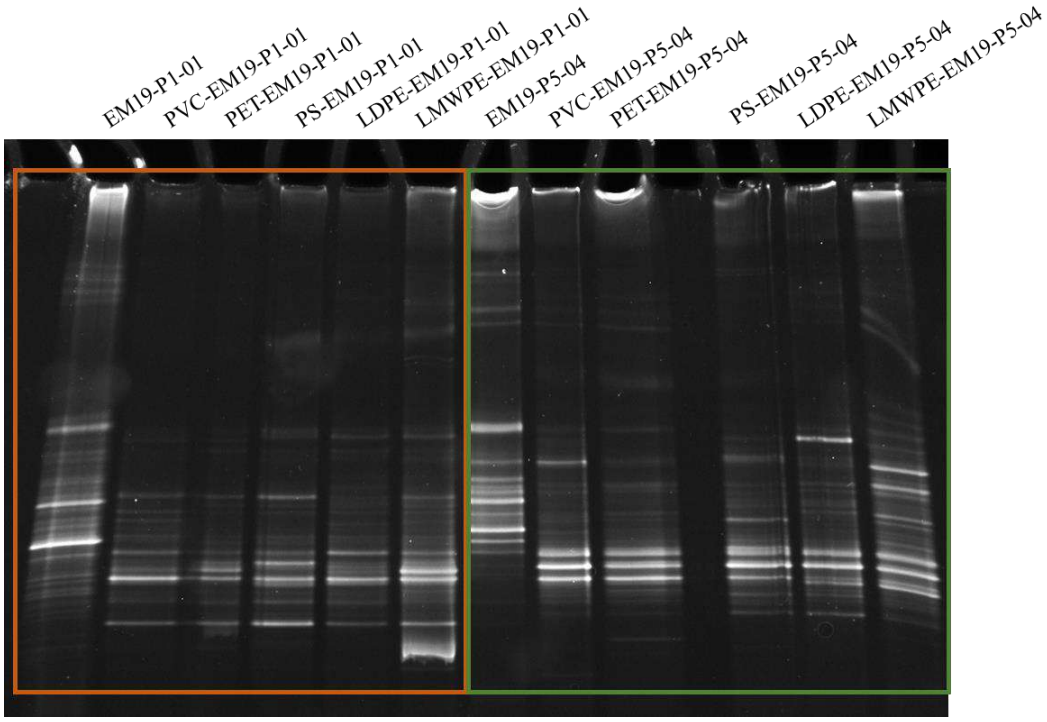

Figure 5: bacterial composition before the enrichment culture (EM19-P1-01 & EM19-P5-04) and after the 80 days of enrichment culture on several plastic (PVC, PET, PS, LDPE and LMWPE).

