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# Écologie microbienne associée à la biodégradabilité des plastiques en milieu marin

Léna Philip

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Léna Philip. Écologie microbienne associée à la biodégradabilité des plastiques en milieu marin. Ecologie, Environnement. Sorbonne Université, 2023. Français. NNT : 2023SORUS505 . tel-04573446

**HAL Id: tel-04573446**

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École doctorale des Sciences de l'Environnement d'Ile de France (ED 129)

SAS Plastic At Sea

Laboratoire d'Océanographie MICrobienne (UMR 7621)

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# Écologie microbienne associée à la biodégradabilité des plastiques en milieu marin

Thèse préparée et présentée par

**Léna PHILIP**

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Thèse soutenue publiquement le 7 Novembre 2023

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## Remerciements

Mes premiers remerciements s'adressent à Marisol Goni et Stéphane Pesce, ainsi que Lise Barthelmebs, Morgan Deroiné, Boris Eyheraguibel et Pierre Galand, d'avoir accepté de faire partie de mon jury et d'évaluer mon travail. Merci également à l'ANRT de m'avoir accordé cette bourse de thèse, et à Fabien Joux de m'avoir accueillie au sein du LOMIC.

Mes prochains remerciements vont à Leila Meistertzheim et Jean-François Ghiglione, merci de m'avoir accueillie d'abord pour un stage, puis en thèse. Merci de m'avoir formée à la recherche, encadrée et suivie tout au long de ce processus, jusqu'à la rédaction de ce manuscrit. Je souhaite également remercier chaleureusement Isabelle Calves pour son accompagnement sans faille depuis 3 ans et demi, ainsi que Karine Lebaron, pour toutes ces manip préparées et lancées ensemble, son soutien, ses conseils et tous les moments partagés.

Le bon déroulement de cette thèse n'a pas été sans compter sur l'aide de nombreuses personnes. Un grand merci au service de verrerie, et en particulier à Laurence, sans qui les expériences n'auraient pas pu être lancées. Je remercie également Mireille Pujo-Pay, Olivier Crispi, Nyree West, Laurent Intertaglia, David Pecqueur, Christophe Salmeron ainsi que l'ensemble du personnel du LOMIC et de Bio2Mar de m'avoir aidée et d'avoir contribué à mon travail. Je souhaite aussi remercier Mounir Traïka de l'ICCF ainsi que Valérie Barbe et le Génoscope pour le traitement de mes échantillons. Merci aux équipages d'Expédition 7<sup>ème</sup> Continent pour cette introduction au monde de la navigation. Merci également à Samuel ainsi que Clément et Eva pour le travail réalisé au cours de leurs stages, mais aussi les moments partagés au-delà du travail. J'aimerais également remercier Valérie Vergé pour sa bienveillance et sa bonne humeur à toute épreuve.

Je souhaite remercier tous mes collègues de Plastic At Sea. Laurie, merci pour ton humour et ta gentillesse, tant au travail qu'en dehors. Tu es une capitaine en or ! Edouard, merci pour ta disponibilité et ton aide. Loïc, merci pour ton travail d'aquario indispensable aux expériences réalisées ces trois dernières années. Leila, merci pour ton aide sur ces derniers mois de thèse. Merci à Elo, Sylvain, Nico, Thomas, Emma, merci à toute l'équipe et tous les anciens collègues avec qui j'ai pris plaisir à travailler.

C'est bien naturellement que je remercie du fond du cœur toutes mes amies sans qui ces années banyulencques n'auraient pas eu la même saveur. Mes mamitas, Doudoule, Mathou, Brunita, Anaël, Milé, Cloclo ; mes camarades de bureau, Max, Emilio et Louis, ainsi que Totoche, Coco et toutes les autres copines et copains, pour les moments passés au labo mais aussi et surtout en dehors ; les cafés du dimanche, les yogas matinaux, les sessions broderie sur la plage, les vendredis au Corsaire, les randos, les déjeuners à la crique... Bien évidemment, merci à Salom et David, mes deux chers colocataires, pour cette vie partagée pendant 3 ans et demi. Enfin, merci à Gabrielle, ma co-thésarde, pour tout, depuis ce déjeuner ambiancé sur la plage centrale, en passant par nos dîners intellectuels à Roscoff, jusqu'à l'écriture de no(tre)s thèse(s). C'est une chance d'avoir eu un binôme comme toi, pour le meilleur et pour le pire !

Je ne pourrai jamais remercier assez mes parents. Maman, Papa, merci de m'avoir permis d'aller jusqu'à la thèse, et de m'avoir toujours supportée et soutenue. Merci à ma famille, Axel, Roman, Rux et Grany, pour leur amour inconditionnel. Merci également à toutes mes amies qui m'ont soutenue de loin.

Pour terminer, merci à toi Thomas. Tu as été mon pilier le plus solide durant ces trois années. Merci pour ta patience, tes encouragements et ton amour.



## **Liste des communications orales**

### **International Symposium on Biopolymers (ISBP, en ligne)**

Léna Philip et Gabrielle Derippe – PHA biodegradation in the marine environment : an innovative approach

### **10<sup>ème</sup> colloque de l'Association Française d'Ecologie Microbienne (AFEM, en ligne)**

Léna Philip, Gabrielle Derippe, Pierre Lemechko, David Leistenschneider, Mireille Pujo-Pay, Pascal Conan, Karine Lebaron, Isabelle Calves, Anne-Leila Meistertzheim, Jean-François Ghiglione, Stéphane Bruzaud – Biodégradabilité en milieu marin de différents plastiques biosourcés à façon

### **Rencontres du groupement de recherche Polymère et Océans**

Léna Philip, Gabrielle Derippe, Isabelle Calves, Karine Lebaron, David Leistenschneider, Mireille Pujo-Pay, Pascal Conan, Boris Eyheraguibel, Alexandra Ter Halle, Anne-Leila Meistertzheim, Jean-François Ghiglione – Évaluation des mécanismes de biodégradation des plastiques en mer par des tests multidisciplinaires et miniaturisés

### **MICRO 2022 (en ligne)**

Léna Philip, Charlène Odobel, Justine Jacquin, Gabrielle Derippe, Emilie Villar, Valérie Barbe, Stéphane Bruzaud, Anne-Leila Meistertzheim, Jean-François Ghiglione – Microbial biodegradation of polyhydroxyalkanoates in the marine environment

### **3<sup>rd</sup> International Conference in Microbial Ecotoxicology - Ecotoxicomic 2022**

Léna Philip, Charlène Odobel, Justine Jacquin, Gabrielle Derippe, Emilie Villar, Valérie Barbe, Stéphane Bruzaud, Anne-Leila Meistertzheim, Jean-François Ghiglione – Microbial role in plastic biodegradation in the marine environment: the case of polyhydroxyalkanoates

### **Symposium métrologie du plastique**

Léna Philip, Maëla Le Picard, Edouard Lavergne, Isabelle Calves, Anne-Leila Meistertzheim, Pascaline Bourgain, Brigitte Sabard, Camille Lacroix, Jean-François Ghiglione – La science participative pour l'analyse de la pollution plastique : points forts et limitations

## **Publications**

Charlène Odobel, Claire Dussud, Léna Philip, Gabrielle Derippe, Marion Lauters, Boris Eyheraguibel, Gaëtan Burgaud, Alexandra Ter Halle, Anne-Leila Meistertzheim, Stéphane Bruzaud, Valérie Barbe, Jean-François Ghiglione – Bacterial abundance, diversity and activity during long-term colonization of non-biodegradable and biodegradable plastics in seawater  
<https://doi.org/10.1016/j.envpol.2022.120463>

Pascal Conan, Léna Philip, Eva Ortega-Retuerta, Charlène Odobel, Clélia Duran, Caroline Pandin, Carolane Giraud, Anne-Leila Meistertzheim, Valérie Barbe, Alexandra Ter Halle, Mireille Pujo-Pay, Jean-François Ghiglione – Evidence of coupled autotrophy and heterotrophy on plastic biofilms and its influence on surrounding seawater  
<https://doi.org/10.3389/fmicb.2021.734782>

Gabrielle Derippe, Léna Philip, Pierre Lemechko, Boris Eyheraguibel, Anne-Leila Meistertzheim, Mireille Pujo-Pay, Pascal Conan, Valérie Barbe, Stéphane Bruzaud, Jean-François Ghiglione – Marine biodegradation of various tailor-made polyhydroxyalkanoates (PHA) influenced by the chemical structure and associated bacterial communities  
Soumis à Journal of Hazardous Materials

Léna Philip, Maëla Le Picard, Edouard Lavergne, Pascaline Bourgain, Brigitte Sabard, Romain Troublé, Anne-Leila Meistertzheim, Wolfgang Ludwig, Alexandra Ter Halle, Camille Lacroix, Jean-François Ghiglione – Comparison of the macro-, meso- and microplastic pollution in French riverbanks and beaches using citizen science with schoolchildren  
Soumis prochainement à Environmental Science and Pollution Research, numéro spécial « Source, fate and effects of plastic litters in the land-sea continuum »

Léna Philip, Leila Chapron, Valérie Barbe, Gaëtan Burgaud, Ika Paul-Pont, Odon Thiebeauld, Alexandra Ter Halle, Boris Eyheraguibel, Wolfgang Ludwig, Stéphane Pesant, Mikael Kedzierski, Anne-Leila Meistertzheim, Jean-François Ghiglione – A pan-European study of plastisphere diversity along the river-sea continuum  
Soumis prochainement à Environmental Science and Pollution Research, numéro spécial « Source, fate and effects of plastic litters in the land-sea continuum »

Léna Philip, Karine Lebaron, Isabelle Calves, Edouard Lavergne, Mireille Pujo-Pay, Boris Eyheraguibel, Anne-Leila Meisterthzeit, Jean-François Ghiglione – The significance of microbial inoculum and test medium for the evaluation of plastic biodegradability in seawater  
Soumis prochainement à Environmental Pollution

Léna Philip, Karine Lebaron, Isabelle Calves, Edouard Lavergne, Gabrielle Derippe, Pierre Lemechko, Stéphane Lemechko, Stéphane Bruzard, Mireille Pujo-Pay, Pascal Conan, Mounir Traïka, Boris Eyheraguibel, Valérie Barbe, Anne-Leila Meistertzzeit, Jean-François Ghiglione – Influence of monomer composition on polyhydroxyalkanoates (PHA) biodegradability in seawater  
Soumis prochainement à Marine Pollution Bulletin

## Liste des abréviations

ADNr 16S : gène codant pour sous-unité 16S de l'ARN ribosomique  
ASV : amplicon sequence variant  
BP : heterotrophic bacterial production  
CO<sub>2</sub> : carbon dioxyde  
DBO : demande biologique en oxygène  
DIC : dissolved organic carbon  
dPhaZ : PHA dépolymérase extracellulaires  
FL : free-living bacteria  
FTIR : spectroscopie infrarouge à transformée de Fourier  
GS : gas chromatography  
HB : acide hydroxybutyrique  
ITS : internal transcribed spacer  
LDPE : polyéthylène à faible densité  
*mcl*-PHA : medium-chain length PHA  
MEB : microscopie électronique à balayage  
MM : (carbon-)minimum medium  
NMDS : nonmetric multidimensional scaling  
MP : microplastic  
*p*MP : pristine microplastic  
NO<sub>3</sub><sup>-</sup> : nitrates  
nPhaZ : PHA dépolymérase intracellulaires  
O<sub>2</sub> : dioxygène  
OCDE : Organisation de Coopération et de Développement Économiques  
PA : particle-attached bacteria  
PBAT : polybutylène adipate téréphtalate  
PCL : polycaprolactone  
PCR : amplification en chaîne par polymérase  
PD : plastic debris  
PE : polyethylene  
PEVA : polyethylene vinyl acetate  
PHA : polyhydroxyalkanoates

PHB : poly(3-hydroxybutyrate)  
PHBHHx : poly(3-hydroxybutyrate-co-3-hydroxyhexanoate)  
PHBHV : poly(3-hydroxybutyrate-co-3-hydroxyvalerate)  
PHHp : poly(3-hydroxyheptanoate)  
PHN : poly(3-hydroxynonanoate)  
PHO : poly(3-hydroxyoctanoate)  
PHOHHp : poly(3-hydroxyoctanoate-co-3-hydroxyheptanoate)  
PHOHHx : poly(3-hydroxyoctanoate-co-3-hydroxyhexanoate)  
PLA : acide polylactique  
PO<sub>4</sub> : phosphate  
PP : polypropylene  
PS : polystyrene  
PVC : polyvinyl chloride  
RMN : résonance magnétique nucléaire  
*scf*-PHA : short-chain length PHA  
SEC : steric exclusion chromatography  
SSW : sterile sea water  
SW : sea water  
UV : ultraviolets

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## *Contexte et objectifs de la thèse*

L'omniprésence de la pollution plastique est maintenant largement reconnue, quel que soit l'environnement considéré (Veidis et al., 2022). Si les effets sur la santé humaine sont encore une question ouverte, les effets délétères de ces contaminations sur les écosystèmes naturels sont indéniables (Carney Almroth & Eggert, 2019). La réduction de la pollution plastique est devenue un enjeu de santé humaine et de santé de l'environnement. Plusieurs lois ont déjà été actées en ce sens au niveau national, interdisant notamment progressivement les plastiques à usage unique, pour une disparition complète des plastiques jetables d'ici 2040 (Loi anti-gaspillage pour une économie circulaire, Ministère de la Transition Ecologique, 2021). Un traité international contre la pollution plastique doit voir le jour en 2025, afin de tenter de coordonner les efforts de lutte au niveau mondial. Le plastique est une composante économique importante, dont les usages sont multiples et quotidiens. Bien que la réduction de la consommation de certains plastiques, tels que les plastiques à usage unique, soit souhaitable et nécessaire, il n'est aujourd'hui pas imaginable d'arrêter complètement leur utilisation.

La durabilité des plastiques conventionnels contribue à leur impact prolongé sur les milieux naturels (Zalasiewicz et al., 2016). Ainsi, le remplacement de ces plastiques récalcitrants au profit de plastiques biodégradables fait partie de la stratégie déployée pour lutter contre cette pollution. Le développement de ces plastiques a d'abord été envisagé pour des applications bien particulières, notamment les emballages alimentaires. Ainsi, la biodégradabilité des plastiques a longtemps été étudiée dans des conditions de compostage industriel, c'est-à-dire des conditions contrôlées non retrouvées dans les environnements naturels (Degli Innocenti & Breton, 2020). Depuis les années 2010, la recherche scientifique sur la pollution plastique dans les océans a augmenté de manière exponentielle, s'accompagnant naturellement d'une volonté de développer des plastiques également biodégradables en milieu marin. Les méthodes d'évaluation de la biodégradabilité en milieu tellurique et en compost industriel ont alors été transposées pour le milieu marin. Cependant, plusieurs études ont pointé du doigt le manque de pertinence de ces protocoles normés au regard des conditions naturelles du milieu marin, dont ils ne sont pas représentatifs (Harrison et al., 2018). La dernière norme de spécification pour les plastiques dans l'environnement marin a été annulée en 2014, et aucune norme ne l'a remplacée depuis.



L'objectif de cette thèse est d'apporter des éléments de compréhension et de discussion sur la biodégradabilité des plastiques en milieu marin, pour identifier une méthodologie pertinente de test dans des conditions proches de celles du milieu naturel et ainsi, accompagner de manière plus robuste le développement de nouvelles matières plastiques.

Ma thèse a bénéficié d'un financement CIFRE entre des laboratoires de recherche public et privé, pour s'inscrire à la frontière entre la science académique et son transfert vers l'industrie :

- La société Plastic At Sea, créée en 2018 par Anne-Leila Meistertzheim et Jean-François Ghiglione, est une PME innovante qui vise à accompagner les industriels dans leur transition plastique en proposant notamment de tester la biodégradabilité et la toxicité de leurs plastiques en conditions naturelles. La mise en œuvre de tests de biodégradabilité dans des conditions plus proches des conditions naturelles est un objectif majeur de cette société car ils doivent permettre d'identifier des alternatives aux polymères conventionnels, plus respectueuses de l'environnement et ainsi permettre de définir des choix stratégiques pour la mise sur le marché de nouveaux produits.
- L'équipe « Ecotoxicologie microbienne marine et ingénierie métabolique » du Laboratoire d'Océanographie Microbienne (LOMIC, UMR7621) travaille depuis plusieurs années sur les communautés bactériennes associées aux débris plastiques en milieu aquatique, ainsi qu'à leur rôle potentiel dans la biodégradation de ces déchets.

Des travaux précédents réalisés par les membres de ces deux entités ont été réalisés avec le Ministère de la transition écologique, pour tester par exemple des substituts biodégradables aux matières plastiques conventionnelles pour les cotons tiges ou encore les microbilles exfoliantes présentes dans les cosmétiques (Cheng et al., 2022; Jacquin et al., 2021). Ils ont participé à l'écriture d'un décret concernant l'interdiction des matières plastiques pour ces usages (décret n°2017-291, du 6 mars 2017).

Ce manuscrit de thèse est organisé en cinq chapitres :

- Le **premier chapitre** dresse un état de l'art sur les plastiques biodégradables, les mécanismes associés à leur biodégradation en milieu marin ainsi que les méthodologies actuelles disponibles pour ces études. Un intérêt est également porté sur la plastisphère, biofilm spécifique associé aux déchets plastiques, jouant un rôle essentiel dans leur biodégradation.

- Le **deuxième chapitre** présente des données issues de la mission *Tara Microplastiques* coordonnée par Jean-François Ghiglione (LOMIC) avec le soutien de la

Fondation Tara Océan, visant à étudier la pollution plastique le long du continuum fleuve-mer. L'étude des communautés bactériennes associées aux plastiques le long de neuf grands fleuves européens y est présentée et discutée.

- Le **troisième chapitre** se concentre sur l'influence des paramètres expérimentaux sur l'étude de la biodégradabilité marine des plastiques en laboratoire. En particulier, plusieurs inocula bactériens à différentes concentrations et plusieurs milieux de culture ont été testés. Ce chapitre donne lieu à une discussion sur les paramètres influençant la biodégradabilité des plastiques en milieu marin, et propose des recommandations quant aux précautions méthodologiques à mettre en œuvre pour l'évaluation de la biodégradabilité marine en milieu contrôlé.

- Le **quatrième chapitre** est une application du protocole présenté dans le chapitre 3 pour un cas d'étude : les polyhydroxyalkanoates (PHA), des polymères biosourcés et biodégradables. Une étude de biodégradabilité a été conduite sur 7 formules de PHA durant 90 jours, et des réponses de biodégradabilité variables ont été observées, relatives à la composition chimique des PHA.

- Le **cinquième chapitre** de ce document dresse une conclusion générale et propose différentes perspectives à ce travail de thèse.



## *Chapitre 1 : État de l'art*

# 1. Pollution plastique en mer

## 1.1. Les chiffres clés

### *1.1.1. Production mondiale de plastique*

Le premier polymère synthétique a été créé au début des années 1900 (Baekeland, 1909), mais c'est à partir des années 1950 qu'une production massive de plastique a été enclenchée, jusqu'à devenir une composante indispensable de nos sociétés actuelles (Geyer et al., 2017). En 2021, ce sont 390,7 millions de tonnes de plastique qui ont été produites mondialement (Plastics - the Facts 2022, Plastics Europe).

Les nombreux avantages des matériaux plastiques expliquent la croissance exponentielle de leur production et de leur utilisation : légers, peu coûteux, durables, ils comprennent une grande diversité de matériaux et donc, de propriétés mécaniques et physiques, permettant leur utilisation dans de nombreux secteurs de la vie quotidienne. En plus de leur versatilité, leur origine synthétique présente un avantage par rapport aux matériaux d'origine naturelle utilisés, tels que le bois ou les fibres textiles, qui constituent des ressources plus instables. Le plastique est aujourd'hui très utilisé dans le secteur de l'alimentation avec les emballages qui représentent presque la moitié de la production mondiale (44 % en 2021 ; Plastics - the Facts 2022, Plastic Europe). Ils sont également très utilisés dans les secteurs du textile, de l'automobile, de l'électronique, du bâtiment ou encore de l'agriculture (Fig. 1.1).

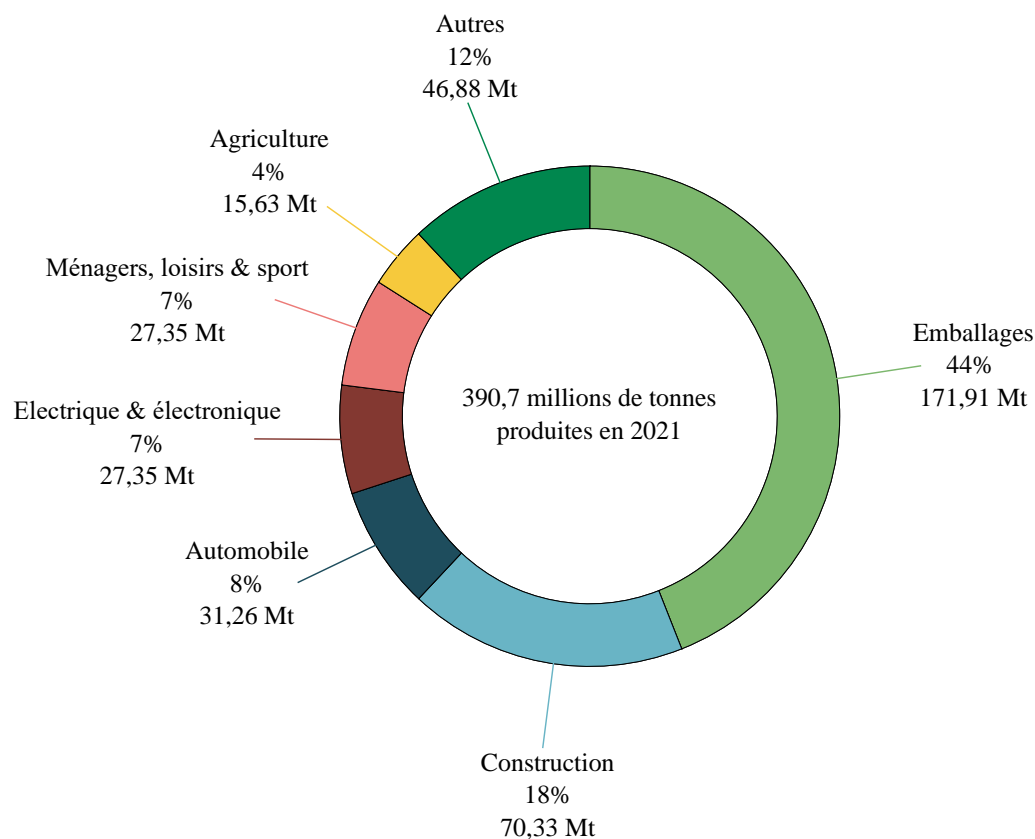


Figure 1.1. Secteurs d'utilisation des plastiques. Adapté de Plastic – the Facts 2022, Plastics Europe.

## 1.1.2. Accumulation des déchets plastiques dans l'environnement

Le cycle de vie des plastiques comprend leur production, leur utilisation et leur traitement après utilisation, c'est-à-dire leur fin de vie. Les emballages et autres objets plastiques à usage unique ont une durée d'utilisation très courte. Avec près de la moitié de la production de plastique mondiale allouée à ces utilisations, ce sont d'importantes quantités de déchets qui sont générées quotidiennement. Une fraction des déchets plastiques est collectée et recyclée mécaniquement pour créer de nouveaux objets plastiques. Si la plupart des plastiques sont recyclables en théorie, très peu sont effectivement recyclés. En 2015, seuls 9 % des plastiques produits depuis les années 1950 avaient été recyclés (Geyer et al., 2017). Actuellement, on considère que moins de 20 % des déchets plastiques sont recyclés chaque année en France, ce qui est inférieur à la moyenne de 30 % en Europe. Une part des déchets plastiques non recyclés mécaniquement sont incinérés pour revalorisation énergétique ; c'est ce

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qui est appelé le « recyclage quaternaire » (Hopewell et al., 2009). Le reste des déchets plastiques collectés qui ne passent pas par ces filières sont enfouis ou stockés en décharges, fermées ou à ciel ouvert ; ils représentent alors une source importante de fuites dans l'environnement (Salahuddin et al., 2023). En plus de cette mauvaise gestion de la fin de vie des plastiques, les pertes accidentelles, la négligence de certains citoyens vis-à-vis de leurs propres déchets, le lessivage des fibres textiles ou encore l'usure des pneus sont autant de sources de contamination plastique dans les milieux naturels. Souvent légers, les débris plastiques peuvent être transportés sur de longues distances, impactant ainsi tous les milieux, urbanisés mais aussi sauvages, des sommets terrestres (Napper et al., 2020) aux profondeurs océaniques (Woodall et al., 2014).

On estime les émissions annuelles de plastiques entre 13 et 25 millions de tonnes dans les écosystèmes terrestres et entre 9 et 23 millions de tonnes dans les écosystèmes aquatiques (MacLeod et al., 2021). Cependant, ces chiffres sont basés sur des modèles dont les résultats varient parfois avec les projections faites sur la base de données de terrain (Weiss et al., 2021). Un effort de quantification de la pollution plastique sur la base de protocoles normés est donc nécessaire afin d'accéder à une estimation plus précise de la quantité de déchets plastiques entrant dans l'environnement, mais les échantillonnages sur le terrain peuvent être limités du fait des ressources opérationnelles qu'elles représentent (Amaral-Zettler et al., 2020). La science participative offre alors la possibilité de déployer des échantillonnages plus importants avec un suivi spatio-temporel, tout en permettant de sensibiliser le grand public aux enjeux environnementaux et de former à la démarche scientifique (Hidalgo-Ruz & Thiel, 2015). Plusieurs actions de science participative ciblant la pollution macro- et mésoplastique sur le littoral existent à travers le monde, comme aux Etats-Unis (Uhrin et al., 2020) ou encore en Chine (Chen et al., 2020). L'action de sciences participatives Plastique à la Loupe, supportée et coordonnée par la fondation *Tara* Océans, et encadrée par le LOMIC et le Centre de Documentation de Recherche et d'Expérimentations sur les pollutions accidentelles des eaux (CEDRE) permet de collecter les données de pollution macro-, méso- et microplastiques sur les berges et littoraux de France métropolitaine et d'outre-mer, en faisant participer des classes de collège et lycée (annexe 1). Une base de données est actuellement en cours de création avec les données collectées depuis 2019 et analysée en collaboration avec la société Plastic At Sea, et vont permettre un suivi spatial et temporel de la pollution plastique en France. L'utilisation d'un protocole normé tiré de la convention OSPAR permet la reproductibilité du protocole et la comparaison des données.

### *1.1.3. Pollution plastique en mer*

L'océan constitue le réceptacle final des déchets à longue durée de vie comme les plastiques. Le flux entrant de déchets plastiques dans l'environnement marin a été récemment évalué entre 470 et 500 kilotonnes par an (Uhrin et al., 2020). Bien qu'une partie de la pollution plastique prenne son origine en mer directement du fait des activités humaines, comme la perte accidentelle de containers lors du transport par cargo ou d'équipements liés à la pêche ou autres activités aquacoles, on considère que 80 % de cette pollution trouverait son origine à terre, transitant par le continuum fleuve-mer. On estime que plus de 3 millions de tonnes de débris plastiques flottent à la surface des océans, ce qui représente une faible proportion de la pollution plastique globale (Kaandorp et al., 2023). Si la dynamique des plastiques dans l'environnement marin reste encore une question ouverte, certaines zones d'accumulation ont cependant été clairement identifiées (Fig. 1.2), au niveau des gyres océaniques notamment, dans lesquels la densité de cette pollution peut atteindre plus de 200 000 particules de plastique par kilomètre carré (Law et al., 2010). Des concentrations équivalentes ont été retrouvées en surface au nord-ouest de la mer Méditerranée, où la masse de plastique est jusqu'à deux fois supérieure à celle du méso-zooplancton dans certaines zones (Collignon et al., 2012).



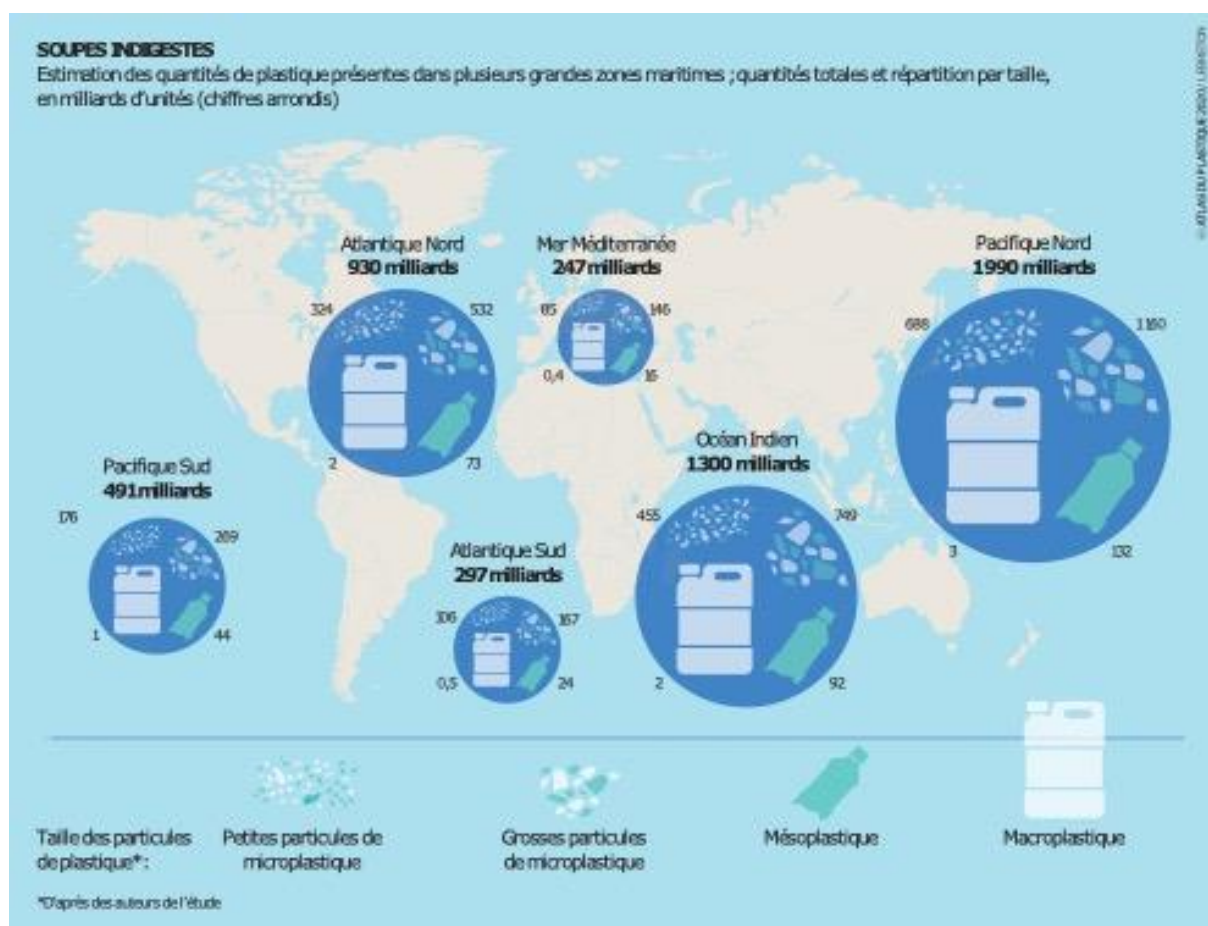


Figure 1.2. Nombre et taille de plastique dans les océans. Les débris plastiques sont présents dans tous les bassins océaniques, et sous une multitude de tailles, allant de l'objet (macroplastique, > 2,5 cm), aux mésoplastiques (entre 2,5 cm et 5 mm) et microplastiques (< 5mm). Tiré de l'Atlas du Plastique, 2020.

### 1.2. Quelques caractéristiques de la pollution plastique

#### 1.2.1. Plastiques majoritaires

Un plastique est défini comme étant composé d'un polymère, macromolécule formée par la répétition d'un ou plusieurs motifs, auquel sont ajoutés des additifs permettant d'améliorer les performances du matériau selon les applications visées (Marturano et al., 2017). Les données sur la nature des plastiques rencontrés en mer concernent souvent les plastiques présents en surface du fait des méthodes d'échantillonnage utilisées (généralement en utilisant un filet manta dont la maille est de 330  $\mu\text{m}$ ). L'analyse de la composition chimique des microplastiques collectés à la surface des océans montre qu'ils sont constitués de trois polymères majoritaires : le polyéthylène (PE), le polypropylène (PP) et le polystyrène (PS) (Andrady, 2011).

Le PE est une polyoléfine obtenue par la polymérisation de monomères d'éthylène (Fig. 1.3). Différents procédés de fabrication permettent d'obtenir des formes de PE variées présentant des densités différentes, influençant sa rigidité et donc ses applications. Toutes formes confondues, le PE est le polymère majeur représentant 26,9 % de la production mondiale de plastiques en 2021 (Plastics – the Facts 2022, Plastic Europe). Le polyéthylène à faible densité (LPDE) est très utilisé dans la fabrication des sacs plastiques. Le PP est également une polyoléfine mais composée de monomères de propylène (Fig. 1.3). Il est le deuxième polymère le plus produit en 2021 avec 75,4 millions de tonnes produites, très utilisé également dans la fabrication des emballages alimentaires. Finalement, le PS est un polymère du styrène (Fig. 1.3) dont la forme la plus connue est le polystyrène expansé, composant des emballages de restauration rapide notamment. Parce que ces polymères sont des hydrocarbures (*i.e.*, des molécules constituées exclusivement d'atomes de carbone et d'hydrogène), ils sont récalcitrants aux attaques microbiennes, donc persistants dans le milieu marin. La stabilité des plastiques dans le milieu naturel couplée au flux important de ceux-ci vers l'environnement conduit à leur accumulation, notamment dans le compartiment océanique.

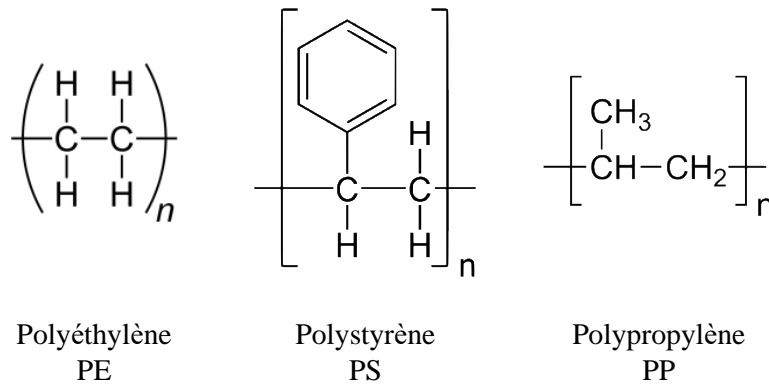


Figure 1.3. Formules chimiques du polyéthylène (PE), du polystyrène (PS) et du polypropylène (PP). Ces trois polymères conventionnels sont constitués d'un enchaînement de monomères (n), et composés uniquement d'atomes de carbone et d'hydrogène.

### 1.2.2. Une pollution à toutes les échelles

La durabilité des plastiques dans l'environnement entraîne des temps de résidence très longs (Ganesh Kumar et al., 2020). S'ils ne sont peu ou pas sensibles aux mécanismes biologiques, ils subissent en revanche de la dégradation abiotique, du fait de la combinaison de plusieurs facteurs tels que les contraintes mécaniques, la température ou encore les ultraviolets (UV) (Welden & Cowie, 2017). Les macroplastiques (*i.e.*, les plastiques de taille supérieure à 20 mm) sont ainsi fragmentés en particules de plastique de plus en plus petites, pouvant descendre jusqu'à des tailles de l'ordre du nanomètre, souvent invisibles mais pourtant bien présentes et toujours persistantes. Certaines zones du gyre du Pacifique Nord, connu comme le 7<sup>ème</sup> continent de plastique, peuvent apparaître comme exemptes de plastiques à première vue. Elles présentent pourtant de très importantes concentrations de plastiques, fragmentés et donc moins visibles (Zettler et al., 2015) (Fig. 1.4). Les microplastiques sont décrits comme les débris plastiques de taille inférieure à 5mm (Sharma & Chatterjee, 2017). Les microplastiques sont maintenant divisés en deux catégories de taille : les petits microplastiques, de taille comprise entre 1 µm et 1 mm, et les grands microplastiques, de taille comprise entre 1 mm et 5 mm (Poulain et al., 2019) (Fig. 1.4). S'ils représentent une faible masse parmi tous les débris plastiques, ils surpassent les macroplastiques en nombre (Poulain et al., 2019). Décrits plus récemment, les nanoplastiques sont définis comme les particules plastiques de taille comprise entre 1 nm et 1 µm. De taille inférieure, ces plastiques nanométriques sont le résultat d'une fragmentation continue et leur nombre devrait augmenter de manière significative dans les

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années à venir (Napper et al., 2020). Les microplastiques issus de la fragmentation des macroplastiques sont appelés microplastiques secondaires (Eerkes-Medrano et al., 2015). Il existe également des microplastiques primaires, qui ont déjà cette taille lorsqu'ils entrent dans l'environnement. Ces microdébris primaires comprennent les microbilles exfoliantes présentes dans les cosmétiques (Napper et al., 2015), les fibres textiles (Dris et al., 2017), ou encore les granulés de plastique industriels issus de la pétrochimie, qui constituent la matière première pour la fabrication des objets plastiques, aussi appelés « larmes de sirène » (Sharma & Chatterjee, 2017).

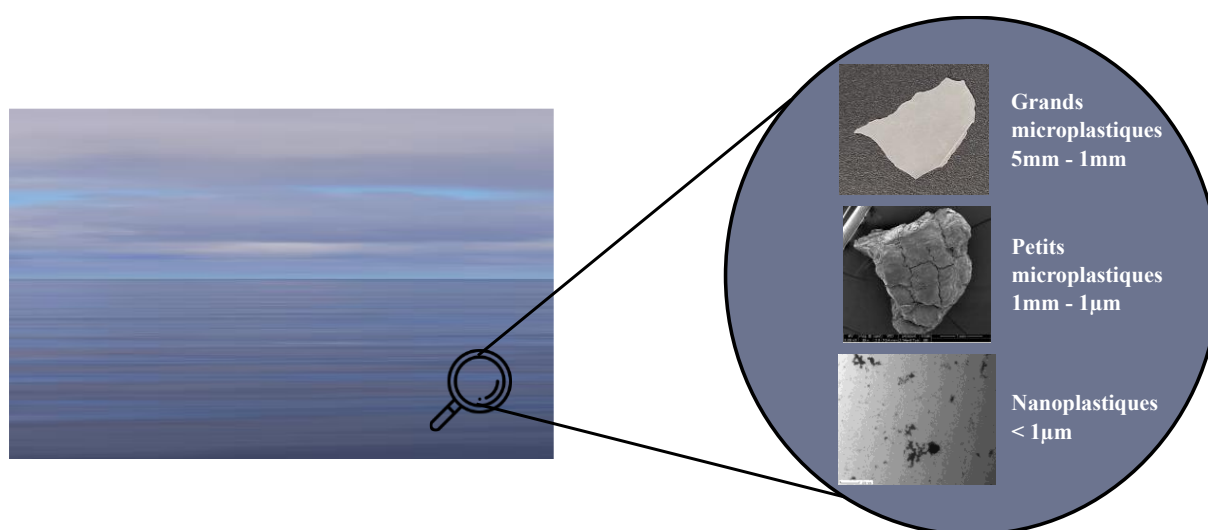


Figure 1.4. Pollution plastique à la surface dans le gyre du Pacifique nord : une soupe de microplastiques de différentes tailles. Adaptée de Amaral-Zettler et al. (2015). La surface semble propre, mais la mer contient en réalité une multitude de grands et petits microplastiques, ainsi que des nanoplastiques.

La diversité de tailles des déchets plastiques entraîne une multitude d'effets sur les écosystèmes marins. L'étranglement des mammifères marins dans les filets de pêche ou l'étouffement des tortues par les sacs plastiques sont des effets largement connus du grand public (Kühn et al., 2015). Les plastiques de plus petite taille favorisent quant à eux l'ingestion de plastique par les organismes plus petits. Colonisés par des microorganismes, ces débris plastiques deviennent appétants pour certains organismes tels que les poissons ou les oiseaux marins, qui peuvent alors les confondre avec leur nourriture, constituant une porte d'entrée dans toute la chaîne alimentaire (Collignon et al., 2012). Au-delà des dégâts physiques sur les organismes marins, les plastiques, de par leur nature hydrophobe, ont été décrits comme des

éponges à polluants et présentent alors un risque chimique pour les organismes avec lesquels ils sont en contact (Abd-Aziz et al., 2019). Les plastiques constituent également un radeau pour de nombreux microorganismes, pouvant conduire à l'introduction d'espèces invasives là où ils sont transportés (Debroas et al., 2017) et peuvent abriter des agents potentiellement pathogènes, ce qui constitue un sujet de recherche actuel (Amaral-Zettler et al., 2020).

## 2. Les bioplastiques, solution partielle à la pollution plastique

### 2.1. Définitions

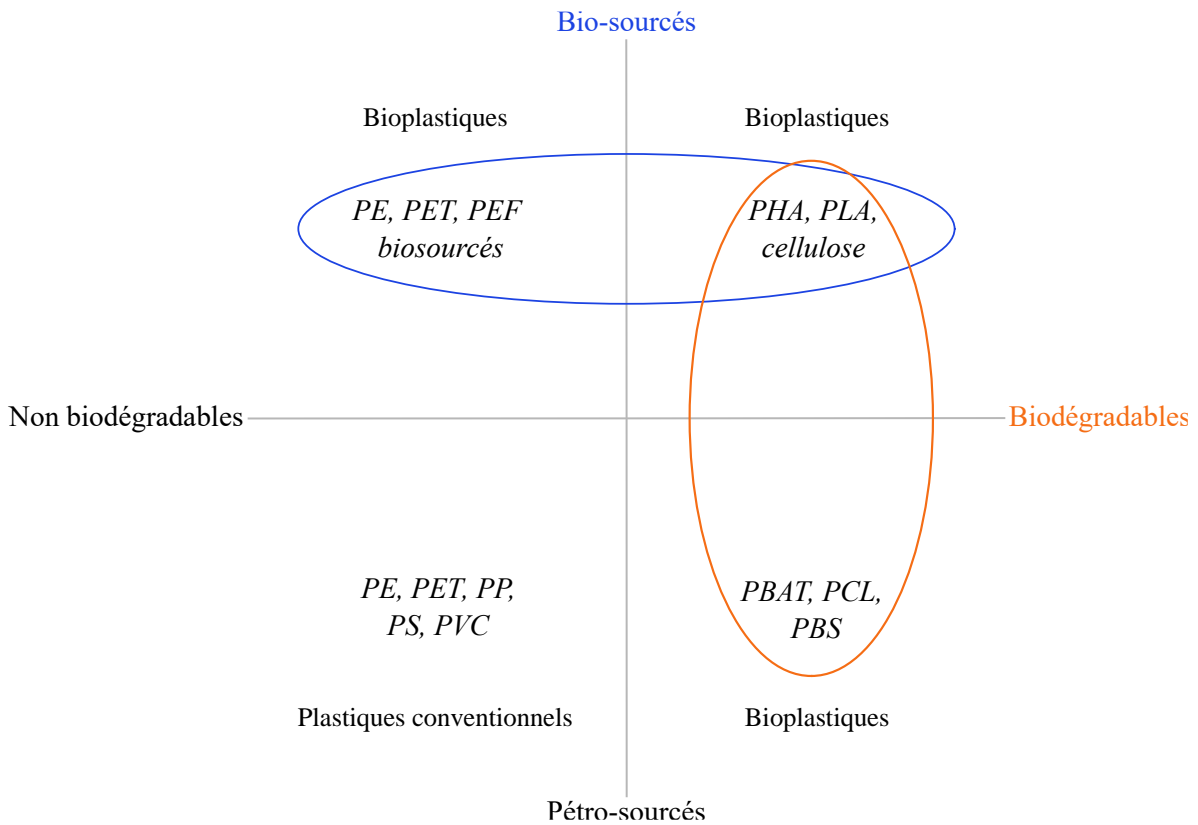


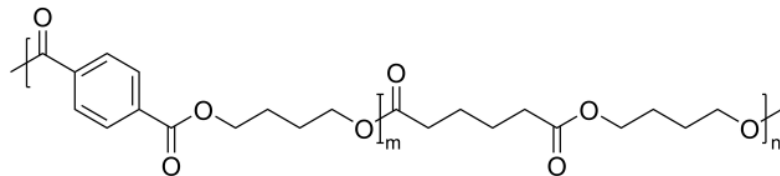
Figure 1.5. Caractéristiques et exemples des plastiques conventionnels et des bioplastiques.  
Adapté de European Bioplastics 2022.

Le terme bioplastique regroupe des plastiques (1) biosourcés, (2) biodégradables ou (3) biosourcés et biodégradables (Fig. 1.5). La possibilité de produire des plastiques à partir de biomasse, telle que le maïs ou la canne à sucre, permet de s'affranchir des matières fossiles, utilisées jusqu'alors pour la fabrication des plastiques conventionnels. Le stock des matières

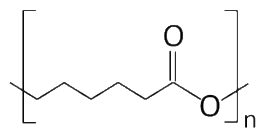
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fossiles est fini, et l'utilisation de ressources renouvelables permet de diminuer les émissions de gaz à effet de serre (Pascoe Ortiz, 2023). Il est cependant important de noter qu'un plastique biosourcé n'est pas nécessairement biodégradable. C'est par exemple le cas du PE biosourcé ; si la ressource de base, comme le maïs, est biodégradable, la structure du produit en revanche reste la même, c'est-à-dire un polymère hydrocarboné non biodégradable (Vikhareva et al., 2021).

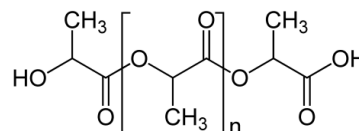
A l'inverse, certains plastiques pétrosourcés sont considérés comme biodégradables, comme le polybutylène adipate téréphtalate (PBAT) ou le polycaprolactone (PCL) (Fig. 1.6). Le PBAT est utilisé dans le secteur de l'emballage, pour remplacer le LDPE (Dammak et al., 2020), ou encore pour la fabrication des paillages d'agriculture (Liu et al., 2022). L'utilisation du PCL est plus limitée, ce plastique trouvant principalement des usages biomédicaux, pour la fabrication de fils de suture notamment. L'acide polylactique (PLA) et les polyhydroxyalkanoates (PHA, voir paragraphe 2.2) sont deux exemples de bioplastiques biosourcés et biodégradables (Fig. 1.6). Le PLA est produit à partir de sucre de betterave ou de maïs (Bogaert & Coszach, 2000). Il trouve de nombreuses applications dans les secteurs de l'emballage (emballage alimentaire, sacs compostables pour les biodéchets) mais aussi du biomédical (Ebrahimi & Ramezani Dana, 2022).



Polybutylène adipate téréphtalate  
PBAT



Polycaprolactone  
PCL



Acide polylactique  
PLA

Figure 1.6. Structures chimiques des polymères PBAT, PCL, et PLA.

### *2.1.1. Marché des plastiques biodégradables*

En 2022, 2,2 millions de tonnes de bioplastiques ont été produites, avec une production prévue de 6,3 millions de tonnes pour l'année 2027 (European Bioplastics, 2022). Si ce marché est en croissance, il représente aujourd'hui moins de 1 % de la production mondiale de plastique (World plastics production, 2021, Plastic Europe, 2022). Les plastiques biodégradables en particulier font partie de la stratégie de lutte contre la pollution plastique. En effet, un plastique est biodégradable s'il est métabolisable par les microorganismes présents dans le milieu (Zumstein et al., 2019). Les études sur la biodégradabilité des plastiques peuvent concerner des expériences en laboratoire avec des souches microbiennes isolées et cultivées, ou la biodégradabilité en milieu plus complexe. Bien qu'il existe une composante intrinsèque des polymères, la biodégradabilité d'un matériau dépend toujours du milieu considéré, comprenant les paramètres physiques, chimiques et biologiques environnant (Degli Innocenti & Breton, 2020). Il n'existe donc pas de biodégradabilité universelle, et le milieu considéré doit être précisé lorsque la biodégradabilité d'un plastique est avancée (Harrison et al., 2018).

### *2.1.2. Applications favorables à la biodégradabilité en milieu ouvert*

Dans un premier temps, les études scientifiques sur la biodégradation se sont surtout intéressées à la biodégradation en sol et en compost (Eubeler et al., 2009). Dans le cas des emballages alimentaires, les matériaux compostables permettent de pallier à l'impossibilité de recycler mécaniquement un plastique souillé par l'alimentaire, ou au contraire, l'introduction de plastique non biodégradable dans les biodéchets (Degli Innocenti & Breton, 2020). Il est important de noter qu'on distingue le compostage industriel, présentant des conditions contrôlées, du compostage ménager (Emadian et al., 2017). Certains plastiques biodégradables présentent généralement une haute biodégradabilité en conditions de compostage industriel, c'est-à-dire, entre autres, thermophiles, soumis à des températures autour de 60°C. Par exemple, le PLA a montré jusqu'à 84 % de biodégradation en 58 jours (Kale et al., 2007), et le PCL 38 % en 6 jours (Nakasaki et al., 2006).

Le constat de l'accumulation des déchets plastiques dans tous les milieux a conduit à s'intéresser à la biodégradabilité des plastiques dans les milieux ouverts, terrestres et aquatiques. Comme évoqué précédemment, la biodégradabilité d'un matériau dépend des paramètres environnementaux. Il n'est donc pas envisageable d'extrapoler des résultats de biodégradabilité à tout milieu qui n'a pas été testé. Des sacs plastiques compostables, étiquetés

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comme biodégradables, ont été retrouvés intacts et fonctionnels après 3 ans d'incubation en sol et en milieu marin (Napper & Thompson, 2019). En sol, les performances de biodégradation sont souvent inférieures par rapport au compostage industriel. La biodégradabilité des plastiques en milieu marin est généralement encore plus lente. Elle sera présentée dans le paragraphe 4 de cette introduction.

L'utilisation des plastiques biodégradables en milieux ouverts a été discutée récemment dans plusieurs articles scientifiques pour tenter de limiter leurs usages en fonction de leur fin de vie potentielle (Paul-Pont et al., 2023). Le développement de plastiques compostables est particulièrement intéressant pour les emballages alimentaires (Degli Innocenti & Breton, 2020). Les plastiques biodégradables en sol présentent un grand intérêt pour des applications agricoles telles que les paillages, en remplacement des plastiques conventionnels utilisés historiquement, et dont la collecte est souvent impossible après utilisation, du fait de la dégradation abiotique (Zumstein et al., 2019). Enfin, la biodégradabilité en mer serait un avantage pour les outils relatifs aux activités maritimes, tels que les filets de pêche ou les bouées météorologiques dont la perte est parfois inévitable (Paul-Pont et al., 2023). Il est important de noter que si des plastiques biodégradables en sol ou en milieu aquatique permettraient de réduire leur persistance dans l'environnement en cas de fuites, qui sont aujourd'hui encore inévitables, le rejet dans l'environnement n'est pas une fin de vie souhaitable (Flury & Narayan, 2021). Il est important d'accompagner la diffusion des solutions biodégradables auprès du grand public afin d'éviter le développement de leur mauvais usage. De plus, le développement de nouveaux matériaux plastiques moins récalcitrants est l'une des composantes d'une stratégie plus globale de lutte contre la pollution plastique, comprenant la réduction ainsi qu'une meilleure gestion des déchets de manière plus générale.



## 2.2. Les polyhydroxyalkanoates : des polymères biosourcés et biodégradables

### 2.2.1. Généralités

Les polyhydroxyalkanoates (PHA) sont des polyesters naturellement synthétisés par de nombreuses bactéries mais aussi certaines champignons, levures et plantes (Luengo et al., 2003). Dans la cellule, ils sont totalement amorphes, stockés sous forme de granules cytoplasmiques, et représentent ainsi une forme de stockage de carbone et d'énergie. Mobilisables en cas de modification de la concentration en nutriments dans l'environnement, les PHA sont synthétisés en présence de carbone et dans des conditions de nutriments limités (Kadouri et al., 2005). Découverts pour la première fois en 1923 par Lemoigne, plus de 150 monomères de PHA ont été décrits à ce jour (Koller et al., 2010). Cette famille de polymères est classiquement divisée en trois catégories, selon la longueur de la chaîne radicale R (Fig. 1.7) : les PHA à courte chaîne (*scl*-PHA), constitués de monomères contenant 3 à 5 carbones, les PHA à moyenne chaîne (*mcl*-PHA), constitués de monomères contenant 6 à 14 carbones et enfin, moins répandus, les PHA à longue chaîne (*lcl*-PHA), constitués de monomères contenant plus de 14 carbones (Vicente et al., 2023).

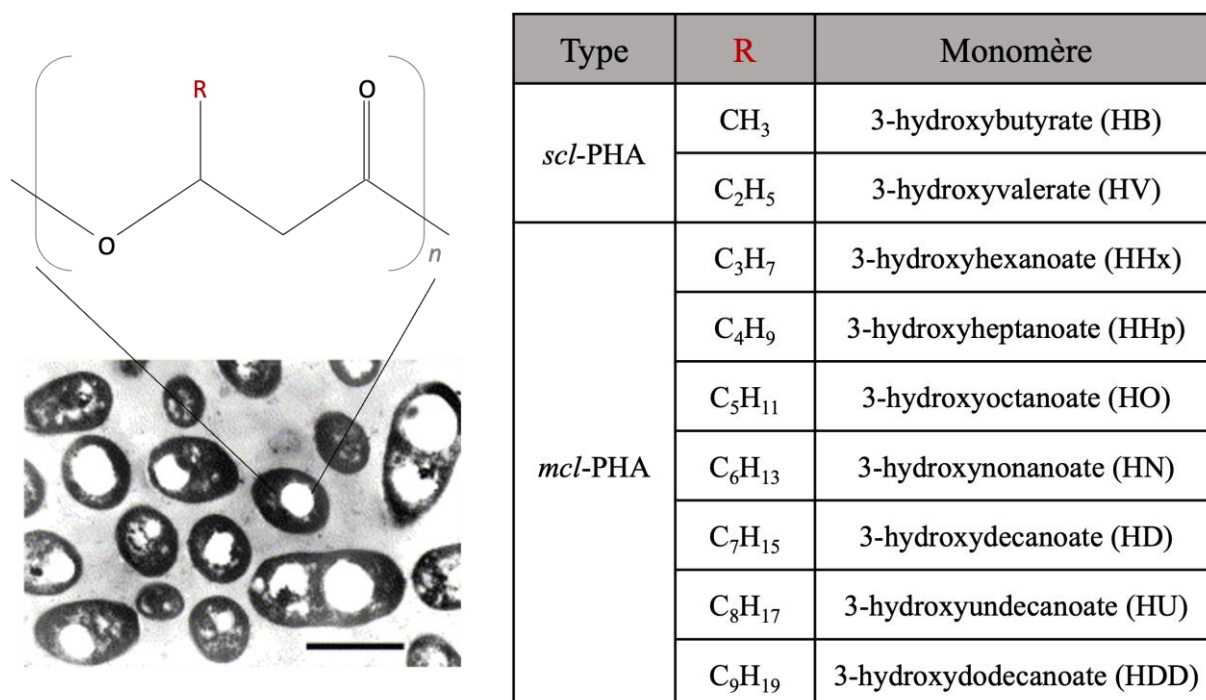


Figure 1.7. PHA : granules cytoplasmiques, formule chimique et composition des monomères.

Photo tirée de Luengo et al. (2003).

