# Microplastics in the marine environment : an ecotoxicological perspective

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# List of publications

1. Dussud, C., Hudec, C., George, M., Fabre, P., Higgs, P., Bruzaud, S., Delort, A.-M., Eyheraguibel, B., Meistertzheim, A.-L., Jacquin, J., **Cheng, J.**, Callac, N., Odobel, C., Rabouille, S., Ghiglione, J.-F., 2018. Colonization of Non-biodegradable and Biodegradable Plastics by Marine Microorganisms. https://doi.org/10.3389/fmicb.2018.01571

2. Jacquin, J., **Cheng, J.**, Odobel, C., Pandin, C., Conan, P., Pujo-Pay, M., Barbe, V., Meistertzheim, A.-L., Ghiglione, J.-F., 2019. Microbial Ecotoxicology of Marine Plastic Debris: A Review on Colonization and Biodegradation by the "Plastisphere." Front. Microbiol. 10. https://doi.org/10.3389/fmicb.2019.00865

3. **Cheng J.,** Jacquin J, Conan P., Pujo-Pay M., Valérie B., Matthieu G., Pascale F., Bruzaud S., Ter Halle A., Meistertzheim A-L and Ghiglione J-F. Relative influence of plastic debris size and shape, chemical composition and phytoplankton-bacteria interactions in driving seawater plastisphere abundance, diversity and activity. Frontiers in microbiology (soon *submitted)*

4. **Cheng J.,** Eyheraguibel B., Jacquin J., Pujo-Pay M., Conan P., Barbe V., Hoypierres J., Deligey G., Ter Halle A., Bruzaud S., Ghiglione J-F & Meistertzheim A-L. Biodegradability under marine conditions of bio-based and petroleum-based polymers as substitutes of conventional microbeads. Environmental pollution (soon *submitted)*

5. **Cheng J,** Meistertzheim A-L, Jacquin J, Valérie B, Escande M-L 4, Bertrand S, Escriva H. and Ghiglione J-F. Beneficial or detrimental effects of microplastics on the marine filter-feeder amphioxus (*Branchiostoma lanceolatum*)? Environmental science & technology *(In prepration)*.

6. Jacquin J., Callac N., **Cheng J.**, Giraud C., Gorand Y., Denoual C., Pujo-Pay M., Conan P., Barbe V., Ter halle A., Meistertzheim A-L., Bruzaud S., Ghiglione J-F. Marine plastisphere activity and diversity during successive colonization and biodegradation phases of various composition of plastic sticks (soon *submitted)*

7. Jacquin J., **Cheng J.**, Conan P., Pujo-Pay M., Bruzaud S., Ghiglione J-F., Barbe V. A new strain capable of synthesizing and degrading PHBV using atypical metabolic pathways (*In preparation*)

8. Jacquin J., Budinich M., **Cheng J**., Barbe V., Pedrotti M-L., Ghiglione J-F. Global diversity and core microbiome of the plastisphere compared to organic-particle attached and free-living planktonic lifestyles from the Tara Oceans expeditions in the Mediterranean Sea and in the North Pacific gyre *(In prepration)*.

# List of scientific communications

1. **ChengJ.** 5th Banyuls-Barcelona interlab meeting, Barcelona. May 2019

2. **Cheng J.**, Jacquin J., Pandin C., Odobel C., Catala P., Pecqueur D., Salmeron C., Pujo-Pay M., Conan P., Eyheraguibel B., Delort A-M., Barbe V., Hoypierres J., Deligey G., Bruzaud S., Fabre P., George M., J-F Ghiglione, A-L Meistertzheim Dégradation des plastiques par les bactéries marines. Poster at Premières Rencontres du GDR Polymères et Océans. Créteil, July 2019.

3. **Cheng J**, Jacquin J, Meisterzheim A-M, Barbe V, Ter Halle A, Matthieu G and Ghiglione J-F. How much is plastisphere driven by the size, composition and incubation time in seawater? ISMS international online conference. Barcelona 2020, July. Oral presentation.

4. Jacquin J., Callac N., **Cheng J.**, Giraud C., Gorand Y., Denoual C., Pujo-Pay M., Conan P., Barbe V., Ter halle A., Meistertzheim A-L., Bruzaud S., Ghiglione J-F. Utilisation des outils "OMICS" pour caractériser la biodégradation des plastiques en mer. Poster au Colloque de l'AFEM. Bussang, Novembre 2019. 1er Prix Poster

5. Jacquin J.**, Cheng J.**, Meistertzheim A-L, Callac C., Lemaire J., Fromageot D., Higgs P., Eyheraguibel B., Delort A-M., Ghiglione J-F., Nouveau test de biodégradabilité des plastiques oxodégradables en milieu marin. Poster au workshop Polymère et océans. Montpellier, Janvier 2018.

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## *Abstract:*

Oceanic plastic pollution is of major concern, with several million tonnes of plastic dumped in the ocean every year that are causing health threat to marine creatures. Impacts have been found at all the trophic chain levels from the zooplankton to the megafauna, but little is known on its impact on the microbial life and its crucial role in the oceanic ecosystem functioning. The objective of this thesis was to study the ecotoxicity of plastics in the marine environment.

The first handled question was: how much the abundance, diversity and activity of bacterial life growing on plastic, i.e. the 'plastisphere' are driven by the chemical properties of the polymer and the environmental changes (Chapter 2)? Polyethylene (PE) and polylactide acid (PLA) together with glass controls in the forms of meso-debris (18mm diameter) and large-microplastics (LMP; 3mm diameter), as well as small-microplastics (SMP; of 100 µm diameter with spherical and irregular shapes) were immerged during 2 months in seawater. We found that the plastic chemical composition, the successive phases of biofilm formation and the phytoplankton-bacteria interactions were more important factors driving the abundance, diversity and activity of the plastisphere as compared to material size and shape.

The second handled question was: would the microplastic (polystyrene PS; 50-100 µm; three concentrations) together with their mature biofilm be toxic for the marine filter-feeder *Branchiostoma lanceolatum* and how much the plastisphere can influence this toxicity (Chapter 3)? We used a large set of complementary techniques to follow the microplastic ingestion (microscopy quantification) and the modification of the gut microbiota (16S rRNA Illumina Miseq sequencing), the gene expression of immune system, oxidative stress and apoptosis (Nanostring) and also histopathology (transmission electron microscopy). No obvious toxicity was observed, while microplastics could be a vector for bacteria to the gut microbiome, can

induce more goblet cell differentiation, and can surprisingly have a positive effect by supplying nutrients to amphioxus in the form of bacteria and diatoms from the plastisphere.

The third handled question was: how much the conventional petroleum-based microbeads classically used in cosmetics can be substituted by other polymers for their biodegradability by the plastisphere in marine environment? (Chapter 4). We used complementary techniques to follow the 4 biodegradation steps including biodeterioration (granulometry, gravimetry and FTIR spectroscopy), biofragmentation (size exclusion chromatography, $^{1}$H nuclear magnetic resonance and high-resolution mass spectrometry), bioassimilation and mineralization ($^{1}$H nuclear magnetic resonance and oxygen measurements). We concluded that microbeads made of polyhydroxybutyrate-co-hydroxyvalerate (PHBV) or rice and in a lesser extent polycaprolactone (PCL) and apricot were good candidates for substitution of conventional microplastics, classically made of PE or polymethyl methacrylate (PMMA) that were not biodegraded under our conditions. Interestingly, the biobased PLA was not biodegradable but the petroleum-based PCL was biodegradable under our marine conditions.

# *Résumé en français :*

## *Contexte :*

### *Plastiques dans l'océan*

Les produits en matière plastique sont légers, peu coûteux et durables. Ces caractéristiques font du plastique un matériau pratique pour les produits quotidiens, y compris l'emballage, la construction, les textiles, le transport, l'électronique, etc. Leur production à grande échelle a débuté environ dans les années 1950 et la production mondiale est passée de 2 millions de tonnes (MT) en 1950 à 380 millions de tonnes en 2015, et elle est estimée atteindre 434 millions de tonnes en 2020 (Geyer et al., 2017).

Son utilisation à grande échelle dans la vie quotidienne, sa résistance à la dégradation naturelle, ainsi que la mauvaise gestion des déchets, font de la pollution plastique un problème mondial, qui se produit non seulement dans les terres mais aussi dans les océans. Aujourd'hui, les politiciens, les scientifiques et le public ont du mal à faire face à ce problème épineux. Le concept de « réduire, réutiliser et recycler » les plastiques devient un leitmotiv classiquement utilisé pour attirer l'attention du public, des industriels et des politiques pour orienter les solutions à cette pollution, alors que des dizaines de millions de tonnes de plastique sont toujours émises dans les océans chaque année, avec un impact important sur l'écosystème marin (Jambeck et al., 2015).

Les plastiques sont classés dans les microplastiques lorsque leur diamètre est inférieur à 5 mm (Thompson et al., 2004). Ils constituent la proportion la plus élevée dans l'environnement marin, échantillonnée avec un filet manta (> 330 µm), où ils représenteraient plus de 90% des plastiques par rapport à ceux de plus grande taille (Eriksen et al., 2014). En général, on estime également qu'environ 75 à 90% des débris de plastique dans le milieu marin proviennent de

sources terrestres et environ 10 à 25% de sources océaniques, telles que les activités de pêche (Duis et Coors, 2016).

Une fois que les plastiques arrivent dans l'océan, ils vont soit flotter à la surface, soit être suspendus dans la colonne d'eau océanique ou se déposer au fond de l'océan, en fonction de leur densité. Le polyéthylène et le polypropylène sont couramment retrouvés dans les eaux de surface et les berges de la mer (Bond et al., 2018). Les plastiques à la surface de l'océan s'accumulent principalement dans les zones de convergence des 5 gyres subtropicaux avec des densités comparable (Pacifique Nord, Atlantique Nord, Pacifique Sud, Atlantique Sud, Océan Indien) et aussi en Mer Méditerranée qui est une mer semi-fermée. En outre, le plastique s'accumule également dans les fonds marins et les sédiments du littoral ou du large, avec des concentrations qui sont beaucoup plus difficile à évaluer.

***Interaction entre les microbes plastiques et marins***

L'une des plus grandes préoccupations de la pollution plastique est l'évaluation de son impact sur les organismes marins allant des microbes à la mégafaune.

Une fois que les plastiques arrivent dans l'océan, les microbes marins, y compris les procaryotes et les microeucaryotes, coloniseront la surface du plastique. Tous les organismes sur la surface en plastique constituent ce qu'on appelle la « plastisphère » (Zettler et al., 2013). Le processus de colonisation est complexe. Auparavant, la formation de biofilm avait été considérée comme régi par des processus chimiques et physiques (comme l'hydrophobicité et / ou la charge de surface)(Teughels et al., 2006). Plus récemment a été pris en compte les mécanismes biologiques du processus de colonisation, comprenant l'attachement initial, la croissance du biofilm et sa maturation (Chapitre 1, Figure 3).

Les bactéries et les diatomées se trouvent sur le plastisphère quel que soit la zone d'échantillonnage considérée, de la côte jusqu'au large (Atlantique Nord ou océan Pacifique).

# *French abstract*

Selon le type nutritionnel, les microbes présents sur la plastisphère pourraient être regroupés en phototrophes, hétérotrophes, prédateurs, symbiotes et saprotrophes. Les phototrophes comprennent les diatomées et les cyanobactéries, qui pourraient utiliser l'énergie lumineuse pour fixer le $CO_2$. Différentes régions océaniques ont une composition de diatomées distincte, les genres de diatomées *d'Amphora* et de *Nitzschia* représentaient une proportion plus élevée. Des cyanobactéries de type *Leptolygbya* ont souvent été signalées sur la plastisphère. Concernant la communauté bactérienne, les Alteromonadaceae et les Rhodobacteraceae sont les principaux colonisateurs en phase de primo-colonisation. Les Planctomycète et les Bactéroïdètes (Flavobactéries) ont tendance à être les seconds colonisateurs sur la plastisphère, avec une proportion plus élevée dans la phase de maturation du biofilm.

La question de la dispersion d'agents pathogènes opportunistes vivant sur les plastiques a été soulevée, tels que le *Vibrio* pathogènes animaux ou humains, qui ont été trouvé jusqu'à 24% du total des séquences d'ARNr 16s d'un échantillon de plastique de l'océan Atlantique (Zettler et al., 2013) ou de la Méditerranée (Dussud et al. 2018), alors qu'il représentent en général quelques pourcents. Les preuves de pathogénicité sur les animaux marins en relation avec le plastisphère n'ont jamais encore été prouvées, et des recherches supplémentaires seront nécessaires avant d'afficher des conclusions alarmistes sur la responsabilité possible des débris plastiques en tant que vecteur de propagation d'organismes pathogènes.

Quel que soit le type de polymère, des études récentes ont souligné la différence entre les bactéries vivant sur les plastiques et celles vivant à l'état libre (Debroas et al., 2017) ou sur des particules organiques dans l'eau de mer environnante (Dussud et al., 2018). Des observations similaires ont été faites pour les communautés fongiques (Kettner et al., 2017). En général, on considère que les facteurs saisonniers et géographiques sont très importants dans la formation de la plastisphère en milieu marin. La propriété de surface du plastique (comme

l'hydrophobicité et la rugosité) et la taille du plastique sont également considérées comme importantes, bien que rarement testées et prouvées.

La quantité de plastique distribué dans les Océans peut également avoir un impact sur les cycles biogéochimiques élémentaire (C, N, P…) dans le milieu marin. Par exemple, en mesurant la production primaire et la respiration du plastique de l'océan Pacifique nord, il a été constaté que la production primaire brute était positive, suggérant que l'augmentation croissante de la production de plastique conduirait à ce que ces déchets deviennent des « points chauds » dans les océans oligotrophes (Bryant et al., 2016). Le biofilm sur la plastisphère rend également les microplastiques sensibles au transport vertical en modifiant leur flottabilité (Rummel et al., 2017). Enfin, une corrélation positive entre la formation de biofilm et la colonisation des larves a été observé, suggérant la possible dispersion d'espèces potentiellement invasives (Salta et al., 2013).

La durée de vie du plastique dans l'environnement marin n'est pas déterminée. Elle est grossièrement estimée à plusieurs centaines d'années d'années. La dégradation du polymère est initiée par une modification des propriétés du polymère due à des réactions chimiques, physiques ou biologiques qui entraînent une scission des liaisons qui forment le polymère et la transformation chimique jusqu'à la biominéralisation finale et sa transformation en $CO_2$ (Singh et Sharma, 2008). La biodégradation du plastique est un processus qui se traduit par une conversion totale ou partielle du carbone organique en biogaz et biomasse associée à l'activité d'une communauté de microorganismes (bactéries, champignons, etc.) capables d'utiliser le plastique comme source de carbone, et elle est considérée comme surviennent après ou en concomitance avec une dégradation abiotique. Le processus se résume en quatre étapes essentielles (Jacquin et al., 2019). La bio-détérioration conduit à la formation de minuscules fragments. La bio-fragmentation réduira le poids moléculaire des polymères et libérera des oligomères sous l'action d'enzymes microbiennes. L'assimilation décrit ensuite l'intégration

des oligomères de moins de 600 Daltons à l'intérieur des cellules et d'être utilisés comme source de carbone, augmentant ainsi la biomasse microbienne. La minéralisation est l'étape ultime de la biodégradation d'un polymère plastique et entraîne l'excrétion de métabolites complètement oxydés et de $CO_2$. Jusqu'à présent, il existe plusieurs normes (AFNOR, ISO) disponibles utilisées pour la détermination de la biodégradabilité des polymères en milieu marin, la plupart des normes prennent la mesure de la respirométrie, tandis que plusieurs auteurs ont critiqué les normes actuelles qui ne pourraient pas prédire de manière réaliste la biodégradabilité en milieu marin notamment sur la base de la préparation des inoculum, la température d'essai sélectionnée (supérieure à la situation), sans essai de toxicité, etc. (Jacquin et al., 2019).

***Interaction entre les animaux marins et les plastiques***

Les activités humaines sont responsables de la principale raison du déclin de la diversité biologique mondiale. Elle est si critique que l'impact de l'homme a accéléré les taux d'extinction actuels de 1,000 à 10,000 fois plus élevés que le taux naturel (Lovejoy, 1997). Une forme particulière d'impact humain constitue une menace majeure pour la vie marine: la pollution par les débris plastiques (Derraik, 2002).

Les rapports fréquents sur l'impact plastique soulignent l'impact de l'ingestion et de l'enchevêtrement chez les organismes. Les observations (ingestion et enchevêtrement) sont passées de 267 espèces en 1997 (Laist, 1997) à 395 espèces en 2015 (Gall et Thompson, 2015). Toutes les espèces connues de tortues de mer (100%), 54% de toutes les espèces de mammifères marins (comme les cétacés, les otaries à fourrure, les baleines…), 56% de toutes les espèces d'oiseaux de mer et 0,68% de toutes les espèces de poissons sont actuellement affectées par l'enchevêtrement ou l'ingestion de débris marins. L'ingestion de microplastiques et nanoplastiques pourraient causer des dommages aux organismes marins au niveau subcellulaire, cellulaire ou organique (Chapitre 1, Figure 6). Par exemple, les effets peuvent être visibles sur les réserves d'énergies, la translocation dans les tissus, l'induction d'un déséquilibre du

microbiote intestinal, du système immunitaire et un stress oxydatif, altérant le processus de reproduction et provoquant des dommages physiques sur l'intestin. En ayant un impact au niveau subcellulaire, cellulaire ou organique, il est prédit que ces effets auraient un impact sur le maintient de certaines espèces et sur l'écosystème.

Récemment, certains auteurs ont souligné des lacunes dans les tests de toxicité associés à la pollution par les microplastiques. Par exemple, la plupart des expériences de laboratoire ont été réalisées avec une concentration de microplastiques de plusieurs ordres de grandeurs supérieurs à ceux trouvés dans le milieu naturel. Deuxièmement, la plupart des auteurs utilisent des microbilles plastique de forme sphérique, qui ne sont pas courante dans le milieu marin. Troisièmement, presque toutes les études utilisent des microplastiques vierges, qui sont différents de ceux trouvés dans les milieux marins qui sont systématiquement colonisés par un biofilm et peuvent avoir adsorbé des polluants à leur surface. Ainsi, la méthodologie associée à cette question devrait également être améliorée

***Objectifs de la thèse***

L'objectif de cette thèse est de mieux comprendre l'écotoxicité de la pollution plastique en milieu marin, en se concentrant principalement sur les microplastiques. Trois questions spécifiques ont été étudiées. Premièrement, comment l'abondance, l'activité et la diversité de la plastisphère réagissent-elles aux changements environnementaux (Chapitre 2) ? Deuxièmement, les microplastiques sont-ils toxiques pour l'organisme filtreur marin *Branchiostoma lanceolatum* (Chapitre 3) ? Troisièmement, pourrait-il être possible de trouver des substituts dégradables aux plastiques conventionnels pour réduire l'impact de la pollution plastique (Chapitre 4) ?

## *Chapitre 2. Influence relative de la taille, de la forme et de la composition chimique des débris plastiques ainsi que des interactions phytoplancton-bactéries sur l'abondance, la diversité et l'activité de la plastisphère en mer*

**Résumé :**

L'objectif de ce chapitre est d'étudier l'impact des facteurs environnementaux sur la plastisphère. Nous avons émis l'hypothèse qu'un large spectre de taille de plastique rencontré dans l'environnement marin peut être un facteur critique de l'abondance, de la diversité et de l'activité de la plastisphère. En parallèle, d'autres facteurs tels que la composition chimique et l'évolution temporelle ont été étudiés et comparés afin de déterminer l'importance relative de chaque facteur. Nos recherches révèlent que la composition chimique du plastique, les phases successives de formation du biofilm et les interactions phytoplancton-bactéries sont des facteurs plus importants qui façonnent l'abondance, la diversité et l'activité du plastisphère par rapport à la taille et à la forme du matériau.

***Configuration de l'expérience :***

Trois types de matériaux (PE, PLA et verre) sous formes de films de 1,8mm, 3mm et sous forme microbilles sphériques ou irrégulières de 100 µm ont été placés dans 12 aquariums en verre identiques d'une capacité de 2 L, dans lesquelles l'eau de mer était continuellement renouvelée par un pompage dans la baie de Banyuls sur mer (Mer Méditerranée nord occidentale). Trois autres aquariums supplémentaires ont été utilisés comme contrôles contenant uniquement de l'eau de mer en circulation. Les aquariums ont été placés sous un rythme de lumière / obscurité de 12h /12h. L'expérience a débuté le 13 août 2019 et des prélèvements ont été effectués après 3, 10, 30 et 66 jours. Tout au long de l'expérience, la température de l'eau de mer était comprise entre 17,5 ° C et 18,5 ° C. Les paramètres suivis ont été l'abondance bactérienne (microscopie

confocale), l'activité bactérienne (incorporation de leucine $^{3}H$) et la diversité bactérienne (séquençage ARNr 16s Illumina Miseq).

***Principaux résultats :***

***Microscopie confocale :*** Des triplicats ont été analysés par microscopie confocale afin de suivre les changements d'abondance bactérienne pour tous les types et tailles de matériaux. Les données ont mis en évidence 3 phases distinctes de la formation du biofilm : la primo-colonisation, la croissance et la maturation (Chapitre 2, Figure 3). Les trois phases distinctes ont été trouvées quel que soit le type ou la taille du matériau. Une ananlyse ANOVA a révélé une différence significative l'abondance bactérienne selon la date d'échantillonnage et le type de matériau, mais pas selon leur taille ($R^2$ = 0,29, 0,29 et 0,01 respectivement). Le PE présentait en moyenne l'abondance bactérienne la plus élevée avec le PLA, alors qu'il était 10 fois plus faible sur le verre. Une primo-colonisation rapide a été observée après 3 jours, avec une abondance moyenne de respectivement $3,0 \times 10^3$, $1,6 \times 10^3$ et $0,3 \times 10^3$ cellules $mm^{-2}$ pour le PE, le PLA et le verre. Une légère croissance a été observée après 10 jours par rapport aux valeurs du jour 3 pour le PE, le PLA et le verre, où l'abondance mesurée sur l'échantillon de verre est restée relativement faible ($8,6 \times 10^3$, $2,8 \times 10^3$ et $0,9 \times 10^3$ cellules $mm^{-2}$, respectivement). L'abondance bactérienne avait une augmentation significative de 10 jours à 30 jours ($p < 0,05$) qui atteignait la phase de maturation correspondant à la stabilisation du nombre de bactéries, sans changement significatif du nombre de bactéries jusqu'au jour 66 ($p > 0,05$). En moyenne, le biofilm mature était de $2,5 \times 10^4$, $1,5 \times 10^4$ et $0,2 \times 10^4$ cellules $mm^{-2}$ pour le PE, le PLA et le verre, respectivement, avec une différence non significative entre la taille et les formes dans chaque type de matériau. L'abondance bactérienne a eu une augmentation significative de 10 jours à 30 jours ($p < 0,05$) qui a atteind la phase de maturation correspondant à la stabilisation du nombre de bactéries, sans changement significatif du nombre de bactéries jusqu'au jour 66 ($p > 0,05$). En moyenne, le biofilm mature avait une abondance de

$2,5 \times 10^4$, $1,5 \times 10^4$ et $0,2 \times 10^4$ cellules $mm^{-2}$ respectivement pour le PE, le PLA et le verre avec une différence non significative entre la taille et les formes dans chaque type de matériau. L'abondance bactérienne avait une augmentation significative de 10 jours à 30 jours ($p < 0,05$) qui atteignait la phase de maturation correspondant à la stabilisation du nombre de bactéries, sans changement significatif du nombre de bactéries jusqu'au jour 66 ($p > 0,05$). En moyenne, le biofilm mature était de $2,5 \times 10^4$, $1,5 \times 10^4$ et $0,2 \times 10^4$ cellules $mm^{-2}$ pour le PE, le PLA et le verre, respectivement, avec une différence non significative entre la taille et les formes dans chaque type de matériau.

Des diatomées n'ont été observées qu'après 66 jours sur tous les matériaux, indiquant une efflorescence de diatomées entre J30 et J66.

***Production bactérienne hétérotrophe*** *:* L'activité spécifique bactérienne a été calculée en divisant la production bactérienne hétérotrophe par l'abondance bactérienne par unité de carbone incorporé par cellule et par heure ($fgC.cell^{-1}.h^{-1}$). Une analyse ANOVA a également montré que tous les facteurs (date d'échantillonnage, type de matériau et taille) pouvaient avoir un impact significatif sur l'activité spécifique, le type de matériau expliquant plus de variation que la date d'échantillonnage et la taille du matériau ($R^2 = 0,08$, 0,27 et 0,07, respectivement). L'activité spécifique du PE s'est montrée significativement plus élevée que celle du PLA et du verre (Chapitre 2, Figure 4B), ce qui a confirmé que le type de matériau pouvait influencer l'activité spécifique bactérienne sur les plastiques. L'activité spécifique du PE, du PLA et du verre dans la moyenne des deux premières dates d'échantillonnage (jour 3 et jour 10) était respectivement de 1,9, 0,3 et 0,4 $fgC.cell^{-1}.h^{-1}$, diminuant au jour 30 (0,45, 0,15 et 0,20 $fgC.cell^{-1}.h^{-1}$, respectivement),

***Indices de diversité*** *:* Les analyses globales de PERMANOVA avec tous les échantillons ont confirmé que la variance était fortement expliquée par la date d'échantillonnage, dans une moindre mesure par le substrat et le facteur de taille (respectivement $R^2 = 0,39$, 0,14 et 0,05 p

<0,01). Les analyses PERMANOVA ont confirmé que l'évolution temporelle du biofilm expliquait de manière significative les changements de structure de la communauté bactérienne au sein de chaque comparaison, avec des valeurs plus élevées trouvées entre la phase de colonisation initiale (primo-colonisation ou phase de croissance) et le biofilm mature à J66 ($R^2$> 0,37, p <0,01). Des différences plus faibles mais significatives ont été trouvées entre les types de substrats, la communauté bactérienne est similaire entre le PLA et le verre que celle du PE. Cependant, aucune différence significative entre la taille du matériau (y compris les formes) n'a pu être trouvée (p> 0,05), ce qui était également étayé par des analyses de dispersion (p> 0,05).

Les analyses taxonomiques ont confirmé la spécificité des structures communautaires formées sur les différents matériaux par rapport à l'eau de mer, la plastisphère bactérienne était principalement composé d' Alphaprotéobactéries, de Gammaprotéobactéries, de Bactéroïdètes et de Planctomycètes tout au long de l'expérimentation (Chapitre 2, Figure 6). Les séquences eucaryotes étaient toujours <4% avant le jour 66 sur ces matériaux et représentaient <0,7% tout au long de l'expérience en eau de mer. Plus de 50% et jusqu'à 86% des séquences eucaryotes appartiennent à la diatomée *Pseudo-nitzschia* sp. au jour 66 pour PE, PLA et verre, alors que les séquences de *Pseudo-nitzschia* sp. dans l'eau de mer est resté relativement très faible (<10%).

***Discussion :***

Au cours des trois phases de son développement, le biofilm a évolué de manière assez différente sur les deux polymères étudiés (PE et PLA) et le verre. Le PLA et le verre étaient plus similaires en termes d'activité et de diversité bactériennes que le PE, tandis que le PE et le PLA avaient une abondance bactérienne similaire et supérieure par rapport au verre. En général, le PE a montré une différence importante par rapport au verre, tandis que le PLA a montré la position intermédiaire entre eux. Les résultats soulignent également que l'activité bactérienne pourrait ne pas être corrélée à son abondance sur la plastisphère, qui est similaire à celle trouvée dans l'eau de mer (Campbell et al., 2011). Cela tend à confirmer le rôle des propriétés de

l’hydrophobicité des surfaces des polymères qui sont beaucoup plus hydrophobes que le verre. L'attachement à la surface est en effet supposée être médié via des interactions spécifiques et non spécifiques, toutes deux dépendant de l'hydrophobicité / hydrophilie de la surface, de la rugosité, de la charge et des groupes fonctionnels.(Caruso, 2020). Le rôle de la dureté ne peut cependant être exclu car il a récemment été démontré qu'il était également un facteur clé par rapport à d'autres propriétés physico-chimiques (Cai et al., 2019). Nos résultats ont également montré que la diversité et l'activité des biofilms matures sur les plastiques peuvent être rapidement et radicalement modifiées en raison de la croissance phytoplanctonique sur les plastiques, quels que soient le type, la taille ou la topographie du polymère.

## *Chapitre 3. Les microplastiques sont-ils nuisibles ou bénéfiques pour le céphalochordé filtreur Branchiostoma lanceolatum ?*

**Résumé :**

L'objectif de ce chapitre est d'étudier l'impact des microplastiques de polystyrène sur le céphalochordé filtreur amphioxus (*Branchiostoma lanceolatum*). Nous avons émis l'hypothèse qu'amphioxus pourrait être sensible à la pollution microplastique. Nos recherches révèlent que les microplastiques de polystyrène ont un impact limité sur amphioxus, voire même être un vecteur de transport de nutriments. Au cours de cette étude, aucune modification significative n'a été observée sur le stress oxydatif de l'amphioxus, le système immunitaire, l'apoptose et la communauté du microbiote intestinal, tandis que le transfert potentiel de taxons bactériens a été observé du microplastique au microbiote intestinal, et le microplastique pourrait également induire plus de cellules de gobelet vers l'intestin pour la sécrétion de mucus.

***Configuration de l'expérience :***

Les amphioxus ont été collectés en octobre 2019. Tous les amphioxus d'une taille de 3,7 ± 0,2 cm ont été répartis uniformément dans des aquariums identiques avec 35 individus pour chaque réservoir, pour lesquels contient 2L d'eau de mer (0,2 μm filtrée), les aquariums ont été placés dans l'obscurité placard et éclairé par le haut avec un rythme clair / sombre de 12/12 h. La température a été maintenue à 16,9℃, au cours de l'expérience, l'eau de mer filtrée a été changée tous les deux jours.

Les amphioxus ont été sacrifiés à partir de 3 aquariums après une semaine de diète, représentant le contrôle avant l'exposition aux microplastiques (Control_D0, ci-après). 12 autres aquariums ont été divisés en 4 groupes, y compris une concentration et un contrôle d'exposition aux microplastiques élevés, moyens, faibles, correspondant à 5000, 500, 50 et 0 particules par litre

(les amphioxus ont été désignés comme High_Con, Middle_Con, Low_Con et Control respectivement, ci-après). Il est à noter que le microplastique de polystyrène (PS ; environ 63 µm) utilisé dans cette étude a été immergé dans l'eau de mer pendant un mois pour mimer le plus possible le biofouling existant en milieu naturel. L'exposition a duré 16 jours avec bullage.

Les paramètres ont été suivis sur l'ingestion de microplastiques (quantification microscopique), l'expression génique sur le système immunitaire, le stress oxydatif et l'apoptose (Nanostring), la modification du microbiote intestinal (séquençage ARNr 16s Miseq) et aussi l'histopathologie (TEM).

***Principaux résultats :***

***Changement de la viabilité de l'amphioxus et de la taille du corps :*** L'intégrité de la peau buccale et abdominale a été vérifiée pour la mortalité de l'amphioxus. La morbidité a été observé pour la première fois pour l'amphioxus lors du 16e jour d'exposition au plastique. Le contrôle avait un nombre de mortalité plus élevé à 3, 5 et 9 individus sur 35 pour les 3 répétitions, respectivement ; 2, 2 et 4 individus pour une concentration d'exposition élevée (High_Con) ; 2, 3 et 6 individus pour une concentration d'exposition moyenne (Middle_Con) ; 0, 2 et 4 individus pour une faible concentration d'exposition (Low_Con) alors que le résultat n'était pas significatif pour le test ANOVA (valeur $p > 0,05$) (Chapitre 3, Figure 3B). L'hypothèse que nous avançons à partir de ces résultats est qu'Amphioxus pourrait profiter de la nutrition du biofilm pour soutenir sa survie. La taille corporelle de l'amphioxus a été mesurée avant et après l'exposition aux microplastiques. Les dimensions médianes de 3,7 cm, 3,5 cm, 3,5 cm, 3,2 cm, 3,2 cm étaient pour le traitement avant exposition microplastique (Control_D0) et après exposition microplastique de High_Con, Middle_Con, Low_Con et Control respectivement. Les traitements Control_D0, High_Con, Middle_Con ont la plus grande longueur de corps par rapport à Low_Con et Control (valeur $p < 0,05$) (Chapitre 3, Figure 3C).

# *French abstract*

***Microbiote intestinal :*** Le test PERMANOVA a montré qu'il n'y avait pas de différence significative pour la comparaison par de paire de 4 groupes de traitement après exposition aux microplastiques (p> 0,05). Les Flavobactéries et les Gammaprotéobactéries constituaient la principale composition taxonomique après exposition aux microplastiques, représentant respectivement 65 ± 5% et 18 ± 4%. Les Gammaprotéobactéries et les Alphaprotéobactéries constituaient la principale composition taxonomique de Control_D0, représentant 52 ± 21% et 39 ± 6% (Chapitre 3, Figure 4). Les séquences eucaryotes ont également été vérifiées pour des échantillons microplastiques et il s'est avéré que les séquences eucaryotes représentées pour 4 ± 2% des séquences totales. *Peudo-Nitzschia* sp. représentaient 84 ± 10% des séquences totales eucaryotes.

***Transfert bactérien potentiel :*** 12 ASV ont été trouvés comme transfert bactérien potentiel du plastique à l'intestinal d'amphioxus, alors que toutes ces ASV appartenaient à une biosphère rare avec une abondance moyenne inférieure à 0,1% (Chapitre 3, Figure 7).

***Expression gènique :*** 62 gènes ont été sélectionnés au cours de cette étude, dont 3 gènes sur le métabolisme, 9 gènes sur l'apoptose, 20 gènes sur la réponse au stress (induction de stress et stress antioxydant), 26 gènes sur le système immunitaire (adaptateur, effecteur, système complémentaire et explosion oxydative) et 4 gènes de ménage. Les résultats ont montré qu'il n'y avait pas de modification évidente après l'exposition aux microplastiques pour l'expression génique de l'apoptose, de la réponse au stress et du système immunitaire (Chapitre 3, Figure 8).

***Observation histopathologique :*** La microscopie électronique à transmission a montré qu'il y avait plus de cellules de gobelet présentées sur les groupes High_Con et Middle_Con par rapport aux groupes contrôle, indiquant que l'ingestion de microplastiques rendra le tissu hépatique pour se différencier en plus de cellules de gobelet et favoriser la sécrétion de mucus (Chapitre 3, Figure 10).

***Discussion :***

Cette étude a montré que les conditions physiologiques des groupes d'amphioxus exposés au plastique présentaient des expressions géniques et des réponses physiologiques similaire à celle du témoin. Aucun gène du stress oxydatif, du système immunitaire et de l'apoptose n'ont été exprimés de manière différentielle après une exposition aux microplastiques. Les microplastiques de polystyrène semblent ne pas agir comme antigène ou facteur pro-inflammatoire, comme ce qui avait été suggéré dans des travaux antérieurs (Ašmonaite et al., 2018). Les microplastiques n'ont pas apporté de modification significative non plus sur le microbiote intestinal. La raison pourrait être due au fait que le microbiote intestinal de l'amphioxus dépend de l'état physiologique de l'hôte, qui n'a pas été impacté comme l'ont révélé les tests d'expression génique. En revanche, le transfert potentiel de taxons bactériens et une cellule de gobelet supérieure a été observé après exposition aux microplastiques. Les résultats suggèrent donc que les microplastiques de polystyrène (63 µm) ont un impact limité sur l'amphioxus.

Amphioxus régurgite les microplastiques de trop grande taille qui pourraient ne pas passer à travers l'intestin (Lacalli et al., 1999). Ainsi, le phénomène aidera amphioxus contre des microplastiques de plus grande taille, évitant potentiellement le blocage intestinal par des microplastiques. Au cours de notre étude, le diamètre du microplastique avait un pic à 63 µm, et amphioxus peut efficacement éliminer les microplastiques. Bien que nous n'excluions pas l'impact négatif sur l'amphioxus, en particulier lorsque le microplastique a une taille inférieure à 2 µm et donc impacter l'organisme par endocytose et/ou transport passif lorsqu'il atteind la taille des nanoparticules (He et al., 2018).

## *Chapitre 4. Biodégradabilité de polymères biosourcés ou non comme substituts aux microbilles conventionnelles utilisées dans la cosmétique*

**Résumé :**

L'objectif de ce chapitre est de démontrer que des polymères biodégradables peuvent être des substituts potentiels aux microbilles conventionnelles utilisées dans les produits cosmétiques et les soins personnels. Nous avons émis l'hypothèse que les polymères biosourcés pourraient être plus dégradables dans l'environnement marin. Cette étude fournit pour la première fois des arguments en faveur de l'utilisation de certains polymères biosourcés (tels que le PHBV, le PCL, les graines de riz et le noyau d'abricot), mais pas tous, comme substituts des microbilles conventionnelles pour soutenir les récentes règles législatives visant à réduire la pollution par les microplastiques primaires dans les océans.

***Configuration de l'expérience :***

Une expérience originale en deux phases a été conçue afin d'évaluer la biodégradabilité des polymères conventionnels à base de pétrole et biosourcés en conditions marines (Chapitre 4, Figure 1). La première étape a consisté à former un biofilm mature dans l'eau de mer naturelle. Brièvement, chaque microparticule (dénommée ci-après « microbilles ») a été incubée pendant une période de 2 mois dans un aquarium de 1,8 L avec une circulation directe vers la mer (Baie de Banyuls sur mer, Méditerranée nord occidentale). Chaque aquarium contenait 12 grammes de chaque type de microbilles (PE, PMMA, PCL, PLA, PHBV, abricot et riz) qui ont été mis le 7 juillet 2017 pour une durée de 2 mois. Tout au long de l'expérience, la température de l'eau de mer (entre 25,3 et 18,3 ° C) et la salinité (38,5) dans les aquariums étaient similaires à l'eau de mer de la baie de Banyuls.

Deuxièmement, environ 10 mg de microbilles sèches ont été transférés dans des conditions stériles dans des flacons en verre fermés de 4,9 ml contenant 2 ml de milieu minimum avec des microbilles comme seule source de carbone (appelé ci-après « MM »). Les flacons ont été incubés à l'obscurité à 18°C sous agitation à 110 rpm (agitateur orbital) pendant une période de 2 mois (ci-après « condition biotique »). De plus, les contrôles ont été incubés et échantillonnés de la même manière en triplicat contenant 2 ml de MM avec des microbilles de la même composition préalablement stérilisées pendant une nuit dans de l'éthanol à 70% et une évaporation sous hotte stérile aux UV (conçue ci-après comme « condition abiotique »). Un total de 130 flacons ont été nécessaire pour suivre les différents paramètres détaillés ci-dessous prélevés en triplicat après 0, 1,3, 7, 15, 30 et 60 jours d'incubation.

Les paramètres suivis ont été la perte de poids (méthode gravimétrique), la consommation d'oxygène (capteur d'oxygène), le changement de poids moléculaire (SEC), le changement d'indice carbonyl (FTIR) et la libération d'oligomères (spectroscopie RMN 1H et spectrométrie de masse).

***Principaux résultats :***

***Dynamique temporelle de la consommation d'oxygène :*** Les 10 premiers jours, le pourcentage de biodégradation (basé sur le rapport de la consommation d'oxygène sur la demande théorique en oxygène correspondante à une dégradation complète) étaient de 4,1 ± 0,4% (moyenne et écart type), 1,0 ± 0,1%, 3,8 ± 1,9% et 0,5 ± 0,2 % pour PHBV, PCL, riz et abricot respectivement. D'autres polymères, dont le PE, le PMMA et le PLA, n'ont montré aucune consommation d'oxygène pendant toute la durée de l'incubation de 2 mois (Chapitre 4, Figure 3)

***Perte de poids :*** La mesure de la perte de poids entre le début et la fin de l'incubation en milieu minimum a séparé les polymères en 2 groupes. Aucune perte de poids n'a été trouvée sur le

polymère pour le PE, le PLA et le PMMA. L'abricot a montré 5,5 ± 8,8% de perte de poids, lorsque PCL, PHBV montrant environ 17,8 ± 7,2% et 17,0 ± 6,1%. Le riz a eu la perte de poids la plus élevée avec 80,1 ± 4,8%.