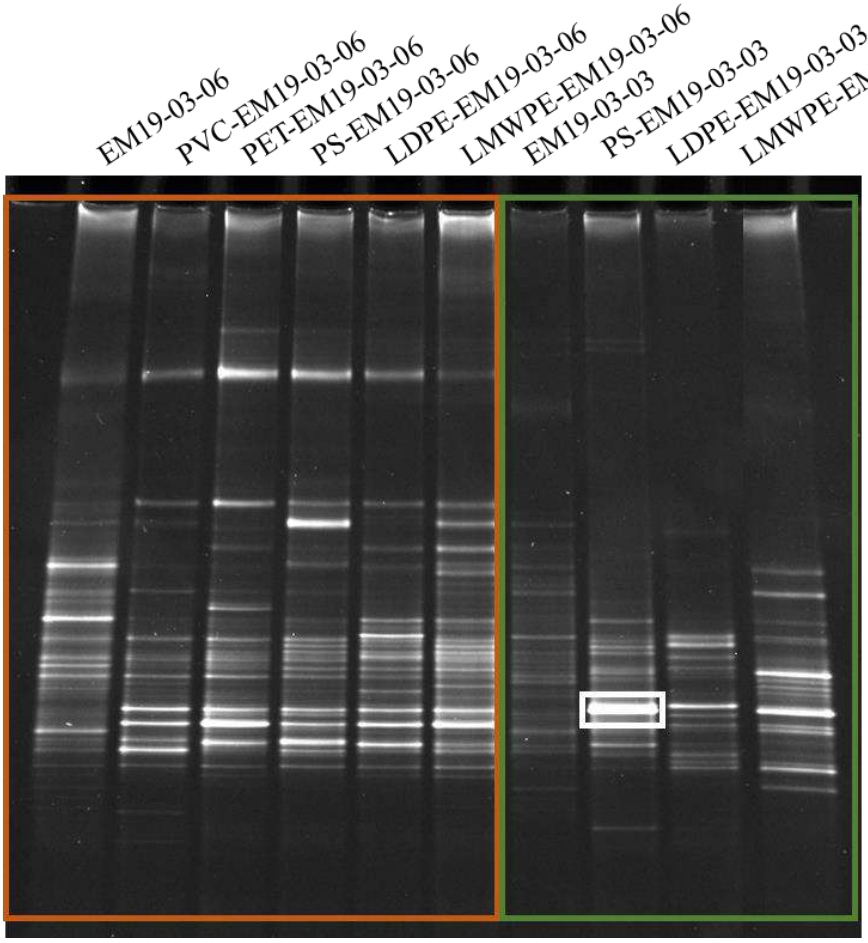

Figure 6: bacterial composition before the enrichment culture (EM19-03-06 & EM19-03-03) and after the 80 days of enrichment culture on several plastic (PVC, PET, PS, LDPE and LMWPE).

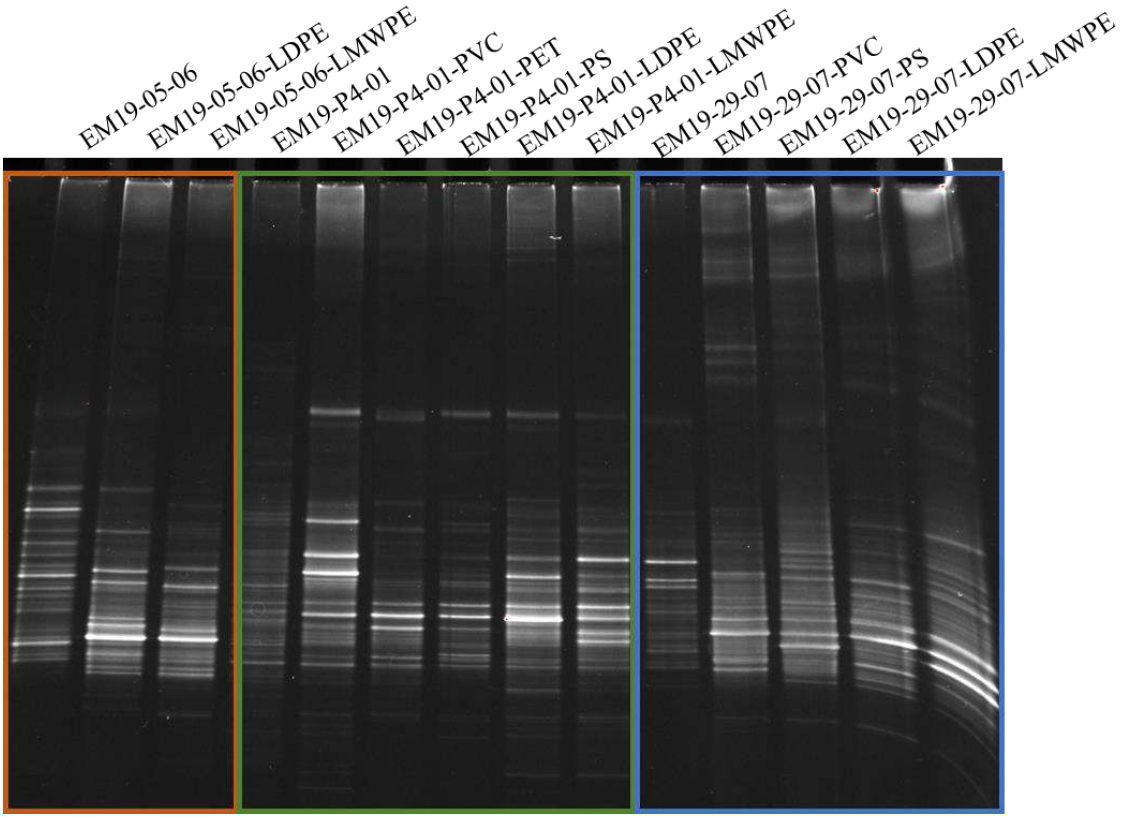

Figure 7: bacterial composition before the enrichment culture (EM19-05-06, EM19-P4-01 & EM19-29-07) and after the 80 days of enrichment culture on several plastic (PVC, PET, PS, LDPE and LMWPE).