### 2.2.2. Propriétés, applications et marché des PHA

Biosourcés, biodégradables et biocompatibles, les PHA sont des polymères attractifs pour le remplacement des polymères utilisés dans la production des plastiques conventionnels. Dans le milieu extracellulaire, les PHA cristallisent (on parle de PHA « dénaturés », par opposition aux PHA natifs, amorphes) et présentent des propriétés similaires aux polymères utilisés dans les plastiques conventionnels (Jendrossek & Handrick, 2002). De par la diversité des monomères existant, cette famille de polyesters présente des caractéristiques physiques et chimiques très variées, offrant une multitude d'applications potentielles (Jendrossek & Handrick, 2002). Le PHA le plus répandu est un homopolymère d'hydroxybutyrate, le poly(3-hydroxybutyrate) (PHB) (Fig. 1.8). Fortement cristallin, les propriétés mécaniques de ce *scl*-PHA sont limitées, du fait notamment de sa rigidité, qui le rend cassant. Des travaux ont donc porté sur la formulation d'autres PHA présentant de meilleures propriétés (Meereboer et al., 2020). Les copolymères du PHB, tel que le poly(3-hydroxybutyrate-co-hydroxyvalérate) (PHBHV), ou le poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) (PHBHHx), présentent une plus faible cristallinité grâce à l'introduction des monomères d'hydroxyvalérate (HV) ou d'hydroxyhexanoate (HHx), et sont moins rigides et cassant (Fig. 1.8). En plus de ces applications industrielles pour le remplacement des polymères conventionnelles, grâce aux propriétés thermoplastiques des PHA dénaturés, la structure des inclusions de PHA natif elle-même (c'est-à-dire des granules cytoplasmiques) présente des intérêts biotechnologiques et médicaux. En effet, ces granules sont composés d'un cœur hydrophobe contenant le PHA amorphe, entouré d'une couronne de phospholipides et de protéines, et pourraient être utilisés pour la purification de protéines ou encore l'administration ciblée de médicaments (Grage et al., 2009).

En 2022, les PHA représentaient 3,9 % des capacités de production mondiale de bioplastiques (European Bioplastics, 2022). Une dizaine d'entreprises commercialisent aujourd'hui différentes formes de PHA, avec des capacités de production de 100 à 10 000 tonnes par an (Koller & Mukherjee, 2022). Parmi les PHA commercialement disponibles, le PHB et PHBHV sont majoritairement retrouvés, ainsi qu'une formulation de PHBHHx. La production de PHA à l'échelle industrielle par fermentation bactérienne est onéreuse, limitant leur diffusion à large échelle (Bedade et al., 2021). D'autres méthodes de production sont aujourd'hui en développement, par synthèse chimique notamment (Westlie et al., 2022).

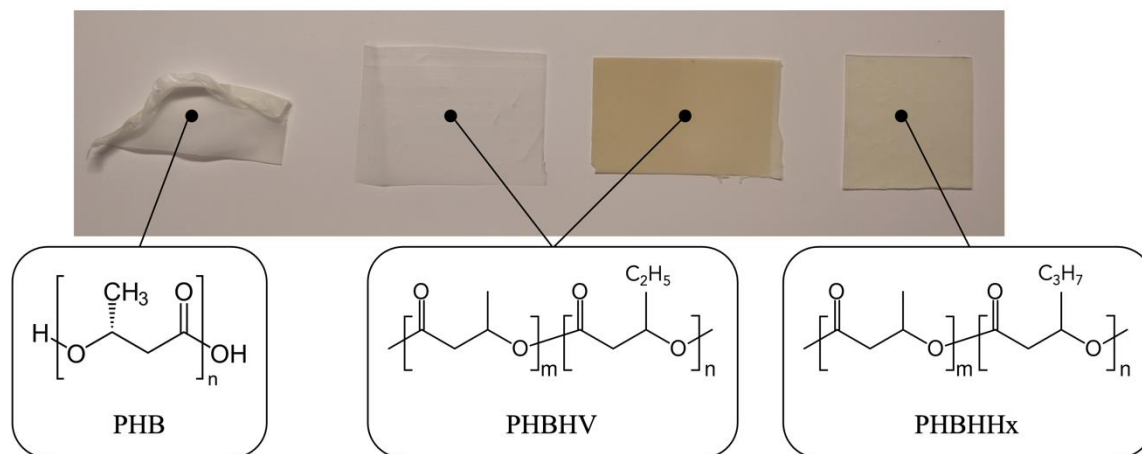


Figure 1.8. Photo de films à partir de différentes formulations de PHA. Photo : Léna Philip

### 2.2.3. Biodégradabilité des PHA en milieu marin

La voie de biodégradation des PHA est relativement simple : le polymère est d'abord hydrolysé par une PHA dépolymérase (PhaZ), qui est une estérase capable de couper les chaînes de PHA en oligomères puis en monomères qui rejoignent alors le métabolisme général de la cellule via le cycle de  $\beta$ -oxydation (Jacquin et al., 2019). Deux types de PHA dépolymérases sont à distinguer : intra- et extra-cellulaires (nPhaZ et dPhaZ respectivement). Les organismes capables de synthétiser les PHA possèdent également des PHA dépolymérases intracellulaires, afin d'utiliser les polyesters stockés lorsque le milieu est appauvri en carbone (Doi et al., 1990). Les PHA dépolymérases extracellulaires, quant à elles, sont des enzymes sécrétées en dehors des cellules car portant un peptide signal afin d'hydrolyser les PHA environnants, qui deviennent alors assimilables ( $< 600$  Da) et disponibles pour le métabolisme microbien (Jendrossek & Handrick, 2002). Seules les PHA dépolymérases extracellulaires sont actives sur les PHA extracellulaires (Sznajder & Jendrossek, 2011). De plus, plusieurs types de PHA dépolymérases extracellulaires existent, et il a été montré qu'elles sont spécifiques du type de PHA considéré, c'est-à-dire *scl*- ou *mcl*-PHA (Jendrossek & Handrick, 2002). La répartition de ces dépolymérases est inégale dans l'environnement ; l'occurrence de ces gènes est plus faible dans le milieu marin qu'en milieu terrestre, et les dPhaZ spécifiques des *mcl*-PHA sont plus rares que pour les *scl*-PHA (Viljakainen & Hug, 2021).

Comme pour d'autres polymères, certaines études mettent en évidence une augmentation de la biodégradabilité des PHA lorsque la cristallinité diminue (Meereboer et al., 2020). La biodégradabilité des PHA a été démontrée en compost (Weng et al., 2011), en sol (Gómez & Michel, 2013), ainsi qu'en milieu marin (Deroiné et al., 2015). Cependant, les études portant sur la biodégradation des PHA ont principalement porté sur le PHB, le PHBHV et plus récemment, le PHBHHx, qui sont les formules commercialement disponibles (Koller & Mukherjee, 2022). La diversité des caractéristiques physiques et chimiques que peuvent présenter les PHA, combinée à l'influence de paramètres intrinsèques tels que la cristallinité sur la biodégradabilité, ainsi que la disparité dans la distribution des PHA dépolymérase laisse penser que la réponse de biodégradabilité des *mcl*-PHA pourrait être différente de celle des PHA étudiés jusqu'alors.

### 3. Les communautés microbiennes associées aux plastiques : la plastisphère

La présence de diatomées et d'hydroïdes sur des débris plastiques a été rapportée pour la première fois en 1972, sur des échantillons collectés dans la mer des Sargasses (Carpenter & Smith, 1972). Du fait de leur nature hydrophobe, la surface des plastiques est recouverte de matière organique et inorganique dès leur entrée dans le milieu marin (Jacquin et al., 2019). Ces débris plastiques sont ensuite rapidement colonisés par des microorganismes capables d'utiliser ces nutriments et/ou matière organique, formant ainsi la plastisphère (Zettler et al., 2013). L'étude de la plastisphère peut être faite à partir de microplastiques environnementaux, prélevés dans le milieu naturel, ou par incubation de plastiques vierges en milieu naturel ou en aquarium avec des points de prélèvement fixés, afin d'étudier la richesse spécifique, la diversité ainsi que la structure des communautés microbiennes associées à ces débris plastiques.

#### 3.1. Séquençage d'ADN haut débit pour l'étude de la diversité bactérienne

Les premières observations de la plastisphère microbienne ont été basées sur des images de microscopie, notamment la microscopie électronique à balayage (MEB) (Fig. 1.9). Si cette technique permet d'avoir une information spatiale de l'agencement de la plastisphère, et de discriminer certains organismes tels que les diatomées ou certaines cyanobactéries, la MEB ne permet pas d'accéder à une information taxonomique précise pour les bactéries

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notamment (De Tender et al., 2017). De plus, cette technique est coûteuse, non automatisée et chronophage. L'hybridation *in situ* fluorescente permet d'avoir des informations taxonomiques, en utilisant des sondes spécifiques des taxons visés. Cependant, cette méthode ciblée suppose d'avoir une connaissance des taxons visés pour l'établissement des sondes.

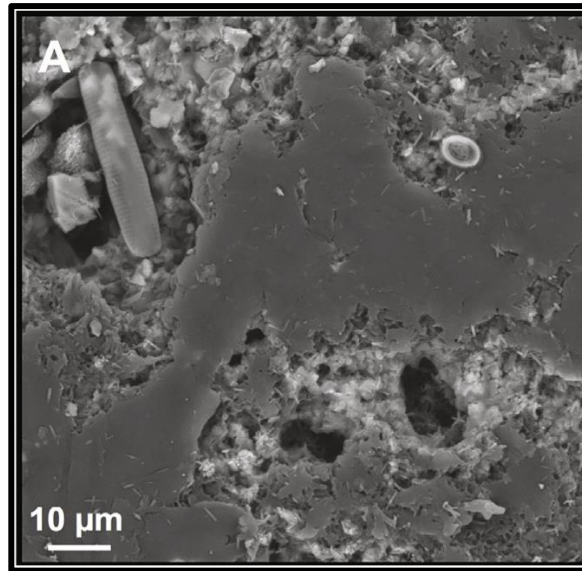


Figure 1.9. Image de microscopie électronique à balayage montrant la colonisation et la détérioration de la surface d'un plastique par divers organismes de la plastisphère. Tirée de Dussud et al. (2018a).

Plusieurs méthodes de biologie moléculaire ont permis d'améliorer les connaissances sur la composition taxonomique et les structures des communautés de la plastisphère, notamment le séquençage de première génération, mais c'est l'arrivée du séquençage haut débit qui a permis la multiplication des études sur le sujet (Amaral-Zettler et al., 2020). La méthode de séquençage la plus utilisée aujourd'hui celle commercialisée par la société Illumina (Goodwin et al., 2016). Un avantage de cette méthode est la possibilité de préparer les bibliothèques d'ADN directement depuis l'échantillon étudié, sans nécessité de clonage dans des cellules hôte. De plus, la capacité de séquençage est augmentée par rapport aux premières technologies (Shendure & Ji, 2008), et la technologie commercialisée par Illumina produit aujourd'hui des séquences de longueur jusqu'à 300 paires de bases.

La plupart des études sur la plastisphère utilisent le séquençage d'amplicons (métabarcoding) (Zettler et al., 2020). Cette méthode consiste à ne séquencer qu'une partie du

génomique, correspondant à un marqueur génétique choisi. Des primers d'amplification en chaîne par polymérase (PCR) sont établis selon la zone génétique d'intérêt, qui est amplifiée sur les fragments d'ADN contenus dans l'échantillon, puis le séquençage est réalisé sur les produits d'amplification (Taberlet et al., 2007). Plusieurs protocoles existent pour le traitement des lectures, séquences obtenues en sortie de séquençage, qui sont assignés taxonomiquement grâce à des bases de données de référence (Glöckner et al., 2017). Le métabarcoding est aujourd'hui très peu coûteux et rapide, et permet de traiter de nombreux échantillons en parallèle, favorisant la comparaison des communautés inter-échantillons (De Tender et al., 2017). Le marqueur génétique le plus fréquemment utilisé pour l'identification des taxons bactériens est le gène codant pour la sous-unité 16S de l'ARN ribosomique (ADNr 16S). Ce gène ubiquiste présente des régions fortement conservées qui permettent l'établissement d'amorces universelles mais également des régions plus variables permettant de discriminer les différents taxons (Head et al., 1998). De plus, l'ADNr 16S est le marqueur pour lequel les bases de données sont les plus fournies (Park & Won, 2018). La longueur des lectures disponibles par séquençage Illumina (jusqu'à 300 paires de base) ne permet souvent pas une assignation taxonomique jusqu'à l'espèce. Cette limite devrait être surmontée grâce au développement du séquençage « long reads » (Krehenwinkel et al., 2019). Au-delà des biais relatifs à la PCR, le métabarcoding ne donne pas d'indication sur le potentiel fonctionnel des communautés, pour lequel le séquençage de l'ensemble de l'ADN, la métagénomique, est nécessaire. Les études métagénomiques de la plastisphère sont encore peu nombreuses. Une difficulté de l'étude génétique de la plastisphère est la quantité d'ADN extraite à partir des échantillons environnementaux (Amaral-Zettler et al., 2020) ; le séquençage pour la métagénomique s'effectue sans amplification par PCR, une faible quantité d'ADN peut donc être limitante. De plus, les analyses des résultats de séquençages métagénomiques sont plus complexes et plus chronophages que le métabarcoding (De Tender et al., 2017).

### 3.2. Dynamique de formation de la plastisphère

#### 3.2.1. *Biofilms*

Les membres de la plastisphère forment un biofilm. Ce mode de vie fixé de certains microorganismes est caractérisé par la formation d'une matrice polymérique extracellulaire constituée principalement de polysaccharides, lipides, protéines et acides nucléiques. Cette matrice compte pour 90% de la masse du biofilm, les 10% restants correspondant aux cellules microbiennes (Flemming & Wingender, 2010). Les biofilms sont un mode de vie avantageux

puisque'ils offrent un microenvironnement protégé, stable, hydraté, contenant des nutriments (Flemming et al., 2016).

La première étape pour la formation d'un biofilm est l'attachement cellulaire, influencé par différents facteurs, biologiques mais aussi environnementaux tels que la température et la concentration en nutriments. L'adhérence des premières cellules au substrat résulte en l'établissement d'une micro-colonie, qui sécrète les substances polymériques extracellulaires et ainsi, la matrice extracellulaire, permettant la croissance des microorganismes pour constituer un biofilm mature (Prakash et al., 2003). Des enzymes extracellulaires sont également sécrétées et stabilisées par la structure du biofilm, pouvant conduire, entre autres, à la dégradation du substrat d'attache du biofilm (Prakash et al., 2003).

### 3.2.2. Formation de la plastisphère

La plastisphère comprend des microorganismes variés, procaryotes et eucaryotes, photoautotrophes et hétérotrophes (Amaral-Zettler et al., 2020) (Fig. 1.10). L'incubation de plastiques en milieu marin naturel ou contrôlé a permis de mettre en évidence différentes étapes successives pour la formation de la plastisphère, au cours de laquelle les groupes dominants changent (Jacquin et al., 2019). Les primo-colonisateurs colonisent rapidement la surface du plastique, et sont dominés par les Gammaprotéobactéries (De Tender et al., 2017). Cet attachement est réversible, l'hydrophobicité de la surface est diminuée (Lobelle & Cunliffe, 2011) et les microorganismes sécrètent des polysaccharides extracellulaires qui favorisent l'attachement des colonisateurs secondaires. Le biofilm primaire est observé dans les premiers jours d'immersion (Quero & Luna, 2017), et comprend également des diatomées et des cyanobactéries (Amaral-Zettler et al., 2015). Lors de la croissance du biofilm, deuxième phase de formation de la plastisphère, la proportion des Bacteroidetes, et en particulier les *Flavobacteriaceae*, augmente (Zhang et al., 2022). D'autres microorganismes colonisent le plastique, grâce notamment aux composés organiques synthétisés par les colonisateurs primaires (Quero & Luna, 2017). La formation du biofilm secondaire peut prendre plusieurs mois (Wright et al., 2020). La troisième phase est la maturation, durant laquelle les membres de la plastisphère deviennent plus stables (Odobel et al., 2021).

Le type de plastique peut influencer la structure des communautés de la plastisphère ainsi que la dynamique de formation du biofilm. En particulier, les plastiques biodégradables présentent une abondance et une activité bactériennes supérieures à celles mesurées sur des

plastiques non biodégradables tels que le PE (Dussud et al., 2018a). La taille et la forme du substrat ne semblent pas influencer l'abondance ni la diversité de la plastisphère associée à ces substrats (Cheng et al., 2021). En revanche, l'occurrence d'un bloom de phytoplancton peut résulter en un changement de diversité et d'activité de la plastisphère (Cheng et al., 2021). Aucun consensus n'a été trouvé quant à la durée de formation des différents stades de la plastisphère parmi les différentes études disponibles, dont les résultats sont influencés par les facteurs environnementaux du lieu d'incubation (Du et al., 2022). L'influence des paramètres environnementaux sur la composition de la plastisphère a été confirmée par l'étude des communautés associées à des débris plastiques environnementaux (Oberbeckmann et al., 2018).

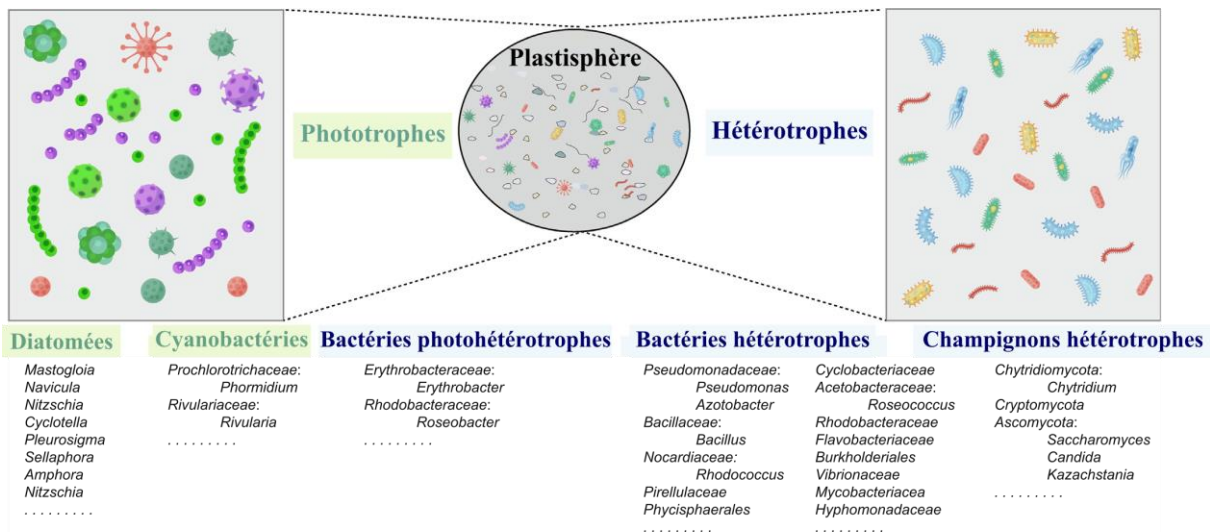


Figure 1.10. Diversité des organismes de la plastisphère. Tirée de Du et al., 2022.

### 3.3. Étude de la plastisphère environnementale

En milieu marin, les communautés bactériennes libres dans la colonne d'eau sont différentes des bactéries qui forment un biofilm associé aux particules organiques (Crespo et al., 2013). Plusieurs études sur la plastisphère associée aux débris plastiques environnementaux ont démontré que ces communautés sont différentes des communautés environnantes, y compris les biofilms associés aux particules naturelles, en milieu marin (Amaral-Zettler et al., 2015; Dussud et al., 2018b) mais également en eau douce (Delacuvellerie et al., 2022). Les débris plastiques constituent donc une nouvelle niche écologique pour les microorganismes marins, et abriteraient entre 0,01 à 0,2% de la biomasse microbienne des eaux de surface de l'océan (Mincer et al., 2016). Cette estimation est basée sur les masses de débris plastiques flottants ; on estime cependant que seulement 1% des plastiques présents en mer sont à la surface. Les



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débris plastiques dans l’océan global représentent donc un potentiel puit de biomasse important. De plus, ils constituent un écosystème à part entière dont les communautés sont distinctes de celles présentes dans l’eau environnantes. Les cycles biogéochimiques étant régis par les communautés microbiennes (Rousk & Bengtson, 2014), il est important de comprendre les interactions entre les microorganismes de la plastisphère et les communautés environnantes, pour tenter de comprendre les impacts potentiels de cette pollution grandissante sur les grands cycles. Une étude en mésocosme a déjà montré un couplage entre la production primaire et hétérotrophe à la surface des microplastiques, influencé par les conditions environnementales (Conan et al., 2022).

Les débris plastiques flottant constituent des radeaux pour les microorganismes qui y sont associés. Plusieurs études ont reporté la présence de groupes de microorganismes comprenant des espèces pathogènes au sein des communautés associées aux débris environnementaux dans différents milieux aquatiques, marin (Frère et al., 2018) et eau douce (Hoellein et al., 2017). Ces potentiels pathogènes pourraient affecter la santé humaine mais également certains organismes marins tels que les mollusques liés à la consommation humaine et avoir un impact économique négatif. Une partie des microplastiques retrouvés dans l’environnement aquatique prend son origine dans les stations de traitement des eaux usées (A. R. McCormick et al., 2016), qui pourraient être une source et des vecteurs de microorganismes pathogènes. L’occurrence de potentiels pathogènes sur la surface de ces particules plastiques est donc aujourd’hui une question clé dans les études épidémiologiques liées aux microplastiques.

Une autre question est le rôle potentiel de certains microorganismes de la plastisphère dans la biodégradation des substrats plastiques auxquels ils sont attachés. Plusieurs études de la plastisphère ont mis en avant un enrichissement de la plastisphère en espèces hydrocarbonoclastes, c’est-à-dire connues pour leur capacité de biodégradation des hydrocarbures (Dussud et al., 2018a). De plus, plusieurs souches bactériennes présentant une activité de biodégradation des plastiques ont été isolées à partir d’échantillons environnementaux (Joshi et al., 2022). L’étude du potentiel de biodégradation de la plastisphère présente plusieurs intérêts, notamment la compréhension du devenir des plastiques en mer ainsi que de potentielles applications pour le recyclage biologique.

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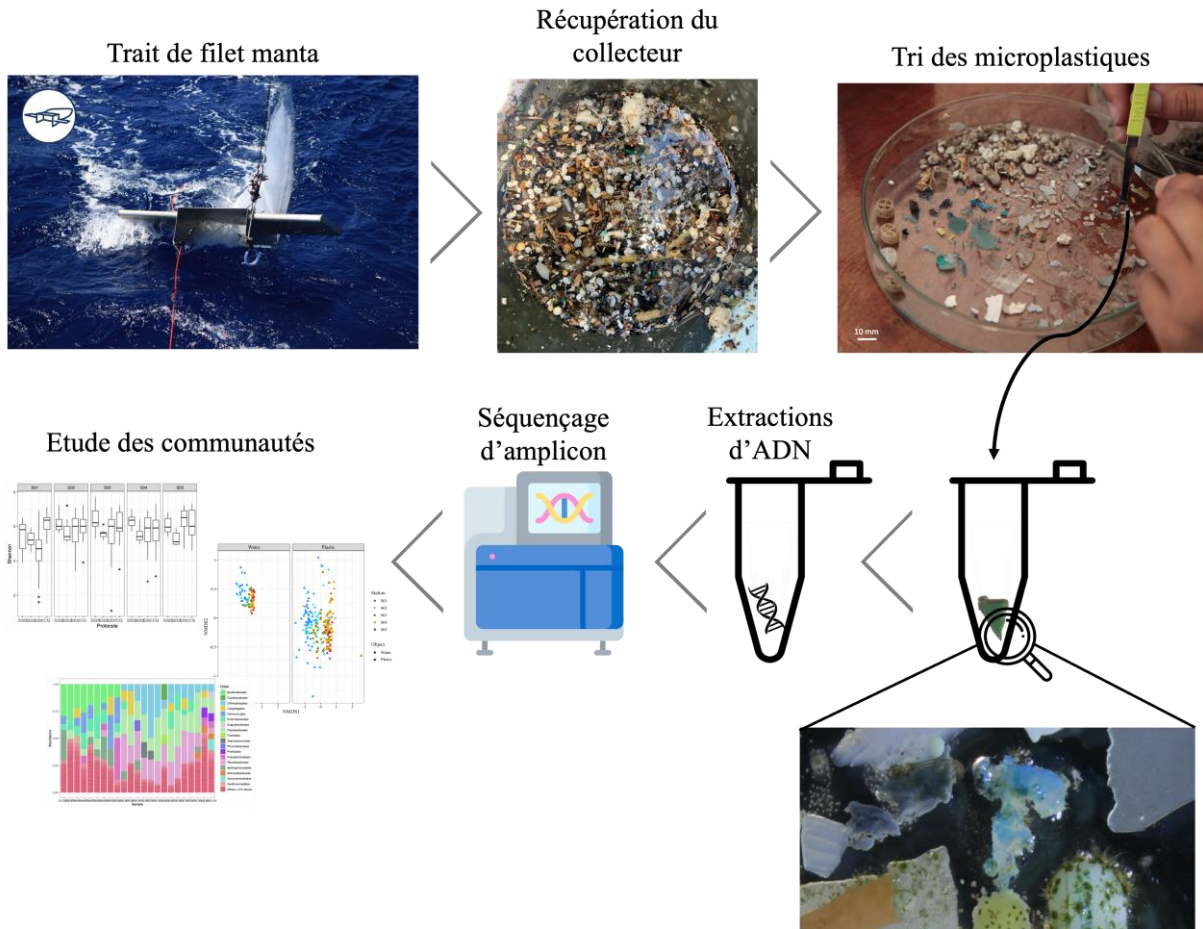


Figure 1.11. Analyse de la diversité des communautés de la plastisphère associées aux microplastiques de surface.

Une étude métagénomique a été conduite sur des débris plastiques collectés dans les eaux de surface du gyre du Pacifique Nord. Cette étude a mis en évidence des différences taxonomiques mais également fonctionnelles entre les communautés associées à ces débris par rapport aux communautés planctoniques environnantes (Bryant et al., 2016). L'incubation en mésocosme simulant l'interface eau/sédiments de morceaux de céramique, de polytéréphtalate d'éthylène (PET) et de PHB a montré un changement de communautés et de leurs potentielles fonctionnalités pour le PHB par rapport aux deux autres matériaux et l'eau environnante, présentant un enrichissement en bactéries sulfo-réductrices notamment (Pinnell & Turner, 2019). Une étude a mis en évidence par une analyse métagénomique la présence de gènes liés à la biodégradation et à de potentiels agents pathogènes sur des échantillons de PE et PP environnementaux ; cependant, l'analyse protéomique de ces échantillons a mis en évidence l'absence de protéines liées à la biodégradation et la pathogénicité (Delacuvellerie et al., 2022). Les études métagénomiques de la plastisphère restent aujourd'hui peu nombreuses.

## 4. Biodégradabilité des plastiques en mer

### 4.1. Mécanismes de biodégradation

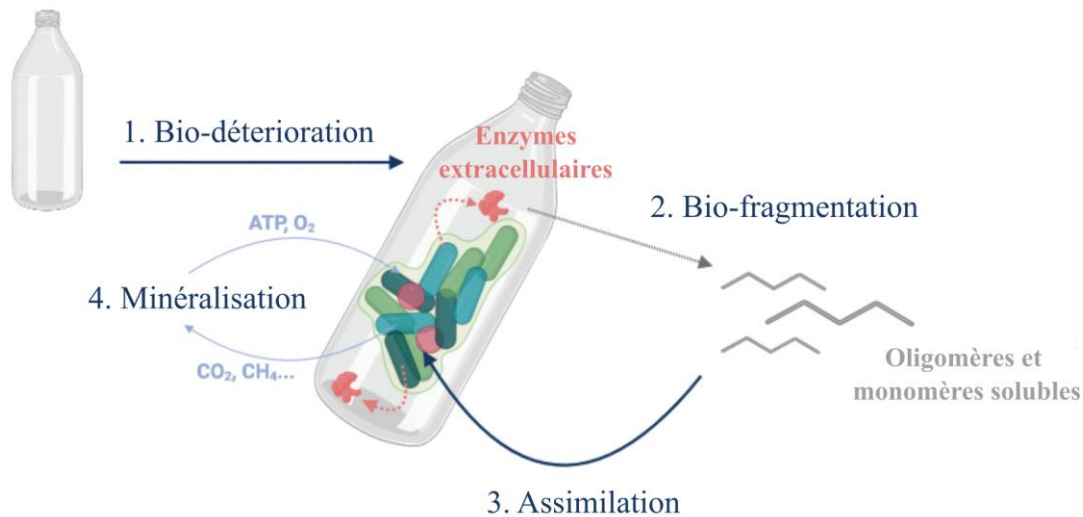


Figure 1.12. Étapes de la biodégradation des plastiques en mer. Adapté de Dussud et Ghiglione (2014).

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Les plastiques entrant dans l'environnement marin sont soumis à des facteurs abiotiques favorisant leur dégradation. Plusieurs phénomènes conduisent à la dégradation des plastiques : l'oxydation, les contraintes mécaniques, la température ainsi que l'hydrolyse par l'eau de mer (Andrady, 2022). La dégradation oxydative concerne les plastiques flottant à la surface, qui sont donc exposés aux UV (Sudhakar et al., 2007). La biodégradabilité d'un matériau correspond à la possibilité qu'il soit utilisé par les microorganismes comme source d'énergie (Lucas et al., 2008). La biodégradation des plastiques en milieu marin est définie en 4 étapes (Dussud et Ghiglione, 2014) (Fig. 1.12) :

- La biodétérioration correspond à des modifications superficielles du plastique du fait de la croissance du biofilm microbien à sa surface, et entraîne des modifications mécaniques, physiques et chimiques. La formation de la matrice extracellulaire du biofilm entraîne des modifications physiques, par la pénétration des constituants de la matrice extracellulaire dans les pores de la surface, et chimiques, par l'action des acides sécrétés notamment (Lucas et al., 2008).
- La biofragmentation est un phénomène lytique, correspondant au clivage des longues chaînes polymérique en oligomères et monomères solubles sous l'action d'enzymes extracellulaires sécrétées par les membres du biofilm. La biofragmentation peut avoir lieu par hydrolyse ou oxydation, selon les enzymes présentes (Lucas et al., 2008).
- L'assimilation est l'entrée dans les cellules microbiennes des produits de biofragmentation solubles d'une taille inférieure à 600 Da. Le carbone organique est alors disponible pour la production de biomasse (Jacquin et al., 2019).
- La minéralisation est l'étape ultime de la biodégradation au cours de laquelle le carbone organique est utilisé pour la production d'énergie, avec la production de métabolites oxydés (Jacquin et al., 2019).

En conditions aérobies, les métabolites sont l'eau et le dioxyde de carbone ( $\text{CO}_2$ ). En conditions anaérobies, les métabolites sont l'eau et le méthane ( $\text{CH}_4$ ) (Goel et al., 2021). Dans le milieu naturel, la dégradation abiotique et la biodégradation sont des phénomènes concomitant (Jacquin et al., 2019).

Il existe aujourd'hui des méthodes analytiques susceptibles de caractériser chacune des 4 étapes de biodégradation. Les changements de surface dus à la biodétérioration peuvent être observés par microscopie électronique. L'étape de biofragmentation peut être suivie par analyse du surnageant pour la détection des produits de dépolymérisation ou encore par le suivi

de la masse molaire et l'indice de polydispersion. L'étape d'assimilation peut quant à elle être suivie par quantification de la biomasse microbienne à la surface des plastiques, ou de l'activité hétérotrophe bactérienne. Enfin, la minéralisation peut être vérifiée par des mesures de respirométrie.

### 4.2. Analyse de la biodégradation en milieu marin

#### *4.2.1. Des travaux peu nombreux sur la biodégradabilité des plastiques en milieu marin*

Bien que les plastiques biodégradables apparaissent aujourd'hui comme des solutions disponibles, les études portant sur leur biodégradabilité sont encore peu nombreuses, en particulier en milieu marin. En effet, une grande partie des articles de recherche contenant le terme « biodégradable » dans leur titre ne contiennent en réalité aucun test de biodégradabilité (Haider et al., 2019). De nombreuses études disponibles actuellement étudient la dégradation marine des plastiques en suivant les modifications physiques et chimiques des échantillons résultant de l'incubation en milieu naturel. Ces analyses comprennent la perte de masse (Volova et al., 2007), les changements de masse molaire et de cristallinité (Volova et al., 2010), le changement des liaisons chimiques (Catarci Carteny & Blust, 2021), ou encore des modifications de surface suivies par microscopie électronique (Sashiwa et al., 2018). Ces études présentent l'avantage d'être réalisées en conditions réelles, puisque les échantillons sont directement immergés dans l'environnement considéré. Toutefois, il est impossible de parler de biodégradation puisqu'aucune activité biologique n'est enregistrée (Folino et al., 2023). A ce jour, le seul moyen de mesurer des activités biologiques est de réaliser des expériences en milieu contrôlé.

#### *4.2.2. Protocoles normatifs pour l'évaluation de la biodégradabilité marine des plastiques*

La biodégradabilité d'un produit est une composante de son impact écologique sur les écosystèmes (Pagga, 1997), et les premières directives pour son évaluation sont apparues dans les consignes de l'Organisation de Coopération et de Développement Economiques (OCDE) dans les années 1980 (Folino et al., 2023). Les protocoles utilisés aujourd'hui dans les publications scientifiques pour évaluer la biodégradabilité d'un plastique en milieu marin suivent les méthodologies proposées par les normes (Folino et al., 2023). Le tableau 1.1 présente des méthodes normées disponibles aujourd'hui. Ces tests sont basés sur des mesures

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de respirométrie, c'est-à-dire en suivant la consommation d'O<sub>2</sub> ou la production de CO<sub>2</sub> dans le cas de la biodégradation aérobie, autrement dit, seulement la dernière étape de la biodégradation (Fig. 1.13). Les normes de biodégradabilité des matériaux plastiques en milieux aquatiques ont récemment été critiquées par la communauté scientifique, notamment pour leurs conditions expérimentales trop peu représentatives de l'environnement naturel (Harrison et al., 2018). De plus, les lignes directrices ne sont pas assez spécifiques, concernant par exemple la température, le conditionnement, ou encore la forme des matériaux testés et de référence, pour lesquelles les normes n'imposent rien ou donnent une large gamme de possibilités (Harrison et al., 2018). Ces tests sont trop simplifiés, le nombre de réplicats insuffisant et peuvent conduire à une surestimation de la biodégradabilité des plastiques testés. De plus, aucun volet de toxicité n'est pour le moment pris en compte quant aux produits de biodégradation relargués durant le processus.

Parmi les normes, il est important de distinguer les normes méthodologiques, qui proposent des protocoles normés avec les conditions assurant que le test est valide, par rapport aux normes de spécification, qui fixent des seuils pour que le produit soit commercialisable avec la mention « biodégradable ». Il n'existe aujourd'hui aucune norme de spécification pour les plastiques en milieu marin, la dernière n'étant plus valable depuis 2014 et n'ayant jamais été renouvelée (ASTM D7081-05, imposant un taux de biodégradation de 90% après 6 mois d'exposition). Les normes actuellement en vigueur proposent d'évaluer le taux de biodégradation d'un plastique dans un temps donné, fixent les conditions de validité du test par rapport au contrôle positif mais ne donnent en aucun cas de critère d'acceptabilité, ni en termes de taux, ni en termes de délais (Harrison et al., 2018). Au sens strict, tout plastique est biodégradable dans l'environnement. Le problème des plastiques conventionnels est le temps nécessaire à leur minéralisation, qui peut prendre plusieurs centaines d'années (Arutchelvi et al., 2008). Dans le cas des plastiques biodégradables en mer, une norme de spécification devrait également inclure un test de toxicité des produits de biodégradation, ainsi que le temps et les seuils de biodégradabilité et de toxicité qui sont acceptables pour considérer que le plastique est biodégradable en conditions marines (Folino et al., 2023).

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Tableau 1.1. Synthèse des normes de spécification actuellement disponibles pour l'étude de la biodégradation des plastiques en milieu marin.

Norme	Nom	Milieu d'incubation	Inoculum	Température	Méthode d'analyse	Durée	Seuil de biodégradabilité
ISO 18830	Détermination de la biodégradation aérobie des matériaux plastiques non flottant à l'interface eau de mer/sédiments sableux	Sédiments marins sableux et eau de mer naturelle ou sédiments marins sableux et eau de mer artificielle	Eau de mer et sédiments	De préférence entre 15 et 25°C Pas au dessus de 28°C	Mesure de l'O <sub>2</sub> consommé	Jusqu'à l'obtention d'un plateau Durée maximale de 2 ans	Le matériau de référence doit avoir atteint 60% de biodégradation après 180 jours d'incubation pour que le test soit valide Pas de seuil à franchir pour le matériau testé
ISO 23977-1	Détermination de la biodégradation aérobie des matériaux plastiques exposés à l'eau de mer	Eau de mer ou eau de mer et un peu de sédiments	Eau de mer et sédiments	De préférence entre 15 et 25°C Pas au dessus de 28°C	Mesure du CO <sub>2</sub> libéré	Jusqu'à l'obtention d'un plateau Durée maximale de 2 ans	Le matériau de référence doit avoir atteint 60% de biodégradation après 180 jours d'incubation pour que le test soit valide Pas de seuil à franchir pour le matériau testé
ISO 23977-2	Détermination de la biodégradation aérobie des matériaux plastiques exposés à l'eau de mer	Eau de mer ou eau de mer et un peu de sédiments	Eau de mer et sédiments	De préférence entre 15 et 25°C Pas au dessus de 28°C	Mesure de l'O <sub>2</sub> consommé	Jusqu'à l'obtention d'un plateau Durée maximale de 2 ans	Le matériau de référence doit avoir atteint 60% de biodégradation après 180 jours d'incubation pour que le test soit valide Pas de seuil à franchir pour le matériau testé
ISO 22404	Détermination de la biodégradation aérobie des matériaux non flottant exposé aux sédiments marins	Sédiments marins sableux et eau de mer naturelle	Eau de mer et sédiments	De préférence entre 15 et 25°C Pas au dessus de 28°C	Mesure du CO <sub>2</sub> libéré	Jusqu'à l'obtention d'un plateau Durée maximale de 2 ans	Le matériau de référence doit avoir atteint 60% de biodégradation après 180 jours d'incubation pour que le test soit valide Pas de seuil à franchir pour le matériau testé
ISO 22403	Evaluation de la biodégradabilité aérobie inhérente des matériaux non flottant en conditions marines	Suivre une des normes: - ISO 18830 - ISO 23977-1 - ISO 23077-2 - ISO 22404 - ISO 19679 - ASTM D6691-17					90% de biodégradabilité du matériau testé
ISO 19679	Détermination de la biodégradation aérobie des matières plastiques non-flottantes à l'interface eau de mer/sédiments	Sédiments marins sableux et eau de mer naturelle ou sédiments marins sableux et eau de mer artificielle	Eau de mer et sédiments	De préférence entre 15 et 25°C Pas au dessus de 28°C	Mesure du CO <sub>2</sub> libéré	Jusqu'à l'obtention d'un plateau Durée maximale de 2 ans	Le matériau de référence doit avoir atteint 60% de biodégradation après 180 jours d'incubation pour que le test soit valide Pas de seuil à franchir pour le matériau testé
ISO 14851	Evaluation de la biodégradabilité aérobie ultime des matériaux plastiques en milieu aqueux	Milieu inorganique artificiel	Boues activées, compost ou sol	De préférence entre 20 et 25°C ou autre température optimale pour l'inoculum	Mesure de l'O <sub>2</sub> consommé	Durée maximale de 6 mois	Le matériau de référence doit avoir atteint 60% de biodégradation après 180 jours d'incubation pour que le test soit valide Pas de seuil à franchir pour le matériau testé
ISO 14852	Evaluation de la biodégradabilité aérobie ultime des matériaux plastiques en milieu aqueux	Milieu inorganique artificiel	Boues activées, compost ou sol	De préférence entre 20 et 25°C ou autre température optimale pour l'inoculum	Mesure du CO <sub>2</sub> libéré	Durée maximale de 6 mois	Le matériau de référence doit avoir atteint 60% de biodégradation après 180 jours d'incubation pour que le test soit valide Pas de seuil à franchir pour le matériau testé
ASTM D6691 09	Détermination de la biodégradation aérobie des matériaux plastiques dans l'environnement marin avec un inoculum bactérien défini ou de l'eau de mer naturelle	Milieu artificiel ou eau de mer naturelle	Minimum 10 microorganismes appartenant à des genres précis ou eau de mer naturelle	30°C	Mesure du CO <sub>2</sub> libéré	Durée maximale de 3 mois	Le matériau de référence doit avoir atteint 70% de biodégradation Pas de seuil à franchir pour le matériau testé
ASTM D7991 15	Détermination de la biodégradation aérobie des plastiques enfouis dans les sédiments marins	Eau de mer et sédiments côtiers	Eau de mer et sédiments	De préférence entre 15 et 25°C Pas au dessus de 28°C	Mesure du CO <sub>2</sub> libéré	Jusqu'à l'obtention d'un plateau Durée maximale de 2 ans	Le matériau de référence doit avoir atteint 60% de biodégradation après 180 jours d'incubation pour que le test soit valide Pas de seuil à franchir pour le matériau testé

### *4.2.3. Difficultés techniques de l'étude de la biodégradabilité en milieu marin*

Si les normes sont importantes pour encadrer le bon développement des matériaux biodégradables, elles nécessitent une uniformisation souvent réductionniste qui peut nuire à sa représentativité en milieu naturel. L'établissement d'une norme pertinente pour l'étude de la biodégradabilité des plastiques en milieu marin présente une multitude de contraintes. D'abord, le milieu marin est un terme général qui comprend des environnements avec des conditions physiques, chimiques et biologiques très variées. En toute rigueur, une norme complète devrait présenter au moins 6 tests qui imposent des conditions expérimentales relatives aux écosystèmes suivants : supralittoral, eulittoral, benthique sublittoral, benthique profond, pélagique et enfoui dans les sédiments (Emadian et al., 2017). La plupart des normes disponibles aujourd'hui représentent l'interface eau/sédiment, probablement par souci de facilité d'obtenir l'inoculum bactérien en prélevant du sable ou du sédiment. L'utilisation de sédiments a récemment été recommandée pour un test rapide de la biodégradabilité des plastiques en mer, car la réponse de biodégradation est plus importante qu'avec l'utilisation d'eau de mer, c'est à dire de communautés pélagiques (Beiras & López-Ibáñez, 2023). Ces conditions ne doivent cependant être utilisées que pour des plastiques posés sur les sédiments, et non pour des plastiques flottants.

Une autre difficulté repose sur la nature du plastique lui-même, qui est un polluant original puisque solide et donc insoluble. La forme du plastique utilisée pour le test de biodégradation reste à définir pour l'établissement de nouvelles normes (état final du produit, film, broyé, poudre...). Aujourd'hui, les tests de biodégradation s'intéressent uniquement à quantifier le taux de biodégradation maximal atteint dans une période maximale de deux ans (tableau 1.1). Ce taux est calculé par le ratio de la consommation d'O<sub>2</sub> ou la production de CO<sub>2</sub> mesurée par rapport à une valeur théorique, basée sur la masse de carbone utilisée dans le test (voir équations A et B, chapitre 5 partie 2 de ce manuscrit). Cependant, parce que le plastique est solide et que les enzymes ne peuvent pas pénétrer la matrice, son hydrolyse ne peut avoir lieu qu'à la surface du matériau (Haider et al., 2019) et la totalité du carbone n'est donc pas disponible pour les micro-organismes, ce qui peut conduire à une sous-estimation de la biodégradabilité du plastique considéré. Une solution à ce problème est de tester le plastique sous forme micronisée, augmentant la surface d'échange, donc la biodisponibilité et ainsi la biodégradation (Beiras & López-Ibáñez, 2023). Cette forme peut être acceptable pour tester la



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biodégradabilité intrinsèque d'un matériau, mais peu représentative de la forme véritable du produit. Le caractère solide du plastique peut également présenter une limite pour l'adaptation de certaines méthodes d'analyses classiquement utilisées en écologie microbienne, qui demandent du travail de développement pour être appliquées à ces échantillons.

Ainsi, la caractérisation de la biodégradabilité marine des plastiques est encore incomplète et présente de nombreuses difficultés. Les données de biodégradation en milieu marin sont encore peu nombreuses, alors qu'elles sont nécessaires pour le développement de protocoles de tests plus pertinents et de matériaux réellement moins récalcitrant (Manfra et al., 2021).



## *Chapitre 2 : Étude des communautés bactériennes de la plastisphère le long du continuum fleuve-mer*

Ce chapitre est rédigé sous la forme d'un article scientifique qui sera soumis en Décembre 2023 au journal « Environmental Science and Pollution Research », dans un numéro spécial dédié à la pollution plastique (Éditeurs en chef : Alexandra ter Halle & Jean-François Ghiglione). Cette partie est une étude des communautés de la plastisphère dans le continuum fleuve-mer. La mission *Tara Microplastiques* est une campagne d'échantillonnage supportée par la fondation Tara Océan d'une durée de 8 mois qui a permis d'étudier la pollution plastique le long de 9 grands fleuves européens : la Tamise, l'Elbe, le Rhin, la Seine, la Loire, la Garonne, le Rhône, l'Ebre et le Tibre (Fig. 2.1). Plusieurs stations ont été échantillonnées le long de ces 9 fleuves : deux stations en eau douce en amont (5) et en aval (4) d'une grande ville, une station à l'estuaire (2), une station en mer au large de l'embouchure (1), et dans certains cas, une station intermédiaire (3) (Fig. 2.1).



Figure 2.1. Plan d'échantillonnage de la mission Tara Microplastiques. A gauche : les 9 fleuves échantillonnés dans le cadre de la mission Tara Microplastiques. A droite : plan des points d'échantillonnage le long de la Seine. A chaque station, l'eau environnante est échantillonnée à l'aide d'une bouteille niskine, ainsi que les débris plastiques flottants (MP) avec un filet manta et les plastiques colonisés pendant un mois (*pMP*).

Les communautés bactériennes associées à trois types d'échantillons ont été analysées :

- Des microplastiques de surface, échantillonnés à l'aide d'un filet manta (désignés par MP = microplastic)
- Des plastiques vierges mis en colonisation pendant un mois aux points d'échantillonnage (désignés par *pMP* = pristine microplastic)

- L'eau environnante, les fractions 3  $\mu\text{m}$  et 0,2  $\mu\text{m}$  ayant été analysées séparément (désignées par AP = organic particle-attached bacteria et FL = free-living bacteria, respectivement)

Les échantillons ont été conservés à  $-80^{\circ}\text{C}$  puis soumis à des extractions d'ADN. Un séquençage d'amplicon a été réalisé par le Génoscope sur les ADN extraits, en ciblant la région V4-V5 du gène codant pour l'ARNr 16S. Les microplastiques sélectionnés pour l'extraction d'ADN ont ensuite été analysés par spectroscopie infrarouge à transformée de Fourier (FTIR) afin de déterminer leur nature chimique.

Les résultats de ces analyses confirment la distinction entre les communautés de la plastisphère et celles de l'eau environnante, libres et attachées aux particules organiques. La zone géographique, ici le fleuve, est également confirmée comme un facteur déterminant de la structure des communautés associées aux débris plastiques, ainsi que de l'eau environnante. De plus, les communautés de la plastisphère aux stations fluviales (4 et 5) ont été montrées significativement différentes de celles associées aux débris plastiques échantillonnés aux stations marines (1), suggérant un faible transfert des communautés de long du continuum fleuve-mer, qui évoluent sous la pression des changements environnementaux. En particulier, seules 74 ASV communs entre la station 1 et les stations 4 et 5 de la Seine ont été mises en évidence. L'étude taxonomique de ces communautés a mis en avant différents groupes dominants selon la station considérée. Enfin, des différences ont été observées entre les communautés des débris plastiques flottant, et les biofilms matures obtenus sur des plastiques après 1 mois de colonisation, en particulier aux stations 4 et 5.

Les résultats de ce travail seront compilés avec l'analyse des communautés fongiques associées aux mêmes échantillons, obtenus grâce au séquençage des régions de l'ADNr 18S et ITS. L'analyse sera réalisée en tenant compte des paramètres environnementaux (température, conductivité, nutriments, matière organique).

## **A pan-European study of plastisphere diversity along the river-sea continuum**

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**Keywords :** Microplastics · Biofilm · Biofouling · Microbial ecotoxicology · River-sea continuum

## Highlights :

- Plastisphere niche partitioning is a common feature in all biota
- Salinity is the main driver of the plastisphere bacterial communities
- Almost complete segregation between seawater and freshwater plastispheres

## Abstract :

Microplastics provide a persistent and buoyant substrate that can act as a raft for microbes. A significant concern regarding the plastisphere is the potential dispersal of freshwater bacteria (including pathogens) into the sea. We explored the plastisphere on plastic debris and on pristine plastics as well as the bacteria living in surrounding waters, along the river-sea continuum in nine major European rivers sampled during the seven months of the *Tara Microplastics* mission. We found a clear niche partitioning in all biota among bacteria living on plastics, and bacteria from the surrounding waters. Among the large dataset, a clear gradient was found from the freshwater to the sea, with a complete segregation in plastisphere composition that is not consistent with a major transfer of pathogens between the two contrasted ecosystems. Finally, we found that biofilm grown during 1-month immersion on pristine plastics presented similar behavior than the plastisphere living on the various plastic debris in the river-sea continuum. These results reinforce the major role played by the environmental conditions in shaping plastisphere biodiversity, rather than the plastic history, polymer chemical composition, size or shape.

# 1. Introduction

Microorganisms living on plastic debris (MP) have received a growing attention since the characterization of a distinct and very diverse community, called the ‘plastisphere’, as compared to the microorganisms living in the surrounding seawater (Zettler et al., 2013). The large number of plastics released in the marine environment is providing a new habitat that is rapidly colonized by microorganisms (Harrison et al., 2014). The microbial biomass harbored on marine MP can be significant, upwards to 6 % of the total mass of a piece of microplastic (Morét-Ferguson et al., 2010). The biomass of the known plastisphere has previously been approximately to 1,000 to 15,000 metric tons, corresponding to a range of 0.01-0.2 % of the microbial biomass in the open ocean surface waters (Mincer et al., 2016), a number that is probably underestimated according to the more recent estimation of microplastic concentration in the surface Ocean (Isobe et al., 2021).

The ecological impact of this human-made ecosystem is largely unknown. Trace nutrients are concentrated onto the plastic surface, making them more bioavailable for microbial phototrophs and heterotrophs that play a crucial role in the carbon biogeochemical cycle (Conan et al., 2022). Interaction with the large diversity of parasitic and saprophytic fungi may also impact the carbon processing within the new plastic habitat as well (Kettner et al., 2019). Recent studies showed that the plastisphere can also affect the animal microbiomes (Lu et al., 2019). MPs provide a durable substrate for marine life that can transport microorganisms for long distances, including potential pathogens (Bowley et al., 2021) and algal species causing harmful blooms (Masó et al., 2003). Physical oceanographic models have shown that plastic can migrate over 1,000 km in less than 2 months (Law et al., 2010). However, the carrying capacity of the community attached to the surface of plastic debris remains largely unknown (Amaral-Zettler et al., 2020). Environmental parameters have been shown to drastically affect the plastisphere composition (Basili et al., 2020), suggesting that plastic-attached communities could overcome changes when transported from one ecosystem to another.

Research efforts have mainly focused on exploring the plastisphere in the marine environment so far, because it is believed that the ocean is the ultimate sink for plastic pollution (Martin et al., 2020). Less attention has been paid to riverine waters despite the fact that about 80% of marine plastic debris are believed to originate from rivers, with an annual transfer of



500 kilotons for the year 2020 (Kaandorp et al., 2023). There is increasing evidence that many rivers across the globe exhibit higher microplastics concentrations than the marine environment (Eriksen et al., 2013; Weiss et al., 2021). The plastisphere in freshwater ecosystems was also demonstrated as a specific niche for microorganisms when compared to the surrounding riverine waters (Yang et al., 2020). In a review paper, Barros and Seena (2021) indicated that some identified plastisphere microbes, including pathogenic bacteria, could be detected in both freshwater and marine systems. Possible pathogen transfer between the two ecosystems has been hypothesized due to the protective environment afforded by the biofilm growing on plastics. It has been evidenced under laboratory conditions (mesocosm) by the survival of human pathogens bound to microplastics during the transfer from freshwater to marine conditions (Metcalf et al., 2023).

Most studies have focused on incubation experiments with known polymer types, and relatively few have examined communities on environmentally collected MPs in freshwater or in marine environments (Amaral-Zettler et al., 2020; Barros & Seena, 2021). Some studies used postconsumer plastic, such as PET bottles or plastic bags (Muthukrishnan et al., 2019; Oberbeckmann et al., 2016), whereas others used industrial pristine plastics from known manufacturing sources such as industrial primary microplastic pellets (Metcalf et al., 2023). The diversity of experimental designs makes it difficult to compare studies directly, but some of them compared results with environmentally collected plastic debris. For example, a minimum of one-month incubation in seawater corresponded to the development of a mature biofilm that presented similarities to environmentally collected plastic debris (Dussud et al., 2018a; Dussud et al., 2018b).

Here, we explored the plastisphere along the river-sea continuum in nine major European rivers sampled during the eight months of the *Tara* Microplastics mission (Ghiglione et al., 2023). We tested the hypothesis of a transfer of microorganisms (including putative pathogens) together with microplastics (MPs) rafting along a salinity gradient from the sea, the outer estuary, downstream and upstream of the first heavily populated city. We also incubated pristine plastics (*p*MPs) at each sampling site for one month, in order to compare mature biofilms to the plastisphere attached to floating MPs and to the bacteria living in the surrounding water at the same sampling site.

## 2. Material and methods

### 2.1. Sampling design

The *Tara* Microplastics mission was conducted during 7 months along nine major rivers in Europe (Ghiglione et al., 2023). Harmonized sampling methodologies were used at 45 sampling sites. Four to five sampling sites along a salinity gradient from the sea (station 1) and the outer estuary (station 2) to intermediate salinity (station 3) and downstream (station 4) and upstream (station 5) of the first heavily populated city located on each river, including London on the Thames, Hamburg on the Elbe, Rotterdam on the Rhine, Rouen on the Seine, Nantes on the Loire, Bordeaux on the Garonne, Tortosa on the Ebro, Arles on the Rhone, and Rome on the Tiber. Only 4 stations were sampled in Thames, with the intermediate-salinity station missing. Water samples and MPs were taken onboard the French research vessel (RV) *Tara* or from a semi-rigid boat in shallow waters.

Water sampling was performed at each sampling station and just before the concomitant 330- $\mu\text{m}$  manta trawl deployments. A 8-L Niskin bottle was triggered just below the surface and water subsamples were transferred to a set of specific devices for nutrients, particulate matter and bacterial diversity analysis. One or two liters (depending on turbidity) of 25  $\mu\text{m}$  prefiltered water (Nylon mesh) were successively filtered onto 3  $\mu\text{m}$  and 0.2  $\mu\text{m}$ -pore size polycarbonate filters (47 mm diameter, Nucleopore) and filters were stored at  $-80^{\circ}\text{C}$  before DNA extraction of both organic-particle-attached bacteria (PA) and free-living bacteria (FL), respectively.

Sampling for MPs was conducted using a 330- $\mu\text{m}$  mesh size manta trawl (aperture of  $30 \times 80$  cm, 2.5 m long nylon net, and  $30 \times 10$  cm<sup>2</sup> weighted cod end). Manta trawl was deployed at an approximate speed of 2.0 knots for 60 min in seawater and 10 min in rivers, in order to avoid clogging (especially in rivers). After careful rinsing of the net with water from the sampling site, macro-debris of natural origin (algae, branches, leaves, etc) were eliminated through rinsing above the collector. MPs with approximately 1 to 5 mm in size accumulated in the final volume of 1.0 L of the collector were transferred in glass petri dishes, observed under a binocular magnifying glass, sorted using alcohol/flame sterilized forceps and immediately frozen in liquid nitrogen for further DNA extraction and chemical identification by attenuated total reflection-Fourier transform infrared spectroscopy (ATR-FTIR).

A team on land was dispatched one month before the arrival of *Tara* for site reconnaissance and for the deployment of cage structures (30 cm x 10 cm cylinder) containing pristine plastics (*p*MPPs) for the *in-situ* colonization experiment. Around 10g of pellets made of polyethylene (PE) and polyoxymethylene (POM) and Nylon mesh (NY, polyamide-6,6) were immersed during one month at the same sampling site as manta trawl deployments, sorted using alcohol/flame sterilized forceps and immediately frozen in liquid nitrogen for further DNA extraction.

## 2.2. Temperature, salinity, nutrients and particulate organic matters

A thermosalinograph (TSG, Seabird SBE45) was installed onboard the RV *Tara* for surface temperature and conductivity measurements at a sampling frequency of 0.1 Hz. Discrete vertical measurements from 0 to 30 m depth (or less in shallow waters) were also done at each sampling station using a portable Sontek CastAway CTD probe (temperature, conductivity, salinity, oxygen and photosynthetically available radiation) (ADCPro, France) attached to the rope holding the 8-L Niskin bottle. The analytical precision was 0.01 °C for temperature and 0.01 to 0.05 for salinity.