***Modifications du poids moléculaire :*** Des signes significatifs de dégradation n'ont été observés que pour les PCL qui ont montré une diminution de 30% du poids moléculaire moyen (Mn) (de 33000 à 23000 g mol-1) et une augmentation de leur indice de polydispersité (1,7 à 2,0) qui indique une dispersion de poids moléculaire plus élevé. Aucun changement significatif n'a été trouvé pour les microbilles de PE, PMMA et PLA. La mesure du poids moléculaire n'était pas applicable pour le PHBV, le riz et l'abricot.

***Modification des propriétés chimiques :*** La composition des microbilles incubées dans des conditions biotiques a été contrôlée par FTIR après 2 mois d'incubation dans MM. Aucune biodégradabilité n'a été trouvée pour le PE, le PMMA et le PLA.

Les spectres FTIR des microbilles PCL ont montré une diminution de l'indice carbonyle et une augmentation concomitante de l'indice de cristallinité. Un tel motif est typique du clivage des liaisons ester dans la région amorphe des chaînes polymères et confirme la réduction du poids moléculaire du polymère. Les microbilles de riz présentaient un spectre caractéristique de l'amidon avec des signaux spécifiques de monosaccharides et de polysaccharides. La forte modification des spectres au cours du temps a révélé une transformation de l'amylopectine constituant l'amidon, l'amylose, en monomère simple de glucose. L'analyse FTIR des microbilles d'abricot a présenté un spectre typique de matière lignocellulosique. Les spectres de ces échantillons n'ont pas changé au fil du temps, indiquant ainsi une faible transformation du matériau. Une augmentation de l'indice de cristallinité a été observée pour les échantillons de PHBV en condition biotique.

***Libération de produits lors de la biodégradation :*** Dans des conditions abiotiques, le signal RMN $^{1}H$ a été enregistré uniquement dans le surnageant d'échantillons de PCL, d'abricot et de

riz incubés dans un milieu minimum alors qu'aucun signal n'a été enregistré pour les autres matériaux (Chapitre 4, Figure 4A). L'attribution du signal confirme l'identification des oligomères PCL à chaînes courtes. Un signal typique d'hydrates de carbone tels que le glucose a été observé pour les oligomères extraits d'échantillons de riz et d'abricot. Fait intéressant, les microbilles de PHA n'ont pas libéré d'oligomères mesurables dans des conditions abiotiques, mais un signal a été enregistré en présence de micro-organismes, suggérant une transformation biotique des polymères en oligomères qui était supérieure à leur consommation (Chapitre 4, Figure 4).

***Spectrométrie de masse OrbitrapTM***

Les oligomères détectés dans le surnageant ont été analysés par spectrométrie de masse pour identifier leur composition moléculaire. L'analyse des surnageants de PCL, d'abricot et de riz confirme la présence d'un composé de faible poids moléculaire correspondant à la dégradation de la matrice polymère en oligomères de différentes tailles. En condition abiotique, l'hydrolyse ester du PCL génère des oligomères de caprolactone qui ont été identifiés comme monomère, dimère et trimère d'oligomères d'acide polycaproïque. Les spectres de masse obtenus à partir du surnageant de riz et d'abricot fournissent un profil complexe de composés de bas poids moléculaire. Les glucides et les acides aminés peuvent être détectés dans les échantillons de riz. Des produits de dégradation de la lignine et des acides gras ont été identifiés dans des échantillons de noyaux d'abricot. Enfin, les microbilles de PHBV incubées avec des micro-organismes libèrent diverses unités de dimères et trimères d'hydroxy-butyrate et d'hydroxy-valérate.

***Discussion :***

Dans cette étude, nous avons combiné le test standard (ISO 14851, 2004) avec d'autres méthodologies afin (i) de confirmer les résultats des tests standard basés sur la biominéralisation processus et (ii) pour donner plus d'informations sur les autres étapes impliquées dans la

biodégradation qui incluent la biodétérioration, la biofragmentation et la bioassimilation des polymères (Dussud et Ghiglione, 2014). Ici, nous avons observé que la spectroscopie RMN et la spectrométrie de masse fournissent des résultats originaux et très informatifs sur les fragments moléculaires des polymères qui peuvent être générés lors de leur biodégradation. Une autre nouveauté de notre étude est de proposer un protocole original en deux étapes pour tester la biodégradabilité du polymère par un biofilm mature naturel. La première étape de formation du biofilm mature a été réalisée en incubant les types de polymères dans de l'eau de mer naturelle. Cette méthode améliore grandement la méthode conventionnelle de préparation des inocula, qui n'est généralement pas représentative des milieux naturels avec des communautés similaires retrouvées pendant plusieurs mois dans l'aquarium par rapport aux conditions complexes du milieu in situ et (ii) pour permettre la formation d'un biofilm mature après 15 à 30 jours, selon les types de polymères. Cette méthode améliore grandement la méthode conventionnelle de préparation des inocula, qui n'étaient généralement pas représentative des milieux naturels.

Un nombre croissant de pays ont mis en œuvre des lois pour restreindre la production de microbilles dans le monde. Nous démontrons ici que les microbilles à base de PHBV ou de riz et dans une moindre mesure de PCL et d'abricot sont de bons candidats pour la substitution des microplastiques classiques classiquement en PE ou PMMA.

## *Chapitre 5 Discussion générale et perspective*

### *1.1 Principaux résultats de la thèse*

Cette thèse a étudié l'écotoxicité des micro-plastiques en milieu marin, principalement dans le domaine biologique. Le chapitre 2 s'est concentré sur les communautés microbiennes colonisant différents types de micro-plastiques (taille, forme et composition) en fonction du temps. Ces communautés dynamiques formant la « plastisphère » ont été étudiés. Le chapitre 3 s'est intéressé à la toxicité du polystyrène (micro-plastiques) sur amphioxus *Branchiostoma lanceolatum*. Le chapitre 4 avait pour objectif de trouver une stratégie de réduction de la pollution plastique par des polymères se substituants aux microbilles conventionnelles utilisées dans les produits cosmétiques et de soins personnels. Leur dégradabilité en milieu marin a été testée par la mesure de différents paramètres biologique, chimique et physique. Les communautés bactériennes vivant sur la plastisphère sont au cœur de cette thèse, puisque nous avons décrit sa composition au chapitre 2, leur rôle dans l'holobionte lors du test de toxicité au chapitre 3, et leur capacité à biodégrader le plastique de composition distincte au chapitre 4 (Chapitre 5, Figure 1).

### *1.2 Résultat de l'expérience du chapitre 2*

Nous avons observé 3 phases de colonisation distinctes : phase de primo-colonisation, phase de croissance et phase de maturation en termes d'abondance, de diversité et d'activité bactérienne (Chapitre 5, Figure 2). Des facteurs influençant la composition de la plastisphère ont été proposés. Les communautés bactériennes de la plastisphère et celles de l'eau de mer environnante ont été régis par des facteurs physico-chimiques dans notre expérience. Ces facteurs ont déjà été mis en évidence dans de précédentes études dans les eaux saumâtres de la mer du Nord et dans l'estuaire de la Chine (Oberbeckmann et al., 2018; Li et al., 2019).

Notre étude a montré que la composition des ASV est restée homogène entre l'eau de mer et les plastiques étudiés. Cependant une modification dans la composition bactérienne a été observée au fil du temps dans l'eau de mer et sur les plastiques. Cela permet d'émettre l'hypothèse qu'il y a un changement de communauté allant dans la même direction. Au cours de notre étude, nous proposons que la taille et la forme du plastique ne sont pas les principaux facteurs impactant la plastisphère, cela a déjà été suggéré dans des études précédentes (De Tender et al., 2017; Frère et al., 2018). Au cours de cette expérience, l'abondance bactérienne de l'eau de mer a été considérée comme non précise, rendant impossible la comparaison de l'activité bactérienne entre le plastisphère et l'eau de mer. À l'avenir s'il y a une expérience similaire à réaliser, je suggère d'utiliser cette méthode couplée à la microscopie à épifluorescence pour mesurer l'abondance bactérienne de l'eau de mer.

### *1.3 Résultat de l'expérience du chapitre 3*

Les expériences réalisées au cours de cette thèse ont montré qu'il est important de ne pas utiliser du plastique vierge, mais plutôt d'utiliser du plastique avec son biofilm mature naturellement formé dans l'eau de mer afin de mieux s'adapter aux conditions environnementales. Cela a souligné l'importance du changement de flottabilité pouvant augmenter la suspension des micro-plastique dans l'eau de mer pendant l'expérience. En effet nous avons observé que les micro-plastiques de polystyrène de 50-100 µm de diamètre avec son biofilm mature présentaient une meilleure flottabilité en eau de mer comparé à aux micro-plastiques de polystyrène vierges (Chapitre 5, Figure 3). Dans notre étude aucun effet toxique sur l'Amphioxus a été observée lorsqu'il a été mis au contact de microbilles de polystyrène. Au contraire le biofilm mature formé à la surface du polymère a probablement servi de source de nutriments à l'amphioxus, un paramètre qui n'a jamais été pris en compte par les études précédentes. Dans le futur, s'il y a une expérience similaire à faire, je proposerai de prendre du micro-plastique d'environ 30 µm de diamètre pour fabriquer tous les micro-plastiques en

suspension dans l'eau de mer. Il est important d'avoir une taille assez conséquente du micro-plastique pour observer un effet significatif du biofilm bactérien. Dans notre étude les amorces utilisées pour le séquençage (V4-5) était satisfaisante pour l'étude des procaryotes et eucaryotes. Cependant à l'avenir, je suggérerai de prendre des amorces spécifiques des procaryotes mais également des eucaryotes pour une profondeur de séquençage plus importante. Ceci dans le but d'obtenir des informations plus précises sur le transfert bactérien.

### *1.4 Résultat de l'expérience du chapitre 4*

Classiquement, la mesure de la respirométrie était réalisée selon le principe de la réduction de la pression atmosphérique (Sashiwa et al., 2018), ou par le titrage sur le $CO_2$ produit (Deroiné et al., 2015). Ces techniques classiques prennent du temps et ne conviennent pas aux expériences à grande échelle. Ici, nous avons souligné le potentiel élevé de l'utilisation d'un capteur d'oxygène (Presens) qui permet la mesure continue de la concentration d'$O_2$ pendant une expérience à court ou à long terme. La mesure est rapide, facile à mettre en place et facilement applicable en laboratoire, pouvant être mise en place pour de futurs tests de biodégradation à grande échelle. Lors de ce test les capteurs d'oxygènes ont été placés en phase liquide dans les flacons pour mesurer l'oxygène dissous. A l'avenir, je proposerai de mettre les capteurs en dehors de l'eau pour avoir la mesure de l'oxygène atmosphérique pour avoir une mesure complémentaire.

### 1.5 Perspective

Actuellement, les préoccupations à propos des micro-plastiques sont en constante augmentation. Dans cette étude, j'ai souligné certains aspects à considérer pour les études futures dans ces domaines de recherche (Chapitre 5, Figure 4). Tout d'abord, pour mieux comprendre la contribution du plastique dans le cycle biogéochimique, il serait nécessaire de réaliser des mesures de production primaire associées à des mesures d'activité bactérienne hétérotrophe et

de respirométrie, afin de tester l'augmentation ou la diminution nette du carbone sur plastisphère. Deuxièmement, les analyses métagénomiques et métatranscriptomique devraient être appliquées aux études futures pour étudier le potentiel métabolique ainsi que le processus métabolique sur la plastisphère. Troisièmement, l'interaction bactérienne entre l'eau de mer et le plastisphère devrait être mieux élucidée. Quatrièmement, la distance de dispersion de la plastisphère bactérienne dans l'océan pourrait être testée à l'aide des bouées dérivantes grâce à l'aide satellite permettant une localisation précise. Cinquièmement, la virulence des pathogènes potentiels sur la plastisphère pourrait être davantage étudiée. Enfin, la méthode par marquage isotopique du carbone du polymère (DNA-SIP) couplé à une analyse métagénomique pourrait donner plus d'informations sur le processus de biodégradation.

# *Introduction*

## 1. Microplastics and its distributions in the ocean

### *1.1 Plastic production and classification*

Plastic is prevalent in our daily life. The plastic with commercial potentiality were first manufactured from 1907 as the Bakelite (Baekeland, 1909). Plastics with the large scale of plastic production was traced back to ~1950, global production of plastic had increased from 2 million metric tons (MT) from 1950 to 380 million MT in 2015, and projected to 434 million MT in 2020. (Figure 1) (Geyer et al., 2017).

FAccording to the raw material source, plastic made from petroleum were classified conventional plastic, which mainly including the polyethylene (PE), polypropylene (PP) and polystyrene (PS). The extensive use of plastics, insufficiency of waste management, together with the high durability of conventional plastic pose a significant threat to the environments, which stimulates the development of the bio-base plastics that made from the renewable resources (Reddy et al., 2013; Ahmed et al., 2018). The manufacturing of bio-based plastic is also under tremendous increasing, total quantity reached to 2 million MT in 2019 (European bioplastics, 2019), mainly including the bio-PE, polylactide acid (PLA) and polyhydroxyalkanoates (PHA).

According to the degradability, the plastic was grouped as non-degradable, such as PE, it could come from oil-based or bio-based materials. The other group is degradable plastics, and it could also come from oil-based material, such as polycaprolactone (PCL), or come from bio-based materials, such as PHA (Ahmed et al., 2018).

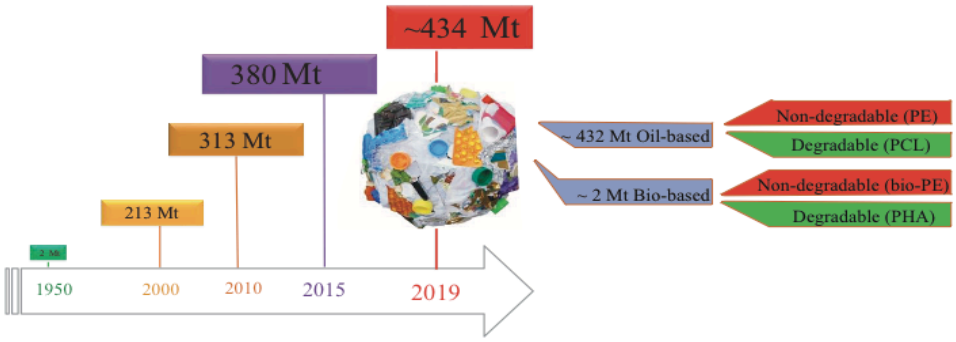


Figure 1. Plastic production and classification (modified from European Bioplastic, and Geyer et al., 2017)

## 1.2 Microplastics and Source of the oceanic microplastics

The largest market for plastic resin is in packaging, that is, materials designed for immediate disposal (PlasticsEurope, Brussels, 2017). As a consequence, considerable amount of plastic ends up into the ocean. The term of microplastic was introduced from 2004, and typically considered to be the size less than 5 mm in diameter (Thompson et al., 2004). To be more specific, the microplastic could be categorized as big microplastic (1mm-5 mm), and small microplastic (1μm-1000μm) (Imhof et al., 2012). Global oceanographical studies estimated that more than 5 trillion plastic pieces over 268,940 tons are afloat at sea, in the mass proportion, macroplastics (> 200 mm) accounts for 75.4%, mesoplastics for 11.4%, large microplastics (1-5mm) for 10.6% and small microplastics for 2.6% (in range of 330 μm-1 mm), when it comes to size fractions, macroplastics only accounts for 0.2%, mesoplastics for 7%, large microplastics for 57.5% , and small microplastics for 34.8% (Eriksen et al., 2014). Recent study demonstrated that small microplastics in the range of 25–1000 μm are much more abundant than large microplastics from the North Atlantic Subtropical Gyre, and also as the main contributor to the plastic mass balance (Poulain et al., 2019).

According to the source, microplastics in the ocean can be grouped as primary microplastics and secondary microplastics. The primary microplastics are directly transported into the marine environment in micrometer size, while the secondary microplastic experienced the fragmentation of big pieces of plastics via UV radiation, mechanical abrasion, biological degradation etc. (Imhof et al., 2012). The release of microplastics into the marine environment occurs through a variety of pathways, including road run-off, beach littering, river and atmospheric transport, or directly from sea via aquaculture, shipping and fishing activities (Duis and Coors, 2016).

According to an estimation, 1.7% to 4.6% of the total produced plastics were entered to the marine environment from the coastal countries, corresponding to 4.8 to 12.7 million MT of plastics in 2010 (Jambeck et al., 2015). Generally, it is also considered that about 75-90% of plastic debris in the marine environment originated as land-based source and about 10-25% are ocean based source, such as fishing activity (Duis and Coors, 2016). The global release of microplastics into marine environment are mainly through wastewater treatment plant (WWTP) and road runoff pathway (direct into the ocean, or entering into the river), with the synthetic textile and tyre as the main composition, respectively (Browne et al., 2011; Boucher and Friot,

2017). Personal care and cosmetic products (so-called "microbeads") could also end up into the ocean through WWTP pathway. According to a survey from 2012, a total amount of 4360 Metric tons of microplastic used across all European Union countries, plus Norway and Switzerland, representing for 0.1-1.5% of total plastic debris emitted to the North Sea marine environment (Gouin et al., 2015). Recent study showed the presence of microplastics in the atmosphere from the remote ocean and coastal area, indicating the atmosphere microplastic could be another important source of microplastic pollution in the ocean (Liu et al., 2019a). Lastly, the microplastic could also come from the raw material of plastic resin pellets mainly due to mismanagement (Mato et al., 2001).

## *1.3 The occurrences of microplastics in the ocean*

After the plastics end up into the ocean, based on plastic density, it could be floated in the surface, suspended in the ocean column or settled to the bottom of the ocean. Polyethylene and polypropylene are main composition in the surface layer and shorelines (Bond et al., 2018). Plastic in the ocean surface is very patchy, and mostly accumulates in the convergence zones of the each of the five subtropical gyres with comparable density (North Pacific, North Atlantic, South Pacific, South Atlantic, Indian Ocean), and enclosed sea (Mediterranean Sea), the microplastic with the highest concentration in the surface water are from the Mediterranean sea and North Pacific with more than $10^8$ pieces per square kilometer (Figure 2) (Eriksen et al., 2014; Van Sebille et al., 2015), around half of the microplastic afloat in subtropical gyres (Van Sebille et al., 2015). Evidence also showed that the subtropical gyre is rapidly accumulating plastics (Lebreton et al., 2018), while any global estimation of total accumulated floating microplastic debris only accounted for 1% or less of the amount of plastic waste emitted into the ocean annually (Jambeck et al., 2015; Van Sebille et al., 2015). The speculation for the plastic distribution is that surface waters are not the final destination for buoyant plastic debris in the ocean. Nano-fragmentation, predation, biofouling, or shore deposition have been proposed as possible mechanisms of plastic removal from the surface (Cózar et al., 2014). Previous study also emphasized that most of the ocean surface is under-sampled for microplastics, uncertainties in the Southern hemisphere basins illustrate the lack of data even in the high concentration subtropical gyre (Van Sebille et al., 2015).

As most of the plastic is missing from the sea surface, it is estimated that seafloor is the area accumulating majority of the plastics (Thompson et al., 2004). The seashore or littoral sediment

is also the accumulating the microplastics, average of 60 and 128 microplastic particles per kg sand (around 0-1.8 cm depth) were found between low tide and high tide line from coast of the southeastern United states and Bohai sea of China, respectively (Yu et al., 2016, 2018). Average of 1445 microplastic particles per kg littoral sediment (0-5 cm depth) were found in the from northeastern Italian coast (Vianello et al., 2013), 141-461microlastics particles per kg littoral sediment (around 0-2 cm depth) were found from the littoral zone of the north Tunisia coast (Mediterranean Sea) (Abidli et al., 2018). In summary, the beach and the littoral sediment are highly contaminated by the microplastics.

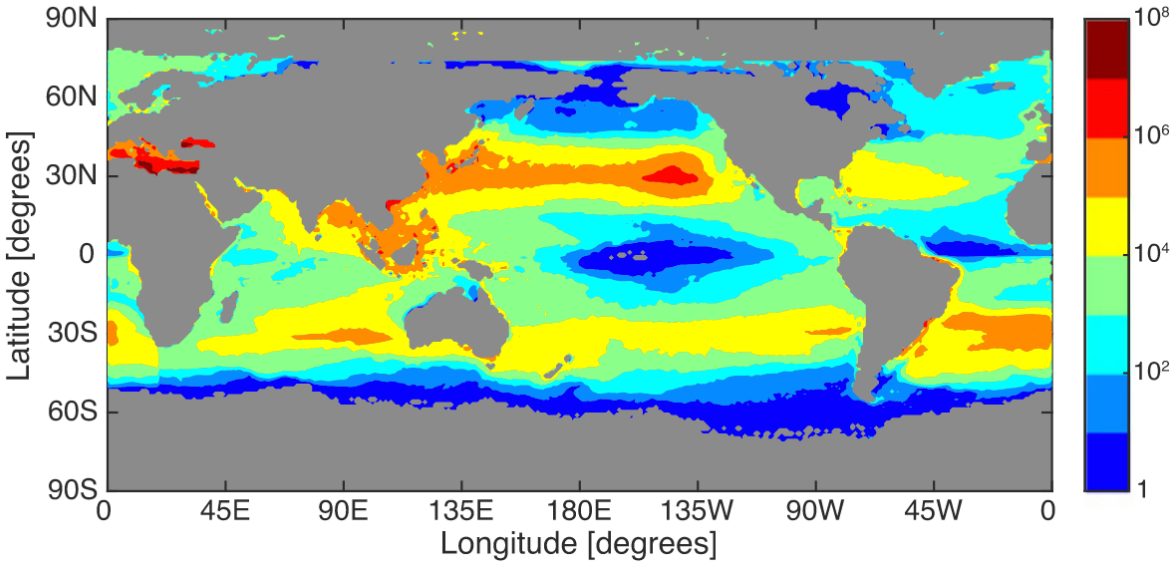


Figure 2. Maps of microplastics distribution in the surface ocean (unites in $km^{-2}$). Lebreton model was used for estimation (Van Sebille et al., 2015)

Deep-sea sediment is also considered as the area for most of microplastic accumulation. Study showed that the microplastics had the average of range of 1 microplastic per 25 $cm^2$ sediment in depth from 1176 to 4844 m depth spanning from Atlantic Ocean to Mediterranean Sea. Another study reported the average of 13.4 microplastics in 50 $cm^2$ from 900 to 3500 m depth sediment of Atlantic Ocean and coastal sea of Spain (Woodall et al., 2014). Recent study also showed that the distribution of seafloor microplastic were controled by the thermohaline-driven currents, the highest concentration for one hotspot reached to 1.9 million pieces $m^{-2}$ from the middle Mediterranean Sea (Kane et al., 2020).

Beside the above-mentioned microplastic accumulation area, biota represent another reservoir due to the microplastic ingestion. Substantial microplastics were estimated to be ingested by

fish from North Pacific ocean with 12 to 24 thousand tons per year (Davison and Asch, 2011).

The microplastics is encountered at nearly all ocean compartments, while we could not draw a clear picture on the microplastic distribution with available research. In addition to the limited data for the ocean surface, the research on the sediment microplastic is just commenced. More field sampling and analyses are required.

# 2 Microbes and "plastisphere"

## *2.1 Microbes in the ocean*

Marine microbial community (consisting of bacteria, archaea, protists, fungi and viruses) process about one-half of the global biogeochemical flux of biologically important elements, such as carbon, nitrogen, phosphorus, sulphur and iron (Arrigo, 2005; Fuhrman et al., 2015). These organisms include phototrophic and chemotrophic primary producers, as well as heterotrophic 'secondary' producers, which recycle dissolved organic carbon and nutrients. A proportion of the fixed carbon is not mineralized but instead stored for millennia as the recalcitrant dissolved organic matter through the microbial loop (Jiao et al., 2010). Remarkable discoveries during the past 30 years have shown that bacteria dominated the abundance, diversity and metabolic activity of the ocean (Azam and Malfatti, 2007). The total bacterial heterotroph cells were estimated to reach $1\times 10^{29}$ in the ocean (Buitenhuis et al., 2012; Flemming and Wuertz, 2019). Considering the microenvironment of 1 $mm^{-3}$, it contains 10,000 viruses, 1,000 bacteria, 100 *Prochlorococcus cells*, 10 *Synechococcus cells*, 10 eukaryotic algae and 10 protists, although the numbers are highly variable (Azam and Malfatti, 2007).

## *2.2 Canonical bacterial colonization process*

Research argues that most bacteria (if not all) are capable of forming a biofilm and a large fraction of their lifetime is probably spent in the biofilm. The biofilm were considered as the microbial development stage, analogous to the microbial spore formation (Monds and O'Toole, 2009). It is estimated that 40-80% of prokaryotes residing in biofilm, and drives all biogeochemical process (Flemming and Wuertz, 2019).

The formation of biofilm had been viewed as the processed governed by the chemical and physical process (such as hydrophobicity and/or surface charge) imposed by the solid surface

and composition of bacterial cell surface (Teughels et al., 2006). The advance of the genetic characterization revealed that it could be an active bacterial colonization process with the genetic pathway dedicated for the surface attachment regulation (Monds and O'Toole, 2009). Recent study strongly support that bacteria can indeed sense the complex surface topographies, and then reside on the favorable area (O'Toole and Wong, 2016).

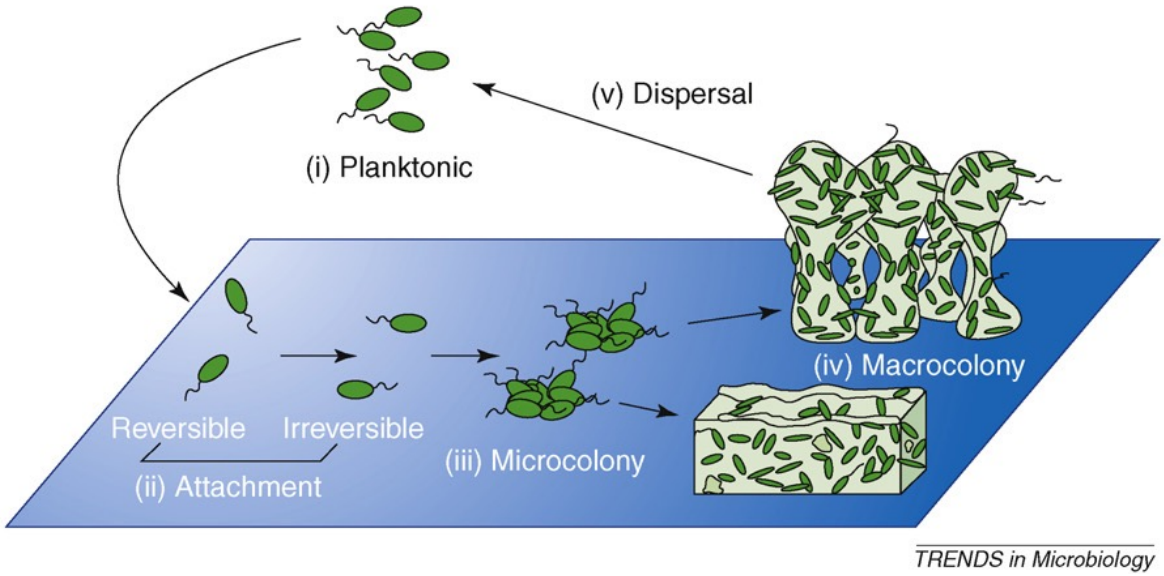


Figure 3. Developmental model of biofilm formation. These stages have been operationally identified as (i) planktonic, (ii) attachment, (iii) microcolony formation, (iv) macrocolony and (v) dispersal (Monds and O'Toole, 2009).

For the formation of biofilm, it is composed of several processes:

- Formation of the conditional film. When a substratum surface is immersed in an aqueous environment, in which the organic material is presented. The surface is immediately (within seconds) become covered by a layer of adsorbed, organic molecules. This is commonly called the 'conditional film', mainly composed of dissolved organic matters and humic substances in seawater environment (Loeb and Neihof, 1975; Teughels et al., 2006).
- The bacteria could use the two-component signal transduction systems and chemotaxis to constantly sense and response to environmental change or stress, such as organic, inorganic nutrients, pH, light intensity, etc. Bacteria could detect the signal gradients between plastic surface and seawater, and further approach the plastic surface (Dang and Lovellc, 2015). Investigators postulated that the inorganic phosphate functionalize as a signal for the 'surface' (O'Toole and Wong, 2016). As an example of active surface colonizer, *Pseudomonas*

*aeruginosa,* as the most researched model for bacterial colonization, utilize the flagella and type IV pili for sensing the surface, after contacting with the surface, the bacteria move forward in the switching motility by the type IV pili, commitment repression of flagellar-dependent swarming motility. During the sensing process, the signal was transduced into the cell with multiple gene expression changes. After arriving at the optimal place, the attachment was switched from reversible to irreversible mode (Toole et al., 2000; O'Toole and Wong, 2016).

- The development of the biofilm includes the formation of the microcolony and macrocolony. The formation of microcolony results from the cell division or the later-joined bacteria. During this phase, bacteria increase the secretion of extracellular polymeric substances (EPS) and gradually form mature biofilm, which could partially explained by the cell to cell communication by quorum sensing (QS) ( Watnick and Kolter, 2000; Monds and O'Toole, 2009). The main composition of biofilm is exopolysaccharides, protein and DNA (Whitchurch et al., 2002). The mature biofilm is a hallmark structure with the interstitial voids or channels. It is north worthy that the composition of biofilm is heterogeneity even for the biofilm coming from the single strain, the bacteria cooperate and perform different metabolite function from the stratified biofilm structure ( Davey and O'toole, 2000; Branda et al., 2005).
- Finally, the bacteria could also detach from the biofilm, one possible signal for detaching may be due to starvation mediated by QS, while available information for the genetic process is limited (Davey and O'toole, 2000).

The biofilm in the nature environment was discovered from 1932 (Henrici, 1932), while the understanding of the genetic mechanism just began from the last three decades. To reduce the complexity, the research on biofilm focus on several model strains, which make it potentially greatly distinct to the process formed in the complicated and changeable marine environments.

## *2.3 Bacterial lifestyle and adaptation*

In the marine conditions, bacteria could live in the life style of free-living, surface-associated (phytoplankton, zooplankton, organic particles or plastics), and holobiont (gut microbiota) (Figure 3) (Flemming and Wuertz, 2019; Hurst, 2019).

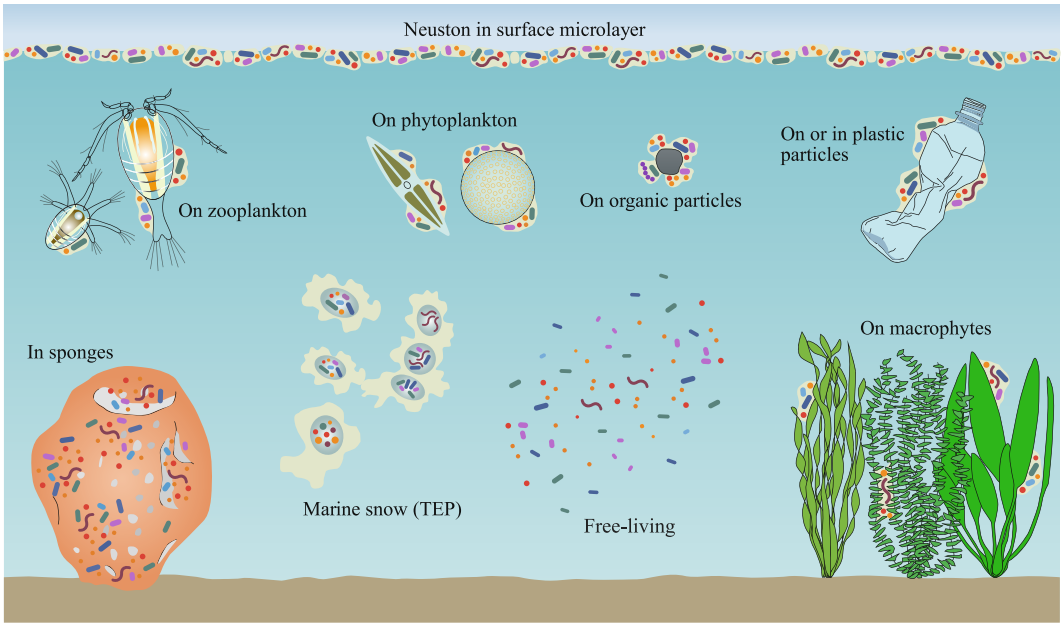


Figure 3. Different bacterial lifestyles in the marine environment (modified from Flemming and Wuertz, 2019).

### 2.3.1 Free-living lifestyle

For free-living bacteria, Grossart *et al.* considered as the bacterial groups which spend their while life cycle as the individual cell (Grossart, 2010). The free-living bacteria have developed several strategies to survival. It tends to be streamlined both in cell size and genome size to reduce the expenditures for the maintenance energy and duplication, and their vulnerability to grazing (Hurst, 2019). SAR11 is the representative for the free-living bacteria. They are the most abundant bacteria (approximately 25% of all bacterioplankton). The SAR11 have the smaller bacterial size, and are generally regarded as the defense generalist. In another point, bacteriochlorophyll and proteorhodopsin are the common feature of free-living bacteria, since these pigments could be involved in getting extra light energy source to cope with extreme oligotrophic condition, the proteorhodopsin was also found to be continuously expressed for bacteria of SAR11 (Kirchman, 2016; Giovannoni, 2017).

### 2.3.2 Surface-associated lifestyle

Surface-associated bacteria have some different traits compared to free-living bacteria (Dang and Lovellc, 2015). Surface-associated bacteria have bigger cell compared to free-living one, it has been proposed that the surface-associated microorganisms are mainly copiotrophic, whereas free-living bacteria are mainly oligotrophic. The mobility and chemotactic behaviors

are considered essential for surface-associated bacteria to reach the nutritious microhabitats.

There are several advantage aspects for surface-associated lifestyle. Surface colonization and the formation of biofilm provides bacteria the shielding matrix, which provide the protection from predators, viruses, antibiotic and the environmental stresses such as UV radiation, pH shifts, osmotic shock (Davey and O'toole, 2000; Matz et al., 2008). Moreover, bacteria with surface-associated lifestyle possess a wild repertoire of genes coding for membrane attachment and extracellular enzyme for digestion of phytoplankton EPS (Hurst, 2019). The elaborate architecture of biofilm could also provide the opportunity of metabolic cooperation, gene exchange (via horizontal gene transfer) (Davey and O'toole, 2000; Madsen et al., 2012).

Cell density in bacterial biofilm tends to be several orders of magnitude higher than the free-living bacteria. In another perspective, there are also competition within the biofilm. To acquire limited resource and space on the surface, more than 50% of bacteria were found to have the antagonistic activity, which was more common for particles-associated bacteria than the free-living bacteria. Members of *Alteromonadales* are one of the most prolific producers for inhibitory materials (Grossart et al., 2004; Long and Azam, 2001). For instance, member of marine *roseobacter* clade were characterized to be an important surface-associated bacteria, and it can produce antimicrobial substance, such as tropodithietic acid (TDA) and indigoidine (Buchan et al., 2014). Some surface-associated bacteria have contact-dependent growth inhibition system (CDI), which is a member of type V secretion system, and could be used for the intra- and interspecies competition or to coordinate the bacteria growth within biofilm. Type VI secretion system (T6SS) is similar to a phage injection system, and it is used for deliver toxins to neighboring bacteria cell, it was presented in more than a quarter of bacteria (mainly in Proteobacteria) (Bingle et al., 2008; Hayes et al., 2010). Thus, it is reasonable to deduce that the cooperation and competition within the biofilm will shape the bacteria diversity, and to be a greater extent, influence the biogeochemical recycling processes.

- In the marine environment, the bacteria could attach the surface of the phytoplankton, which is called the 'phycosphere', the bacteria could form mutualism or competition relationship with the phytoplankton. The phytoplankton-associated bacterial community are often restricted within a handful of groups, including the specific number of the Roseobacter clade, Flavobacteraceae, and Alteromonadaceae (Seymour et al., 2017). For the mutualism point of view, the phytoplankton such as diatoms will provide the essential organic compounds to the bacteria, such as dimethylsulfoniopropionate (DMSP), inversely, the bacteria will provide

vitamin (such as $VB_{12}$, nitrogen or iron) to diatoms (Seymour et al., 2017). Furthermore, the bacteria could also facilitate the diatom division (Amin et al., 2015). In another point, the mutualistic bacteria such as some Roseobacter could secret the TDA and kill the algicidal bacteria (Geng and Belas, 2010). While the interaction could become competitive or antagonistic, for example, the Bacteroidetes *Kordia algicida* could infect and lead to cell lysis of diatoms (Paul and Pohnert, 2011). In addition, The diatom could also impact on the seawater bacteria community, it is revealed that diatoms explained 30% of the variance in the prokaryotic community composition in early spring in the Southern Ocean (Liu et al., 2019). Thus, the sophisticated interaction between phytoplankton and bacteria could have significant impact the biogeochemical cycle for carbon, iron, sulphur, nitrogen, etc. (Amin et al., 2012).

- Bacteria is predominant on macroaggregates (> 500 μm, such as marine snow) composed of living, senescent and dead algae, protozoan, other materials scavenged from surrounding seawater. the bacteria could also presented on microaggregates (5-500 μm, such as transparent exopolymer particles, TEP), the TEP is considered to be secreted by diatoms during their exponential growth or stationary phase (Simon et al., 2002). Bacteria were identified as the key contributor for organic matter decomposition (Fenchel, 2002). The organic particle-attached bacteria represent for 10-20% of total abundance (compared with free-living bacteria) in euphotic zone, for 4-5% of the total abundance in mesopelagic zone (~1500 m), while it has higher bacterial activity compared to free-living bacteria, notably from the Northwest Mediterranean Sea, the heterotrophic bacterial production could account for up to 78% under mesotrophic condition (Ghiglione et al., 2007; Mével et al., 2008; Nagata, 2008).
- For the detailed information of plastic-associated bacteria, we will elaborate in the following parts of the introduction.

### 2.3.3 gut-associated lifestyle

Marine invertebrate and vertebrate often host diverse gut microbial community, which could be distinguished as the allochthonous and autochthonous. The allochthonous microbiota are the transient microbiota associated with the digesta, whereas, the autochthonous microbiota colonizes the mucosal surface of the digestion tract and make up the core community (Egerton et al., 2018). The invertebrate has simpler taxonomy composition compared with vertebrate, the gut microbiota species from invertebrates span from dozens (ascidian) to thousands (coral, sponge) compared to vertebrates spanning hundreds to thousands (fish). Hence, it is proposed to take the specific invertebrate groups to investigate the interaction between the host and gut

microbiota (Chaston and Goodrich-Blair, 2010; Ghanbari et al., 2015; O'Brien et al., 2019). *Prochloron* sp. were dominant the gut microbiota of ascidian (*Lissoclinum patella*) (Tianero et al., 2015), *Endozoicomonas* sp. (*Gammaproteobacteria*) is generally the highest abundance genus in coral microbiota (Hernandez-Agreda et al., 2017). For the vertebrate of fish microbiota, *Vibrio* and *Photobacterium* were the most reported in the gut microbiota for carnivorous species, *Clostridium* is more related to the herbivorous species. Multiple factors could shape the intra- and inter-species gut bacterial community diversity, which include the life stage, trophic level, diet, season, habitat and phylogeny (Egerton et al., 2018).

In the cooperation relationship between the host and gut microbiota, each symbiont provides the goods or services to other members, while receiving the benefits that balance this cost (Incurs et al., 2004). The host could provide the nutrition for the bacteria growth and proliferation. In turn, the gut microbiota play a role in nutritional provisioning, metabolic homeostasis and immune defense (Pérez et al., 2010; Egerton et al., 2018). For instance, the bacteria could serve the vitamins to and host and provide the enzymes contributing to the breakdown of indigestible products, such as chitin, cellulose and collagen (Ringø et al., 1995). The bacteria could also aid the host to avoid the pathogens' invasion, thus, multiple bacteria stains were proposed as the probiotics in aquaculture (Balcázar et al., 2006).