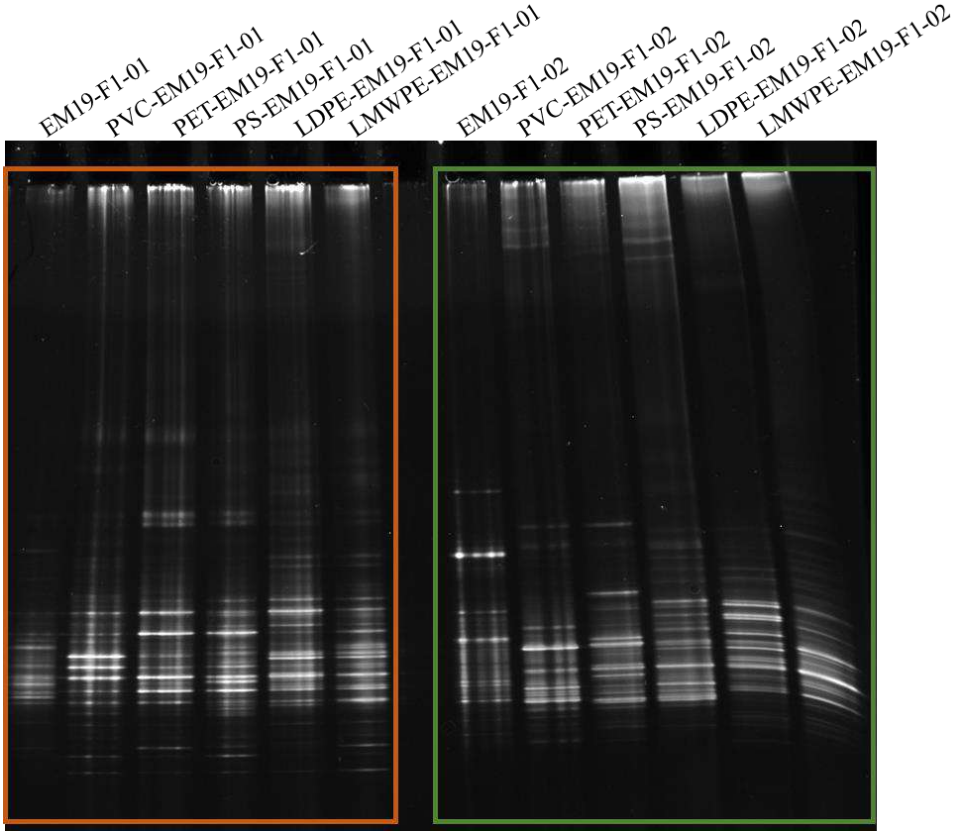

Figure 8: bacterial composition before the enrichment culture (EM19-F1-01 & EM19-F1-02) and after the 80 days of enrichment culture on several plastic (PVC, PET, PS, LDPE and LMWPE).

After these first DGGE, we compare the bacterial community after the 80 days of enrichment culture on the same plastic nature (Fig.9-13). Despite the fact that the starting cultures come from different plastics and therefore are composed of different bacterial communities, microbial community developed on LDPE for example is composed by a common bacterial core. However, due to the difference of the initial bacterial community, some bands are present only in one sample. To better understand the role of these bacteria selected by the enrichment culture on several plastic, it will be necessary to analyze the 16S rRNA amplicon sequencing results.