Nutrients were analyzed from a 18-mL subsample of water filtered through a glass syringe fitted with a Whatman Anodisc-Paradisc 0.45- $\mu$ m filter and placed in a 20-mL polyethylene scintillation vial. Another 8-mL were placed in a 20-mL polyethylene scintillation vial for ammonium ( $\text{NH}_4^+$ ) analysis. The samples were frozen at -20 °C and stored until analysis. Upon return to the laboratory, nitrate ( $\text{NO}_3^-$ ), nitrite ( $\text{NO}_2^-$ ), phosphate ( $\text{PO}_4^{3-}$ ), and dissolved silica ( $\text{Si}(\text{OH})_4^-$ ) concentrations were measured on a continuous flow Seal-Bran luebbe® AutoAnalyzer III, whereas  $\text{NH}_4^+$  determinations were performed by fluorimetry on a Jasco FP-2020 fluorimeter (Holmes et al. 1999). The analytical precision of  $\text{NO}_3^-$ ,  $\text{NO}_2^-$ ,  $\text{PO}_4^{3-}$ , and  $\text{Si}(\text{OH})_4^-$  is  $\pm 0.02 \mu\text{M}$ ,  $\pm 0.01 \mu\text{M}$ ,  $0.02 \mu\text{M}$ , and  $\pm 0.05 \mu\text{M}$ , respectively, and  $\pm 5 \text{ nM}$  for  $\text{NH}_4^+$ .

Suspended particulate matter (SPM) was determined from water subsamples (from 100 to 500 mL) filtered on pre-combusted glass fiber filters (Whatman GF/F, 25 mm, 450 °C, 12 h) and then dried at 60 °C and stored in a desiccator until further analysis. SPM concentrations were determined by differences between the dry weights of the respective filters before and after filtration. Particulate organic matter was measured by dry combustion on a

CHN 2400 Perkin Elmer analyzer (detection limit: 0.1 mg of C) after decarbonatization through subsequent rinsing of the filters with phosphoric (1 M) and hydrochloric (2 M) acid.

### 2.3. DNA extractions, PCR and sequencing

The same DNA extraction protocol was used for organic-particle-attached bacteria (PA), free-living bacteria (FL) in waters and for the plastisphere of plastic debris (MP) and pristine plastics (*p*MP) made of polyethylene (PE) and polyoxymethylene (POM) and Nylon (NYL). DNA extractions were performed using a classical phenol-chloroform method with slight modifications, as previously described (Jacquin et al., 2019). Polymerase Chain Reaction (PCR) amplification of the V4-V5 region was done using universal 16 rRNA primers (515Y and 926R) (Parada et al., 2016) with Illumina-specific primers and barcodes. Sequencing was performed on Illumina MiSeq by Genoscope (Evry, France). Sequences analysis was done using the DADA2 pipeline (Callahan et al., 2016) for ASV establishment and taxonomy assignment. Taxonomic assignment was done using the SILVA 138 SSU database. ASV that did not belong to the *Bacteria* kingdom as well as ASV from chloroplasts and mitochondria were removed from the dataset. The number of sequences per sample was normalized by rarefaction ( $n=15,657$ ) for sample comparison. All further analyses were performed in the resampled ASV table containing 137,948 ASV in 316 samples.

### 2.4. ATR-FTIR analysis

An attenuated total reflection-Fourier transform infrared spectrometer (ATR-FTIR Vertex70v, Bruker, ATR Golden Gate) was used to determine the polymer composition and chemical characteristics of the sorted microplastics and FTIR spectra were identified using POSEIDON software (Kedzierski et al., 2019). For each of the polyethylene (PE) infrared spectra, the carbonyl index (CI), hydroxyl index (HI), and fouling index (FI) were determined, as previously described (Kedzierski et al., 2022). Analyses were performed using the following parameters: 32 scans, 4 of resolution and large scale from 4000 to 600  $\text{cm}^{-1}$ .

### 2.5. Data management

Data were treated with R version 4.3.1. Graphical representations were done using the *ggplot2* version 3.4.2 (Wickham, 2016) and the *vegan* version 2.6.1 (Dixon, 2003) packages. Kruskal-Wallis and Dunn tests (Holm-Bonferroni correction) were done with the *stats* version 4.3.1 and *rstatix* version 0.7.2 packages respectively. Resampling and calculation of alpha-diversity indexes were done using the *phyloseq* package version 1.44.0 (McMurdie & Holmes,

2013). The Bray-Curtis dissimilarity matrix, NMDS and PERMANOVA tests were done using the *vegan* package. Post-hoc pairwise analyses were done using the *pairwiseAdonis* package version 0.4.1 with Holm-Bonferroni correction. Similarity Percentage analysis (SIMPER) was performed to identify the contribution of each ASV, using PRIMER 6. Clustering was done using UPGMA method with the *stats* package version 4.3.1. Venn diagrams were done using the *MicEco* package version 0.0.19.

### 3. Results

#### 3.1. Chemical nature of microplastics

We visually sorted 115 MPs in order to obtain 0 to 12 pieces per station (mean = 2.6, SD=3.4, n=43 sampling sites). With sizes ranging from 1.6 to 17.8 mm, some of the pieces did not correspond exactly to the arbitrary limit of microplastics (size <5 mm), but were representative of the majority of MPs recovered in all manta nets. ATR-FTIR analysis revealed that polyethylene (PE) dominated the composition of the MPs (38%), followed by polypropylene (13%), polystyrene (12%), and polyethylene vinyl acetate (6%). Unidentified polymers, representing 29% of the collected pieces, were categorized as “unknown” (appendix 2.1). Polyethylene (PE) was present in most of the sampling sites (83% of the sampling sites with MPs), and polypropylene (PP) was more prevalent than Polystyrene (PS) and PEVA (38%, 29% and 17% of the sampling sites with MPS, respectively).

#### 3.2. Alpha-diversity

Alpha-diversity was assessed by calculation of Chao1, Pielou (appendix 2.2) and Shannon indexes (Fig. 2.1). Overall, free-living bacteria (FL) present the lowest Shannon diversity values (median =  $4.8 \pm 0.58$ , n=42) as compared to the other samples (median =  $5.4 \pm 0.78$ , n = 39 and median =  $5.4 \pm 0.86$ , n = 104, for PA and pMPs, respectively), except for MPs sampled at sea (stations 1) (median =  $4.0 \pm 1.2$ , n = 28). Regardless of the sampling site, highest Chao1 values were systematically found for the PA bacteria samples (median values between 1,323 and 3,013, n = 39) as compared to other samples (median values between 602 for MPs at station 1 and 2,177 for pMPs at station 5, n = 28 and n = 11 respectively).

Nonparametric pairwise multiple comparisons in independent groups using Dunn’s test showed no significant difference in all diversity indexes when compiling water (FL and PA)

and plastic (MPs and *p*MPs) samples at any of the stations (Holm-Bonferroni corrections,  $p > 0.5$ ). All diversity indexes were significantly lower for MPs sampled at sea (stations 1) as compared to those sampled in freshwater, upstream from the city (station 5) (Dunn tests with Holm correction;  $p$ -value =  $2.2 \times 10^{-4}$ ,  $p$ -value =  $1.7 \times 10^{-3}$  and  $p$ -value =  $3.9 \times 10^{-4}$  for Chao1, Pielou and Shannon indexes, respectively). This was not the case for *p*MPs samples, which did not show significant difference of diversity indexes among stations, all rivers comprised nor when considering rivers individually. No significant difference in diversity indexes was found between MPs and *p*MPs groups, except for sea samples (station 1), for which significantly lower Chao1, Pielou and Shannon diversity were observed for MPs ( $p$ -value = 0.021,  $p$ -value =  $2.1 \times 10^{-6}$  and  $p$ -value =  $7.9 \times 10^{-6}$  respectively).

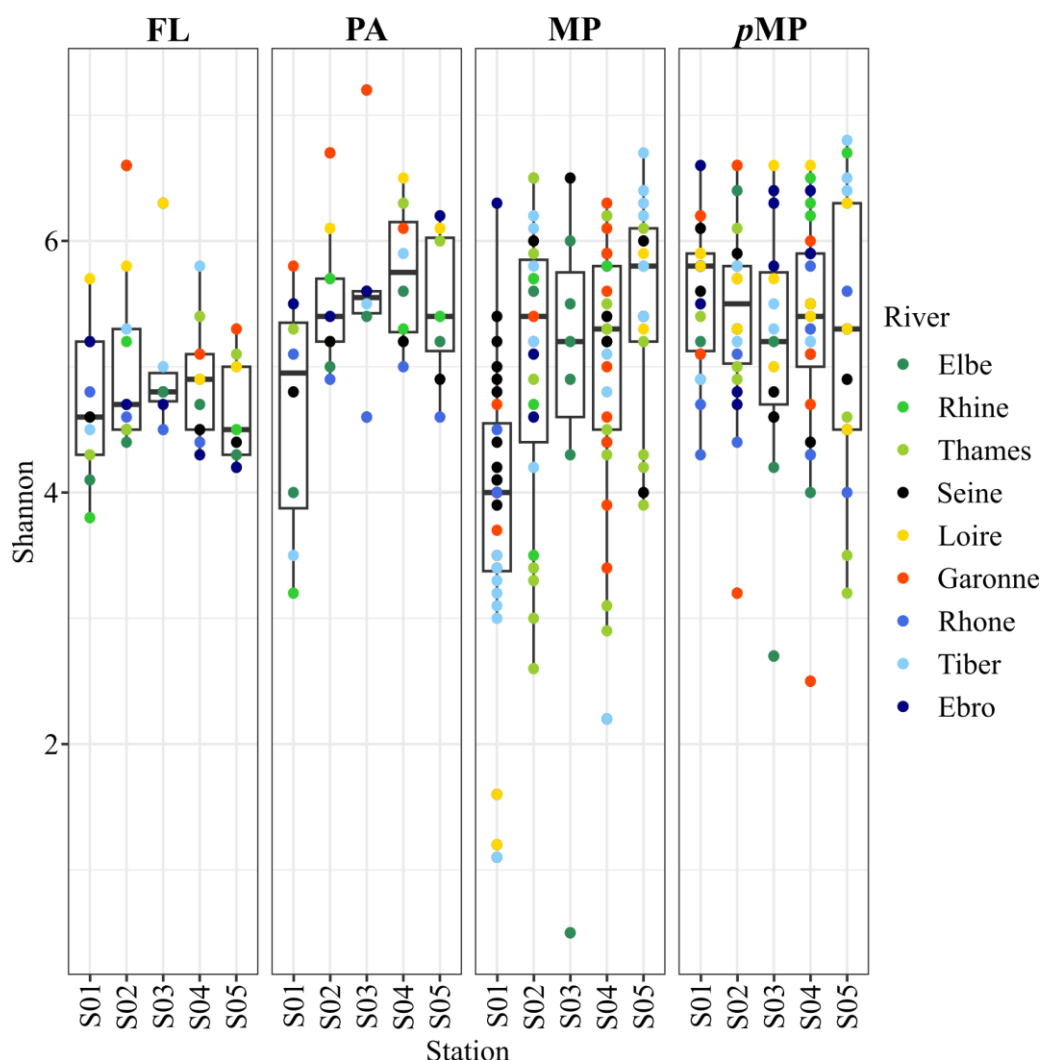


Figure 2.1. Shannon indexes at each station, all rivers considered. PA refers to the particle-attached bacteria, FL to free-living bacteria, MP to floating microplastics and *p*MP to 1-month plastispheres colonized on pristine plastics.

### 3.3. Beta-diversity

NMDS based on Bray Curtis similarity showed clear distinction in community structure between samples taken at sea (station 1) and freshwater samples (stations 4 and 5), with intermediate similarities for samples originating from the estuarine (station 2) and intermediate salinity stations (station 3) (Fig. 2.2). A PERMANOVA test confirmed that the sampling station / river-to-sea continuum drove the entire dataset of bacterial community structure ( $R^2 = 0.050$ ,  $p$ -value = 0.001), with lower difference between stations 4 and 5. Bacterial communities associated with plastics (both MPs and *p*MPs) differed significantly from the surrounding water communities (both FL and PA fractions) at each station. No significant difference was found between FL and PA community structures at each different station ( $p > 0.05$ ) and within the rivers ( $p > 0.05$ ). Another driving factor was the river origin that significantly explained the community structures (PERMANOVA  $R^2 = 0.087$ ,  $p$ -value = 0.001).

Bacterial communities associated with plastics (both MPs and *p*MPs) differed significantly from the surrounding water communities (both PA and FL fractions) at each station. Significantly higher dissimilarity was found in the freshwater (post-hoc multiple comparisons from stations 4 and 5;  $R^2 = 0.12$ ,  $p$ -value= 0.001 and  $R^2 = 0.17$ ,  $p$ -value= 0.001 respectively) as compared to seawater samples (post-hoc multiple comparisons from station 1;  $R^2 = 0.084$ ,  $p$ -value= 0.001). No significant difference was found between PA and FL community structures at each different station ( $p$ -value > 0.05) and within the rivers ( $p$ -value > 0.05).

Some differences were highlighted between communities associated to MPs compared to *p*MPs at different sampling sites, when the number of samples allowed the statistical comparison. In particular, significant differences were identified between MP and *p*MP plastispheres sampled at seawater station 1 of the Seine and the Garonne rivers (post-hoc multiple comparisons with Holm-Bonferroni correction;  $R^2 = 0.23$ ,  $p$ -value = 0.004 and  $R^2 = 0.27$ ,  $p$ -value = 0.022, respectively). Except for some stations in the Seine (station 5) and Garonne (station 2) rivers, we always found significant dissimilarities between MPs and *p*MPs plastisphere community structure at the same sampling station (post-hoc multiple comparisons with Holm-Bonferroni correction,  $p$ -value < 0.05). The composition of the polymers was also not significantly driving the community structures, since no difference was found between MPs

made of PE, PP and PS communities ( $p$ -value > 0.05) and  $p$ MPs made of PE, POM or NYL ( $p$ -value > 0.05) within the same stations of all rivers.

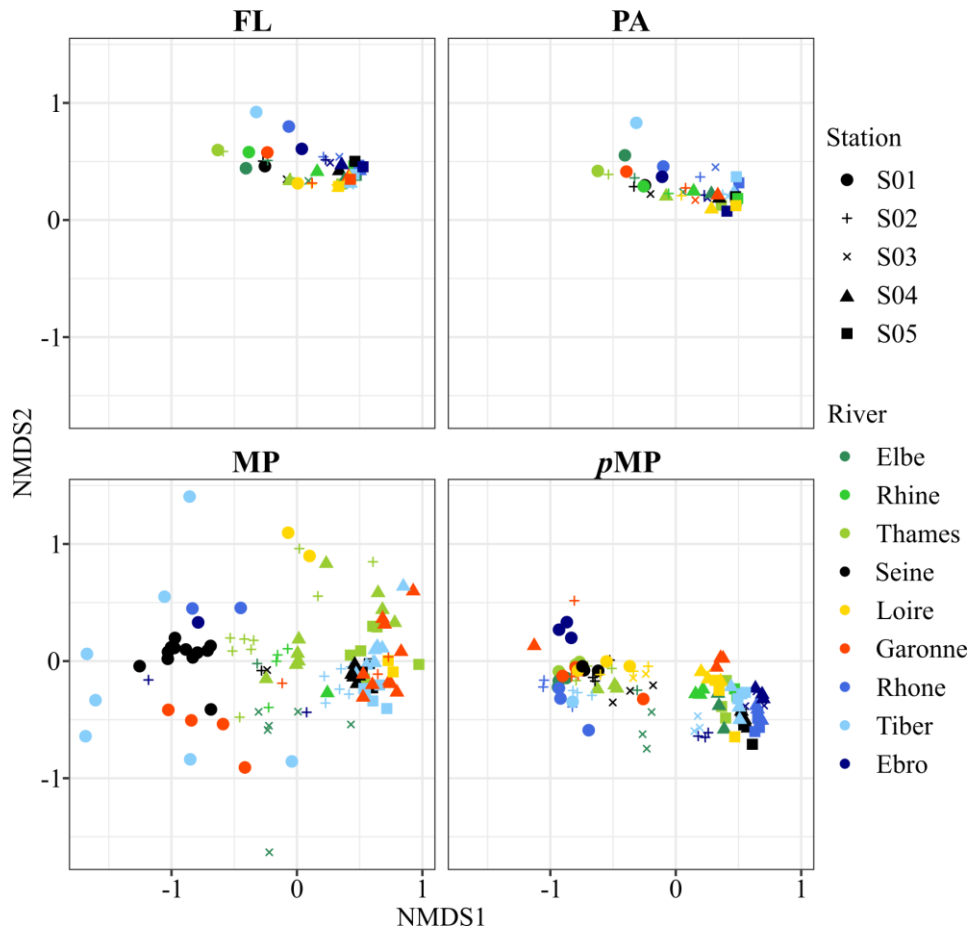


Figure 2.2. Nonmetric multidimensional scaling (NMDS) plot showing dissimilarities among PA, FL, MP and  $p$ MP communities. Each category has been sorted from the same NMDS for better visualization (stress=0.19). Colors indicate the station, i.e., sea stations (1) in blue, estuarine stations (2) in light gray, intermediate stations (3) in dark gray, downstream riverine stations (4) in orange and upstream riverine stations (5) in red.

### 3.4. Focus on MP plastisphere communities

We decided to focus on the bacterial diversity associated to MPs samples for the Seine River, because sufficient MPs sequencing data were available for all sampling stations. As previously mentioned for the entire dataset, MPs plastispheres in the Seine River differed significantly when sampled in seawater (station 1) or freshwater environments (stations 4 and 5) (post-hoc multiple comparisons with Holm-Bonferroni correction;  $R^2 = 0.34$ ,  $p$ -value = 0.001



and  $r^2 = 0.23$ ,  $p$ -value = 0.003 respectively). UPGMA dendrogram based on Bray Curtis similarities confirmed the sample organization into two clusters: the first one including samples from MPs communities from freshwater stations (stations 4 and 5), and the second comprising samples from seawater (station 1) separated from the estuarine and intermediate sampling stations (stations 2 and 3) (Fig. 2.3). Taxonomy associated to MPs from stations 4 and 5 showed high relative abundance of Burkholderiales and Deinococcales (mean relative abundance of 21 % and 12 %, respectively), whereas these groups had minor contributions at station 1 (only 0.14 % and 0.05 %, respectively). In the later station, samples presented high abundance of bacterial taxa belonging to Chitinophagales, Flavobacteriales and Rhodobacteriales (mean relative abundance of 17 %, 26 % and 23 % respectively, against 1.2 %, 5.7 % and 3.3 % at stations 4 and 5, respectively) (Fig. 2.3).

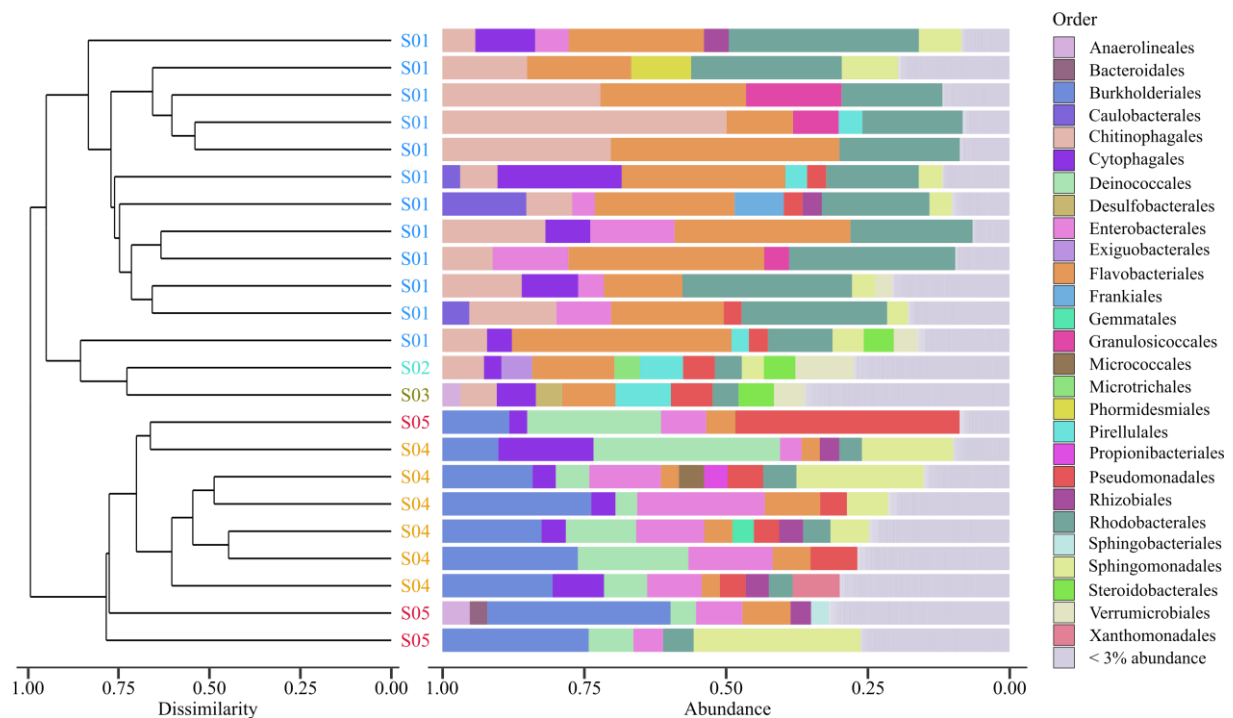


Figure 2.3. Spatial variation of taxonomic abundances and bacterial communities structure associated to MP across the Seine River. Stations 1 are represented in blue, station 2 is represented in cyan, station 3 is represented in green, stations 4 are represented in orange and stations 5 are represented in red. The dendrogram was made by UPGMA method based on a Bray-Curtis dissimilarity matrix and bar charts represent cumulative abundances of taxa at the order level.

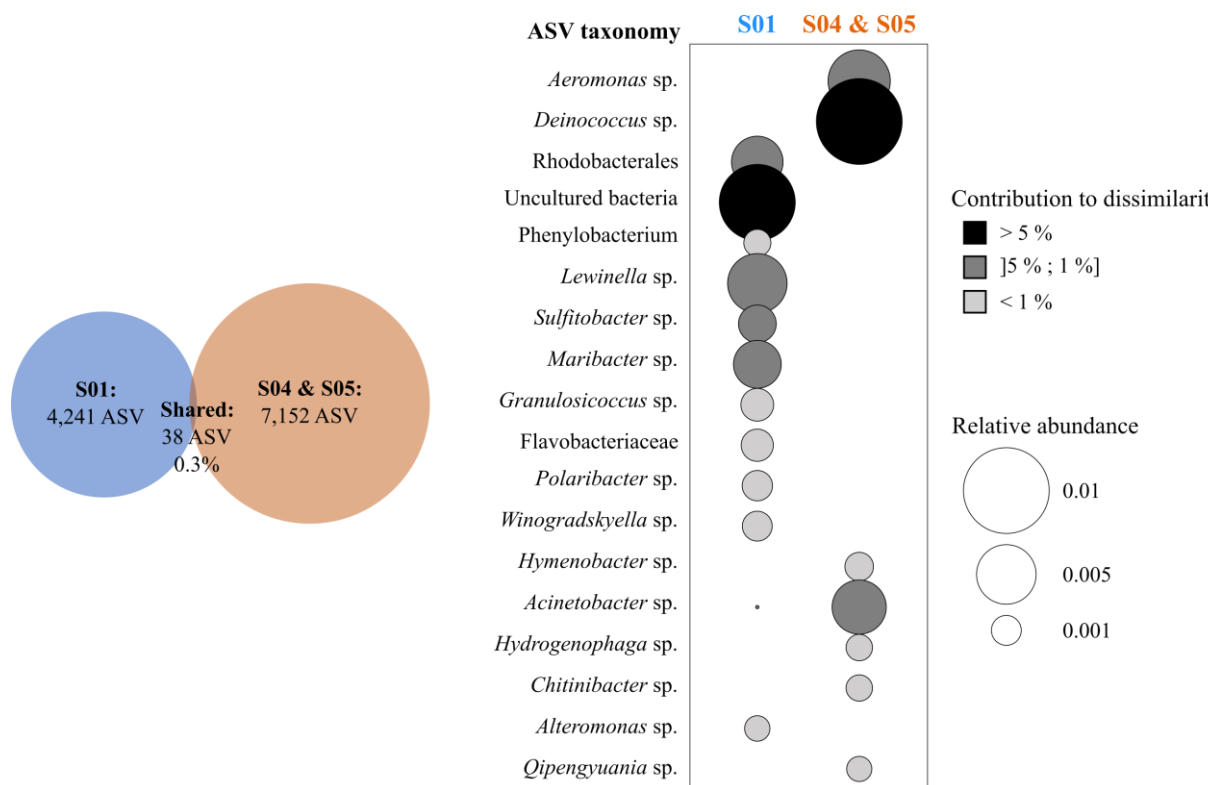


Figure 2.4. Comparison between communities associated to MP at sea stations (1) and riverine stations (4 and 5). Up: Venn diagram identifying the shared and unique ASV for the considered stations. Down: Bubble plot showing the relative abundance and taxonomy of the ASVs contributing up to 30% of the difference between the stations, based on a SIMPER analysis. Bubbles are sized according to the relative abundance and colored according to their contribution to the global dissimilarity.

Venn diagram showed that only 0.3 % of the total ASV (38 ASV) were shared between sea and freshwater MPs communities (Fig. 2.4), whereas all the other ASV were unique to one or the other aquatic compartment (river or sea). The shared ASV happened to be either abundant in samples from freshwater stations, or belonging to the rare biosphere (average relative abundance < 0.01 of the total ASV) of sea or freshwater samples. Among 30 genera identified as containing pathogenic taxa, only 3 were found in these 38 common ASV, e.g., *Psychrobacter*, *Massilia* and *Acinetobacter* genera, corresponding to 5 ASV with mean relative abundances below 0.01% in sea samples (MPs collected at station 1). SIMPER analysis highlighted 18 dominant ASV contributing to a cumulative 30 % of the dissimilarity between MPs in seawater and freshwater fractions (Venn diagram showed that only 0.3 % of the total ASV (38 ASV) were shared between sea and freshwater MPs communities (Fig. 2.4), whereas all the other ASV were unique to one or the other aquatic compartment (river or sea). The shared ASV happened to be

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## 4. Discussion

### 4.1. Plastisphere niche partitioning is a common feature in all biota

Plastisphere has been extensively studied in the marine environment since 2013 (Zettler et al., 2013), whereas plastisphere in freshwater is an emerging concept (Barros & Seena, 2021). The “plastic life cycle” is based on the fact that plastics mainly originate from terrestrial sources and are transported via rivers to reach the ocean (Jambeck & Walker-Franklin, 2023; Sonke et al., 2022). It is therefore crucial to study the plastisphere sampled across a transect that includes rivers, estuary and inshore seawater from the same geographical zone, but also across different river types. Mission *Tara* Microplastics was undertaking the first pan-European study of the plastisphere diversity across nine of the main European rivers together with their river-sea continuum. We chose four to five sampling sites along a salinity

gradient from the sea (station 1), the outer estuary (station 2), intermediate salinity (station 3) and to downstream (station 4) and upstream (station 5) of the first heavily populated city located on each river. Alpha-diversity (richness, evenness and diversity) within the plastisphere followed the same order as the surrounding seawater, with the highest diversity indexes observed in the PA and the lowest in the FL. However, we found significant differences in beta-diversity between the plastisphere (MP and *p*MP) and the bacteria living in the surrounding waters (FL and PA) in all the aquatic biota. Similar niche partitioning was consistently found in various marine ecosystems (Jacquin et al., 2019) and more recently in the highly urban river in Chicago, USA (McCormick et al., 2014). This is also in accordance with a study which compared bacterial communities living on microplastics and their surrounding water samples in freshwater and seawater ecosystems in the Shandong Province, China (Li et al., 2021). Similar niche partitioning was also found in several lakes (Di Pippo et al., 2022). Thus, the extension to a pan-European approach given by our study reinforces the general feature of microplastics providing a unique habitat for microorganisms in aquatic ecosystems.

It has been previously shown that organic particle-attached bacteria (PA) generally differ from free-living bacteria (FL) in all aquatic biota, including marine (Dussud et al., 2018b), lakes (Zhao et al., 2017) and riverine environments (Zhao et al., 2021). Here, we found that dissimilarities between PA and FL community structures were smoothed when compiled together with the plastisphere from 45 sampling stations (within and between stations) along the river-sea continuum of nine of the major European rivers. This is in line with the conclusion by Li et al. (2021), that showed that niche-based processes (deterministic) govern the structure of the anthropogenic plastisphere community, while neutral-based processes (stochastic) dominate the planktonic community structure going beyond the difference between PA and FL communities. These authors speculated that these results resulted from the high heterogeneity as well as fragmented and disconnected nature of the plastisphere as a habitat.

We could have expected that the difference in composition and load of organic particles between rivers and seawaters may affect the plastisphere communities. Plastics are constantly submitted to sorption of hydrophobic and hydrophilic organic materials (Liu et al., 2019) together with the particle-attached bacteria that may have taken the opportunity to colonize plastics. Clear dissimilarities were already observed between plastisphere and organic particle-attached bacterial communities in the marine environment (Dussud et al., 2018b), but no equivalent study was made in freshwater. Despite the heterogeneous environments

encountered along the river-sea continuum, the same conclusion was found, thus indicating little interaction between bacteria living in the two co-existing particle types whatever the sampling zone.

#### 4.2. Salinity is the main driver of the plastisphere bacterial communities

Several studies conducted in seawater demonstrated the importance of environmental factors in shaping the plastisphere diversity and community structure (Basili et al., 2020). Geographical location and seasons (Amaral-Zettler et al., 2015; Coons et al., 2021; Oberbeckmann et al., 2014) as well as chemical polymer composition, plastic shape, size (Cheng et al., 2021; Delacuvellerie et al., 2022) or even colors (Wen et al., 2020) were shown to influence the bacterial community structure living on MPs in the marine environment. Other factors were shown to play a substantial role in shaping the early plastic colonizers, such as hydrophobicity, topography, roughness, crystallinity, and surface charge (Rummel et al., 2017), whereas these factors may play a limited role when the biofilm become mature (Cheng et al., 2021; Dussud et al., 2018a).

Interestingly, the polymer chemical composition was not shown as driving forces of the plastisphere community structure in this work. In our study, we found that the sorted 115 MPs were mainly composed of polyethylene (PE) followed by polypropylene (PP) and polystyrene (PS). These results are in accordance with MP characterization in seawater (Auta et al., 2017) and in freshwater (Li et al., 2020), thus rendering our dataset representative of the large microplastics (LMP, from 500  $\mu\text{m}$  to 5mm) encountered in the environment. Our study provides further evidence that environmental conditions rather than polymer properties determine the plasisphere at the global (Amaral-Zettler et al., 2015), oceanic basin (Dussud et al., 2018b) or regional scales (Basili et al., 2020). This result is contradiction with other studies showing clear differences between biofilms grown on different polymers (Dussud et al., 2018a; Oberbeckmann et al., 2018; Pinto et al., 2019). Such discrepancy could be explained by the fact that later studies focused on the long-term colonization of pristine plastics (*pMP*), which is different from sampling MP directly into the environment.

In rivers, sediment movement is characterized using the concept of “spiraling”, including downstream transport, deposition, bed load transport, and resuspension. Analogous processes in estuarine environments include tidal movements and storm surges which strand plastic on intertidal or wrack zones (Eerkes-Medrano et al., 2015), whereas river floods are

particularly important processes for the delivery, deposition and dispersal of riverine organic matter in river dominated coastal oceans (Wheatcroft et al., 1997).

Based on our nine European river-sea gradient sampling strategy, estuaries were associated with a complex plastisphere that differed from freshwater and seawater communities. We are aware that this particular ecosystem needs more studies to evaluate the influence of tidal movements or storm surges which strand plastic on intertidal zones that may strongly affect the plastisphere composition.

#### 4.3. Complete segregation between seawater and freshwater plastisphere

Because rivers are major sources of plastic to the sea, their associated plastisphere has been thought as a vector of bacterial species from freshwater to the ocean, including potentially harmful microorganisms for human and marine animal health (Barros & Seena, 2021). Freshwater and marine habitats share a number of features, but there are also strong differences between them that affect the plastisphere consortia. All along the nine European rivers and whatever the various plastic types (MP and pMP) and characteristics (chemical composition, shape, size, color), we found a strong selective pressure exerted between freshwater and the marine environments, with very few examples of resilience. We found only 7% of common ASV between freshwater and seawater across the Seine River, with none of them belonging to putative pathogens (Amaral-Zettler et al., 2020). All the other ASV were unique to one or the other biota, thus suggesting that common ASV between freshwater and seawater (including putative pathogens) were rather an exception than a rule. Deinococcales and Burkholderiales dominated the freshwater plastisphere and had minor contribution in seawater plastisphere, which is consistent with a recent study in a small river that flows into the Mediterranean Sea (Var, France) (Delacuvellerie et al., 2022). Inversely, Rhodobacterales, Flavobacteriales and Chitinophagales dominated coastal seawater and were minor in the freshwater. These groups have been classically found on plastics in previous studies in marine environments (Dussud et al., 2018b; Oberbeckmann et al., 2016). In particular, SIMPER analysis highlighted *Lewinella* sp. as a major contributor to the difference between bacterial communities found at sea and at the riverine stations of the Seine River, abundant on samples from station 1 and absent on stations 4 and 5. This taxon was previously found on plastic debris in the North Pacific Subtropical Gyre (C. Li et al., 2021) and on PET bottles incubated for 6 weeks in the North Sea (Oberbeckmann et al., 2016). *Saprospiraceae* sp. was also found on MP floating at the surface of the Mediterranean Sea (Vaksmas et al., 2021). The SIMPER analysis

also highlighted *Yoonia-Loktanella* sp. as a major contributor to the difference between the samples, abundant in marine samples but absent from riverine ones. This taxon was found on plastics incubated at sea in New Zealand, increasing in abundance in the mature biofilm (Wallbank et al., 2022). Interestingly, two of the taxa highlighted with the SIMPER analysis are groups containing strains previously identified as plastic degraders, isolated from insect larvae. In particular, two strains of *Acinetobacter* were identified as PE (Kim et al., 2023) and PS (Wang et al., 2020) degraders. Finally, some strains of *Aeromonas* sp., which was identified as a contributor to the difference between marine and riverine communities across the Seine River, were previously identified as putative pathogens. In particular, *Aeromonas salmonicida* has been identified on floating MP in the North Adriatic and described as a fish pathogen (Viršek et al., 2017). It is noteworthy that in our study, *Aeromonas* sp. were abundant in riverine samples but absent from sea samples. However, the analysis conducted here (16s rDNA amplicon sequencing with short reads) does not allow to identify precisely the species, nor to highlight pathogenicity.

We also noted that the richness, evenness and diversity on marine MP were significantly lower than from the riverine stations. Overall, our results are in accordance with the only other study compiling the plastisphere in the river-sea continuum, focusing on the Shandong Province, China (Li et al., 2021). We confirm the strong dispersal limitation for the plastisphere microorganisms in the river-sea continuum at a European level, with freshwater plastisphere communities being almost completely reshaped when entering the sea, with very few bacteria able to adapt to these fragmented habitats.

#### 4.4. Similar but different plastisphere in plastic debris and in pristine plastics

Most of the studies on the plastisphere were based on incubation experiments with plastics of known composition and under controlled conditions, but only a few explored the plastic debris sampled in the aquatic environment, with most of them in marine waters and very few in freshwaters (Amaral-Zettler et al., 2020). One study investigating the plastisphere associated to plastic debris (MP) (Dussud et al., 2018b) and another work on colonization of pristine plastics (*p*MP) (Dussud et al., 2018a) in seawater from the same geographical zone were compared thanks to a uniform processing of samples. It highlighted that a minimum of one-month incubation in seawater was necessary to reach a mature biofilm in *p*MP that presented similarities with MP. The same colonization period was chosen in this study, with various polymer types (PE, POM and NYL) immersed all along the river-sea continuum. We

observed that both MP and *p*MP plastisphere presented similar diversity index values and showed the same clear niche partitioning when compared to the surrounding planktonic bacteria (PA and FL). They also followed the same geographical gradient, all along the river-sea continuum. Such similarities between MP and *p*MP suggest that the heterogeneous environmental conditions of the river-sea continuum were more important than the plastic history, chemical composition, shape or size in driving changes in plastisphere diversity. Likewise, *p*MP composition (PE, POM or NYL) had no effect on the plastisphere diversity and composition after 1-month incubation, as previously observed with other conventional polymer types (Dussud et al., 2018; Odobel et al., 2021).

However, a more subtle distinction could be noticed between the two plastics. First, UPGMA analysis showed that MP and *p*MP plastisphere community structure slightly differed within each station, with less dissimilarity dispersion in *p*MP samples that always grouped together. This is probably the signature of the large heterogeneity of plastic types in the MP fraction, that was not a driving factor at the river-sea continuum scale, but exerted a selective pressure at the local scale. This is in line with a recent study on a small river (Var, France) that found that the most important drivers of plastisphere community structure along the river-sea continuum were mainly the sampling site, and in a lesser extent the polymer chemical composition (Delacuvellerie et al., 2022). Second, one exception was found in the seawater samples, with lower diversity in MP compared to *p*MP plastisphere and dissimilar community structures between the two groups. This may be due to the different locations between the sampling area, since MP were sampled in coastal seawater in front of the river, whereas the *p*MP were immersed on the closest beach (less than 3 km away from the estuary). We verified that salinity was similar between both locations, but it may not be sufficient to ensure comparable environmental conditions. It was not the case for all the other MP and *p*MP, that were sampled at the exact same location. Further studies are needed to confirm or infirm these last results, by using *p*MP immersion in cage structure attached to buoys, for example.

## 5. Conclusion

Given the complexity and ubiquity of microplastics and their associated microbial assemblages, this pan-European research initiative gathered scientists from interdisciplinary scientific fields to address the serious concerns regarding freshwater plastisphere transfer to seawaters. Our study presents a large set of data consistently measured with standardized



methods at all sites during the *Tara Microplastics* mission. It allowed us to confirm that the niche partitioning between the plastisphere and the surrounding aquatic bacteria was a common feature all along the river-sea continuum. Another important aspect of this study was to prove the complete segregation between seawater and freshwater plastisphere. The very few common ASV to both ecosystems did not include any putative pathogens in any of the nine European rivers, thus discrediting the hypothesis of a possible transfer of bacterial pathogens by microplastics from the rivers to the sea. Plastisphere is a new anthropogenic ecosystem that was found to be very sensitive to environmental changes, especially in heterogeneous environments. In the river-sea continuum, the drastic changes in salinity were shown here as a major barrier for the freshwater plastispheres to survive in the coastal seawaters.

Our results present the first pan-European set of data associated with the plastisphere along the river-sea continuum. To date, the biodiversity of microbial assemblages on the freshwater plastisphere was mainly limited to few geographic locations. Further studies are needed to evaluate the temporal changes of a plastisphere that can be greatly challenged between the low water period and flood events. There is also a substantial need for studies on plastisphere eukaryotes, and especially fungal communities, as they too are vital parts of the ecosystem.

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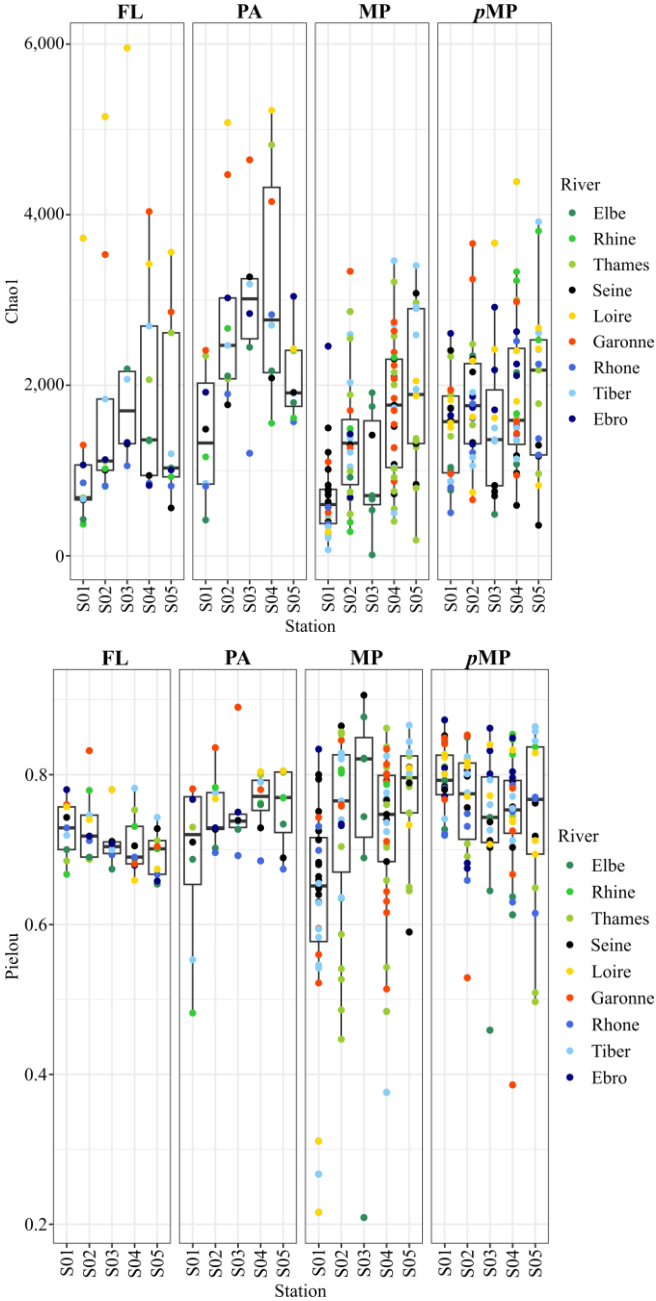
## Appendix 2.1. Proportion of polymers among the surface MP collected at the different sampling sites.

PE: polyethylene; PP: polypropylene; PS: polystyrene; PEVA: polyethylene vinyl acetate; PA: polyamide; PVC: polyvinyl chloride.

River	Station	n	PE	PP	PS	PEVA	PA	PVC	Unknown
Ebre	S01	1	0	0	100	0	0	0	0
	S02	2	0	0	0	0	50	0	50
	S03	0	NA	NA	NA	NA	NA	NA	NA
	S04	0	NA	NA	NA	NA	NA	NA	NA
	S05	0	NA	NA	NA	NA	NA	NA	NA
Elbe	S01	0	NA	NA	NA	NA	NA	NA	NA
	S02	1	0	0	0	0	0	0	100
	S03	6	17	33	0	0	0	0	50
	S04	0	NA	NA	NA	NA	NA	NA	NA
	S05	0	NA	NA	NA	NA	NA	NA	NA
Garonne	S01	4	75	0	25	0	0	0	0
	S02	3	100	0	0	0	0	0	0
	S03	0	NA	NA	NA	NA	NA	NA	NA
	S04	10	20	20	30	10	0	0	20
	S05	0	NA	NA	NA	NA	NA	NA	NA
Loire	S01	2	100	0	0	0	0	0	0
	S02	0	NA	NA	NA	NA	NA	NA	NA
	S03	0	NA	NA	NA	NA	NA	NA	NA
	S04	0	NA	NA	NA	NA	NA	NA	NA
	S05	2	100	0	0	0	0	0	0
Rhin	S01	0	NA	NA	NA	NA	NA	NA	NA
	S02	4	25	0	0	0	0	0	75
	S03	1	100	0	0	0	0	0	0
	S04	0	NA	NA	NA	NA	NA	NA	NA
	S05	0	NA	NA	NA	NA	NA	NA	NA
Rhone	S01	2	100	0	0	0	0	0	0
	S02	0	NA	NA	NA	NA	NA	NA	NA
	S03	0	NA	NA	NA	NA	NA	NA	NA
	S04	0	NA	NA	NA	NA	NA	NA	NA
	S05	0	NA	NA	NA	NA	NA	NA	NA
Seine	S01	12	25	8	8	17	0	8	33
	S02	2	0	0	0	0	0	0	100
	S03	1	100	0	0	0	0	0	0
	S04	7	29	29	29	0	0	0	14
	S05	3	33	0	0	0	0	0	67
Tamise	S01	0	NA	NA	NA	NA	NA	NA	NA
	S02	9	22	22	0	22	0	0	33
	S03	9	44	11	0	0	0	0	44
	S04	6	50	17	0	0	0	0	33
Tibre	S01	8	75	0	0	25	0	0	0
	S02	7	29	0	57	0	0	0	14
	S03	0	NA	NA	NA	NA	NA	NA	NA
	S04	7	29	29	0	0	0	0	43
	S05	6	17	33	33	0	0	0	17

0	
]0;25]	
]25;50]	
]50;75]	
]75;100]	

Appendix 2.2. Chao1 and Pielou indexes for water and plastic samples.





*Chapitre 3 : Influence de l'inoculum bactérien et du milieu d'incubation sur l'étude de la biodégradabilité des plastiques en milieu marin*

## Chapitre 3

Ce chapitre est rédigé sous la forme d'un article scientifique qui sera soumis prochainement au journal « Environmental Pollution ». Cette partie s'intéresse à l'influence des paramètres expérimentaux sur l'étude de la biodégradabilité marine des plastiques en milieu contrôlé. En effet, il existe peu d'études à ce sujet, qui sont pourtant importantes pour l'établissement des conditions expérimentales des protocoles normés permettant la réalisation de tests robustes.

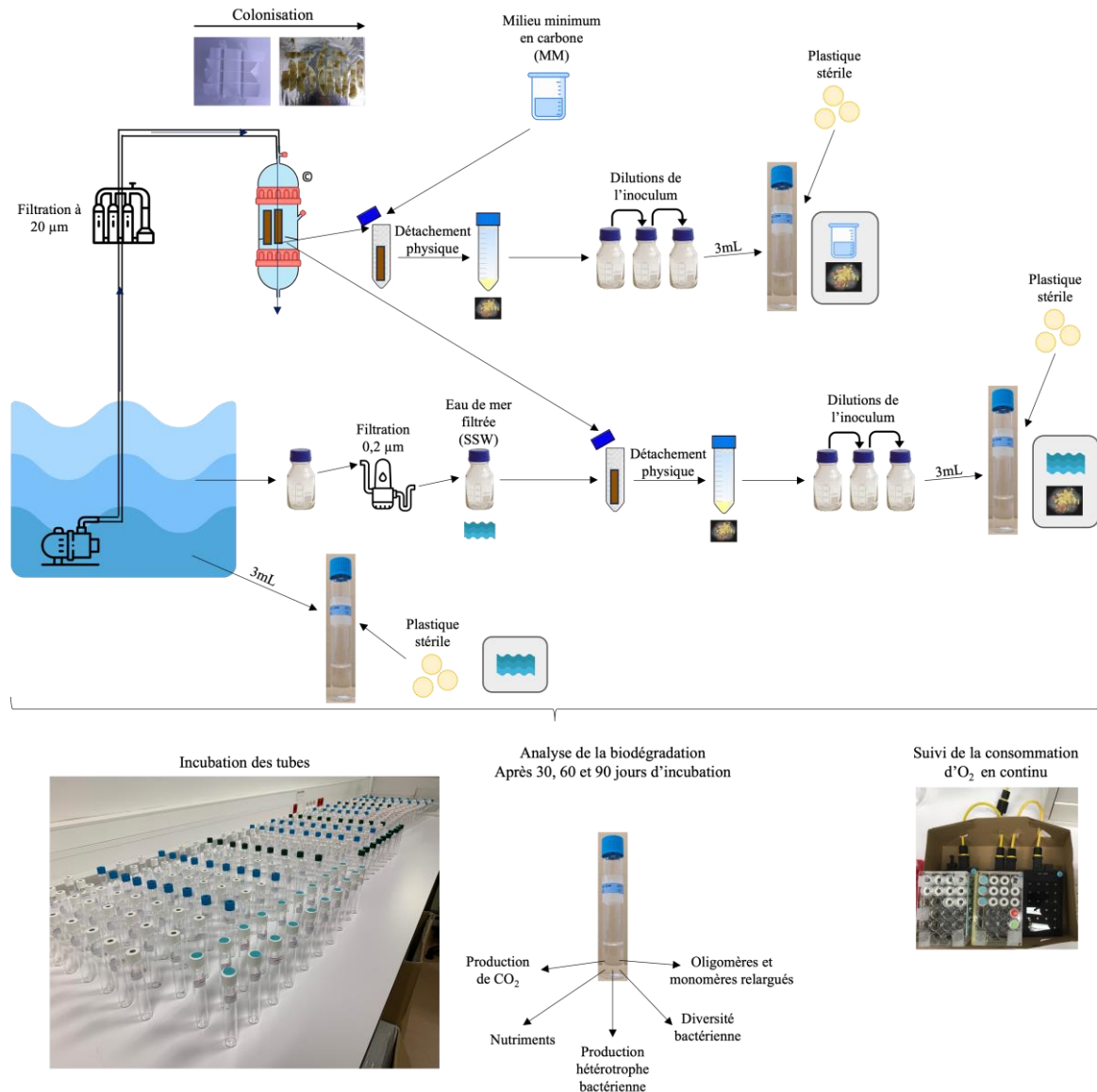


Figure 3.1. Protocole d'incubation de l'expérience. La colonisation en circulation ouverte a été faite pour chacun des matériaux (cellulose, PHBHV, PE), et le détachement des inocula fait séparément. Les aquariums ont été placés dans le noir. Les tubes ont été incubés dans le noir à 18°C sous 120 rpm d'agitation.

## Chapitre 3











































































Les protocoles normatifs disponibles actuellement proposent d'utiliser des conditionnement volumineux, tels que des bouteilles de 250mL. Dans ce travail, un modèle miniaturisé a été utilisé afin de pouvoir multiplier les conditions expérimentales, permettre des analyses pluridisciplinaires et multiplier les contrôles (Fig. 3.1). Les matières utilisées sont la cellulose, référence positive proposée dans les normes, le PHBHV, biopolymère biodégradable en condition marine, ainsi que le PE, qui constitue dans cette étude un contrôle négatif puisque ce polymère conventionnel n'est pas biodégradable dans des temps raisonnables en milieu marin. Deux milieux ont été comparés : un milieu marin synthétique (appelé MM dans la suite du chapitre), limité uniquement en carbone, et de l'eau de mer naturelle échantillonnée en surface dans la baie de Banyuls sur Mer. Deux types d'inocula ont été utilisés : un biofilm mature, présélectionné sur chaque matière testée au travers d'une incubation d'un mois en aquarium ouvert sur la mer, et les communautés de l'eau de mer. L'inoculum sélectionné a été testé à différentes concentrations ( $10^4$ ,  $10^5$ ,  $10^6$  cellules par mL), pour étudier leur effet sur la biodégradabilité (ce facteur n'est pas discuté dans les normes actuelles). Des contrôles abiotiques (plastiques seuls, sans bactéries) et des blancs (bactéries seules, sans plastique) ont été ajoutés pour chacune des analyses à chacun des temps. Les contrôles abiotiques permettent d'observer la dégradation sans effet des communautés bactériennes. Les blancs (inocula seuls) permettent d'avoir le bruit de fond de l'activité de la communauté en l'absence d'une source de carbone. Les combinaisons de conditions expérimentales sont présentées dans le tableau 3.1.

Le protocole expérimental pour l'inoculation des échantillons est schématisé sur la figure 3.1. La consommation d'oxygène a été suivie en continu. Des échantillons ont été fixés après 30, 60 et 90 jours pour suivre différents paramètres : la production de  $\text{CO}_2$ , la production hétérotrophe bactérienne, la diversité bactérienne, les concentrations en phosphate et en azote, et enfin, les produits de biodégradation. Les résultats mettent en évidence la pertinence d'utiliser des communautés présélectionnées pour les tests de biodégradabilité. Aucun signe de biodégradabilité n'a été enregistré pour la cellulose et le PHBHV incubés avec les communautés d'eau de mer. De plus, aucun effet de concentration n'a été observé pour ces deux matières en conditions riches en nutriments (MM). Enfin, ces résultats mettent en avant l'importance de suivre les nutriments pour les tests de biodégradation. En effet, aucun signal de biodégradation n'a été enregistré en conditions oligotrophes (SW) pour le PHBHV, malgré des communautés spécifiques. L'étude de la diversité et de la structure des communautés est en cours, et ne sera pas présentée dans ce manuscrit.



## Chapitre 3

Tableau 3.1. Bilan des conditions expérimentales.

		Milieu	Inoculum	Concentration	Plastique	
<b>Echantillons</b>	Cellulose	Milieu minimum (MM) 	Biofilm spécifique 	10 <sup>4</sup> cellules/mL	Cellulose	
		Milieu minimum (MM) 	Biofilm spécifique 	10 <sup>5</sup> cellules/mL	Cellulose	
		Milieu minimum (MM) 	Biofilm spécifique 	10 <sup>6</sup> cellules/mL	Cellulose	
		Eau de mer filtrée (SSW) 	Biofilm spécifique 	10 <sup>4</sup> cellules/mL	Cellulose	
		Eau de mer filtrée (SSW) 	Biofilm spécifique 	10 <sup>5</sup> cellules/mL	Cellulose	
		Eau de mer filtrée (SSW) 	Biofilm spécifique 	10 <sup>6</sup> cellules/mL	Cellulose	
		Eau de mer (SW) 	Eau de mer		Cellulose	
	PHBHV	Milieu minimum (MM) 	Biofilm spécifique 	10 <sup>4</sup> cellules/mL	PHBHV	
		Milieu minimum (MM) 	Biofilm spécifique 	10 <sup>5</sup> cellules/mL	PHBHV	
		Milieu minimum (MM) 	Biofilm spécifique 	10 <sup>6</sup> cellules/mL	PHBHV	
		Eau de mer filtrée (SSW) 	Biofilm spécifique 	10 <sup>4</sup> cellules/mL	PHBHV	
		Eau de mer filtrée (SSW) 	Biofilm spécifique 	10 <sup>5</sup> cellules/mL	PHBHV	
		Eau de mer filtrée (SSW) 	Biofilm spécifique 	10 <sup>6</sup> cellules/mL	PHBHV	
		Eau de mer (SW) 	Eau de mer		PHBHV	
	PE	Milieu minimum (MM) 	Biofilm spécifique 	10 <sup>4</sup> cellules/mL	PE	
		Milieu minimum (MM) 	Biofilm spécifique 	10 <sup>5</sup> cellules/mL	PE	
		Eau de mer filtrée (SSW) 	Biofilm spécifique 	10 <sup>4</sup> cellules/mL	PE	
		Eau de mer filtrée (SSW) 	Biofilm spécifique 	10 <sup>5</sup> cellules/mL	PE	
		Eau de mer (SW) 	Eau de mer		PE	
	<b>Contrôles abiotiques</b>	Cellulose	Milieu minimum (MM) 	-	-	Cellulose
			Eau de mer filtrée (SSW) 	-	-	Cellulose
PHBHV		Milieu minimum (MM) 	-	-	PHBHV	
		Eau de mer filtrée (SSW) 	-	-	PHBHV	
PE		Milieu minimum (MM) 	-	-	PE	
		Eau de mer filtrée (SSW) 	-	-	PE	
<b>Blancs</b>		Cellulose	Milieu minimum (MM) 	Biofilm spécifique 	10 <sup>4</sup> cellules/mL	-
			Milieu minimum (MM) 	Biofilm spécifique 	10 <sup>5</sup> cellules/mL	-
	Milieu minimum (MM) 		Biofilm spécifique 	10 <sup>6</sup> cellules/mL	-	
	Eau de mer filtrée (SSW) 		Biofilm spécifique 	10 <sup>4</sup> cellules/mL	-	
	Eau de mer filtrée (SSW) 		Biofilm spécifique 	10 <sup>5</sup> cellules/mL	-	
	Eau de mer filtrée (SSW) 		Biofilm spécifique 	10 <sup>6</sup> cellules/mL	-	
	PHBHV	Milieu minimum (MM) 	Biofilm spécifique 	10 <sup>4</sup> cellules/mL	-	
		Milieu minimum (MM) 	Biofilm spécifique 	10 <sup>5</sup> cellules/mL	-	
		Milieu minimum (MM) 	Biofilm spécifique 	10 <sup>6</sup> cellules/mL	-	
		Eau de mer filtrée (SSW) 	Biofilm spécifique 	10 <sup>4</sup> cellules/mL	-	
		Eau de mer filtrée (SSW) 	Biofilm spécifique 	10 <sup>5</sup> cellules/mL	-	
		Eau de mer filtrée (SSW) 	Biofilm spécifique 	10 <sup>6</sup> cellules/mL	-	
	PE	Milieu minimum (MM) 	Biofilm spécifique 	10 <sup>4</sup> cellules/mL	-	
		Milieu minimum (MM) 	Biofilm spécifique 	10 <sup>5</sup> cellules/mL	-	
		Eau de mer filtrée (SSW) 	Biofilm spécifique 	10 <sup>4</sup> cellules/mL	-	
		Eau de mer filtrée (SSW) 	Biofilm spécifique 	10 <sup>5</sup> cellules/mL	-	
-	Eau de mer (SW) 	Eau de mer		-		

## **The significance of microbial inoculum and test medium for the evaluation of plastic biodegradability in seawater**

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**Keywords:** biodegradability tests • standards • plastisphere • plastics

### Highlights:

- Relevance of pre-adapted plastic-specific microbial communities rather than seawater inoculum
- Nutrient availability may limit biodegradability tests under controlled conditions
- Inoculum cell concentration higher than  $10^4$  cells does not impact biodegradation under nutrient-rich condition

### Abstract:

Biodegradable plastics have been developed for years as one of the possible solutions to tackle the plastic pollution. Here, we investigated the influence of experimental conditions on marine plastic biodegradability tests, with special emphasis on the microbial inoculum and the test medium. Biodegradability was monitored during 90 days on cellulose, poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBHV) and polyethylene (PE) through bacterial activity measurements ( $O_2$  consumption,  $CO_2$  production and heterotrophic bacterial activity). First, we support the use of mature biofilm grown in seawater on the corresponding plastic type as test inoculum, rather than free-living seawater bacteria. Second, we found that synthetic carbon-minimum medium with enriched nutrients is more adequate for biodegradation tests than seawater only, especially in oligotrophic conditions. Such medium also facilitates comparison between studies. Overall, this work showed that both microbial inoculum and test medium are important factors that should be taken into consideration for an accurate evaluation of plastic biodegradability in seawater.