The surface and gut-associated bacteria have sophisticated network, the first point could be illuminated as the gut microbiota, and it is imperative to further clarify the communication mechanism between the host and bacterial cell, and also illuminate the mechanism for the bacterial cooperation and competitiveness. Furthermore, in the marine environments, it is also important to find out the contribution of surface-associated bacteria to the biogeochemical process.

## *2.4 Zooming in on "Plastisphere"*

The term of "plastisphere" was first suggested by Zettler on 2013, and it refers to the organisms living on plastics, including the diverse microbial community, predators, and symbionts (Zettler et al., 2013). Most of the research on the plastisphere came from the plastic incubation in natural seawater (Oberbeckmann et al., 2018; Muthukrishnan et al., 2019), laboratory aquaria (Kirstein et al., 2018) or static laboratory system in containers (Ogonowski et al., 2018), and few came from the sampling in situ (Bryant et al., 2016; Dussud et al., 2018).

2.4.1 Technique for the research on the microbial community

Concerning on the technique on determination of bacterial abundance and identification of microbial eukaryotes living on plastisphere, microscopy such as scanning electron microscopy (SEM) is a powerful technique widely used on the research on plastisphere, flow cytometry is less used, because it is difficult to detach all the microbes from plastisphere due to the sticky nature of EPS (Salta et al., 2013). For the identification of microbial composition, biomarkers of 16S and 18S rRNA gene were used more common nowadays (Jacquin et al., 2019). Only one study used the metagenomic sequencing to study the metabolite potential on plastisphere from North Pacific subtropical gyre (Bryant et al., 2016).

2.4.2 A new niche for the marine microorganisms

Whatever the polymer type, recent studies emphasized the difference between the bacteria living on plastics and living in free-living state (Debroas et al., 2017) or on organic particles in the surrounding seawater (Dussud et al., 2018; Oberbeckmann et al., 2018). Similar observations have been made for fungal communities (Kettner et al., 2017).

2.4.3 Microbial community abundance and composition

The SEM and next generation sequencing data revealed distinct microbial groups on plastisphere, including bacteria, diatoms, fungi, bryozoans, dinoflagellates, radiolarians, barnacles, isopods, marine worms and marine larvaes etc. (Table 1). During the surveys by SEM, bacteria and diatoms presented on plastisphere wherever the sample from the coastal area or pelagic ocean (North Atlantic or Pacific Ocean). The diatoms had different abundance, ranging from several counts to thousands per square millimeter (Table 1). The bacteria had the counts of thousands per square millimeter (Carson et al., 2013; Dussud et al., 2018). In some situation, the bacteria and diatom could have the comparable abundance (table 1）(Carson et al., 2013).

According to the nutritional type, the microbe on plastisphere could be grouped as phototrophs heterotrophs, predators, symbionts and saprotrophs (Figure 4).

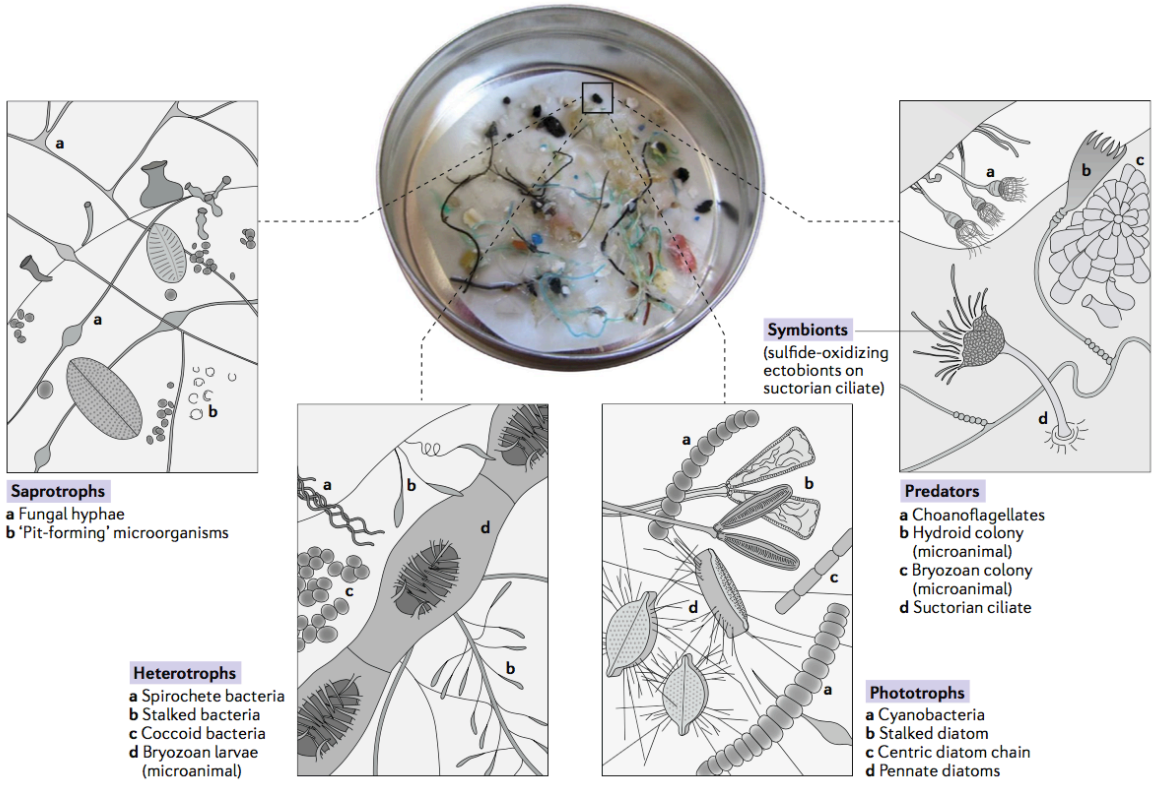


Figure 4. The plastisphere community. Conceptual model of the diverse plastisphere community, showing a microbial ecosystem of bacteria, protists and animals in the oligotrophic open ocean. Members include cyanobacteria and diatom primary producers, predatory ciliates and hydroids, grazers including ciliates and bryozoans, symbiotic relationships and heterotrophs (Amaral-Zettler et al., 2020).

- Phototrophs

***Diatoms**:* The genera of *Navicula*, *Nitzschia*, *Sellaphora*,*Stauroneis*, and *Chaetoceros* were found as plastic-associated taxa based on DNA sequencing data from the North Pacific gyre (Zettler et al., 2013). Genera of *Navicula*, *Amphora*, *Nitzschia*, *Pleurosigma* and *Thalassionema* were more omnipresent on the 4-day-old biofilm from the fiberglass and glass coupons immersed from the coastal water of India (Patil and Anil, 2005). Genera of *Nitzschia*, *Cylindrotheca*, *Navicula* and *Amphora* have been identified on 10-day-old biofilm from polystyrene petri dishes from coastal China (Chiu et al., 2008). Genus of *Nitzschia* was found as the most frequent diatom sampling from the coastal Australia sea (sample occurrence: 42.6%), followed by *Amphora* (13.2%), *Licmophora* (11.8%), *Navicula* (8.8%), *Microtabella* (5.9%), *Cocconeis* (4.4%), *Thalassionema* (2.9%), and *Minidiscus* (2.9%) (Reisser et al., 2014)., Besides, it was found that the diatom genera of *Ceratoneis* (73%), *Cocconeis* (54%), *Navicula* (50%), *Thalassionema* (42%), *Achnanthes* (23%) and *Amphora* (23%) more presented on plastic from the sampling from the Mediterranean Sea (Maso et al., 2016). Lastly, study carried

out from Caribbean Sea showed that *Cocconeis, Fragilara and Navicula* appeared on all samples, to a less extent, by *Mastogloia, Striatella and Amphora* (Dudek et al., 2020). In summary, the diatom genera of *Amphora* and *Nitzschia* could have a worldwide distribution on plastisphere from the coast to the open ocean from the surface water.

***Cyanobacteria**:* The cyanobacteria of *Synechococcus* and *Prochlorococcus* often dominate the phytoplankton biomass and primary production in the tropical and subtropical gyres from the seawater (Kirchman, 2016). Cyanobacteria species could also thrive on the plastisphere compared with seawater (10-30% of total 16s RNA sequences). *Phormidium*, and an unsigned cyanobacteria (subsection III) contributed the one of the highest sequences from the north pacific gyres (Zettler et al., 2013). Cyanobacteria were also consistently one of the highest bacteria in phylum level on plastisphere from the metagenomic SSU rRNA gene analyses carried out from the North Pacific gyres, with the taxonomy composition of *Phormidium*, *Rivularia*, and *Leptolyngbya* (Bryant et al., 2016). In addition, study from the sampling of the Mediterranean Sea showed similar results, the cyanobacteria could account for the bacterial abundance up to 40%, with the main composition of *Pleurocpsa*, *Calothrix*, *Oscillatoriales*, *Scytonema* and *Leptolyngbya* (Dussud et al., 2018). At least, it could be concluded that the genera of *Leptolygbya* will be more presented the samples from the enclosed sea to the open ocean.

From the metagenomic study, it is shown that the plastisphere have more phycobilisome antenna protein-encoding genes compared to the Chl a/b-binding light-harvesting protein-encoding genes in the surrounding water column, that means that the cyanobacteria from the plastisphere and the seawater column could have distinct light-harvesting machinery (Bryant et al., 2016).

Table 1. Observation different organisms living on plastic by scanning electron microscopy

| Studied area | Sample type | Species observed | Observation | Reference |
|---|---|---|---|---|
| North Atlantic Ocean | Sampling at sea | pennate diatoms, filamentous cyanobacteria, coccoid bacteria, and bryozoans | 50 distinct morphotypes, the most observed morphotypes were diatoms, filaments and 2µm round bacteria | (Zettler et al., 2013) |
| Northeast Pacific Ocean | Sampling at sea | Bacillus bacteria, pennate diatoms, coccoid bacteria, centric diatoms, dinoflagellates, coccolithophores, and radiolarians | Bacillus bacteria (mean 1664 ± 247 individuals $mm^{-2}$) and pennate diatoms (1097 ± 154 $mm^{-2}$) were most abundant | (Carson et al., 2013) |
| Costal Australia | Sampling at sea | Diatoms, bacteria, cyanobacteria, coccolithophores, bryozoans, barnacles, dinoflagellate, isopod and fungi | Diatoms were the most abundant, 1188 per $mm^{-2}$ (78%) of plastic were observed on this group, followed by rounded bacteria, with 1833 per $mm^{-2}$ (72%) plastic occurrence | (Reisser et al., 2014) |
| Urbanized river in North America | Sampling at river | Bacteria | No fungal hyphae or algal cells were found, the bacterial abundance was approx. 30000 individuals $mm^{-2}$ | (McCormick et al., 2014) |
| Costal Mediterranean | Sampling at sea | Bacteria, Diatoms, Fungi, dinoflagellates, Coccolithophores, Protozoa, and Bryozoan | Bacteria were observed for all samples, different eukaryotes composition for benthic and pelagic plastic | (Maso et al., 2016) |
| Tropical bay in Panama | Incubation at Sea | diatoms, dinoflagellates, red, green, and brown algae, ciliates, and apicomplexans | diatoms were the most abundant eukaryotes, accounting for 10 $mm^{-2}$ | (Dudek et al., 2020) |

- Heterotroph

For the field collected sample, in heterotrophic bacterial plastisphere, the main taxa composition is Proteobacteria (mainly *Alphaproteobacteria* with genus of *Roseobacter, Erythrobacter* and *Hyphomonas;* and *Gammaproteobacteria* with the genus of *Alteromonas*) and Bacteroidetes (mainly composed of *Flavobacteriaceae* and *Sphingobacteria*) in the open ocean (Zettler et al., 2013; Bryant et al., 2016; Dussud et al., 2018).

For the sample from laboratory incubation, there is a rapid bacterial colonization that could happen within hours (Foulon et al., 2016), primary colonizers were mainly *Gammaproteobacteria* and *Alphaproteobacteria*. The results were also recognized by the studies from the estuaries (Jones et al., 2007), the Arabian Gulf (Abed et al., 2019), the Mediterranean sea (Elifantz et al., 2013; Dussud et al., 2018a), and ocean coast (Dang and Lovell, 2002; Dang et al., 2008). In family level, *Alteromonadaceae* and *Rhodobacteraceae* were the main components. The observation of *Alteromonadaceae* as primary colonizer could be cross-ocean whatever the substrates, as this group has also been found from the Mediterranean Sea (Briand et al., 2017; Dussud et al., 2018a), from the Indian ocean coast (Rampadarath et al., 2017; Rajeev et al., 2019), the Pacific Ocean coast (Dang et al., 2008; Lee et al., 2008). The Planctomycete and Bacteroidetes (*Flavobacteria*) are prone to be the second colonizers on plastisphere (De Tender et al., 2017; Abed et al., 2019).

Except for the heterotrophic bacteria, the fungi were also frequently observed (Table 1), but rarely explored, studies have shown that the *Chytridiomycota*, *Cryptomycota* and *Ascomycota* are the main composition (Kettner et al., 2017).

As we mentioned above, *Roseobacter* clade, *Flavobacteraceae*, and *Alteromonadaceae* were the most common number from the phycosphere, while, we could also observe that these taxa are also abundant on bacterial plastisphere. Considering the diatoms is also abundant on plastisphere, we could imagine the interation between these taxa and the diatoms on plastisphere.

- Predators

Bacterial predators were also often observed from the SEM, such as bryozoans, ciliates, radiolarians, which could potential impact the bacterial community, and also transportation for long distance using the plastic as the vehicles (Reisser et al., 2014; Amaral-Zettler et al., 2020), In addition, The numerous type IV genes secretion and T6SS components were also more

abundant in plastic metagenomes compared to seawater, indicating the competition within the bacteria in plastisphere (Bryant et al., 2016).

- Potential pathogens

Interests have been raised about opportunist pathogens dispersal on plastics, such as animal or human pathogenic *Vibrio* with 24% total 16S rRNA sequences on one plastic sample from Atlantic Ocean. (Zettler et al., 2013). Marine plastic debris as vector of harmful species was first suggested by Masó et al. (Masó et al., 2003), who identified potential harmful dinoflagellates such as *Ostreopsis* sp. and *Coolia* sp. Putative pathogen of fish (*Tenacibaculum* sp.) and of invertebrates (*Phormidium* sp. and *Leptolyngbya* sp.) were found more common on plastic compared to surrounding seawater (Dussud et al. 2018). Some bacterial taxa considered as putative pathogen for human, corals and fishes were also found in the intertidal zone of the Yangtze Estuary, at relatively low abundance (<1.6%) (Jiang et al., 2018). A putative pathogen for coral *Halofolliculina* spp. was found abundant on some western Pacific plastic debris (Goldstein et al., 2014). Some toxic eukaryotic species were also mentioned by Debroas et al. (Didier et al., 2017) at low abundance (<0.04%), but might be regarded as hitchhiker organisms. Nevertheless, caution should be taken since the 16S rRNA metabarcoding approach used in all these studies were not an appropriate method for describing bacterial virulence. The recent coupling of 16S rRNA metabarcoding technique with the detection of virulence-associated genes may be an interesting option to answer this question (Kirstein et al., 2016). Pathogenicity evidence on marine animals in relation to the plastisphere has never been proven, and further researches are required before displaying alarmist conclusions on the possible responsibility of plastic debris as vector for the spreading of disease-causing organisms. Apart from those results, microplastics colonized by pathogen may also pose threats to humans who are exposed to contaminated beach and bathing environments (Keswani et al., 2016a). Evidences are still missing to determine if plastic debris could lead to the spread and prolonged persistence of pathogenic species in the Oceans.

In summary, the advent of next generation sequencing greatly unveil the bacterial plastisphere, and improve our understanding on plastisphere, while some points are still poorly investigated. For instance, the research on the open ocean is restrained on the limited research. In another point, the interaction between the diatom and bacteria is also poorly understood, the diatom's impact on the bacterial plastisphere is needed to be further solved.

## 2.5 Factors driving the formation of bacterial plastisphere

In general, it is considered that seasonal and geographical factors are very important in shaping bacterial plastisphere from the study from North Sea and North Pacific gyre *vs*. North Atlantic gyre (Amaral-Zettler et al., 2015; Oberbeckmann et al., 2016). Inversely, one study based on a large number of microplastics sampled in the Mediterranean Sea showed no effect on geographical location (Dussud et al., 2018).

The substrate could be also important factor determining the bacterial plastisphere, while inconsistency results were often reported. For instance, no difference was found for different material type from the study of hydrothermal vents (PVS, PS, Aluminum) (Lee et al., 2014), the Mediterranean Sea (PE, PP, PS) (Dussud et al., 2018), the North Sea (PET, glass) (Oberbeckmann et al., 2016), Baltic sea (PS, wood) (Ogonowski et al., 2018) and the Bohai Sea (PE, PP, PS) (Wu et al., 2020). In contrast, the substrate will significant change the bacterial community when comparing the degradable *vs*. non-degradable substrate (Dussud et al., 2018a), polymer *vs*. polymer with coating material (Catão et al., 2019) and PET, PE and steel from Arabic Gulf (Muthukrishnan et al., 2019).

Lastly, several reviews considered that the plastic surface property, such as surface topography, plastic size etc. are important, but in the marine environment, it is seldomly tested (Harrison et al., 2018b; Oberbeckmann et al., 2015).

By now, there is no clue on how long the geographical distance will change the bacterial plastisphere. For the plastic in Mediterranean Sea, there is no significant difference on bacterial plastisphere, it could be indicating that the bacterial plastisphere could disperse for a long distance especially in the Mediterranean Sea. For the substrate, it appears that the polymer substrate composition, such as polyester vs. carbon-strain-skeleton-based polymer (such as PE, PP) have some difference in some point. The impact from complex network is still not interpreted. A clearer picture will hopefully emerge from extensive investigations.

## 2.6 Impact of plastic and plastic biofilm in ecosystem

The plastic impact on oceanic elemental biogeochemical cycle is not quantitative so far, while available literature tends to show that the microplastic could change the marine ecosystem in some points. There is no doubt the plastic input into the ocean has been increasing the carbon

reservoir. The plastic leachate from plastic was estimated up to 23600 metric tons of DOC annually, and the 60% of leachate could be assimilated within 5 days, importantly, it could stimulate the microbial activity and the biomass (Romera-Castillo et al., 2018). By measuring of the primary production and respiration of the plastic from the north Pacific Ocean, it was found the gross primary production (NCP) is positive, whereas NCP in bulk seawater was close to zero, the net increase of production on plastic could be the "hot spots" in the oligotrophic ocean (Bryant et al., 2016). For the carbon cycle, it has showed that the heterotrophic bacterial bacteria activity on plastisphere is more active than the water column (Dussud et al., 2018a).

The higher nitrogenase genes in plastisphere also suggested that the nitrogen fixation could be reducing the nitrogen limitation on the plastisphere (Bryant et al., 2016), this could be due to the biofilm hallmark structure forming the anaerobic microenvironment. As we have mentioned above, the diatom abundance could reach the thousands per square millimeter (table 1), this could also contribute on the sulphur cycle, since the diatoms and dinoflagellate as the main dimethylsulfoniopropionate (DMSP) producers (Zhang et al., 2019). Finally, the plastic will also increase the trace metal net input into the ocean, such as As, Ti, Ni, Cd, Fe, interestingly, the plastic could also enrich the Fe from the ocean water column (Prunier et al., 2019), which potentially influence the microbial metabolic activity in seawater and also on plastisphere, for instance, the ocean close to equator area and Southern Ocean is often Fe-limited for diatoms and small phytoplankton (Moore et al., 2001), the plastic could be more attractive due to the higher Fe on plastic surface, and further influence the carbon and Fe cycle in the ocean.

After the formation of biofilm, there could be some other impacts on the ecosystem. For instance, the increase of biofouling could increase the particle's density for lower density plastic, in contrast, the buoyancy of particles that had a higher density than seawater may increase the density as the results of biofouling, rendering microplastics susceptible on the vertical transport (Rummel et al., 2017). Another impact is that most studies identified a positive correlation with biofilms inducing larvae's settlement and metamorphosis (Salta et al., 2013).

Coupling primary production and heterotrophic production measurements over large temporal and spatial scales will be necessary to obtain a better view of the role of the plastisphere.

## *2.7 Plastic degradation*

Plastic degradation are the change in polymer properties due to chemical, physical or biological

reaction resulting from bond scission and subsequent chemical transformation (Singh and Sharma, 2008). Degradation has been reflected in changes of material properties such as mechanical, optical or electrical characteristics, resulting in crazing, cracking, erosion, discoloration, phase separation or delamination (Shah et al., 2008). For abiotic factors, degradation initiated by solar UV-B radiation is very efficient mechanism in plastic exposed in air lying on a beach surface, while degradation is severely retarded in seawater (Andrady, 2011). Temperature, ozone and mechanochemistry (such as salinity) could also contribute to the abiotic degradation of plastic (Da Costa et al., 2018; Singh and Sharma, 2008). The microplastic degradation analysis carried by FTIR (short for Fourier transform infra-red spectroscopic) had shown that the flat pieces of debris (2-5 mm length) typically have one face that is degraded, and other face with more biofilm, suggesting the film floating in the ocean with preferred orientation, smaller cubic shaped debris (<2mm) seems to roll at sea (Ter Halle et al., 2016).

The plastic persistence in seawater is not fully determined, it is estimated that the plastic longevity in seawater could be hundreds to thousands of years (Barnes et al., 2009). Recent study also suggested the plastic fragmentation could be faster than expected in the nature environment, as it is shown that smaller microplastics is prone to be more oxidized than the bigger size (Ter Halle et al., 2017).

(Zettler et al., 2013)

The formation of pits and grooves fit with the shape of the microbes in the ocean, suggesting that the microbes also contribute to the plastic degradation (Zettler et al., 2013; Reisser et al., 2014), which refer to the biodegradation.

Biodegradation of plastic is a process that results in total or partial conversion of organic carbon into biogas and biomass associated with the activity of a community of microorganisms (bacteria, fungi, etc.) capable of using plastic as a carbon source, it is considered to occur after or concomitant with abiotic degradation. It is summarized in four essential steps (Jacquin et al., 2019):

- Bio-deterioration relates to the biofilm growing on the surface and inside the plastic, which increases the pore size and provokes cracks that weaken the physical properties of the plastic (physical deterioration) or releases acid compounds that modify the pH inside the pores and results in changes in the microstructure of the plastic matrix (chemical deterioration), the

bio-deterioration leads to the formation of tiny fragments from the plastic surface.

- Biofragmentation corresponds to the action of extracellular enzymes (oxygenases, lipases, esterases, depolymerases and other enzymes that may be as diverse as the large spectrum of polymer types) released by bacteria colonizing the polymer surface. These enzymes will reduce the molecular weight of polymers and release oligomers and then monomers that can be assimilated by cells.
- Assimilation allows oligomers of less than 600 Daltons to be integrated inside the cells to be used as a carbon source, thus increasing the microbial biomass.
- Mineralization is the ultimate step in the biodegradation of a plastic polymer and results in the excretion of completely oxidized metabolites ($CO_2$, $CH_4$, and $H_2O$).

There are a couple of techniques could determine the polymer degradability or the biodegradability (Table 2). The color change and surface observation could be used for the first indices for the abiotic degradation and/or biodeterioration. After the initial degradation, the crystalline spherulites appear on the plastic surface, which could explain the preferential degradation of the amorphous polymer fraction (Shah et al., 2008). FTIR is widely used in the marine polymer identification and also for the determination of polymer oxidation (Bond et al., 2018). FTIR results showed that the polymer degradation is limited to the top 100 μm in the marine environment from the north Atlantic Ocean gyre (Ter Halle et al., 2017). The index of crystallinity is also important, because it could provide the degree of degradability, as the oxygen or the microbes is prone to degrade the amorphous than the crystalline area (Andrady, 2017). The measurement of crystallinity could be conducted by X-ray diffraction or differential scanning calorimetry, also be inferred from the characterized bonds from FTIR, because the higher degree of crystallinity result in correspondingly higher density of MPs rendering them negatively buoyant (Andrady, 2017). This could be particular important for the small microplastic's hydromechanics in the marine environment, it may explain some extent of the microplastic discrepancy in the marine environment (the marine oceanographers found that there is a plastic debris percentage drop off when the plastic size is less than 2 mm).

liquid chromatography coupled to mass spectrometry (LC-MS) and gas chromatography coupled to mass spectrometry (GC-MS) could be used for the identification of nanoplastics, additive or plastic surface-associated chemicals. For instance, the (Pyrolysis) GC-MS were used for identification of nanoplastics, which found that nanoplastics were mainly made of polyvinyl chloride, polyethylene terephthalate, polystyrene and polyethylene from the North Atlantic subtropical gyre (Ter Halle et al., 2017), in another point, the technique could also identify the

assimilation process.

Gravimetric were more used for test the polymer degradation in the laboratory (Shah et al., 2008). It is noteworthy that all the above-mentioned techniques cannot detect the mineralization, which could be just measured by respirometry method, and the mineralization is the proxy of completely removal of the polymer from the ocean environment (Andrady, 2017).

Table 2. (Bio)degradability estimation: analytical techniques

| | Analytical technique | Parameter description | AB | BD | BF | A |
|---|---|---|---|---|---|---|
| **Morphological** | Yellowness | Color change | X | | | |
| | Microscopy | Surface property measurement | X | X | | |
| **Rheological** | Tensile | Mechanical strength | X | X | X | |
| | X-ray diffraction | Crystallinity | X | X | X | |
| | Differential scanning calorimetry | Crystallinity, phase transition temperature | X | X | X | |
| **Gravimetric** | Balance | Weight change | X | X | X | |
| **Spectroscopic and Chromatographic** | FTIR | Functional group change | X | X | X | |
| | Nuclear magnetic resonance (NMR) | Molecular quantification and identification | X | X | X | |
| | Gel permeation chromatography | Molecular weight | X | X | X | |
| | (Pyrolysis) GC-MS | Polymer and additive identification | X | X | X | |
| | LC-MS | Polymer and additive identification | X | X | X | |
| **Respirometry** | Titrimetric method | Oxygen or $CO_2$ titrimetric method | | | | X |
| | oxygen or $CO_2$ sensor | Oxygen consumption or $CO_2$ evolution | | | | X |
| | Pressure change | Oxygen consumption | | | | X |

AB: Abiotic degradation, BD: Biodeterioration, BF: Biofragmentation, A: Assimilation, X: applicable. Table is adapted from (Lucas et al., 2008)

Hitherto, there are several available norms used for determination of the polymer biodegradability in the marine environment, most of the which take the respirometry measurement (such as ISO 18830:2016 and ISO 19679:2016). While several authors criticized the current norms could not realistically predict the biodegradability in the marine environment (Harrison et al., 2018a; Jacquin et al., 2019), such as inocula preparation, selected test temperature (higher than the situ), without toxicity testing etc. Thus, the norms are expected to

be improved to meet the criteria of the polymer's degradability in real natural condition.

# 3. Toxicity of plastics to marine creatures

Human activities are responsible for the major reason for the decline of the world's biological diversity, it is so critical that the impact from the human accelerated the present extinction rates to 1000-10,000 times higher than the natural rate (Lovejoy, 1997). One particular form of human impact constitutes a major threat to the marine life: the pollution from plastic debris (Derraik, 2002).

## *3.1 Plastics ingestion and entanglement*

Encounters between creatures and plastic debris were first reported in the 1960s (Holgersen, 1961), when the plastic production was only 9 MT compared to ~ 400 MT now (Geyer et al., 2017). The reported plastic encounters (ingestion and entanglement) had increased from 267 species on year 1997 (Laist, 1997) to 395 species on year 2015 (Gall and Thompson, 2015). On 2015, all known species of sea turtle, 54% of all species of marine mammal (such as cetaceans, fur seal, sea lion), and 56% of all species of seabird and 0.68% of fish were affected by entanglement or ingestion of marine debris. Species with the greatest number of individuals ingesting debris were the northern fulmar (n= 3444), (*Procellariiformes*, *Fulmarus glacialis*), species with the greatest number of individuals becoming entangled in debris were the northern fur seal (*Callorhinus ursinus*), (n = 3835) (Gall and Thompson, 2015). According to a survey for 20, 852 individuals among 25 *Procellariiformes* species, 25% burrow-nesting species and 8.56% surface-nesting species were reported on plastic ingestion (Savoca et al., 2016). *Procellariiformes* could be more vulnerable to plastic debris, in some point, it do not have the ability to regurgitate the ingested plastics, the one manages to regurgitate plastic debris could pass them into their chicks during the feeding activity, making chicks also susceptible to the plastic debris (Derraik, 2002). It is also showed that the *Procellariiformes* species having the dimethyl sulfide (DMS) sensory ability, tend to ingest more plastic than the one do not have the ability, DMS emitted from plastic is a keystone infochemical to attract birds to ingest the plastics (Savoca et al., 2016). On the other hand, the fishing gear is a serious threat to the sea

turtle, marine mammals. Firstly, sea turtle is regarded as the most vulnerable species to the 'ghost netting', because they use the floating objects for shelter to avoid predation or as the foraging station (Li et al., 2016). The plastic entanglement were also frequently reported on fur seals, who is curious and playful and attracted to the floating debris, and then the fur seal dive and roll about in the fishing gear, unfortunately, scientist estimated that up to 40, 000 fur seal were killed by the plastic entanglement in 1976 (Derraik, 2002).

The growing studies recorded the ingestion of microplastic by fish (Jovanović, 2017; Wang et al., 2020). While for now, there is no consensus on the microplastic susceptibility on the fish in different trophic levels (Jovanović, 2017; Wang et al., 2020). The microplastic occurrence in fish is substantial, a survey from the North Pacific Central Gyre showed that around 35% of planktivorous fish contained microplastics in the gastrointestinal tracts out of six species (n=670) (Boerger et al., 2010). For another study from English Channel, all the 10 examined carnivorous contained the plastic in the gut with the occurrence of 36.5% (Lusher et al., 2013). The catfish from Brazilian Northeast coast were also examined on the plastic ingestion, it found that, 33% had ingested plastic (n=122), nylon fragments from cable used in fishery activity played a major role in this contamination, it is speculated the ingestion of plastic on catfish came from low trophic level transfer, since the catfish is carnivorous (Figure 5) (Possatto et al., 2011). The plastic fibres may be particularly hazardous, because they potentially clump and knot and eventually preventing egestion (Cole et al., 2011). The plastic occurrences from mesopelagic fishes from North Pacific Subtropical Gyre was 9.2% (n=141), it is estimated that the mesopelagic fishes could ingest 12000 to 24000 tons per year (Davison and Asch, 2011).

For the species in lower trophic level, such as zooplankton and invertebrates, there is less report on the field research compared to the quantity from the laboratory, 39 zooplankton species were reported on the ingestion of microplastics, equivalent to 28 taxonomic order, including copepods, salps and fish larvae (Botterell et al., 2019). The encounter rate, for two zooplankton species from Northeast Pacific Ocean, showed that 3% for copepods (*Neocalanus cristatus*) and 6% for euphausiids (*Euphausia pacifia*), considering that the density of zooplankton abundance (28 and 2 per $m^3$ respectively), the quantity of microplastic ingestion could be substantial, besides, it was also found that the quantity of microplastic ingestion decreased from shore to the open ocean in euphausiids, indicating the increased plastic- euphausiids encounter rate closed to the land (Desforges et al., 2015). Another study from the East China Sea showed that the occurrences of copepoda and Pteropoda were 13% and 35% respectively, the microplastic occurrences were influenced by the feeding mode with the order of omnivore >

carnivore > herbivore (Sun et al., 2018). The invertebrate or the zooplankton with the filter-feeding lifestyle, are considered to more be more susceptible to the microplastic, since these species filter high amount of seawater that increase the possibility of plastic ingestion (Desforges et al., 2015). Comparison between commercial and wild type mussel (*Mytilus edulis,* and *M. galloprovincialis*) showed that there was no significant difference concerning on the microplastic occurrences between these two kinds of mussels (2.6-5.1 fibres per 10 g), thus, the microplastics could be transferred from the mussel to humans (De Witte et al., 2014).

The microplastic ingestion were considered to be attributed to several factors, main based on the marine creatures feeding behavior as well as plastics properties, such as plastic size, color, shape, polymer density and composition (Paul-Pont et al., 2018; Botterell et al., 2019).

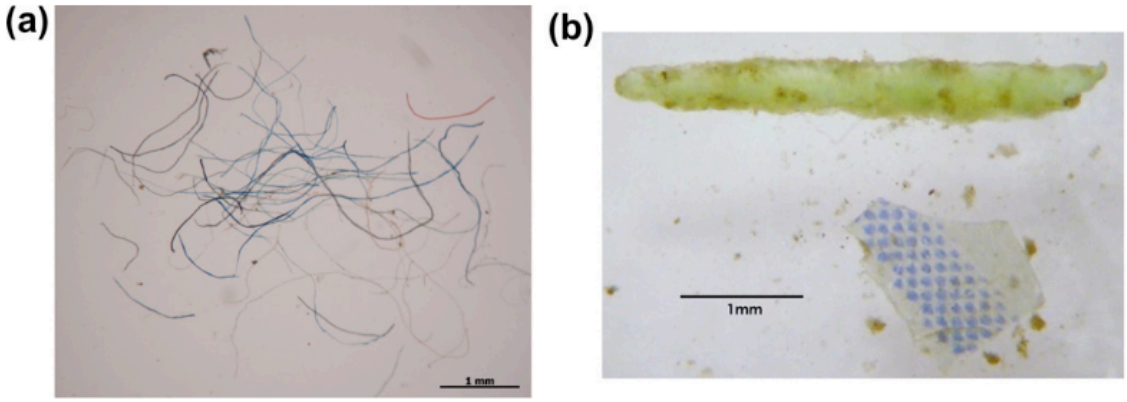


Figure 5. Observation of plastic ingestion by catfish. a: nylon fibers b: hard plastic (Possatto et al., 2011)

## *3.2 Toxic aspects of microplastics*

### 3.2.1 Effect of microplastic on subcellular, cellular and organic level

After the microplastic ingestion, it could cause harm in subcellular, cellular or organic level to marine organisms (Figure 6).

- Impact on energy reserve

Energy reserve is one of the highest concerns on microplastic effect. For instance, it has shown that the pristine polyvinyl chloride (PVC) microplastic ingestion by marine lugworm *Arenicola marina* could reduce 50% of its energy reserve (Wright et al., 2013a). The pristine polystyrene

microplastic could also reduce the energy intake on the copepod *Calanus helgolandicus* (Cole et al., 2015), the clam *Atactodea striata* (Xu et al., 2017) and the pearl oyster *Pinctada margaritifera* (Gardon et al., 2018), the reduction of energy reserve could be due to the false satiation or the accumulation of microplastics in the gut (Guzzetti et al., 2018). In contrast, there was also the reports which did not observe the microplastic energy budget impact on isopod *Porcellio scaber* with polyethylene microplastics extracted from commercial plastic bag and facial cleanser (Jemec Kokalj et al., 2018), and also on blue mussel *M. edulis* and lugworm *A. marina* (Van Cauwenberghe et al., 2015a).

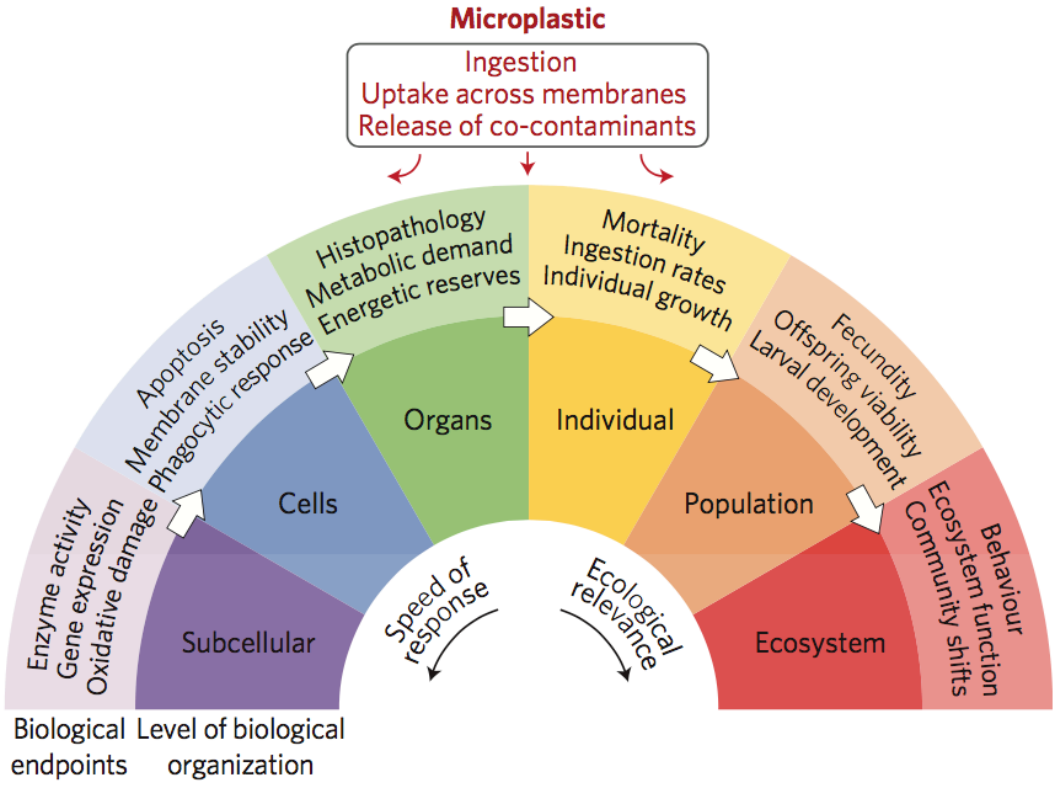


Figure 6. Scheme illustrating potential impacts of exposure to microplastic across successive levels of biological organization (Galloway et al., 2017).

- Impact in terms of translocation

The fine microplastics could also translocated into tissues. It has shown microplastic of polystyrene (3.0 and 9.6 µm) and polyethylene (0-80 µm) could translocated into the circulatory system of mussel *M. edulis*, the smaller particles, the more potential accumulated in the tissue (Browne et al., 2008; Von Moos et al., 2012). Similarly, the polystyrene (5 µm) was also observed in the liver of zebrafish, indicating the translocation (Lu et al., 2016).

- Impact on the transfer of toxic pollutants

The hydrophobic property of microplastic make it prone to adsorb the persistent organic pollutant, metal, with the higher concentration reaching to 5000 ng/g for polycyclic aromatic hydrocarbon (PAH), 2000 ng/g for polychlorinated biphenyls (PCBs), and $10^4$ μg/g in the marine environment (Guo and Wang, 2019). The adoption of organic pollutant and metal pose another threat to marine organisms. In this case, the microplastic act as the vector for the toxic pollutants. For instance, the microplastic could transfer the pollutants (nonylphenol and phenanthrene) and additives (Triclosan and PBDE-47) to lugworms *A. marina* (Browne et al., 2013), while some other study commented that the microplastic ingestion could pose less risk compared to the other natural prey or organic matter (Koelmans et al., 2016), while things are still in the argument concerning on the impact from metal and additive.

- Impact on gut microbiota dysbiosis

Recently, there are also report that microplastic could induce the gut microbiota dysbiosis. After the exposure of polystyrene microplastics to zebrafish, increase of relative abundance has been observed on Firmicutes, decrease on Bacteroidetes and Proteobacteria (Jin et al., 2018). Significant decrease was observed on the Actinobacteria from mice after polystyrene microplastic exposure (Jin et al., 2019). As we mentioned above, the gut microbiota homeostasis is crucial for host in terms of the nutrients, immune system and protection of pathogen. Thus, the gut microbiota dysbiosis rise another concern for the microplastic impact.