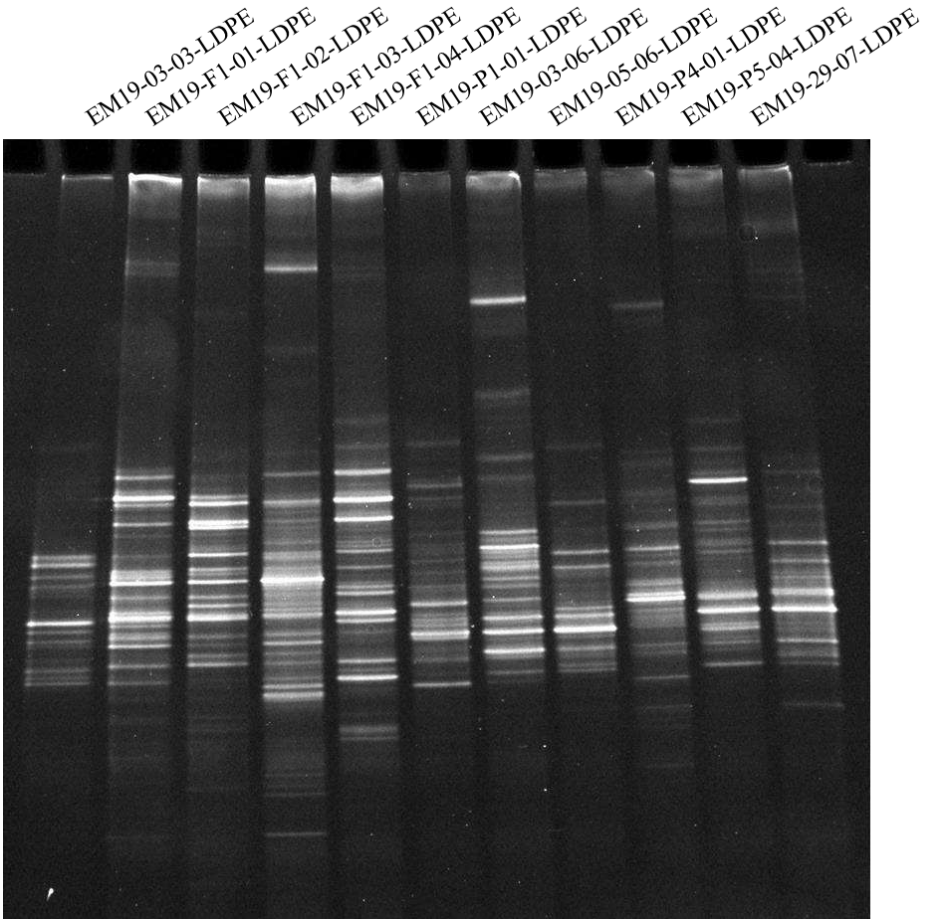

Figure 9: bacterial composition after the 80 days of enrichment culture in presence of LDPE film.

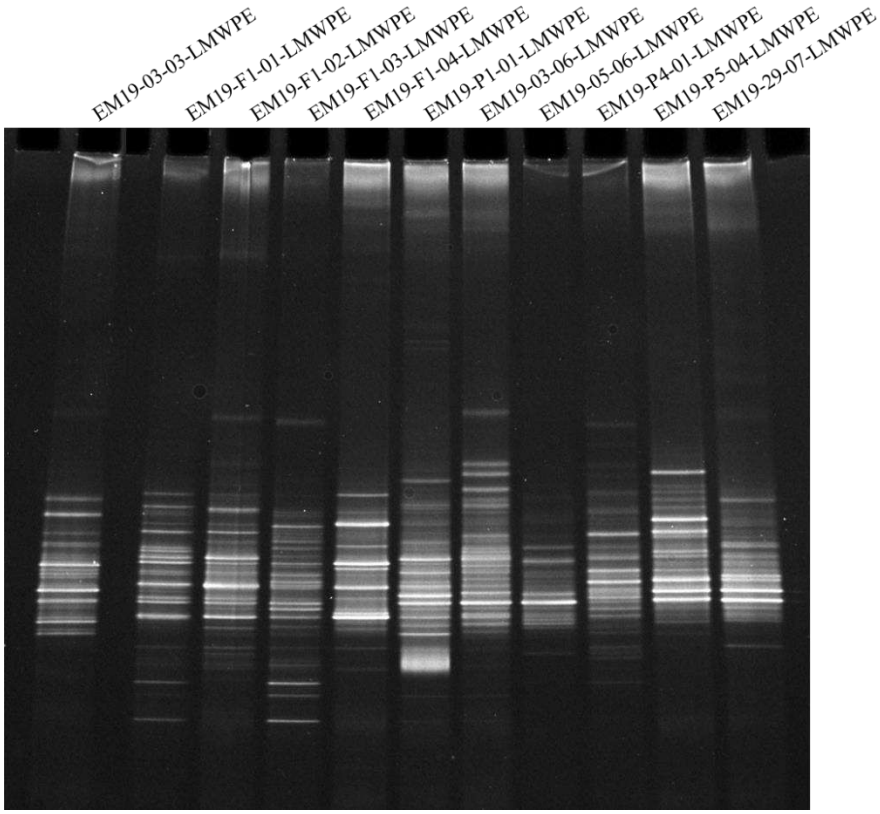

Figure 10: bacterial composition after the 80 days of enrichment culture in presence of LMWPE pellets.