### 1. Introduction

It is now admitted that conventional plastics, made from fossil fuel, accumulate in all natural ecosystems, due to their persistence together with insufficient capabilities of waste treatment leading to accidental loss. Therefore, the plastic industry has started its transition for several years, seeking for alternatives to these materials. Bioplastics are composed of polymers that are either biosourced, *i.e.*, made from biomass, biodegradable, or both (Lambert & Wagner, 2017). The possibility of using renewable resources to produce plastics is an opportunity not to rely on fossil resources that are limited, thus reducing emissions linked to their use (RameshKumar et al., 2020). The use of biodegradable materials would help to optimize the end of life of plastics, and contribute to circular economy for selected applications with respect to their uses and ends of life (Paul-Pont et al., 2023).

Biodegradation refers to the use of a substance by microorganisms as a substrate for biomass and energy production. The biodegradability of a substance is a component of its ecological impact on natural ecosystems (Pagga, 1997), and the first guidelines for such evaluation appeared in the Organization for Economic Co-operation and Development (OECD) guidelines in the 1980s (Folino et al., 2023). However, these recommendations apply to chemical substances, in other words to small, soluble or dispersible molecules. Plastics are solid, not soluble materials, composed of long polymeric chains, often hard to depolymerize for microbes, which biodegradation is thus more complex than for other pollutants (Shah et al., 2008). The capability of biodegradation of a plastic depends on its chemical composition and structure, corresponding to its intrinsic biodegradability (Degli Innocenti & Breton, 2020), but also on the environmental parameters, comprising physical, chemical and biological parameters (Zumstein et al., 2019). So far, plastic biodegradation has been widely studied in industrial composting conditions, because it offers the potential of waste conversion to a valuable soil resource. However, industrial composting requires controlled conditions favorable for material degradation, such as high temperatures (>60°C) that are not found in the natural environment. Therefore, a plastic material which biodegradability was proved in such conditions might turn out to be persistent in natural environments, such as the marine environment (Manfra et al., 2021).

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Plastic biodegradation is a four-step process: (i) the biodeterioration is induced by the formation of a microbial biofilm, in which some of the microorganisms produce extracellular enzymes to make the (ii) biofragmentation of the polymeric chains, leading to the formation of soluble oligomers and monomers who can be (iii) bioassimilated by organisms of the plastisphere to produce biomass and finally be (iv) biomineralized to produce energy (Dussud & Ghiglione, 2014; Lucas et al., 2008). For years, many studies focused on plastic degradation at sea, mainly monitoring weight loss and changes in the physical properties during field experiments. More recently, several standards were developed as guidelines for the monitoring of plastic biomineralization by microbial communities, which is more of a proof of biodegradation (Folino et al., 2023). However, some works recently pointed out weaknesses of these protocols, notably experimental conditions poorly representative of the natural environments (Harrison et al., 2018). To our knowledge, only few studies worked to improve the evaluation of plastic biodegradability since then (Beiras & López-Ibáñez, 2023; Briassoulis et al., 2020). Overall, studies of marine plastic biodegradation are based on standard methods, aiming to quantify the biodegradation level of a substance, generally based on respirometry measurement. Very few of them focused on protocol improvements to adapt tests to the specific case of plastic materials. Moreover, the specificity of the marine environment was not taken into account so far, for example recent knowledge on the natural plastisphere showing their difference compared to surrounding bacterial communities. Test inoculum and medium should be discussed to improve their representativity of the natural environment (Paul-Pont et al., 2023). Rigorous evaluation of the methodology to perform plastic biodegradability tests is mandatory to avoid the development of so-called biodegradable materials that would not be realistic to the environment conditions (Napper & Thompson, 2019).

In this study, we tested the influence of several experimental conditions on the monitoring of plastic biodegradability under seawater conditions. We tested different bacterial inocula and test mediums. First, we used natural mature plastispheres grown on plastics as inocula for biodegradability tests performed on sterile seawater (SSW) or on a synthetic carbon-minimum medium with nutrient amendment (MM). Second, comparison was made with free-living bacteria living in raw seawater (SW) as another inoculum and medium types. The biodegradability tests were performed during 90 days through the monitoring of bacterial activities ( $O_2$  consumption,  $CO_2$  release and heterotrophic production), as well as biodegradation products (oligomers and monomers). Nutrients concentrations (nitrate, nitrite, phosphate) were also monitored during the whole experiment, as well as the dynamics of the

associated bacterial communities (Illumina Miseq Sequencing). Tests were performed on three plastic types: the both biodegradable cellulose and poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBHV), and the non-biodegradable polyethylene (PE). The hypothesis was that the inoculum biodiversity, cell concentration and origin (plastisphere vs. seawater) as well as the test medium may be of great influence on the biodegradability results. Finally, this article is advising several recommendations for further studies using plastic biodegradability tests in the marine environment.

## 2. Material and methods

### 2.1. Experimental design

3 types of inocula were used for the biodegradation tests. The first type of inoculum consisted in the free-living bacteria sampled in seawater collected from Banyuls bay (called “SW” hereafter), close to the SOLA observatory station (NW Mediterranean Sea, France) (Lambert et al., 2018). The two other inoculum types originated from a two-phase stepwise approach in order to favour the use of inoculum from the natural plastisphere growing on each polymer type, as previously described (Cheng et al., 2022). The first step consisted in the formation of a marine natural mature biofilm growing on cellulose (Whatman® filter paper Grade 42, Merck, United States), PHBHV (Enmat Y1000P, Tianan Biological, China), and PE (LDPE, Symphony Environmental Technology, United Kingdom). Plastic films were incubated for a period of 1 month in aquaria with direct circulation to the sea, as previously described (Dussud et al., 2018a). Briefly, sterilized 6.75 cm<sup>2</sup> pieces (addition of ethanol 70 % and 15 min under UV light) of the three polymer types (cellulose, PHBHV or PE) were placed in 0,5 L glass aquariums (Plasticell©), in which total seawater was continually renewed by direct pumping at 14 m depth in Banyuls bay. A flow rate of 200 mL.min<sup>-1</sup> was chosen to ensure a sufficient renewal of natural bacteria (every 30 min) and a homogeneous distribution of the plastic pieces in the aquariums. Second, the plastic pieces were sampled using sterile forceps and placed in two medium conditions: (1) sterile seawater (called “SSW” hereafter) collected from the Banyuls bay and filtered through 0.2 µm-pore size (47 mm diameter, Nucleopore) to remove most bacteria and (2) a synthetic carbon-minimum medium (called “MM” hereafter, adapted from Eguchi et al. 1996). The mature biofilms formed on plastic pieces were carefully detached in SSW and MM separately (called “detached biofilm inoculum” hereafter) using 3 cycles of 1 minute of vortex followed by 3 minutes in ultrasonic bath (Conan et al., 2022).

Immediately after cell detachment, the concentration of the detached biofilm inoculum was measured by flow cytometry (BD FACSCanto™ II, BD Biosciences, United States), as previously described (Odobel et al., 2021). Bacterial heterotrophic production was measured in triplicate for the detached biofilm inoculum by using the <sup>3</sup>H-leucine incorporation method (Céa et al., 2015), in order to ensure that the cells were still active after cell detachment (heterotrophic activity was about). The detached biofilm inoculum of each polymer type (cellulose, PHBHV, PE) were then diluted in SSW and MM at concentrations of 10<sup>4</sup>, 10<sup>5</sup> and 10<sup>6</sup> cells.mL<sup>-1</sup> for further biodegradation tests on each polymer, except for PE where incubation was not possible for the 10<sup>6</sup> cells.mL<sup>-1</sup> condition (the cell numbers recovered from the mature biofilm grown on PE was not sufficient with respect to the volume needed for incubation of all tubes). Three sterile 5 mm-diameter plastic pieces were placed in sterile individual 12 mL Exetainer tubes with hermetic screw caps (Exetainer®, Labco, United Kingdom) for each plastic type (cellulose, PHBHV, PE), and 3 mL of inoculum (SW or detached biofilm inoculum) in each medium (SSW, MM) was added to each tube. Two types of controls were also tested: abiotic controls, containing sterile plastic pieces and sterile medium (SSW and MM without inoculum) and blanks, containing the microbial inoculum only (SW or detached biofilm inoculum) without plastic. A total of 2043 tubes were placed in an incubator at 18°C under agitation of 120 rpm (Innova® S44i, Eppendorf, Germany) in the dark. Analyses of the biodegradation were conducted on samples after 30, 60 and 90 days.

### 2.2. Continuous oxygen measurement

Duplicate vials for each polymer type in the different inoculum and medium, as well as vials for abiotic controls and blanks were equipped with luminescent oxygen sensor spots (SP-PSt5, Presens, Germany) that were placed at the bottom of the 12 mL Exetainer vials. Dissolved oxygen concentrations were measured every hour during 90 days using 24-channel readers (SDR SensorDish® Reader, Presens, Germany). Additionally, data were acquired for more than 100 days after the end of the experiment. In order to compare with other measurements, data from days 30, 60 and 90 were averaged and the difference with day 0 was calculated at each day for all the conditions.

### 2.3. Carbon dioxide production measurement

Dissolved inorganic carbon concentration was measured in the liquid compartment of tubes to monitor plastic mineralization. On days 0, 30, 60 and 90, mercury chloride (37g.L<sup>-1</sup>) was added to the corresponding tubes in order to block bacterial activity. Mercury chloride

was injected directly in the tubes with a syringe through the septa of the caps and silicon was applied on the caps' septa to ensure the good sealing of the tubes. Tubes were kept at room temperature and analyzed altogether at the end of the experiment, using a TOC analyzer (TOC-L Series, Shimadzu). The DIC production was calculated as the difference of DIC concentrations between the sampling days and day 0.

### 2.4. Heterotrophic bacterial production

Heterotrophic bacterial production was monitored after 30 and 60 days of incubation, on all samples and controls. Plastic pieces were placed on 1.5 mL of MM, then soft cell detachment was performed with 3 cycles of vortex (1 minute) and ultrasound bath (3 minutes), and the measurement of  $^3\text{H}$  leucine incorporation was performed as previously described (Dussud et al., 2018a). Briefly,  $^3\text{H}$  leucine (specific activity 100 Ci.mmol $^{-1}$ ) and cold leucine were added (final concentrations of 1 nmol.L $^{-1}$  and 150 nm.L $^{-2}$  respectively), samples were incubated for 3 hours at 18°C, then the bacterial activity was blocked by addition of trichloroacetic acid (TCA) 50% and samples were washed with TCA 5% then ethanol 70%. The number of disintegrations per minute (DPM) was acquired using a scintillation counter (Hidex 300 SL), after addition of a liquid scintillation cocktail (Cocktail LSC Ultima Gold, Sigma-Aldrich, United States). DPM were converted to rates of carbon incorporation (ngC.mm $^{-2}$ .h $^{-2}$ ) using the empirical conversion factor of the leucine, 1.55 ngC.pmol $^{-1}$  (Simon & Azam, 1989).

### 2.5. Nutrients concentrations

After 30, 60 and 90 days of incubation, 12mL of medium culture were pooled for each condition, filtered on 0.4µm syringe filter (UptiDisc™, interchim) in a scintillation vial (S220 SNAPTWIST® 20 mL, Simport Scientific), then frozen at -20°C until the analysis. Nitrate, nitrite and phosphate concentrations were simultaneously measured in samples, on a continuous flow Autoanalyser III Seal-Bran&Luebbe (Aminot & K erouel, 2007).

### 2.6. DNA extractions, PCR, sequencing and data analysis

Plastic pieces were sampled after 30, 60 and 90 days with a sterile tweezers, fixed with liquid nitrogen and stored at -80°C for further analysis. DNA extractions were performed using a classical phenol-chloroform method (Dussud et al., 2018b). Polymerase Chain Reaction (PCR) amplification of the V4-V5 region was done using universal 16 rRNA primers (515Y and 926R) (Parada et al., 2016). Illumina MiSeq sequencing was performed at Genoscope (Evry, France). Sequences analysis will be performed using the DADA2 pipeline (Callahan et



al., 2016) for ASV establishment and taxonomy assignment, and the SILVA 138 SSU database for taxonomic assignment.

### 2.7. Data management

Data normality was evaluated with a Shapiro-Wilk test, using the *stats* package version 4.3.1. Data did not follow a normal distribution; thus, non-parametric tests were used. Kruskal-Wallis and Dunn tests (Holm-Bonferroni correction) were done separately for each date, with *stats* package version 4.3.1 and *rstatix* package version 0.7.2 respectively.

## 3. Results

### 3.1. Oxygen consumption

Dissolved oxygen concentrations were continuously measured in the medium culture during the whole experiment (Fig. 3.2). No oxygen consumption was recorded in abiotic controls nor in blanks during the 90 days of incubation. In all conditions, oxygen concentration did not fall below  $50 \mu\text{mol.L}^{-1}$ , ensuring aerobic conditions.

Starting from polymers incubated with the free-living bacteria in raw seawater (SW), no oxygen consumption was found during the 90 days of incubation either for cellulose nor PHBHV. The signal recorded for PHBHV in SW on day 90 (mean =  $7.7 \times 10^{-3} \mu\text{mol}$ ) was not significantly different from PE incubated in MM at  $10^5 \text{ cells.mL}^{-1}$  (mean =  $5.0 \times 10^{-3} \mu\text{mol}$ ; Kruskal-Wallis test,  $p$  value = 0.12). No oxygen consumption was found either for PHBHV incubated with its detached biofilm inoculum in sterile seawater (SSW), whatever the initial inoculum cell counts (from  $10^4$  to  $10^6 \text{ cells.mL}^{-1}$ ). As for PHBHV samples incubated in SW, the signal recorded in SSW with its specific inoculum was equivalent to the signal recorded in the non-biodegradable PE samples. However, cellulose samples incubated in SSW with its specific inoculum at  $10^5$  and  $10^6 \text{ cells.mL}^{-1}$  presented oxygen consumption during the course of the experiment. Oxygen consumption was significantly higher for a detached biofilm inoculum concentration of  $10^6 \text{ cells.mL}^{-1}$  of SW (mean =  $8.9 \times 10^{-2} \mu\text{mol}$  after 90 days of incubation) than  $10^5 \text{ cells.mL}^{-1}$  in the same medium (mean =  $2.5 \times 10^{-1} \mu\text{mol}$  after 90 days of incubation; Kruskal-Wallis test with Holm correction,  $p$  value = 0.0040). Surprisingly, no oxygen consumption was monitored on cellulose incubated in SSW with detached biofilm inoculum concentration of  $10^4 \text{ cells.mL}^{-1}$ .

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Overall, high oxygen consumption was recorded when using MM as test medium for biodegradable polymers, except for PE with similar low oxygen consumption. Contrary to cellulose incubated in SSW, we found no effect of the detached biofilm inoculum concentration on the oxygen consumption, with high levels during the course of the experiment whatever the cell concentration (mean =  $2.1 \times 10^{-1} \mu\text{mol}$  and mean =  $2.4 \times 10^{-1} \mu\text{mol}$  for incubation at  $10^5$  and  $10^6$  cells.mL<sup>-1</sup> respectively). Likewise, PHBHV samples incubated in MM presented high oxygen consumption with the three concentrations of detached biofilm inoculums, significantly higher after 90 than 30 days of incubation (Kruskal-Wallis test with Holm correction,  $p$  value = 0.013) with no correlation between the bacterial activity levels and the inoculum concentrations (mean = 0.34, 0.26 and 0.30 after 90 days of incubation for  $10^4$ ,  $10^5$  and  $10^6$  cells.mL<sup>-1</sup>, respectively).

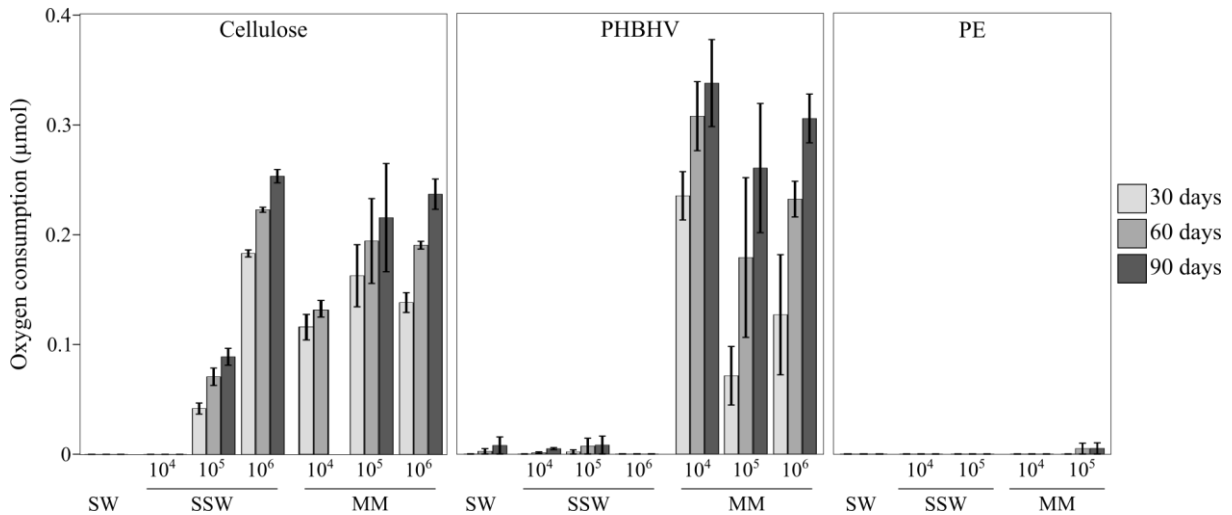


Figure 3.2. Oxygen consumption on sampling days. SW refers to raw seawater, without specific inoculum. SSW refers to sterile seawater and MM refers to synthetic carbon-minimum medium.  $10^4$ ,  $10^5$  and  $10^6$  are the concentrations of the detached biofilm inoculum. The barplots represent the average values and the bars correspond to minimum and maximum values.

### 3.2. Carbon dioxide production

Dissolved inorganic carbon (DIC) was measured after 0, 30, 60 and 90 days of incubation in the liquid phase of the samples (Fig. 3.3). No DIC production was observed in abiotic controls nor in blanks after 90 days of incubation.

Contrary to oxygen measurements, a slight DIC production was recorded for cellulose and PHBHV incubated in SW from day 30 (mean = 0.90  $\mu\text{mol}$  and mean = 0.34  $\mu\text{mol}$  respectively). The DIC production on day 90 was not significantly different from values on day 30, neither for cellulose nor PHBHV incubated in SW (Kruskal-Wallis test with Holm correction,  $p$  value = 0.137).

As for oxygen consumption, no significant DIC production was observed during the 90 days of incubation for PHBHV samples incubated in SSW with the detached biofilm inoculum. No significant difference was observed between data from days 30 and 90 on these conditions, whatever the detached biofilm inoculum concentrations (mean =  $0.43 \pm 0.14$  and mean =  $0.49 \pm 0.10$   $\mu\text{mol}$  on days 30 and 90 respectively; Kruskal-Wallis test,  $p$  value = 0.533). For cellulose samples incubated in SSW with its detached biofilm inoculum, DIC production was recorded from day 30. In cellulose incubated at a bacterial concentration of  $10^4$  cells.mL<sup>-1</sup> in SSW, a slight increase of the DIC production was observed at 90 compared to 30 days, with values equivalent to samples incubated in SW. However, for cellulose incubated at bacterial concentrations of  $10^5$  and  $10^6$  cells.mL<sup>-1</sup> in SSW, DIC production was significantly higher on day 90 than on day 30 (Kruskal-Wallis tests with Holm correction,  $p$  value = 0.022 and  $p$  value = 0.034 for bacterial concentrations of  $10^5$  and  $10^6$  cells.mL<sup>-1</sup> respectively). As for oxygen consumption, we observed an increase of DIC production when increasing the detached biofilm inoculum concentration.

Overall, the highest DIC production was recorded when using MM as test medium for biodegradable polymer, except for PE. Both cellulose and PHBHV showed significant DIC productions from day 30, and values were significantly higher at day 90, whatever the detached biofilm inoculum concentration (Kruskal-Wallis tests with Holm correction,  $p$  value =  $3.4 \times 10^{-4}$  and  $p$  value =  $2.1 \times 10^{-4}$  for cellulose and PHBHV respectively). As observed with the measurement of oxygen consumption, in MM, the increase of detached biofilm inoculum concentration did not increase DIC production.

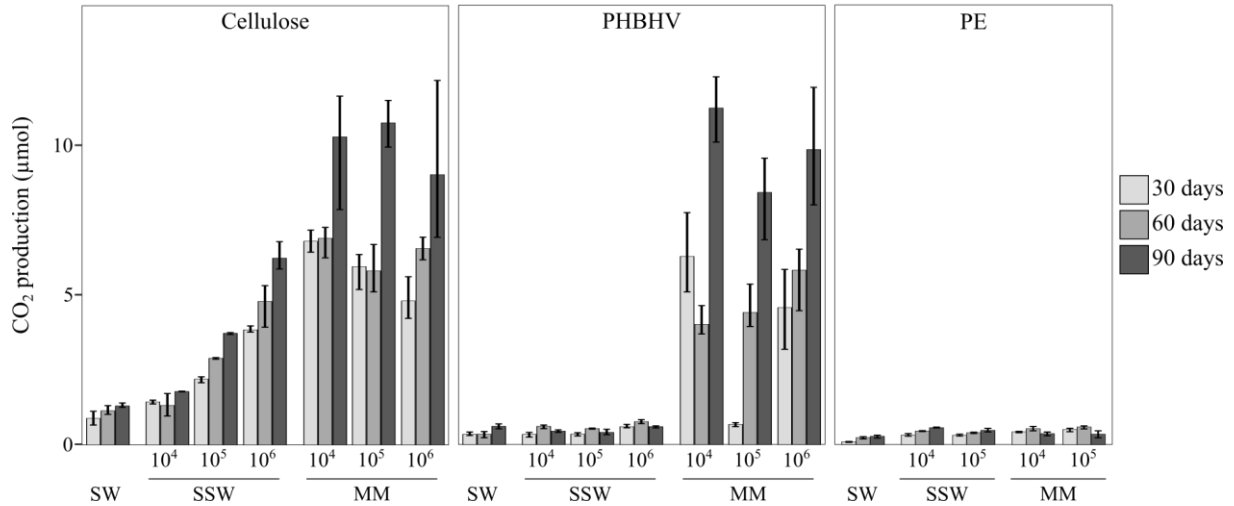


Figure 3.3. Carbon dioxide production on sampling days. SW refers to raw seawater, without specific inoculum. SSW refers to sterile seawater and MM refers to synthetic carbon-minimum medium. 10<sup>4</sup>, 10<sup>5</sup> and 10<sup>6</sup> are the concentrations of the detached biofilm inoculum. The barplots represent the average values and the bars correspond to minimum and maximum values.

### 3.3. Heterotrophic bacterial production

Heterotrophic production was measured in triplicates after 30 and 60 days of incubation in all the conditions by measurement of leucine incorporation (Fig. 3.4). Data were overall more dispersed than for oxygen consumption and DIC production.

The both biodegradable plastics, cellulose and PHBHV, exhibited low heterotrophic bacterial production in raw SW, without specific inoculum, that were not significantly different from activities measured in the not biodegradable PE samples (Kruskal-Wallis with Holm correction, p value = 0.51). Cellulose samples incubated in SSW with the detached biofilm inoculum at a concentration of 10<sup>4</sup> cells.mL<sup>-1</sup> showed low heterotrophic activity, as well as PHBHV samples incubated in SSW, whatever the detached biofilm inoculum concentration (from 10<sup>4</sup> to 10<sup>6</sup> cells.mL<sup>-1</sup>). As observed for both oxygen consumption and DIC production, the increase of the detached biofilm inoculum concentration from 10<sup>5</sup> to 10<sup>6</sup> cells.mL<sup>-1</sup> in cellulose incubated in SSW led to a significant increase of the heterotrophic bacterial production (mean = 0.18 ± 0.057 ngC.mm<sup>-2</sup>.h<sup>-1</sup> and mean = 0.63 ± 0.21 ngC.mm<sup>-2</sup>.h<sup>-1</sup> for bacterial concentrations of 10<sup>5</sup> and 10<sup>6</sup> cells.mL<sup>-1</sup> respectively; Kruskal-Wallis test with Holm correction,

p value =  $3.4 \times 10^{-3}$ ). Cellulose incubated in MM with its specific inoculum presented high and increasing heterotrophic activity after 30 and 60 days of incubation, whatever the detached biofilm inoculum concentration (mean =  $0.75 \pm 0.18 \text{ ngC} \cdot \text{mm}^{-2} \cdot \text{h}^{-1}$  and mean =  $0.81 \pm 0.28 \text{ ngC} \cdot \text{mm}^{-2} \cdot \text{h}^{-1}$  after 30 and 60 days of incubation, respectively). The same results was observed for PHBHV samples incubated in MM with detached biofilm inoculum concentrations ranging from  $10^4$  to  $10^6 \text{ cells} \cdot \text{mL}^{-1}$  (mean =  $0.73 \pm 0.13 \text{ ngC} \cdot \text{mm}^{-2} \cdot \text{h}^{-1}$  and mean =  $0.98 \pm 0.16 \text{ ngC} \cdot \text{mm}^{-2} \cdot \text{h}^{-1}$  after 30 and 60 days of incubation, respectively).

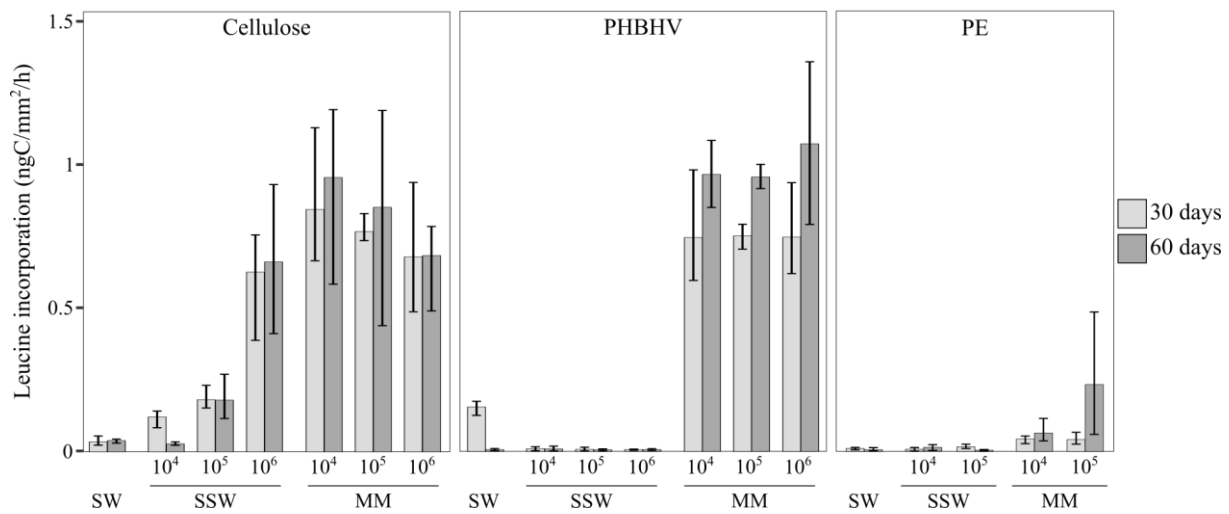


Figure 3.4. Heterotrophic bacterial production on sampling days. SW refers to raw seawater, without specific inoculum. SSW refers to sterile seawater and MM refers to synthetic carbon-minimum medium.  $10^4$ ,  $10^5$  and  $10^6$  are the concentrations of the detached biofilm inoculum. The barplots represent the average values and the bars correspond to minimum and maximum values.

### 3.4. Nutrients concentration

Phosphates ( $\text{PO}_4$ ) and nitrates ( $\text{NO}_3^-$ ) concentrations were measured in the different media throughout the incubations. The two nutrients were in very low concentration in seawater (SW and SSW) compared to the synthetic carbon-minimum medium (MM). At the beginning of the experiment, phosphates measurements were  $[\text{PO}_4] = 0.013 \mu\text{mol} \cdot \text{L}^{-1}$  in seawater, against  $[\text{PO}_4] = 26 \mu\text{mol} \cdot \text{L}^{-1}$  in the MM. As for nitrates, we measured  $[\text{NO}_3^-] = 0.36 \mu\text{mol} \cdot \text{L}^{-1}$  in seawater, against  $[\text{NO}_3^-] = 1,028 \mu\text{mol} \cdot \text{L}^{-1}$  in the MM. However, in samples with biodegradation activities ( $\text{O}_2$  consumption,  $\text{CO}_2$  production, bacterial heterotrophic activity), we observed an

increase in  $\text{PO}_4$  and  $\text{NO}_3^-$  concentrations, suggesting that if communities could start biodegradation with low nutrient concentrations, these two nutrients were not limited after 90 days of incubation.

### 3.5. Bacterial diversity

*Work in progress*

### 3.6. Degradation products

*Work in progress*

## 4. Discussion

### 4.1. The importance of using specific biofilm for marine biodegradability tests

In addition to its intrinsic properties, the choice of the experimental inoculum is a critical parameter for biodegradation studies (Harrison et al., 2018). Three types of inocula are proposed by the currently available standards for biodegradation tests in the marine environment: seawater (ISO 23977-1, ISO 23977-2), sediments (ISO 18830, ISO 19679) or a defined mixture of culturable bacterial strains (ASTM D6691). Culture bacteria represent less than 0.1% of the number of bacteria in the marine environment (the so-called “great plate count anomaly”) and a very small proportion of its very large diversity (Wu et al., 2009), thus rendering irrelevant their use for biodegradability tests aiming to mimic environmental conditions. Marine biodegradation of plastics has been generally conducted using sediments to provide the bacterial inoculum, which was shown to bring better biodegradation levels for the biodegradable PHB than seawater only, and thus recommended for rapid biodegradation testing (Beiras & López-Ibáñez, 2023). However, using sediments is not representative of the seawater, since the pelagic and benthic ecosystems differ greatly in terms of bacterial communities and nutrient availability (Wang et al., 2020). Using sediments can also bring uncertainties because of the possible use by bacteria of the large amount of natural organic matter in the sediment rather than plastics (Harrison et al., 2018). Here, we used two types of inocula, *i.e.*, the free-living bacteria from seawater (SW) and detached biofilm inoculum grown on each polymer type during one-month incubation in natural seawater. After 90 days of incubation, we did not detect any sign of effective biodegradation with SW inoculum for the biodegradable cellulose and PHBHV polymers. All the microbial activity measurements ( $\text{O}_2$ ,  $\text{CO}_2$ , heterotrophic activity) showed

congruent results, with similar signals as for the non-biodegradable PE under the same conditions. Several studies underlined the clear differences between the plastisphere and the bacterial communities living in the surrounding seawater (Zettler et al., 2013). This clear niche partitioning has evident consequences on the functional ecology of the two distinct communities, with enriched potential plastic degraders on the plastisphere as compared to seawater bacteria (Amaral-Zettler et al., 2020; Odobel et al., 2021). Therefore, the use of the free-living bacteria from seawater as test inoculum does not reflect the complex community living on plastics and is therefore not so relevant to mimic natural conditions in plastic biodegradation tests in the marine environment (Jacquin et al., 2019).

In our conditions, no sign of biodegradation was observed when using free-living bacteria from seawater (SW) as test inoculum, whereas the use of adequate plastisphere community as inoculum resulted in positive response to biodegradability tests. It may be argued that the incubation time of 90 days in our study may be not sufficient to observe biodegradation using SW as inoculum. However, thanks to the use of a continuous, non-destructive method for oxygen concentration measurement, we were able to keep monitoring oxygen consumption on the samples after 90 days and we did not observe biodegradation signs in any of the samples incubated in SW after 200 days of incubation. Other studies showed positive signs of PHBHV biodegradation by using SW as inoculum in the literature (Komiya et al., 2021). Such discrepancy may be explained by the bigger volumes in the experimental design used in these studies, which might increase the probability to form a biofilm with putative plastic degraders. Nevertheless, none of these studies gave information on the bacterial diversity composing the biofilm growing on plastics, which may differ greatly from a natural biofilm in the natural environment. Indeed, several studies indicated that a minimum of 15-30 days was necessary for a mature biofilm to grow on plastics in seawater, which clearly differed with free-living seawater bacterial diversity (Dussud et al., 2018a). We therefore recommend the use of a pre-selected mature bacterial biofilm growing on the selected materials for the study of marine biodegradability in seawater. So far, this method appears to offer the best compromise to mimic the natural conditions of plastic colonization while working under controlled conditions for the biodegradability tests.

## 4.2. Nutrient availability may limit biodegradation tests under controlled conditions

Available works on plastic biodegradation using seawater proposed the addition of nutrients to avoid nitrogen or phosphorous limitations during the tests (Beiras & López-Ibáñez, 2023). Indeed, nutrients are known to be a classical limiting factor in biodegradation processes, especially in oligotrophic seawaters (Pulido-Villena et al., 2012). Two methodological standards for aerobic biodegradation of plastic exposed to seawater impose to report the nitrogen content of the medium and propose addition of nutrients (ISO 23977-1, ISO 23977-2), but without clear setpoints, and no mention of the phosphorus content. While nutrient composition appears to be critical for plastic biodegradability, little attention is paid to this parameter in the development of methodologies. In this study, we monitored the biodegradation of cellulose and PHBHV with their specific biofilm inoculum using two types of medium: sterile natural seawater (SSW) and a synthetic carbon-minimum medium (MM). We hypothesized that the presence of organic matter in SSW compared to MM could enhance the biodegradation, known as the ‘priming effect’ (Blanchet et al., 2017; Eubeler et al., 2009). We did not record any biodegradation signals for PHBHV incubated in SSW within the 90 days of incubation. Biodegradability testing of this polymer during laboratory incubations in natural seawater previously showed good results using waters of the Atlantic Ocean (Deroiné et al., 2015), as well as the Pacific Ocean (Komiya et al., 2021). Our work was conducted in the Mediterranean Sea during summer, which corresponded to classical oligotrophic conditions (Siokou-Frangou et al., 2010). Low phosphorus concentration was proved to be a limitation for heterotrophic bacteria in the NW Mediterranean Sea (Pulido-Villena et al., 2012), which may explain the absence of biodegradation of PHBHV in SSW conditions, despite the addition of the specific inoculum. Bottom-up control by nutrient limitation has been intensively described for hydrocarbon biodegradation in marine conditions (Röling & van Bodegom, 2014; Sauret et al., 2014), but such limitation for plastic biodegradation has been poorly investigated so far (Beiras & López-Ibáñez, 2023). In contrast to SSW results, clear signs of biodegradation were always detected when the specific biofilm inoculum was incubated with cellulose and PHBHV in the MM. The low concentration of inorganic nitrogen and phosphorus in the SSW compared to MM was confirmed by measurement of nutrients in the media and across the experiment. We therefore recommend the use of a synthetic medium with nutrient enrichment for plastic biodegradability tests under controlled conditions. Because of the wide variability of the nutrients content in seawaters across space (Moore et al., 2013) and times (Pasquero De



Fommervault et al., 2015), such precaution may render the comparison between different biodegradation tests more relevant. However, one should keep in mind that such laboratory constraint diminishes the environmental reliability, and caution must be taken when extrapolating biodegradability results to the natural conditions.

### 4.3. Impact of increasing bacterial inoculum concentration on bacterial activities on plastics

Adding a specific bacterial inoculum from a preselected natural biofilm requires the consideration of the bacterial concentration for inoculation. Intuitively, we could have predicted that the more bacterial cells inoculated, the higher biodegradation will be. We observed an influence of the bacterial inoculum concentration only for cellulose samples incubated in SSW, with increased activity when increasing bacterial cells (from  $10^4$  to  $10^6$  cells.mL<sup>-1</sup>). However, no influence of inoculum concentration was observed for cellulose and PHBHV in MM test medium. Similar results were found on the only one study testing the impact of the inoculum concentration on PHBHV biodegradation on marine conditions, with  $1.30 \times 10^5$  and  $1.14 \times 10^6$  CFU/mL of biofilm added originated from fish tanks (Deroiné et al., 2015). Bacterial growth was probably limited in the low nutrient SSW condition, whereas the MM offers optimal conditions leading to rapid growth, explaining the similar activity for the three bacterial concentrations. Based on the metabolic theory of ecology (Browne et al., 2010), the carrying capacity of a community refers as the maximum abundance reached at the plateau stage of an experiment that reflects limiting resources (Huete-Stauffer et al., 2015). Our result indicated that a concentration of  $10^4$  cells.mL<sup>-1</sup> measured by cytometry approach was sufficient to reach the carrying capacity of the tested plastics under nutrient-rich conditions. It is noteworthy that our detachment procedure was soft enough to keep the microbial cells activity, but a large number of plastic pieces were needed for the pre-selection of the specific biofilm inoculum to reach enough cells for the incubation with  $10^6$  cells mL<sup>-1</sup>. This was not feasible for the non-biodegradable PE polymer that presented much less detached cells than for cellulose and PHBHV pre-selected biofilms, as counted by flow cytometry. Hence, we recommend the addition of detached biofilm inoculum concentration of  $10^5$  cells.mL<sup>-1</sup> for the biodegradability tests, which is similar to bacterial concentration found in the natural seawaters (Pulido-Villena et al., 2012).

### 5. Conclusion

In this study, we tested the influence of experimental conditions on marine plastic biodegradability tests, with special emphasis on the microbial inoculum and test medium. We demonstrated that both of these parameters impact the biodegradation in our conditions and we propose some recommendations.

First, we recommend to perform a one-month incubation of the tested material in seawater in order to dispose of a mature natural biofilm as inoculum. It ensures a realistic colonization of the material, that would not be satisfied by using free-living bacteria from natural seawater as inoculum, since it was demonstrated that the plastisphere is clearly distinct from the surrounding seawater communities (Dussud et al., 2018b; Zettler et al., 2013). We also recommend to use a concentration of detached natural biofilm inoculum of  $10^5$  cells.ml<sup>-1</sup>.

Second, we recommend to use a test medium with enriched nutrients in order to avoid nutrient limitation during the biodegradability test. It ensures sufficient nutrient disposal for cells that are necessary for biodegradation, that would not be always satisfied by using natural seawater and especially under oligotrophic conditions. This was our case in this study, where phosphorus limitation of the oligotrophic NW Mediterranean seawater resulted in an absence of biodegradation detected for PHBHV in sterile seawater with pre-selected biofilm, whereas biodegradability was confirmed when incubated a synthetic medium.

Third, we recommend to evaluate the bacterial diversity during the course of the biodegradability experiment, which reflects the relevance of the tests in relation to natural conditions. Following changes in alpha diversity are of interest to evaluate the loss of richness or evenness under laboratory conditions that may not be realistic of the natural conditions, where a large diversity of microorganisms continue to colonize plastic materials during the course of the biodegradation processes.

Finally, we believe that such experiment should be repeated in other location and seasons, *i.e.*, other inoculum communities and seawater conditions, to sharpen the comprehension of the biodegradation processes. We insist that laboratory conditions needed for biodegradation tests give putative information on biodegradability, but may suffer for lack of

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representability of the large variety of conditions encountered in the natural environment. Therefore, conclusions on seawater plastic biodegradability must be formulated with prudence, since universal biodegradability in any marine ecosystem does not exist due to the limitless combinations of environmental conditions (*e.g.*, organic matter, oxygen levels, temperature, pH, turbulence). Overall, this work improves our understanding on the importance of microbial inoculum and test medium on the evaluation of plastic biodegradability in seawater and paves the way for deepening the understanding of the underlying mechanisms.

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***Chapitre 4 : Influence de la composition en monomère  
sur la biodégradabilité des polyhydroxyalcanoates  
(PHA) en milieu marin***

## Chapitre 4

Ce chapitre est rédigé sous la forme d'un article scientifique qui sera soumis prochainement au journal « Marine Pollution Bulletin ». Cette partie présente un test de biodégradabilité de 7 formules de PHA en conditions marines en utilisant le modèle expérimental présenté dans le chapitre 3. Parmi ces 7 PHA, 2 sont des *scl*-PHA, 3 sont des *mcl*-PHA et 2 sont des mélanges de monomères courtes et moyennes chaînes. Les PHA sont une famille de biopolymères présentés comme biodégradables dans plusieurs milieux, incluant le milieu marin. Plus de 150 monomères de PHA ont été identifiés à ce jour, et cette famille de polymères présente des propriétés physico-chimiques très variées, permettant d'envisager des applications très variées. Les PHA sont aujourd'hui de bons candidats pour remplacer les polymères conventionnels utilisés dans la manufacture des plastiques. Cependant, les études sur leur biodégradabilité en milieu marin n'ont utilisé jusqu'à aujourd'hui que du PHB et deux de ses copolymères, qui sont aujourd'hui les trois formes de PHA commercialement disponibles.

Un biofilm mature colonisé en milieu naturel sur du PHBHV a été utilisé comme inoculum expérimental. 2 mL de cet inoculum dilué à  $10^4$  cellules/mL ont été ajoutés à 6 mg de PHA, et incubés pendant 90 jours. La consommation d'O<sub>2</sub> a été suivie en continu, et des analyses de la biodégradation ont été faites après 15, 30 et 90 jours d'incubation, en mesurant la production de CO<sub>2</sub>, la production hétérotrophe bactérienne, ainsi que les produits de biodégradation. L'évolution des communautés bactériennes le long de l'expérience a aussi été suivie.

Après 90 jours d'incubation, tous les PHA ne présentent pas la même biodégradabilité. En particulier, les 3 *mcl*-PHA, *i.e.*, les PHOHHx, PHOHHp et PHHp n'ont montré aucun signe de biodégradation. Seuls les PHA contenant des monomères de HB ont montré des signes de biodégradation significatifs après 90 jours d'expérience. L'analyse des milieux de culture en RMN a montré la présence de monomères d'HB dans les échantillons de deux des PHA présentant des signes de biodégradation, à 90 jours d'incubation uniquement. Ces résultats suggèrent que la biodégradabilité des PHA en milieu marin est conditionnée par leur composition en HB, en lien avec les PHA dépolymérase disponibles dans l'environnement.

La diversité des communautés bactériennes est restée stable au cours des 90 jours, indiquant que l'incubation en milieu fermé n'a pas induit une perte de diversité au profit de

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quelques taxons dominants. De plus, l'étude des communautés a montré une séparation des consortia bactérien impliqués dans leur biodégradation vs consortia colonisant les autres PHA. *Marinobacter sp.* a été identifié comme un taxon contribuant significativement à cette différence observée entre les deux groupes, particulièrement abondant dans les échantillons biodégradables. Ce groupe contient des souches identifiées comme des producteurs de PHA dépolymérase extracellulaires dégradant le PHB. Enfin, le suivi de la diversité permet de mettre en avant de nouveaux groupes taxonomiques potentiellement capables de biodégrader les *scl*-PHA.

## **Influence of monomer composition on polyhydroxyalkanoates (PHA) biodegradability in seawater**

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**Keywords:** Biosourced and biodegradable polymers • biodegradability tests • plastisphere

### Highlights :

- Not all PHA present rapid biodegradability in marine seawaters
- Monomer composition drives PHA biodegradability at sea
- Specific bacterial communities associated with the biodegradable vs. more recalcitrant PHA
- *Marinobacter* sp. was a key degrader for short chain length-PHA in our conditions

### Abstract :

Polyhydroxyalkanoates (PHA) are natural biodegradable polyesters which could replace conventional, recalcitrant polymers in plastic material for selected application. So far, most studies focusing on PHA biodegradation were conducted on homo- or copolymers of poly(3-hydroxybutyrate) (PHB), the most naturally widespread form of PHA. Here, we investigated the biodegradability under marine conditions of 7 types of PHA, two *scl*-PHA, three *mcl*-PHA and two PHA containing both *scl*- and *mcl*-PHA monomers. A mature biofilm grown on PHA films in seawater was used for the test inoculum to mimic natural conditions. Polymer biodegradability was monitored through respirometry measurements (O<sub>2</sub> consumption and CO<sub>2</sub> production), together with the characterization of biodegradation products and associated bacterial diversity. After 90 days of incubation, all PHA containing hydroxybutyric acid monomers (HB) showed clear biodegradability, contrary to the three *mcl*-PHA. After 90 days of incubation, NMR spectroscopy and mass spectrometry confirmed the release of HB monomers in samples exhibiting biodegradation signals. Illumina sequencing highlighted *Marinobacter* sp. and *Alcanivorax* sp. as putative *scl*-PHA degraders. We believe that in our conditions, the chemical composition of the PHA, *i.e.*, the monomer type, is a more important driver of their biodegradability than intrinsic properties such as molecular mass or crystallinity.

## 1. Introduction

Biosourced and biodegradable plastics are gaining a growing interest due to fossil resources depletion and plastic pollution (RameshKumar et al., 2020). They have been proposed to replace traditional plastics such as single-use plastics, since they present similar properties as their petroleum-based counterparts. They offer the advantages of being derived from renewable resources, contributing to circularity, reducing carbon footprint, and exhibiting complete biodegradation within a reasonable timeframe (Silva et al., 2023). Relevance of biodegradable plastics have been recently discussed for selected applications only, with respect to their use and end of life (Paul-Pont et al., 2023). Biodegradable plastics have been recommended when collection at the end of life is not possible or extremely difficult, such as plastic items widely used in the agriculture (strings, clips, bale, mulching film), in care products (cosmetics, detergents), microfibers used in textiles, and for fishery gears and aquaculture equipment.

Plastic biodegradation has been described as a four steps process including (i) *bio-deterioration* associated to biofilm formation, (ii) *bio-fragmentation* by extracellular enzymes, forming soluble oligomers and finally monomers that (iii) can be *bio-assimilated* by cells to produce biomass and (iv) *bio-mineralized* until the excretion of completely oxidized metabolites (CO<sub>2</sub>, N<sub>2</sub>, CH<sub>4</sub>, and H<sub>2</sub>O) (Jacquin et al., 2019). The current standards to evaluate the biodegradability of materials propose tests based on respirometry measurements (O<sub>2</sub> uptake or CO<sub>2</sub> release), susceptible to describe the mineralization of plastic, which is the ultimate step of biodegradation. For the marine environment, different methodological standards exist to evaluate the level of plastic biodegradation at the interface seawater/sediments (ISO 19679, ISO 18830, ASTM D7991-15) or in the water column (IDO 23977-1 and -2, ASTM D6691), generally within a maximum of 24 months. Recent papers have pointed out that the current standards were insufficient in their ability to reliably predict plastic biodegradability in natural conditions (Harrison et al., 2018; Napper & Thompson, 2019; Paul-Pont et al., 2023). In particular, they mentioned the lack of relevance of the experimental microbial inoculum towards the marine environment, and the need for a multidisciplinary approach rather than over-simplified methods to estimate biodegradability.



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Polyhydroxyalkanoates (PHA) are listed as the most common bioplastics commercially available, together with polycaprolactone (PCL), polylactic acid (PLA), polybutylene adipate-co-terephthalate (PBAT) and polybutylene succinate (PBS) (RameshKumar et al., 2020). They are polyesters naturally produced by many bacteria and fungi (Jendrossek & Handrick, 2002). In carbon-rich but nutrient-limited environments, these microorganisms synthesize PHA-based polymers to store carbon in inclusion bodies, that they can depolymerize when carbon becomes limited. PHA are classically divided into different groups depending on the size of their radical chain: short-chain length PHA (*scl*-PHA), containing 3 to 5 carbons per monomer, medium-chain length PHA (*mcl*-PHA), containing 6 to 14 carbons per monomer, and long-chain length PHA (*lcl*-PHA) containing more than 14 carbons per monomer (Vicente et al., 2023). More than 150 monomers of PHA have been described so far, displaying a large set of properties, hence offering the possibility of various usages (Jendrossek & Handrick, 2002). However, to date, most studies on PHA marine biodegradation focused on two commercially available polymers, *i.e.*, poly(3-hydroxybutyrate) (PHB) and poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBHV). Very efficient biodegradation was observed under marine conditions for the two polymer types (minimum of 70% of carbon mineralization after 40 days of incubation) (Thellen et al., 2008), but these studies are not sufficient to guarantee the biodegradability of all forms of PHA. Moreover, these two polymers present drawbacks compared to other conventional plastics, such as limited impact strength and flexibility and high brittleness, which limit their commercial applications (Meereboer et al., 2020). Other biodegradability tests showed the biodegradability of the copolymer poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) (PHBHHx) (Wang et al., 2018), but no comparison exist with other *scl*-PHA. Furthermore, to our knowledge, *mcl*-PHA biodegradability was never tested under marine conditions so far.

Here, we compared for the first time the biodegradability under marine conditions of seven formulas of PHA, including two *scl*-PHA, three *mcl*-PHA and two PHA containing both *scl*- and *mcl*-PHA monomers. Tests were performed during 3 months using a multidisciplinary approach, to characterize the degradation products (oligomers and monomers using NMR spectroscopy and mass spectrometry) together with the biodegradation activities (O<sub>2</sub> uptake and CO<sub>2</sub> release, by using respirometry measurements). Special care was taken on the selected marine microbial inoculum and on the description of the bacterial diversity associated with the PHA biodegradation (16S rRNA Illumina sequencing). We hypothesized that not all PHA types present rapid signs of biodegradability under marine conditions, and that the monomer

composition drives the biodegradability of PHA rather than other physico-chemical properties such as crystallinity or molecular mass.

## 2. Material and methods

### 2.1. PHA films characteristics and preparation

Seven formulas of polyhydroxyalkanoates (PHA) were chosen for this study: (1) two *scl*-PHA including poly(3-hydroxybutyrate) (PHB) and poly(3-hydroxybutyrate-co-3-hydroxyvalerate) with HB content of 95% (PHBHV95) (2) three *mcl*-PHA including poly(3-hydroxyoctanoate-co-3-hydroxyhexanoate) with HO content of 92% (PHOHHx), poly(3-hydroxyhexanoate-co-3-hydroxyheptanoate) with HO content of 60% (PHOHHp) and poly(3-hydroxyheptanoate) (PHHp) and (3) two PHA containing both *scl*- and *mcl*-PHA monomers referred as poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) containing 93 and 89% of HB (PHBHHx94 and PHBHHx89, respectively).

Tableau 4.1. Composition et propriétés des PHA.

PHA	Composition	T <sub>g</sub> (°C)	T <sub>m</sub> (°C)	ΔH <sub>m</sub> (J.g <sup>-1</sup> )	MW (g.mol <sup>-1</sup> )	PDI
PHB	100% HB	2.7	178	101	263,000	1.7
PHBHV95	95% HB 5% HV	4	165	93	256,000	1.8
PHBHHx94	94% HB 6% HHx	1	143	47	112,000	2.0
PHBHHx89	89% HB 11% HHx	0	130	39	123,000	2.0
PHOHHx	92% HO 8% HHx	-35	52	23	216,000	1.8
PHOHHp	60% HO 40% HHp	-33	48	15	146,000	2.0
PHHp	100% HHp	-30	NA	NA	124,000	2.0

## 2.2. Experimental design

We used a two-phase stepwise approach in order to use a natural inoculum for biodegradability tests (called ‘test inoculum’ hereafter), as previously described (Cheng et al., 2022). The first step was the formation of a mature biofilm growing naturally on PHA in seawater, where PHBHV films (50 pieces of 9 mm diameter and 200  $\mu\text{m}$  thick, supplied by Tianan Biological Materials Co. Ltd., under the trade name ENMAT Y1000P, China) were incubated for several months in 1.8 L aquarium with direct circulation to the sea. A flow rate of  $50 \text{ ml}\cdot\text{min}^{-1}$  was chosen to ensure a sufficient renewal of natural microorganisms (Odobel et al., 2021). Seawater was pumped at 14 m depth and at 30 m from the coastline in the Banyuls bay, close to the SOLA observatory station (NW Mediterranean Sea, France). In the second step, the mature biofilm formed on PHBHV pieces was carefully detached using 3 cycles of 1 minute of vortex followed by 3 minutes in ultrasonic bath and transferred in a synthetic carbon-minimum medium (called “MM” hereafter, adapted from (Eguchi et al., 1996)) (Conan et al., 2022). Immediately after cell detachment, the concentration of the inoculum was measured by flow cytometry (BD FACSCanto™ II, BD Biosciences, United States), as previously described. Bacterial heterotrophic production was measured in triplicate for the detached biofilm by the  $^3\text{H}$ -leucine incorporation method (Céa et al., 2015), in order to ensure that the bacterial community was still active after cell detachment ( $397\pm 140 \text{ ngC}\cdot\text{L}^{-1}\cdot\text{h}^{-1}$ ). The inoculum was then diluted in MM at the concentration of  $10^4 \text{ cells}\cdot\text{mL}^{-1}$  for incubation with the different types of PHA. Replicate of plastic samples for each PHA film type were placed in sterile individual 12 mL tubes (Exetainer®, Labco, United Kingdom) to reach 6 mg of plastic per tube (corresponding to between 5 to 15  $\text{mm}^2$  depending on the PHA type). Plastic samples were then sterilized by addition of 500  $\mu\text{L}$  of ethanol 70% and exposure to UV light for 15 minutes. Tubes were placed under a sterile hood until complete evaporation of ethanol and 2mL of diluted inoculum was added on each tube. Two types of controls were included: abiotic controls containing sterile plastic samples in sterile MM, and blanks containing the test inoculum only without plastic. A total of 711 tubes were placed in an incubator at  $18^\circ\text{C}$  under agitation of 120 rpm (Innova® S44i, Eppendorf, Germany) in the dark. Biodegradation of the samples was monitored on days 5, 15, 30 and 90.

### 2.3. Continuous oxygen measurement

Oxygen concentration was measured in the culture medium as a proxy of plastic biodegradation. Oxygen sensor spots (SP-PSt5, Presens, Germany) were placed at the bottom of the Exetainer tubes, and dissolved oxygen concentrations were measured continuously during the 90 days of the experiment, using a small 24-channel reader (SDR SensorDish® Reader, Presens, Germany). One measurement was acquired automatically every hour in triplicates for biotic conditions as well as in abiotic and blank conditions.

### 2.4. Carbon dioxide production

Dissolved inorganic carbon concentration was measured in the culture medium to monitor plastic mineralization. On days 5, 15, 30 and 90, mercury chloride ( $37\text{g}\cdot\text{L}^{-1}$ ) was added to the corresponding tubes in order to block bacterial activity. Mercury chloride was injected directly in the tubes with a syringe through the septa of the caps and silicon was applied on the caps' septa to ensure the good sealing of the tubes. Tubes were kept at room temperature and analyzed altogether at the end of the experiment, using a TOC analyzer (TOC-L Series, Shimadzu).