- Impact on physical damage

After 90 days of PVC microplastic exposure, half of the individuals has been observed on physical damage from European sea bass intestine (Pedà et al., 2016). Similarly, physical damage was also observed on zebrafish intestine with polystyrene microplastics after 7 days exposure (Lu et al., 2016).

- Impact on immune system

Research on the immune system is scare. We could glimpse the impact from some studies. 18-days polyethylene microbeads exposure showed the repressed immune system gene expression on mussel *M. galloprovincialis*, elevated apoptosis gene expression was also determined (Détrée and Gallardo-Escárate, 2018a). Acute polystyrene exposure (12h) also showed the suppressed immune gene expression on coral *Pocillopora damicornis* (Tang et al., 2018). The first vitro study of the impact of microplastic to fish head-kidney leucocyte showed that the microplastic could impair the phagocytosis and increase the respiratory burst (the respiratory

burst refer to rapid release of ROS by immune cell to kill the pathogen), (Espinosa et al., 2018).

- Impact on oxidative stress

One of the frequently report is the induction of oxidative stress, which is defined as the imbalance of the production of reactive oxygen species (ROS) (like peroxide and free radicals) and the cell ability to detoxify these reactive intermediates (like catalase, superoxide dismutase). The free radical could permanent damage the cell structure, such as DNA, protein and lipids (Cook and Petrucelli, 2012). The ROS signal transduction could be mediated by the phosphorylation of N-terminal kinase (p-JNK) or phosphorylation of p38 mitogen-activated protein kinase (p-p38 MAPK) pathway in the monogonont rotifer by polystyrene microbeads (*Brachionus koreanus*) (Jeong et al., 2016). The ROS signal could also induce the expression of nuclear factor erythroid 2-related factor 2 (Nrf2) pathway, and then the Nrf2 will activate the expression of antioxidant gene in the marine copepod (*Paracyclopina nana*) (Jeong et al., 2017). There are several possibilities that the microplastic trigger the production of ROS. Firstly, the microplastic could cause damage to the marine creatures which in turn induce the oxidative stress (Anbumani and Kakkar, 2018). Secondly, it could also due to the toxicity of microplastic monomers such as styrene (Mögel et al., 2011). Thirdly, The ROS could be produced during the detoxification process of the toxic compounds (such as organic pollutants) with the cytochrome P450 and other enzymes (Ron van der et al., 2003). Lastly, the modification of gut microbiota could be another reason, which is also linked to oxidative stress (Cani et al., 2008).

### 3.2.2 Microplastic impact from individual to ecosystem

Directly linking the sub-organism impacts to the ecosystem level is hugely challenging for any kinds of environmental pollutant, while it could be inferred with the available investigation.

For the microbes, it has shown that the microplastics is the new niche for the bacteria, and other microbial eukaryotes (Dussud et al., 2018). More importantly, the microplastics could provide the niche for the bacterial ‘rare biosphere’ (Oberbeckmann and Labrenz, 2020). As mentioned above, the microplastic could also impact the primary and secondary production, and further impact the biogeochemical cycle.

The impact to the ecosystem could also come from the energy reserve (as mentioned above), which could impact the energy harvest for higher trophic level of marine organisms. Besides, the microplastic could also impact marine organism’s growth rate and reproduction. For example, the oyster reproduction is also impacted by exposure polystyrene microplastic, with

decreases in oocyte number (−38%), diameter (−5%), and sperm velocity (−23%), the larval yield and larval development of offspring decreased by 41% and 18%, respectively, this study reinforce the significant impact of microplastics on the offspring (Sussarellu et al., 2016). The polystyrene microplastic suppress the growth and fecundity for the copepod *C. helgolandicus* (Cole et al., 2015), which could have the impact on the general copepod population in the marine environment, which is very important in the food chain, as it is the predator of the phytoplankton and microbes. On the other hand, it is also the prey for the fish and higher trophic level organisms. Thus, the impact on the population of lower trophic level organism will finally impact the population of higher organisms.

The impact of microplastic could be also in marine benthic ecosystem. For example, it has showed that the microplastic could reduce the feeding activity of marine lugworm *A. marina.* The impairment of feeding activity on these kinds of bioturbator organisms could reduce the surface area availability for the sediment-water exchange, and further impact the inorganic nutrients (Galloway et al., 2017).

The microplastics could directly impact the population of low trophic level organisms and high trophic level organisms. On the other hand, the low tropic level organisms could serve as the prey and indirectly impact the high trophic level organisms, and further impact the ecosystem.

To conclude the toxic impact of plastic, recent results provide great understanding of the toxic impact. The detrimental impact is definitely existed in the marine creatures, while this could be dependent on the species and properties of different polymers. As the new emerged research topic, the reports are limited to draw a consistent view, there are also some contradictory results. Thus, more tests are required. Recently, there is also author commenting on the microplastic's toxicity test. For example, most laboratory experiment have been performed with the microplastic concentration of a higher order of the magnitude than those found in the marine environment. Secondly, most of the authors use the uniform plastic microsphere which are not common in the marine environment. Thirdly, almost all studies use the pristine microplastics, which is different from the one in the marine environments characterized by the biofilm and also the absorbed metal and organic pollutants. Thus, the modern methodology is also expected to be improved (Phuong et al., 2016).

## Summary of introduction:

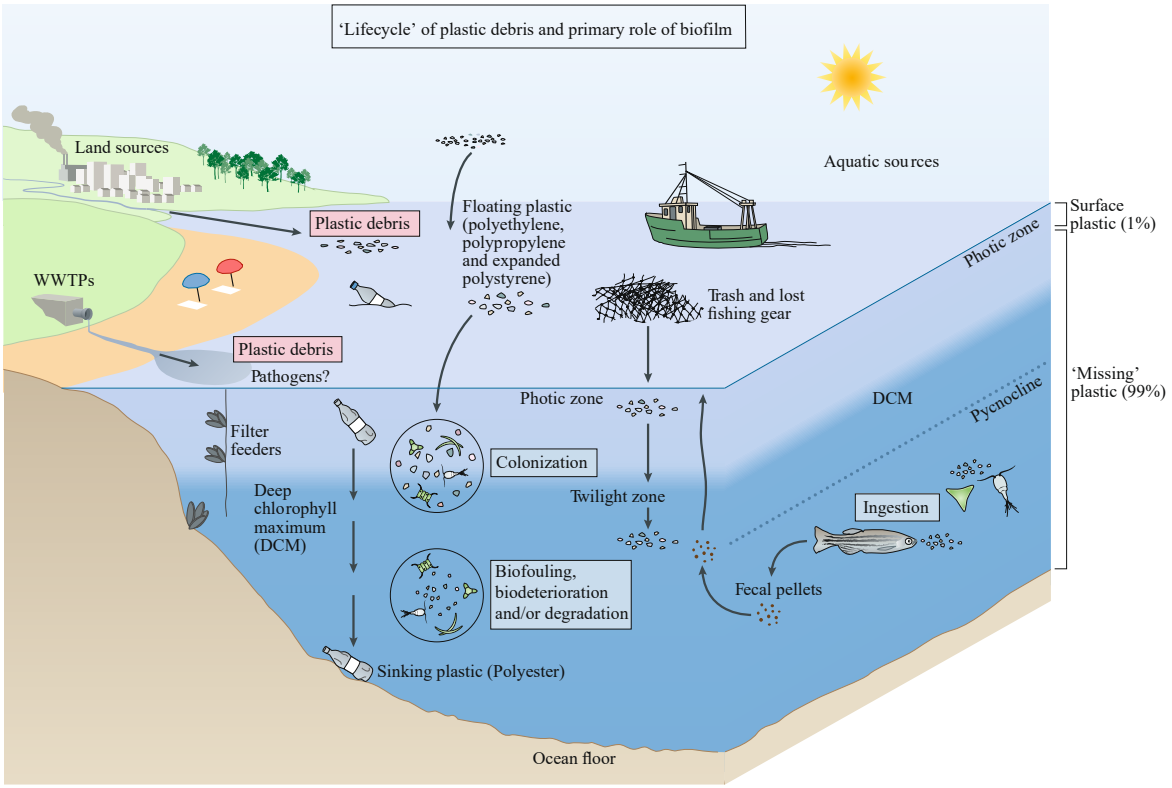


Figure 7. Graphic summary of introduction.

I: Majority of field work focus on floating microplastic, which is mainly composed of PE and PP, the microplastics accumulate in the five subtropical gyres, the enclosed sea and the coastal sediment. Surface floating plastics only represent 1% plastic annually input into the ocean, the water column and benthic ocean could be the main microplastic distribution area.

II: When a new substrate was introduced into the ocean, it will rapidly form the biofilm, which include the process of the formation conditional film, bacterial attachment, development, and finally the detachment.

III: The microplastics in the ocean are characterized by the biofilm, or the so-call 'plastisphere', the bacteria and diatom are the main composition. The bacterial plastisphere is important in the elemental biogeochemical cycle, plastic's vertical transport and larval's development.

IV: The microbes in the ocean have different lifestyle, composing of free-living, surface-associated and gut associated lifestyle, different lifestyles develop different survival strategies.

V: After the plastic introduced into the ocean, degradation is commenced by the abiotic and

biotic factor, several techniques could be used to follow the degradation or biodegradation, while the longevity of plastic in the ocean is uncertain now, methodology to follow the biodegradation is still in development.

VI: The great concern of microplastic in the ocean is the its toxicity for the marine organisms. Ingestion and entanglement are widely reported by the higher trophic level organisms. In subcellular, cellular or organic level, microplastics could induce the energy reserve reduction, oxidative stress, etc. The fine microplastic could also the translocated in to other tissues apart from the gut, and impact the marine creature's reproduction, which could have a big impact on the ecosystem.

## *Thesis Objectives:*

The objective of this thesis manuscript was to be illuminating and advancing the 'microplastic ecotoxicity' in the marine environment: the impact of microplastics on microbial community and other marine creatures and inversely, and we also attempted to find the solution to reduce the impact of microplastic pollution. This thesis focuses on, firstly, detecting the plastic impact of microplastic on microbial community (chapter 2); secondly, detecting toxicity of microplastics on marine filter-feeder invertebrates (amphioxus) (chapter 3); and lastly, find a strategy to reduce the microbead pollution in the marine environments (chapter 4). The three aspects were studied in this thesis manuscript and are briefly described below.

**Chapter 2:** Microplastics in the marine environment were characterized by the 'plastisphere' (Zettler et al., 2013), which has found containing hundreds to thousands bacterial species. Recent studies pointed out that the temporal and geographical factors are the main drivers for the bacterial community on plastisphere (Oberbeckmann and Labrenz, 2020). While, some other factors pre-described potentially influencing the plastisphere are still unknown (Jacquin et al., 2019). Thus, **the aim of chapter 2** was to further characterize the bacterial plastisphere and clarify the relative importance of different environmental factors driving the plastisphere, including plastic size and shape, chemical composition and interaction with phytoplankton. The experiment was carried out and sampled at the three colonization phase as revealed previously: primo-colonization phase, growing phase and mature phase (Dussud et al., 2018a).

*Chapter Contributions*

*Ghiglione Jean-François and Cheng Jingguang conceived the experiment plan. Alexandra Ter Halle and Bruzaud Stéphane prepared the microplastics. Conan Pascal and Pujo-Pay Mireille prepared the data on SOLA station. Meistertzheim Anne-Leila provided aquaria working platform. Jacquin Justine and Cheng Jingguang performed the sampling. Matthieu Georges and Cheng Jingguang carried out the data analysis on bacteria abundance. Ghiglione Jean-François coordinated the study. Jingguang Cheng was responsible for the rest of experimental work, interpretation and statistical analysis of the data.*

*The work is in preparation for submission to a peer-review journal of Frontiers in Microbiology or Science of Total Environment.*

**Chapter 3:** Ingestion and entanglement of large plastic items have been reported on 395 species. While identification of microplastic ingestion for marine wildlife were relative less, mainly due to invisibility (Gall and Thompson, 2015). The amphioxus is a marine invertebrate, having the

filter-feeding lifestyle and living in shadow water sediments, for which is a highly contaminated region by microplastics in the marine environments. As a consequence, amphioxus could be susceptible to microplastics pollution. By now, there is still no report on microplastic ingestion on amphioxus. Thus, **the aim of chapter 3** is to take amphioxus as model organism, and to test the toxicity impact by polystyrene microplastics. Several parameters were followed, such as the impact on gut microbiota, oxidative stress, immune system and apoptosis.

*Chapter Contributions*

*Ghiglione Jean-François, Meistertzheim Anne-Leila and Cheng Jingguang conceived the experiment plan, Bertrand Stephanie and Hector Escriva provided technical support on amphioxus husbandry and sampling. Bertrand Stephanie and Cheng Jingguang prepared the rRNA probe sequences on gene expression assay, Marie-Line Escande performed transmission electron microscopy. Jacquin Justine and Cheng Jingguang performed the sampling. Ghiglione Jean-François coordinated the study. Cheng Jingguang was responsible for the rest of experimental work, interpretation and statistical analysis of the data.*

*The work is in preparation for submission to a peer-review journal of Environmental science & technology.*

**Chapter 4:** Microplastics is ubiquitous in the marine environment. One of the important sources come from the "primary microplastics" purposefully manufactured to be millimeter size, which were used in personal care and cosmetic products. It has been suggested that trillions of microbeads were emitted into the aquatic habitats per day from the United State (Rochman et al., 2015). To reduce the impact of microbeads in the marine environments, **the aim of the chapter 4** is to test different materials degradability (PE, PMMA, PCL, PLA, PHBV, rice seeds and apricot kernel) in the marine environment, and find out the potential degradable substitutes compared to the conventional non-degradable plastics of PE.

*Chapter Contributions*
*Ghiglione Jean-François and Meistertzheim Anne-Leila conceived the experiment plan. Bruzaud Stéphane, Hoypierres Julia and Deligey Gaëlle prepared the microplastics and performed the measurement on SEM and granulometry. Alexandra Ter Halle performed the measurement of polymer's molecular weight. Conan Pascal and Pujo-Pay Mireille, Jacquin Justine, Meistertzheim Anne-Leila and Cheng Jingguang performed the oxygen measurement. Eyheraguibel Boris performed the FTIR, 1H NMR spectroscopy and mass spectrometry. Ghiglione Jean-François and Meistertzheim Anne-Leila coordinated the study. Meistertzheim Anne-Leila, Jacquin Justine, and Cheng Jingguang performed the samplings. All authors contributed on the data analysis.*

*The work is in preparation for submission to a peer-review journal of Environmental Pollution.*

# *CHAPTER 2: Relative influence of plastic debris size and shape, chemical composition and phytoplankton-bacteria interactions in driving seawater plastisphere abundance, diversity and activity*

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**Keywords:** plastic litters, plastisphere, biofilm, biofouling, microbial ecotoxicology

# *Chapter 2*

**Abstract:**

The thin film of life that inhabits all plastics in the oceans, so-called "plastisphere", has multiple effects on the fate and impacts of plastic in the marine environment. Its composition was shown to be influenced by plastic type, geographical distance and environmental changes between seasons. Here, we hypothesized that the large spectrum of plastic sizes encountered in the environment may be also a critical driver of the plastisphere. Polyethylene (PE) and polylactide acid (PLA) together with glass controls in the forms of meso-debris (18mm diameter) and large-microplastics (LMP; 3mm diameter), as well as small-microplastics (SMP) of 100 µm diameter with spherical or irregular shapes were immerged during 2 months in seawater. Results of bacterial abundance (confocal microscopy), diversity (16S rRNA Illumina sequencing) indicated that the three classical colonization phases, including primo-colonization (after 3 days), growing phase (after 10 days) and maturation phase of the biofilm (after 30 days), were not influenced by the size and the shape of the materials, even when a diatom bloom (*Pseudo-nitzschia* sp.) occurred after the first month of incubation. Influence of plastic size and shape was only visible on bacterial activity ($^{3}$H leucine incorporation), where SML showed higher activity than the rest material sizes, irregular 100µm particles showed higher activity than the regular one. A mature biofilm was visible after 30 days for all material types, with significantly higher abundance in the plastics (PE and PLA) compared to glass, with distinct bacterial assemblages found on each material type. The diatom bloom event had a great impact on the plastisphere of all materials, resulting in a drastic change in diversity and activity. Our research reveals that the plastic chemical composition, the successive phases of biofilm formation and the phytoplankton-bacteria interactions are more important factors than the material size and shape in shaping the abundance, diversity and activity of the plastisphere.

# *Chapter 2*

## 1. Introduction:

Plastic pollution has become a global environmental problem affecting all parts of oceans worldwide, including the most remote areas such as deep seafloor or polar regions where the longevity of the plastics is estimated to be hundreds to thousands of years (Barnes et al., 2009; Lusher et al., 2015; Kane et al., 2020). Vast accumulation zones have been identified in the five subtropical oceanic gyres (Van Sebille et al., 2015), but also in the Mediterranean Sea that has been proposed as the sixth great accumulation zone for marine litter (Cózar et al., 2015). Variation in quantities and compositions were observed throughout the different environmental compartments: polyethylene (PE) and polypropylene (PP) were mostly observed in epipelagic waters, whereas polyamide and polyester dominated in sediments. These variations have been explained through the differences in density, surface area, and the size of plastic litters (Chubarenko et al., 2016; Kowalski et al., 2016; Schwarz et al., 2019).

Once entering the environment, plastic litters are subjected to degradation caused by a combination of mechanical abrasion, photo- or thermal-oxidation, hydrolysis and biodegradation (Andrady, 2003). Plastic degradation results in the formation of tiny plastic fragments of <5mm size, so-called "secondary microplastics" to be distinguished from the "primary microplastics" designed and produced as purpose, for example in industrial cleaners and personal care products. Larger plastics are classically categorized into meso-debris (5mm-2cm) and macro-debris (>2cm) for large-scale and long-term monitoring of plastic litters across countries and environments (Thompson et al., 2009). Over the estimated 5.25 trillion particles afloat in the global ocean, 34.8% are small microplastics (SMP; 330 µm-1mm), 57.5% large microplastics (LMP; 1-5mm), 7% meso-plastics, and 0.2% macro-plastics (Eriksen et al., 2014). Large debris have been shown to have adverse effects on fish, seabirds, and other top consumers, whereas microplastics make it suitable for ingestion by smaller organisms at lower trophic levels (Wang et al., 2019b).

When directly released at sea, plastics are primarily colonized by microorganisms that form dense biofilms on their surfaces, the so-called "plastisphere" (Zettler et al., 2013). The plastisphere has multiple effects on the fate and impacts of plastic in the marine environment. First, the biofilm growing on the surface and inside plastic cracks can contribute to a loss of physical integrity, a phenomenon called "biodeterioration" that play a significant role on the breakdown of large plastic debris into microplastics when coupled with abiotic degradation

(Sabev et al., 2006; Dussud and Ghiglione, 2014). Second, the biofouling may increase or decrease the buoyancy of the plastic particles, rendering them susceptible to upward transport (Kooi et al., 2017; Kane et al., 2020). Third, extracellular polymeric substances produced by the biofilm contribute to co-aggregation of microorganisms and detritus together with microplastics, thus resulting in an increase or decrease of sedimentation rates of algal bloom (such as diatoms or cryptophytes) with important impact on ecosystem functioning (Long et al., 2015; Severin et al., 2017). Fourth, biofilms alter the physico-chemical properties of plastics and increase further colonization by metazoan larvae (Hadfield, 2011; Ghiglione and Laudet, 2020). Fifth, biofilms can host pathogens species that can be transported across the marine environment by plastic dispersion and thus participate to the diffusion of infectious diseases (Keswani et al., 2016; Frère et al., 2018). And finally, plastic biodegradation is promoted by the biofilm by secreting extracellular enzymes able to transform polymers into oligomers and monomers ("biofragmentation"), which can serve as carbon source for microbial growth ("bio-assimilation") that may result in the complete mineralization of polymers into $CO_2$ and $H_2O$ ("biomineralization") (Jacquin et al., 2019).

A growing literature is reporting the large diversity of microorganisms composing the plastisphere, which differed from the surrounding communities living in a free-living state (Zettler et al., 2013; Bryant et al., 2016; Pinto et al., 2019), or attached to organic particles (Dussud et al., 2018), sediment particles (Basili et al., 2020) or other substrates such as wood, cellulose or glass (Kirstein et al., 2018; Oberbeckmann et al., 2018; Ogonowski et al., 2018). The reasons for the preferential attachment of specific communities to plastic particles is still enigmatic. Within the plastisphere communities directly sampled at sea, several factors such as plastic type (PE, PP, PS), geographical location or seasons appeared to differentiate the biofilm communities (Amaral-Zettler et al., 2015; Oberbeckmann et al., 2018). Other factors such as hydrophobicity, topography, roughness, crystallinity and surface charge may play a role in the selection of bacterial community in the early stages of colonization, which is a crucial step for the following colonizing communities by modifying the material-specific surface properties (Rummel et al., 2017). However, most of the above studies focused on microbial diversity and abundance, but only one evaluated the corresponding activity of the microorganisms that form the biofilm (Dussud et al., 2018a). Moreover, only one observation based on field study tested the influence of plastic size and shape (Frère et al. 2018) that is often mentioned as having a crucial role in shaping the biofilm (Oberbeckmann et al., 2015; Harrison et al., 2018), but no specifically designed experiment was dedicated to this question so far.

# *Chapter 2*

The aim of this study was to test how much plastisphere was influenced by different polymer composition (polyethylene PE and polylactic acid-PLA), different sizes (SMP, LMP and meso-plastics of 100µm, 3mm and 1.8cm in diameter, respectively) and topography (spherical *vs*. irregular SMP). During a 2-months incubation in natural seawater from the NW Mediterranean Sea, we also evaluated the impact of phytoplankton bloom on mature biofilms. Temporal variations of bacterial abundance (confocal microscopy), diversity (16S rRNA sequencing) and heterotrophic activity (radiolabeled leucine incorporation) were measured on all plastic types, but also compared to glass of similar size and shape, as well as to the surrounding seawater.

## 2. Material and Methods

### *2.1. Preparation of polymers of different composition, size and roughness*

High density-polyethylene HDPE and Poly L lactic acid PLA were supplied by Good Fellow company (Avilés, Spain) in a form of film of 10 and 50 µm thickness (± 20%), respectively. Circular pieces of respectively 3 mm and 1.8 cm in diameter were cut using hole puncher. Glass coverslip (soda lime composition) were supplied by Verres Vagner company (Toulouse, France) in circular form with 1.8 cm and 3mm diameter and 170 µm thickness.

Irregular PLA and glass microbeads were obtained by cryo-grinding the polymer and glass films described above (SPEX sample Prep), which were further wet sieved with ethanol in order to recover the microparticles for which the size was ranging from 90 to 125 µm. Material for HDPE irregular microbeads were obtained from Good Fellow films with 1 mm thickness to ensure the 3-dimensional structure, and then reduced in size by cryo-grinding as described above.

Spherical HDPE microbeads of size distribution between 96-125um were commercially available (CPMS-0.96, Cospheric$^{TM}$). Spherical PLA microbeads were obtained as pellets and transformed in spherical microbeads by solvent emulsion-evaporation technique. It consisted in dissolving the polymer in a volatile organic solvent immiscible with water (dichloromethane), then introducing this solution into an aqueous solution containing an emulsifier as poly(vinyl alcohol) (PVA, 2%). The emulsion was finally placed under moderate magnetic stirring for 24 hours at atmospheric pressure and ambient temperature, in order to allow the microbeads to harden, until complete evaporation of the organic solvent. The spherical PLA microbeads were collected by wet sieving between 90 and 125 µm, rinsed with permuted water and lyophilized until further use. Spherical glass microbeads were mainly made up with soda lime and commercially available from Good fellow.

Granulometry analysis using a laser diffraction particle size analyzer (Malvern Mastersizer 2000 model with a Scirocco 2000 module) showed a gaussian distribution of the microbeads that always peaked at 100 µm for all polymers and spherical or non-spherical beads. Before the experiment, all the materials (including irregular microbeads (IR), spherical regular microbeads (RE) of average 100 µm diameter as well as films of 3 mm and 1.8 cm in diameter) were washed for 1 hour with ethanol followed by 3 round of vortex (1 min) and sonic bath (3 min) and then dried under sterile hood.

*2.2. Experiment setup*

Each material type (spherical or irregular 100 µm microbeads, 3 mm and 1,8 cm films of PE and PLA, respectively) were placed in triplicate in 12 identical glass tanks of 2 L capacity (Plastic@Sea, Banyuls-sur-mer, France), in which seawater was continually renewed (flow rate was set on 20 mL·min$^{-1}$) by direct pumping at 14 m depth in Banyuls bay closed to the SOLA observatory station (NW Mediterranean Sea, France). Another 3 extra tanks containing circulating seawater only were used as controls. Seawater was pre-filtered with 20 µm porosity filters (Dutscher$^{TM}$) to remove inorganic matter and potential predator before each tank. The tanks were placed in a dark room and illuminated from above in a 12/12 h light/dark rhythm by Lumivie LED RAL G2-SBM lamps (42934, Zoomalia, France) with a nominal luminous flux of 1860 lm each. The experiment started from 13 August 2019, and samples were taken after 3, 10, 30 and 66 days.

*2.3. Seawater environmental variables*

Temperature, salinity, nutrients, chlorophyll a, and particulate organic carbon and nitrogen were *in situ* weekly recorded at the SOLA station (0.5 milles off coast) in the framework of the French national coastal monitoring program "Service d'Observation en Milieu Littoral" (SOMLIT) according to protocols previously described (Ghiglione et al. 2005) and available on the SOMLIT website (http://somlit.epoc.u-bordeaux1.fr/). All samples were processed after sampling within 30 min.

*2.4. Confocal microscopy and flow cytometry*

For each sampling date, triplicate samples were fixed into 1 % (v/v) glutaraldehyde for 30 min before freezing. Confocal microscopy observations were done using Leica TCS SP8 Confocal laser scanning microscope after DAPI staining (final concentration 10% [v/v], Sigma Aldrich). PMT 3 detectors were used for detecting the fluorescence signal and TLD detectors were used to capture the white light signal. The light intensity was compensated for the microbeads, and the Z-Step size were set on 1 µm to get regularly spaced cross sections of the bacteria covered beads. For each sample, 3 beads or 3 pieces of films were used for counting the bacterial abundance using the image J software (Abràmoff et al., 2004). For the regular spherical microbeads, the surface area was calculated using a simple geometrical formula. For irregular microbeads, the surface area was estimated using two different methods. First, a simple geometrical calculation based on the overall shape of the particle, i.e. ellipsoidal, cylindrical or

conic. The main dimensions used for the surface area calculation were measured from optical images. In addition, for a series of samples, the surface was calculated via a full reconstruction of the particle surface using 1 µm separated confocal microscopy cross-sections, and image-J software. A 10% agreement was found between the two methods (geometrical estimation and 3D volume reconstruction) for the samples studied, thus validating the use of a simple geometrical method for irregular beads and giving us the surface measurement uncertainty. Cell counts were verified using Gwyddion software (Nečas and Klapetek, 2012) threshold filter and grains numbering. Manual counting was performed on a series of samples to double check the cell counts accuracy, which was found to be of 10%. Cell counts were then expressed as the number of cells over surface area (in cells.mm$^{-2}$) with an accuracy of 20%.

In parallel, 1 mL of seawater from the control aquarium were also fixed using the same procedure. A 500-µL control seawater was mixed with the nucleic acid dye SYBR Green I (final concentration 0.05% [v/v], Sigma Aldrich) for 15 min at room temperature and in the dark. Cell counts were performed with a FACSCanto II flow cytometer (BD Bioscience, San Jose, CA, United States) equipped with a blue laser (488-nm, air-cooled, 20-mW solid state), as previously described (Mével et al. 2008).

*2.5. Heterotrophic bacterial Production*

Bacterial production was measured for each material at each sampling time by $^{3}$H-leucine incorporation (Dussud et al., 2018a). In brief, the films with the size of 3 mm and 18 mm were rinsed with sterile filtered seawater using wash bottle before transferring to the microtubes containing 1.5 mL sterile filtered seawater. Microbeads were collected on a membrane filter with 10 µm pore size membrane filter (LCWG02500, Mitex$^{TM}$) and then rinsed with sterile filtered seawater, and the seawater was removed by air pumping for 30 seconds to get accurate sample weight. Afterwards, the microbeads were weighed with 15 mg for PE, 18 mg for PLA and 33 mg for glass before adding the sterile filtered seawater for bacterial production assay. The bacteria were detached from plastic with 3 rounds of (1 min vortex and 3 min sonic bath). Immediately after cell-detachment, $^{3}$H-leucine (125.6 Ci·mmol$^{-1}$, Perkin Elmer$^{TM}$) were added at 1 nmol·L$^{-1}$ final concentration (completed with cold leucine to 150 nmol·L$^{-1}$), which consisted of 1.5 ml sterile seawater containing the film or microbeads and detached bacteria. For seawater samples from the control aquarium, $^{3}$H-leucine was added at a final concentration of 4.3 nmol L-1 to 1.5 mL of control seawater. All the samples were incubated in the dark at 18 °C for 3 h. The empirical conversion factor of 1.55 ng C pmol$^{-1}$ of incorporated leucine was

used to calculate the bacterial heterotrophic production (Simon and Azam, 1989; Kirchman, 2001). For each microbeads on each sampling date, the percentage of hydration of the beads were assessed by weighting 3 additional aliquots (wet weight), that were frozen, lyophilized and weighted a second time to measure dry weight. Knowing the wet weight and the percentage of hydration of each material during the kinetic, the bacterial activity was expressed per dry weight.

Knowing the number of cells (N) per unit area for each sample and the average specific surface $R_{SV}$ (i.e. surface to volume ratio) for each type of microbeads, we expressed the heterotrophic bacterial production as the carbon produced per unit area per unit time ($ngC.dm^{-2}.h^{-1}$) or the specific bacterial activity as the carbon produced per cell per unit time ($fgC.cell^{-1}.h^{-1}$). This data representation allows to meaningfully compare materials having different shapes, i.e different surface to volume ratios.

Additionally, we used bacterial production data derived from leucine incorporation to estimate the bacterial growth rate on plastisphere, using a conversion factor of 12 fg C per cell as previously described (Fukuda et al., 1998).

*2.6. DNA extraction PCR and sequencing*

On each sampling date, triplicates of plastic or glass were harvested from each sample by using the same method described above for the bacterial production and immediately stored at -80 °C. Triplicates of 2-L seawater were also obtained using 0.2 µm pore size polycarbonate filters (47 mm diameter, Nucleopore). The microbial genomic DNA extraction were followed with classic phenol-chloroform protocol (Ghiglione et al., 1999). PCR amplification of 16S rDNA V4-5 region was done using 515F-Y and 926 R primers, which has been shown well-suited for marine sample (Parada et al., 2016). Illumina MiSeq sequencing were performed at Genoscope (Evry, France) for the 156 samples, corresponding to the 144 samples of PE, PLA and glass (3 substrate * 4 sampling date * 4 size fraction * 3 replicates) and the 12 seawater samples (4 sampling date * 3 replicates).

*2.7. Data analysis*

Processing of 16S rDNA sequences was performed with DADA2 pipeline (Callahan et al., 2016) using R 3.6.1 version (Bunn and Korpela, 2008). The primers were trimmed off before error correction and denoising step. Paired reads were merged (average length from 367 to 377 bp) and all the singletons were discarded. The chimeras were checked and removed for the

merged reads. The amplicon sequence variants (ASVs) were assigned with SILVA release 128 database (Quast et al., 2013). The taxonomic affiliation of ASVs of interest were further verified against sequences from the NCBI database using BLASTnt. The ASVs corresponding to eukaryotes, archaea, chloroplast and mitochondria were removed and all the sample were rarified to the same number (rngseed=1) before the analyses on bacteria using phyloseq R package (McMurdie and Holmes, 2013). The α-diversity was performed using *alpha()* function from microbiome R package. Square root transformation was performed for β-diversity analyses and hypotheses test. The taxonomy compositions were visualized using histogram and bubble plot with ggplot2 R package (Wickham, 2016).

To investigate the unique and shared bacterial community between biofilm and seawater, the reads from plastic or glass with different size fractions were pooled and rarefied into the same number to that of seawater. Two levels of comparison were conducted: in ASV level (presence or absence) and in tags levels.

### *2.8. Statistical analyses*

An unweighted-pair group method with arithmetic (UPGMA) dendrogram based on Bray-Curtis similarities was used for visualization of beta-diversity. A similarity profile test (SIMPROF, PRIMER 6) was performed on the null hypothesis that a specific sub-cluster can be recreated by permuting the entry species and samples. The significant branch was used as a prerequisite for defining bacterial cluster.

The significance of the factor size (including irregular and regular microbeads), the substrate and the date were analyzed using global or pairwise permutational multivariate analysis of variance (PERMANOVA) (Anderson and Walsh, 2013) using *adonis()* function with vegan R package (Oksanen et al., 2008), the homogeneity of variances was tested using *betadisper()* function. The p value was adjusted with Benjamin-Hochberg method. To test the bacterial community relationship between seawater and plastic, Mantel test was performed in Vegan using *mantel()* based on Pearson correlation method.

One-way ANOVA or multi-way ANOVA were performed for statistical analyses with the type I sum of square applied. Tukey's post-hoc test was used when necessary. Relative importance of each predictor (in R square) for ANOVA results was determined with function of *calc.relimp()* from relaimpo R package (Grömping, 2006). When the data did not meet homogeneity, the Welch's ANOVA was chosen for the analyses. Games-Howell test was used as post-hoc test.

## 3. Results

### *3.1. Environmental conditions*

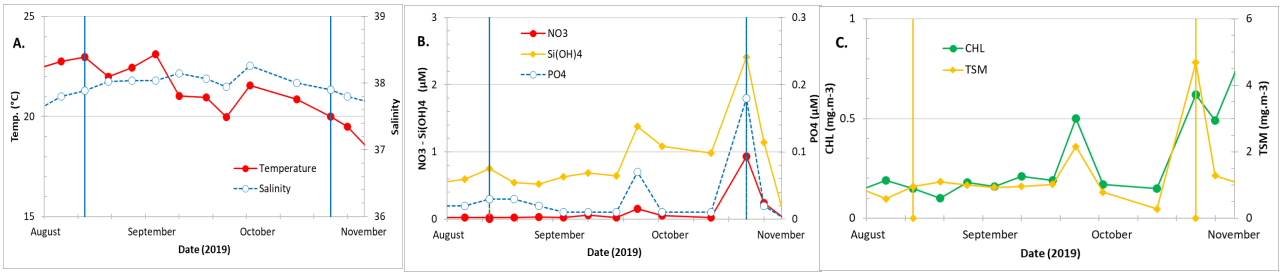


Figure 1. Time series at the SOLA station (bay of Banyuls) between August and November 2019 at 3 m depth for A. Temperature (°C) and Salinity, B. Nitrate, silicate and phosphate (in µM) and C. Chlorophyll a and Total Suspended Matter (in mg.m-3). Data from SOMLIT data base (http://somlit.epoc.u-bordeaux1.fr/)

During the studied period, the environmental conditions in the Bay of Banyuls were characteristic of an autumn situation in a temperate Mediterranean area. Indeed, between the beginning of the experiment (13 August) and the end (20 October) the surface water temperature decreased from 23°C to 20°C and the decrease continued during November (Figure 1A). Salinity was relatively high and constant over the same period with a value of about 38.

Concerning the mineral compartment, as expected in late summer, concentrations were low (~0.5 µM of silicate) or often close to the detection limit (<0.05 µM and <0.02 µM respectively for nitrate and phosphate (Figure 1B). Two events were clearly identified during the period and marked by an enrichment of the water column in nutrients. The first had a limited magnitude on 24th September and the second was more important at the very end of the experiment on 25th October with concentrations of 2.4, 0.93 and 0.18 µM in $Si(OH)_4$, $NO_3$ and $PO_4$ respectively.

These nutrient inputs due to the first mixing inducing the disruption of the water column stratification were responsible for an increase in particulate matter in the water column as shown in Figure 1C in terms of Total Suspended Matter, and especially in terms of chlorophyll. After a low and homogeneous concentration during August and the beginning of September (range between 0.1 and 0.2 $mg.m^{-3}$ of chlorophyll a), we observed 2 peaks (0.5 and 0.6 $mg.m^{-3}$ of chlorophyll a) characteristic of a coastal autumn bloom situation.

*3.2. Microbial cell counts and shape*

Confocal microscopy revealed a large diversity of morphological forms including spherical, rod-shaped or spiral-shaped bacterial like structure at the surface of PE, PLA and glass, for which rod-shaped and spiral shaped structure was more observed on Day 3 and Day 10 compared to Day 30 and D66 (Figure 2). Typical morphotypes of diatoms appeared at day 66 and were not visible before. Confocal microscopy was also useful to confirm the size distribution of microbeads between 90-125 µm, as well as their shape (regular versus irregular).

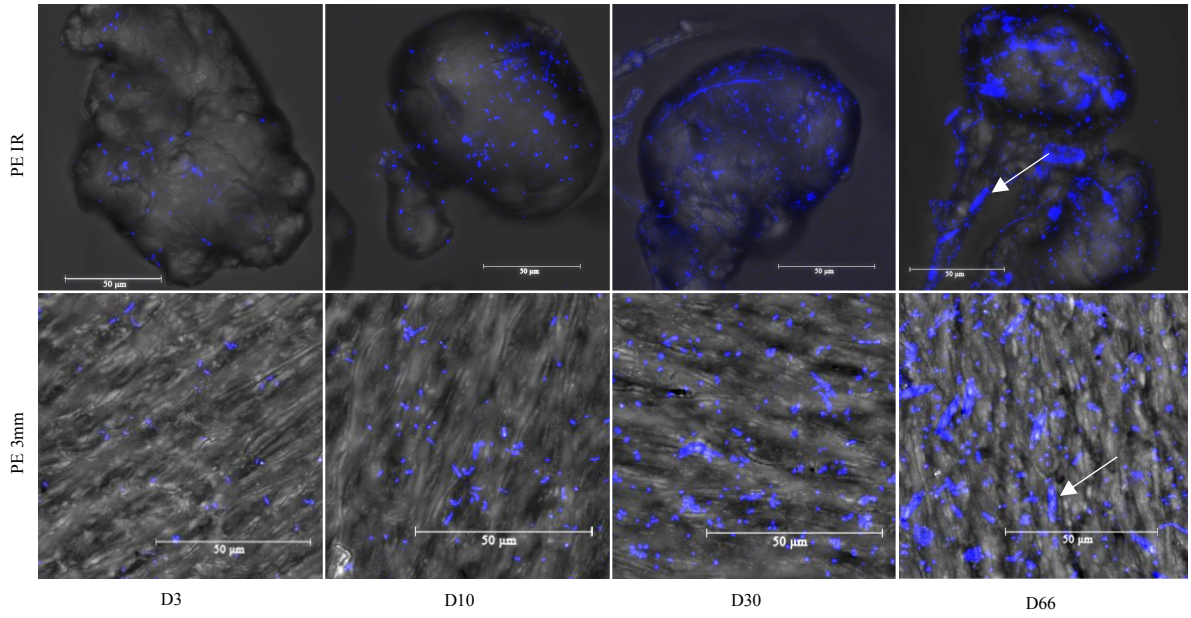


Figure 2. Confocal microscopy for polyethylene irregular microbeads of around 100 µm diameter (PE IR) and 3mm film (PE 3mm) at days 3 (D3), 10 (D10), 30 (D30) and 66 (D66). Scale bar: 50 µm. Arrows are pointing typical shape of diatoms that appeared at D66.