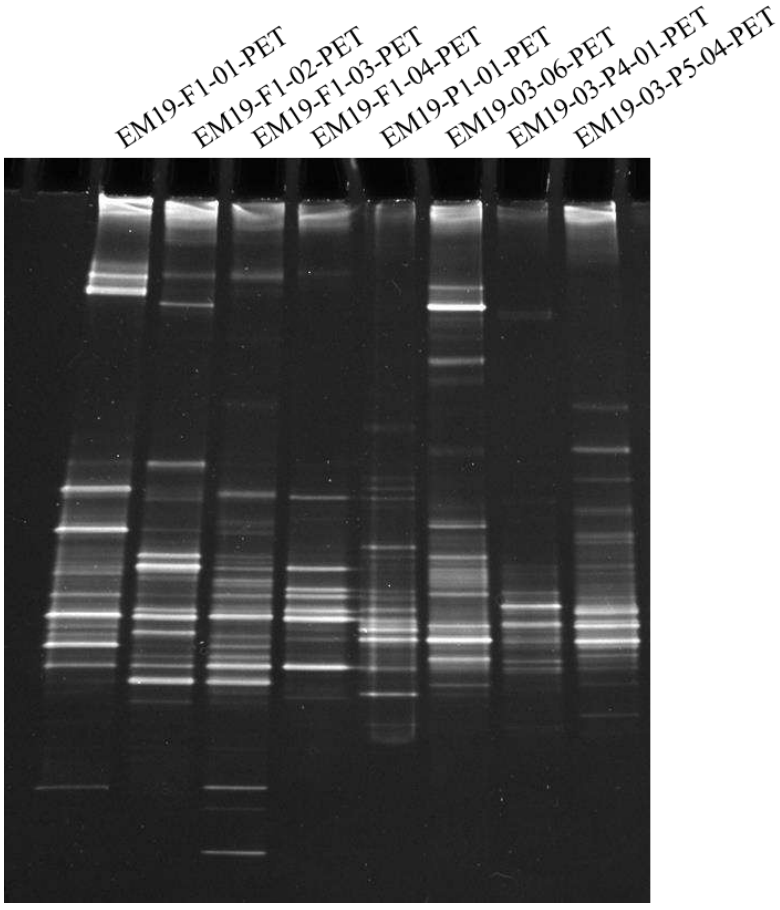

Figure 11: bacterial composition after the 80 days of enrichment culture in presence of PET film.

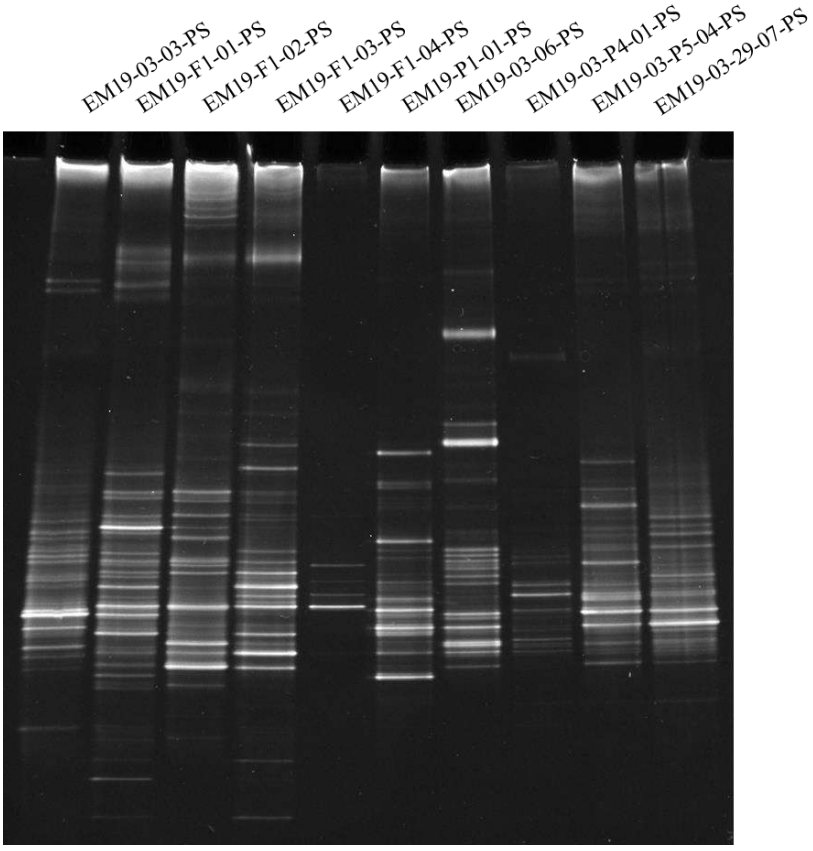

Figure 12: bacterial composition after the 80 days of enrichment culture in presence of PS film.