### 2.5. Characterization of the degradation products

#### 2.5.1. NMR spectroscopy

The same volume of culture medium from biodegradability tests ( $600\mu\text{l}$ ) were aliquoted and lyophilized until further analysis. The dry samples were resuspended in  $600\mu\text{l}$  of  $300\text{mM}$  phosphate buffer (pH 2.6) in Deuterated water ( $\text{D}_2\text{O}$ , Eurisotop), and  $0.2\text{ mM}$  of deuterated trimethylsilylpropanoic acid sodium salt (TSPd<sub>4</sub>, Eurisotop).  $\text{D}_2\text{O}$  was used for locking and shimming while TSPd<sub>4</sub> constituted a reference for chemical shifts ( $^1\text{H}$ ,  $\delta$  0.00 ppm) and quantification.  $^1\text{H}$  NMR spectra were recorded on a Bruker Avance 500 MHz spectrometer, equipped with a 5 mm inverse-triple tuned (TXI)  $^1\text{H}/^{13}\text{C}/^{15}\text{N}$  with z-gradient coil probe (Bruker Biospin Wissenbourg, France), with 5 mm-diameter tubes containing  $600\mu\text{l}$  of sample. 128 scans were collected ( $90^\circ$  pulse, 3.24 s acquisition time, 4.0 s relaxation delay, 4789.272 Hz SW, 65536 data points).  $^1\text{H}$ -NMR spectra were processed using Topspin software version 4.2 (Bruker Biospin, Germany). An exponential filter was applied before Fourier transformation. All spectra were manually phased, and a baseline correction was performed on spectra before integration. Each spectrum was aligned by shifting the TSPd<sub>4</sub> signal to zero to perform quantification. Under these conditions, the limit of quantification is in the range of  $0.01\text{ mM}$ .

The spectral peaks were assigned by comparing chemical shift and multiplicity with 3-hydroxybutyric acid and 3-hydroxyvaleric acid standards and the literature. To compare the samples, the NMR signals were calibrated according to the internal deuterated standard TSPd<sub>4</sub> and the whole spectrum signal was integrated to determine the relative quantity of degradation products (oligomers) in the samples (oligomers). Quantification was performed based on the proton from 3-hydroxybutyric acid methyl (CH δ 4.2 ppm) signals according to the following formula.

$$C_x = C_{tsp} \times \frac{n_{tsp}}{n_x} \times \frac{A_x}{A_{tsp}}$$

where  $C_x$  and  $C_{tsp}$  are the concentration (mol.L<sup>-1</sup>),  $n_x$  and  $n_{tsp}$  the numbers of protons and  $A_x$  and  $A_{tsp}$  the integral values of the analyte and the standard, respectively.

$$n = C \times V$$

Where  $n$  is the number of mole of the analyte,  $C$  the concentration (mol.L<sup>-1</sup>) and  $V$  the volume of the sample.

### 2.5.2. Mass spectrometry

The supernatants (300 μL) from biodegradation experiments were freeze dried and resuspended in 100 μL of water / acetonitrile mixture (50:50 v:v) to provide 3-fold concentrated samples. HRMS analyses were performed with an Ultimate 3000 RSLC chromatographic system (ThermoScientific) coupled to an Exactive Orbitrap mass spectrometer (Thermo Fisher Scientific) equipped with an electrospray (ESI) source. The chromatographic separations were performed using a Kinetex EVO C18 column (2,1 x 100 mm; 1,7μm-Phenomenex) operating at 30°C and an injection volume of 5μl. The flow rate was fixed at 0.45 mL/min with 0.1% of formic acid in water (A) and 0.1% of formic acid in acetonitrile (B) for mobile phases at the following gradient: initial, 95% A; 0–7.5 min linear, 1% A linear; 7.5–8.5 min, 1% A; 8.5–9 min, 95% A linear, 9-11 min 95% A following by washing and reconditioning of the column. The mass spectrometer was operated in positive ion mode with capillary voltage at 3,2 kV and a capillary temperature set at 320 °C. The detection was performed with full scan from  $m/z$  50 to 750 using a resolution set at 70 000 at  $m/z$  200. HRMS raw data were processed with Xcalibur Software (version 4.1) considering the  $m/z$  values with a peak intensity strictly greater than 10<sup>4</sup>. Identification of monomer and oligomer were obtained using analytical standard of 3-hydroxybutyric acid.

### 2.6. DNA extractions, PCR, sequencing and data analysis

Plastic pieces were sampled after 15, 30 and 90 days with sterile tweezers and stored at -80°C for further analysis. DNA extractions were performed using a classical phenol-chloroform method (Jacquin et al., 2021). Polymerase Chain Reaction (PCR) amplification of the V4-V5 region was done using universal 16 rRNA primers (515Y and 926R) (Parada et al., 2016). Illumina MiSeq sequencing was performed at Genoscope (Evry, France). Sequences analysis was done using the DADA2 pipeline (Callahan et al., 2016) for ASV establishment and taxonomy assignment. Taxonomic assignment was done using the SILVA 138 SSU database. ASV that did not belong to Bacteria kingdom as long as ASV from chloroplasts and mitochondria were removed from the dataset. The number of sequences per sample was normalized by rarefaction (n=39,027) for sample comparison. The final ASV table contained 17,004 ASV in 51 samples.

### 2.7. Data management

Data were treated on R version 4.3.1 and PRIMER6 softwares. Graphical representations were done using the *ggplot2* package version 3.4.2 and the *vegan* package version 2.6.4. Data normality was evaluated with a Shapiro-Wilk test, using the *stats* package version 4.3.1. Data did not follow a normal distribution; thus, non-parametric tests were used. Kruskal-Wallis and Dunn tests (Holm-Bonferroni correction) were done separately for each date, with *stats* package version 4.3.1 and *rstatix* package version 0.7.2 respectively. Resampling and calculation of alpha-diversity indexes were done using the *phyloseq* package version 1.44.0. The Bray-Curtis dissimilarity matrix, the ANOSIM test and the NMDS were done using the *vegan* package version 2.6.4. The SIMPER analysis was performed on the PRIMER6 software.

## 3. Results

### 3.1. Oxygen consumption

Oxygen consumption was monitored during the 90 days of the experiment by continuous measurement of dissolved oxygen concentrations in the culture medium, abiotic controls and blanks. For further comparison with other destructive sampling for the monitoring of other parameters, data recorded on days 15, 30 and 90 of incubation were averaged. None of the measured oxygen concentrations fell above 50  $\mu\text{m.L}^{-1}$ , ensuring that aerobic conditions

were maintained throughout the experiment. No oxygen consumption was detected in abiotic controls.

Overall, a higher oxygen consumption was measured for *scl*-PHA and the two PHBHHx, as compared to the *mcl*-PHA (Fig. 4.1). After 90 days of incubation, PHBHHx89 showed the highest oxygen consumption ( $0.17\pm 0.016$   $\mu\text{mol}$ ), followed by PHBHV95 ( $0.15\pm 0.055$   $\mu\text{mol}$ ), PHB ( $0.073\pm 0.032$   $\mu\text{mol}$ ) and PHBHHx94 ( $0.078\pm 0.022$   $\mu\text{mol}$ ). Oxygen consumption was lower for PHOHHx, PHOHHp and PHHp after 90 days ( $0.039\pm 0.0042$   $\mu\text{mol}$ ,  $0.051\pm 0.0022$   $\mu\text{mol}$  and  $0.032\pm 0.015$   $\mu\text{mol}$ , respectively). Evolution with time was less dispersed (from 15 to 90 days of incubation) for the latter three polymers compared to the first four ones. Oxygen consumptions in *scl*-PHA and PHBHHx 94 and 89 were significantly higher after 90 days of incubation compared to day 15 ( $p = 0.0099$ , Holm-Bonferroni correction), whereas the change in oxygen consumption was not significant for *mcl*-PHA ( $p = 0.086$ , Dunn test with Holm-Bonferroni correction).

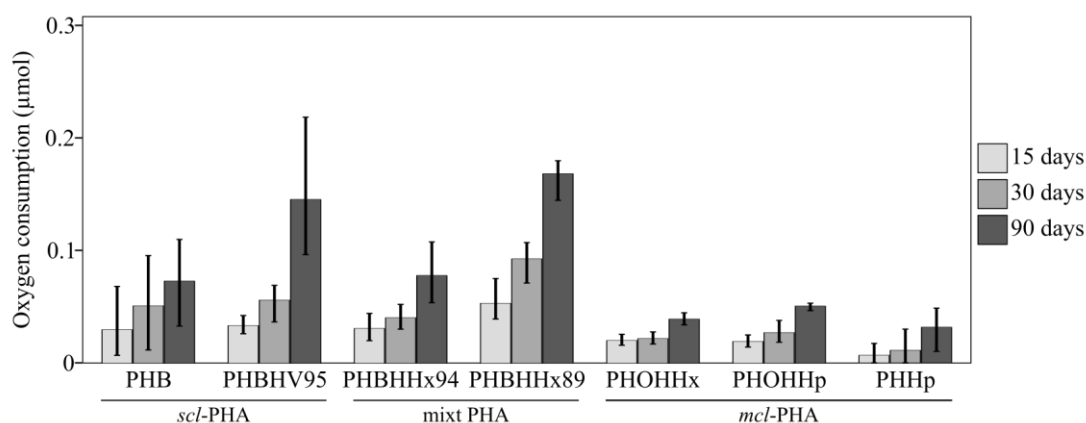


Figure 4.1. Oxygen consumption on sampling days. The barplots are the mean values and the bars correspond to minimum and maximum values. Oxygen consumption on day 90 was significantly higher than on day 15 for *scl*- and mixt PHA. No such significance was found between oxygen consumption for *mcl*-PHA.

### 3.2. Carbon dioxide production

Concentrations of dissolved inorganic carbon (DIC) were measured after 15, 30 and 90 days of incubation in the culture medium, abiotic controls and blanks to monitor CO<sub>2</sub> production corresponding to the mineralization step of the biodegradation. No production of CO<sub>2</sub> was recorded in abiotic controls, and the signal was too low in blanks to be detected. Again, CO<sub>2</sub> production was higher after 90 days of incubation in *scl*-PHA and PHBHHx samples than in the PHOHHx and PHHp samples (Fig. 4.2). However, unlike for oxygen consumption, it was equivalent between PHOHHp, PHBV and the two PHBHHx (Fig. 4.2). PHB samples presented the highest CO<sub>2</sub> production after 90 days of incubation ( $3.2 \pm 0.55$   $\mu\text{mol}$ ), followed by PHBV95 ( $1.04 \pm 0.092$   $\mu\text{mol}$ ), PHBHHx94 ( $0.90 \pm 0.31$   $\mu\text{mol}$ ) and PHBHHx89 samples ( $0.87 \pm 0.096$   $\mu\text{mol}$ ). CO<sub>2</sub> production after 90 days of incubation was lower for PHOHHp ( $0.70 \pm 0.22$   $\mu\text{mol}$ ), PHHp ( $0.40 \pm 0.18$   $\mu\text{mol}$ ) and PHOHHx samples ( $0.17 \pm 0.12$   $\mu\text{mol}$ ). Significant difference was found between CO<sub>2</sub> production of the two *scl*-PHA and the two PHBHHx and the *mcl*-PHA after 30 and 90 days of incubation ( $p = 0.0058$  and  $p = 0.0070$  respectively, Dunn test with Holm-Bonferroni corrections). Higher dispersion with time was observed for PHB than for the other polymer types.

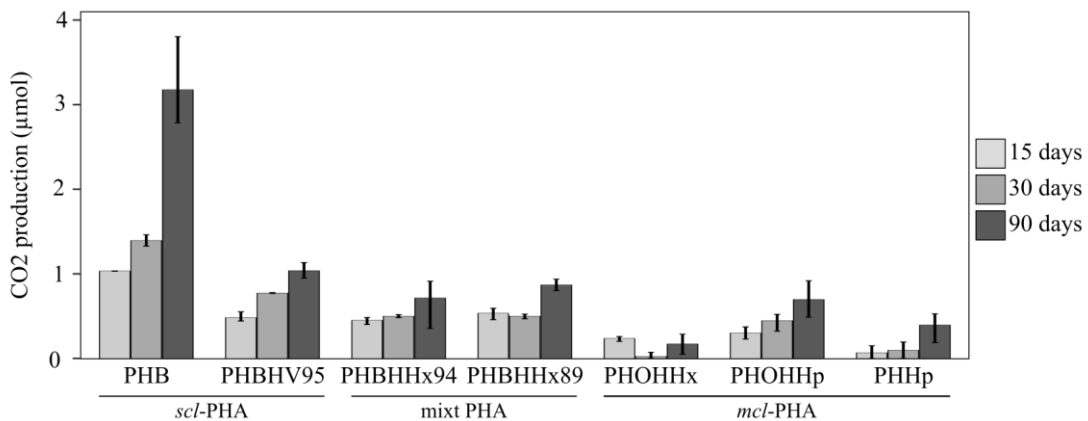


Figure 4.2. Carbon dioxide production on sampling days. The barplots correspond to the mean values and the bar to the minimum and maximum values. CO<sub>2</sub> production was significantly lower for *mcl*-PHA than the other types after 30 and 90 days of incubation.



### 3.3. Oligomer and monomer release

The formation of degradation products such as oligomers or monomers was monitored by NMR spectroscopy and mass spectrometry in the culture medium after 15, 30 and 90 days of incubation. A clear signal, characteristic of 3-hydroxybutyrate ( $\text{CH}_3\text{-HB}$   $\delta$  1.2 ppm,  $\text{CH}_2\text{-HB}$   $\delta$  2.4-2.5 ppm,  $\text{CH-HB}$   $\delta$  4.1-4.2 ppm,) was detected in the NMR spectra of PHB and PHBHHx89 supernatant samples while no such signal was detected in other samples, blanks nor in abiotic controls (Fig. 4.3). This signal was present only after 90 days of incubation but not detected after 15 and 30 days and the concentration of HB by-products was estimated to  $0,079 \pm 0,009 \mu\text{Mol}$  and  $0,027 \pm 0,002 \mu\text{Mol}$  for PHB and PHBHHx89 samples respectively. The identification of HB was confirmed by the detection of HB monomer ( $m/z$   $\text{H}^+$ : 105,0549) in the corresponding mass spectra of the samples.

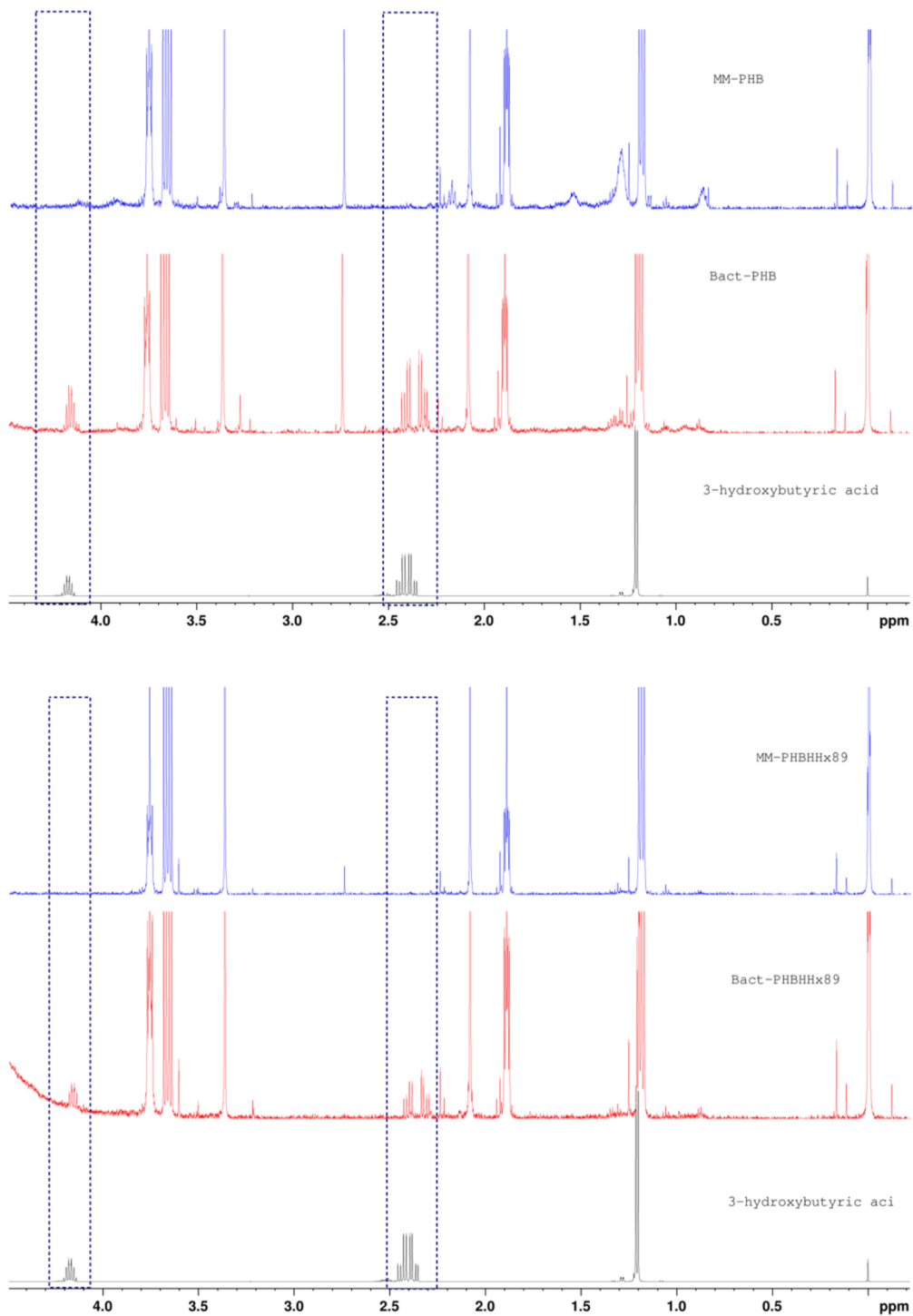


Figure 4.3. NMR spectra of PHB and PHBHHx samples after 90 days of incubation. The blue spectra are the abiotic controls (without bacterial inoculum). The red spectra are the samples (with bacterial inoculum). The gray spectra are the reference spectra of the hydroxybutyric-acid (HB).

### 3.4. Microbial diversity and community structure

Illumina Miseq sequencing was performed in duplicate after 15, 30 and 90 days of incubation, in order to evaluate the changes in microbial diversity and community structure during the biodegradability test and to identify the putative ASV involved in PHA biodegradation.

Alpha-diversity indexes (Chao1 richness, Pielou evenness, Simpson and Shannon diversity) showed no significant changes during the 90 days of experiment.

Beta-diversity showed significant dissimilarities after 90 days of incubation between the bacterial communities living on samples presenting clear biodegradability after 90 days of incubation (PHB, PHBHV95, PHBHHx94 and PHBHHx89) as compared to the other PHA types (PHOHHx, PHOHHp and PHHp) (ANOSIM  $R = 0,5271$ ,  $p = 0,005$ ) (Fig. 4.4). Interestingly, such a distinction could not be made after only 15 and 30 days of experiment.

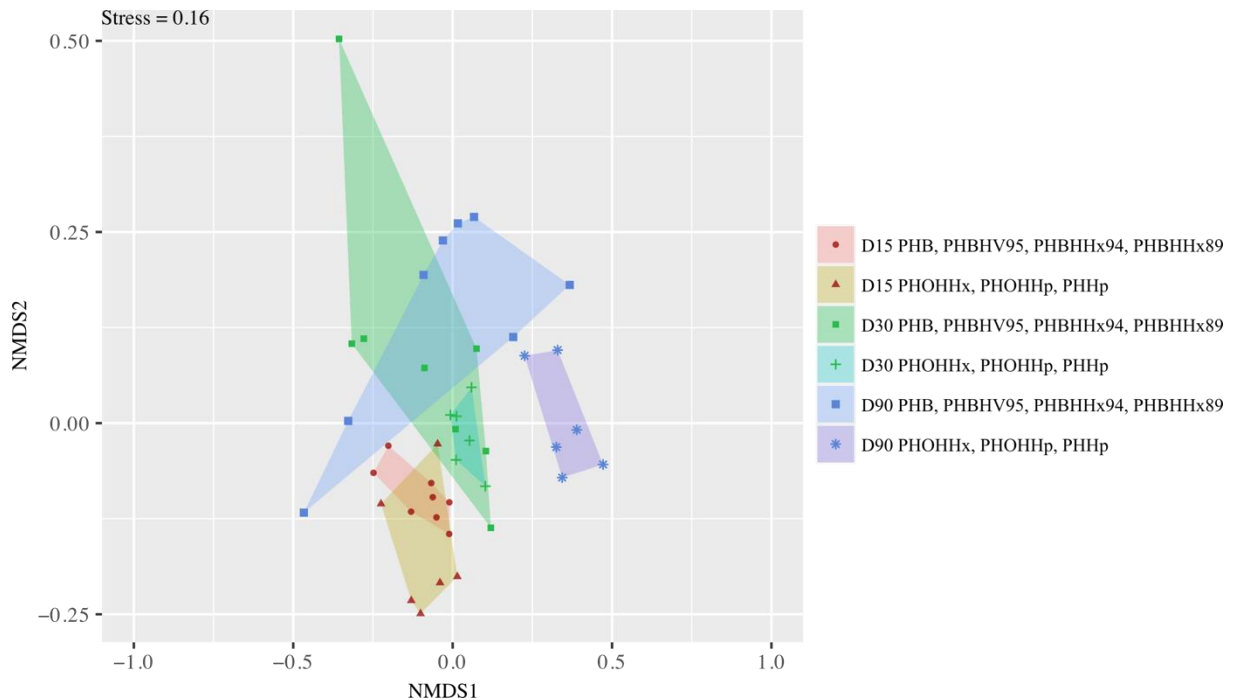


Figure 7. Nonmetric multidimensional scaling plot of the bacterial communities on sampling days. The plot is based on Bray Curtis distance.

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Specific attention was given on the taxonomic composition of samples after 90 days of incubation, by using a SIMPER analysis to identify the ASVs with cumulative contribution of 50% to explain the difference between bacterial communities living on biodegradable PHA (PHB, PHBHV95, PHBHHx94 and PHBHHx89) and on the more recalcitrant *mcl*-PHA (PHOHHx, PHOHHp and PHHp) (Fig. 4.5). Interestingly, *Marinobacter* sp. together with an ASV affiliated to Flavobacteriaceae were abundant in the first biodegradable PHA group and minority on the *mcl*-PHA group, explaining 10.42% and 7.13% of the dissimilarity between the two groups, respectively. Other ASVs belonging to *Flagellimonas* sp., *Maritalea* sp., *Alcanivorax* sp. and to Stapiaceae contributed also between 2.5 and 5% of the dissimilarity between the two groups.

In the opposite, an ASV belonging to Polyangiales was not detected from samples of the first group (PHB, PHBHV, PHBHHx94 and PHBHHx89) but became abundant in the other *mcl*-PHA group and contributed to 10.48% of the dissimilarity between these two groups.

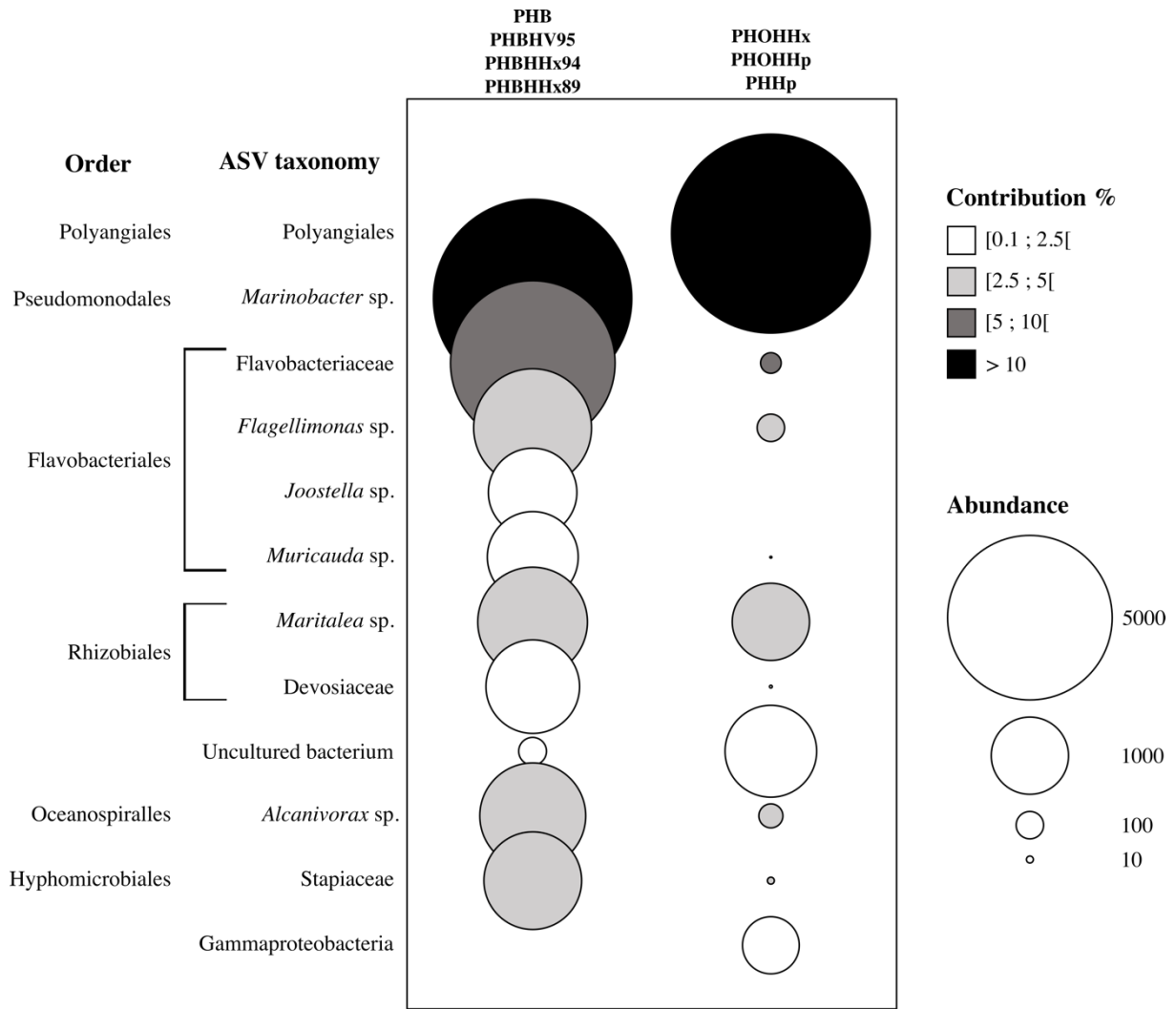


Figure 4.5. Bubble plot of the relative abundance and taxonomy of the ASV contributing to 50% of dissimilarity. PHA were grouped according to the signal of biodegradation recorded after 90 days of incubation: PHB, PHBV95, PHBHHx94 and PHBHHx89 vs PHOHHx, PHOHHp and PHHp. Bubbles are sized according to the abundance of the ASV and colored according to their contribution to dissimilarity.

## 4. Discussion

### 4.1. PHA with different monomer composition present various biodegradability in marine conditions

PHA-based plastics are presented as biodegradable in various environments, including marine conditions (Paul-Pont et al., 2023). However, PHA is a huge family of polymers that display very broad physical and chemical characteristics. Most PHA biodegradation studies in the marine environment were conducted on PHB (Nakayama et al., 2019), PHBHV (Thellen et al., 2008) and also on PHBHHx (Sashiwa et al., 2018), but to our knowledge, no study used PHA without HB monomers so far.

Proof of PHA degradation in seawater was previously given by measuring weight loss (Sridewi et al., 2006; Voinova et al., 2008), changes in physical properties (tensile strength, molecular mass, polydispersity, crystallinity) (Doi et al., 1992; Volova et al., 2010) or macro-deformations (cracks, roughness, scratches, holes) (Deroiné et al., 2015). If they give information on the deterioration (biotic or abiotic) of the material, such methodologies are not sufficient to give proof of complete biodegradation. Biomineralization is generally considered as the only stage capable of indicating the material's complete biodegradation, that must be estimated through respirometry methods (Silva et al., 2023). A few studies conducted biodegradation experiments using either O<sub>2</sub> consumption or CO<sub>2</sub> production on PHB (López-Ibáñez & Beiras, 2022), PHBHV (Komiyama et al., 2021) or PHBHHx (Wang et al., 2018). However, comparison between studies is sometimes difficult, because the biodegradability tests may be influenced by the experimental conditions and the methods used. For the first time, our study tested the biodegradability of PHA with different *scl*- and *mcl*-PHA monomers in marine conditions using the same protocols. This was facilitated by the miniaturization of the experimental setup, as previously described (Cheng et al., 2022). A total of 711 tubes were incubated simultaneously in the same conditions, which would not have been possible by following the current standards that generally propose flask volumes of 250 or 300 ml (ISO 19679, ISO 18830, ISO 23977-1, ISO 23977-2). In our study, we combined both O<sub>2</sub> consumption and CO<sub>2</sub> production measurements as a proof of mineralization during a 3-month period. Overall, our results confirmed the previous findings showing rapid signs of degradation for *scl*-PHA (PHB, PHBHV95) and PHBHHx (PHBHHx94 and PHBHHx89) (Sashiwa et al., 2018; Wang et al., 2018). Interestingly, our 3-month experiment was not sufficient to prove the

biodegradability of PHA composed only of *mcl*-PHA monomers (PHOHHx, PHOHHp and PHHp), that may need longer time to biodegrade. Further studies with longer incubation time (up to 24 months, as proposed in ISO 19679, ISO 18830, ISO 23977 and ASTM D7991) are needed to give a more definitive conclusion about *mcl*-PHA biodegradation in seawater. The different patterns of biodegradability between the *scl*- and *mcl*-PHA questioned the importance of the polymer composition but also chemical and physical properties as key drivers of the PHA biodegradation. Previous reviews on PHA biodegradation suggested that high crystallinity may decrease biodegradation capabilities, and that amorphous regions of a polymer may be preferred for microbial degradation (Meereboer et al., 2020; Mukai et al., 1993). However, previous incubation of PHBHV films in freshwater conditions suggested that amorphous and crystalline zones of the polymers were simultaneously biodegraded (Komiyama et al., 2021). In our study, the highest biodegradation signs were found for the most crystalline polymer, the PHB, whereas the amorphous PHHp did not show clear signs of biodegradability in our conditions.

Our results show that among *scl*-, *mcl*-PHA and PHA containing both types of monomers, the highest biodegradation signals were for HB-containing after 90 days of incubation. These results suggest that prior to physical properties, the monomer composition of the polymers has a major influence on PHA biodegradability in marine conditions. The only type of monomer detected by the analysis of oligomers and monomers released in the supernatant after 90 days of incubation was 3-hydroxybutyrate, even for co-polymers samples containing other monomers than HB. Moreover, no signal was recorded in any of the abiotic controls, confirming that the monomers detected in samples are due to biological action rather than abiotic degradation. The presence of such soluble molecules is a good proxy of the PHA hydrolysis under the action of PHA depolymerases. This result allows a better understanding of biodegradation mechanisms in natural conditions and reinforces the hypothesis that the mixed biodegradability observed for the different PHA formula may be more related to whether they contain HB than their intrinsic physico-chemical properties. This hypothesis is in line with the specificity of PHA depolymerases for a given PHA type (Jendrossek & Handrick, 2002) and with the observation that HB monomers are the most widespread products among PHA-producing microorganisms (Suzuki et al., 2021). It has also been shown that *mcl*-PHA depolymerases are globally scarcer in the environment than *scl*-PHA depolymerases (Viljakainen & Hug, 2021).

## 4.2. Tracking the bacterial communities throughout the experiment

Several authors mentioned that one of the drawbacks of current plastic biodegradation standards is the lack of representativity of experimental conditions towards the natural environment, especially when considering the microbial inoculum and its evolution during the biodegradability tests (Harrison et al., 2018; Paul-Pont et al., 2023). Recent works on the microorganisms living on plastics (the ‘plastisphere’) in the environment revealed very abundant and diverse communities, with a clear niche partitioning on plastics as compared to the surrounding seawater bacteria (Dussud et al., 2018b; Zettler et al., 2013). The proposition of using some isolated bacterial strains (a ‘mock’ community, as proposed in ASTM D6691) is therefore not receivable, as it does not represent the bacterial diversity that presents the plastisphere. The use of natural seawater as an inoculum (as proposed in ISO 23977-1 and -2, and ASTM D6691) or even seawater and sediments (as proposed in ISO 19679, ISO 18830 and ASTM D7991-15) is not relevant either, as these communities are far from the natural plastisphere communities (Oberbeckmann et al., 2016; Wright et al., 2021). Moreover, recent works underlined that the plastisphere growing on conventional plastics differed from biodegradable plastics (Dussud et al., 2018a; Odobel et al., 2021). These authors also described that a minimum one-month colonization was a prerequisite for the formation of a mature biofilm. In order to be as close as possible to natural conditions, these studies proposed that the inoculum should come from a mature biofilm growing on the material to be tested and under natural conditions. In our study, we took special attention to select a mature biofilm growing for several months on PHA as test inoculum, in order to mimic what would happen in seawater environment. Soft biofilm detachment was applied on colonized PHA, with no consequence on bacterial activity (as confirmed by  $^3\text{H}$ -leucine incorporation), which allowed an uniform inoculation of  $10^4$  cells  $\text{mL}^{-1}$  on each pre-sterilized polymer types. The use of new PHA samples for the study of biodegradation, instead of using directly the colonized pieces, prevented the introduction of organic matter susceptible to be easily used by the test inoculum rather than the studied polymers. Interestingly, we confirmed the presence of a very diverse community on all PHA types after 15 days of incubation ( $\alpha$ -diversity), that remained stable all along the biodegradation tests. This result reinforces the proof of concept of using miniaturized tubes with a well characterized MM for plastic biodegradability tests (Cheng et al., 2022), since diverse bacterial communities were maintained despite the confinement (as known as the ‘bottle effect’) (Pernthaler & Amann, 2005). Note that the volume of MM used in this study (2 mL of MM in 12mL hermetic tubes) sufficiently supplied the inorganic nutrient consumption and the



oxygen demand during the whole experiment (dissolved oxygen concentrations were above 50  $\mu\text{m.L}^{-1}$  to ensure aerobic conditions).

Another strength of tracking bacterial communities throughout the experiment is to seek for putative bacteria involved in PHA biodegradation. After observing clear signs of biodegradation on HB-containing PHA, we explored the communities at the end of the experiment (3 months), to highlight bacterial taxa that explained the dissimilarity between *scl*-PHA and the more recalcitrant *mcl*-PHA (Bray-Curtis and SIMPER analysis). Putative PHB degraders were dominated by *Marinobacter* sp., contributing of 10.42% the difference between the two PHA types. *Marinobacter* sp. are known hydrocarbonoclast bacteria, often described as members of the plastisphere (Amaral-Zettler et al., 2020; Dussud et al., 2018a), and putative plastic degraders (Delacuvellerie et al., 2019), on conventional plastics but also PHA. In particular, *Marinobacter* sp. NK-1 was isolated from PHB placed on the seafloor in Sagami Bay, and described as a PHB producer and degrader (Kasuya et al., 2000). Moreover, the predicted PHA-depolymerase activity of *Marinobacter algicola* DG893 was confirmed on PHB films (Martínez-Tobón et al., 2018). We also detected *Alcanivorax* sp. as a putative contributor to *scl*-PHA biodegradation (contributing to 3% of the difference between *scl*- and *mcl*-PHA communities). The study of the genome of *Alcanivorax* sp. 24, isolated from a marine plastic debris, showed the presence of an esterase susceptible of hydrolyzing PHB (Zadjelovic et al., 2020). This same strain further showed clear biodegradation ability on PHB and PHBHV (Zadjelovic et al., 2020). Other putative contributors to *scl*-PHA biodegradation were not described in the literature for *scl*-PHA biodegradation (*Flagellimonas* sp.) or not affiliate at sufficient taxonomy level (Flavobacteriaceae and Stapiaceae families) to build links with known species in the literature involved in *scl*-PHA biodegradation. These results pave the way for the description of new putative *scl*-PHA degraders in natural environment.

## 5. Conclusion

In this study, we monitored the biodegradability in seawater of PHA with various chemical composition, using a multidisciplinary experimental design. We show that not all PHA were rapidly biodegradable in marine conditions, with congruent signs of biodegradation for *scl*-PHA and two PHBHHx compared to the more recalcitrant *mcl*-PHA types. We are aware that we chose short biodegradation test period on the basis of a 3-month experiment, and longer period (up to 24 months, as recommended by ISO 23977-1 and -2, ISO 18830, ISO 19679) may be needed for further studies to describe the biodegradability of *mcl*-PHA in marine conditions. We also identified several ASVs (including *Marinobacter* sp. and *Alcanivorax* sp.) with potential role in HB-containing PHA biodegradation, which may be good candidates to further explore the metabolic processes involved in PHA degradation that is currently underexplored. The analysis of samples by NMR spectroscopy and mass spectrometry confirmed the biodegradation for two of the PHA, and could further help to better understand the biodegradation process in natural conditions for understudied materials. Finally, we propose that the PHA monomer composition prior to other physico-chemical parameters (crystallinity, melting temperature, molecular weight and polydispersity) drove the biodegradability of the PHA in marine conditions. We believe that this study is the ground for a better comprehension of the fate of biodegradable polymers in case of release in the environment.

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## *Chapitre 5 : Discussion, perspectives et conclusion de la thèse*

### Discussion et perspectives

Cette dernière partie a pour objectif d'ouvrir une discussion et de proposer des perspectives pour de futurs travaux. Elle est organisée en trois points qui font écho aux trois grandes parties expérimentales de cette thèse.

#### 1. Étude de la plastisphère environnementale dans le continuum fleuve-mer

La mission *Tara* Microplastiques nous a permis de travailler sur des échantillons environnementaux collectés sur 9 des plus grands fleuves européens, représentant un échantillonnage à méso-échelle. Au sein de chaque fleuve, un échantillonnage à l'échelle locale a permis de couvrir un gradient allant des eaux douces vers les eaux marines situées au large de l'estuaire de chacun des fleuves. La force de cet échantillonnage tient à l'utilisation des mêmes protocoles expérimentaux durant les 8 mois de la mission. Si les études décrivant la diversité de la plastisphère sont aujourd'hui relativement nombreuses, elles ne couvrent en général que des zones géographiques restreintes, du fait des contraintes logistiques importantes associées à un échantillonnage à grande échelle (Du et al., 2022). L'inter-comparaison entre les différentes études de la plastisphère n'est pas forcément aisée du fait de la diversité des protocoles utilisés, depuis le protocole d'échantillonnage jusqu'au traitement des données de séquençage (De Tender et al., 2017).

Les analyses réalisées sur les échantillons de la mission *Tara* Microplastiques ont permis de confirmer que les bactéries de la plastisphère sont composées de communautés distinctes des communautés des eaux environnantes, que ce soit en milieu marin ou dans les fleuves. Ces travaux ont permis de montrer que ce résultat, déjà observé dans l'environnement marin (Dussud et al., 2018b; Zettler et al., 2013), est transposable à toutes les zones pélagiques des milieux aquatiques. Nos résultats ont également permis de confirmer l'importance des conditions environnementales sur la diversité et la structure des communautés de la plastisphère. La localisation géographique en lien avec la salinité (gradient de salinité fleuve vs. mer) sont des facteurs influençant significativement les communautés de la plastisphère. La distinction claire entre les communautés des stations marines et des stations fluviales suggère que le transfert de communautés bactériennes des fleuves vers la mer reste limité.

- **Perspective 1 : élargissement des résultats aux communautés eucaryotes**

Les données présentées dans cette partie ne concernent que les communautés bactériennes. Cependant, les communautés de la plastisphère contiennent des organismes variés (Amaral-Zettler et al., 2020). En effet, de nombreuses études sur la plastisphère associées à des plastiques de surface ont reporté la présence d'organismes phototrophes comme les diatomées, parfois dominantes dans les communautés de la plastisphère (Eich et al., 2015). L'activité de ces producteurs primaires est associée à une activité hétérotrophe forte au sein des communautés liées aux débris plastiques. De plus, certaines analyses des communautés de la plastisphère ciblant les organismes eucaryotes ont mis en évidence la présence de champignons, dont le rôle fonctionnel au sein de la plastisphère reste encore à déterminer. L'étude des communautés eucaryotes associées à du PE et du PS en eaux saumâtres et douces a révélé que les séquences fongiques représentaient 4% des séquences eucaryotes totales (Kettner et al., 2019). Des cas de symbiose ont également été mis en évidence au sein de la plastisphère, entre un organisme cilié et une bactérie ectosymbiotique (Amaral-Zettler et al., 2020). Les débris plastiques abritent donc des communautés complexes, dont le fonctionnement reste sous étudié mais susceptible d'altérer le cycle du carbone océanique.

Les concentrations d'ADN qui ont été extraites pour le séquençage haut débit de l'ADNr16S ont été suffisantes pour réaliser le séquençage de la région hypervariable V4 de l'ADNr 18S et la région ITS2 qui permettront de décrire les communautés eucaryotes attachées aux plastiques échantillonnés lors de la mission *Tara* Microplastique. Une collaboration avec le Laboratoire Universitaire de Biodiversité et d'Écologie Microbienne de l'Université de Brest (Gaëtan Burgaud) va nous permettre d'étudier les communautés fongiques associées aux mêmes déchets plastiques, et de rendre compte des liens avec les communautés bactériennes au sein de la plastisphère. Ces analyses sont actuellement en cours et devraient faire l'objet d'un article soumis avant le 15 Décembre 2023 au numéro spécial « Source, fate and effects of plastic litters in the land-sea continuum (Acronym-PLASTRANSFER) » 2023 du journal *Environmental Science and Pollution Research* (Editeurs en chef : Alexandra Ter Halle et Jean-François Ghiglione).

- **Perspective 2 : suivi des taxons potentiellement pathogènes de la plastisphère dans le continuum fleuve-mer.**

Les stations d'eau douce des fleuves ont été choisies en amont et en aval de la plus grande ville à proximité des estuaires, afin de voir si l'impact anthropique des grandes villes pouvaient être une source de taxons particuliers, potentiellement pathogènes. En effet, le passage des débris plastiques par les zones urbanisées le long des rivières a été identifié comme une source potentielle d'enrichissement en taxons pathogènes (Li et al., 2021). De plus, les stations de traitement des eaux usées constituent des sources importantes de microplastiques (McCormick et al., 2016). Dans notre étude, les communautés bactériennes associées aux plastiques de ces deux stations n'ont pas été montrées comme significativement différentes, et la présence de groupes contenant des microorganismes reconnus comme des agents pathogènes n'a pas été flagrante. D'autre part, les quelques ASV identifiées comme communes entre l'eau douce et l'eau de mer le long de la Seine ne contenaient pas de taxons potentiellement pathogènes, dans les limites de détection de la méthode de métabarcoding 16S rRNA utilisée. Plusieurs études ont précédemment observé la présence de microorganismes potentiellement pathogènes sur des microplastiques flottants prélevés en mer (Frère et al., 2018), ainsi qu'en rivière (A. McCormick et al., 2014). Ces potentiels pathogènes sont par exemple du groupe *Vibrio splendidus*, pouvant présenter une pathogénicité pour les huîtres, détectés sur 77% des microplastiques prélevés dans la baie de Brest (Frère et al., 2018), ou encore *Escherichia coli*, détectés sur des microplastiques flottants dans la baie Guanabara, et peu abondants dans l'eau environnante (Silva et al., 2019). Ces taxons potentiellement pathogènes représentent donc un risque pour les organismes aquatiques, pouvant engendrer des dégâts écologiques et économiques, ainsi que pour l'Homme. En parallèle, le rôle éventuel de ces microplastiques dans la dispersion de virus entériques humains, connus pour une stabilité importante en milieu marin, n'a, à notre connaissance, pas encore été évalué. Si la présence de microorganismes potentiellement pathogènes a été observée sur de nombreux échantillons de microplastiques en milieu aquatique, la compréhension des facteurs favorisant leur attachement sur les plastiques (colonisateur primaire) ou au sein du biofilm mature de la plastisphère (colonisateur secondaire), ainsi que leur persistance potentielle sous forme viable et infectieuse restent largement sous étudiées. Ces informations sont pourtant cruciales pour évaluer le risque que représentent les microplastiques comme vecteurs d'agents pathogènes.

## Chapitre 5

Il est important de noter que la méthode d'analyse utilisée dans cette étude limite les conclusions que l'on peut tirer sur la présence de bactéries pathogènes. Le séquençage « short read » sur une partie restreinte de l'ADNr 16S permet difficilement une assignation précise à l'espèce. Une des perspectives de ce travail consisterait en un couplage entre des analyses de séquençage haut débit de l'ARNr16S à des analyses en PCR (qPCR et/ou ddPCR) (Frère et al., 2018; Silva et al., 2019), ciblant certains microorganismes ou gènes d'intérêt, afin de mieux appréhender le risque associé à leur détection sur les microplastiques flottants (Tableau 5.1), voire en la détection de plasmide de virulence présents dans les ADNx extraits (Schikorski et al., 2013). La même approche pourrait également être employée sur les microorganismes eucaryotes. D'autre part, les outils de biologie moléculaire classiquement utilisé *in situ* pour détecter la présence de pathogènes sur des microplastiques collectés en mer (séquençage haut débit, qPCR, ddPCR) ne permettent pas de prédire le potentiel pathogénique réel, car celui-ci dépend souvent de l'état biologique de ces micro-organismes dans la plastisphère et de facteurs de virulence qui peuvent être présents ou non au sein d'une même espèce (Sakib et al. 2018). Le développement d'une approche expérimentale serait ainsi nécessaire pour évaluer à la fois la transmission et la pathogénicité d'agents infectieux associés aux microplastiques vers des espèces hôtes sensibles. Enfin, cette voie de transmission nécessiterait d'être comparée aux processus de transmission non plastiques (pathogènes libres dans l'eau ou associés à des particules organiques ou inorganiques) afin d'évaluer si elle offre de plus grandes possibilités de transmission d'agents pathogènes.

Tableau 5.1. Exemples de pathogènes humains et marins à cibler dans les études de plastisphère. Ces agents pathogènes sont ceux proposés dans le projet PATHOPLASTIC, coordonné par Ika Paul-Pont (CNRS Brest) qui sera soumis à l'ANR 2024.

Règne	Pathogènes	Hôtes	Matrice	Gène cible	Référence
Virus	Norovirus	Humain	ARN	VP1	Loisy et al. 2005
	Virus de l'hépatite A	humain	ARN	5'NC	Costafreda et al. 2006
	Ostreid herpesvirus	Huitre ( <i>Crassostrea gigas</i> )	ADN	Region B	Paul-Pont et al. 2013
Bactéries	<i>Vibrio vulnificus</i>	Humain	ADN	Vv	Cantet et al. 2013
	<i>Vibrio cholerae non O1 non O139</i>	Humain	ADN	1GS, ctxA et ctxB	Sharma & Chaturvedi, 2006
	<i>Vibrio parahaemolyticus</i>	Humain	ADN	pR72H, tdh et trh	Byappanahalli et al. 2015
	<i>Vibrio crassostreae</i>	Huitre ( <i>Crassostrea gigas</i> )	ADN	16S	Paul-Pont (pers.Com.)
	<i>Vibrio splendidus</i>	Moule ( <i>Mytilus edulis</i> et <i>M. galloprovincialis</i> )	ADN	16S	Saulnier et al. 2017
Eucaryotes	<i>Bonamia sp (B. ostreae et B. exitiosa)</i>	Huitre ( <i>Ostrea edulis</i> )	ADN	18S	Canier et al. 2020
	<i>Marteilia refringens</i>	Huitre ( <i>Ostrea edulis</i> ), Moule ( <i>Mytilus edulis</i> et <i>M. galloprovincialis</i> )	ADN	18S	Canier et al. 2020
	<i>Haplosporidium costale</i>	Huitre ( <i>Crassostrea gigas</i> )	ADN	18S	Arzul (pers. Com.)
	<i>Haplosporidium nelsoni</i>	Huitre ( <i>Crassostrea gigas</i> )	ADN	18S	Arzul (pers. Com.)
	<i>Perkinsus olseni</i>	Palourde ( <i>Ruditapes philippinarum</i> et <i>R. decussatus</i> )	ADN	ITS	Itoiz et al (2021)
	<i>Perkinsus chesapeakei</i>	Palourde ( <i>Ruditapes philippinarum</i> et <i>R. decussatus</i> )	ADN	ITS	Itoiz et al (2022)

- **Perspective 3 : variation temporelle de la plastisphère dans le continuum fleuve-mer.**

La campagne *Tara* Microplastiques a permis d'atteindre une dimension à l'échelle Européenne, en comparant 9 des plus grands fleuves Européens. Néanmoins, les fleuves sont des écosystèmes particulièrement dynamiques, soumis à de grandes variations saisonnières et à des événements extrêmes, tels que les crues. Pour évaluer les variations temporelles de la plastisphère le long du continuum fleuve-mer, un projet de recherche est en cours sur le fleuve du Rhône. Ce projet, intitulé « Etat des lieux de la pollution plastique dans le Rhône » (acronyme PLASTIC-RHONE), est coordonné par la société Plastic At Sea et financé par la Compagnie Nationale du Rhône (CNR) et l'Agence de l'eau Rhône Méditerranée Corse (AERMC). Des échantillonnages ont été conduits sur 5 stations le long du Rhône, allant de Pyrimont, en aval de la source, jusqu'à Port Saint Louis du Rhône, situé à l'embouchure du fleuve. Les extractions d'ADN des échantillonnages menés en janvier et en mai sur trois années consécutives (2021 à 2023) ont été réalisées et sont en cours de séquençage. Ils devraient permettre d'étudier la saisonnalité des communautés bactériennes de la plastisphère le long du continuum Rhône-Méditerranée.

## 2. Étude de la biodégradabilité marine des plastiques en milieu contrôlé

La synthèse bibliographique présentée dans le chapitre 1 a souligné le manque de travaux sur la biodégradabilité des plastiques en mer (Manfra et al., 2021). Nous avons également souligné que les protocoles actuellement disponibles pour évaluer le niveau de biodégradabilité des plastiques dans l'environnement naturel sont insuffisants (Harrison et al., 2018), et les mécanismes associés encore peu investigués en conditions naturelles. Dans cette thèse, l'utilisation d'un modèle miniaturisé (tubes de 12 mL) pour l'étude de la biodégradabilité a permis de tester de nombreuses conditions expérimentales (plus de 2000 tubes), ce qui n'aurait pu être faisable avec des flacons plus volumineux. A l'issue de cette étude, nous proposons que l'inoculum utilisé pour tester la biodégradabilité d'un plastique soit issu d'un biofilm mature et spécifique, obtenu par colonisation de la matière testée en milieu naturel. De plus, les nutriments sont montrés comme étant un paramètre limitant pour la biodégradabilité des plastiques en milieu marin. Cette limitation a déjà été démontrée pour d'autres substrats, comme les hydrocarbures (Sauret et al., 2016). Nous recommandons donc l'utilisation d'un milieu synthétique pour les tests de biodégradabilité en milieu fermé, afin de surmonter la potentielle limite nutritive que représente l'utilisation d'eau de mer naturelle, exempte de

carbone. Les communautés bactériennes associées aux différentes conditions d'incubation seront également analysées, afin de vérifier la réponse des communautés naturelles aux différentes conditions de milieu au cours des 90 jours d'incubation.

- **Perspective 4 : amélioration des protocoles des tests de biodégradabilité des plastiques en mer.**

Les tests normés actuels préconisent l'utilisation de flaconnage volumineux, comme des bouteilles de 250mL, pour évaluer la biodégradabilité aérobie des plastiques en milieu marin, ce qui limite grandement la reproductibilité et le nombre de mesures réalisées lors du test. Si les travaux de cette thèse ont démontré la possibilité de miniaturisation des tests dans des volumes de 12 mL, la concordance des réponses de biodégradabilité obtenues par les tests miniaturisés et les tests normés reste à étudier. Une expérience de biodégradation préparée dans le cadre de ma thèse et réalisée par les équipes de Plastic At Sea est en cours d'analyse afin de comparer le modèle miniaturisé présenté ici aux conditionnements plus gros utilisés dans le cadre des normes. De plus, le suivi de la consommation d'oxygène dans le modèle miniaturisé présenté dans cette thèse est réalisé dans le milieu de culture, par la mesure de l'oxygène dissous dans le surnageant. En revanche, les méthodes proposées par les normes reposent sur des mesures de pression dans l'espace de tête pour le suivi de la consommation d'oxygène, ou par titration du dioxyde de carbone piégé dans l'espace de tête. Pour répondre à cette question, cette expérience a été conduite en suivant la consommation d'oxygène à la fois dans le milieu aqueux et dans l'espace de tête. Enfin, une comparaison de différents systèmes de mesure d'oxygène a été entreprise dans la même expérience entre les sondes Presens® et le système Oxitop® (VWR), classiquement utilisé pour évaluer la charge organique des cours d'eau ou dans les stations de traitement des eaux usées (aujourd'hui préconisé pour le suivi par respirométrie de la biodégradation d'un plastique). Ces données permettront de répondre à plusieurs questions :

- Quel est l'impact de la miniaturisation sur l'activité de biodégradation de l'inoculum expérimental ?
- Le suivi de la consommation d'oxygène par mesure dans le milieu de culture est-il robuste compte tenu des équilibres avec l'espace de tête ?
- Les systèmes de mesures Presens® et Oxitop® sont-ils concordants ?



- **Perspective 5 : comparaison de la forme des matériaux testés (sous forme de poudre, de film, de coupon ou de produits commercialisés)**

L'expérience en cours décrite ci-dessus devrait permettre de fournir des éléments techniques pour l'établissement de nouveaux protocoles normatifs sur les plastiques biodégradables en mer. Il est important de noter que ces résultats ne seront pas basés sur des objets plastiques mis dans le commerce, mais sur des films de polymères. La transformation d'un polymère pour la manufacture d'un objet plastique peut faire varier sa biodégradabilité (Lambert & Wagner, 2017). De plus, la fabrication des objets en plastiques implique l'ajout d'additifs, qui leur confèrent des propriétés adéquates selon l'application visée, *e.g.*, des colorants, des anti-oxydants, ou encore des retardateurs de flamme (Leistenschneider et al., 2023). Ces additifs peuvent également être une barrière à la biodégradabilité d'un plastique, et ils devront être pris en compte dans l'établissement de nouveaux protocoles normatifs (Polman et al., 2021).

Les normes actuelles préconisent l'utilisation de plastique sous forme de poudre pour les tests de biodégradation, ce qui n'est pas représentatif de l'objet final mis dans le commerce. En effet, la biodégradabilité des plastiques étant généralement un phénomène de surface, le temps de biodégradation d'un objet en plastique sera certainement dépendant de son épaisseur et de sa forme. Décrire un polymère comme biodégradable à 60% en 6 mois par exemple n'est donc pas représentatif de tous les objets qui seront réalisés avec ce polymère, comme le sous-entendent plusieurs utilisateurs. Nous soulignons ici l'importance de tenir compte de la forme des produits finaux (sous forme de poudre, de film, de coupon ou de produits commercialisés) pour relativiser les tests de biodégradation des plastiques en mer.

- **Perspective 6 : améliorer l'évaluation du taux de biodégradation des plastiques en mer.**

Les protocoles normatifs méthodologiques actuels visent à quantifier le niveau de biodégradation d'un matériau plastique en milieu marin. Ce calcul est basé sur la mesure de minéralisation du plastique, à partir de la production de CO<sub>2</sub> (équations A) ou de la consommation d'O<sub>2</sub> (équations B) selon la méthode utilisée.

**Équations A (ISO 23977-1 :2020)**

$$(1) ThCO_2 = m \times TOC \times \frac{44}{12}$$

- (1) Calcul du CO<sub>2</sub> théoriquement produit pour la minéralisation complète de la substance, ThCO<sub>2</sub>, exprimée en mg, avec : m la masse de substance exprimée en mg ; TOC la teneur en carbone organique de la substance divisée par 100, exprimé en % ; 44 et 12 les masses molaires du CO<sub>2</sub> et du carbone respectivement, exprimées en g.mol<sup>-1</sup>.

$$(2) Dt = \frac{CO_2 \text{ produit}}{ThCO_2} \times 100$$

- (2) Calcul du taux de biodégradation, Dt, exprimé en %, avec : CO<sub>2</sub> produit la masse de CO<sub>2</sub> produite, exprimée en mg ; ThCO<sub>2</sub> la masse théorique de CO<sub>2</sub> produite pour la minéralisation complète de la substance.