Triplicate samples analyzed by confocal microscopy allowed us to follow the changes in bacterial counts for all material types and sizes. The data highlighted three distinct phases of biofilm formation: primo-colonization, growth and maturation (Figure 3). Interestingly, the three distinct phases were found whatever the material type or size. Three-way ANOVA revealed significant difference in bacterial counts according to the sampling date and material type, but not within material sizes ($R^2$ = 0.29, 0.29 and 0.01 respectively). PE presented the largest bacterial abundance on average together with PLA, whereas it appeared to be ten-fold smaller on glass. A rapid primo-colonization was observed after 3 days, with average abundance of $3.0.10^3$, $1.6.10^3$ and $0.3.10^3$ cells.mm$^{-2}$ for PE, PLA and glass, respectively. Slight growth was observed after 10 days as compared to day 3 values for PE, PLA and glass, where

glass samples remained relatively low ($8.6.10^3$, $2.8.10^3$ and $0.9.10^3$ cells.mm$^{-2}$, respectively). The bacterial abundance had a significant increase from 10 days to 30 days ($p<0.05$) that reached the maturation phase corresponding to the stabilization of bacterial counts, with no significant changes in bacterial counts until day 66 ($p>0.05$). On average the mature biofilm was of $2.5.10^4$, $1.5.10^4$ and $0.2.10^4$ cells.mm$^{-2}$ for PE, PLA and glass, respectively, with non-significant differences between size and shapes within each material type. It should be noted however, that a different behavior was observed for PLA regular beads with an abundance significantly lower than for other PLA samples and much closer to those of glass samples. The use of surfactants in the home-made synthesis of PLA regular beads could be responsible for this behavior, as surfactant could be able to remain at the beads surfaces and considerably modify their hydrophobicity. Thus, PLA regular microbeads was excluded from the statistical analysis for bacterial abundance and activity.

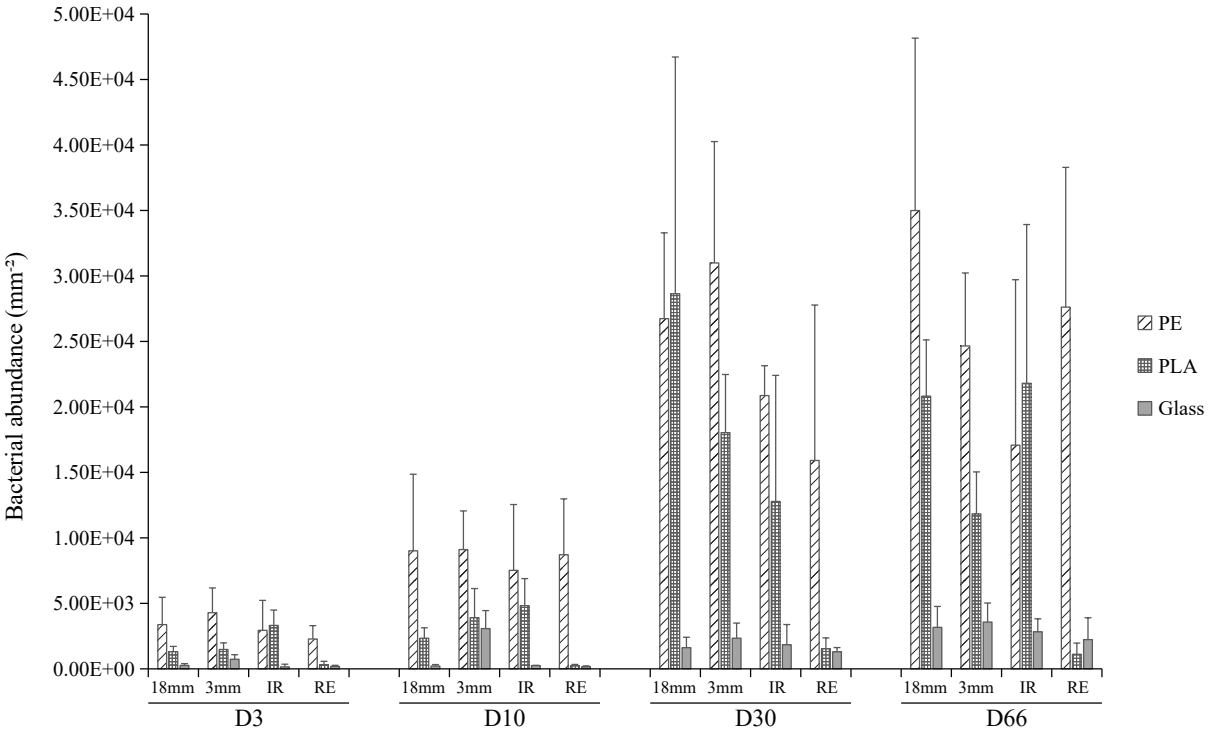


Figure 3. Bacterial cell counts per surface area (mm$^{-2}$) for PE, PLA and Glass on meso-plastics (18 mm diameter), large microplastics (3 mm diameter) and small microplastics microbeads (100 µm diameter) with irregular (IR) or regular spherical shapes (RE) during the course of the experiment at days 3 (D3), 10 (D10), 30 (D30) and 66 (D66).

Diatoms with the average length around 11 µm were observed only after 66 days on all materials, with higher abundance for 3 mm and 1.8 cm sizes on PE ($3.5.10^3$ cells.mm$^{-2}$) and

PLA ($2.4.10^3$ cells.mm$^{-2}$) compared to glass ($0.8.10^3$ cells.mm$^{-2}$). They were rarely visible on spherical or regular microplastics.

*3.3. Heterotrophic bacterial production*

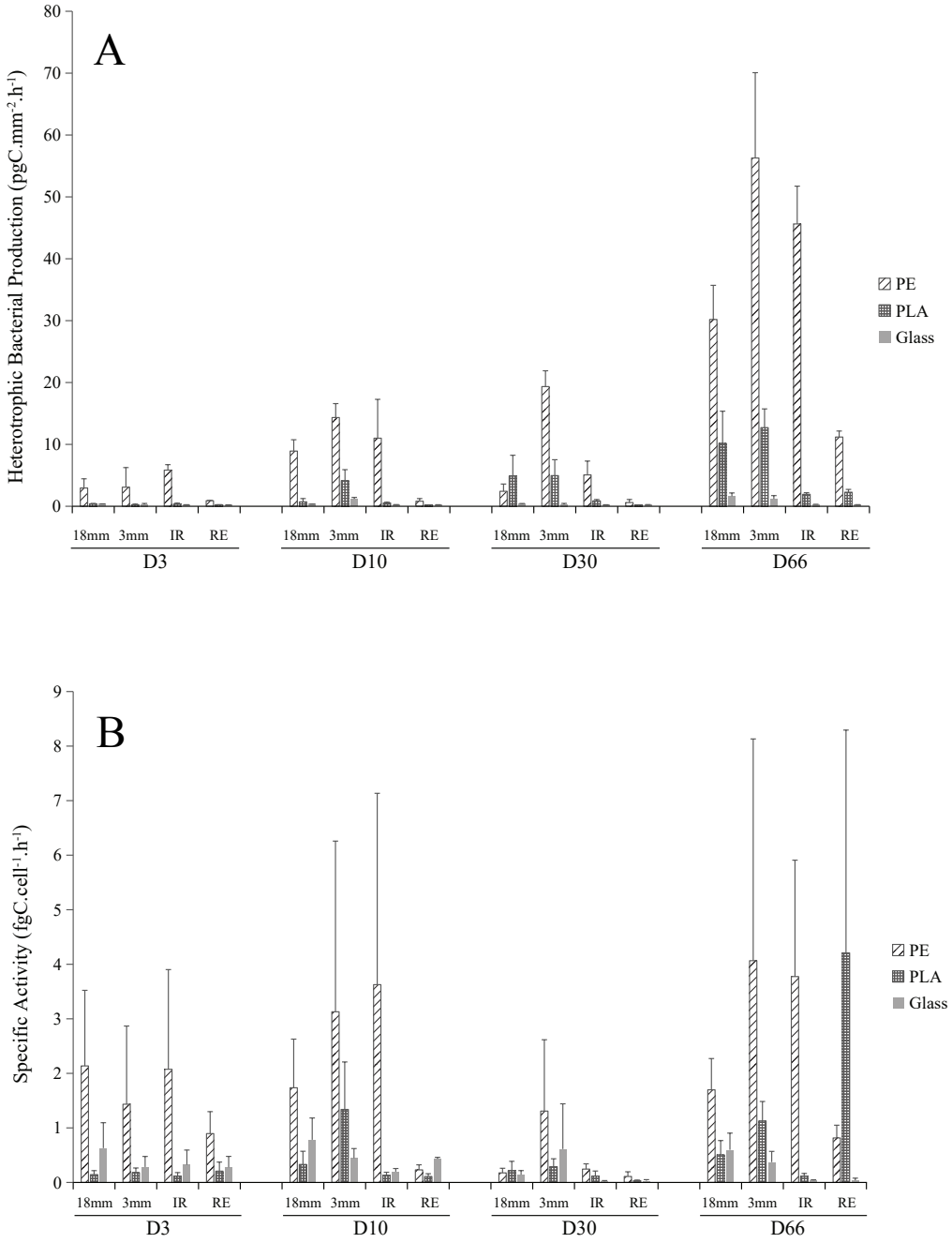


Figure 4. Bacterial heterotrophic production (BP, pgC mm$^{-2}$ h$^{-1}$, panel A) and bacterial activity (fg C cell$^{-1}$ h$^{-1}$, panel B) for PE, PLA and glass on larger pieces (18 mm diameter), microplastics (3 mm diameter) and irregular (IR) or regular spherical (RE) microbeads (100 µm diameter) during the course of the experiment at days 3 (D3), 10 (D10), 30 (D30) and 66 (D66).

Heterotrophic bacterial production (BP) was measured alongside the time course of the experiment and it was expressed in units of incorporated carbon per square millimeter per hour ($pgC.mm^{-2}.h^{-1}$) (Figure 4A). Three-way ANOVA showed that all the factors (sampling date, material type and size) could significantly impact the BP, the sampling date and material type explaining more variation than the material size ($R^2$ = 0.20, 0.25 and 0.07, respectively). Significant increase has been observed from the primo-colonization phase (on day 3) to the growing phase (on day 10). Interestingly, the BP did not show significant differences between day 10 and day 30, even though there was a significant increase in bacterial abundance (Figure 3). The maturation phase showed a significant increase from day 30 to day 66. Differences in BP were also observed on the three sample types, with PE being significantly higher than PLA and glass. In average, BP of PE is 16 and 110 times higher than that of PLA and glass by comparing within each material size. The BP of PE, PLA and glass have increased from 3.2, 0.2 and 0.1 $pgC.mm^{-2}.h^{-1}$ at the primo-colonization phase (day 3) to 35.8, 6.8 and 0.7 $pgC.mm^{-2}.h^{-1}$ at the maturation phase (day 66), with intermediate maturation phase showing 6.8, 2.7 and 0.1 $pgC.mm^{-2}.h^{-1}$, respectively (day 30). Besides, BP difference from material type was also observed, with LMP of 3mm size being higher than the rest of the samples, and the regular SMP microplastics of 100 µm size presenting the lowest values. Generally, PE and glass in regular SMP were 11 and 6 times lower than other material sizes (also containing form). PLA regular SMP were 150 times lower than other sizes or shape, probably due to the surfactant used during synthesis. The material size or shape could have an effect on the BP, as the PE in 3 mm size was in slight curly shape after the manufacture, whereas the PE in 18 mm size was flat. The BP for seawater ranged from 88 to 150 $pgC.mL.h^{-1}$ without significant difference between sampling dates.

Bacterial specific activity was further calculated by dividing the BP by the bacterial abundance in the unit of incorporated carbon per cell per hour ($fgC.cell^{-1}.h^{-1}$) (Figure 4B). Three-way ANOVA also showed that all the factors (sampling date, material type and size) could significantly impact the specific activity, the material type explaining more variation than the sampling date and material size ($R^2$ = 0.08, 0.27 and 0.07, respectively). Interestingly, BP on the primo-colonization phase of day 3 and growing phase of day 10 and maturation phase of day 66 were significantly higher than during the maturation phase at day 30 ($p<0.05$). When it came to the material type, specific activity of PE showed significantly higher levels than PLA and glass, which confirmed that the material type could influence the bacterial specific activity on plastics. The BP of PE, PLA and glass were in the average of the first two sampling dates

(day 3 and day 10) with 1.9, 0.3 and 0.4 fgC.cell$^{-1}$.h$^{-1}$ respectively, decreased to day 30 (0.45, 0.15 and 0.20 fgC. cell$^{-1}$.h$^{-1}$, respectively), and increased until day 66 (2.5, 1.5 and 0.2 fgC.cell$^{-1}$.h$^{-1}$, respectively). The material size and shape could also impact the specific activity, no difference was found between the size of 18 mm and irregular microplastic of 100 µm, while BP of the samples in 3mm showed higher activity, and regular microbeads of 100 µm showing the lowest.

Accordingly, the bacterial growth rate on PE was higher than on PLA and glass. The bacterial growth rate of PE, PLA and glass had the average of the first two sampling dates (day 3 and day 10) with 3.8, 0.6 and 0.8 day$^{-1}$ respectively, decreased to day 30 (0.9, 0.3 and 0.4 day$^{-1}$), and drastically increased until day 66 (5.2, 3.0 and 0.5 fgC. cell$^{-1}$.h$^{-1}$, respectively). The growth rate measured in seawater was 2.8, 6.4, 2.0 and 5.2 day$^{-1}$ for day 3, day10, day30 and day66, respectively.

*3.4. Diversity indexes*

Illumina MiSeq DNA sequencing generated 3 823 342 sequences tags, falling into 5293 ASVs after randomly resampling to 5177 sequences per sample to provide statistical robustness when comparing diversity measures among samples. DNA quantity were not sufficient to allow the sequencing of several samples at the early colonization stage (Day 3) that failed for glass microbeads and 1.8 mm diameter size samples, and for 3mm-PE, PLA and Glass. A total of 123 samples were used for the following analyses, excluding also two samples (irregular PE microbeads and seawater) with low number of reads at day 30. Rarefaction analysis suggested that all samples approached an asymptote (data not shown).

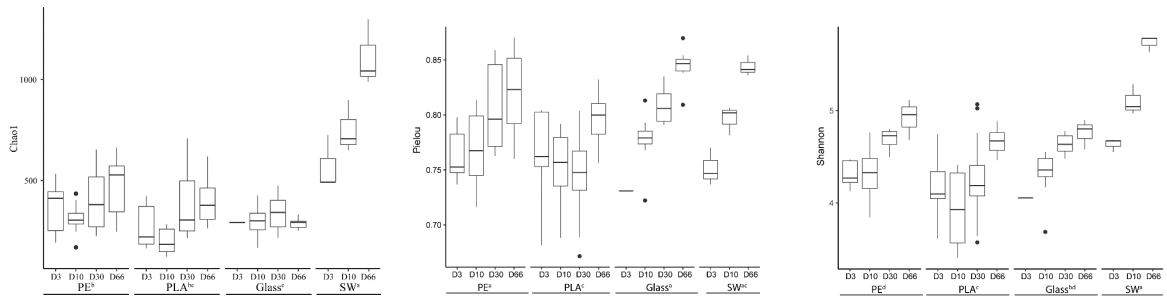


Figure 5. Alpha-diversity indices of richness (Chao1), eveness (Pielou) and diversity (Shannon) of the materials (PE, PLA, glass) and surrounding seawater during the course of the experiment at days 3 (D3), 10 (D10), 30 (D30) and 66 (D66). The boxplots show the median between triplicate samples of different sizes

for each material (heavy horizontal line inside the boxes), the box represents the first and third quartiles and unfilled circles indicate outliers. Lowercase letters (a, b, c) denote statistically different groups ($p<0.05$).

Chao 1 estimator showed that the richness increased drastically in seawater during the course of the experiment, ranging on average from 570 to 1110 ASVs at day 3 and day 66, respectively. A slighter Chao 1 estimator increased was also found from day 3 (average of 363, 265 and 267, respectively) to day 66 (468, 395 and 288, respectively) for PE, PLA and glass with average values of 380, 286 and 283 ASVs, respectively, indicating that on the primo-colonization phase, a handful of bacterial species already colonizing the plastic surface (Figure 5). Seawater sample showed significant higher Chao1 richness than PE, PLA and glass. No difference was found on the Pielou index between seawater and PE, PLA or glass (Welch's $p$ >0.05), where evenness increased for all materials and seawater samples at the end of the experiment. Shannon diversity index of PE and glass was significantly higher than PLA (average of 4.6, 4.5 and 4.3, respectively) and no significant difference could be found in relation to the size of the different materials (Welch's ANOVA test). High correlation of the temporal dynamic of Shannon index and Pielou evenness was found between 3 material types and seawater diversity (Pearson correlation, p=0.018, r=0.75; and p=0.03, r= 0.71 respectively).

*3.5. Bacterial community structure*

UPGMA dendrogram based on Bray-Curtis dissimilarities showed that each of the triplicate samples in each sampling time and condition clustered together (except for one sample D10-PE-IR), confirming the homogeneity within the triplicates and also the proper sampling strategy, rigorous DNA extraction, sequencing and data processing (Figure 6).

Clear dissimilarities were found between bacterial communities in seawater and material types (PA, PLA, glass) (dissimilarity >85%) and between samples corresponding to before (days 3 to 30) and after the diatom bloom (day 66) within each cluster (dissimilarity >75%). Similarity profile testing (SIMPROF) showed these groups to be highly significant ($p < 0.001$).

Samples from the primo-colonization (day 3) and the growing phases of the biofilms (day 10) grouped in separated clusters for PLA and glass (dissimilarity 59 %), and also for PE but with less dissimilarity among samples (48 %). A clear shift in bacterial community was observed when the biofilms became mature (within day 30 – dissimilarity 66%) followed by a drastic change after the diatom bloom (within day 66 – dissimilarity 77 %), whatever the material type (PE, PLA, glass) and size. All the above cited clusters were significantly different when using SIMPROF tests ($p<0.05$).

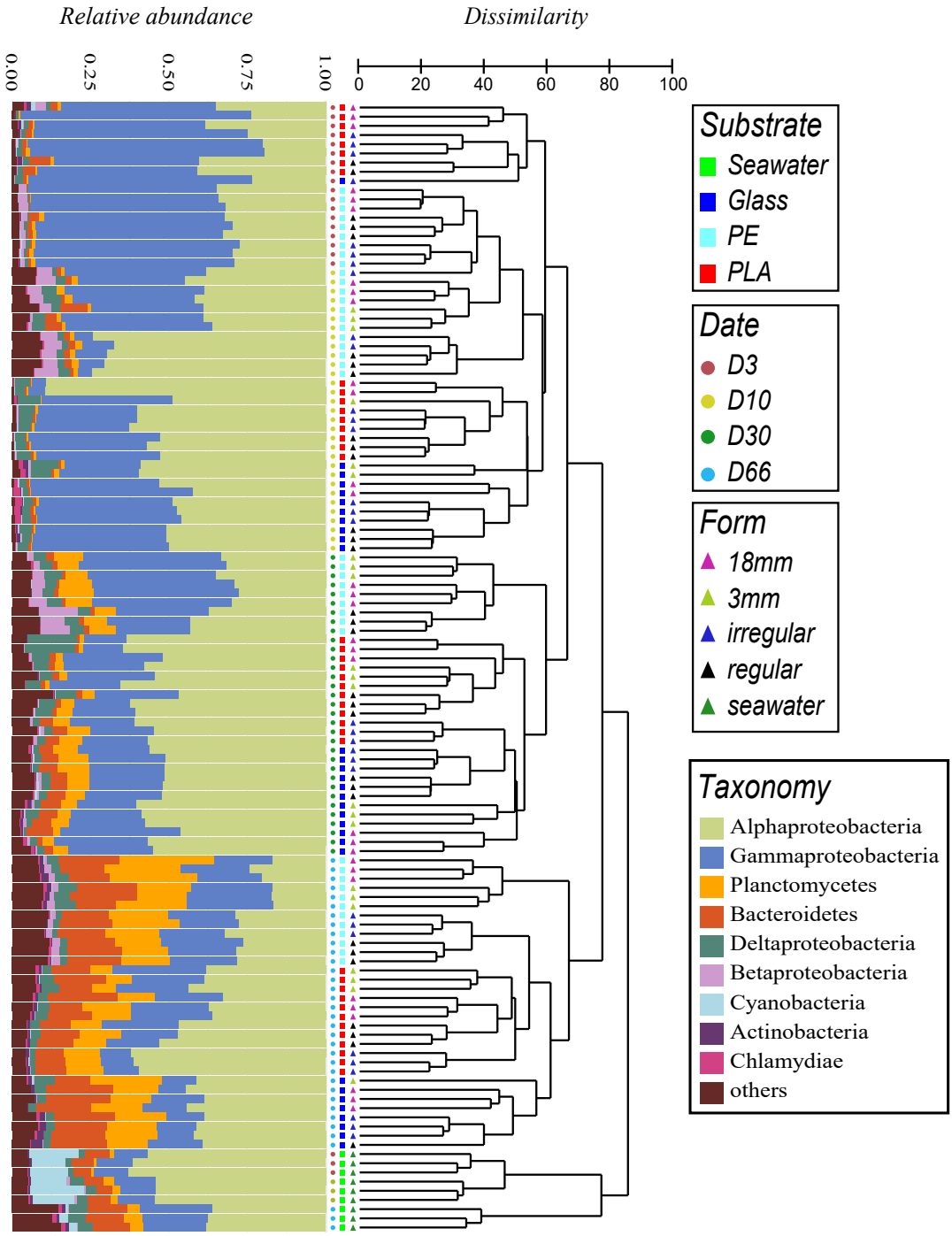


Figure 6. Comparison of temporal variation of taxonomic abundances and community structure of bacteria in seawater (SW) and biofilms of different materials (PE, PLA and Glass) according to immersion time in days (D), by cumulative bar charts comparing relative abundances (left) and by UPGMA dendrogram based on Bray–Curtis dissimilarities between 16S rRNA-based sequencing profiles (right).

Global PERMANOVA analyses with all samples confirmed the variance were highly explained by sampling date, to a less extent by chemical composition and material size ($R^2$= 0.39, 0.14 and 0.05 for PE, PLA and glass respectively, $p<0.01$) (Table S1). Pairwise PERMANOVA analyses confirmed that the factors of sampling date or material chemical composition can significantly explain the changes of bacterial community structure within each group, with higher values found between the primo-colonization or the growing phase compared to the mature biofilm influenced by the diatom bloom ($R^2 > 0.37$, $p<0.01$). Smaller but significant differences were found between substrate types ($R^2 = 0.07$ between PLA and glass and $R^2 =$ 0.11 and 0.14 between PE and PLA or glass, respectively; $p<0.01$), indicating that the bacterial community is more similar between PLA and glass than with PE. However, no significant difference between material size or shape could be found ($p>0.05$), which was also supported by dispersion analyses ($p>0.05$) (Table S2).

The time was the main factor driving the bacterial communities, the material type showed also different patterns. At each sampling date, PE formed always a specific cluster with all the size fractions. This was also the case for PLA and glass, except for the primo-colonization (Day 3) where they grouped in a same cluster.

Interestingly, Mantel tests showed a significant relation between temporal changes in the seawater communities and in the biofilms growing on the different material types (Table S3). Permutating correspondence between seawater and biofilms communities showed high correlation (Spearman rank=0.84; $p<0.05$) within dates (D3, D10, D66 seawater samples compared to biofilms at the same dates) that decreased drastically when permutating dates and when permuting day 66 in particular (Spearman rank < 0.5; $p<0.05$). Shared ASVs between seawater and biofilms were <25% for PE, <17% for PLA and glass, but these ASVs were abundant in the samples since they represented >48% of the tags in all cases, and maximum reach 62% for PE on day 3 (Figure S1). SIMPER analyses based on presence and absence data showed high dissimilarity among each substrate alongside the temporal evolution (61%, 65%, 63% and 60% for PE, PLA, glass and seawater respectively), while the shared ASVs and shared tags remained constant, suggesting that the bacterial community from the biofilm and seawater shift in the same direction.

*3.6. Taxonomic composition*

Taxonomic analyses confirmed the specificity of the community structures formed on the different materials compared to seawater, the latter being dominated by *Alphaproteobacteria*

and composed mainly of *Gammaproteobacteria*, Cyanobacteria, Bacteroidetes and Actinobacteria throughout the experimentation (Figure 6).

The distinct phases of biofilm formation were also clearly visible. The primo-colonization phase (Day 3) was dominated by *Gammaproteobacteria* (61±0%, 61±12%, and 71% for PE, PLA and glass, respectively) and *Alphaproteobacteria* (31±2%, 31±9% and 23% for PE, PLA and glass, respectively). The growing phase resulted in a decrease of *Gammaproteobacteria* (27 ±18%, 29 ±15%, and 40 ±8% for PE, PLA and glass, respectively) and a concomitant increase of *Alphaproteobacteria* (53 ±17%, 64 ±15%, and 51 ±5% for PE, PLA and glass, respectively). Dominant family were *Alteromonadaceae* for *Gammaproteobacteria* and *Rhodobacteriaceae* for *Alphaproteobacteria*. The main change for the maturation phase (Day 30) was the increase of Planctomycetes (8 ±1%, 4 ±2% and 6 ±2% for PE, PLA and glass, respectively) and of Bacteroidetes mainly for glass but not for PE and PLA (5 ±2% for glass). At this stage, *Gammaproteobacteria* (39 ±9%, 22 ±5% and 25 ± 5%) became as abundant or even less abundant as *Alphaproteobacteria* (34 ±6%, 58 ±5% and 54 ±4% for PE, PLA and glass, respectively).

The mature biofilm changed drastically in the presence of diatoms with a continuous increase of Planctomycetes (20 ±5%, 10 ±5% and 16 ±3% for PE, PLA and glass, respectively) and Bacteroidetes (16 ±2%, 12 ±3% and 18 ±3% for PE, PLA and glass, respectively). The presence of diatoms at this stage was confirmed when looking at the eukaryote sequences that were initially removed for the bacterial diversity analysis. Note that the number of eukaryotic sequences increased at the end of the experiment (day 66), especially for glass where eukaryotic sequences could reach until 19% of the total reads per sample, but also for PE and PLA where they can reach until 12% and 9%, respectively (data not shown). Interestingly, eukaryotic sequences were always <4% before the day 66 on these materials and represented <0.7% all along the experiment in seawater. More than 50% and until 86% of the eukaryotic sequences belong to the diatom *Pseudo-nitzschia* sp. on day 66 for PE, PLA and glass, whereas the sequences of *Pseudo-nitzschia* sp. in seawater remained relative very low (<10%).

We followed in particular 21 dominant bacterial ASVs that accounted for >5% of the sequences in each substrate for individual sampling date (Figure 7). Among those top 21 ASVs, 5 were unique for PE, PLA or glass (Figure 7). We found the SAR11_surface_2 and *Rhodobacteraceae* more abundant in seawater compared to biofilms on the different material types.

During the primary colonization phase (Day 3), one ASV belonging to *Alterononadaceae* was abundant in all material types, and distinction could be made between *Neptuniibacter* sp. and

*Thalassobius* sp. that were more abundant on PE, whereas *Cellvibrionaceae* were more abundant in PLA, and *Alteromonas sp.* in glass samples. During the growing phase (Day 10), *Thalassobius* sp. were more represented on both PE and glass, while *Ponticaulis* sp. and *Oleibacter* sp. were more found for PLA. Changes between the maturation phase (day 30) and the diatom bloom event (day 66) varied according to the material types. The ASVs SSI-B-06-26 was more abundant on PE at day 30 and *Gammaproteobacteria* and *Portibacter* sp. increased drastically at D66. *Ponticaulis* sp. dominated PLA at day 30, while it switched to *Gammaproteobacteria* ASV at day 66. Less changes were found for glass where *Sphingobium* sp. remain abundant for glass both at day 30 and day 66.

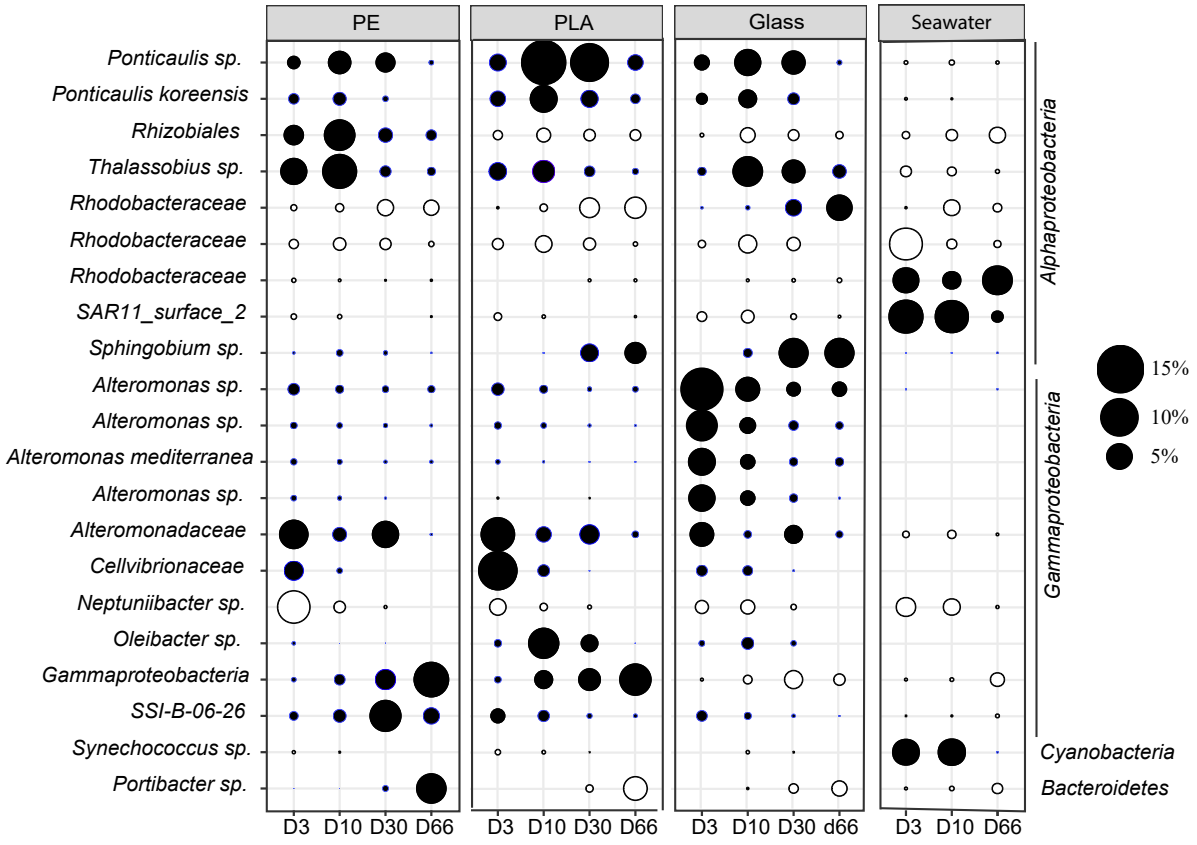


Figure 7. Bubble plot showing the relative abundance of the majority ASVs (>5%), each dot is the average result among different size (18mm, 3mm, IR and RE), the closest classification of each ASV was shown on the left of the panel, black filled color indicates significant different between seawater and corresponding substrate (considering all sampling date from Welch's ANOVA test).

## 4. Discussion:

The experiments have provided data on the time evolution of bacterial abundance, diversity and activity, allowing us to discuss the role of the chemical nature of the material (PE, PLA or glass) and the samples shape and size on these three colonization indicators.

### *4.1 Effect of size and shape of the substrate and its chemical composition on the plastisphere*

Raw measured values of abundance were found to differ between diverse size (meso-plastics of 18 mm diameter, LMP of 3mm diameter and SMP of 100 µm diameter) and shapes (irregular IR and regular RE) for a given material. However, once the abundance was expressed *per unit surface* as in Figure 3, the differences disappeared between mesoplastics, LMP and SMP of RE or IR shapes. This shows, for the first time, that the apparent size effect on raw data is only due to the difference in specific surface (i.e. surface to volume ratio) for different particle shapes. For a same mass of material, an irregular surface has a larger available surface than a regular one, which therefore leads to a higher abundance hence a higher activity. In the present experiments, the excess surface is typically around 1.5 fold higher for IR compared to RE.

Besides, there was also no effect on the bacterial diversity from the different material size or shape, while bacterial activity was dissimilar depending on the material size or shape. For instance, PE in 3 mm (slight curly) was higher than other 18 mm, and IR was higher than RE, which could be explained by very large roughnesses at the typical scale of a bacteria, one could expect in addition, a different packing of bacteria or a different biofilm structure - due for example to adhesion differences - which could induce differences in density of bacteria and/or in their heterotrophic production. In this study though, the roughness of all samples (including IR beads) was of the order of 100 nm over 100x100 squared microns areas, so that such effects -if any- would not be visible for the bacterial abundance or diversity. More studies on roughness at a very small scale need to be undertaken if one wants to conclude on this aspect.

The present experiments also seem to show that in the present case, the temporal dynamic of biofilm formation together with the material type were always more important factors than the material size and shape for shaping the bacterial abundance, diversity and activity. This is an interesting result since it has been shown that the bacterial spatial position on natural plastisphere is not totally even distributed, meaning that the bacterial community could be size-dependent for small enough particles (Schlundt et al., 2019).

During the three phases of its development, the biofilm evolved in a fairly different way on the two polymers studied (PE and PLA) and glass. PLA and glass were more similar in terms of bacterial activity and diversity compared to PE, while PE and PLA had similar and higher bacterial abundance compared to glass. In general, PE showed drastic differences compared to glass, while PLA showed intermediate values between them. The result is also pointing out that the bacterial activity could be not correlated to its abundance on plastisphere, which is similar to that found in seawater (Campbell et al., 2011). This tends to confirm the role of wetting properties: polymer surfaces being far more hydrophobic than glass. Attachment to surface are indeed supposed to be mediated via specific and non-specific interactions, both depending on surface hydrophobicity/hydrophilicity, roughness, charge and functional groups (Caruso, 2020). The role of hardness cannot however be excluded since it was recently shown to also be a key factor compared with other physicochemical properties (Cai et al., 2019).

Somewhat surprisingly, PE showed on average a higher abundance and activity than PLA and also presented specific bacterial community structure. These small but significative differences between the two polymers could be an expression of the chemical differences between their surfaces. However, it should be noted that they also presented a large difference in their buoyancy: HDPE (0.95 $g.cm^{-3}$) was floating, whereas PLA (1.25 $g.cm^{-3}$) sinked in our experimental conditions, which could lead to a different oxygenation of water or a different exposure to bacteria (composition, light intensity, contact frequency, exposition to air versus water).

Even though PLA is a compostable polymer and degradable in human body (Pillai and Sharma, 2010), it is known, like PE, not to be biodegradable in marine environment (Karamanlioglu et al., 2017), and certainly not in the relatively short time frame of this experiment. Colonization, heterotrophic activity as well as the specificity of the species observed are therefore certainly not related to any biodegradation of the polymers. It seems more relevant to attribute the colonization, activity and specialization of bacteria to the formation of a conditioning film and subsequently the chemical composition of extracellular polymeric substance and which could also be surface properties dependent.

### *4.2. Presence of three following phases of the biofilm development in all substrates.*

Confocal microscopy was a powerful tool to follow the biofilm formation on the various samples tested in our study. For all studied material, the triplicate samples out of all the

sampling dates highlighted three classical successive phases (primo-colonization, growth and maturation) of biofilm formation, already observed on natural (rocks and algae) or artificial surfaces (glass, acryl and steel and plastics) immerged in seawater (Caruso, 2020).

First, the primo-colonization designates the pioneer bacteria that shape the first layer of initial biofilm (Lorite et al., 2011). After 3 days of immersion in natural seawater, we observed that the primo-colonizers presented a higher abundance and heterotrophic activity ($^{3}$H leucine incorporation) on PE compared to PLA and glass. MiSeq 16S rRNA sequencing revealed that bacterial richness was already high after 3 days with a minimum average Chao1 estimation of 265 ASVs whatever the material types. We observed distinct but closed bacterial communities in PE, PLA and glass, dominated by Gammaproteobacteria (61%, 61% and 71% for PE, PLA and glass, respectively) and Alphaproteobacteria (31%, 31% and 23% for PE, PLA and glass, respectively). Previous studies showed that the *Roseobacter* clade and *Alteromonas* were the main bacterial primary colonizers (Dang and Lovell, 2000; Dang and Lovellc, 2015; Salta et al., 2013). From our results, we reported that *Thalassobius* sp. of the Roseobacter clade could be also the primary colonizer on plastisphere. *Thalassobius* sp. was also found on 3-day-old polymethyl methacrylate (PMMA) panel immersed the Arabian Gulf with relatively low abundance compared to our study (Abed et al., 2019) as well as on PE plastisphere from North Atlantic (Zettler et al., 2013). *Alteromonas* sp. was extremely abundant on glass samples compared to PE and PLA, where the three materials occupied different ASVs from Alteromonadaceae (Figure 7). *Neptuniibacter* sp. was also one of the main taxa revealed in our study, but also on 7-day-old colonized poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) from the Banyuls Bay, France (Dussud et al., 2018a). Even though it is considered that the *Neptuniibacter* sp. is an hydrocarbonoclastic bacteria (Dombrowski et al., 2016), we showed here that it might be just a primary colonizer, while not participating to the plastic degradation.

Second, the growing phase of the biofilm is described growth by secondary species, which induce modifications in the properties of the substratum (Lorite et al., 2011). After 10 days immersion in seawater, we have seen an increase in bacterial abundance and heterotrophic activity, together with significant changes in bacterial community structure for all material types. In particular, Alphaproteobacteria became more abundant (53%, 64%, and 51% for PE, PLA and glass, respectively) compared to Gammaproteobacteria (27%, 29%, and 40% for PE, PLA and glass, respectively). *Thalassobius* sp. was still abundant in the growing phase after the primo-colonization phase. In addition, we firstly reported the *Ponticaulis* sp. as an important group of primary colonizers on plastisphere, strikingly on PLA. Besides, previous study also

showed that *Ponticaulis* sp. was one of the main colonizers for the metallic alloys (Procópio, 2020). It suggested that the *Oleibacter* sp. could be one of the pioneer bacteria for plastic colonization within hours (Pollet et al., 2018), while we showed that it was more obvious on PLA in the growing phase after 10 days.

Third, the "maturation phase" occurs through diverse, competitive or synergistic interactions between cells, with either further recruitment or loss of species (Lorite et al., 2011). This has led to a stabilization of bacterial abundance and heterotrophic bacterial production, together with a drastic shift in bacterial community structure in all material types. PE still presented significantly higher abundance and activity compared to glass, whereas PLA showed similar bacterial abundance compared to PE and similar bacterial activity compared to glass. At this stage, Gammaproteobacteria (39%, 22% and 25%) were as abundant or even less abundant as Alphaproteobacteria (34%, 58% and 54% for PE, PLA and glass, respectively). Bacteroidetes and Planctomycetes were also found as secondary colonizers in other studies (Dang and Lovellc, 2015; Pinto et al., 2019). We observed noticeable increases of Planctomycetes (8%, 4% and 6% for PE, PLA and glass, respectively) and also Bacteroidetes (mainly for glass (5%) but not for PE and PLA). Interestingly, we observed a similar evenness associated to an increase of richness during the growing and maturation phases, which is characteristic of spatial heterogeneity, abundant and heterogeneous resources and nutrients offered by plastic particles compared to the nutrient-depleted oceanic deserts (Zhou et al., 2002).

During this study, we found that the Roseobacter and Alteromonas were important clades for whatever the three-colonization phase, while the two clades were also found as bacterial 'phycosphere' (bacterial taxa colonizing on phytoplankton) (Seymour et al., 2017). Thus, we suspect that the plastic surface and phytoplankton surface could have some similar trait to be the environmental cue for these two clades, otherwise these two clades were simple surface-colonizers whatever the material surface types.

### *4.3. Influence of phytoplankton bloom on the mature biofilm.*

In our study, the diatoms were presented in seawater during the entire course of the experiment course, while a diatom bloom was observed at day 66 on plastisphere. It suggested that a bacterial biofilm would be a prerequisite for the diatom bloom on plastisphere. Most of the microalgae sequences on plastisphere belonged to *Pseudo-Nitzschia* sp., which is in accordance to the observation of their typical morphotype on day 66 with confocal microscopy technique

on all material surfaces. The diatoms were more observed on the film other than SML, suggesting that the rigidity morphology of diatoms require more flatter surface area for the colonization comparing to bacteria. *Pseudo-nitzschia* is a global distributed diatom genus in the marine environment (Lelong et al., 2012). It has not only been reported in the Mediterranean Sea of marine observatory stations in the Banyuls Bay (France), but also in the 150 km-south Blanes Bay (Spain) where the phytoplanktonic bloom in seawater were consistently attributable to chromophytes, the most abundant taxa being *Pseudo-nitzschia calliantha* (Mura and Agustí, 1996; Charles et al., 2005).