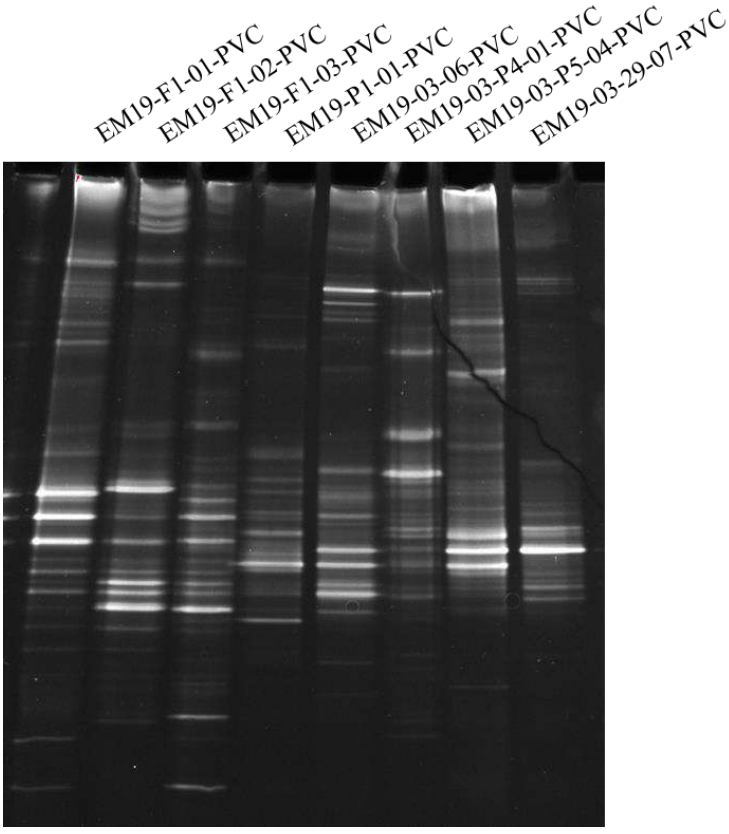

Figure 13: bacterial composition after the 80 days of enrichment culture in presence of PVC film.

3.3. DNA extraction and 16S rRNA amplicon sequencing

The DNA extraction were carried out on all the enrichment culture and on these samplings: EM19-01; EM19-03, EM19-05, EM19-08, EM19-10, EM19-11, EM19-12, EM19-13, EM19-14, EM19-15, EM19-16, EM19-17, EM19-P1, EM19-P2 and EM19-F1. The PCR for the 16S rRNA amplicon sequencing were performed on: EM19-01, EM19-03, EM19-05, EM19-08, EM19-10, EM19-F1 and EM19-P1. These first samples have been sent to the GIGA platform in Liège to be sequenced.

4. Perspectives and timing

Gantt diagram for the rest of the analysis:

	2020						2021				
	7	8	9	10	11	12	1	2	3	4	5
DNA extraction (continuation and end)											
PCR for the sequencing (continuation and end)											
Plastic identification by ATR-FTIR (continuation and end)											
Analysis of 16S rRNA amplicon sequencing											
Article writting with 16S rRNA amplicon sequencing results											
Optimisation of the metaproteomic protocol											
Metagenomic and metaproteomic analysis											
Article writting with metaproteomic and metagenomic results											

Bacterial community will be study by 16S rRNA amplicon sequencing to better understand the plastisphere. For that, the bacterial composition will be studied in comparison of different parameters: (1) plastic natures (Polypropylene, polyethylene, polystyrene, …); (2) plastic size (micro-, meso-, microplastic), (3) plastic colors; (4) sampling places (inshore, offshore, harbor, river); (5) water temperature; (6) sampling geography. Moreover, the bacterial community selected by the enrichment culture will be studied by 16S rRNA amplicon sequencing to find potential candidate for the plastic degradation. With these results and the results of the plastic nature, a first article could be written. We try to optimize the protocol for the metaproteomic analysis. When the protocol will be well developed, metaproteomic analysis and metagenomic analysis will be carried out on the macroplastics. If the results are conclusive, a second article could be written.

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