**Équations B (ISO 23977-2 :2020)**

$$(1) DBO_s = \frac{DBO_t - DBO_b}{\rho}$$

- (1) Calcul de la DBO spécifique, DBO<sub>s</sub>, au temps t, exprimée en milligramme par gramme de matériau d'essai, avec : DBO<sub>t</sub> la DBO des fioles contenant le matériau d'essai au temps t, en milligrammes par litre ; DBO<sub>b</sub> la DBO du blanc au temps t, en milligrammes par litre ; ρ la concentration du matériau d'essai, en milligrammes par litre.

$$(2) DThO = \frac{16[2c + 0,5(h - cl - 3n) + 3s + 2,5p + 0,5na - o]}{Mr}$$

- (2) Calcul de la demande théorique en oxygène, DThO, exprimée en milligrammes par gramme de substance ou milligrammes par milligramme de substance, pour une substance C<sub>c</sub>H<sub>h</sub>Cl<sub>cl</sub>N<sub>n</sub>P<sub>p</sub>Na<sub>na</sub>O<sub>o</sub>, avec : Mr la masse moléculaire relative de la substance en g.mol<sup>-1</sup>

$$(3) Dt = \frac{DBO_s}{DThO} \times 100$$

- (3) Calcul du taux de biodégradation, Dt, au temps t, exprimé en %, avec : DBO<sub>s</sub> la demande biologique en oxygène spécifique et DThO la demande théorique en oxygène.

Une première limite de cette méthode est l'impossibilité de quantifier le carbone réellement disponible à la surface des matériaux testés, qui permettrait de déterminer la valeur théorique par laquelle relativiser la donnée mesurée. De plus, en ce qui concerne la mesure de production de CO<sub>2</sub>, une partie du carbone assimilé est incorporé dans la biomasse microbienne, et non minéralisé sur la durée du test. Il n'existe pas aujourd'hui de protocole pour la mesure de cette biomasse microbienne (Degli Innocenti & Breton, 2020). Des tests ont été réalisés sur les échantillons présentés dans ce travail. Les microorganismes de la plastisphère ont été marqués avec un agent fluorescent intercalant de l'ADN (4',6-diamidino-2-phenylindole, DAPI) puis imagés par microscopie optique à épifluorescence. Si le marquage permet d'obtenir des images exploitables pour les échantillons de PE, les échantillons de cellulose et de PHBV, des indicateurs de biodégradation positifs, présentaient un bruit de fond trop important pour discerner les cellules de manière à les dénombrer. Nous avons également testé une autre méthode classiquement utilisée en écologie microbienne, la cytométrie en flux. Une limite importante de cette méthode est l'efficacité de détachement du biofilm, nécessaire pour la mise en suspension des cellules du biofilm et le passage de l'échantillon en cytométrie en flux. Nous avons pu observer que l'étape de détachement est très variable en fonction du matériau considéré, du degré de maturation du biofilm et du développement des exopolysaccharides. Des travaux menés actuellement par la société Plastic At Sea s'orientent vers un dénombrement par qPCR, avec des premiers résultats qui semblent prometteurs, mais qui demandent d'être confirmés en fonction du type de polymère et du temps de colonisation. Une autre proposition est l'utilisation de polymères radiomarqués, dont le carbone est traçable et quantifiable dans la biomasse microbienne (Zumstein et al., 2019), mais la production de ces substrats est si onéreuse que cette méthode n'est pas envisageable en routine.

- **Perspective 7 : comparaison des mesures d'O<sub>2</sub> et de CO<sub>2</sub>.**

Les expériences présentées dans ce manuscrit ont été réalisées dans le noir afin de prévenir l'activité autotrophe, qui interférerait avec les mesures de production de CO<sub>2</sub> et produirait de la matière organique disponible pour la communauté. Or, la plastisphère marine est composée de nombreux microorganismes phototrophes qui transforment le CO<sub>2</sub> en matière organique en présence de lumière (Amaral-Zettler et al., 2020). De la même manière, les incubations sont faites en milieu synthétique minimum en carbone, afin que le plastique soit la seule source de carbone pouvant être utilisé pour l'activité microbienne, non limitant en nutriments. Dans l'environnement naturel, d'autres sources de carbone plus facilement mobilisables seraient évidemment disponibles. Les niveaux de biodégradabilité peuvent donc

être surestimés dans les tests de biodégradation du fait que le plastique constitue la seule source de carbone disponible. Nous proposons de comparer les tests réalisés en présence et en absence de lumière et, de la même manière, comparer des tests réalisés avec ou sans ajout d'autres sources de carbone, pour encore mieux appréhender les mécanismes en place en conditions naturelles.

- **Perspective 8 : évaluer l'importance de la diversité des inocula.**

Nos résultats ont confirmé l'importance des paramètres environnementaux sur la diversité de la plastisphère (voir chapitre 2). Or les tests réalisés dans le chapitre 3 ont été menés à partir d'incubation de plastiques en mer Méditerranée nord occidentale. Les résultats auraient-ils été différents si les biofilms sélectionnés provenaient d'autres bassins océaniques ? Des résultats de notre équipe (Jacquin et al., en préparation) ont montré que des plastisphères de bassins océaniques différents (mer Méditerranée vs. Océan Pacifique) ne partageaient que très peu de taxons communs. Ce résultat ne va pas dans le sens d'un 'core microbiome' plastique global, mais plutôt de spécificités qui peuvent être dépendantes de contraintes environnementales locales. Pour évaluer la biodégradabilité des plastiques « en milieu pélagique », des tests doivent être mis en place pour comparer les capacités de biodégradation des plastiques de plusieurs zones d'un même bassin ou entre bassin. Conformément aux résultats présentés dans le chapitre 3, le plastique testé pourra être incubé pendant une période d'au moins 1 mois sur différents sites géographiques, puis rapatrié au laboratoire dans les délais les plus brefs pour réaliser les tests de biodégradabilité. De plus, la saisonnalité pourrait être étudiée en répétant ces expériences à des temps de l'année différents.

- **Perspective 9 : dépôt d'une nouvelle norme méthodologique de la biodégradabilité des plastiques en milieu marin.**

Les résultats de cette thèse ont permis de proposer différentes améliorations pour le développement de tests de biodégradabilité pour qu'ils soient plus représentatifs des tests normés actuels. Ces travaux devraient permettre à la société Plastic At Sea de déposer une proposition de norme méthodologique de biodégradabilité des plastiques en milieu marin. Cette société a déjà participé avec le LOMIC au dépôt d'une norme de spécification de la biodégradabilité des plastiques en milieu marin. Néanmoins, cette norme de spécification ne pouvait pas tenir compte des récents résultats de la littérature et de cette thèse, car elle devait s'appuyer sur des normes méthodologiques existantes. Cette norme de spécification s'appuyait sur des tests de production de CO<sub>2</sub>, qui ont été moins utilisés dans cette thèse. Au regard des

résultats encourageant sur la miniaturisation des tests obtenus dans cette thèse, la société Plastic At Sea peut envisager le dépôt d'une norme méthodologique sur la base des tests de consommation d'O<sub>2</sub> incluant les tests miniaturisés qui permettrait ensuite le dépôt d'une nouvelle norme de spécification.

### 3. Biodégradabilité marine des PHA

Le travail présenté dans le quatrième chapitre de cette thèse présente les résultats d'une étude de la biodégradabilité utilisant la miniaturisation décrite précédemment de sept formules de PHA en conditions marines. Les biopolymères de type PHA sont généralement présentés comme biodégradables en mer, bien qu'ils représentent une famille de nombreux polymères ayant des propriétés physiques et chimiques très variées. La majorité des études sur la biodégradation des PHA en milieu marin ne présentent que des résultats de dégradation des matériaux, n'apportant aucune preuve de l'assimilation du carbone organique par les communautés microbiennes. De manière générale, les seules formules de PHA ayant été testées en milieu naturel sont le PHB (Nakayama et al., 2019) et deux copolymères de ce dernier, le PHBHV (Deroiné et al., 2015) et le PHBHHx (Komiya et al., 2021). Notre étude prenant en compte différentes classes de PHA (*scl*- et *mcl*-PHA) a permis de mettre en évidence que tous les PHA ne présentent pas une biodégradabilité rapide en milieu marin. En effet, après 90 jours d'incubation dans nos conditions, seulement 4 des 7 formules de PHA présentent des signes de biodégradation.

Plusieurs travaux suggèrent que la biodégradabilité des plastiques est fortement liée à la cristallinité des matériaux (Eubeler et al., 2009). De précédentes études de biodégradation de ces polymères menées en laboratoire suggéraient que la diminution de la cristallinité entraînait une meilleure biodégradabilité, en lien avec une meilleure affinité enzymatique des PHA dépolymérase sur les zones amorphes (Mukai et al., 1993). Pourtant, dans notre étude, les PHA présentant la plus faible cristallinité n'ont pas présenté de biodégradabilité après 90 jours d'incubation. Nos travaux suggèrent que la biodégradabilité observée des PHA dépend étroitement de sa composition en monomères HB, qui est le monomère le plus répandu. L'analyse des produits de biodégradation nous a permis de n'identifier que ce monomère libéré sous l'action bactérienne dans le milieu, même pour le PHBHV et les deux formules de PHBHHx. Il a été montré que les dPhaZ, responsables de l'hydrolyse de ces polymères dans l'environnement, sont spécifiques de la nature des polymères (Jendrossek & Handrick, 2002). De plus, la répartition des dPhaZ dans les différents environnements naturels semble inégale,

et leur occurrence en milieu marin est plus faible, en particulier celles spécifiques des *mcl*-PHA (Viljakainen & Hug, 2021). Nos travaux suggèrent que la composition en monomère HB des PHA est un facteur de biodégradabilité prépondérant, avant même ses caractéristiques intrinsèques telles que la masse molaire ou la cristallinité. Ces travaux soulignent l'importance de ne pas porter de conclusion hâtive sur des familles de polymères sous prétexte que certains d'entre eux présentent des tests positifs de biodégradabilité en milieu marin et force la vérification systématique de la biodégradation des matières dans des conditions les plus proches des conditions naturelles.

- **Perspective 10 : étudier la biodégradabilité marine des *mcl*-PHA en milieu contrôlé avec un inoculum spécifique.**

Les résultats présentés dans le chapitre 4 de cette thèse ont mis en évidence une différence de biodégradabilité des différentes formes de PHA, suggérant une meilleure biodégradabilité pour les PHA contenant des monomères HB. Cependant, dans cette étude, un seul inoculum bactérien sélectionné sur une PHBHV, *scl*-PHA, a été utilisé pour tous les types de PHA. La spécificité des dPhaZ, responsables de l'hydrolyse de ces polymères dans le milieu, a été démontrée au regard du type de monomère du PHA considéré, *i.e.*, les *scl*- ou *mcl*-PHA (Jendrossek & Handrick, 2002). De plus, l'isolation de souches bactériennes et la caractérisation de leurs activités sur les PHA ont montré que certaines souches n'ont que des dPhaZ spécifiques d'un type de PHA. Ainsi, une étude de la biodégradabilité de 6 formules de PHA a été menée dans le cadre de la thèse de Gabrielle Derippe, en prenant soin de sélectionner un inoculum sur un PHBHV, incubé avec les *scl*-PHA (PHB, PHBHV 6% HV et PHBHV 11% HV) ainsi qu'un inoculum sur un PHO, incubé avec les *mcl*-PHA (PHO, et deux formes de PHN). Après 90 jours d'incubation, les *mcl*-PHA ont montré une faible biodégradabilité, tandis que les *scl*-PHA ont présenté une biodégradabilité supérieure à la cellulose, contrôle positif de biodégradabilité en milieu marin (annexe 2). Ces résultats confirment ceux présentés dans ce travail, et suggèrent que la biodégradabilité des PHA en milieu marin est conditionnée par leur composition en monomères.

- **Perspective 11 : coupler la génomique et la métagénomique pour une meilleure compréhension des mécanismes moléculaires de la biodégradation des PHA en milieu marin.**

Cette thèse avait pour objectif général d'améliorer la représentativité des tests de biodégradation des plastiques en milieu naturel, en focalisant sur les PHA comme modèle d'étude. En revanche, la compréhension des mécanismes moléculaires de la biodégradation des PHA en milieu marin n'a pas été abordée dans ce travail. Des analyses de métagénomique permettraient d'accéder au pool génétique de la plastisphère pour décrire la présence de gènes impliqués dans la biodégradation des PHA. Néanmoins, il serait difficile de distinguer le pool génétique spécifiquement associé à la dégradation des PHA au sein de la plastisphère globale, pour répondre à la question de « qui fait quoi au sein de la plastisphère, et par quels mécanismes ? ». Pour répondre à cette question d'écologie fonctionnelle, des travaux préliminaires menés par Charlène Odobel (post-doctorante au LOMIC) ont permis d'envisager l'utilisation d'un marquage par des isotopes stables du carbone ( $^{13}\text{C}$ -PHA) pour réaliser des analyses de DNA-stable isotope probing (Sauret et al., 2014). Cette technique permet de séparer les communautés fonctionnelles capables d'incorporer les  $^{13}\text{C}$ -PHA dans leur ADN (donc, des dégradeurs) par rapport aux communautés opportunistes (dont l'ADN reste avec l'isotope naturel  $^{12}\text{C}$ ) grâce à la séparation physique des ADN extraits sur un gradient de densité de chlorure de césium, et ainsi de cibler les analyses métagénomiques sur les fractions lourdes d'ADN, marqués au  $^{13}\text{C}$ . Des premiers travaux réalisés en utilisant un  $^{13}\text{C}$ -PHB (*i.e.*, un *scl*-PHA) sont prometteurs et doivent être étendus à des *mcl*-PHA marqué au  $^{13}\text{C}$  dans le cadre de l'ANR PLASTIMAR (2024-2028), coordonnée par Jean-François Ghiglione.

En parallèle, des analyses génomiques sur des cultures pures restent nécessaires pour renforcer les bases de données génétiques relatives aux enzymes impliquées dans la biodégradation des PHA. La base de données DED dédiée aux PHA dépolymérase (Knoll et al., 2009) comprend très peu d'enzymes isolées de bactéries du milieu marin. Des travaux de génomique et transcriptomique menés par le LOMIC en collaboration avec le Génoscope ont montré l'originalité d'une souche marine, *Alteromonas plasticoclasticus*, dans ses mécanismes de biodégradation des PHA en conditions marines (Barbe et al. *soumis*). Le même type d'approche pourra être mené sur d'autres bactéries marines isolées à partir de différents types de polymères présentant des taux de biodégradation variables, incluant les PHA, pour renforcer la liste des gènes impliqués dans la biodégradation des polymères biosourcés par les bactéries

## Chapitre 5

marines. Ces travaux sont indispensables pour pouvoir améliorer les bases de données qui permettront ensuite une meilleure affiliation des métagénomés lors de tests réalisés en milieu naturel.



### Conclusion

Ce travail de thèse est pluridisciplinaire, couplant des outils d'écologie microbienne, de biologie moléculaire, de physiologie et de chimie analytique. Il est basé sur des données environnementales issues de campagne d'échantillonnages de grande envergure (*Tara Microplastiques*), mais également d'analyses en conditions contrôlées. Le dispositif d'aquariologie de recherche de l'Observatoire Océanologique de Banyuls sur mer, permettant la mise en circulation ouverte sur la mer des aquariums brevetés par Plastic At Sea ont largement contribué à la démonstration de l'importance de l'utilisation d'inocula naturels pour les tests de biodégradation en milieu marin. Les collaborations avec les laboratoires de l'Institut de Chimie de Clermont Ferrand, du Génoscope d'Evry et de l'Institut de Recherche Dupuy de Lôme de Lorient ont permis la production de polymères précis, l'étude des communautés associées au plastiques en mer ainsi que la caractérisation des produits de biodégradation. La collaboration entre la société Plastic At Sea et le LOMIC a permis de valider l'utilisation d'un système de test miniaturisé, permettant de répondre au besoin de répliquabilité et de complémentarité des méthodes de chimie et de biologie, aboutissant à une approche originale de mesure de la biodégradabilité des polymères en milieu marin.

En conclusion, ce travail de thèse a permis d'apporter de nouvelles connaissances sur l'écologie microbienne associée aux plastiques en milieu marin et en particulier, à leur biodégradabilité. Nous avons montré que les débris plastiques sont de nouvelles niches écologiques qui abritent des communautés distinctes des communautés bactériennes environnantes dans différents milieux aquatiques (eau douce, eau saumâtre et eau marine). Ces communautés évoluent lors du transfert le long du continuum fleuve-mer sous la pression des changements environnementaux et particulièrement ceux associés au changement de salinité. Ces différences devront être prises en compte pour l'évaluation de la biodégradabilité des plastiques en milieu aquatique. Les tests robustes de biodégradabilité des plastiques en milieu marin sont rares. Il est nécessaire de multiplier les études de biodégradabilité en conditions contrôlées afin de mieux comprendre les mécanismes qui y sont associés, et de pouvoir établir des conditions plus pertinentes au regard du milieu naturel, tout en s'appuyant sur des méthodes d'analyses prenant en compte les récentes avancées de la science dans ce domaine. La normalisation des tests de biodégradabilité marine des plastiques présente de nombreux challenges, du fait de l'originalité de ce polluant ainsi que de la complexité du milieu naturel.

## Chapitre 5

L'établissement d'une norme de spécification est nécessaire pour encadrer le développement de nouveaux matériaux, mais il est important de noter que les tests de biodégradabilité en milieu contrôlé ne présentent que des activités potentielles sur des temps relativement courts. L'importance des facteurs environnementaux sur la structure des communautés de la plastisphère souligne également l'importance de réaliser des tests dans plusieurs conditions environnementales avant d'affirmer sa biodégradabilité dans tous les milieux marins. Finalement, les plastiques biodégradables restent une solution de niche, à ne recommander que pour certains produits dont la fin de vie est prédite dans l'environnement. De nombreux scientifiques s'accordent aujourd'hui sur l'objectif prioritaire de réduction de la production mondiale de plastique, dont les déchets sont encore très mal gérés et continuent d'engendrer une pollution croissante dont aucun écosystème naturel n'est préservé.



*Annexe 1 : La science participative au service de la quantification et du suivi de la pollution macro-, méso- et microplastiques sur les berges et plages françaises : Plastique à la Loupe*

## **Comparison of the macro-, meso- and microplastic pollution in French riverbanks and beaches using citizen science with schoolchildren**

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**Keywords:** pollution • macrolitter • beaches • riverbanks • microplastics • citizen science

## Abstract:

Rivers are the major source of anthropogenic litters entering the ocean, especially plastic debris that accumulate in all ecosystems around the world and pose a risk to the biota. Reliable data on distribution, abundance and types of stranded plastics are needed, especially on riverbanks that have received less attention than beaches. Here, we present the citizen science initiative *Plastique à la loupe* (Plastic under the magnifier), that compares for the first time the distribution of different litter sizes (macrolitter, meso- and microplastics) over 81 riverbanks and 66 beaches sampled in France between 2019 and 2021. A total of 149 classes (3,113 schoolchildren) from middle class to high school collected, sorted and enumerated 55,986 pieces of plastic to provide a baseline of current pollution by stranded debris at the national level. Single-use plastics (mainly food-related items) were very abundant on riverbanks (43%), whereas fragmented debris dominated the macrolitter on beaches (28%). Microplastics were always higher in number compared to mesoplastics and macrolitter, with polystyrene and polyethylene in equivalent proportions on riverbanks while polyethylene dominated on beaches. Tracing the source of plastic items was possible only for a small proportion of the numerous collected items, mainly for identifiable macrolitter and microplastic pellets. This study lays out the foundations for further works using *Plastique à la loupe* citizen science initiative in France and additional comparisons to other studied habitats worldwide, which can be used by scientists and policy-makers for future litter monitoring, prevention and clean-up strategies.

## 1. Introduction

Plastic pollution has been documented in all major ocean basins and a growing number of freshwater and terrestrial environments (Bucci et al., 2020). Despite a growing literature in the last decade, the ultimate fate of plastic debris and its transport mechanisms in terrestrial, freshwater, and marine environments are poorly understood, both on regional and global scales (Zhu et al., 2021). Still, there is a peculiar, several orders of magnitude mismatch between projected litter emissions into the ocean (Jambeck et al., 2015) and global estimates based on field data (van Sebille et al., 2015), indicating hitherto insufficiently accounted sinks such as remote beaches and riverbanks (Bergmann et al., 2017).

The importance of tackling plastic litter worldwide has been globally recognized in the context of the 2030 agenda for sustainable development, adopted by all United Nations Member States in 2015 (see target 14.1 in United Nations, 2015). In the marine environment, plastic litter is one of the 11 descriptors of Good Environmental Status (GES) of the European Marine Strategy Framework Directive (2008/56/EC, MSFD) (Galgani et al., 2013). In freshwater, contamination by plastic litters has not yet been considered as a descriptor of good environmental status, including for example the European Water Framework Directive (2000/60/EC, WFD). This gap could be explained by the lack of data relating the occurrence and associated effects of plastic contamination in freshwater ecosystems (Dris et al., 2015). Several studies recognized that plastics with terrestrial usages are the main sources of marine plastic pollution, either by direct emission from coastal zones (Li et al., 2016) or transport through rivers (Lebreton et al., 2017, Schmidt et al., 2017, Weiss et al., 2021). Riverine plastic transport remains understudied and better understanding of the sources and pathways of plastics in freshwater ecosystems is a prerequisite to develop effective prevention and collection strategies.

Gathering sufficient data for scientific research is challenging, with limited sampling time and human resources involved in classical scientific projects (Zettler et al., 2017). Because marine litters are easily identifiable and their quantification requires relatively little scientific training, it is particularly well suited for engaging citizen scientists to expand our knowledge of the spatial and temporal distribution of marine litter, especially in remote, under-sampled areas (Hidalgo-Ruz and Thiel, 2015). In addition to data provisioning, citizen engagement

serves at the same time as an outreach mechanism to inform and involve the general public on scientific progress (Silvertown 2009). An increasing number of citizen science initiatives exist on plastic litter, mainly focusing on macro- and microplastics washed or deposited on beaches or shorelines (beach litter) in the United States (Barrows et al., 2018, Uhrin et al., 2020), China (Chen et al., 2020), Indonesia (Syakti et al., 2017), United Kingdom (Nelms et al., 2020), Denmark (Syberg et al., 2020), Netherlands and Antilles Islands (Bosker et al., 2017), British Columbia and Canada (Harris et al., 2021), Chili (Hidalgo-Ruz, 2013), Australia (Carbery et al., 2020, van der Velde et al., 2017), Svalbard (Bergmann et al., 2017), and Lofoten Island (Haarr et al., 2020). Other initiatives with focus on floating plastic debris were carried out in the United States (Davis et al., 2015), Sweden (Gewert et al., 2015) and Taiwan (Chiu et al., 2020). Studies on plastic debris on the seafloor were conducted in the United Kingdom (Nel et al., 2020) and across 13 countries in Europe (Lots et al., 2017). Surprisingly, none of these initiatives considered riverbanks despite the need of data on plastic quantification at the source of the pollution. Plastic debris encompass a wide size distribution, from large abandoned and derelict consumer litters (often single-use products) to unrecognizable fragments of meso- (from 25 mm to 5 mm) and microplastics (5 mm to 500  $\mu\text{m}$ ) (Hinata et al., 2017). Several methodologies for monitoring marine litter already exist. Among them, the OSPAR beach litter protocol is one of the most used to monitor macrolitter on beaches (OSPAR, 2020) and it has been adapted to monitor macrolitter on riverbanks (van Emmerik et al., 2020).

This study presents the first citizen science initiative dedicated to the comparison between macro-, meso- and microplastic debris on riverbanks and beaches. *Plastique à la loupe* is also the first initiative conducted in France, engaging nearly 4,500 teenagers and their teachers from 149 schools across the nation collecting and extracting samples since 2019. Here, we focused on assessing the composition, distribution and abundance of plastic debris on riverbanks and beach surveys in France from data collected between 2019 and 2021 (**Fig. 1**). Schoolchildren have used the same scientific protocol as developed during the Tara Microplastic expedition, adapted from the OSPAR protocol (Ghiglione et al., submitted). We ended up with 43,571 macro- and mesoplastic items that were characterized together with 12,415 microplastics for analysis by Fourier transform infrared spectroscopy (FTIR). This study confirms the potential of using citizen science for relevant analysis of macro-, meso- and microplastic pollution on riverbanks and beaches.



## 2. Materials and methods

### 2.1. Study sites

Study sites were first chosen by teachers based on local experience and further validated by the scientific committee. Selection criteria were defined from OSPAR and MSFD recommendations and adapted to the citizen science format in order to guarantee the safety of participants and quality of the data collected (OSPAR, 2020, MSFD TG ML, 2013), including: (1) absence of danger, (2) easy access, (3) presence of deposited litter, (4) absence of cleaning in the 15 days before the sampling, (5) minimum length of 10 m for riverbanks and 50 m for beaches, (6) presence of sand for microplastic sampling. A total of 81 riverbanks and 66 beaches were visited by 149 classes from middle to high school (11 to 18 years old). Sampling sites were spread over the whole French metropolitan territory, in 17 of the 30 academies of the French Ministry of National Education (Fig. A1.1). The classes sampled in the field between September and March 2019-2020 and 2020-2021. Each class was asked to fill a site description form (adapted from the OSPAR beach litter monitoring form; OSPAR, 2010). Information collected includes orientation of the site, sand granulometry, uses (seasonal/annual), accessibility and nature of the site's surroundings (town, village, port, estuary, landfill sites, sewage treatment plant, etc.) as well as the frequency and method of cleaning (if relevant) (Appendix A1.1).

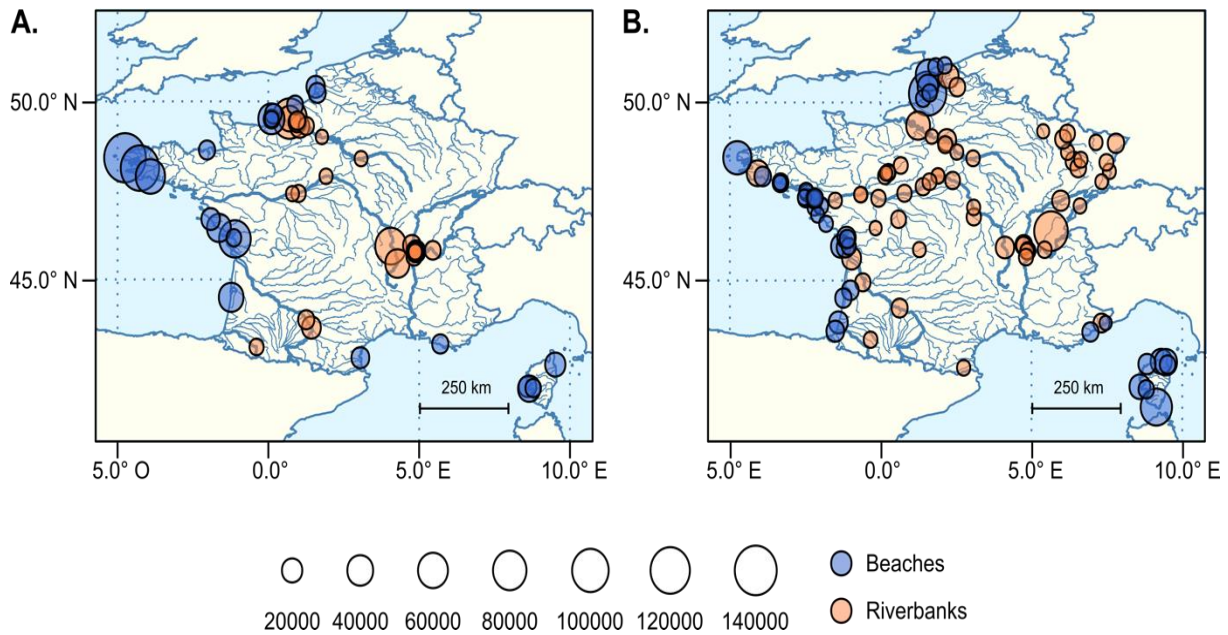


Figure A1.1. Total number of litters per site samples in 2019-2020 (A) and 2020-2021 (B). Beaches are represented with blue dots; riverbanks are represented with orange dots. The number of litters correspond to the sum of macrolitter, meso- and microplastics, normalized for 100 linear meters.

## 2.2. Stranded macrolitters

Stranded macrolitters were surveyed using a method adapted from the OSPAR methodology (OSPAR 2020), with a slight difference depending on whether sites presented wrack lines. On a section with a known length ranging from 10 to 100 m and located from the first wrack line to the back of the riverbank or beach, classes collected all visible litters larger than 2.5 cm (Fig. A1.2 (A)). In the absence of a visible wrack line, sampling was made on the whole width of the riverbank or beach across the section (Fig. A1.2 (B)).

The collected litter was placed in bin bags, except for items that were too large or too heavy which were left on site and noted for later counting. Collected litter was brought back to the classroom, for estimation of the volume (L) and measurement of the mass (kg) of total macrolitter. Each litter was sorted, identified and counted according to the OSPAR beach litter survey data form enriched with additional litter types such as Covid19 crisis related items (disposable mask etc) and reorganized by item uses categories (fishing related, medical etc) to assist the macrolitter identification. Some items including single used plastic items (straws,

lolly sticks etc) and foamed polystyrene fragments were separated for a better focus. The survey data form describing the different categories, types and uses, is presented in supplementary data. A picture was taken once all litters were sorted, and litters were then disposed according to their composition in the appropriate waste disposal center. Results obtained were expressed in volume, mass and number for 100 linear meters of riverbank or beach, 100 meters being the survey unit adopted for OSPAR and MSFD assessments of beach litter (OSPAR, 2020, MSFD TG ML, 2023). These results were obtained by dividing the data obtained by the number of linear meters surveyed and multiplying by 100. Data normalization was done relative to 100 linear meters rather than surfaces in order to avoid the bias induced by tides, which changes the surface of the sampling area depending on the time of the sampling and therefore, the density of items on the beach.

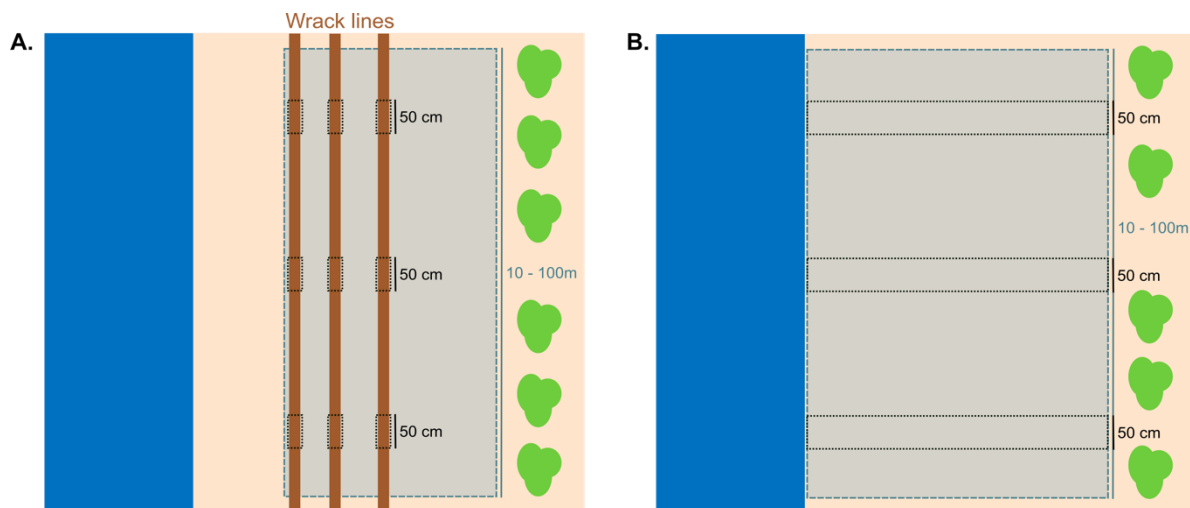


Figure A1.2. Sampling units for macrolitter, meso- and microplastics on riverbanks and beaches in situation with (A) and without (B) wrack lines. Grey shaded areas represent the sampling space of macrolitter and black dashed lines the sampling spaces of meso- and microplastics. Adapted from Vriend et al., 2020.

### 2.3. Stranded meso- and large microplastics

Stranded mesoplastics and large microplastics were assessed using a method proposed at the European level to monitor mesolitter fragments and pellets on the coastline (MSFD TG ML, 2023). The protocol consists in delimiting three 50 cm-wide bands evenly

distributed over the macrolitter section, and sampling along these three bands, perpendicularly to the water line, either on each wrack line (Fig. A1.2 (A)) or on the whole riverbank or beach width in the absence of visible wrack line (Fig. A1.2 (B)). Materials used for sampling were made of metal or glass to prevent any sample contamination. Samplings were done by collecting sand surface using a trowel on the three bands. To limit organic matter and sand collection, meso- and microplastics were extracted directly on site, by flotation in either seawater (for coastal sites) or freshwater (for river sites). Floating particles were recovered with a metallic cooking sieve with a mesh size of 1 mm, then stored in a metal tray and brought back to class for sorting. Once in classroom, samples from the three subsamples were treated separately, by visually sorting organic debris and plastics. Plastics were then sorted according to their size class: mesoplastics [0.5; 2.5 cm] and large microplastics [0.1; 0.5 cm].

The particles were then counted according to their type (fibers, pellets, fragments...) and color based on a list adapted from MSFD microlitter monitoring guidelines (MSFD TG ML, 2023) as presented in the survey data form. Meso- and microplastic lists were the same except for the industrial plastic pellets (IPP) category (also known as nurdle or pellet), which is only included in the microplastic list. Results were expressed in number of particles by 100 linear meters of riverbank or beach, by dividing the number of particles by 1.5 m (corresponding to 3x50 cm-wide bands) and multiplying by 100 m. After sorting, a picture of each sample was taken. 96 microplastics were then selected randomly in the sample and sent to the Observatoire Océanologique de Banyuls sur mer (OOB, France) or to the Cedre (Brest, France), for polymer composition analysis by Fourier transformed infrared spectroscopy (FTIR). FTIR analyses were performed with following parameters: 32 scans, 4 of resolution and large scale from 4000 to 600  $\text{cm}^{-1}$ . Polymer identification was performed using the POSEIDON tool that contains a spectra bank obtained from microplastics collected during the Tara Mediterranean (2014) and Tara Microplastic (2019) expeditions (Kedzierski et al., 2019, Ghiglione et al., in prep).

#### 2.4. Data management and analyses

Survey data forms were gathered for riverbanks and beaches, and raw data were normalized for 100 linear meters. Some sites were excluded from the analysis when the length of the sampling section was not mentioned by schools. Data from riverbanks and beaches were treated separately. For figures 3C, 4, 5 and 6, proportions were calculated from normalized data for each site, and the mean of these proportions was calculated to have information on the dispersion of data. For plastic litter size analysis (Fig. A1.6), only the sites with complete sampling of macro-, meso-, and microplastics on a known section were studied.

Correlations between numbers of macro-, meso-, and microplastics were calculated for riverbanks and beaches separately. Data normality was tested using a Shapiro test. Correlation indexes were calculated using the non-parametric Spearman test.

### 3. Results

#### 3.1. Number, mass and composition of macrolitters on riverbanks and beaches

A total of 81 riverbanks from large and small rivers were sampled in France, together with 66 beaches located along the French coastline either on the coast of the Mediterranean Sea, the Atlantic Ocean or the English Channel. Among all the sites that were studied, only two were not polluted with macrolitter on the sampling zone; however, for one of these two sites, the area surrounding the sampling section was highly polluted, mainly with glass debris. The median number of macrolitters per 100 linear meters collected on riverbanks was approximatively twice lower (median = 232 for 100 linear meters,  $n = 81$ ) than on beaches (median = 443 for 100 linear meters,  $n = 66$ ) (Fig. A1.3 (A)). Mean numbers of macrolitters per 100 linear meters were much higher than median numbers, on both riverbanks and beaches, due to extreme sites ( $1032 \pm 2147$  macrolitter for 100 linear meters and  $1276 \pm 2623$  respectively). The opposite tendency was observed when expressing macrolitters by weight, in kg of litter per 100 linear meters (median = 10.0 kg,  $n = 67$  sites and median = 5.1 kg,  $n = 52$  sites per 100 linear meters on riverbanks and beaches, respectively) (Fig. A1.3 (B)).

Plastic was the most dominant debris type in number of items compared to the total number of collected litters, with a lower proportion on riverbanks than on beaches ( $55.1 \pm 30.4$  and  $80.0 \pm 22.4$  % respectively). Other debris were composed of glass ( $16.3 \pm 20.8$  and  $8.4 \pm 17.8$  % respectively), metals ( $13.0 \pm 15.3$  and  $2.7 \pm 4.5$  % respectively), papers and cardboards ( $5.3 \pm 9.1$  and  $1.2 \pm 2.2$  % respectively), ceramics ( $2.7 \pm 7.9$  % and  $1.4 \pm 3.9$  % respectively), textiles ( $2.7 \pm 5.0$  % and  $1.1 \pm 2.6$  % respectively), wood ( $1.5 \pm 4.5$  % and  $3.0 \pm 6.7$  % respectively) and rubber ( $0.9 \pm 2.7$  and  $2.2 \pm 4.0$  % respectively) (Fig. A1.3 (C)).

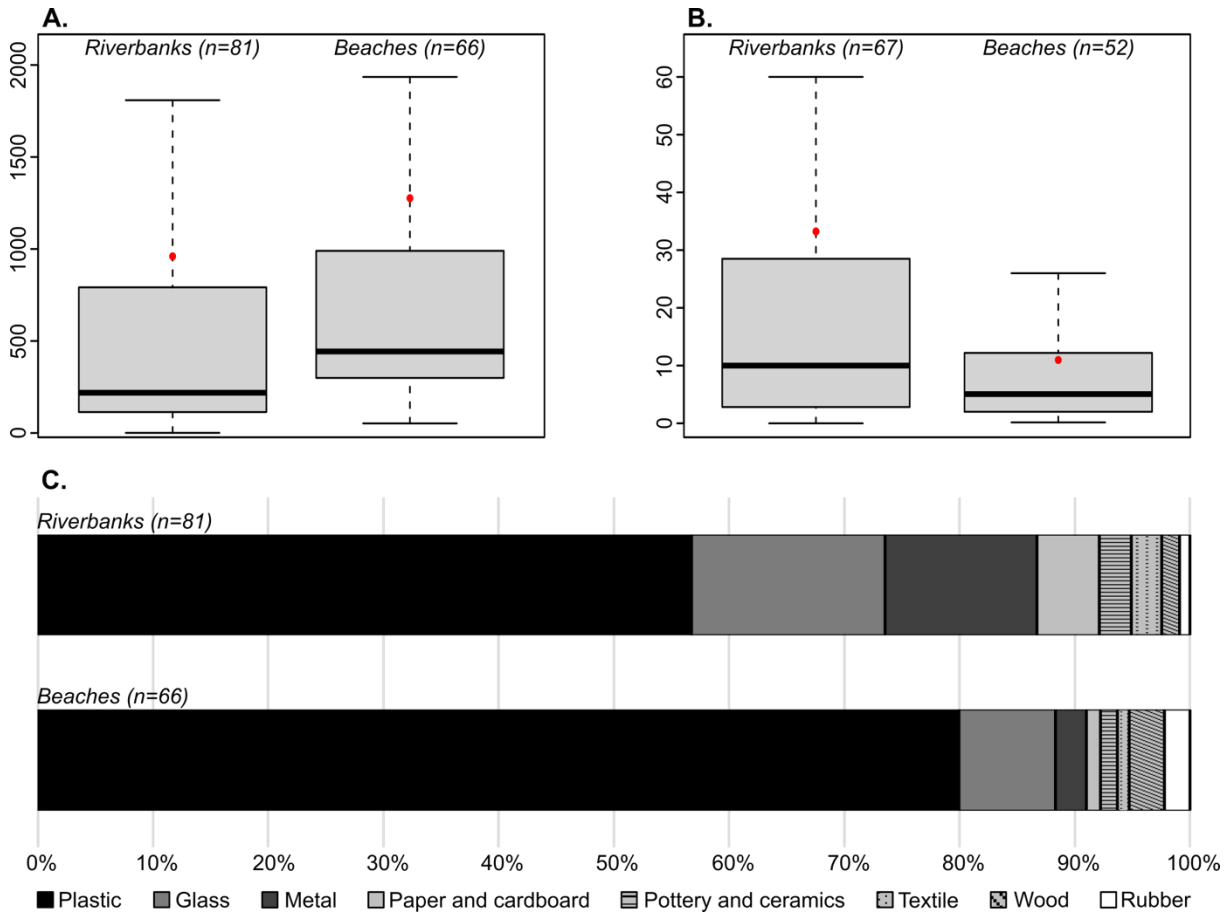


Figure A1.3. Number, mass and proportion of macrolitters on riverbanks and beaches. (A) Number of macrolitters for 100 linear meters. (B) Mass of macrolitters in kg for 100 linear meters. (C) Proportion of types of macrolitters.

### 3.2. Common macroplastic types and composition on riverbanks and beaches

Macroplastic debris found across all riverbanks (n=81 sites) were dominated by single-use disposable plastics ( $43.4 \pm 26.2$  %), whereas it represented only  $27.6 \pm 17.4$  % on beaches (n=66 sites) (Fig. A1.4 (A) and A1.4 (C)). Plastic fragments were the second dominant plastic type collected on riverbanks ( $23.2 \pm 24.8$  %), despite it dominated the plastic debris on beaches ( $28.7 \pm 24.5$  %). Marine activities-related items (fishing, aquaculture and maritime gears) were more present on beaches ( $24.9 \pm 21.7$  %) than on riverbanks ( $4.5 \pm 9.8$  %). Inversely, bags and wrappers were more abundant on riverbanks ( $11.5 \pm 16.5$  %) compared to beaches ( $5.3 \pm 7.9$  %). The same trend was found for sanitary and medical items ( $3.0 \pm 10.1$  % and  $2.7 \pm 12.0$  % respectively on riverbanks and  $<0.6$  % on beaches). A significant number of

## Annexe 1

unclassified items (recognizable items that were not listed in the survey data form) were found on both riverbanks ( $10.5 \pm 16.9\%$ ) and beaches ( $10.8 \pm 17.9\%$ ) (Fig. A1.4 (A) and A1.4 (B)), limiting the description of macrolitters on the studied sites.

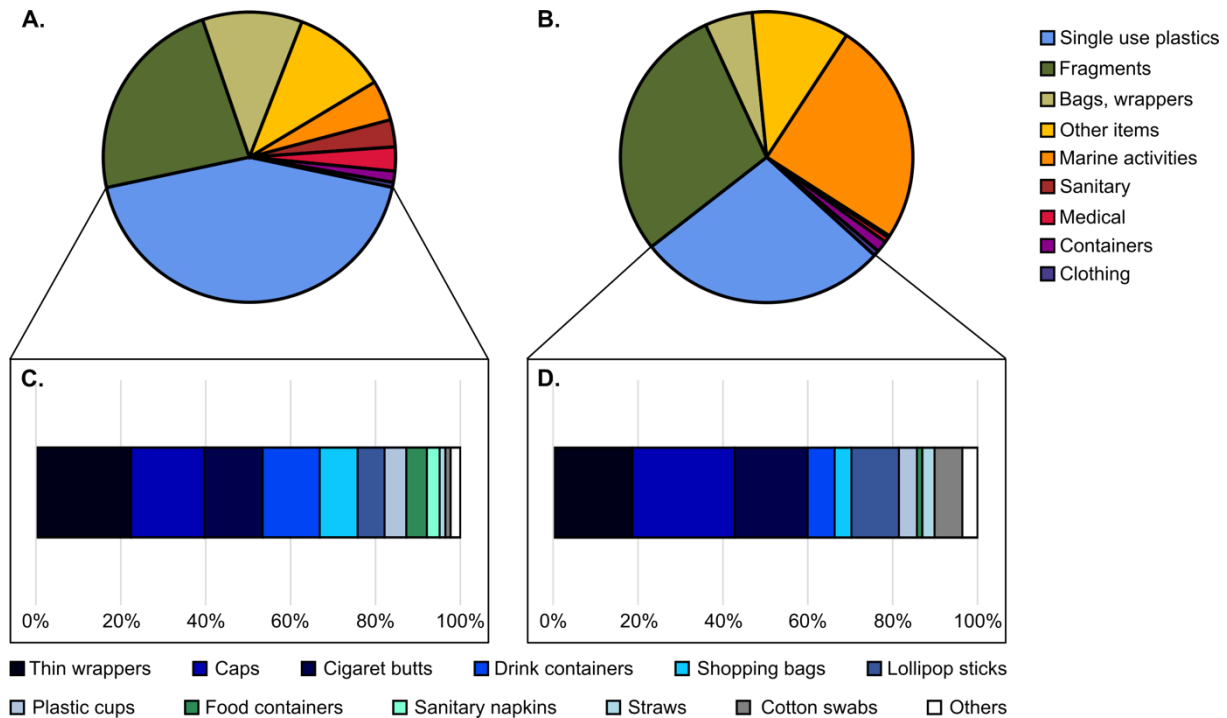


Figure A1.4. Types of macroplastics and single-use plastics on riverbanks and beaches. A and C present riverbanks,  $n=81$ ; B and D represent beaches,  $n=66$ . The mean proportion are represented. The category “other items” in A and B correspond to recognizable items which were not detailed in the survey form. The category “others” in C and D comprises foamed polystyrene food containers and cups, tampons and applicators, disposable plates and cutlery, and stirrers. These former items were found in proportions  $<1\%$ , excepted for foamed polystyrene food containers on beaches.

### 3.3. Focus on single-use disposable plastics on riverbanks and beaches

Single-use disposable plastics represented  $43.3 \pm 26.2\%$  and  $27.6 \pm 17.4\%$  of all macrolitters on riverbanks and beaches respectively. They were dominated by thin wrappers and caps on both riverbanks ( $20.5 \pm 28.1\%$  and  $15.7 \pm 23.1\%$ , respectively) and beaches ( $18.7 \pm 19.8\%$  and  $24.1 \pm 23.2\%$ , respectively) (Fig. A1.4 (C) and A1.4 (D)). Drink containers, shopping bags and food containers were found in higher proportions on riverbanks ( $12.3 \pm 18.8$

%,  $8.2 \pm 19.3\%$  and  $4.5 \pm 9.5$  respectively), despite they were also present on beaches ( $6.4 \pm 15.3\%$ ,  $3.9 \pm 10.9\%$  and  $1.2 \pm 3.9\%$ , respectively). Inversely, cigarette butts, lollipop sticks and cotton swabs were found in higher proportions on beaches ( $17.2 \pm 21.6\%$ ,  $11.2 \pm 13.0\%$  and  $6.5 \pm 15.2\%$ , respectively) compared to riverbanks ( $12.6 \pm 24.0\%$ ,  $5.8 \pm 16.8$  and  $1.1 \pm 4.7\%$ , respectively) (Fig. A1.4 (C) and A1.4 (D)).

### 3.4. Comparison between macro-, meso- and microplastics on riverbanks and beaches

Microplastics represented a major part of the number of plastics found on both riverbanks ( $47.0 \pm 34.2\%$ ,  $n=67$  sites) and beaches ( $45.7 \pm 25.5\%$ ,  $n=51$  sites) (Fig. A1.5). It is noteworthy that the industrial plastic pellets (IPP) itself represented around a quarter of the microplastics found on riverbanks and beaches ( $22.5 \pm 28.1\%$  and  $25.2 \pm 28.1\%$  of sampled microplastics respectively, corresponding to  $13.1 \pm 22.4\%$  and  $13.3 \pm 19.5\%$  of total plastics, respectively), while the rest of microplastics was mostly dominated by fragmented pieces. The second most dominant plastics on riverbanks were fragmented mesoplastics ( $21.7 \pm 25.9\%$  and  $35.9 \pm 22.0\%$  of total plastics on riverbanks and beaches, respectively). On beaches, macroplastics were found in lowest proportion ( $16.8 \pm 24.7\%$ ), whereas it represented  $31.3 \pm 31.4\%$  of total plastics on riverbanks. Here, it is noticeable that a large proportion of macroplastics found on beaches were too fragmented to be identified (representing  $6.6 \pm 13.6\%$  of total plastics) (Fig. A1.5), whereas the other part were recognizable macroplastics as depicted in Fig. A1.4 (A) and Fig. A1.4 (B).



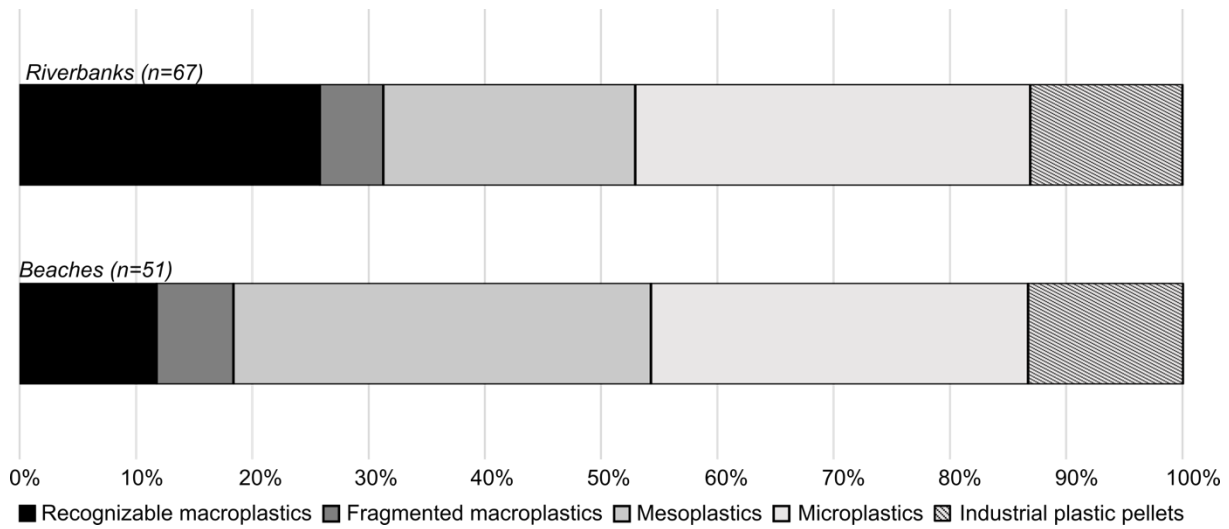
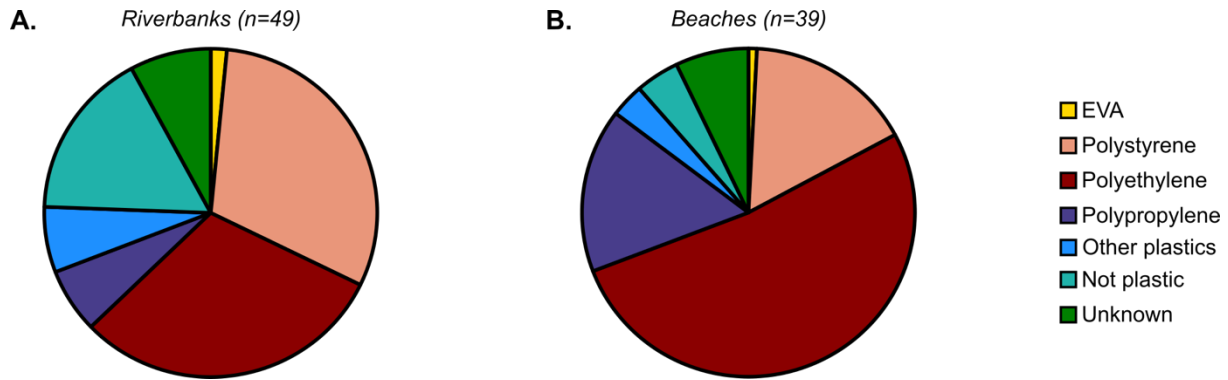


Figure A1.5. Size fraction of plastic litters on riverbanks and beaches. The mean proportions for each size category are represented for all riverbanks on top (n=67) and all beaches on the bottom (n=51).

### 3.5. Chemical composition of microplastics on riverbanks and beaches

Similar polymer types were found for microplastics collected both on riverbanks (n=49 sites) and on beaches (n=39 sites), but with a clear difference in their relative proportions (Fig. A1.6). On riverbanks, most of the microplastics were made of polystyrene and polyethylene ( $30.7 \pm 36.8$  % and  $30.6 \pm 27.7$  % respectively) whereas beaches were clearly dominated by polyethylene ( $52.1 \pm 26.2$  %). On beaches, polystyrene and polypropylene were found in similar proportions ( $16.3 \pm 23.8$  % and  $16.1 \pm 17.5$  % respectively). Among microplastics studied on riverbanks, only  $6.4 \pm 12.7$  % were made of polypropylene. Ethylene-vinyl acetate (EVA) represented only  $1.6 \pm 5.2$  % and  $0.8 \pm 2.4$  % on riverbanks and beaches respectively.

It is noteworthy that a non negligible proportion of sampled micro-particles were natural ones, certainly mistaken for plastics when sorted out, representing the sampling error for microplastics ( $16.5 \pm 21.6$  % and  $4.3 \pm 8.1$  % on riverbanks and beaches respectively). A proportion of microplastics was not identified ( $7.9 \pm 13.6$  % and  $7.1 \pm 13.7$  % on riverbanks and beaches respectively) (Fig. A1.6).



FigureA1.6. Chemical composition of microplastics found on riverbanks (A) and beaches (B). The mean proportions for each chemical composition are represented for riverbanks (n=49) and beaches (n=39).

## 4. Discussion

### 4.1. Adding riverbanks to beaches citizen science monitoring for macrolitter, meso- and microplastics

Monitoring efforts for stranded debris have mostly focused on beaches. Riverbanks are constantly supplied with plastic debris from the rivers, driving the need for more research and management of marine debris. Riverbanks were poorly investigated, with generally very few numbers of studied sites per river (Rech et al., 2014, Bruge et al., 2018). Only one study involved a large number of sites in German rivers, by involving citizen science with schoolchildren (Kiessling et al., 2019).

Citizen science monitoring provides a baseline understanding of debris composition, concentration and sources, and helps inform policies to reduce environmental impacts of plastic debris. Numerous initiatives exist all around the world but this study provides the first citizen science initiative for a comparison between debris found on riverbanks and beaches. This baseline study presents the application of debris citizen science monitoring called “*Plastique à la loupe*” to establish the first large-scale and long-term debris dataset for France, making it accessible to facilitate cost effective research efforts. In this study, conscientious collection by 4,500 schoolchildren from 149 classes removed a total of 48,023 macrolitters on riverbanks (n=81 sites) and beaches (n= 66 sites) in two years. This labor-intensive monitoring effort would not have been feasible by a group of scientists, thus underlying the power of the *Plastique à la*

*loupe* citizen science initiative for a French national survey. Since 2022, this dataset is used as complementary data in national assessments of aquatic litter pollution conducted by Cedre for French authorities in the context of the MSFD or other international monitoring programs.

Another originality of this work was to consider all plastic debris, including visible microplastics (between 5 mm to 500  $\mu\text{m}$ ). Most of the monitoring efforts so far have focused on macro- and mesoplastics, and none of them studied microplastics, despite their importance in the total number of plastic particles and on their impacts on ecosystem health.

### 4.2. Data quality controls

Participants were provided with support documents and visioconferences twice a year (by groups of 10 to 20 classes), allowing to gain confidence in their data-collection skills which was critical. The support document tool kit for teachers included (i) a support guide to explain the general concepts and objectives of the *Plastique à la loupe* initiative together with answer to frequently asked questions (FAQ), (ii) an easy and straightforward protocol guide slightly adapted from the OSPAR beach litter monitoring form (OSPAR, 2010), (iii) a photoguide for the macrolitter identification and (iv) a video guide for *in situ* training. The reliability of the sampling area chosen by the teachers was also verified by the scientists for each class. In addition, at the beginning of the schoolyear, the teams of teachers involved benefited from a one-day formation to the project in the presence of the educational team of the Tara Ocean Foundation.

A main concern regarding citizen-science studies is whether the collected data are reliable and comparable to professional studies. In order to test the reliability of the sampling, sorting and data acquisition, 8 sampling sites (6 on riverbanks and 2 on beaches) were first analyzed by scientists (without removing plastics) before the on-site visit of schoolchildren and comparison showed no or very little difference for mesoplastics and macrolitter (data not shown). No significant difference with results gathered by experienced scientists was found in other citizen science studies performing similar data quality control (Thiel et al., 2013). However, more errors were found by the schoolchildren for the microplastics with non-plastic particles representing around 7 % of the total number of microplastics on riverbanks and beaches. As previously observed, it was found that glass shards for example had been misidentified as small plastic debris (Hidalgo-Ruz and Thiel, 2013). This error can be easily corrected by the FTIR analysis that helps to detect non-plastic particles, which mitigates the impact of such error on the results.