Diatoms have been found as omnipresent and sometimes dominant colonizers on plastic debris (Oberbeckmann et al., 2014; Maso et al., 2016; Michels et al., 2018; Kettner et al., 2019). Morphological identification by microscopy together with new chloroplast databases from bacterial amplicon surveys (Decelle et al., 2015) included *Mastogloia*, *Cyclotella*, *Pleurosigma, Amphora* and *Pseudo-Nitzschia* genera in the Arabian Gulf, Grenada Island, Atlantic and Pacific gyres (Amaral-Zettler et al., 2020). *Pseudo-nitzschia* spp. has been identified as the dominant diatoms on 10-day-old biofilm developed on polystyrene Petri dishes immersed at the low intertidal zone, Hong Kong (China) (Chiu et al., 2008). To our knowledge, it is the first time that *Pseudo-nitzschia* spp. were identified as dominant phototrophs on plastic debris on Mediterranean plastisphere. The diatoms evens happened on plastisphere could be also related to the diatom bloom events happened on Banyuls Bay (Figure 1C).

Interaction between phytoplankton and bacteria are known to play key roles in mediating biogeochemical cycling and the food web structure in the ocean, including the microbial loop (Mayali, 2018). Diatom blooms are also known to be one of the main drivers of the temporal dynamics of bacterial abundance, diversity and activity in the Mediterranean Sea and elsewhere (Ghiglione et al., 2005; Lambert et al., 2019), with consistent taxonomic association between specific bacteria and diatom taxa (Behringer et al., 2018). Our results confirm that such association exist also within the biofilms associated with plastic, as it has been observed elsewhere (Amaral-Zettler et al., 2020). We found co-associated bacterial epibionts on the mature biofilms at day 66 that were related to the specific biofilm of each polymer type. For example, we found common colonizers of diatom detritus, such as *Portibacter* sp. (Crenn et al., 2018), and *Sphingobium* sp. (Ramanan et al., 2015) and Rhodobacteraceae (previously mostly assigned as Roseobacter clade) (Simon et al., 2017). The interaction between diatoms and bacteria within the mature biofilms was accompanied with a drastic increase of bacterial heterotrophic activities in PE and PLA. This is a typical response of nutrient recycling

heterotrophs to primary producing photoautotrophs, where bacterial activity per cell increases drastically together with changes in community structure (Mayali, 2018). Our results showed that the diversity and activity of the mature biofilms on plastic can be rapidly and drastically changed due to phytoplanktonic growth on plastics, whatever the polymer type, size or topography. To our knowledge, only one study so far measuring chlorophyll *a* and net primary production in the North Pacific gyre showed that microplastic particles were creating net autotrophic hot-spots in the oligotrophic ocean (Bryant et al., 2016). In parallel, another unique study in the Mediterranean Sea revealed higher bacterial heterotrophic activity on plastic compared to the surrounding seawater (Dussud et al., 2018a).

It has been reported that the average bacterial growth rate in seawater is 0.1 $day^{-1}$ (Kirchman, 2016), whereas we found here that the growth rate was higher than 0.1 $day^{-1}$ whatever the material type. During this study, we cannot really compare the bacterial activity or growth rate between plastisphere *vs.* our seawater samples, because of the lower bacterial abundance numeration on seawater samples. While we could propose the possible scenario, the bacterial growth rate on the primo-colonization and growing phase should be higher than that of seawater, at least on PE samples, as previous study showed that the Roseobacter and Alteromonadaceae have relative high growth rate compared to the bulk bacterial community (Ferrera et al., 2011). The bacterial activity or growth rate on the maturation phase in the marine environment could be higher than seawater considering that autotroph microbes such as diatoms were omnipresent on plastics.

Further works coupling both primary and heterotrophic production measurements are needed to determine the bacterial activity difference between plastisphere and seawater, but also test if the microscale algal-bacterial interactions on the large amount of plastic floating in sea surface have consequences on ecosystem functioning and/or biogeochemical cycling. Our work also showed links between bacterial plastisphere and seawater community, but future works should also consider this point other than only being checking the community difference.

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***Conflict of interest.*** *All of the reported work is original, and authors have seen and approved the final version submitted. The material has not been submitted for publication elsewhere while under consideration for Environmental Microbiology journal. The authors declare that there are no conflicts of interest. Likewise, consent is given for publication in the Environmental Microbiology journal, if accepted.*

# Chapter 2 supporting information

Table S 1: Global PERMANOVA on the factor of sampling date, chemical composition and material size

| | Df | Sum of square | Mean of square | F value | $R^2$ | Pr(>F) | p.betadisper |
|---|---|---|---|---|---|---|---|
| Size | 3 | 1.2434 | 0.41447 | 1.842 | 0.04783 | **0.011** | 0.05165 |
| chemical composition | 2 | 3.5863 | 1.79314 | 8.8822 | 0.13796 | **0.001** | 0.4128 |
| sampling date | 3 | 10.149 | 3.3828 | 23.483 | 0.3904 | **0.001** | **0.0001397** |
| Total | 113 | 25.995 | | | 1.0000 | | |

Table S 2: Pairwise PERMANOVA on the factor of sampling date, chemical composition and material size

| | Pairs | $R^2$ | *p*.value | *p*.adjusted | *p*.betadisper |
|---|---|---|---|---|---|
| Sampling date | D10 vs D3 | 0.155 | **0.001** | **0.001** | **0.027** |
| | D10 vs D30 | 0.207 | **0.001** | **0.001** | 0.961 |
| | D10 vs D66 | 0.375 | **0.001** | **0.001** | **0.020** |
| | D3 vs D30 | 0.308 | **0.001** | **0.001** | **0.027** |
| | D3 vs D66 | 0.416 | **0.001** | **0.001** | **0.001** |
| | D30 vs D66 | 0.236 | **0.001** | **0.001** | **0.020** |
| Chemical composition | Glass vs PE | 0.138 | **0.001** | **0.001** | 0.968 |
| | Glass vs PLA | 0.068 | **0.001** | **0.001** | 0.432 |
| | PE vs PLA | 0.109 | **0.001** | **0.001** | 0.432 |
| Material size | 18mm vs 3mm | 0.029 | 0.128 | 0.140 | 0.184 |
| | 18mm vs IR | 0.031 | **0.049** | 0.122 | 0.081 |
| | 18mm vs RE | 0.023 | 0.140 | 0.140 | 0.081 |
| | 3mm vs IR | 0.054 | **0.009** | 0.054 | 0.774 |
| | 3mm vs RE | 0.035 | 0.061 | 0.122 | 0.774 |
| | IR vs RE | 0.025 | 0.140 | 0.140 | 0.901 |

Table S 3: Mantel test between bacterial plastisphere and seawater community.

| | Correspondence | Spearman correlation (Rho) | Note |
|---|---|---|---|
| Biofilm community | D3, D10, D66 | 0.845* | No exchange[#] |
| Seawater community | D3, D10, D66 | | |
| Biofilm community | D3, D10, D66 | 0.2727* | exchange of D10 and D66[†] |
| Seawater community | D3, D66, D10 | | |
| Biofilm community | D3, D10, D66 | 0.7227* | exchange of D3 and D10 |
| Seawater community | D10, D3, D66 | | |
| Biofilm community | D3, D10, D66 | 0.4511* | exchange of D3, D10 and D66 |
| Seawater community | D10, D66, D3 | | |
| Biofilm community | D3, D10, D66 | 0.4583* | exchange of D3, D10 and D66 |
| Seawater community | D66, D3, D10 | | |
| Biofilm community | D3, D10, D66 | 0.4975* | exchange of D3 and D66 |
| Seawater community | D66, D10, D3 | | |

Notes：Seawater community permutation were taken before Mantel test for some groups. for instance, the symbol of # indicates no permutation. † indicates that one matrix of seawater community was permutated before mantel test for D10 and D66. * indicating *p* value less than 0.05.

From the results one can note that the correlation is the highest for the group without permutation, that means that the bacterial community from plastisphere and seawater were highly linked to each other.

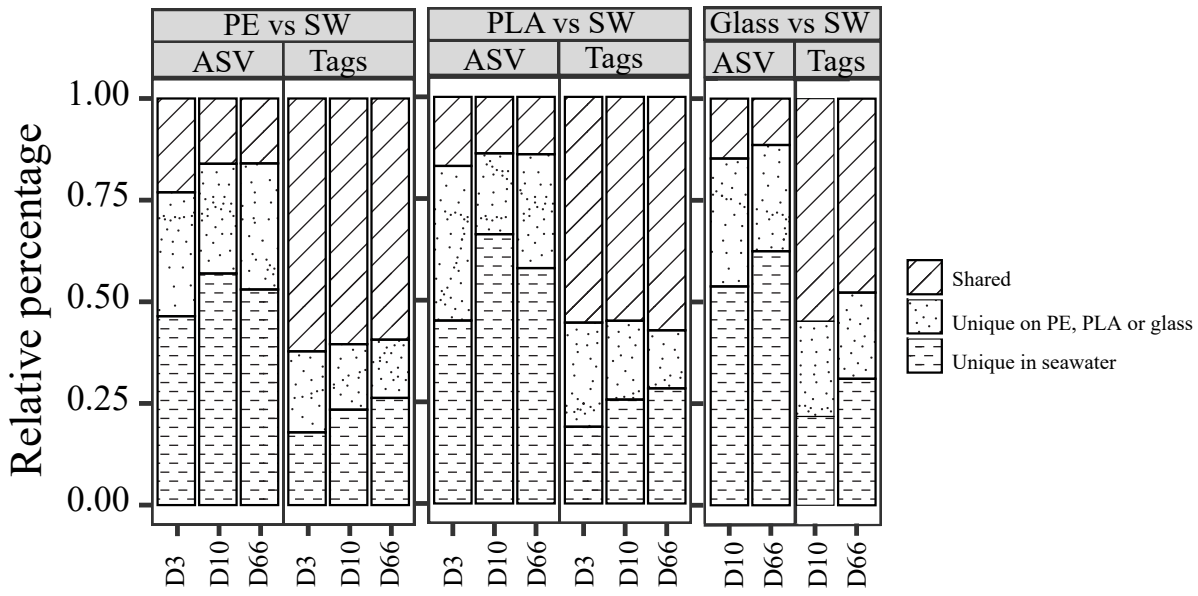


Figure S 1: The shared and unique ASV and reads between the bacterial community on biofilm and seawater during the course of the experiment at days 3 (D3), 10 (D10), 30 (D30) and 66 (D66).

# *CHAPTER 3: Beneficial or detrimental effects of microplastics on the marine filter-feeder amphioxus (Branchiostoma lanceolatum)?*

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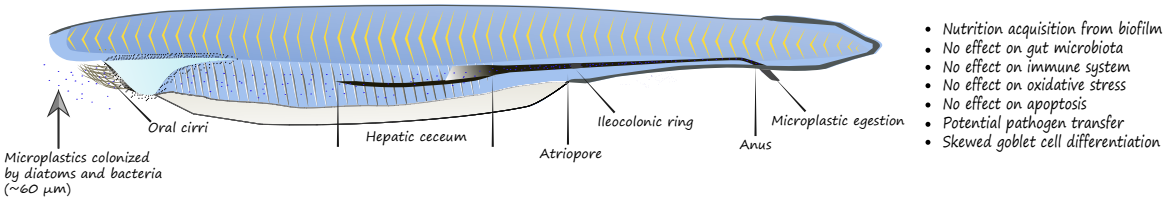
**Keywords:** microplastics, toxicity, gut microbiome, plastisphere, oxidative stress, amphioxus

# *Chapter 3*

**Abstract:**




Microplastics are ubiquitous in the marine environment and accumulate in the coastal regions, which especially pose the health threats to marine wildlife living in the microplastic accumulation hotspots. Here, we evaluate the effect of microplastics on amphioxus (*Branchiostoma lanceolatum*), a marine invertebrate with filter-feeding lifestyle living in shallow water sediments. First, gut microbiome from different treatments (with or without starvation) were tested and selected to obtain the most homogenous group prior microplastic exposure. Second, polystyrene microplastics were immersed in natural circulating seawater to mimic its natural presence. Third, the microplastic exposure on amphioxus were carried out for 16 days with three different concentration (50, 500 and 5000 particles. $L^{-1}$). The results showed that microplastics did not modify the gene expression on oxidative stress, immune system and apoptosis (Nanostring technology). No impact on the gut microbiota was observed (16S rRNA sequencing), even if transfer of potential harmful algae and pathogen could be possible for the highest and rather unrealistic concentration of microplastics. Within microplastic exposure treatments, we observed skewed cell differentiation into more goblet cells by transmission electron microscopy. A rather positive impact of microplastics was found by transfer the nutrition (mainly diatoms) from microplastics to amphioxus. This study provides a multidisciplinary approach to prove the low toxicity of microplastic and its biofilm on a marine filter-feeder.

# *Chapter 3*

## 1. Introduction:

Plastic production is continually increasing, with 380 million tonnes in 2015 (Geyer et al., 2017), plastic waste entering the oceans was estimated at 4–12 million tonnes from 192 coastal countries in 2010 (Jambeck et al., 2015). Microplastics were defined as plastic particles smaller than 5 mm, generated from fragmentation of larger plastic items or directly manufactured to be of microscopic size (Barnes et al., 2009; Andrady, 2011). Microplastic accumulation were observed on surface water of enclosed seas and subtropical gyres (Eriksen et al., 2014; Cózar et al., 2015). The sediment of littoral and subtidal zones, as well as deep sea ocean were also highly polluted by microplastics (Browne et al., 2011; Woodall et al., 2014; Van Cauwenberghe et al., 2015; Kane et al., 2020).

Given the ubiquitous plastics in the marine environment, it poses the health threats to the marine life. The expected effects of microplastic on marine life are combined and include (i) direct physical mechanical damage, (ii) ingestion and gut blockage, (iii) introduction of pathogenic agents colonizing plastics and (iv) exposure to chemical contaminants including plasticizers and organic pollutants concentrated on the plastics out of the seawater column (Mato and Isobe, 2001; Sweet et al., 2019).

Ingestion and entanglement have been reported on 395 species by large piece of plastic on higher organisms, typically vertebrates as turtle, fish, birds and sea mammals (Gall and Thompson, 2015). However, the process of microplastic ingestion is relatively less studied, related species are mainly on the invertebrates of bivalves, crustacea and vertebrates of fish. Information regarding on biological impacts of microplastic is just emerging (Wright et al., 2013; Wesch et al., 2016). Physiological impact of microplastics on marine life have shown the reduction feeding activity on deep water corals with 500 µm polyethylene microplastics (Chapron et al., 2018), translocation to circulation system on mussel with 3 µm polystyrene microplastics (Browne et al., 2008), reproduction impairment on oyster with 6 µm polystyrene microplastics (Sussarellu et al., 2016) and potential trophic chain transfer to the fish with the size around 6 cm from the Northern Pacific Ocean (Boerger et al., 2010). At the cellular and molecular levels, alteration of immunological response has been observed on mussel with 1-50 µm polyethylene microplastics (Détrée and Gallardo-Escárate, 2018b), modification of bacterial microbiome on zebrafish with 5 µm polystyrene microplastics (Qiao et al., 2019) and oxidative stress on rotifer with 6 µm polystyrene microplastics (Jeong et al., 2016). Lastly,

another concern is that plastics could act as vector for transferring organic pollutant and additive to marine life (Browne et al., 2013; Koelmans et al., 2016; Lamb et al., 2018). The effect of microplastics on the microbiome composition *via* the holobiont concept, which involves assessing the health of a host organism in the context of its associated microbiome has never been investigated (Thompson, 2015; Lartaud et al., 2020) Gut microbiome contributes to the physiology, development, immunity, and behavior of their host, and may respond very rapidly to changing environmental conditions, providing a powerful mechanism for acclimatization and also possibly rapid adaptation of meta-organisms (Rosenberg and Zilber-Rosenberg, 2014).

Except the one on deep water coral (Chapron et al., 2018), all the toxicity tests mentioned above were carried out with pristine materials, which could lead to divergences compared to real conditions in the nature environment. Indeed, once plastic has been immerged into the seawater, the microbial colonization process was considered to be initiated to reach a stable and mature biofilm after one month immersion in seawater. The microbial community presented on plastic surface were the so-called "plastisphere", with bacteria and diatoms being the most observed species (Zettler et al., 2013; Dussud et al., 2018). These microorganisms were shown to be different from the surrounding seawater or organic particles (Dussud et al., 2018) and also different from microbiome associated to macro-organisms. Therefore, the extent to which the plastisphere influences the organism microbiome in natural environment and its putative transfer of microorganisms from the plastisphere to the host microbiome need to be investigated.

Owing to much abundance of microplastics and more accessible to wide range of organisms, the impact of microplastics could be even more detrimental for invertebrates of suspension filter-feeding species, which filter large water volumes, thus may ingest high quantities of microplastics (Galgani, 2013; Eriksen et al., 2014). Amphioxus, as the invertebrates, is one of the closest living relatives of the vertebrates, also one kind of filter-feeding marine animals that burrow in sand in tropical or temperate waters around the world, almost all amphioxus species were found in shallow water close to the seashore (0.5 m to 40 m deep) (Bertrand and Escriva, 2011). The amphioxus existence is susceptible to human activity and environment changes, the population decline was recorded for some habitats (Holland et al., 2017). Considering coastal areas are more contaminated by plastics (Pedrotti et al., 2016), the potential toxicity effects on amphioxus are required to be determined.

In this study, special care was taken on the gut microbiome stability of amphioxus in order to ensure the most homogenous condition between individuals before toxicity tests, including

immediate use of animals after sampling, after one-week starvation or after one-week feeding with algae followed by one-week starvation. In order to perform toxicity tests as close as possible to environmental conditions, another concern was to provide microbeads together with the same mature microbial biofilm to amphioxus all along the 16 days toxicity tests. The aim of this study was to answer the following questions: what is the impact on the gut microbiota, including transfer of microorganisms growing on plastics to amphioxus? Does microplastic ingestion result in gene expression of immune system and apoptosis in amphioxus or in gene expression and protein activities of oxidative stress response? Complementary technologies were used to answer these questions *via* the holobiont approach, including next generation 16S rRNA sequencing, nanostring technology, biochemical tests and histopathological observation using transmission electron microscopy.

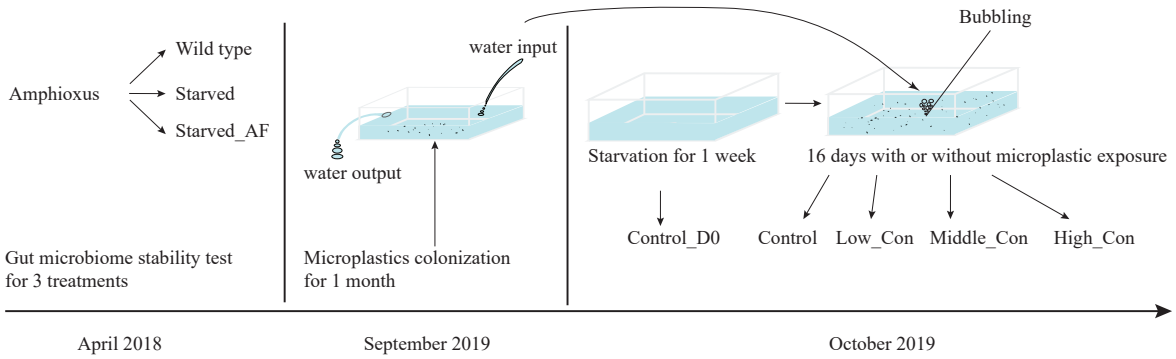



Figure 1. **Experimental design.** Schematic presentation of the three phases stepwise experiment of (1) the stability of gut microbiome was tested within three treatments on April 2018 (2) Polystyrene microplastics were immersed in natural seawater for 1 month from September 2019 (3) Microplastic colonized with the microbial community were used for the toxicity test after 1 week starvation on amphioxus from October 2019, with three concentration of 50, 500, 5000 particles. $L^{-1}$ designated as the Low_Con, Middle_Con and High_Con.

## 2. Materials and methods

### *2.1 Gut microbiome stability test*

Adult amphioxus (*Branchiostoma lanceolatum*) were collected in coastal waters closed to Leucate (France) at a depth around 1 m on April 2018. After transporting to the laboratory, 10 amphioxus with the size 3.0 ± 0.3 cm (mean ± SD) were anesthetized with 7% magnesium chloride for 5 min before dissection with sterile tools (designating as "wild type", hereafter). Gut specimen between the area closed to atriopore and anus was harvested under stereomicroscope, rinsed with phosphate-buffered saline (PBS) and snap-freezed with liquid nitrogen before stored in -80°C. Another 10 amphioxus with the size of 3.1 ± 0.2 cm were starved for one week in 0.2 µm filtered seawater changed every two days, and then dissect as above (designating as "starved", hereafter). Lastly, Another 10 amphioxus with size at 3.0 ± 0.1 cm were fed with algae for one week, and starved for another week before dissection (designating as "starved_AF" for "starvation after feeding", hereafter). The three treatment groups were used to test the stability of amphioxus gut microbiome before toxicity tests (Figure 1).

### *2.2 Microplastics preparation*

Commercially available polystyrene microbeads of 106-125 µm (Polysciences) were first used in this study. As the microplastics in the natural environment were mainly in irregular form, therefore, irregular microplastics were prepared by cryo-grinding (SPEX sample Prep) and further sieved in order to recover the microplastics with the diameter ranging from 50-100 µm, corresponding to the size of feeding particles. Granulometry analysis using a laser diffraction particle size analyzer (Malvern Mastersizer 2000 model with a Scirocco 2000 module) showed a gaussian distribution of the microbeads having the pick at 63 µm. The microplastics were immersed into the glass tanks on September 2019 with 2 L capacity (Verres Vagner, France), in which seawater was continually renewed (flow rate was set on 20 $mL \cdot min^{-1}$) by direct pumping at 14 m depth in Banyuls bay closed to the SOLA observatory station (NW Mediterranean Sea, France). Before plastic exposure to amphioxus, the microplastics that pre-colonized by microbes for one month were tested for its suspension in seawater with bubbling flow at 35 mL $second^{-1}$, aliquots of seawater samples were transferred on membrane filter, and counted by microscopy with 10 random views. The bubbling flow were used for all the tanks for toxicity test.

*2.3 Experiment design for microplastics toxicity tests*

Amphioxus were collected on October 2019 at the same location mentioned above, 630 adult amphioxus with the size at 3.7 ± 0.2 cm were distributed into 18 identical plastic tanks with 35 individuals for each tank, for which contains 2 L seawater after filtered with 0.2 μm Sterivex (51563, dominique Dutscher), the tanks were placed in a dark closet and illuminated from above with a 12/12 h light/dark rhythm. Temperature was kept at 16.9 ℃ by controlling circulating seawater temperature outside the tanks. All the amphioxus were starved for one week before plastic exposure, during which 0.2 μm filtered seawater were changed for every two days.

After one-week starvation, amphioxus were sacrificed from 3 tanks representing 3 replicates as the control before microplastic exposure (control_D0, hereafter). For each tank, amphioxus was anesthetized, and specimen was rinsed with PBS buffer. 5 gut or hepatic caecum specimens were pooled for biochemical or gene expression test and individual amphioxus specimen was used for gut microbiome. After collection, samples were snap-freezed with liquid nitrogen before stored in -80°C. Gut and hepatic tissue were individually fixed for histopathological tests before stored at 4 ℃ in an incubator.

12 tanks after starvation were divided into 4 groups of 35 amphioxus in triplicate tanks, including high microplastic exposure concentration, middle concentration, low concentration and control, corresponding to 5000, 500, 50 and 0 particles. $L^{-1}$ (Amphioxus were designated as High_Con, Middle_Con and Low_Con, respectively hereafter). The results were mathematically converted to weight at 0.42, 0.042, 0.0042 and 0 mg. $L^{-1}$ for the 4 groups according the granulometry results. The plastic exposure was lasted for 16 days with bubbling, with the filtered seawater change for every two days (Figure 1). No food was provided during this process to reduce the potential impact. For each tank, and oral cirri and skin from belly were checked for its health condition, and the one without morbidity sign were used for dissection as mentioned above, for which the gut contents were gently removed if there were some.

The remaining 3 tanks after starvation were used to test the quantity of microplastic ingestion with the three concentration, after the microplastics were introduced into tanks for two hours, 5 random individuals from each tank was distributed into petri dishes with filtered seawater inside, and placed in dark for overnight to allow all the ingested microplastic be egested, all the feces were collected, and lysed with proteinase K with final concentration at 0.2 mg/mL

(Sigma-Aldrich) for 1 hour at 55 ℃. The pellet with supernatant were transferred on filter and stained with Nile Red (0.01 mg/mL) (Sigma-Aldrich) (Maes et al., 2017), microplastics were counted under microscope.

*2.4 Amphioxus body size change*

Pictures were taken from 3 tanks at the beginning and all the tanks at the end of plastic exposure, body length were measured for 10 random individuals for each tank with the software ImageJ (Schneider et al., 2012).

*2.5 Sequencing of gut microbiome*

The genomic DNA from gut tissue in triplicate were extracted with classic phenol-chloroform protocol (Ghiglione et al., 2005). PCR amplification of 16S V4-5 region was done using 515F-Y and 926 R primers (Parada et al., 2016). Next generation 16S rRNA sequencing were performed on Illumina MiSeq platform, samples from 2018 and 2019 were sequenced by MR DNA (Canada) and Genoscope (France) separately.

Processing of 16S rRNA sequences for the dataset of 2018 and 2019 were performed with DADA2 pipeline (Callahan et al., 2016) using R 3.6.1 version (Bunn and Korpela, 2008). The primers were trimmed off before error correction and denoising step. Paired reads were merged (average length from 364 to 378 bp) and all the singletons were discarded. The chimeras were checked and removed for the merged reads. The amplicon sequence variants (ASVs) were assigned with SILVA release 128 database (Quast et al., 2013). The taxonomic affiliation of ASVs of interest were further verified against sequences from the NCBI database using BLASTnt. The eukaryotes, archaea, chloroplast and mitochondria were removed and all the sample were rarified to the same depth (rngseed=T) before the analyses on bacteria using phyloseq R package (McMurdie and Holmes, 2013). The α-diversity were performed with Microbiome R package. Taxonomy composition were analyzed with web-based platform of microbiomeanalyst (Chong et al., 2020). Relative abundance of ASVs (> 5% for each library) within each treatment were averaged, and visualized by heatmap with online tool of ClustVis (Metsalu and Vilo, 2015). Potential bacterial transfer was defined as the ASVs absent in control (without microplastic exposure after 16 days) and Control D0, and present on plastic and gut samples after plastic exposure.

*2.6 Gene expression assay*

Triplicate RNA samples were extracted with RNeasy Mini Kit following manufacturer instruction (74104, Qiagen), RNA quality and quantity were verified with DeNovix, RiboGreen dye (R11490, Invitrogen) using Victor3™ and also Agilent 2100 bioanalyzer.

Nanostring technology were applied to gene expression assay (Kulkarni, 2011), 62 genes were selected concerning on immune system, stress response, apoptosis and housekeeping gene based on the literature (Huang et al., 2011; Zhang et al., 2016) and also online database: UCSC database (Karolchik et al., 2003), Amphiencode database (Marlétaz et al., 2018), JGI database (Putnam et al., 2008), and LanceletDB database (You et al., 2019). The Coding Sequence (CDS) regions were compared between *B. lanceolatum* and *B. belcheri* with BioEdit software (Hall, 1999), conservative region were chosen to ensure the sequence's accuracy, selected nucleotide sequences were translated into protein to reconfirm the accuracy with online tool of ExPASy (Artimo et al., 2012), probe specificity were achieved by local BLAST by BioEdit software. The probes were synthesized by Nanostring company, and the probe hybridization were carried out with nCounter Analysis System from CRCT platform (Toulouse, France).

After the raw data was generated, background and housekeeping gene normalization were carried out with the "advance analysis" module from nsolver software (Nanostring), algorithm of geNorm were used to determine housekeeping gene's superior. For gut and hepatic tissue, the comparisons were performed separately with its respective control sample, to be more exact, Control_D0, High_Con, Middle_Con and Low_Con were compared to Control treatment.

*2.7 Biochemical analysis*

Enzymatic biomarkers of functional responses in gut and hepatic tissues were measured using superoxide dismutase (SOD) and Catalase (CAT) assay kits commercially available (000070, Sigma-Aldrich; ab83464, Abcam. respectively). The measurements were measured in triplicates according to the manufacturer instructions with some modification. Briefly, gut or hepatic tissues were disrupted and homogenized with sterile stainless steel beads (69989, Qiagen) using TissueLyser (Qiagen) in PBS buffer at 30 Hz for 2 min, and then centrifuged for 5 mins at 13000 g, the supernatant were divided into aliquots used for biochemical test and protein quantification. Protein content of samples was determined with Bradford method

(Bradford, 1976). The enzyme activity was expressed as the relative activity to its gut Control or hepatic Control.

*2.8 Histopathological observation*

Two kinds of fixatives as paraformaldehyde and glutaraldehyde were successively used to fix amphioxus gut and hepatic tissue (He et al., 2018). Duplicate samples were then imbedded into epoxy resin before transmission electron microscopy observation. Percentage of goblet cell were expressed by its number divided by total examined cell.

*2.9 statistical test*

For gene expression assay, student t-test were conducted on pairwise comparisons to test the differential gene expression, *p*-value were adjusted with method of Benjamini & Yekutieli (Benjamini and Yekutieli, 2001).

For 16s RNA sequencing data, an unweighted-pair group method with arithmetic (UPGMA) dendrogram based on Bray-Curtis similarities was used for visualization of beta-diversity with the software of PRIMER 6. A similarity profile test (SIMPROF, PRIMER 6) were performed on the null hypothesis that a specific sub-cluster can be recreated by permuting the entry species and samples. The significant branch was used as a prerequisite for defining bacterial cluster. Permutational multivariate analysis of variance (PERMANOVA) was used to test the significance difference for different treatment (Anderson, 2017) using *adonis()* function with vegan R package (Oksanen et al., 2008), the homogeneity of variances was respected with *betadisper()* function.

Other statistical analyses were performed with one way or multiway ANOVA in R software, tukey's test was used for post-hoc analyses.

## 3. Results:

### *3.1 Stability of the gut microbiome*

All the three amphioxus groups sampling on 2018 were chosen with similar size (~ 3 cm) to reduce the potential size effect, and separated into 3 groups: wild type, starved group and starved after feeding group (starved_AF). Illumina MiSeq DNA sequencing were resampled to 1012 tags and samples with the tags < 1012 were removed to provide more robust analysis. Rarefaction curve revealed that all sample reached an asymptote (data not shown). Finally, 7 samples from wild group, 2 samples from starved group and 4 samples from starved_AF group were discarded.

A high microbial community heterogeneity was found for each of the three treatments (Figure 2). Starved group formed a cluster together with wild_3 and starved_AF_3 with average dissimilarity at 74%. The starved group was less dispersed compared to wild type group and starved_AF group. SIMPER analyses showed that the wild type group, starved group and starved_AF group presented an average dissimilarity of 82%, 69% and 80%, respectively. The results turned out that the bacterial community from starved group was more stable compared to wild group and starved_AF group.

*Alphaproteobacteria* and *Gammaproteobacteria* were the main taxa for all the 3 treatment groups, *Alphaproteobacteria* accounted for 13 ±1% (mean value and standard deviation), 29 ±6% and 43 ±14% for wild type, starved and starved_AF groups, respectively. *Gammaproteobacteria* accounted for 25 ±7%, 25 ±7% and 30 ±6% for wild type, starved and starved_AF group respectively.

In conclusion, these results showed that one week starvation prior microplastic addition was the best condition to ensure microbial stability needed for running further toxicity tests.

### *3.2 Microplastic suspension and ingestion*

It turned out that about 52% of microplastics could be suspended with bubbling flow at 35 mL $sec^{-1}$ and the counts were stable after 2 hours. Microplastic with bigger size were descended to the tank bottom. The high, middle and low concentrations were adjusted at 50, 500 and 5000 particles. $L^{-1}$ according to the microplastics in suspension.

Preliminary results have shown that about 2.6 hours were necessary for the microplastics to be egested by amphioxus of 3.2 cm size. The bigger the amphioxus size, the more time is needed for the microplastic to be egested (data not shown). Therefore, 2 hours were used for the microplastic ingestion test. We detected 8-42 microplastic particles for high exposure concentration, 2-18 particles for middle exposure concentration, and 0-2 particles for low exposure concentration (Figure 3A).

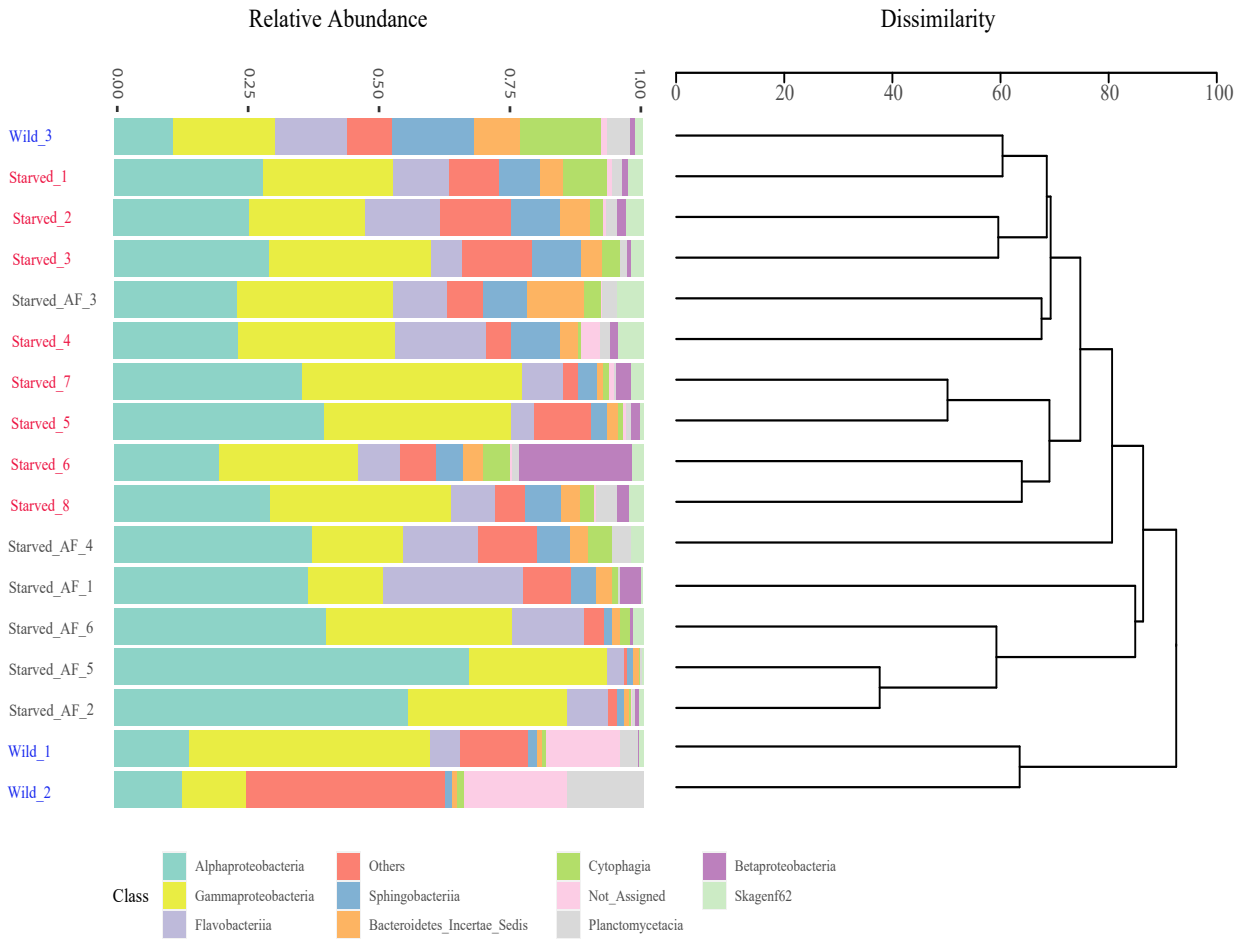



Figure 2. Comparison of variation of taxonomic abundances and community structure of gut microbiome in wild type (text in blue color), one-week starvation (red color) and starvation after feeding with algae (black color) by cumulative bar charts comparing relative abundances in class level (left) and by UPGMA dendrogram based on Bray–Curtis dissimilarities between 16S rRNA-based sequencing profiles (right)

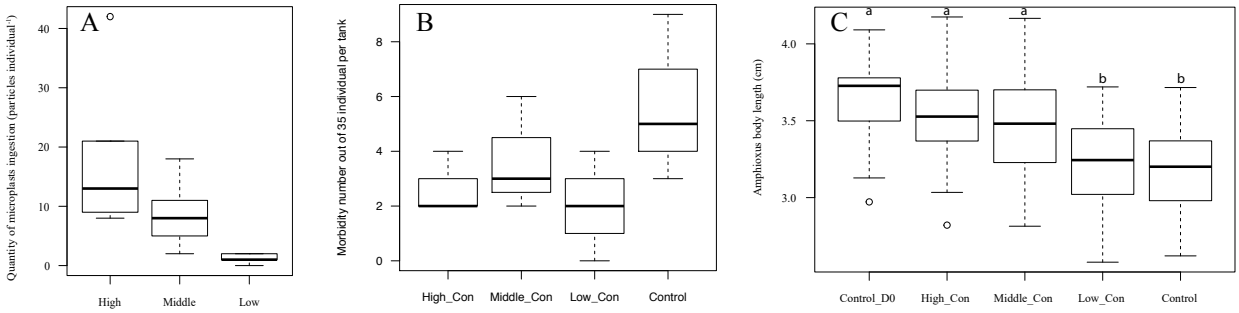



Figure 3. Microplastic ingestion, amphioxus viability and amphioxus body length. (A) Boxplot showed the number of microplastics ingested within 2 hours for high, middle and low exposure concentration, 5 individuals were checked for each treatment, the box represents the first and third quartiles, heavy horizontal line inside the boxes indicate the median value and unfilled circles indicate outlier. (B) Morbidity number that were observed out of 35 individuals, each box indicates the 3 replicates. (C) Amphioxus body length change before and after microplastic exposure, 10 random individuals from each tank were pooled for each treatment, the difference of small case letter indicates significant difference.

Abbreviation:
Control_D0: amphioxus treatment after one-week starvation and before microplastic exposure.
High_Con: amphioxus treatment after 16 days' high concentration exposure (5000 particle.$L^{-1}$).
Middle_Con: amphioxus treatment after 16 days' middle concentration exposure (500 particle.$L^{-1}$).
Low_Con: amphioxus treatment after 16 days' low concentration exposure (50 particle.$L^{-1}$).
Control: amphioxus treatment after 16 days' without microplastic exposure.

### *3.3. Amphioxus viability and body size change*

Integrity of oral cirri and belly skin were checked. Morbidity signs were first observed for amphioxus on the 16$^{th}$ days plastic exposure. Controls had morbidity of 3, 5 and 9 out of 35 individuals for the 3 replicates respectively; 2, 2 and 4 individuals for high exposure concentration (High_Con); 2, 3 and 6 individuals for middle exposure concentration (Middle_Con); 0, 2 and 4 individuals for low exposure concentration (Low_Con). The result was not significant for pairwise group test (Tukey test, *p*-value > 0.05) (Figure 3B).