Once the sampling site validation step was performed by scientists, only one site had to be excluded from the analysis because the sampling section length was missing (*i.e.*, data normalization was not possible), thus underlying the high levels of coordination and personal motivation. Here, we underlined the importance of several steps including encouraging schoolchildren and teachers to describe any uncertainties to researchers, data autoevaluation and communication of results as a concluding activity to enhance their commitment to the activity. Globally, this study demonstrates the great potential of using the *Plastique à la Loupe* citizen science initiative with schoolchildren for the distribution and composition of debris from microplastics to macrolitters on riverbank and beaches.

### 4.3. Distribution and composition of all debris on riverbanks and beaches

We observed that around 55% of all debris collected on riverbanks for 100 linear meters were plastic, which was much lower than on beaches (around 80% for 100 linear meters). This result is consistent with another study conducted in Chili showing that plastics were the prevailing litter items and were more frequently found on beaches than at the riversides (Rech et al., 2015). Another national study conducted on German riverbanks found similar proportion of plastics among all debris (51%, including 20% of cigarette butts) (Kiessling et al., 2019). Other studies at local or regional scales found much higher proportions of plastics among all debris in the Adour riverbank (94%) and closed beaches (95%) (Bruge et al., 2018) or in the riverbanks of the Dutch Rhine-Meuse delta (85%) (van Emmerik et al., 2020). Such discrepancy may be explained by local or regional disparities on the amount of other type of debris (glass, metal, ceramics, paper, wood, rubber and textile) and on the modest sampling effort. In our case, a significant percentage of all debris on riverbanks were made of glass and metals, thus explaining the higher weight of all debris on riverbanks compared to beaches (median of 10 kg and 5 kg for 100 linear meters, respectively). These non-buoyant litter items are frequently attributed to non-riverine sources like direct litter dumping (Bravo et al., 2009), by opposition to the high abundance of plastic items that in addition can be transported by rivers and deposited on riverbanks due to their buoyancy and extreme persistence (Derraik et al., 2002; Moore et al., 2008).

#### 4.4. Detailed plastic litters analysis in relation to their origin

Single-use plastics together with packaging (bags and wrappers) dominated most of the riverbanks (around 44.4 %), in a higher proportion than on beaches (around 32.9 %). In particular, food-related items dominated the top 10 single-use plastics. It was dominated by caps (mainly from plastic bottles) and thin wrapper on both riverbanks and beaches. Drink containers, shopping bags and food containers were found in higher proportions on riverbanks, despite they were also present on beaches. Most of these items are typically used by individuals and are classically found on riverbanks and beaches (Lacroix et al., 2022). Either thrown away because of incivility (close to “take-away” restaurants), involuntary loss, or mismanagement (discarded during collection operations or transport by local authorities), they are ending up on city grounds, pushed away by the wind and runoff to rainwater collection systems which take them either straight to the closest river or to the next Waste Water Treatment Plant (WWTP) (Bruge et al., 2018). Cigarette butts, lollipop sticks and cotton swabs were found in higher proportions on beaches compared to riverbanks, mainly due to incivility. Indeed, it has been shown that cigarette butts may not be considered littering by many smokers (Rath et al., 2012). As for the former three items, marine activities-related items (rope, buoys, floats, lures/lines, packaging straps) were much more present on beaches ( $24.9 \pm 21.7$  %) than on riverbanks ( $4.5 \pm 9.8$  %), probably reflecting the importance of higher losses from professional and recreative fishing activities in the marine environment in France. Together with fishing gears lost at sea during storms, discarding damaged nets is a common practice that results in debris accumulation on beaches or seafloor, close to zones of high fishing activity such as the north and south-west of the Gulf of Lion, and in the South Brittany region (Galgani et al., 2000). Here, we observed that around 87% of marine litter originated from land-based uses, which is consistent with classically found at a global scale (GRID-Arendal, 2016; Conservancy, 2017). Together with the numerous broken glass and sharp metal objects, sanitary and medical litters represented a smaller portion of all the riverbanks litters (around 5%), but higher than uncounted on beaches (around 0.8%). They represent potentially dangerous items to human health, together with other items that were found less frequently such as decomposing food leftovers (which could attract disease-carrying animals or harm small children upon accidental ingestion) and litter items containing chemicals (e.g. aerosol cans, batteries, paint containers) (Kiessling et al., 2019). A specific awareness was given in the support guide, in the protocol guide and in the photoguide of *Plastique à la Loupe* initiative, to prevent risks for schoolchildren participants during sampling and sorting.

Litter types classified as “others” represented a significant proportion of all debris ( $10.5\pm 16.9\%$  and  $10.8\pm 17.9\%$  on riverbanks and beaches, respectively). They included car parts, electronics, oil drums, batteries, etc. Attribution to this category is part of the OSPAR data collections scheme (OSPAR, 2020) and we decided to retain these data in our analyses. It diminished our ability to identify the source of litters, and we recognize that there are challenges regarding the source allocations for this category; yet, it gives information on macrolitters fragmentation, since the corresponding items are still recognizable. Photographs could have been used to go deeper in one specific item, but it is time consuming.

Interestingly, macroplastic fragments ( $>2.5\text{ cm}$ ) was the second dominant plastic type collected on riverbanks ( $23.2\pm 24.8\%$ ) despite it dominated the plastic debris on beaches ( $28.7\pm 24.5\%$ ). Fragmented plastic is a direct result of weathering and photodegradation, resulting in surface embrittlement and microcracking, yielding particles that are carried into the closest river or the next WWTP by wind and runoff to rainwater collection systems and also by wind and wave action when transported to beaches (Andrady, 2011). They mainly consist of foam, hard and soft fragments, of which their original item identity remains unknown. Overall, the detailed litter analysis provided more information to identify specific sources of (plastic) litters, and support policy-makers to implement prevention measures targeted at specific items.

### 4.5. Macro-, meso- and microplastics

To date, studies on microplastics mainly concerned ones floating at sea, while land-based studies of the stranded plastic litters on riverbanks and beaches focused more on macro- and mesoplastics (Vriend et al., 2020). Very few data exist on the comparison of all plastic sizes, despite a growing interest on understanding the “plastic cycle” (Hoellein et al., 2021). The *Plastique à la loupe* initiative offers the possibility of tracking the different plastic sizes in a large set of riverbanks and beaches data. Tracing the source of plastics was possible only for a small proportion of the numerous collected items, mainly for identifiable macroplastics ( $25.8\pm 29.7\%$  of all plastic size on riverbanks and  $11.7\pm 19.0\%$  on beaches) and microplastic pellets ( $13.1\pm 22.4\%$  on riverbanks and  $13.3\pm 19.5\%$  on beaches). Most of the plastic items were non identifiable, resulting from the fragmentation of macroplastics into meso- and microplastics by breaking down in smaller sizes after exposure to ultraviolet light or mechanical forces once lost in the environment (Weinstein et al., 2016). Mesoplastics, originating from macroplastics fragmentation, represented a lower proportion of total plastic items on riverbanks

than on beaches ( $21.7 \pm 25.9$  % and  $35.9 \pm 22.0$ % respectively). However, it was difficult to conclude on any relation between the abundance of fragmented plastic litters and the distance to the sea from our current dataset, because of the lack of sufficient number of sites per river. We observed that abundances of meso- and micro-plastics were the most strongly correlated in both riverbanks and beaches.

Numbers of macro- and microplastics, and meso- and microplastics were positively correlated on both riverbanks and beaches. On beaches, there was a higher correlation between the abundances of meso- and microplastics than between macro- and microplastics ( $r_s=0.4$ ,  $p=6.2 \times 10^{-3}$ ;  $r_s=0.7$ ,  $p=7.7 \times 10^{-9}$ ), which is congruent with previous studies (Lee et al., 2013). Numbers of mesoplastics was proposed to serve as a better proxy of microplastic pollution than macroplastics, thus helping easier surveys to identify hot spots of microplastic pollution in large geographical areas with limited resources (Lee et al., 2013). That was not the case on riverbanks, where correlations between meso- and microplastics gave the same values than between macro- and microplastics ( $r_s=0.51$ ,  $p=1.1 \times 10^{-5}$  for both tests).

Microplastics represented a major part of the number of plastics found on both riverbanks ( $47.0 \pm 34.2$  % of all plastic debris) and beaches ( $45.7 \pm 25.5$  %). On riverbanks, a large proportion of microplastics were made of polystyrene (43%), which is congruent with previous results showing that such floating plastics tend to beach sooner and accumulate on riverbanks or lake beaches due to wind effects (Corcoran et al., 2015; Faure et al., 2015). On marine beaches, polyethylene dominated the microplastics (61.1%), as classically found in seawaters (Erni-Cassola et al., 2019). Interestingly, we observed on both riverbanks and beaches that a quarter of the microplastics were made of industrial pellets (primary microplastics, also known as virgin pellets or nurdles, recognized by their regular shape, usually cylindrical or ovoid), which form the feedstock of the plastics industry. These pellets enter the environment when they are spilled accidentally, either on land or at sea. Previous observation mentioned the clear link between the presence of industrial pellets and the vicinity to urban-industrial centers or in relation to accidental leakage during transport (Ryan et al., 2018).

## 5. Conclusion

Previous mathematical model based on estimations of river discharge and mismanaged plastic waste resulted in a total global riverine emission of plastics into the ocean in the range of million metric tons per year (Lebreton et al., 2017, Schmidt et al., 2017). A recent study based on in-depth statistical reanalysis of updated data on microplastics demonstrated that current river flux assessments are overestimated by two to three orders of magnitude (Weiss et al., 2021). Such discrepancy demonstrates the need of more field data to improve the modeling estimation to quantify land-based marine debris transport into the ocean. Monitoring all plastics sizes (macro-, meso- and microplastics) both in riverine and marine environments is a prerequisite for understanding how plastic is transported and where it accumulates, as well as how fragmentation occurs. This study presents the power of the *Plastique à la loupe* initiative that follows the recent recommendation for harmonization of monitoring efforts on riverbanks (Vriend et al., 2020), as previously done for floating macroplastics through the RIMMEL project (González-Fernández and Hanke, 2017). Consistent and harmonized sampling and quantification methodologies are required to gather comparable data from the increasing number of scientific and citizen science initiatives around the world. This study presents the first two years data of the *Plastique à la loupe* initiative in France that is still running for the next coming years with the same protocol and with higher national coverage, both in metropolitan and overseas territorial departments. Schoolchildren removed more than 55,980 pieces of plastic from riverbanks and beaches in two years and prevented the formation of millions of micro- and nanoplastics through degradation over time (Ryan et al., 2020). The increasing number of classes per year (50 in 2019, 150 in 2020, 300 in 2021, 450 in 2022) in the *Plastique à la loupe* initiative will undoubtedly contribute to the incredibly valuable litter collecting by citizens over the world (European Environment Agency, 2018) and to detect meaningful trends in litter volumes over time on riverbanks and beaches. Engagement went beyond riverbanks or beach clean-ups and instead the *Plastique à la loupe* initiative was used as a tool to bridge gaps between communities and scientists, while also raising awareness of the plastic pollution, increasing schoolchildren interest for science and inspiring solutions to act.



### **Acknowledgments**

We thank the commitment of the following institutions, persons and sponsors: Agnès b., Ademe, Casden, Rothschild Foundation, Philgood foundation and the French Ministry of National Education. We are grateful to Guigui PA, VF, JS, JP for insightful comments on the manuscript.

### **Statements and Declarations**

This work was supported by the European Union's Horizon 2020 research and innovation project AtlantECO under grant agreement No 862923, and by the project PLASTRANSFER founded by the Ademe and the French Biodiversity Agency (OFB).

The authors have no relevant financial or non-financial interests to disclose. The authors have no competing interests to declare that are relevant to the content of this article. All authors certify that they have no affiliations with or involvement in any organization or entity with any financial interest or non-financial interest in the subject matter or materials discussed in this manuscript. The authors have no financial or proprietary interests in any material discussed in this article.

**Ethical Approval:** This article follows the Committee on Publication Ethics (COPE) guidelines, including the ethical responsibilities of authors. The authors declare that they obtained study-specific approval by the appropriate ethics committee for research content of this article.

**Consent to Participate:** All authors agreed to participate to the co-authorship. The authors have no competing interests to declare that are relevant to the content of this article.

**Consent to Publish:** All co-authors agreed with the content of this article and they all gave explicit consent to submit. They obtained consent from the responsible authorities at the institute/organization where the work has been carried out, before the work has been submitted.

**Availability of data and materials:** The datasets and materials used and/or analyzed during the current study are available on reasonable request.

### **Author Contributions (CRediT taxonomy)**

**Léna Philip:** Investigation, Methodology, Visualization, Writing - original draft, review & editing; **Maëla Le Picard:** Investigation, Methodology, Visualization, Writing - review & editing; **Edouard Lavergne:** Investigation, Methodology, Visualization, Writing - review & editing; **Pascaline Bourgain:** Conceptualization, Project administration, Resources, Supervision, Writing - review & editing; **Brigitte Sabard:** Conceptualization, Funding

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acquisition, Project administration, Resources, Supervision, Writing - review & editing; **Romain Troublé:** Funding acquisition, Project administration, Supervision, Writing - review & editing; **Anne-Leila Meistertzheim:** Conceptualization, Funding acquisition, Investigation, Methodology, Supervision, Visualization, Writing - review & editing; **Wolfgang Ludwig:** Conceptualization, Methodology, Visualization, Writing - review & editing; **Alexandra ter Halle:** Conceptualization, Methodology, Visualization, Writing - review & editing; **Camille Lacroix:** Conceptualization, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Visualization, Writing - review & editing; **Jean-François Ghiglione:** Conceptualization, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Visualization, Writing - original draft, review & editing.

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## Appendix A1.1. Study site characterization

Year of sampling :		
The sampling site is located on the coastline or on a river bank?		
Specify the name of the river or sea associated with the sampling site?		
The participants	School name :	
	School municipality :	
	Grade level of the class :	
	Number of students :	
	Academy name :	
Study site	Study site name :	
	Study site code :	
	Study site region :	
	Study site municipality :	
Date and time	Collect date :	
	Hour :	
Sea tide	Tidal coefficient :	
	High tide hour :	
Collect	Number of person who participated to the collect :	
	Collect time (in h) :	
Sorting	Number of person who participated to the sorting :	
	Sorting time (in h) :	
Length of the beach or river bank studied section (in m) :		
Back of your study site (example: dunes/forest/road/...) :		
GPS coordinates of the beginning of the studied section :	Latitude (Decimal degrees) :	
	Longitude (Decimal degrees) :	
GPS coordinates of the end of the studied section :	Latitude (Decimal degrees) :	
	Longitude (Decimal degrees) :	
Collect date (day/ month/ year) :		/ /
Direction of prevailing currents : (during the sampling season)		

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Direction of prevailing winds : (during the sampling season)		
How is oriented your study site :		
What type of material covers the study site, in % coverage (e.g. 60% fine sand and 40% rocks)?	Rocks [20mm : 200mm]	
	Gravels [2mm : 20mm]	
	Coarse sands [0,2mm : 2mm]	
	Fine sands [20µm : 2mm]	
	Silt [2µm : 20µmm]	
	Clays [<2µm]	
Are there elements in the sea or in the river that are likely to influence the pollution (e.g. a dike that could trap waste)? If so, which ones?		
What are the 3 main uses of your site? (check the corresponding boxes, with an "x")	Walk (seasonal)	
	Walk (annual)	
	Swimming (seasonal)	
	Swimming (annual)	
	Water activity (seasonal)	
	Water activity (annual)	
	Fishing (seasonal)	
	Fishing (annual)	
	Other (please specify)	
Accessibility of the site by foot (walking distance required from the road): (check the corresponding box, with an "x")	Direct (<200m)	
	200m < d < 1km	
	1 km < d < 5 km	
	> 5km	
What is the nearest urban area?		
What is the size (number of inhabitants) of the nearest agglomeration?		
Distance of the agglomeration from the study site (km)?		
Is there any infrastructure in the immediate vicinity of your site?		
Are there any takeaway businesses in the immediate vicinity of your site?		
What is the distance between your site and the nearest shipping line (in km)?		
The nearest harbour	Distance (in km)	
	Name	
	Type of harbour (fishing/yachting...)	

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	Harbour size	
The nearest estuary (for coastal sites)	Distance (in km)	
	Name	
	Name of the nearest river	
	Orientation of the estuary	
Landfills and wastewater discharges	Is your study site located near a landfill or a wastewater discharge?	
	Distance (in km)	
	Orientation	
Cleaning of the study site	Was a site cleanup performed within 15 days prior to sampling?	
	How often is your study site cleaned?	
	Which method is used (manual or mechanical)?	
	Who is in charge of the cleaning?	
	Is there a tidal tank near your site?	
Special weather events	Could particular events (heavy rain, flooding, storms, etc.) have influenced the quantity of waste on the site?	
	If yes, which ones? (heavy rain, flood, storm,...)	
	If so, how do you interpret this (more or less waste,...)?	
Additional comments:		



*Annexe 2 : Influence de la composition en monomère  
sur la biodégradabilité marine de 6 formules de PHA  
faites à façon*

## **Marine biodegradation of tailor-made polyhydroxyalkanoates (PHA) influenced by the chemical structure and associated bacterial communities**

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**Keywords:** Polyhydroxyalkanoate (PHA), Biosynthesis, Biodegradation, Plasticsphere

## Abstract

Over recent years, biodegradable polymers have been proposed to reduce environmental impacts of plastics for specific applications. Production of polyhydroxyalkanoates (PHA) by using diverse carbon sources provides further benefits for the sustainable development of biodegradable plastics. Here, we present the first study evaluating the impact of physical, chemical and biological factors driving the biodegradability of various tailor-made PHAs in the marine environment. Our multidisciplinary approach demonstrated that the nature of the chemical structure of the polymer (*i.e.*, the side chain size for *short-* vs. *medium-chain* PHA) which are intrinsically correlated to the physico-chemical properties, together with the specificity of the biofilm growing on plastic films (*i.e.* the associated ‘plastisphere’) were the main drivers of the PHA biodegradability in the marine environment. Environmental implication:

Plastic pollution is a critical problem that has the potential for long-lasting impact. While all plastics eventually break down to at least some degree, they can remain in different transition states for extended periods of time, such as microplastics and nanoplastics, that represent different types of hazards. PHA currently occupy a growing portion of the biodegradable plastics market and relevant studies on their biodegradation are needed to address potential environmental problems.

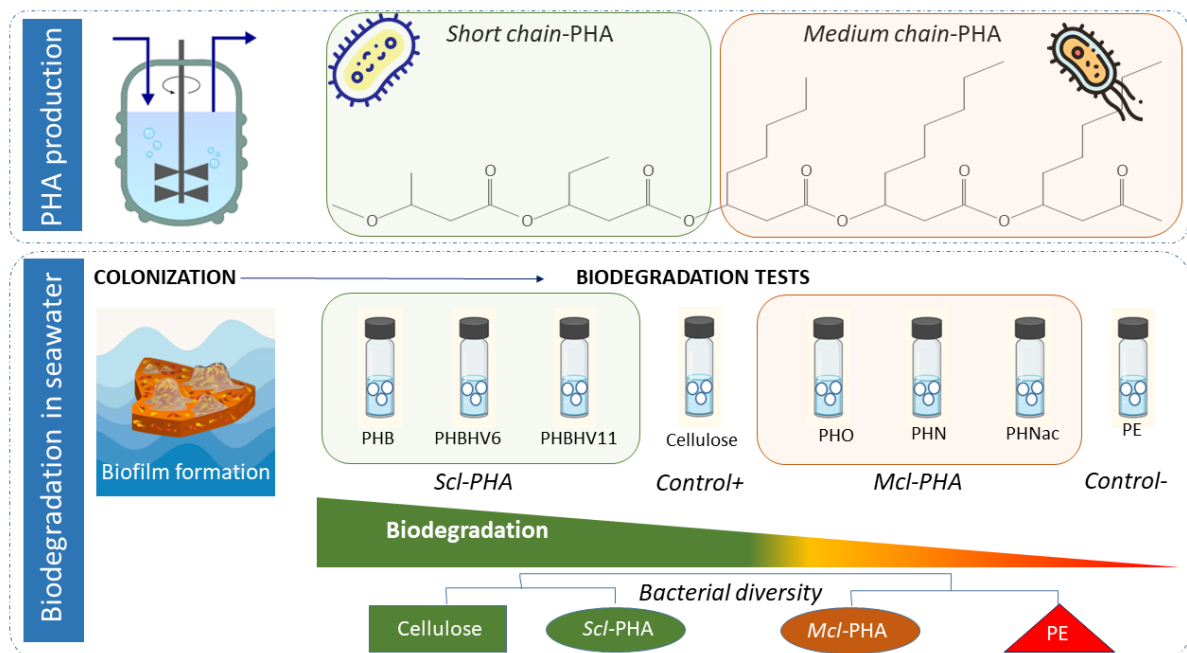


Figure A2.1. Graphical abstract

## 1. Introduction

Plastic pollution is nowadays a global and evident crisis that is of major concern to marine organisms (Deudero and Alomar, 2015), but also contributes to raise global greenhouse gas emissions and climate change (Shen et al., 2020). The marine environment constitutes a large reservoir of mismanaged plastic waste, with 4.8 to 12.7 million metric tons of plastic entering the oceans every year (Jambeck et al., 2015). As a partial solution, it has been proposed to manufacture plastics that would be both bio-based, i.e. made from renewable resources, and biodegradable (totally transformed into biomass and CO<sub>2</sub>) in a given environment (compost, soil, water) over a reasonable amount of time (weeks, months). The biodegradable plastics have been considered relevant for selected applications with respect to their use and end of life (Paul-Pont et al., 2023). Among the bio-sourced and biodegradable polymers, polyhydroxyalkanoates (PHA) are considered as a promising alternative to petroleum-based or non-biodegradable polymers. Mainly but not only from bacterial origin, the PHA constitute a large family and display a wide range of chemical compositions and properties according to the producing strain, the source of carbon used for feeding and the fermentation process. PHA can be divided into two subgroups: short chain-length PHA (*scl*-PHA) composed of 3 to 5 carbon atoms, and medium chain-length PHA (*mcl*-PHA) composed of 6 to 14 carbon atoms. The physico-chemical properties differ between the *scl*-PHA, that are rigid and brittle polymers and the *mcl*-PHA that are usually more rubbery and ductile (Pérez-Rivero and Hernandez-Raquet, 2017). Poly(3-hydroxybutyrate) (PHB) is one of the most widespread and best characterized among the PHA. With high crystallinity (>50%), it is a relatively brittle and stiff polymer (Corre et al., 2012; Koller et al., 2010). Production of the copolymer with valeric acid triggers the incorporation of 3-hydroxyvalerate (HV) and results in the less stiff and brittle copolymer poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBHV), easier to process for commercial applications (Lemechko et al., 2019; Pachekoski et al., 2009). *Mcl*-PHA display properties that could replace elastomers. They are rubbery, soft and showed a lower degree of crystallinity, melting temperature and glass transition (Abe et al., 2012). Despite the recent interest of their properties for biomedical or cosmetic applications, *mcl*-PHA are not produced in industrial quantities and the relationships between their biodegradation and their physico-chemical properties have been poorly explored (Abe et al., 2012).



Biofilm growing on plastic is characterized by very diverse and niche-specific microbial communities called the “plastisphere” (Zettler et al., 2013) that can play a predominant role in plastic degradation (Jacquin et al., 2019). Previous studies showed the great abilities of microbial communities to biodegrade *scl*-PHA in the marine environment (Deroiné et al., 2014; Volant et al., 2022). The microbial communities colonizing commercial PHA (PHBHV) under marine conditions have been studied during semi- and long-term colonization, but no clear relation was made with rate of biodegradation (Dussud et al., 2018a; Odobel et al., 2021). Due to the limited commercial availabilities, no studies have ever reported microbial activity and diversity on *mcl*-PHA, thus resulting in a lack of comparison between the environmental “end of life” of plastics made of *scl*- or *mcl*-PHA families.

In this study, we describe the bacterial production of 6 different tailor-made *scl*- and *mcl*-PHA by *Halomonas sp. SF2003* (Thomas et al., 2019) and *Pseudomonas putida KT2440* (DSM 6125), respectively, together with their physico-chemical characterization. We also analysed their biodegradation by using a two steps protocol including a one-month pre-colonisation step for each PHA group (*scl*- and *mcl*-PHA) and controls (cellulose and Polyethylene, PE) to mimic the growth of marine natural biofilms and another step in minimum medium with plastics as sole carbon source to test biodegradation. We hypothesized that various PHA types and their related chemical and physical properties, as well as the associated natural biofilms, are driving the biodegradation activities in seawater. We used a multidisciplinary approach to produce (bioreactor) and characterize six tailor-made PHA (gas chromatography, steric exclusion chromatography, contact angles, differential scanning calorimetry) and to evaluate the bacterial diversity (16S rDNA Illumina sequencing) associated to the biodegradation activity (oxygen consumption, heterotrophic bacterial production) of each polymer type.

## 2. Materials and methods

### 2.1. Production of *scl*- and *mcl*-PHAs

Pre-cultures of *Halomonas sp. SF2003* for *scl*-PHA production and *Pseudomonas putida* KT2440 for *mcl*-PHA production were both performed in 500 mL Erlenmeyer flasks at 30 °C and 200 rpm, with incubations for 8 h in Zobell media (Thomas et al., 2019) or for 16 h in mineral medium (Maclean et al., 2008), respectively. The preculture was then transferred into

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a 5 L bioreactor (GPC-BIO, MINIPROLAB, France) containing a final volume of 2 L of Zobell medium or mineral medium for *scl*- and *mcl*-PHA production, respectively. Cultivation temperature was 30 °C, pH  $7.0 \pm 0.2$ . Agitation was at a minimum of 400 rpm to maintain a dissolved oxygen concentration above 30 %, as measured with optical dissolved oxygen sensors (Hamilton company, Switzerland).

Prior to PHA accumulation from different carbon sources, *scl*-PHA fermentation started with 10 g.L<sup>-1</sup> of glucose to promote growth. After 12 h, one pulse of 5 g.L<sup>-1</sup> of glucose was added every 4 hours until 24 h for the PHB accumulation. A mix of glucose and valeric acid (50/50, %mol) was continuously dropped in the bioreactor at a rate of 4 mL.h<sup>-1</sup> for 24 h for the poly-(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV6) fermentation. A mix of glucose and valeric acid (70/30, % mol) was continuously dropped at a rate of 6 mL.h<sup>-1</sup> for 24 h for the PHBV11 fermentation. *Mcl*-PHA fermentation also started with glucose implementation to promote high cell density before PHA accumulation, as previously described (Sun et al., 2006). Briefly, this included a first phase of 24 h growth with a feeding strategy based on pulses of exponential quantity of glucose over 7 h (12.5 g.L<sup>-1</sup> of glucose) followed by a linear feeding strategy from 7 to 24 h by adding a pulse of 1 g of glucose additionally to the previous quantity of glucose pulsed every hour. After 24 h, 1 g of fatty acids (octanoic, heptanoic or nonanoic acid to produce a poly(3-hydroxyoctanoate) (PHO), a poly(3-hydroxynonanoate) (PHN) and another PHN called "PHNac", respectively) was added when the dissolved oxygen concentration was above 30% (approximately every 15 minutes).

At the end of the fermentation, bacterial cells were recovered by centrifugation and PHA were separated from bacterial biomass using an incubation with an excess of solvent under stirring in a glass bottle (50 mL of chloroform for approximately 1 g of *scl*-PHA at 60°C overnight and 40 mL of dichloromethane at room temperature overnight for approximately 1 g of *mcl*-PHA). For *scl*-PHA extraction, distilled water (V/V) was added after cooling. The suspension was mixed and centrifuged to recover the organic layer before filtration on glass cotton and casting in a glass Petri dish. *Mcl*-PHA solutions were filtered through a 1.2 µm glass microfiber paper and the concentrated *mcl*-PHA solution was precipitated in cold ethanol (10% v/v) and stored at 6 °C for two days before collection. The casting process consisted of dissolving PHA in their respective solvents, pouring PHA solutions into a glass Petri dish covered with lids that were opened briefly twice a day until constant weight to allow slow solvent evaporation. Films were stored at room temperature and in the dark for three weeks

before any characterizations. Thickness of the films ranged from 80 to 120  $\mu\text{m}$  for all PHA films.

## 2.2. Physico-chemical characterization of the six tailor-made PHA films

Compositions of the produced PHA were determined by gas chromatography (GC). *Scl*-PHA and *mcl*-PHA underwent a propanolysis (Riis and Mai, 1988) and a methanolysis (Furrer et al., 2007), respectively. Briefly, 10 mg of PHA were dissolved in 1 mL of chloroform or dichloromethane and further propyl esterified (*scl*-PHA) with 1 mL of a solution of 1-propanol/37% HCl (8/2, V/V) or methyl esterified (*mcl*-PHA) with 1 mL of MeOH/BF<sub>3</sub> (10% BF<sub>3</sub>, V/V) at 80°C for 20 h. After cooling down, distilled water (V/V) was added and the solutions were vortexed. The organic phase was retrieved, dried on MgSO<sub>4</sub>, filtered on glass cotton and samples were injected on a Perkin Elmer Clarus 480 gas chromatograph equipped with a 30 m x 0.32 mm DB-5 column (HP) with splitless injector and flame ionization detector (FID). Oven temperature, ramp and nitrogen flow were measured according to Riis and Mai (1988) and Furrer et al. (2007).

Molecular weights were measured by steric exclusion chromatography (SEC) using an Agilent Technologies 1200 Infinity II containing an isocratic pump, a column oven at 35 °C and a RI detector. PHA sample separations were performed by two columns PLgel (Mixed-E, 3  $\mu\text{m}$  and Mixed-D, 5  $\mu\text{m}$ ) from Polymer Laboratories for *scl*-PHA and by two columns from Malvern Panalytical technologies (LT4000L, 4 $\mu\text{m}$  and LT5000L, 10  $\mu\text{m}$ ) for *mcl*-PHA and a column guard. About 10 mg of PHA were first dissolved into 2 mL of chloroform (*scl*-PHA) or THF (*mcl*-PHA) then filtered with PTFE filter (0,45  $\mu\text{m}$ ) before a 50  $\mu\text{L}$  injection. The calibration was done with polystyrene standards from Agilent Technologies.

Contact angles were measured on each PHA film using a drop shape analyser from KRÜSS scientific technologies with a 2  $\mu\text{L}$  droplet of water placed on the top surface-air side of the solvent-casted films that have been previously stabilized for 3 weeks at room temperature. More than 5 measurements were carried out for a single sample and the resulting values were averaged.

Thermal properties were determined by differential scanning calorimetry (DSC) using a Mettler-Toledo DSC-882 equipment. About 6 mg of PHA were taken from the cast films of PHA. Particular attention was given so that all the PHA samples experienced the same

thermal history (3 weeks ageing at room temperature in the dark for all solvent-casted films) in order to avoid the induction of different structures, as reported elsewhere (Crétois et al., 2016; Laycock et al., 2014; Xie et al., 2008). PHA samples were equilibrated to 25°C then heated to 190 °C for *scl*-PHA or 80°C for *mcl*-PHA at 10 °C.min<sup>-1</sup> and kept isothermal for 2 min followed by a cooling down to -40 °C for *scl*-PHA or -70 °C for *mcl*-PHA at 10 °C.min<sup>-1</sup>. A second isotherm at -40 °C or -70 °C was kept for 2 min then the sample was heated from -40 to 190 °C or -70 °C to 80 °C at 10 °C.min<sup>-1</sup> according to the PHA type. Melting temperatures and melting enthalpies were measured from the first heating ramp while glass transition temperatures were measured on the second heating ramp and values correspond to the inflection point (Appendix A).

### 2.3. Experimental setup of the biodegradation assay

A two phase stepwise experiment (Appendix B) was designed in order to evaluate the biodegradability of the polymers under marine conditions, as previously described (Cheng et al., 2022). Briefly, the first step consisted of the formation of a mature biofilm on each PHA groups : PHBHV (from Tianan biological materials, China, 40 µm thickness) was used for the colonization of the *scl*-PHA group including PHB, PHBHV6 and PHBHV11; PHO (produced as described above, 120 µm thickness) was used for the colonization for *mcl*-PHA including PHO, PHN and PHNac) together with a positive control (Cellulose filter colonization, CELLU, Whatman 42, thickness 200 µm) and a negative control (Blow Low Density polyethylene colonization, PE, Symphony Environmental Technology, UK, thickness 50µm). Large rectangular pieces of 13.5 cm<sup>2</sup> of each polymer type mentioned above were incubated for one month (5 August to 6 September 2021) in separate 2.4 L aquarium with continuous circulating seawater (flow rate ranged from 8 to 12 mL.min<sup>-1</sup>) pumped in the Banyuls bay (NW Mediterranean Sea). Throughout the experiment, seawater temperature (between 19 °C and 24 °C) and salinity (38.5 g.L<sup>-1</sup>) in the aquarium were similar to seawater from the Banyuls bay. Secondly, individual biofilms were detached from two pieces of each PHA or PE or Cellulose colonized films by three cycles of 1 min vortex and 3 min sonication and resuspended in a 40 mL of carbon-minimum medium called “MM” hereafter (Appendix C). Cell counts was verified by flow cytometry (FACSCanto II flow cytometer, BD Bioscience, San Jose, CA) and adjusted to the exact same concentration 10<sup>5</sup> cells.mL<sup>-1</sup> by dilution of each detached biofilm in MM, in order to ensure comparable inoculum concentration between biodegradation tests. Previous tests using different detached biofilm concentration (from 10<sup>4</sup> to 10<sup>6</sup> cells.mL<sup>-1</sup>) showed that it was an optimal concentration under our experimental conditions, according to the carrying

capacity of the biofilm on plastics (data not shown) and also in accordance to the classical bacterial concentration found in seawater (Pulido-Villena et al., 2012). Three sterile discs of 6 mm<sup>2</sup> diameter each of each PHA solvent-casted films (PHB, PHBHV6, PHBHV11, PHO, PHN and PHNac, total surface of  $60 \pm 0.5$  mm<sup>2</sup>), PE (total surface of 59 mm<sup>2</sup>) and Cellulose (total surface of 64 mm<sup>2</sup>) were then placed in sterile 12 mL Exetainer tubes (Exetainer flat bottom 12 mL, Labco, Lampeter, UK) together with 3 mL of the corresponding inoculum previously detached. The tubes were incubated in the dark at  $18 \pm 0.25^\circ\text{C}$  under agitation at 110 rpm (orbital agitator, Innova® S44i, Eppendorf, Germany) for a 2-month period (called “biotic conditions” hereafter). In addition, similar incubation and sampling procedure were used for abiotic controls, which consisted of triplicate vials containing 3 mL of MM with plastics of the same composition (called “abiotic condition” hereafter). A total of 887 tubes were needed to follow the different parameters detailed below, with triplicates samples taken after 0, 1, 15, 30 and 60 days of incubation.

#### 2.4. Continuous oxygen measurement

During the second step of the experiment, duplicate vials with each plastic type were equipped with an optical fiber luminescent oxygen sensor (SP-PSt5, Presens, Germany) and oxygen concentration was monitored using a small 24-channel reader (SDR SensorDish®, Presens, Germany). Oxygen sensors were placed in the liquid phase to obtain the concentration of dissolved oxygen recorded every hour over 60 days. Oxygen consumption was expressed in  $\mu\text{mol}(\text{O}_2).\text{mm}^2$ . Total surface of the three discs were taken into account:  $3 \times (\text{top and bottom: } \pi \times r^2 \text{ and exposed edges: } 2 \times \pi \times r \times h)$ .

#### 2.5. Heterotrophic Bacterial Production

Heterotrophic Bacterial Production (BP) was measured on triplicate samples for each PHA type at 15, 30 and 60 days by <sup>3</sup>H-leucine incorporation into proteins, as previously described (Dussud et al., 2018a). Briefly, a soft cell detachment pre-treatment based on three cycles of vortex and sonication was first performed. Then, <sup>3</sup>H-leucine (specific activity of 112 Ci.mmol<sup>-1</sup>) was added onto PHA samples (final concentration of 1 nmol.L<sup>-1</sup> after addition of cold leucine). Radioactivity was measured using a Beckman Scintillation Counter (LS 5000CE) after addition of trichloroacetic acid (TCA) 50% and resuspension in a liquid scintillation cocktail (Ultima Gold). An empirical conversion factor of 1.55 ng C.pmol<sup>-1</sup> of incorporated leucine was used to calculate BP (Simon and Azam, 1989). Blanks followed the same protocol but bacterial activity was stopped by the introduction of 50% TCA prior to the addition of the

radioactive mix. BP was expressed in  $\text{ng(C).mm}^2.\text{h}^{-1}$ . Total surface of one disc was taken into account: (top and bottom:  $\pi*r^2$  and exposed edges:  $2*\pi*r*h$ ).

## 2.6. DNA extraction and sequencing

Plastic pieces were sampled at 15, 30 and 60 days and stored at  $-80^\circ\text{C}$  until DNA extraction. One litre seawater was sampled from the control aquarium, then successively filtered through 3- $\mu\text{m}$  and 0.2- $\mu\text{m}$  pore size polycarbonate filters (PC, 47 mm diameter, Nucleopore), and filters were stored at  $-80^\circ\text{C}$ . We also sampled the initial biofilm previously detached after the first one-month colonisation step on PHBHV, PHO, cellulose and PE films, which was stored at  $-80^\circ\text{C}$  after filtration onto 0.2- $\mu\text{m}$  pore size polycarbonate filters (PC, 47 mm diameter, Nucleopore). DNA extractions were realized on all samples using the same phenol-chloroform method, as previously described (Odobel et al., 2021). Primers used for PCR amplification of the 16S V3–V5 region were 515F-Y and 926R (Fuhrman et al., 1989), previously shown as well-suited for marine samples (Parada et al., 2016). Sequencing was performed on Illumina MiSeq by Genoscope (Evry, France), generating 3,060,721 paired sequences in the 29 samples. Raw FASTA files were deposited at EBI under the accession number ERP148254. Sequence analysis was processed using the package DADA2 (Version 1.24.0) into R studio software (R Core Team, 2022, version 4.2.2). A standard pipeline was applied with the following parameters: `trimLeft= c(19,20)`, `truncLen= c(240,240)`, `maxN=0`, `maxEE=c(2,2)`, `truncQ=2`. The sequences were therefore filtered, dereplicated, denoised by removing sample interference and chimeras before merging. Clusters were assigned with the Silva 128 16S rRNA database (Quast et al., 2013) and clusters that did not belong to Bacteria kingdom were removed as well as chloroplast and mitochondrial sequences. The number of sequences per sample was normalized by rarefaction ( $n= 21,324$ ) and a table with 29 samples and 5,053 amplicon sequence variants (ASV) was obtained.

## 2.7. Statistical analysis

All graphical representations and statistical analysis were performed on R studio software (R Core Team, 2022, version 4.2.2) using the packages `ggplot2` (Wickham, 2016), `vegan` (Oksanen et al., 2007) and `phyloseq` (McMurdie and Holmes, 2012) and `PRIMER6` (Clarke and Gorley, 2006). Data were compared with Kruskal-Wallis tests followed by post hoc tests at D60 for oxygen consumption and heterotrophic bacterial production. Sequences were analyzed with the `phyloseq` package. The alpha diversity indexes were calculated and compared with Wilcoxon tests. Differences in microbial community structure among samples were tested

by ANOSIM based on Bray-Curtis distances (PRIMER6 software). The ASVs that contributed most to differentiate microbial community structures between *scl*-PHA vs. PE, *scl*-PHA vs. *mcl*-PHA and *mcl*-PHA vs. PE were tested with a similarity percentage analysis (SIMPER, PRIMER6) (Clarke, 1993).

### 3. Results

#### 3.1. Physico-chemical characterization of the six tailor-made PHA

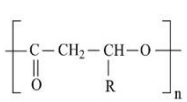
Three fermentation processes in a bioreactor with *Halomonas sp. SF2003* growing on different substrates resulted in the production of three *scl*-PHA: PHB, PHBHV6 and PHBHV11 (Table 1). *Halomonas sp. SF2003* growing on glucose accumulated a homopolymer of PHB composed at 100% of 3-hydroxybutyrate units (HB) (Table 1) with 4.00 carbons per monomer. A mix of glucose and valeric acid (70/30, % mol) resulted in PHBHV11 production composed of 89% of HB and 11% of HV, leading to an average number of 4.11 carbons per monomer. PHBHV6 was produced using another mix of glucose and valeric acid (50/50, % mol) resulting in a copolymer composed of 94% of HB and 6% of HV with an average number of 4.06 carbons per monomer. Fermentations from *Pseudomonas putida KT2440* from two different fatty acids (octanoic and nonanoic acid) and a  $\beta$ -oxidation pathway inhibitor (acrylic acid) (Jiang et al., 2013) led to three *mcl*-PHA: PHO, mainly composed of 3-hydroxyoctanoate (HO) monomer (89%), then 3-hydroxyhexanoate (HHx) and 3-hydroxydecanoate (HD) (5.5% each) for an average number of 7.78 carbons per monomer, PHN was composed of 3-hydroxynonanoate (HN) monomer units (58%) plus HD (24%), 3-hydroxyheptanoate (HHp) (14%) and HO (4%) units with an average number of 8.92 carbons per monomer and PHNac composed of HN (73%), HHp (23%) and HD (2%) with an average number of 8.47 carbons per monomer.

Thermal properties of all PHA were characterized by DSC (Table A2.1). *Scl*-PHA displayed glass transition temperatures between  $-7^{\circ}\text{C}$  and  $4^{\circ}\text{C}$ . *Scl*-PHA with HV units (PHBHV6 and PHBHV11) showed slightly lower peaks of melting temperatures ( $171$  for PHBHV6 and  $172^{\circ}\text{C}$  for PHBHV11) compared to PHB ( $177^{\circ}\text{C}$ ). Melting enthalpies also differ according to the chemical composition. Higher melting enthalpy is observed for PHB ( $\Delta H_m = 89 \text{ J.g}^{-1}$ ) then followed by PHBHV6 ( $\Delta H_m = 60 \text{ J.g}^{-1}$ ) and finally by PHBHV11 ( $\Delta H_m = 30 \text{ J.g}^{-1}$ ) due to the presence of HV units which partially limit the chain crystallisation. Number

## Annexe 2

average molecular mass could not be measured for PHB due to solubility difficulties. PHBHV6 and PHBHV11 displayed  $\overline{M}_n$  of 340 000 and 325 000 g.mol<sup>-1</sup> with a dispersity index (Đ) of 2.8 and 2.9, respectively. Among *scl*-PHA, contact angles with distilled water revealed that PHB is a less hydrophobic polymer with a contact angle of 64 ± 1.6 °, followed by PHBHV11 (74 ± 1 °) and PHBHV6 (76 ± 2 °). On the other hand, *mcl*-PHA displayed lower glass transition temperatures (PHO: -36 °C, PHN: -40 °C and PHNac: -39 °C) and lower melting temperatures (PHO: 57 °C, PHN: 49 °C and PHNac: 52 °C) than all *scl*-PHA. Melting enthalpies is also far lower than *scl*-PHA with a melting enthalpy of 20 J.g<sup>-1</sup> for PHO and PHN and a lower one of 13 J.g<sup>-1</sup> for PHNac.  $\overline{M}_n$  of *mcl*-PHA ranged from 60 000 to 84 000 g.mol<sup>-1</sup> with a Đ ranging from 2.2 to 2.7. High hydrophobicity is displayed by *mcl*-PHA, especially for PHN (90 ± 2 °) then for PHO and PHNac (82 ± 2 ° and 80 ± 1 °, respectively). Overall, physico-chemical properties of the PHA produced differed greatly according to the type of PHA (*scl*- or *mcl*-) while slight but noticeable differences were found within both PHA types.

Tableau A2.1. Composition of the 6 tailor-made PHA and their associated thermal characteristics, average molecular mass and contact angle.

	Chemical composition	Average number of carbons per monomer	$\overline{M}_n$ (g.mol <sup>-1</sup> )	Đ	T <sub>g</sub> (°C)	T <sub>m</sub> (°C)	ΔH <sub>m</sub> (J.g <sup>-1</sup> )	Contact angle (°)
<b>PHB</b>	100% HB	C <sub>4.00</sub>	-	-	4	177	89	64 ± 2
<b>PHBHV6</b>	94% HB 6% HV	C <sub>4.06</sub>	340 000	2.8	-7	171	60	76 ± 2
<b>PHBHV11</b>	89% HB 11% HV	C <sub>4.11</sub>	325 000	2.9	-7	172	30	74 ± 1
<b>PHO</b>	5.5% HX 89% HO 5.5% HD	C <sub>7.78</sub>	84 000	2.2	-36	57	20	82 ± 2
<b>PHN</b>	14% HHp 4% HO 58.1% HN 24% HD	C <sub>8.92</sub>	60 000	2.7	-40	49	20	90 ± 2
<b>PHNac</b>	23% HHp 74% HN 2%HD	C <sub>8.47</sub>	70 000	2.2	-39	52	13	80 ± 1



### 3.2. Biodegradation activities

Several parameters were used to evaluate the biodegradability of the various PHA. Firstly, abiotic controls (PHA, CELLU or PE) did not show signs of contamination and chemical oxygen demand was negligible in our conditions. Secondly, continuous oxygen consumption (Presens sensors) by microorganisms with PHA as sole carbon and energy source showed a clear distinction between *scl*- and *mcl*-PHA. During the first 15 days, oxygen consumption rapidly increased and tended to reach a plateau from 15 to 60 days for the *mcl*-PHA while it kept increasing to a greater extent for *scl*-PHA (Fig. A2.1 (A)). At day 60, PHB (mean =  $4.47 \pm 0.23 \times 10^{-3} \mu\text{mol}(\text{O}_2).\text{mm}^{-2}$ ), PHBHV6 (mean =  $4.37 \pm 0.57 \times 10^{-3} \mu\text{mol}(\text{O}_2).\text{mm}^{-2}$ ), and PHBHV11 ( $3.39 \pm 0.23 \times 10^{-3} \mu\text{mol}(\text{O}_2).\text{mm}^{-2}$ ) presented a much higher oxygen consumption than PHO ( $0.18 \pm 0.02 \times 10^{-3} \mu\text{mol}(\text{O}_2).\text{mm}^{-2}$ ), PHN ( $0.70 \pm 0.11 \times 10^{-3} \mu\text{mol}(\text{O}_2).\text{mm}^{-2}$ ), and PHNac ( $0.31 \pm 0.02 \times 10^{-3} \mu\text{mol}(\text{O}_2).\text{mm}^{-2}$ ). Significant difference was found between *scl*- and *mcl*-PHA biodegradation ( $p < 0.05$ ), as well as with between *scl*- and the PE control ( $0.72 \pm 0.09 \times 10^{-4} \mu\text{mol}(\text{O}_2).\text{mm}^{-2}$ ,  $p < 0.05$ ). No significant difference was found between the oxygen consumption on *scl*-PHA compared to Cellulose ( $2.08 \pm 0.12 \times 10^{-3} \mu\text{mol}(\text{O}_2).\text{mm}^{-2}$ ,  $p > 0.05$ ). Likewise, a kinetic comparison between within *scl*-PHA or within *mcl*-PHA did not show any statistical differences. Among *mcl*-PHA, small but noticeable oxygen consumption was observed for PHN, while PHO and PHNac had a similar trend to the PE negative control.

Thirdly, cell incorporation of  $^3\text{H}$ -leucine into proteins showed similar trends, with significantly higher heterotrophic activities for *scl*-PHA as compared to *mcl*-PHA. Within the first 15 days of incubation in minimum medium, the activities of the biofilms were high and then decreased until day 60 for all the PHA (Fig. A2.1 (B)). At day 60, maximum activities were found for the positive control cellulose ( $2.35 \pm 1.08 \times 10^{-1} \text{ng}(\text{C}).\text{mm}^{-2}.\text{h}^{-1}$ ) and *scl*-PHA, including PHBHV6 ( $2.67 \pm 0.97 \times 10^{-1} \text{ng}(\text{C}).\text{mm}^{-2}.\text{h}^{-1}$ ), PHB ( $2.58 \pm 0.31 \times 10^{-1} \text{ng}(\text{C}).\text{mm}^{-2}.\text{h}^{-1}$ ) and PHBHV11 ( $2.28 \pm 0.85 \times 10^{-1} \text{ng}(\text{C}).\text{mm}^{-2}.\text{h}^{-1}$ ). Much lower bacterial activities were observed for the negative control PE ( $0.95 \pm 0.58 \times 10^{-2} \text{ng}(\text{C}).\text{mm}^{-2}.\text{h}^{-1}$ ) and *mcl*-PHA, including PHN ( $0.77 \pm 0.30 \times 10^{-1} \text{ng}(\text{C}).\text{mm}^{-2}.\text{h}^{-1}$ ), PHNac ( $0.49 \pm 0.20 \times 10^{-1} \text{ng}(\text{C}).\text{mm}^{-2}.\text{h}^{-1}$ ), PHO ( $0.21 \pm 0.10 \times 10^{-1} \text{ng}(\text{C}).\text{mm}^{-2}.\text{h}^{-1}$ ), that significantly differed from cellulose ( $p < 0.05$ ).

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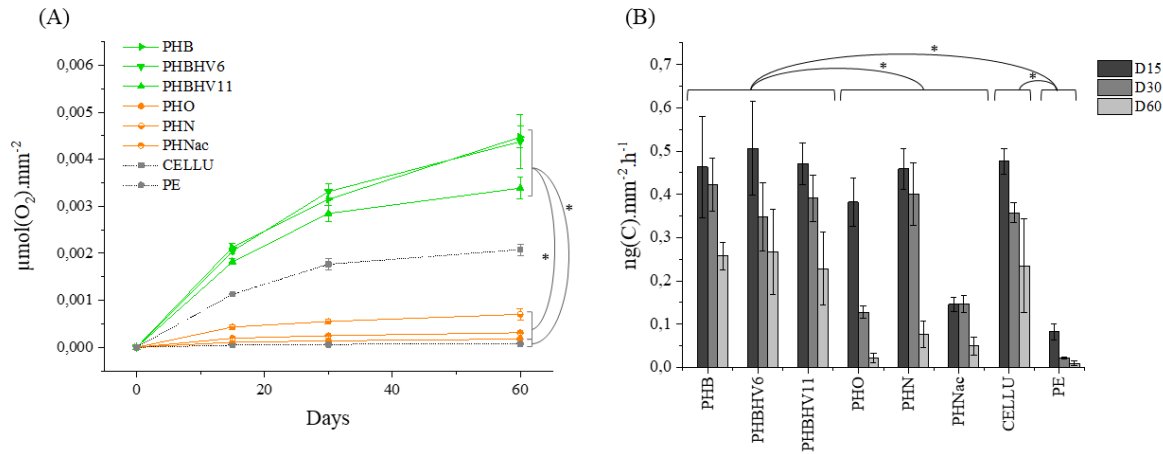


Figure A2.2. Oxygen consumption (A) and bacterial heterotrophic production (B) on sampling days. The green lines are the PHB, PHBHV11 and PHBHV6. The orange lines are the PHO, PHN and PHNac. The grey are the cellulose and the PE. Error bars are the standard deviations. The \* indicates significant difference by Kruskal-Wallis test on day 60 ( $n=24$ ).

### 3.3. Bacterial diversity

During the 60-days of experiment, no significant change in alpha-diversity was observed in all polymer types over time, including all the measured diversity indexes (Chao1 richness, Pielou evenness, Shannon and Simpson diversity) ( $p > 0.05$ ) (Table A2.2). However, significant differences were found between the polymer groups, including cellulose and *sc*-PHA (PHB, PHBHV6 and PHBHV11), as compared to another group including PE and *mc*-PHA (PHO, PHN and PHNac) ( $p < 0.05$ ). Lower Chao1 richness and Shannon diversity were found for *sc*-PHA ( $244.4 \pm 8.8$  and  $3.3 \pm 0.3$ ;  $n = 9$ , respectively) as compared to *mc*-PHA ( $606.8 \pm 19.4$  and  $4.4 \pm 0.4$ ;  $n = 9$ , respectively) ( $p = 1.6 \times 10^{-4}$ ). Higher diversity on the free-living bacteria and on the initial inocula for each polymer type was also observed (Table A2.2).

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Tableau A2.2. Total number of ASV and alpha diversity indexes. SW refers to seawater, INOC refers to microbial inocula and D is the sampling time.

<b>Sample</b>	<b>Total ASV</b>	<b>Chao1</b>	<b>Pielou</b>	<b>Shannon</b>	<b>Simpson</b>
SW-FREE	939	1313	0.716	4.9	57.8
SW-ATT	497	541	0.644	4	18.3
INOC_CELL U	707	889	0.671	4.4	25.5
CELLU_D60	207	226	0.656	3.5	15.5
CELLU_D30	288	322	0.636	3.6	12.7
CELLU_D15	128	129.	0.556	2.7	5.8
INOC_SCL	651	656.	0.818	5.3	75.4
INOC_MCL	1276	1425	0.755	5.4	42.9
PHBHV11_D 15	307	331	0.559	3.2	8.2
PHBHV11_D 30	166	171	0.548	2.8	6.3
PHB_D15	313	343	0.574	3.3	11.4
PHB_D30	243	264	0.564	3.1	11.4
PHB_D60	213	246	0.653	3.5	18
PHBHV11_D 60	266	294	0.591	3.3	10.9
PHBHV6_D3 0	212	237	0.672	3.6	14.8
PHBHV6_D6 0	156	163	0.733	3.7	27
PHBHV6_D1 5	147	148	0.561	2.8	8
PHN_D60	423	528	0.678	4.1	23.5
PHNac_D15	435	470	0.691	4.2	23.8
PHNac_D30	517	560	0.640	4	14.5
PHNac_D60	403	472	0.650	3.9	20.7
PHN_D15	434	498	0.692	4.2	25.4
PHN_D30	290	302	0.776	4.4	45.3
PHO_D15	689	767	0.719	4.7	48.7
PHO_D30	753	879.	0.740	4.9	58.4
PHO_D60	822	983	0.775	5.2	74.7
PE_D60	711	828	0.701	4.6	31.3
PE_D30	616	676	0.747	4.8	65.5
PE_D15	547	614	0.793	5	82.7

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Beta-diversity analysis showed four distinct groups between bacterial communities living on cellulose, *scl*-PHA (PHB, PHBHV6 and PHBHV11), *mcl*-PHA (PHO, PHN and PHNac), PE and in seawater (Fig. A2.2). Interestingly, inoculum grown on cellulose before the experiment grouped with the bacterial communities living on cellulose as sole carbon source. It was not the case for *scl*-PHA and *mcl*-PHA inocula, that changed when incubated with the different polymer types. ANOSIM analysis showed significant differences between *scl*-PHA and *mcl*-PHA samples ( $R = 0.964$ ,  $p < 0.05$ ). Within these groups, no clear distinction could be made between samples, except for PHBHV6 (day 15 and 60) for *scl*-PHA and PHO-D60 for *mcl*-PHA.

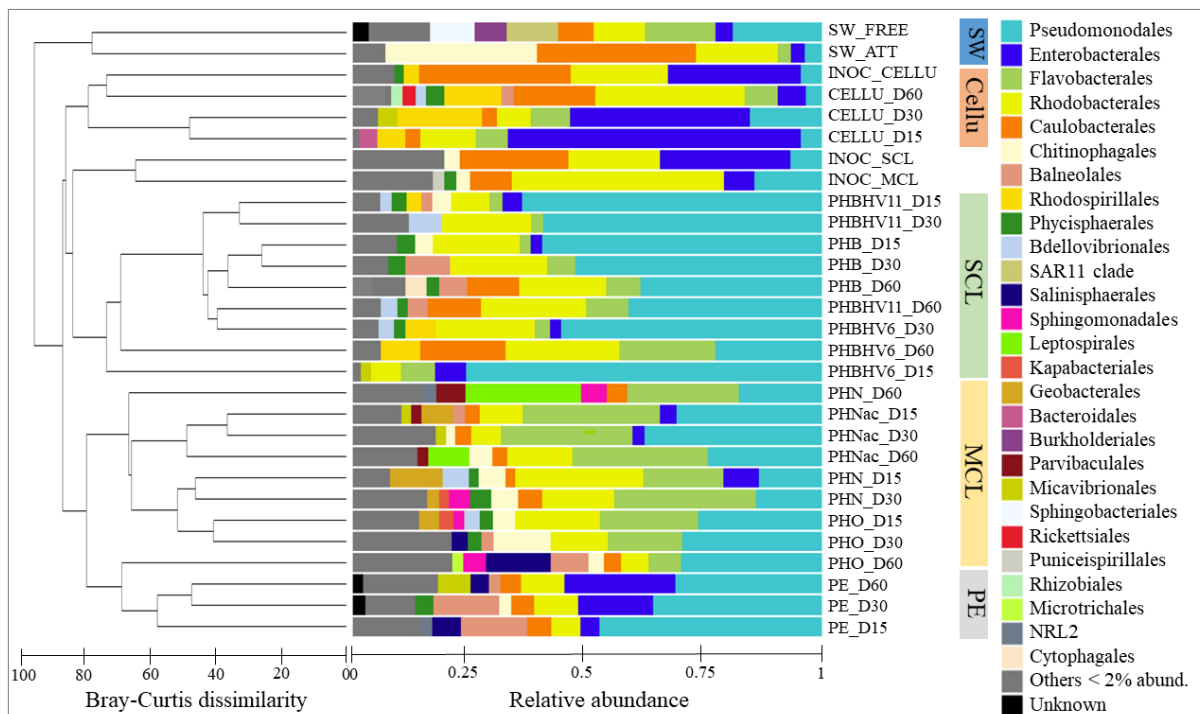


Figure A2.3. Comparison of community structures and taxonomic abundances of bacteria in seawater (SW), on the microbial inoculum (INOC), and different samples according to sampling days (D). Clustering was made by UPGMA method based on Bray-Curtis dissimilarities. Bar charts represent cumulative relative abundances at the order level.

Taxonomic composition confirmed the niche-partitioning between the bacterial communities living in seawater compared to the plastisphere of the different polymer types. Free-living bacteria were composed of Pseudomonadales (19%), Flavobacteriales (15%),

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Rhodobacterales (11%), SAR11 clade (11%), Sphingobacteriales (9%) and Caulobacterales (8%), while organic particle-attached bacteria were dominated by Caulobacterales (34%), Chitinophagales (32%) and Rhodobacterales (17%). The inoculum pre-grown on cellulose in seawater was mainly composed of Caulobacterales (32% for INOC\_CELLU), Rhodobacterales (21% for INOC\_CELLU), Enterobacterales (28% for INOC\_CELLU) and to a lesser extent of Pseudomonadales (5% for INOC\_CELLU). The same groups were also found for the inocula pre-grown on *scl*-PHA and *mcl*-PHA but with different proportions, including Caulobacterales (23% for INOC\_SCL and 9% for INOC\_MCL), Rhodobacterales (20% for INOC\_SCL and 45% for INOC\_MCL), Enterobacterales (28% for INOC\_SCL and 7% for INOC\_MCL) and Pseudomonadales (5% for INOC\_CELLU, 7% for INOC\_SCL and 14% for INOC\_MCL). After inoculation with the different polymer types as sole carbon source, the proportion of different taxa was different between the different polymer types. Pseudomonadales were high on *scl*-PHA ( $52 \pm 15\%$ ,  $n=9$ ), *mcl*-PHA ( $25 \pm 2\%$ ,  $n=9$ ), PE samples ( $28 \pm 7\%$ ,  $n=3$ ) and cellulose ( $8 \pm 5\%$ ,  $n=3$ ) together with Rhodobacterales (mean =  $18 \pm 6\%$ ,  $13 \pm 7\%$ ,  $8 \pm 1\%$ ,  $17 \pm 10\%$ ,  $n=9$  for *scl*-PHA, *mcl*-PHA, PE and cellulose, respectively). *Mcl*-PHA exhibited high proportions of Flavobacteriales (mean =  $22 \pm 7\%$ ,  $n = 9$ ) compared to other polymers (between 0% on PE and 10% on others polymer). The main taxa found on cellulose was Enterobacterales (mean =  $35 \pm 23\%$ ,  $n = 3$ ), especially on CELLU\_J15 and CELLU\_J30 (Fig. A2.2).

SIMPER analysis on the Bray Curtis dissimilarity index highlighted two species contributing to the differences between *scl*-PHA and PE. First, ASV affiliated to *Marinobacter sp.* showed a high relative abundance in *scl*-PHA (more than 37%) and a high contribution (20%) (Fig. A2.3) compared to PE. *Cobetia sp.* is the second most specific species found on *scl*-PHA in a lesser abundance (10%) and contributes to 6% on *scl*-PHA. Those two species that display a clear distinction between *scl*-PHA and PE microbial communities also contributed to the differences found between *scl*-PHA and *mcl*-PHA. Indeed, *Marinobacter sp.* and *Cobetia sp.* were poorly represented on *mcl*-PHA, while a major relative abundance and contribution were found for *Pseudomonas sp.* (12% and 7% for relative abundance and contribution, respectively) and Flavobacteriaceae (11% and 6% for relative abundance and contribution, respectively) for this polymer type. Additionally, *Pseudomonas sp.* and Flavobacteriaceae were poorly represented on *scl*-PHA, as well as on PE. Bacterial communities observed on *mcl*-PHA and PE seemed richer and more scattered. With the exception of Rhodobacterales that were abundant on all polymer types, taxa belonging to *Alcanivorax sp.* (13% and 8% of relative

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abundance and contribution, respectively), Balneolaceae, Pseudomonadales, Alloanivorax, Alcanivoraceae and Alteromonadaceae found on PE were poorly abundant on *scl*-PHA. Taxa belonging to Alloanivorax and Alcanivoraceae were also present on *mcl*-PHA but presented lower contribution (2%) when compared to PE.

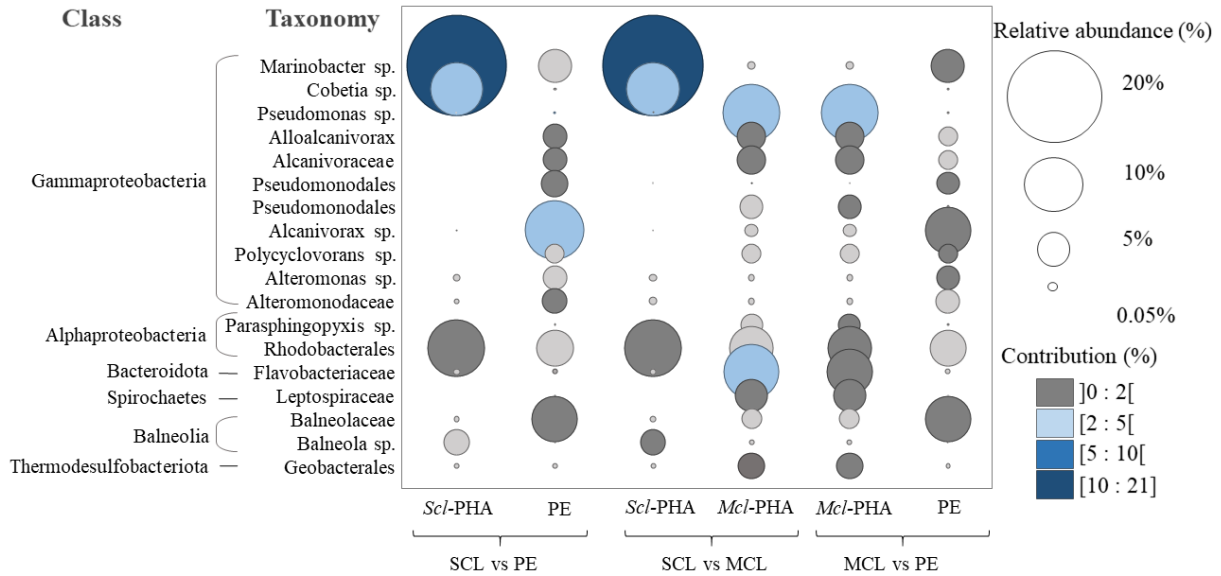


Figure A2.4. Bubble plot of the relative abundance and taxonomy of ASV contributing up to 50% to the dissimilarity. Bubbles are sized according to their relative abundance and colored according to their contribution to dissimilarity.

## 4. Discussion

### 4.1. Different physico-chemical characteristics of the six tailor-made PHA

The first step of this study was to produce three *scl*-PHA and three *mcl*-PHA with various intrinsic properties in order to estimate the influence of the physico-chemical characteristics in the PHA biodegradation in the marine environment. The bacterial strain *Halomonas sp.* SF2003 has been used for the production of *scl*-PHA using glucose and/or valeric acid to generate PHB, PHBV6 and PHBV11. Surprisingly, an increase of valeric acid in the substrate feeding led to a lower HV content in PHBV6. Valeric acid was added to the medium under the form of a mix of valeric acid and glucose. Since it was continuously dropped at a slow rate ( $4 \text{ mL}\cdot\text{min}^{-1}$ ), it conducted to the permanent presence of low valeric acid concentration in the bioreactor but also glucose concentration. *Halomonas sp.* SF2003 cells seem, in these particular conditions (on two permanently available substrates), metabolize glucose for HB accumulation at the expense of valeric acid and therefore HV incorporation in the polymer. The use of different substrates induced different chemical compositions that affect thermal properties. HV incorporation tended to slightly lower the glass transition temperatures changed from  $-7$  to  $4$  °C with 11 and 6% of HV proportions. Melting temperatures dropped from  $177$  °C to  $172$  °C and  $171$  °C with 11% and 6% of HV incorporation, respectively. Overall, DSC analysis showed common features found in other *scl*-PHA produced and characterized in the literature (Koller et al., 2010; Lemechko et al., 2019; Możejko-Ciesielska and Kiewisz, 2016). The melting enthalpy, comparable to the PHA crystallinity, was a parameter influenced by the HV incorporation due to a higher steric hindrance generated by the HV units compared to that of HB units. It tended to decrease as much as the HV content increased to reach  $30 \text{ J}\cdot\text{g}^{-1}$  with 11% of HV unit content. The modulation of HV unit proportion, even at low incorporation, is a promising way to modify the PHA properties, for instance to bring softness and elasticity to the homopolymer PHB, which can show some difficult features to process due to its high crystallinity and a melting temperature close to its degradation temperature (Pachekoski et al., 2013). Regarding *mcl*-PHA, different chain lengths and/or monomer proportions have been achieved, thus impacting to a certain extent the intrinsic properties of each of these *mcl*-PHA. PHO was mainly composed of HO (89%), PHN was mainly composed of HN (58%) and the addition of acrylic acid improved the HN content in PHNac (74%). Improving the HN content in PHNac seemed to mainly modify the crystallinity, since the melting enthalpy drops from 20

to 13 J.g<sup>-1</sup> in this latter polymer. Generally, thermal properties between *mcl*-PHA slightly differ and are in accordance with PHA composed of these monomers (Abe et al., 2012; Możejko-Ciesielska and Kiewisz, 2016). Tailor-made production with different strains and carbon sources allowed to produce PHA displaying clear and significant distinctions between *scl*- and *mcl*-PHA, including thermal properties, molecular weights or hydrophobicity.

#### 4.2. Various biodegradation activities on the different polymer types

The main originality of our study is to present pioneer results of microbial biodegradation activities of the various tailor-made PHA under natural marine conditions. Particular attention has been made here to produce PHA samples through the same process and of the same shape and size. Special care was also taken to mimic the polymer biodegradation capabilities of natural mature biofilm growing on plastics, by using pre-colonized biofilms on each *scl*- and *mcl*-PHA as test inoculum (or biofilm growing on PE and cellulose for controls), as previously described (Cheng et al., 2022). Previous studies underlined the lack of environmental representability of standard test methodologies, which was due in particular to the inadequate test inoculum (Harrison et al., 2018; Napper and Thompson, 2020; Paul-Pont et al., 2023). In particular, the use of seawater as test inoculum is not representative of the biofilm growing on plastic, since the two communities were shown to clearly differ in terms of biodiversity and functions (Bryant et al., 2016; Zettler et al., 2013). Moreover, mature biofilm formed on conventional plastic (such as PE) were shown to be different from biodegradable plastics (such as PHA or cellulose) (Odobel et al. 2021). This is the reason why the first step consisted of the formation of a mature biofilm on each PHA groups (*scl*- and *mcl*-PHA), as well as on PE and cellulose controls. In addition, a minimum medium with no carbon source was used to avoid false positive signals, together with the addition of nutrient according to Redfield N:P ratio classically used in marine biodegradation tests (16:1) (Van Wambeke et al., 2009). Most of the evidence for PHA biodegradability in marine environment focused previously on weight loss (Deroiné et al., 2015, 2014; López-Ibáñez and Beiras, 2022; Volova et al., 2011). Weight loss provides a proof of the plastic disintegration only, which may or may not be associated to the complete mineralization by bacteria (Haider et al., 2019). There is a consensus in using the last mineralization step as a relevant proof of plastic biodegradability, either estimated by O<sub>2</sub> uptake or CO<sub>2</sub> release (Jacquin et al. 2019). Here, we measured the O<sub>2</sub> uptake directly on the aqueous phase by using the ‘plastic-free’ Presens® optical dissolved oxygen sensors, which have been proven to give similar response and with less abiotic losses compared



to other commercially available manometric test systems, such as the Oxitop® device (Brown et al. 2018). It was particularly well suited for the large number of replicate samples tested in this study, and allowed the use of 12mL Exetainer tubes with perfect sealing that fitted in only one incubator for better reproducibility and with strict thermal regulation ( $\pm 0.25^{\circ}\text{C}$ ) that reduced variation in  $\text{O}_2$  values. With PHA being the sole carbon source in our biodegradation tests, the trend of oxygen consumption and microbial activities on *scl*-PHA clearly demonstrated their biodegradability in seawater, thus confirming previous observations by using other techniques (Deroiné et al., 2015, 2014; Volant et al., 2022).

Under laboratory conditions and by using pure bacterial culture, PHA biodegradation processes were depicted to be as the result of specialized extracellular enzymes called PHA depolymerases (Leathers et al., 2000; Mukai et al., 1993). The enzymes are capable of hydrolysing PHA chains into smaller water-soluble compounds ( $< 600$  Da) that can cross the membranes for further bacterial degradation and assimilation (Azam and Malfatti, 2007). In our study, respiration associated to the *scl*-PHA assimilation by bacteria resulted in a regular increase in oxygen consumption during the 60 days of biodegradation tests. The respiration rates were higher than with the cellulose positive control in the same experimental conditions, whereas it was almost undetectable on PE negative control. Bacterial heterotrophic activity ( $^3\text{H}$ -Leucine incorporation) on *scl*-PHA as sole carbon source showed the same trend, with significantly higher activity on *scl*-PHA than for *mcl*-PHA and PE. Both oxygen consumption and bacterial heterotrophic activities were high during the first 15 days of tests (even for PE films to a lesser extent), likely due to the organic matter that was detached together with the pre-colonized biofilm or due to mortality, thus rendering this period of the biodegradation tests less adequate for biodegradation measurement under our conditions. A similar difference in bacterial heterotrophic production between PHBHV and PE films was previously found during long-term colonization and biodegradation (Dussud et al., 2018b; Odobel et al., 2021). Within the *scl*-PHA group, we observed slight but significantly higher oxygen consumption on PHB and PHBHV6 compared to PHBHV11 after 60 days. Such a difference was not found for bacterial heterotrophic activities, rendering the difference in biodegradability within the *scl*-PHA less robust. Contrasting results found in the literature confirmed the possible but not consistent difference in biodegradation rates for these two polymers. A hypothesis of better biodegradation abilities of the PHBHV copolymer was linked to an increase of amorphous regions which are more susceptible to enzymatic attack compared to the homopolymer PHB (Meereboer et al.,

2020; Numata et al., 2008). Other studies of *in vitro* enzymatic degradation showed the opposite, with better degradation capacities on PHB compared to PHBHV (Mukai et al., 1993). Slight differences in terms of biodegradation between *scl*-PHA are therefore difficult to explain since biodegradation is a combination of physical, chemical and biological factors (Dilkes-Hoffman et al., 2019). As a consequence, we conclude that the intrinsic differences within the *scl*-PHA properties (hydrophobicity, crystallinity, molecular weight) were not sufficient to induce a difference in biodegradation activities in our marine experimental conditions.

In the opposite, signs of biodegradation were very low or almost undetectable for the tested *mcl*-PHA types. By comparison to *scl*-PHA, very few studies tested the biodegradability of *mcl*-PHA in marine ecosystems, probably because no *mcl*-PHA are commercially available (Lott et al., 2021; Suzuki et al., 2021). The tailor-made *mcl*-PHA produced in this study showed clear distinct chemical differences between PHO, PHN and PHNac. Although PHN showed a slightly higher oxygen consumption and bacterial heterotrophic activities after 60 days than PHO and PHNac, no statistical difference was shown. It is to be noted that oxygen consumption and bacterial heterotrophic activities of the *mcl*-PHA group were similar to the negative control PE, which was a sign of very low or no biodegradability in our marine experimental conditions. We are aware that the 2-month timing of tests was probably not sufficient and we propose to perform further studies with a longer test period before giving a firm conclusion of the absence of biodegradability (in a reasonable period of time) for the *mcl*-PHA.

Interestingly, our study offers a large set of analyses to compare the physico-chemical characteristics of *scl*- and *mcl*-PHA and assess their impact on PHA biodegradation. Although polymers with low number average molecular weight, low crystallinity and low hydrophobicity are expected to show better sign of biodegradation (Kumar et al., 2020), it does not seem to fully explain the difference found in *scl*- and *mcl*-PHA biodegradability with natural inoculum. Indeed, *scl*-PHA produced in this studies were more crystalline with higher number average molecular weight than the *mcl*-PHA but they still showed far greater biodegradation abilities. Then, the differences in physico- chemical characteristics between *scl*- and *mcl*-PHA might not be sufficient to explain the difference observed on biodegradation. As mentioned in previous studies of PHA biodegradation in seawater (Deroiné et al., 2015), no significant changes were observed in molecular weight at the end of the experiment, thus confirming a enzymatic process of degradation that resulted in surface erosion rather than bulk erosion (Appendix D). We

hypothesize that biodegradation might also result from the specificity of the extracellular PHA depolymerase to the *scl*- or to the *mcl*-PHA. Indeed, it has been previously shown that the catalytic domain activity differed between *scl*- and *mcl*-PHA depolymerase, rendering the *mcl*-PHA depolymerase ineffective on *scl*-PHA, and inversely (Kim et al., 2000). Moreover, the carbon chain length of *mcl*-PHA (which present a higher molecular mobility compared to those of *scl*-PHA) could inhibit enzymatic degradation by impeding the catalytic domain with longer side chain length and steric hindrance interferences (Numata et al., 2009). Finally, *mcl*-PHA depolymerases are less abundant than *scl*-PHA depolymerases in several types of environments including the marine environment (Viljakainen and Hug, 2021). These results suggest that the type of PHA mainly influences the biodegradation rate.

#### 4.3. Dissimilar microbial community associated to the various polymer types

The biodegradation process is a complex process involving intrinsic (relative to the polymer) but also extrinsic factors (relative to the environment). In this study, we decided to keep the same temperature, agitation, light and nutrients constant to focus on the impact of bacterial diversity as a key factor in plastic biodegradation. First, we paid specific attention to performing the biodegradation tests with realistic biofilm living in the natural marine environment. While there is no consensus today for the preparation of the microbial inoculum in the ISO or ASTM standards for polymer biodegradability tests, convergent views indicated that complex natural marine inoculum made of biofilm growing on the corresponding plastics under natural conditions are recommended (Cheng et al., 2022). A colonisation phase for a minimum of one month in natural seawater has been shown to be a pre-requisite to mimic a mature biofilm in seawater (Jacquin et al., 2019; Odobel et al., 2021), as has been done in this study. Bray-Curtis similarity showed that the biofilms growing during one month in natural seawater were similar in *scl*-PHA and *mcl*-PHA, but different from cellulose or PE films. As previously described in other studies, free-living and particle-attached bacteria living in the seawater presented very different communities compared to the plastisphere of the mature biofilms (Dussud et al., 2018b; Wright et al., 2020), thus rendering the use of seawater as inoculum for biodegradation tests irrelevant. The transfer of pre-formed biofilm from natural seawater to minimum medium resulted in bacterial community changes for *scl*-PHA and *mcl*-PHA, but not for cellulose that remained stable during the entire 60-day incubation. Following the evolution of the bacterial community changes during the biodegradation tests has been recommended by previous studies (Jacquin et al., 2019; Kowalczyk et al., 2015), but this

recommendation has been poorly followed thereafter. Changes in bacterial diversity may be used as a signal of the lack of representability of the biodegradation tests to mimic the natural environment. By following the bacterial diversity for all the tested plastics, we showed that the alpha-diversity remained stable during the course of the second step of the experiment for all plastic types, which is a prerequisite for the biodegradability tests in natural conditions (Jacquin et al., 2019).

The study of the bacterial communities also permitted to describe the potential of some ASVs to be involved in the biodegradation of the *scl*-PHA. SIMPER analysis on Bray-Curtis 16S rRNA dissimilarities showed the importance of *Marinobacter* sp. and *Cobetia* sp. in explaining the difference between the *scl*-PHA and the negative control PE. *Marinobacter* sp. has been previously shown to present abilities to degrade PHB and PHBHV (Kasuya et al., 2000; Martínez-Tobón et al., 2018). These authors demonstrated PHB and PHBHV depolymerase activities of *Marinobacter* isolated strains, and identified the *scl*-PHA depolymerase *PhaZ* gene. *Cobetia* sp. has never been observed as PHA-degraders, but it is a well-known producer of PHA (Christensen et al., 2021; Moriya et al., 2020). Further studies are needed to evaluate its potential to perform both the production and the degradation of *scl*-PHA, as it has been shown for other species (Martínez-Tobón et al., 2018; Nygaard et al., 2021). These two strains were much less abundant in PE but also in *mcl*-PHA, thus suggesting a selection in *scl*-PHA associated to its biodegradation under marine conditions. Some specific species were detected in *mcl*-PHA that presented low abundance in PE. This is particularly the case for *Pseudomonas* sp., which were previously shown as very effective producers of *mcl*-PHA (Prieto et al., 2016), with the ability to also produce extracellular *mcl*-PHA depolymerase (Schirmer et al., 1993; Schirmer and Jendrossek, 1994; Young et al., 2005). *Pseudomonas* sp. was also very low in abundance in *scl*-PHA, confirming the selection of different species depending on the *scl*-PHA vs. *mcl*-PHA groups. Further long-term studies will be needed to evaluate if the *Pseudomonas* sp. selected on *mcl*-PHA may be involved in their biodegradation. This first study on *mcl*-PHA opens new routes for further studies to better understand the bacterial diversity involved in their biodegradability in the marine environment.

## 5. Conclusion

The PHA are generally cited as one solution among others to replace conventional plastics, that would be both bio-sourced and biodegradable. Most of the studies so far have proven the rapid biodegradability of *scl*-PHA that are already commercially available, but very few of them investigated the fate of *mcl*-PHA in the environment. To our knowledge, this is the first study comparing the biodegradability of *scl*- and *mcl*-PHA in the marine environment. One strength of our work was to produce six tailor-made PHA with different physico-chemical characteristics, in order to estimate the main drivers of their biodegradability in the marine environment. The physico-chemical properties of the PHA studied might not be enough scattered to point out an impact of these characteristics on biodegradation signals within a PHA type. However, this study showed that the chemical nature of the polymer (*short*- vs. *medium-chain* length PHA) together with the diversity of microorganisms living on the plastic films (and probably the associated enzymes, *i.e.*, PHA depolymerase) were the main drivers of the PHA biodegradability in the marine environment. These results are of importance for further application of PHA with different rates of biodegradability for commercial purpose, such as the production of fishing nets, buoys or cosmetic products that end their life in the marine environment (Paul-Pont et al., 2023). This study also showed that *mcl*-PHA biodegradation takes longer time than *scl*-PHA, which could orientate the use of this PHA group for longer-lifetime products. Further biodegradation tests with longer period of time (more than 2 months) are needed to better explore the biodegradation of the more recalcitrant *mcl*-PHA, and we believe that this study open new routes for a better understanding of *scl*-PHA and *mcl*-PHA biodegradation in the marine environment.

### **Acknowledgements**

This project was supported by the PEPS-CNRS “Development of New Biodegradable Biopolymers” in the project “Biodegradability of biosourced polymers such as PolyHydroxyAlkanoates (PHA) in the marine environment (acronym: PHABIO), coordinated by S. Bruzaud & J.F. Ghiglione. It is also part of the EU-funded AtlantECO project (Atlantic Ecosystems Assessment, Forecasting & Sustainability, Horizon 2020 No 862923). We thank David Leistenschneider and Maxime Beauvais for their help launching the whole experiment and we are grateful to Guigui PA, VF, JS, JP for insightful comments on the manuscript. This work was part of the Ph.D thesis of G. Derippe supported by a financial support from the CNRS through the MITI interdisciplinary programs (AAP 80|PRIME-2020) and of the Ph.D thesis of L. Philip supported by Plastic@Sea company and the CIFRE program.

Conflict of 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.

### **Author Contributions (CRediT taxonomy)**

**Gabrielle Derippe:** Conceptualization, Formal analysis, Investigation, Methodology, Visualization, Writing - Original Draft, review & editing, **Léna Philip:** Conceptualization, Formal analysis, Investigation, Methodology, Visualization, Writing - review & editing, **Pierre Lemechko:** Formal analysis, Investigation, Writing - review & editing, **Boris Eyheraguibel:** Visualization, Writing - review & editing, **Anne-Leïla Meistertzheim:** Methodology, Visualization, Writing - review & editing, **Pascal Conan:** Methodology, Visualization, Writing - review & editing, **Mireille Pujó-Pay:** Methodology, Visualization, Writing - review & editing, **Valérie Barbe:** Supervision, Formal analysis, Visualization, Writing - review & editing, **Stéphane Bruzaud:** Conceptualization, Funding acquisition, Methodology, Project administration, Resources, Supervision, Visualization, Writing - review & editing, **Jean-François Ghiglione:** Conceptualization, Funding acquisition, Methodology, Project administration, Resources, Supervision, Visualization, Writing - review & editing.

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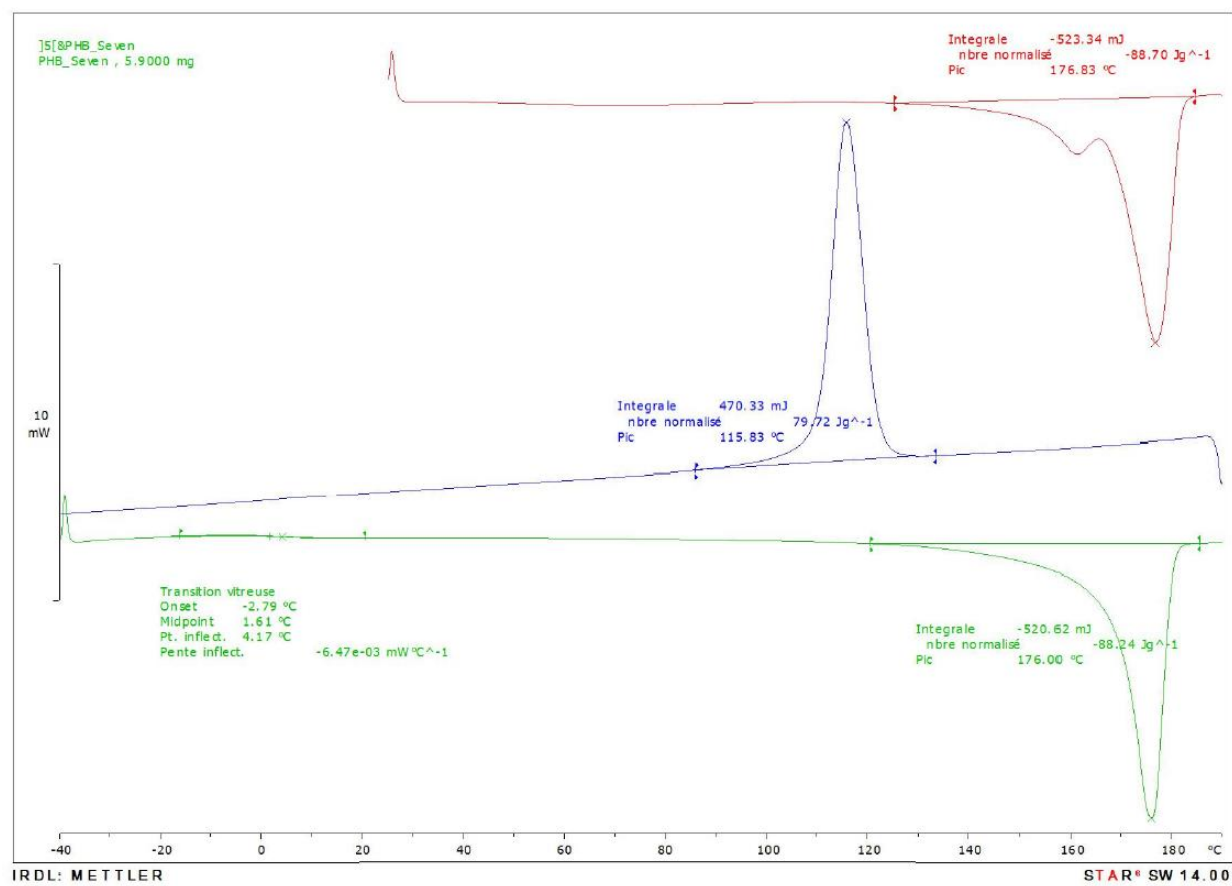
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## Appendix A: DSC curves of the 6 PHA solvent-casted films

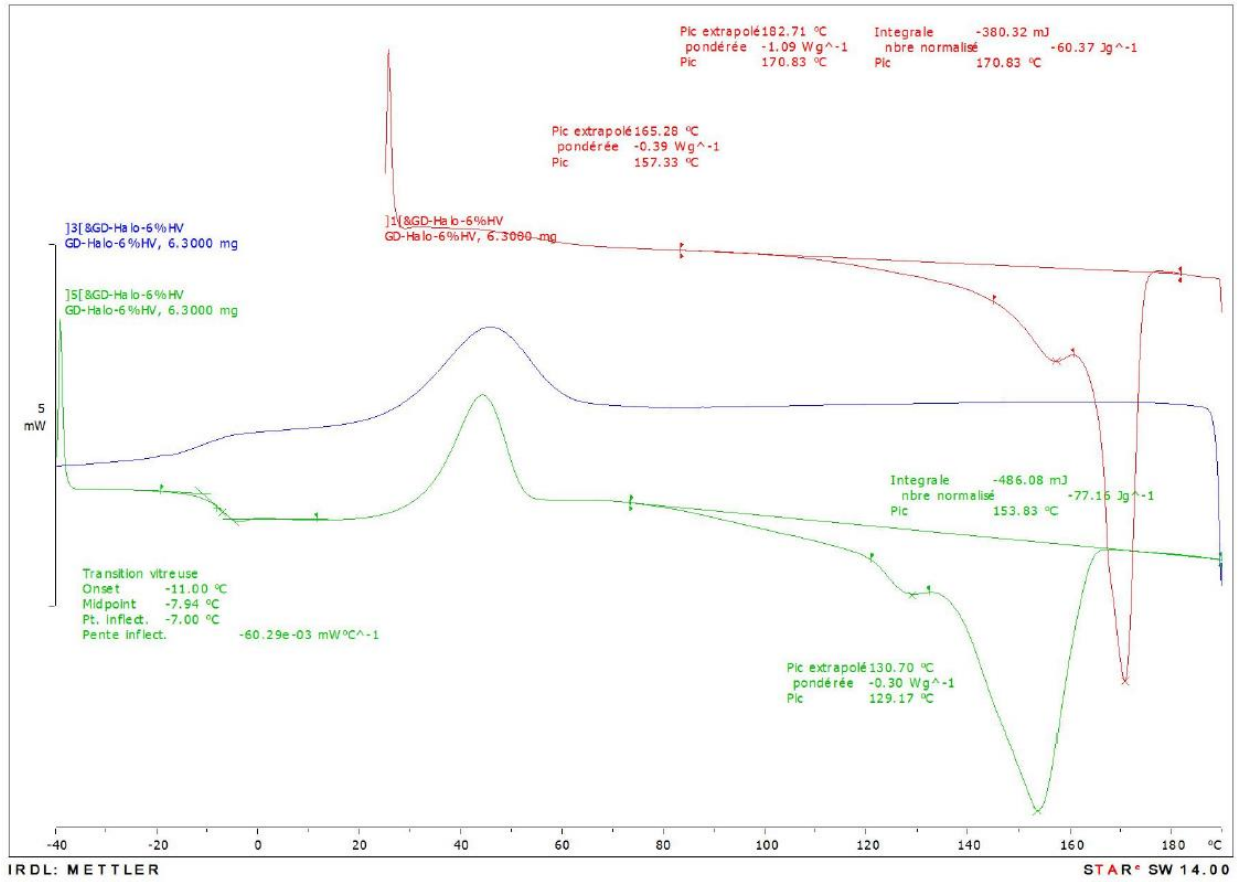
## PHB





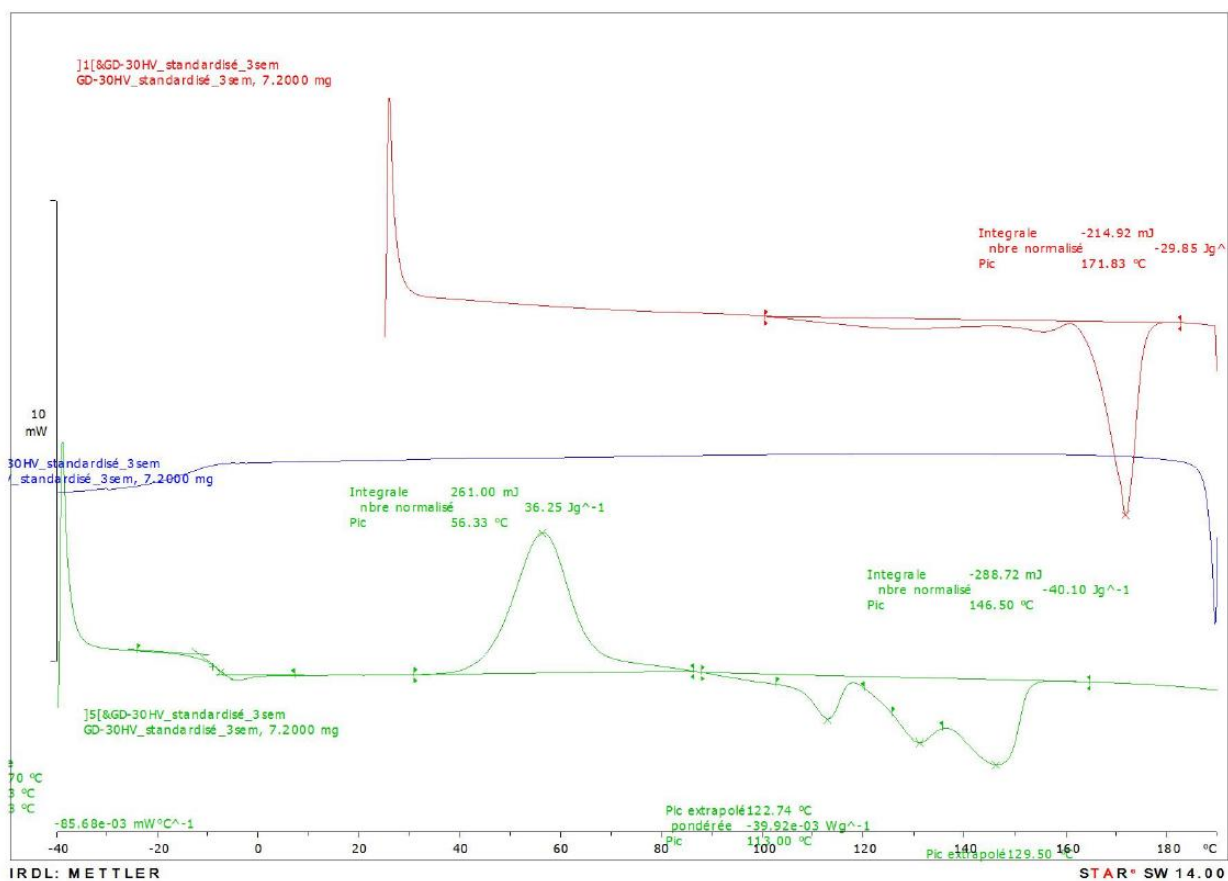
# Annexe 2

## PHBHV6



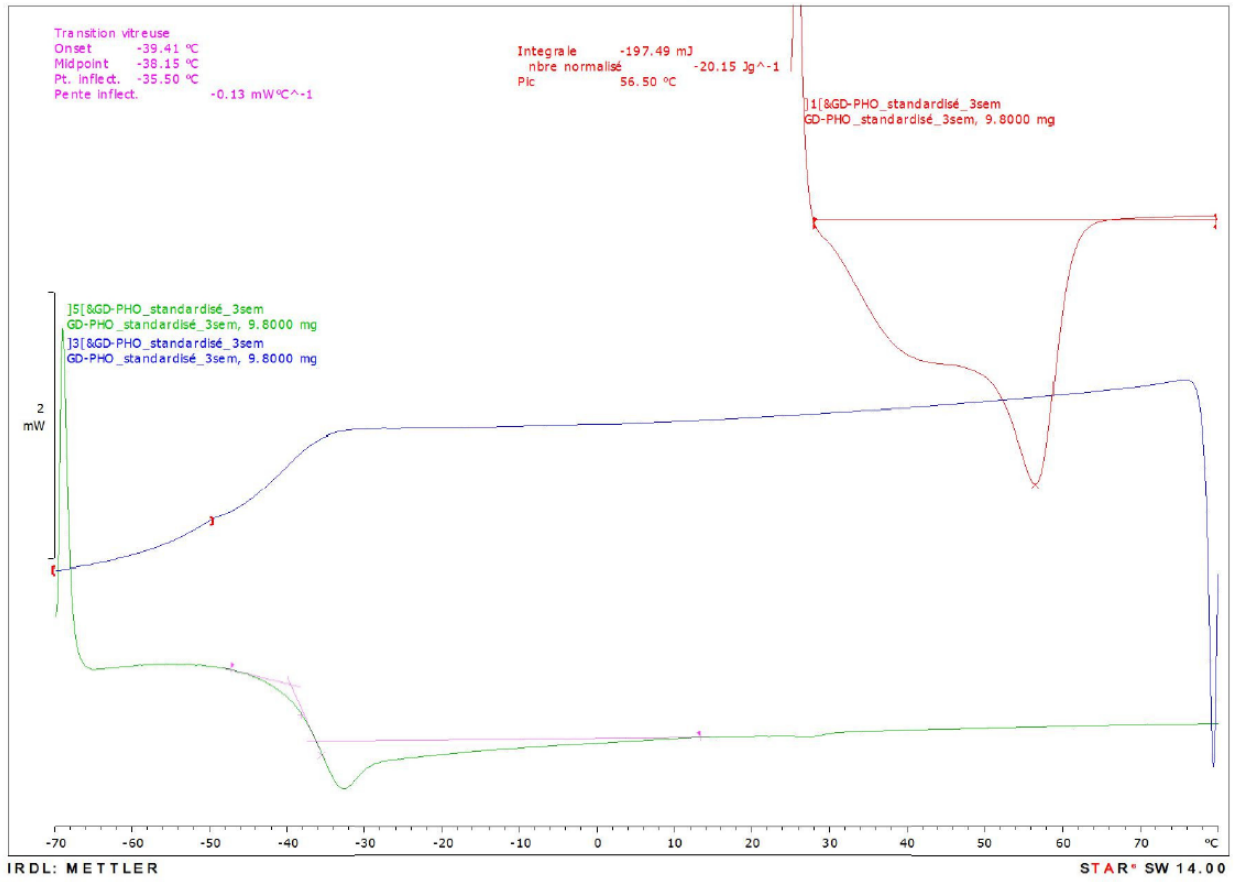
# Annexe 2

PHBHV11



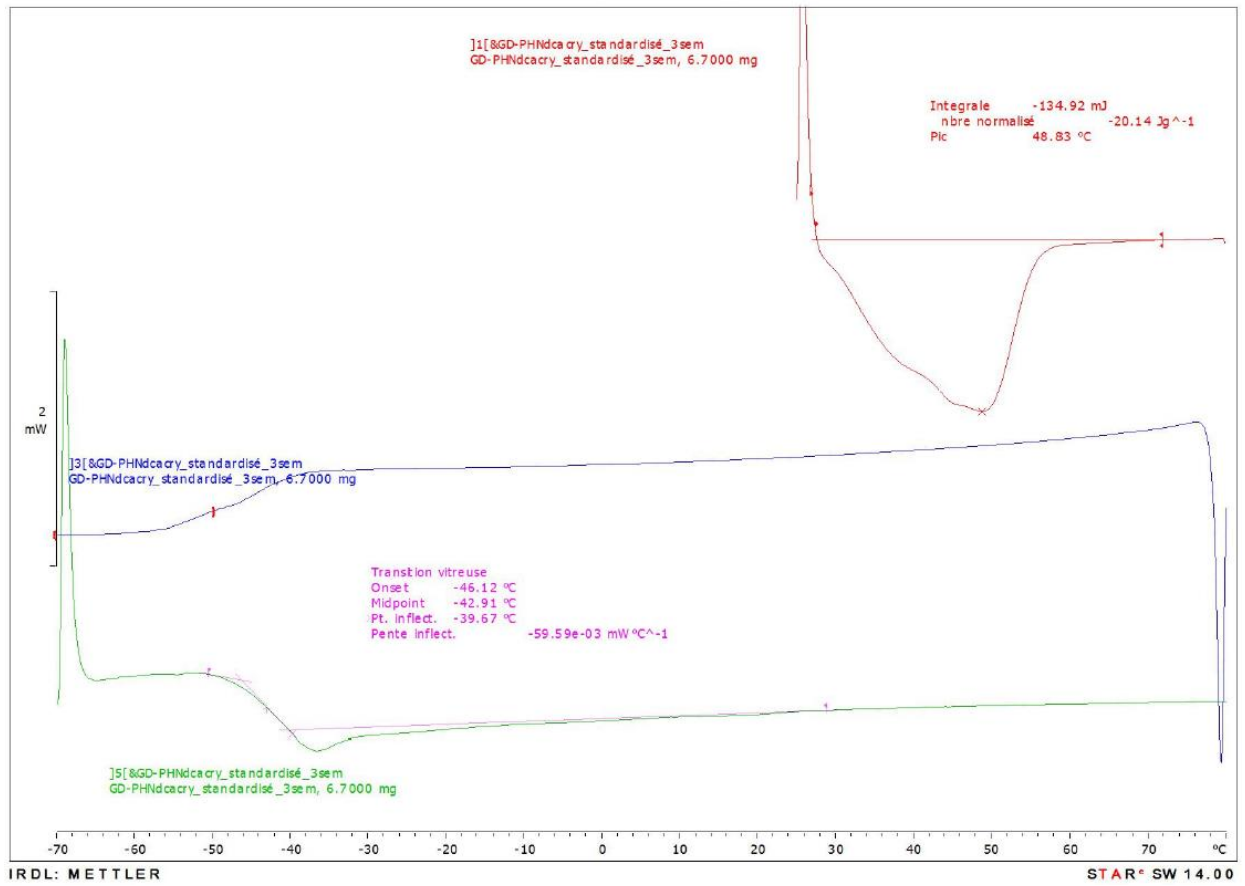
# Annexe 2

## PHO



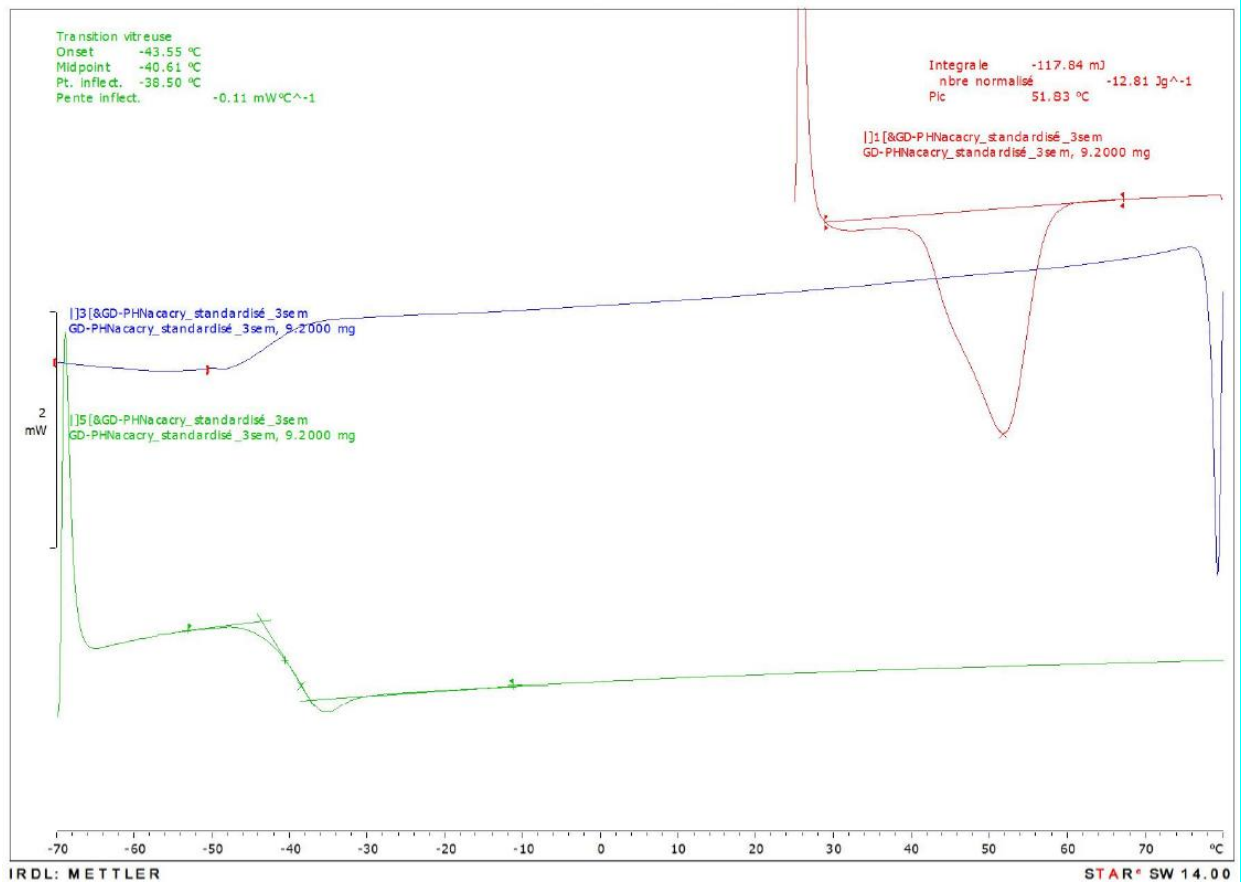
# Annexe 2

PHN

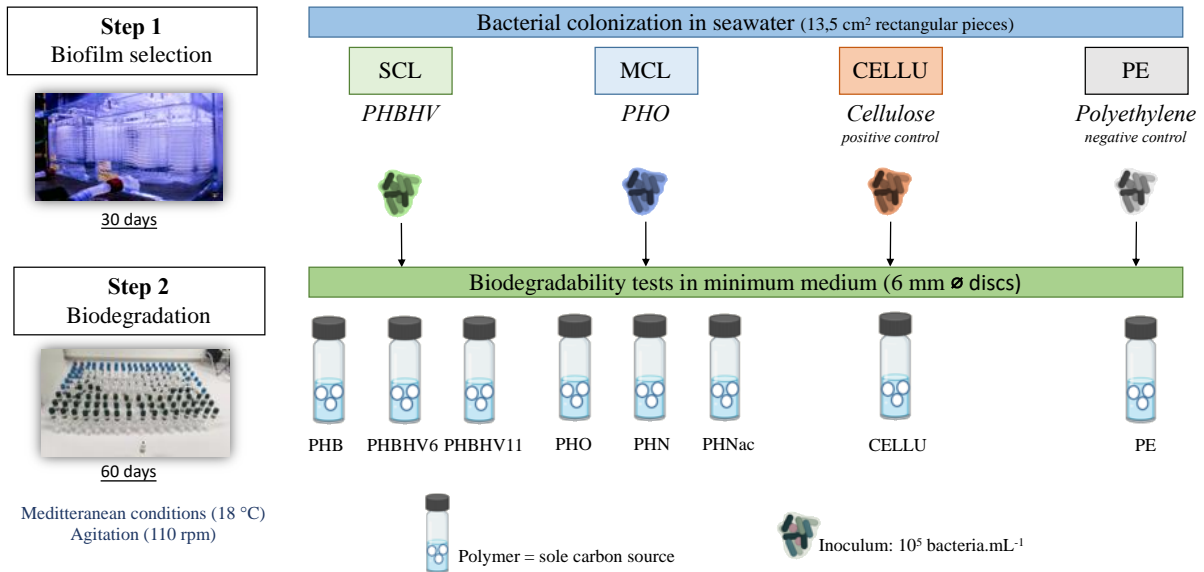


# Annexe 2

## PHNac



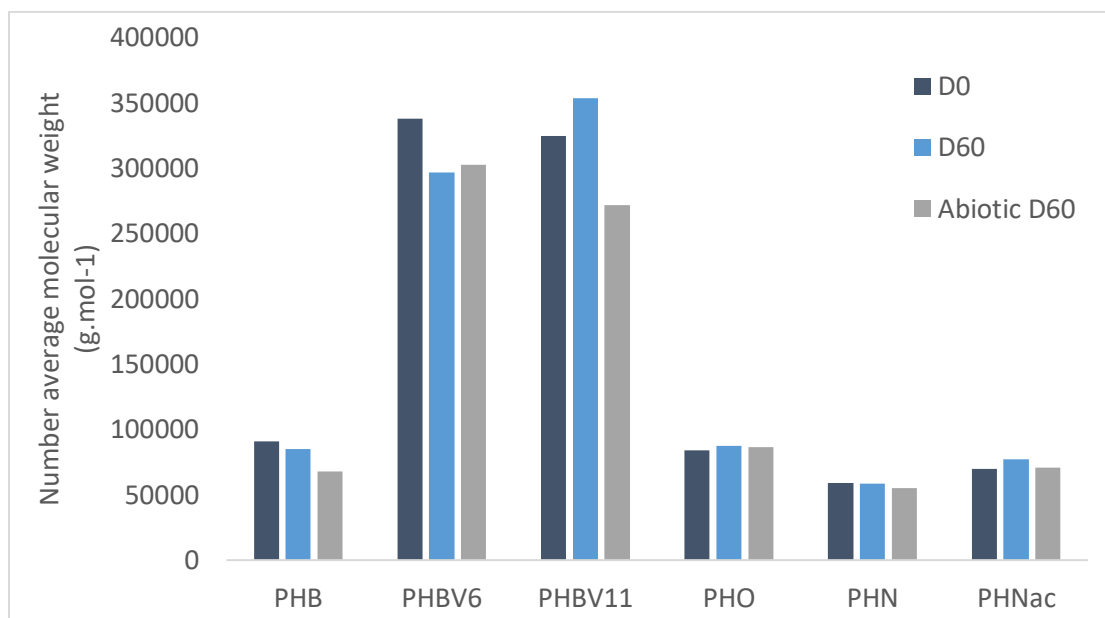
## Appendix B:



## Appendix C:

Minimal medium composition : : NaCl 24 g.L<sup>-1</sup>, Na<sub>2</sub>SO<sub>4</sub> 4 g.L<sup>-1</sup>, KCl 0.68 g.L<sup>-1</sup>, KBr 0.1 g.L<sup>-1</sup>, H<sub>3</sub>BO<sub>3</sub> 0.025 g.L<sup>-1</sup>, NaF 0.002 g.L<sup>-1</sup>, MgCl<sub>2</sub>·6H<sub>2</sub>O 10.8 g.L<sup>-1</sup>, CaCl<sub>2</sub>·2H<sub>2</sub>O 1.5 g.L<sup>-1</sup>, SrCl<sub>2</sub>·6H<sub>2</sub>O 0.024 g.L<sup>-1</sup>, NaHCO<sub>3</sub> 0.2 g.L<sup>-1</sup>, NaHPO<sub>4</sub> 0.04 g.L<sup>-1</sup>, NH<sub>4</sub>Cl 0.5 g.L<sup>-1</sup>, FeCl<sub>3</sub> 4 g.L<sup>-1</sup>, EDTA 2 g.L<sup>-1</sup>, 1 mL of traces elements for 1 L of medium composed of: CuCl<sub>2</sub>·2H<sub>2</sub>O 0.015 g.L<sup>-1</sup>, NiCl<sub>2</sub>·H<sub>2</sub>O 0.025 g.L<sup>-1</sup>, Na<sub>2</sub>MoO<sub>4</sub>·2H<sub>2</sub>O 0.025 g.L<sup>-1</sup>, ZnCl<sub>2</sub> 0.07 g.L<sup>-1</sup>, MnCl<sub>2</sub>·4H<sub>2</sub>O 0.1 g.L<sup>-1</sup>, CoCl<sub>2</sub>·6H<sub>2</sub>O 0.12 g.L<sup>-1</sup> and 1 mL of a vitamin solution for 1 L of medium composed of: p-aminobenzoic acid 0.005 g.L<sup>-1</sup>, pyridoxine-HCl 0.1 g.L<sup>-1</sup>, thiamine-HCl 0.05 g.L<sup>-1</sup>, riboflavin 0.05 g.L<sup>-1</sup>, nicotinic acide 0.05 g.L<sup>-1</sup>, D-Ca-pantothenate 5.10<sup>-9</sup> g.L<sup>-1</sup>, lipoic acid 0.05 g.L<sup>-1</sup>, nicotinamide 0.05 g.L<sup>-1</sup>, B12 vitamin 0.05 g.L<sup>-1</sup>, biotine 0.02 g.L<sup>-1</sup> and folic acid 0.02 g.L<sup>-1</sup>.

Appendix D : Evolution of the number average molecular weight ( $\text{g}\cdot\text{mol}^{-1}$ ) at Day 0, Day 60 and of abiotic control (PHA but no bacteria) at Day 60.







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## Résumé

La lutte contre la pollution plastique est devenue une priorité afin de limiter les impacts déjà visibles sur les écosystèmes naturels. La production de plastiques dits biodégradables est aujourd'hui une composante de la stratégie globale envisagée par les états pour lutter contre cette pollution. Cependant, les tests normatifs (ISO, AFNOR, ASTM) qui valident la biodégradabilité des plastiques en milieu aquatique ont été largement critiqués dans la littérature scientifique, notamment pour leur manque de représentativité du milieu naturel.

L'objectif de cette thèse est de renforcer les connaissances sur la biodégradabilité des plastiques en milieu aquatique et de proposer des pistes d'amélioration des normes actuelles. Une attention particulière est portée sur les communautés microbiennes qui colonisent les plastiques en milieu naturel, la « plastisphère ». La première partie de cette thèse porte sur l'analyse des communautés bactériennes associées aux débris plastiques flottant le long du continuum fleuve-mer, associée à une campagne d'échantillonnage de 8 mois réalisée le long de 9 grands fleuves européens (mission *Tara Microplastiques*, soutenue par la fondation Tara Océans). Nous avons mis en évidence des distinctions claires entre la diversité des plastisphères en eau douce et en mer, suggérant un faible transfert des communautés du fleuve vers la mer (y compris pathogènes). La deuxième partie de ce travail concerne les méthodes d'évaluation de la biodégradabilité des plastiques en milieu marin. La miniaturisation des tests nous a permis d'augmenter le nombre de conditions et de répliques analysés pour tenir compte de l'effet du milieu utilisé, mais également de la diversité de l'inoculum bactérien et de sa concentration sur différents types de polymères (polyéthylène-PE, polyhydroxyalcanoate-PHA et cellulose). L'évolution des activités bactériennes, des produits de biodégradation et des concentrations en sels nutritifs ont été analysés pendant 90 jours d'incubation. Ces expériences ont permis de mettre en évidence l'importance d'utiliser un inoculum présélectionné et de suivre les limitations possibles en éléments nutritifs pour réaliser ces tests dans des conditions les plus proches possible du milieu naturel. La troisième partie de cette thèse est une application de la méthode miniaturisée à l'étude de la biodégradabilité de sept formulations de PHA ayant des compositions chimiques et des propriétés physiques différentes. Nos tests indiquent que la biodégradabilité des PHA est étroitement liée à la présence de monomère d'acide hydroxybutyrate (HB), qui est à la fois le monomère le plus produit par les organismes qui synthétisent des PHA, mais aussi le plus ciblé par les enzymes des microorganismes responsables de leur dégradation. Ces travaux de thèse contribuent à une meilleure compréhension l'écologie microbienne associée à la biodégradabilité des plastiques en mer et ouvrent de nouvelles perspectives pour l'amélioration des méthodes utilisées dans les normes de biodégradation des plastiques dans l'environnement.

**Mots clés :** débris plastiques • plastisphère • biodégradabilité • milieu marin • normes