Amphioxus body size were measured before and after microplastic exposure. Higher median size were obtained for amphioxus before microplastic exposure (Control_D0), and after microplastic exposure of High_Con, Middle_Con (with 3.7 cm, 3.5 cm, 3.5 cm, respectively). Significant lower sizes were obtained for amphioxus in controls tanks or exposed to low concentration of plastics (3.2 cm) (Tukey test, *p*-value < 0.05) (Figure 3C).

*3.4. Bacterial gut microbiome response on plastic exposure*

To make the dataset 2019 (microplastic exposure) comparable to the dataset 2018 (gut microbiome stability test), dataset of 2019 was all rarefied to the same number of 1012 reads per samples. Rarefaction curve confirmed the sufficient resampling depth (data not shown).
Hierarchical clustering depicted 3 main clusters: microplastic samples with 50.0% average dissimilarity, a group before microplastic exposure with 53.6% average dissimilarity (Control_D0) and a group after microplastic exposure with 47.6% average dissimilarity. Amphioxus of Control_D0 had a sub-cluster with microplastic samples other than that after microplastic exposure. In addition, the triplicate samples from High_Con, Middle_Con, Low_Con and Control did not form separate cluster within each treatment, but more dispersed among the 4 treatments (Figure 4). PERMANOVA test showed that there was no significant difference for the pairwise comparison of 4 treatment groups after microplastic exposure ($p>0.05$).
*Flavobacteriia* and *Gammaproteobacteria* were the main taxa after microplastic exposure, accounting for 65 ±5%, 18 ±4%, respectively. *Gammaproteobacteria* and *Alphaproteobacteria* were the main taxonomy composition for Control_D0, accounting for 52 ±21% and 39 ± 6%, and also as the main composition for microplastic groups, accounting for 46 ± 7% and 36 ± 3%, respectively.

The eukaryote sequences were also checked for microplastic samples. It turned out the eukaryotic sequences represented for 4 ±2% total tags, and also in genus level, *Peudo-Nitzschia* accounted for 84 ±10% total tags of eukaryotes.

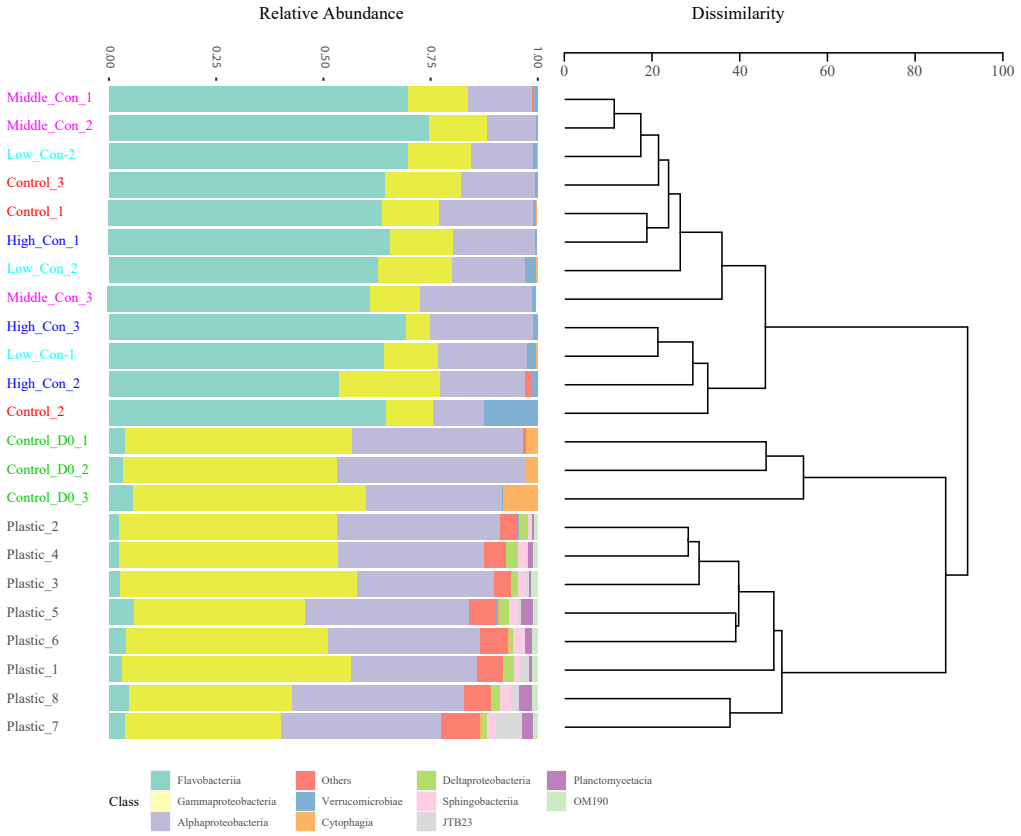



Figure 4. Comparison of taxonomic abundances and community structure of gut microbiome in samples before plastic exposure (Control_D0), High exposure microplastic concentration (High_Con), middle plastic exposure concentration (Middle_Con), low microplastic exposure concentration (Low_Con) and microplastics (number indicating the sequential microplastic exposure order for every two days) for samples by cumulative bar charts comparing relative abundances in class level (left) and by UPGMA dendrogram based on Bray–Curtis dissimilarities between 16S rRNA-based sequencing profiles (right).

### *3.5. Characterization bacterial gut microbiome*

Chao1 estimator showed that significant decrease from amphioxus sample of 2018 to 2019, with average ASVs of 90 and 45 respectively (Figure 5). Significant decreases were also observed for Pielou and Shannon index (Tukey test, $p < 0.05$), indicating that amphioxus from the dataset of 2019 had less bacterial diversity and few bacterial species dominating their gut microbiome.

When comparing the sample within the dataset of 2019, no difference was found on Chao1, Pielou and Shannon diversity for High_Con, Middle_Con, Low_Con to the Control group (Tukey test, $p > 0.05$), suggested that there was less plastic impact on the α-diversity. When compared the Control_D0 sample to the rest amphioxus sample, the Pielou showed significant decrease, meaning that including Control, few ASVs become more abundant after 16 days plastic exposure test. Plastic sample used for exposure had higher indices compared to amphioxus.

It should be highlighted that 3 ASVs of *Polaribacter* sp., *Aquibacter* sp. and *Sulfitobacter* sp. dominated the amphioxus samples after plastic exposure (including Control), with the highest abundance reaching at 43.0%, 37.8, and 11.7%, respectively (Figure 6). *Polaribacter* sp., *Aquibacter* sp. belongs to Bacteroides, and *Sulfitobacter* sp. belongs to roseobacter clade, α-*proteobacteria* sp. As we may have observed that Control_D0 had higher evenness, for the taxonomy composition, it was abundant for *Pseudophaeobacter* sp., *Paraglaciecola* sp., *Alteromonas* sp., *Vibro* sp., and *Ruegeria* sp. For the amphioxus sample on the dataset of 2018, *Acinetobacter* sp. and one unassigned ASV from Proteobacteria were abundance for Wild group. Two ASVs from *Pseudophaeobacter* genus were abundant for the Starved_AF groups. No ASVs had the relative abundance above 5% for Starved group, and *Ruegeria* sp. had the relative higher abundance at 1.3%. For the plastic samples, *Alteromonas* sp. had the highest abundance at 7.8%.

It is also important to point out that the *Sulfitobacter* sp., *Pseudophaeobacter* sp., and *Alteromonas* sp. were presented on all amphioxus groups, and it can be regarded as the core microbiome of amphioxus (the same results for the core microbiome using 1% cutoff, data not shown). In addition, *Pseudophaeobacter* sp. *Sulfitobacter* sp. and *Ruegeria* sp. were more predominant in Starved_AF group, potentially reflecting the situation in the natural environment.

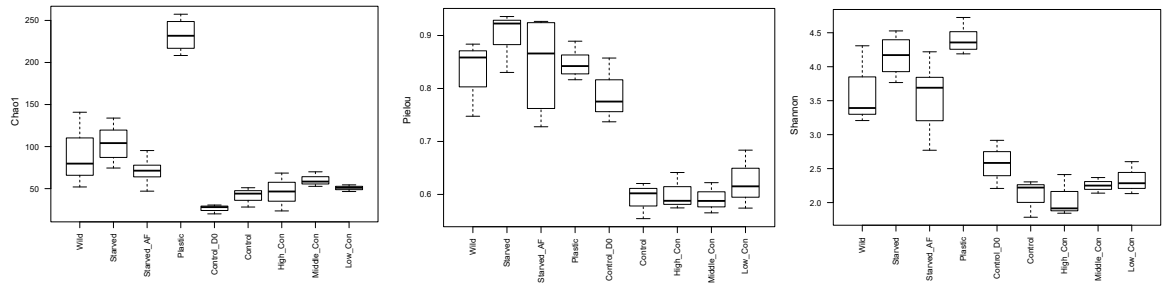



Figure 5. Alpha-diversity indices of richness (Chao1), evenness (Pielou) and diversity (Shannon) of plastic and amphioxus gut microbiome. As mentioned above, sample size was 3 (Wild), 8 (Starved), 6 (Starved_AF), 8 (plastic) and 3 (Control, High_Con, Middle_Con and Low_Con, respectively) for the different treatment.

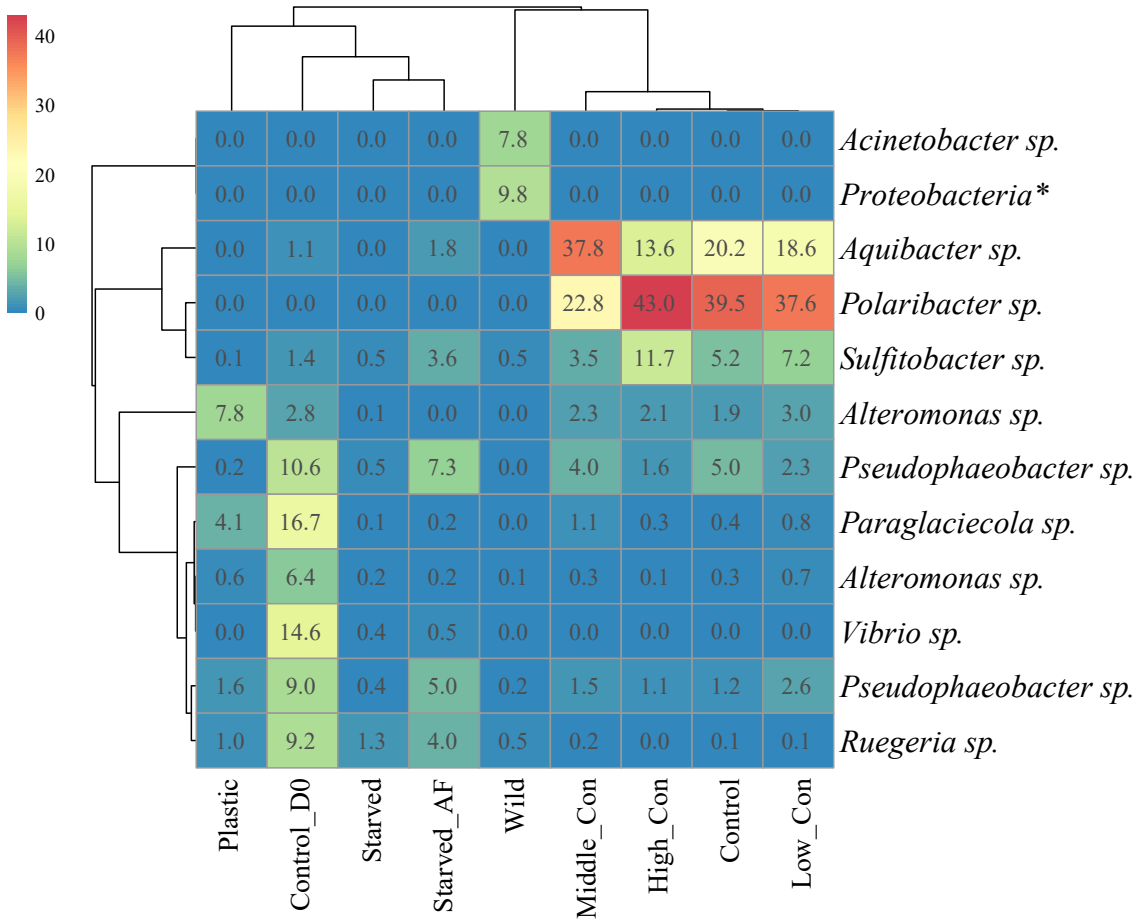



Figure 6. Heatmap of the main ASVs. ASV with relative abundance over 5% in each library of the 16S rRNA gene sequences were selected. Rows and columns are clustered using correlation distance and average linkage, color key represents relative average abundance, * indicates the annotation in phylum level, while the rests in genus level.

*3.6. Potential bacterial transfer*

We found 12 ASV as potential bacterial transfer from plastic to bacterial gut microbiome after 16 days of plastics exposure, while all these ASVs belonged to rare biosphere, with the average abundance less than 0.1% (Figure 7). *Staphylococcus* sp. were presented associated to amphioxus both on High_Con and Low_Con, *Glaciecola* sp. and another ASV from *Gammaproteobacteria* were presented both on Middle_Con and low_Con.

*Elizabethkingia* sp., *Enhydrobacter aerosaccus*, *Ponticaulis* sp. and ASV of OCS_116 clade was just presented on High_Con. *Alteromonas* sp., *Pseudoteredinibacter* sp., *Spongiispira norvegica* and *Ponticaulis koreensis* was just presented on Middle_Con. *Litorimicrobium* sp. was just presented on Low_Con.

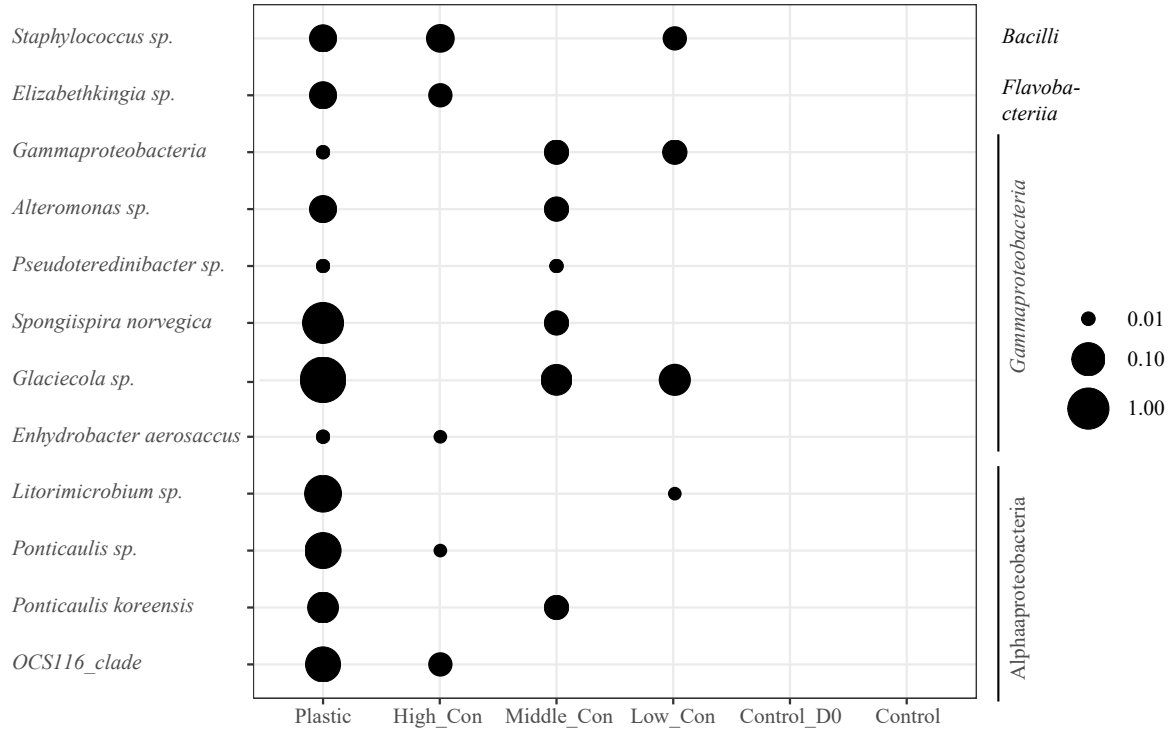



Figure 7. Bubble plot showing the presence and absence of ASVs to determine potential bacterial transfer from plastic to gut microbiome. The most closed annotation was labelled on the left of the figure, and the class level annotation were labelled on the right of the figure, bubble size represents the average of relative abundance for each treatment.

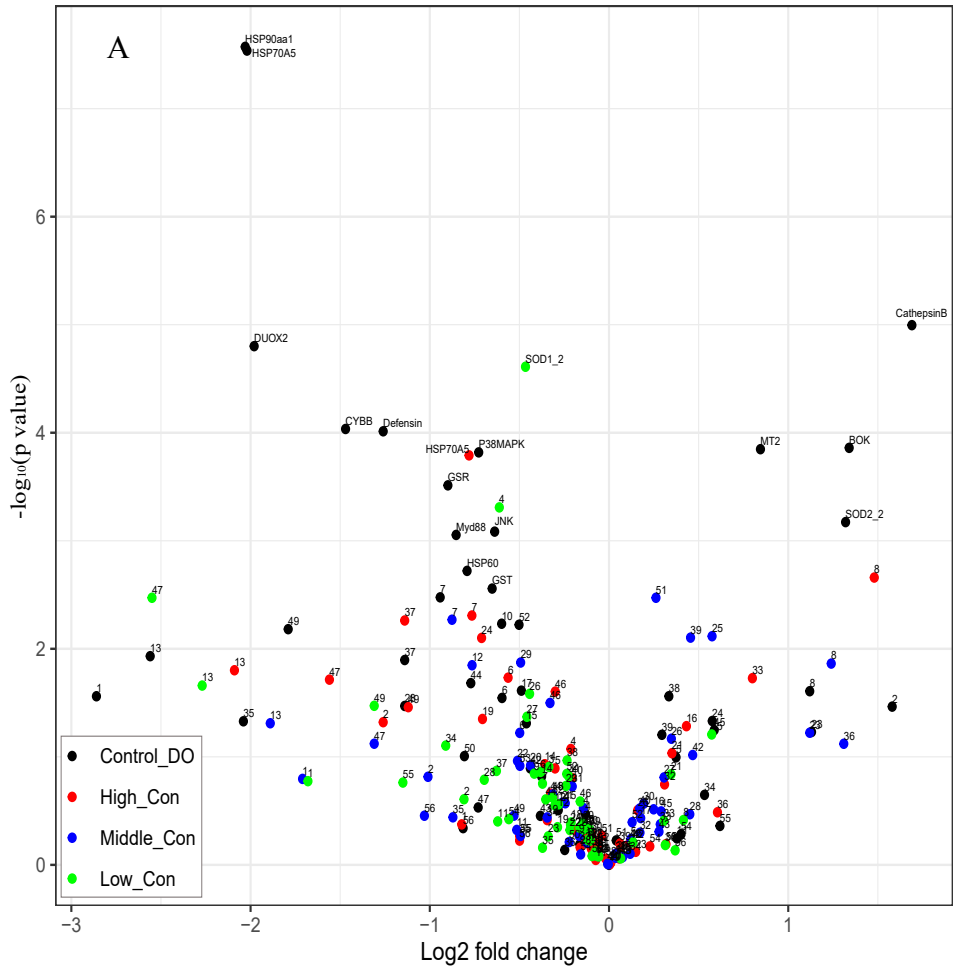
*3.7. Gene expression*

Expressions of 62 genes were selected during this study, including 3 genes involved on metabolism regulation, 9 genes linked to apoptosis, 20 genes involved on stress response (stress induction and antioxidative stress) and 26 genes of the immune system (adaptor, effector, complementary system and oxidative burst). We chose the Elongation factor 1 alpha (*EEF1A1*) and ribosomal protein S20 (*S20*) as housekeeping genes for gene normalization, glucose 6-phosphate dehydrogenase (*G6PDH*) and 18S ribosomal RNA (*18S*) being discarded due to lower performance. Immune genes of *C1q* and Lysozyme (*Lysozyme_4*) were removed for gut and tissue sample, and *PGRP3* were removed for gut sample due to low abundance.

Among the significant results of the gut samples, heat shock protein 70 (*HSP70A5*) was significantly down-regulated on High_Con treatment compared to the other treatments and superoxide dismutase 1 (*SOD1_2*) were significant up-regulated on Low_Con condition (Figure 8A). For hepatic samples, immune genes of *Bf2* and cytochrome b alpha (*CYBA*) were significantly down-regulated on High_Con treatment, *CYBA* gene was significantly down-regulated for Middle_Con treatment. Immune gene of apextrin1 was found down-regulated on Low_Con treatment (Figure 8B).

More significant results were observed on Control_D0 compared to Control treatment. For gut sample, a significant up-expression on stress response gene for Control_D0 was just observed on superoxide dismutase 2 (*SOD2_2*) and metallothionein (*MT2*). Significant down-regulations were observed for heat shock protein 90 (*HSP90aa1*) and heat shock protein 70 *HSP70AA5*, heat shock protein 60 (*HSP60*), and Glutathione S-transferase (*GST*) and p38 mitogen-activated protein kinases (*P38MAPK*), glutathione-disulfide reductase (*GSR*), c-Jun N-terminal kinases (*JNK*). Gene expression of *HSP90aa1* and *HSP70AA5* showed the highest significant down-regulation with 4 folders change, indicating an induction of genes involved in stress responses in amphioxus after 16 days in starvation condition. In addition, significant up-regulation of gene involved on apoptosis were observed for Bcl-2 related ovarian killer (*BOK*) associated to a down-regulation of genes involved on immune system as oxidase 2 (*DUOX2*), cytochrome b beta (*CYBB*), *Defensin*, myeloid differentiation primary response 88 (*MyD88*). It is noteworthy that significant higher expression was observed for *CathepsinB* involved in the general metabolism in Control treatment group compared to group during starvation.

For the hepatic samples, significant up-regulation of genes involved on the stress response were observed for Control_D0, including catalase (*CAT*), *MT2* and *SOD_2*, and a down-regulation were observed for *HSP90aa1*, *HSP70A5*, *CST*, *GSR*, *JNK*. Significant induction of *Defensin*, *Lysozyme_5* combined with a down-regulation of *Apextrin1*, *Lysozyme_3*, *CYBA* and neutrophil cytosolic factor 2 (*NCF2*), all involved on immune system. The 2 chosen metabolism (*Ferritin2* and *CathepsinB*) genes have significant higher level of expression on Control_D0.



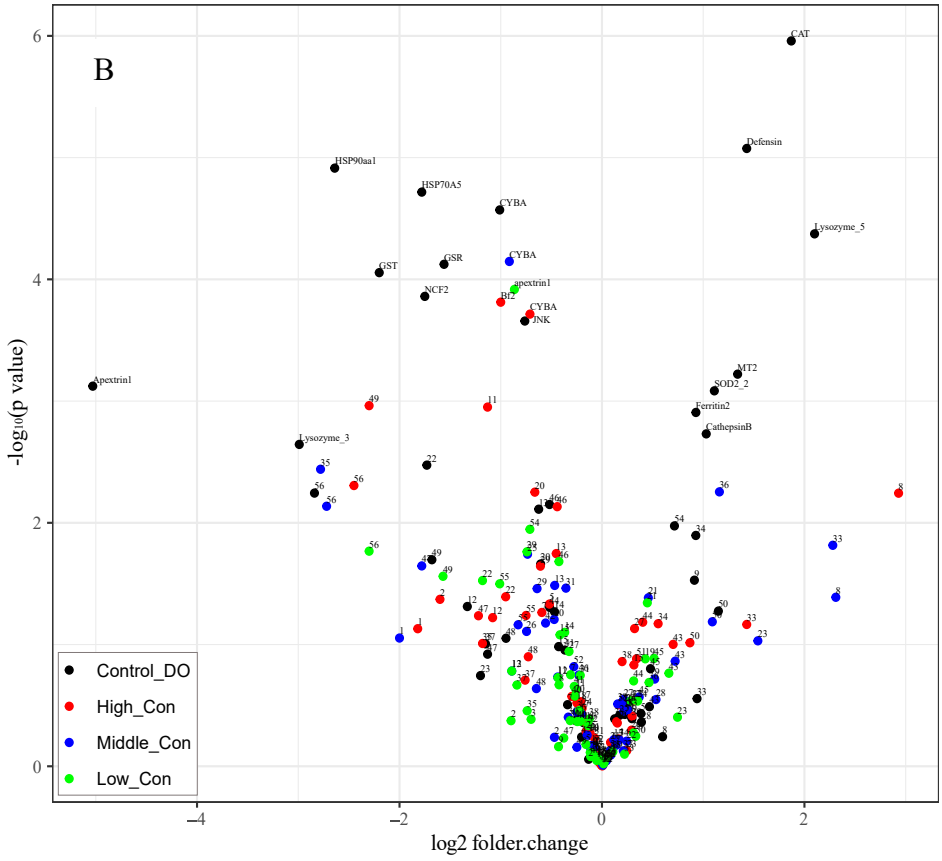



Figure 8. Volcano plot showing gene expression for gut (A) and hepatic (B) out of 58 genes, including oxidative response, immune system, apoptosis and metabolism. x-axis is the log2 fold change of gene expression level to the Control of gut or hepatic tissue, Y-axis is the negative log10 fold change of the *p*-value, significant adjusted *p*-value (Benjamini & Yekutieli) ($p < 0.05$) were labelled with gene names in figures. Gene labels: 1, apextrin1; 2, apextrin2; 3, BCL2L; 4, BCL2L1; 5, Bf1; 6, Bf2; 7, Bf3; 8, big defensin; 9, BOK; 10, BOK like (BOKL) ;11, component C3 (C3);12, Complement C6 like (C6L); 13, Caspase 3 like (CASP3L); 14, CASP6; 15, CASP7;16, CASP8; 17, CAT; 18, CathepsinB; 19, CYBA; 20, CYBB; 21, Defensin; 22, DUOX2; 23, Ferritin; 24, Ferritin2;25, GSR; 26, GST; 27, HSP60; 28, hsp70.1; 29, HSP70A5; 30, HSP90aa1; 31, JNK; 32, LPS-induced TNF-α factor (LITAF); 33, Lysozyme_1; 34, Lysozyme_2; 35, Lysozyme_3; 36, Lysozyme_5; 37, mucosa-associated lymphoid tissue lymphoma translocation protein 1 (MALT1); 38, MAPK; 39, MT1; 40, MT2; 41, Myd88; 42, NCF2; 43, Nuclear Factor Kappa B Subunit 1 (NFKB1); 44, Nuclear factor-κB (NFKBs); 45, The nuclear factor erythroid 2–related factor 2 (Nrf2); 46, P38MAPK; 47, peptidoglycan recognition protein (PGRP_1); 48, PGRP_2; 49, PGRP3; 50, SOD1_1; 51, SOD1_2; 52, SOD2_1; 53, SOD2_2; 54, V region containing chitin-binding protein (VCBP_1); 55, VCBP_2; 56, VCBP1. Function category, expression folder change and gene reference, are presented in the supplementary table S1.

*3.8. Biochemical test*

Control samples showed higher gut SOD activity, while it was not significantly different with the High_Con, Middle_Con, Low_Con and Control_D0 groups, which had the relative activity of 46, 60, 42 and 48%, respectively. For the hepatic SOD activity, significant values were only found between High_Con group and Control_D0 group, with the relative activity of 141% and 41% respectively, but were not different from Control samples ones (Figure 9).

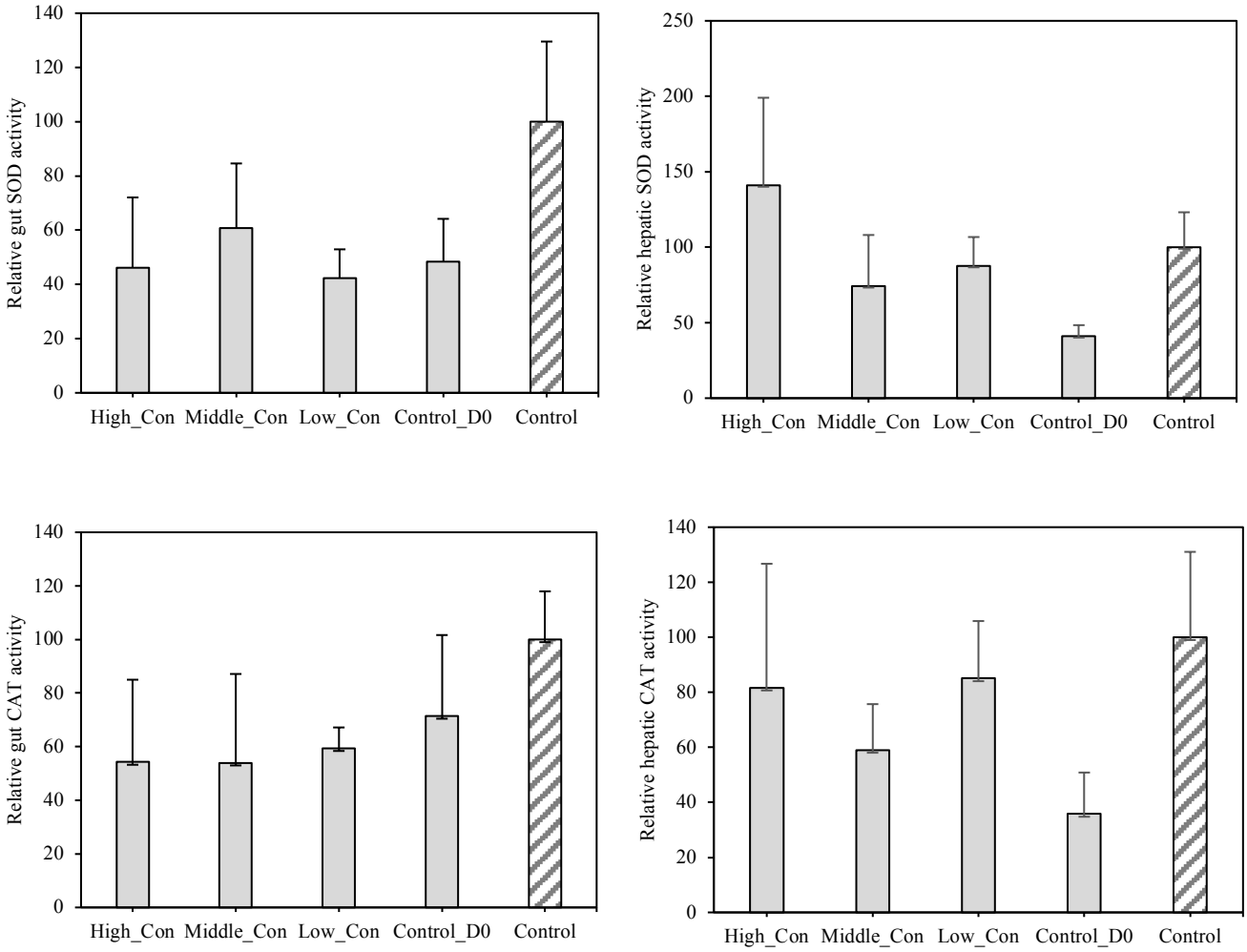



Figure 9. Histogram showing the relative SOD or CAT activity. Y-axis is the relative activity to its Control treatment and expressed in percentage.

Gut and hepatic sample from Control groups also had the higher CAT activity compared to the rest groups, while results were also not significant, indicating that the microplastic exposure was not such toxic that trigger the oxidative stress.

*3.9. Histopathological observation*

Transmission electron microscopy showed that the gut samples were more spoiled, and only hepatic samples were allowed to do the comparison. No goblet cell were detected on the Control group, while it represented for (0.28% and 0%), (17% and 0%) and (5% and 0%) for the duplicates of Low_Con, Middle_Con and High_Con, respectively, indicating the ingestion of microplastics will make the hepatic tissue differentiate into more goblet cell to promote the mucus secretion (Figure 10).

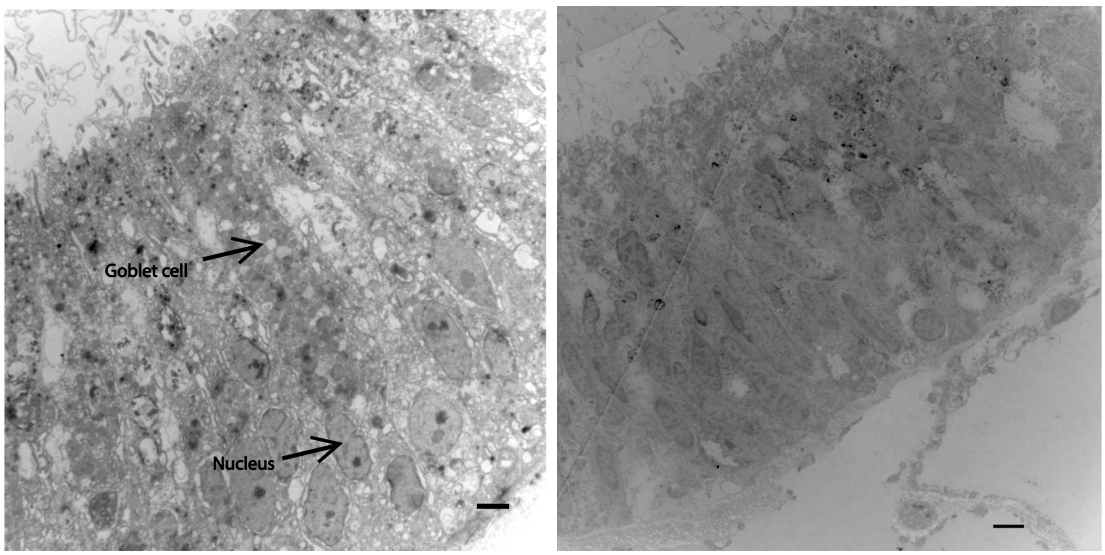



Figure 10. Transmission electron microscopy observation of amphioxus hepatic tissue, scale bar: 2 μm, left panel is the figure from High_Con, right panel is the figure from Control.

## 4. Discussion:

Amphioxus has the geographical distribution overlapped with the microplastic pollution hotspots, and the filter-feeding lifestyle making it susceptible to ingest microplastics. It is the first investigation of the microplastic's toxicity impact on amphioxus. During this study, the amphioxus used for plastic exposure was starved for one-week to keep the highest initial gut microbiota homogeneity, and potential also for other physiological status.

### *4.1. Microplastic exposure has limited impact on the filter-feeder amphioxus*

During this study, to understand the microplastic impact on amphioxus in molecular level, activities of anti-oxidative enzymes and regulation of multiple genes selected involved in different biological processes highlighted the limited effect of microplastics on the health status of amphioxus after 16 days of exposure. No oxidative stress was measured on amphioxus through gene expression nor enzyme activity in exposed organisms. 18 out of 20 oxidative response gene did not show the significant differential expression, the *HSP70A5* from High_Con and *SOD1_2* from Low_Con were down-regulated; therefore, it should not be regarded as more stressed on this two group. Besides, there was also no significant differential expression on the 9 apoptosis gene after plastic exposure treatment. Furthermore, 22 out of 24 selected immune system gene did not show significant differential expression, Bf2 and CYBA from High_Con hepatic and CYBA from Middle_Con hepatic were also down-regulated, indicating that plastic exposure treatment did not pose more threats on amphioxus. Polystyrene microplastics may not act as the antigen or pro-inflammatory factor that suggested aforehand (Ašmonaite et al., 2018).

Microplastics did not pose significant modification on gut microbiota after 16 days of exposure in contradiction with observation on zebrafish exposed for 21 days with 5 μm polystyrene microplastics (Qiao et al., 2019), the difference could be the gut microbiome research on zebrafish was actually from the fecal samples which was transient, while we used the mucosal samples which more considered as the core microbiome (Egerton et al., 2018). The microbiome contributes to health status of their host and inversely, it is dependent on the physiological condition of the host. The bacterial community from the treatment after one-week starvation were more clustered together, and the groups after plastic exposure were more clustered together. The gut microbiota profile was supported by previous studies, which showed that the gut microbiota was linked to the gut nutrition availability, starvation condition and host

oxidative stress condition (Schluter & Foster, 2012; Qiao et al., 2013; Tran et al., 2018). Even though the amphioxus were exposed with the microplastic that pre-colonized by microbes, while the dominant species were shared between High_Con, Middle_Con, Low_Con and Control, indicating the gut microbiome were specifically selected by the host. It might be not the abundance-dependent of taxa from plastisphere. For instance, the one taxon of *Sphingobium sp.* was abundant on plastisphere with the average abundance of 4.27%, while this taxon was not found on the gut microbiota after microplastic exposure on amphioxus. On the other hand, the taxon of *Staphylococcus sp.* (~0.02% on plastisphere) was found to be transferred to the gut microbiota of amphioxus.

### *4.2. Gut microbiota characterization*

After the plastic exposure experiment, *Sulfitobacter* sp. affiliating *Roseobacter* clade, species of *Aquibacter* sp., *Polaribacter* sp. affiliating to Bacteroidetes, became dominant in plastic exposure treatment group and also Control group. These genera were not restrained on amphioxus gut microbiota, the species were also found from the gut microbiota of mussel, salmon, and sea cucumber (Ciric et al., 2018; Auguste et al., 2019; Zhao et al., 2019). *Sulfitobacter* sp. was capable to produce antibiotic of tropodithietic acid (TDA), which might provide the survival strategy (Sharifah and Eguchi, 2012). *Aquibacter* sp. and *Polaribacter* sp., as the Bacteroidetes, can break down high molecular weight materials, which could harvest additional energy source from the intestine (Buchan et al., 2014), Besides, these two species were also antibiotic resistant, which could glimpse the reason of its high abundance (Wang et al., 2019a; Zhao et al., 2019).

In the natural condition, *Pseudophaeobacter* sp., *Sulfitobacter* sp. and *Ruegeria* sp. could be more presented in the amphioxus gut microbiota, these three species also belong to *Roseobacter* clade, the clade is also known to produce Vitamin B1, B7 and B12, thus forming the potential mutualism relationship with amphioxus (Luo and Moran, 2014). *Ruegeria* sp. *Sulfitobacter* sp. could also produce TDA and may also function as probionts that deter potential pathogens (Sharifah and Eguchi, 2012; Sonnenschein et al., 2018).

Modification of the microbiome during captivity was previously described in marine organism with different speeds depending on the species (Galand et al., 2018). We can also hypothesize that the differences of the microbiomes observed between amphioxus at different dates (year

2018 vs. 2019 after one week starvation) could be explained by diets, amphioxus size and the seasonality (David et al., 2014; Miyake et al., 2015; Egerton et al., 2018; Galand et al., 2020).

### *4.3. Beneficial or detrimental nature of microplastics to amphioxus?*

According to the observation of body length change, we can infer that amphioxus could take advantage of the nutrition from biofilm, that means that the digestion system, especially the ileocolonic ring (for twisting ingested food), could detach the diatoms and the biofilm from plastisphere (Urata et al., 2007). It did not significantly improve the health condition, as shown from the morbidity data, probably due to the imbalance between the obtained nutrition and the high energy requested for mucus secretion (Reichert et al., 2019). Another hypothesis could be the toxical effect of poisoning induced by diatom of *Pseudo-nitzschia* sp., as nearly half of *Pseudo-nitzschia* spp. can produce domoic acid, which could lead to the death of seabirds, see lions and whale, and even pose a health threat to human beings by eating the molluscan infected by *Pseudo-nitzschia* sp. (Trainer et al., 2012).

Microplastics could be also a vector for pathogen transfer to the host. We found 12 ASVs potentially transferred from the microplastic to the gut microbiota of amphioxus. Even though all the 12 ASVs had low abundance (<1%), it is noteworthy that the *Staphylococcus* sp. is a typical pathogen for amphioxus (Huang et al., 2007). Low concentration of the pathogen could be not severe to amphioxus, while it could be fatal if the immune system from the host were suppressed, such as in reproduction period or injured condition or thermal stress (Travers et al., 2010). Microplastics could also act as a pathogen reservoir and favor the exchange of virulence plasmids among putative pathogens presented in the plastisphere leading to an increase virulence of opportunistic pathogens (Nasfi et al., 2015). Besides, coral bleaching were also associated the pathogen transfer from microplastics (Lamb et al., 2018).

Overall, we found that the microplastics could improve the survival of amphioxus compared to the group without plastic exposure when looking at the data from the morbidity results. The amphioxus is the filter feeding creatures, while not all microplastics can enter the digestion system even passing the oral cirri, amphioxus is known to have the ‘cough’ effect to spit out too big size microplastic that might not pass through the gut (data not shown) (Lacalli et al., 1999). Thus, the phenomenon will help amphioxus against bigger size of microplastics, potentially avoid the gut blockage of microplastics.

**Conclusion:**

This study showed that natural polystyrene plastics might have limited impacted on the amphioxus after 16 days of exposure of microplastics with the size range around 63 μm, including gut microbiome, oxidative impact, immune system and apoptosis. The gut microbiome was also characterized in the first time, the *Pseudophaeobacter* sp., *Sulfitobacter* sp. and *Ruegeria* sp. being the main natural microorganisms associated to amphioxus gut microbiome. Microplastic can transferred microorganisms to amphioxus used for nutrition, microplastics can potentially transfer the pathogens and also harmful algae to amphioxus. As far as we know, it is the first research of the microplastic impact on the gut microbiome on marine animals. Further studies are required to reinforce this result and other marine animals on the mucosal gut microbiome. This work shed light on microplastics impact in different biological parameters, taking into consideration the whole holobiont concept and pave the road on using the amphioxus as the model organism on the toxicity research of plastics at sea.

# Chapter 3 supporting information

Table S1: Gene information used for Nanostring gene expression assay. Function category, expression folder change and gene reference

| Gene Name | Functional category | Functional subcategory | Hepa control_DO | Hepa High_Con | Hepa Middle_Con | Hepa Low_Con | Gut control_DO | Gut High_Con | Gut Middle_Con | Gut Low_Con | Probe_ID | Reference |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| BCL2L | Apoptosis | - | 0.25 | -0.0809 | -0.246 | -0.7 | -0.0604 | -0.121 | 0.0363 | -0.335 | BL11889 | (Huang et al., 2011) |
| BCL2L1 | Apoptosis | - | -0.202 | 0.165 | 0.0867 | -0.212 | -0.131 | -0.213 | -0.143 | -0.612 | BL01041 | (Huang et al., 2011) |
| BOK | Apoptosis | - | 0.913 | 0.298 | 0.521 | -0.429 | 1.34** | -0.078 | -0.167 | -0.417 | BL10588 | (Huang et al., 2011) |
| BOKL | Apoptosis | - | -0.125 | 0.144 | 0.212 | -0.0518 | -0.599 | 0.00774 | -0.139 | -0.104 | BL22709 | (Huang et al., 2011) |
| CASP3L | Apoptosis | - | -0.625 | -0.452 | -0.467 | -0.891 | -2.56 | -2.09 | -1.89 | -2.27 | BL21627 | (Huang et al., 2011) |
| CASP6 | Apoptosis | - | -0.468 | 0.152 | 0.0306 | -0.368 | -0.376 | -0.358 | -0.124 | -0.281 | BL22445 | (Huang et al., 2011) |
| CASP7 | Apoptosis | - | -0.428 | 0.316 | -0.103 | -0.415 | -0.461 | -0.0755 | -0.244 | -0.163 | BL21827 | (Huang et al., 2011) |
| CASP8 | Apoptosis | - | -0.183 | -0.157 | 0.218 | -0.0449 | 0.125 | 0.432 | 0.249 | -0.0755 | BL05237 | (Huang et al., 2011) |
| MALT1 | Apoptosis | - | -1.15 | -0.759 | 0.211 | -0.84 | -1.14 | -1.14 | 0.0759 | -0.628 | BL00110 | (Huang et al., 2011) |
| CathepsinB | metablism | - | 1.03* | -0.247 | -0.0495 | -0.0454 | 1.69*** | 0.0626 | -0.314 | -0.211 | BL05273 | (He et al., 2018) |
| Ferritin | metablism | - | -1.2 | 0.245 | 1.54 | 0.746 | 1.13 | 0.149 | 1.12 | -0.339 | BL11377 | (Huang et al., 2007) |
| Ferritin2 | metablism | - | 0.928* | -0.196 | 0.239 | 0.0487 | 0.576 | -0.711 | -0.0578 | -0.215 | BL06687 | (Huang et al., 2007) |
| Myd88 | immune system | Adapter | -0.366 | -0.117 | -0.08 | -0.274 | -0.853* | -0.0597 | -0.00366 | -0.106 | BL08792 | (Huang et al., 2011) |
| apextrin1 | immune system | Effector | -5.03* | -1.82 | -2 | -0.865 | -2.86 | -0.822 | -1.71 | -1.68 | BL08998 | (Huang et al., 2014) |
| C1q | immune system | Recognition receptor | - | - | - | - | - | - | - | - | BL12233 | (Huang et al., 2011) |
| apextrin2 | immune system | Recognition receptor | -0.132 | -1.6 | -0.47 | -0.898 | 1.58 | -1.26 | -1.01 | -0.809 | BL08995 | (Huang et al., 2014) |
| Bf1 | immune system | Complement systems | 0.0531 | -0.519 | 0.0367 | -0.158 | 0.374 | -0.301 | 0.122 | -0.119 | BL09735 | (Huang et al., 2011) |
| Bf2 | immune system | Complement systems | 0.102 | -1* | -0.201 | -0.0411 | -0.597 | -0.563 | -0.498 | -0.382 | BL22154 | (Huang et al., 2011) |
| Bf3 | immune system | Complement systems | -0.34 | -0.594 | 0.138 | -0.31 | -0.942 | -0.765 | -0.876 | -0.371 | BL17291 | (Huang et al., 2011) |
| C3 | immune system | Complement systems | -0.26 | -1.13 | -0.438 | -0.256 | 0.379 | -0.074 | -0.515 | -0.621 | BL09813 | (Huang et al., 2011) |
| C6L | immune system | Complement systems | -1.33 | -1.08 | -0.891 | -0.435 | -0.283 | -0.129 | -0.764 | -0.302 | BL00451 | (Huang et al., 2011) |
| Lysozyme_1 | immune system | Recognition receptor | 0.939 | 1.43 | 2.28 | 0.222 | -0.163 | 0.8 | 0.286 | 0.306 | BL14669 | (Huang et al., 2011) |
| Lysozyme_2 | immune system | Recognition receptor | 0.927 | 0.555 | 0.171 | 0.353 | 0.533 | -0.345 | 0.118 | -0.911 | BL05503 | (Huang et al., 2011) |
| Lysozyme_3 | immune system | Recognition receptor | -2.99* | -1.18 | -2.78 | -0.739 | -2.04 | -0.498 | -0.871 | -0.371 | BL05743 | (Huang et al., 2011) |
| Lysozyme_4 | immune system | Recognition receptor | - | - | - | - | - | - | - | - | BL27735 | (Huang et al., 2011) |
| Lysozyme_5 | immune system | Recognition receptor | 2.1** | -0.026 | 1.16 | 0.465 | -0.247 | 0.605 | 1.31 | 0.314 | BL18647 | (Huang et al., 2011) |
| PGRP_1 | immune system | Recognition receptor | -1.13 | -1.22 | -1.78 | -0.377 | -0.73 | -1.56 | -1.31 | -2.55 | BL08659 | (Huang et al., 2011) |
| PGRP_2 | immune system | Recognition receptor | -0.948 | -0.728 | -0.648 | -0.111 | -1.79 | -1.12 | -0.53 | -1.31 | BL00379 | (Huang et al., 2011) |
| PGRP3 | immune system | Recognition receptor | -1.68 | -2.3 | -0.248 | -1.57 | - | - | - | - | BL04461 | (Huang et al., 2011) |
| VCBP_1 | immune system | Recognition receptor | 0.716 | -0.0237 | -0.146 | -0.712 | 0.402 | 0.228 | -0.158 | -0.559 | BL05899 | (Huang et al., 2011) |
| VCBP_2 | immune system | Recognition receptor | 0.385 | -0.75 | -0.828 | -1.01 | 0.619 | 0.318 | -0.494 | -1.15 | BL05900 | (Huang et al., 2011) |
| VCBP1 | immune system | Recognition receptor | -2.84 | -2.45 | -2.72 | -2.3 | -0.814 | -0.499 | -1.03 | 0.369 | BL14855 | (Huang et al., 2011) |
| Bigdefensin | immune system | Oxidative burst | 0.599 | 2.93 | 2.31 | -0.424 | 1.12 | 1.48 | 1.24 | 0.415 | BL12629 | (Huang et al., 2011) |
| DUOX2 | immune system | Oxidative burst | -1.73 | -0.951 | 0.244 | -1.18 | -1.98*** | -0.187 | -0.511 | -0.218 | BL19081 | (Huang et al., 2011) |
| CYBA | immune system | Oxidative burst | -1.01** | -0.711* | -0.915* | 0.428 | 0.000266 | -0.706 | -0.345 | -0.287 | BL07269 | (Huang et al., 2011) |
| CYBB | immune system | Oxidative burst | -0.606 | -0.663 | -0.472 | -0.267 | -1.47** | 0.0053 | -0.438 | -0.0563 | BL22566 | (Huang et al., 2011) |
| defensin | immune system | Oxidative burst | 1.43** | 0.0845 | 0.458 | 0.448 | -1.26** | 0.351 | -0.261 | 0.346 | BL18783 | (Huang et al., 2011) |
| NCF2 | immune system | Oxidative burst | -1.75** | -0.00565 | -0.557 | 0.285 | -0.439 | 0.117 | 0.467 | 0.134 | BL11216 | (Huang et al., 2011) |
| CAT | Stress response | Antioxidative | 1.87*** | -0.209 | 0.117 | -0.222 | -0.489 | 0.0108 | 0.177 | -0.142 | BL09457 | Online database |

| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| GSR | Stress response | Antioxidative | -1.56** | -0.156 | -0.735 | -0.0443 | -0.899** | 0.0445 | 0.575 | -0.144 | BL05308 | Online database |
| GST | Stress response | Antioxidative | -2.2** | -0.0861 | -0.745 | -0.116 | -0.652* | 0.16 | 0.348 | -0.443 | BL96886 | Online database |
| SOD1_1 | Stress response | Antioxidative | 1.15 | 0.869 | 1.09 | 0.334 | -0.807 | -0.0704 | -0.222 | -0.0946 | BL09017 | Online database |
| SOD1_2 | Stress response | Antioxidative | 0.225 | 0.341 | 0.0806 | 0.0222 | 0.0401 | -0.0403 | 0.262 | -0.466** | BL04387 | Online database |
| SOD2_1 | Stress response | Antioxidative | -0.0789 | 0.164 | -0.278 | -0.137 | -0.502 | -0.0603 | 0.129 | -0.236 | BL13154 | Online database |
| SOD2_2 | Stress response | Antioxidative | 1.11* | -0.063 | 0.255 | 0.0159 | 1.32* | -0.0992 | -0.497 | -0.0657 | BL03632 | Online database |
| HSP60 | Stress response | Antioxidative | 0.174 | 0.322 | 0.215 | -0.325 | -0.792* | -0.0951 | 0.307 | -0.458 | BL08136 | Online database |
| hsp70.1 | Stress response | Antioxidative | 0.39 | 0.292 | 0.534 | 0.312 | -1.14 | -0.161 | 0.451 | -0.696 | BL21182 | Online database |
| HSP70A5 | Stress response | Antioxidative | -1.78** | -0.608 | -0.641 | -0.741 | -2.02*** | -0.781* | -0.493 | -0.24 | BL18316 | Online database |
| HSP90aa1 | Stress response | Antioxidative | -2.64** | 0.00276 | -0.333 | -0.314 | -2.03*** | -0.0983 | 0.194 | -0.145 | BL18399 | Online database |
| MT1 | Stress response | Antioxidative | 0.296 | 0.0087 | 0.0436 | 0.108 | 0.295 | 0.0608 | 0.455 | -0.108 | BL11229 | Online database |
| MT2 | Stress response | Antioxidative | 1.34* | -0.298 | -0.111 | -0.243 | 0.845** | -0.207 | 0.171 | -0.123 | BL14103 | Online database |
| Nrf2 | Stress response | Antioxidative | 0.479 | 0.0868 | 0.368 | 0.515 | 0.587 | 0.0602 | 0.291 | 0.573 | BL11654 | Online database |
| LITAF | Stress response | inflammation induction and Cell survival | 0.0424 | 0.302 | 0.00858 | -0.0887 | -0.093 | 0.31 | 0.173 | -0.315 | BL00758 | Online database |
| MAPK | Stress response | inflammation induction and Cell survival | 0.125 | 0.199 | 0.155 | -0.125 | 0.334 | -0.0185 | 0.0363 | -0.234 | BL15950 | Online database |
| NFKB1 | Stress response | inflammation induction and Cell survival | 0.469 | 0.703 | 0.723 | 0.661 | -0.383 | 0.0565 | 0.279 | 0.0652 | BL18808 | Online database |
| NFKBs | Stress response | inflammation induction and Cell survival | -0.517 | 0.403 | 0.0345 | 0.313 | -0.771 | -0.326 | -0.00805 | -0.353 | BL04343 | Online database |
| JNK | Stress response | inflammation induction and Cell survival | -0.762** | -0.0728 | -0.356 | -0.214 | -0.638* | -0.0653 | -0.205 | -0.224 | BL14252 | Online database |
| P38MAPK | Stress response | inflammation induction and Cell survival | -0.518 | -0.442 | -0.229 | -0.425 | -0.727** | -0.3 | -0.329 | -0.161 | BL95334 | Online database |
| EEF1A1 | Housekeeping gene | - | - | - | - | - | - | - | - | - | BL08018 | (Zhang et al., 2016) |
| G6PDH | Housekeeping gene | - | - | - | - | - | - | - | - | - | BL24542 | (Zhang et al., 2016) |
| 18S | Housekeeping gene | - | - | - | - | - | - | - | - | - | BL08675 | (Zhang et al., 2016) |
| S20 | Housekeeping gene | - | - | - | - | - | - | - | - | - | BL07569 | (Zhang et al., 2016) |

# *CHAPTER 4: Biodegradability under marine conditions of bio-based and petroleum-based polymers as substitutes of conventional microbeads*

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# *Chapter 4*

**Abstract:**

Plastic microbeads have been produced at very large scales since several decades for cosmetics and personal care products, a large amount of which being directly transported in the oceans as 'primary microplastics' and emerging pollutants. To better understand their biodegradability in marine environment and evaluate their possible replacement by biodegradable polymers, seven polymer types were studied including three conventional petroleum-based polymers (PE, PMMA and PCL), two bio-based polymers (PLA and PHBV) and two natural products (rice seeds and apricot kernel). We used several innovative approaches by both the experimental design and the set of multidisciplinary techniques that allowed us to follow the successive steps of biodegradation together with abiotic degradation under seawater conditions. We found that microbeads made of PHBV or rice and in a lesser extent PCL and apricot are good candidates for substitution of conventional microplastics, classically made of PE or PMMA that were not biodegraded under our conditions. Congruent signs of biodegradability were first observed by oxygen measurement and weight loss. Mass spectrometry and $^{1}$H NMR confirmed the biodegradation by identifying ester bound cleavage for PHBV, with 3-hydroxybutyric acid, 3-hydroxyvaleric acid and its dimer and heterotrimer as degradation products. Similarly, ε-caprolactone and glucose were identified as the corresponding oligomers of PCL and rice. This study provides the first time arguments for the use of some bio-based polymers, but not all, as substitutes of conventional microbeads to support the recent legislative rules aiming to reduce the pollution by primary microplastics in the oceans.

# *Chapter 4*

## 1. Introduction

Accumulation of plastics in the marine environment has been observed in ocean gyres, on beaches and in sediments worldwide (Barnes et al., 2009). The major source of plastic at sea arises from mismanaged plastic waste and from riverine inputs (Schmidt et al., 2017). Beside entanglement and ingestion of macro debris by large vertebrates (birds, turtles etc.) (Derraik, 2002), adverse effects on marine organisms were particularly due to microplastics with proven transfer along the food chain (Harry and Cecilia, 2003). Within microplastics, distinction has been made between small microplastics (25–1000 µm) that can be as abundant as large microplastics (1–5 mm) in the oceans (Poulain et al., 2019). Another distinction has been made between "primary microplastics" purposefully manufactured to be of millimeter size (e.g. microbeads and plastic pellets) and "secondary microplastic" that result from breaking down of larger plastic pieces due to UV light, waves or abrasion (Xanthos and Walker, 2017).

Microbeads are primary microplastics commercially available in particle sizes ranging from 10 µm to 1 mm, which are used in hundreds of products, often as abrasive scrubbers, including face washes, body washes, cosmetics, and cleaning supplies (Rochman et al., 2015). After use, microbeads included in personal care products are poured down the drain and cannot be collected for recycling. Wastewater treatment plant is generally not a sufficient solution to prevent microbeads pollution (Fendall and Sewell, 2009). Microbeads from personal care and cosmetics products could be a significant source of microplastics pollution in the ocean (Fendall and Sewell, 2009; Bhattacharya, 2016; Cheung and Fok, 2017; Ding et al., 2020). It has been estimated that about 8 trillion microbeads were emitted into aquatic habitats per day from the United States (Rochman et al., 2015).

Several governments have implemented legislation on the rinse-off microbeads over the last decade, such as the United States and some European countries, but restriction is at its infancy stage (Xanthos and Walker, 2017). As an alternative way to relieve the problem, it has been also advised to replace the conventional microplastic, generally made of polyethylene (PE) or polymethyl methacrylate (PMMA) by 'biodegradable' microparticles. Biodegradable plastics can be petroleum-based polymers such as Polycaprolactone (PCL), or bio-based polymers (so-called 'biosourced') such as poly(D-lactide) (PLA) and polyhydroxyalcanoates (PHAs). Other natural compounds such as rice seeds and apricot kernels have already been used as exfoliating material by some manufacturers and are considered as bio-based polymers (Wardrop et al., 2016).

Most of scientific studies on plastic biodegradation were conducted under controlled laboratory conditions by using soil or compost, but very few studies investigated the aquatic media and especially the marine environment (Dilkes-Hoffman et al., 2019). By now, few standard methods could be applied to assess the biodegradability of plastics under marine conditions, but several scientists underlined the drawbacks and biases of these methods (Krzan et al., 2006; Harrison et al., 2018; Jacquin et al., 2019). Several authors underlined the need of convergent results from combined approaches, including proof of complete mineralization by microorganisms together with tests of chemical structure alteration in order to conclude on the biodegradability of a polymer (Lucas et al., 2008; Shah et al., 2008). Another concern is the necessity to mimic biological, physical and chemical conditions of the marine environment during the tests. For example, biodegradability standards for aquatic environments are using microbial inoculum that do not mimic the natural conditions, such as wastewater and field-collected or laboratory-prepared sludge to soil, compost, livestock faeces or even non-specific 'organic waste' (Harrison et al. 2018). It is well-known that microbial communities living on marine environments are very different from soil, compost, sludge or freshwater (Lozupone and Knight, 2007; Tamames et al., 2010), and that biodegradation capabilities are very different according to the microbial communities and the expression of their metabolic enzymes that differ from one environment to another (Pathak and Mohan, 2017). More generally, the successful implementation of standards is severely hindered by a lack of relevant primary research for biodegradation tests of several types of polymers in marine conditions (Harrison et al. 2018).

The aim of this study was to test the biodegradability under marine conditions of bio-based (PLA, PHBV, apricot kernels and rice seeds) and petroleum-based polymers (PCL), as substitutes of conventional microbeads (PE, PMMA) for cosmetics. We used microbeads of 50 to 250 µm diameter, which were commercially available or laboratory-made by solvent emulsion-evaporation technique. Biodegradability tests were performed in a two-steps protocol including a first 2-months incubation of each polymer type with natural seawater communities, followed by a transfer of the plastic together with its natural microbial biofilm to a marine minimum medium with no other carbon source than the plastic for another 2-months. We hypothesized that bio-sourced polymers may have a greater biodegradation capability compared to petroleum-based polymers under marine conditions. Here, we combined different methodological approaches including weight loss, continuous biological oxygen consumption (optical fiber luminescent sensor), changes in molecular weight (high temperature size

exclusion chromatography), size and surface properties (laser diffraction particle size analyzer and scanning electron microscopy), carbonyl index (Fourier transform infrared spectroscopy) and release of oligomers ($^{1}$H nuclear magnetic resonance spectroscopy and *Liquid Chromatography-High Resolution Mass Spectrometry*).

## 2. Materials and Methods:

### *2.1. Production of petroleum-based and bio-based microbeads*

The petroleum-based microbeads PE and PMMA were directly supplied by Good Fellow company (Lille, France). Natural microparticles were manufactured by cryogrinding of the rice seeds (Rice Exfoliator 200, Lessonia, Saint Thonan, France) so called "rice" hereafter and crushed apricot kernels (Apricot Exfoliator 200, Lessonia, Saint Thonan, France) so called "apricot" hereafter. PE, PMMA, rice seeds and crushed apricot were sieved in order to recover the microparticles for which the diameter was ranging from 50 to 250 µm.

PCL (CAPA 6800, Perstorp Company, Sweden), PLA (7001D, Nature Works, Blair, USA) and PHBV (ENMAT Y1000P with a HV molar ratio of 3%, Tianan Biological Materials Co. Ltd., China) were obtained as pellets and transformed in spherical microbeads by solvent emulsion-evaporation technique. This technique consists in dissolving the polymer in a volatile organic solvent immiscible with water, then introducing this solution into an aqueous solution containing an emulsifier as poly(vinyl alcohol) (PVA). Several parameters can influence the formation of microbeads: the quantities and concentrations of organic and aqueous solutions, the type of emulsifier used, the organic solvent used, the technique and speed of agitation and even the polymer itself can have an influence on the size, size distribution and surface appearance of the microbeads obtained (Bouza et al., 2016; Hong et al., 2005). The PCL, PLA or PHBV pellets were dissolved separately in 300 mL of dichloromethane. Each solution was then added in a stream to an aqueous 2% PVA solution, with mechanical stirring at 2000 rpm. The mixture was then emulsified by stirring at 4000 rpm for 15 min. 300 mL of deionized water was then added to the mixture. The emulsion was finally placed under moderate magnetic stirring for 24 hours at atmospheric pressure and ambient temperature, in order to allow the microbeads to harden, until complete evaporation of the organic solvent. The microbeads were collected by wet sieving between 50 and 250 µm, rinsed with permuted water and lyophilized. The quantities of material and the volumes of aqueous solution used for each polymer are listed in Table S1.

*2.2. Microparticle characterization*

The microparticle surface morphologies were observed at the microbeads production step by using a Jeol JSM-6031 scanning electron microscope (SEM). Prior to any observation, the fracture surfaces were coated with a thin gold layer by means of a Polaron sputtering apparatus.

*2.3. Experimental setup*

A two phases stepwise experiment was designed in order to evaluate the biodegradability of the conventional petroleum-based and bio-based polymers under marine conditions (Figure 1). The first step consisted in the formation of a mature biofilm in natural seawater, as previously described (Dussud et al., 2018a). Briefly, each microparticle (called "microbeads" hereafter) was incubated for a period of 2-months in 1.8L aquarium (Sodispan, Spain) with direct circulation to the sea. Seawater was pumped at 14m depth in the Banyuls Bay close to the SOLA observatory station of the Laboratoire Arago (SOLA station - NW Mediterranean Sea). A flow rate of 50 $ml.min^{-1}$ was chosen to ensure a sufficient renewal of natural bacteria (every 30 min) and the surface outlet water was filtered at 50 µm. Each aquarium contained 12 grams of each microbead types (PE, PMMA, PCL, PLA, PHBV, Apricot and Rice) that were put on the 7th July 2017 for a 2-months period. Throughout the experiment, seawater temperature (between 25.3°C and 18.3°C) and salinity (38.5) in the aquariums were similar to seawater from Banyuls bay (Figure 1).

Second, about 10 mg of dry microbeads (after assay ratio between wet and dry weight) were transferred under sterile conditions to 4.9 mL closed glass vials (Interchim, Montluçon, France) containing 2 mL of minimum medium (called "MM" hereafter) with microbeads as sole carbon source. Vials were incubated in the dark at 18°C under agitation at 110 rpm (orbital agitator) for a period of 2 months (called "biotic condition" hereafter). In addition, controls were incubated and sampled in the same way and consisted in triplicate vials containing 2 mL of MM with microbeads of the same composition but previously sterilized overnight in 70% ethanol and evaporation under UV sterile hood (called "abiotic condition" hereafter). A total of 130 vials were needed to follow the different parameters detailed below in triplicate samples taken after 0, 3 7, 15, 30 and 60 days of incubation (Figure 1). The marine minimum medium (MM) utilized throughout the study had the following composition: NaCl, 420 mM ; $Na_2SO_4$, 28.8 mM; KCl, 9.39 mM ; NaBr, 0.84 mM ; $H_3BO_3$, 0.485 mM ; $MgCl_2 \cdot 6H_2O$, 54.6

mM ; $CaCl_2$, 10.5 mM ; $NH_4Cl$, 9.35 mM ; $SrCl_2 \cdot 6H_2O$, 0.0638 mM ; NaF, 0.0714 mM ; $NaNO_3$, 0.88 mM ; $NaH_2PO_4 \cdot H_2O$, 0.036 mM ; $KH_2PO4$, 0.106 mM ; $CuSO_4 \cdot 5H_2O$, 0.04 µM ; $ZnSO_4 \cdot 7H_2O$, 0.08 µM ; $CoCl_2 \cdot 6H_2O$, 0.04 µM; $MnCl_2 \cdot 4H_2O$, 0.91 µM ; $Na_2MoO_4 \cdot 2H_2O$, 0.03 µM ; $FeCl_3$, 1.85 µM ; thyamine, 33.24 nM ; biotin, 2.0 nM ; and Cobalamin, 0.32 nM.

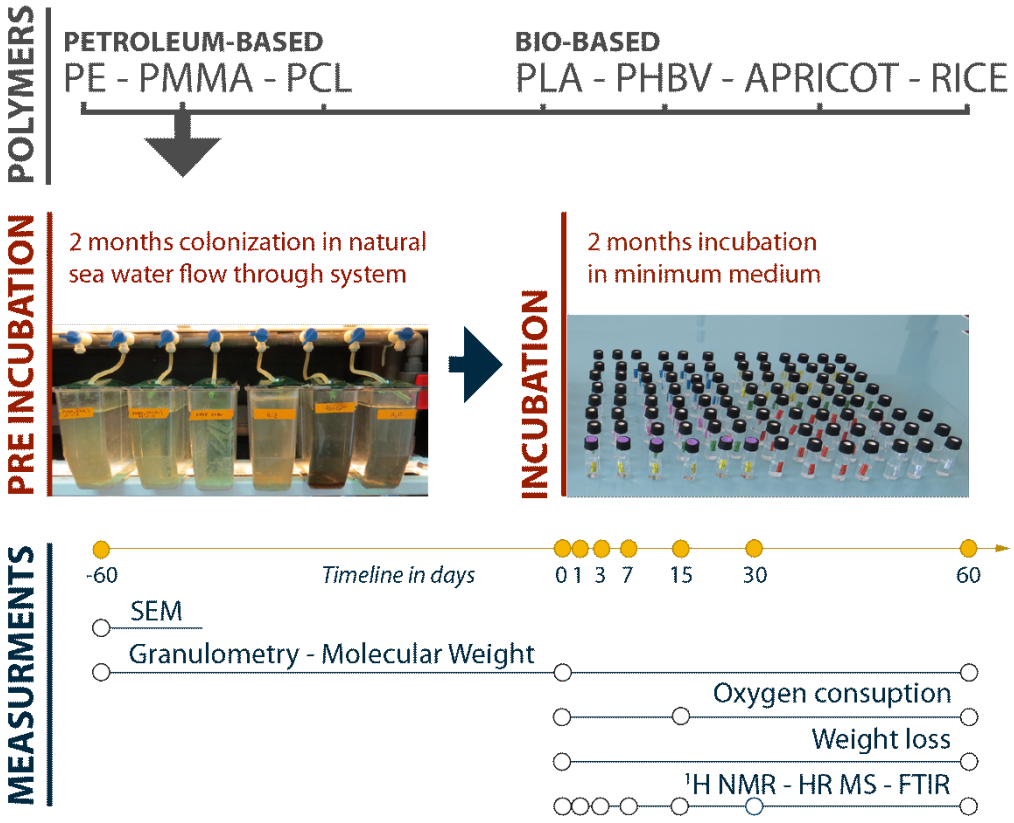



Figure 1. **Experimental design.** Schematic presentation of the two phases stepwise experiment of (1) a 2-months incubation in natural seawater flow through system (flow rate 50ml.min$^{-1}$) for petroleum-based (PE, PMMA, PCL) and biobased microbeads (PLA, PHBV, apricot kernels, rice seeds) followed by (2) a transfer into 4 mL glass vials containing 2mL minimum medium with microbeads as sole carbon source for another 2-months. Lines indicate when measurement were possible for respective assays and dots indicate each measurement during the course of the experiment.

*2.4. Continuous oxygen measurement and percentage of biodegradation*

During the second step of the experiment, triplicate vials for each microbeads type were equipped with an optical fiber luminescent oxygen sensor (Presens, SP-PSt3-YAU) using the instrument of Stand-alone Fiber Optic Oxygen Meter (Fibox 4, Germany). Oxygen concentration was manually measured daily during the 2-months in minimum medium, oxygen was converted from mole concentration to total oxygen in vial with the henry's law (oxygen equilibrium confirmed between the two phases) and ideal gas law. In the case of PHBV only,

vials were opened under a sterile laminar flow hood after 15 days to ensure that oxygen was not limiting for bacterial growth and closed again. In this case, oxygen concentration was always maintained >1.5µmole and the re-opening for 10 minutes was enough to return to initial values (around 23 µmole). Bacterial oxygen demand (BOD) was computed from the difference of oxygen concentration versus time. The consumption of oxygen with the different polymer types but without any bacteria served as a negative control. The first 10 days oxygen consumption was used to calculated the biodegradability, considering the oxygen consumption kinetic, especially for PHBV that could be the oxygen limitation happened for some vials between day10-day15. The percentage of biodegradation was expressed as the ratio of the oxygen consumption over the theoretical amount of consumed oxygen for complete degradation (ThOD), as previously described (Sashiwa et al., 2018).

*2.5. Size change*

The sizes and the size distributions of microparticles were measured at the beginning and at the end of the experiment (Figure 1) with a laser diffraction particle size analyzer (Malvern Mastersizer 2000 model with a Scirocco 2000 module) through granulometry analyses. The particles were analyzed according to a model of spherical particles of polystyrene and according to the Fraunhofer diffraction theory (Hirleman, 1988). Each measurement was an average of 15 scans and each sample was analyzed, thus providing the number, volume and length of the particles.

*2.6. Weight loss*

Microbeads weight was measured at the beginning and at the end of the incubation in MM in triplicate (Figure 1) after 1 min centrifugation at 13000 rpm. The pellet was washed twice with distillated water to remove salt, then lyophilized and immediately weighed in analytical balance with the precision of 0.1 mg (ENTRIS1241-1S, Sartorius). Weight loss was estimated as the weight difference between values obtained at the beginning (*D0*) and the end of the incubation in MM (*D60*).

*2.7. Molecular weight*

Molecular weight was determined for each polymer before colonization and at the end of the incubation in MM (Figure 1). High temperature size exclusion chromatography (HT-SEC) analyses were performed using a Viscotek system (Malvern Instruments) equipped with a

combination of three columns (Polefin 300 mm × 8 mm I. D. from Polymer Standards Service, porosity of 1000 Å, 100 000 Å and 1 000 000 Å). Samples were dissolved in the mobile phase (tetrahydrofuran) with a concentration of 5 mg $mL^{-1}$ and 200 µL of sample solutions were injected and eluted in tetrahydrofuran using a flow rate of 1 mL $min^{-1}$ at 25 °C. The same procedure was applied to polyethylene samples using 1,2,4-trichlorobenzene at 150 °C to dissolve the sample and as mobile phase. The mobile phase was stabilized with 2,6-di(tert-butyl)-4-methylphenol (200 mg $L^{-1}$). Online detection was performed with a differential refractive index detector, a multichrom right angle light scattering detector, and a viscometer detector. A universal calibration curve, obtained with polystyrene standards (Polymer Standards Service, Mainz, Germany) in the range of 0.5–7.106 kg $mol^{-1}$, was used to calculate the molar mass distribution of the samples. OmniSEC software version 5.12 (Malvern Panalytical, UK) was used for the calculations. The typical evaluated accuracy for this technique is close to 10%.

*2.8. Fourier Transform Infrared (FTIR) spectroscopy*

Fourier Transform Infrared (FTIR) analyses were performed on microbeads incubated in minimum medium for 0 and 60 days (Figure 1). After collection, the supernatant was removed and samples were dried at 60° in an aerated oven until constant weight to eliminate trace of water. FTIR measurements were carried out using a Nicolet™ 380 FT-IR (ThermoFisher Scientific) in ATR (Attenuated Total Reflectance) mode. For each sample, the total amount of microbeads (10mg or less) was compressed against a diamond crystal with a constant force. FTIR spectra were collected using 16 scans from 4000 $cm^{-1}$ to 400 $cm^{-1}$, with a resolution of 4 $cm^{-1}$. To assess the variation between spectra, the characteristic absorption bands were realized using Omnic Specta software (Thermo Fisher Scientific). For each polymer, manual assessment of absorption band was done and compared to the literature. The calculations for carbonyl and crystallinity index are detailed for each polymer in the supporting material (table S2-8).

*2.9. Characterization of the degradation products by $^{1}H$ NMR spectroscopy*

$^{1}H$ NMR spectroscopy analysis were carried out to assess the release of degradation products (oligomers) from microbeads as sole carbon source for the biofilms incubated in minimum medium for 0, 1, 3, 7, 15, 30 and 60 days in abiotic and biotic condition (Figure 1). For each point, samples (microbeads + liquid media) were transferred to 2 mL Eppendorf tube and centrifuged to remove the microbeads. Supernatants (540 µl) were supplemented with 60 µL of

a 2 mM solution of $TSPd_4$ (sodium tetra deuterated tri methylsilyl propionate, Eurisotop) in deuterated water ($D_2O$, Eurisotop). $D_2O$ signal was used for locking and shimming while TSPd4 constituted a reference for chemical shifts (0 ppm) and quantification. $^1H$ NMR spectra were recorded on a Bruker Avance 500 MHz spectrometer, equipped with a 5 mm inverse-triple tuned (TXI) $^1H/^{13}C/^{15}N$ with z-gradient coil probe (Bruker Biospin Wissenbourg, France), with 5 mm-diameter tubes containing 600 µl of sample. 128 scans were collected (90° pulse, 3.24 s acquisition time, 4.0 s relaxation delay, 4789.272 Hz SW, 65536 data points). Water signal was eliminated by pre-saturation. An exponential filter was applied before Fourier transformation, and a baseline correction was performed on spectra before integration. Under these conditions, the limit of quantification is in the range of 0.01 mM. The relative quantity of oligomers was expressed using the $TSPd_4$ as the reference.

*2.10. Liquid Chromatography- High Resolution Mass Spectrometry*

When detected by NMR spectroscopy, the degradation products were then identified by High Resolution Mass Spectrometry. The extracts were analyzed using Electrospray Ionization (ESI) with a Q-Exactive Orbitrap™ mass spectrometer (Thermo Scientific) providing a mass accuracy lower than 3 ppm and an ultra-high resolution over 100 000. Briefly, 5 µl of supernatant from extracted samples were analyzed using positive and negative ion mode. The ion source and the capillary heater were set to operate at 3.2 kV and 320 °C respectively. Nitrogen gas was used for nebulizing and as the damping and collision gas in the mass analyzer.

**3. Results**

*3.1. Size changes of microparticles*

At the beginning of the experiment the SEM images of the particles showed significantly different size, sphericity and roughness aspects depending on how the material was obtained (Figure 2). The production of microbeads by cryo-grinding increased the roughness and the size distribution of particle compared to the solvent emulsion-evaporation technique. The size distribution of the PLA, PCL and PHBV microparticles was centered around approximately 100 µm (median diameter between 80 and 125 µm depending on the types of bioplastics) (Table 1). The PE microparticles were the largest with a median diameter around 210 µm. On the other hand, those made of PMMA were the smallest since their median diameter is around 50 µm. Natural microparticles made from rice or apricot kernels had much more heterogeneous

dimensions over a wide range of sizes confirming SEM observations. The median diameter for these microparticles was estimated to be around 120 and 160 µm, respectively.

After 2-months of incubation in natural seawater followed by 2-months incubation in minimum medium, sample aggregation was observed for most samples except for PLA due to the formation of the extracellular polymeric substance, no change of the size distributions was observed for the PMMA. For apricot and PLA, values of the median diameter were similar but smaller particles disappeared. For rice and PE, median diameter was higher after 4 months, with no smaller particles. For PCL and PHBV, decreases of the median diameters were observed with the decrease of higher particles (Figure S1), caution should be taken when interpreting the granulometry results and the technique is still under development, especially the samples after the degradation process.

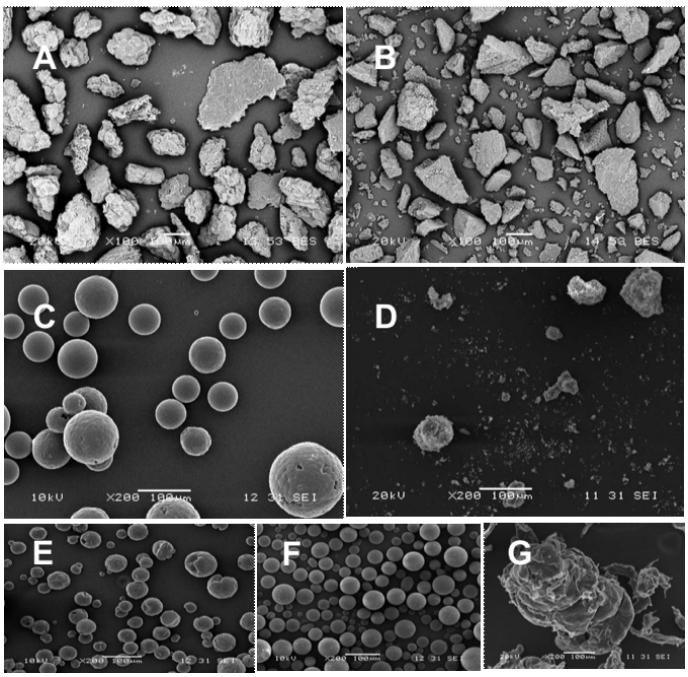



Figure 2. Scanning electron microscopy for the microbeads made of seven polymer types: (A) ground apricot kernels, (B) ground rice particles, (C) PLA, (D) PHBV, (E) PCL (F) PMMA, (G) PE. The scale bar in all panels are 100 µm.

*3.2. Temporal dynamic of oxygen consumption*

The continuous evolution of oxygen consumption by bacteria in minimum medium with each polymer as sole carbon source showed different patterns according to the polymer types. Oxygen consumption by bacteria growing on PHBV was so high that it was necessary to re-

open the tube after 15 days in order to ensure a minimum oxygen concentration of 1.5 µmole for bacterial growth (Figure 3). When a decrease of 19 µmole was observed for PHBV during the first 15 days, PCL, Rice and Apricot also showed significant oxygen consumption of 12.7, 6.8, 2.4 µmole for the same period, respectively. Due to the potential limitation of oxygen on PHBV during 10-15 days, the first 10 days of percentage of biodegradation (based on the ratio of oxygen consumption over the theoretical oxygen demand for complete degradation) were 4.1±0.4% (average and standard deviation), 1.0±0.1%, 3.8±1.9% and 0.5±0.2% for PHBV, PCL, Rice and Apricot respectively. It has to be noted that vials with rice became visually very turbid, which may affect the transmission of luminescence and then underestimate the oxygen consumption and resulting percentage of biodegradation.

Other polymers including PE, PMMA and PLA did not show any oxygen consumption during the entire course of the 2-months incubation (Figure 3).

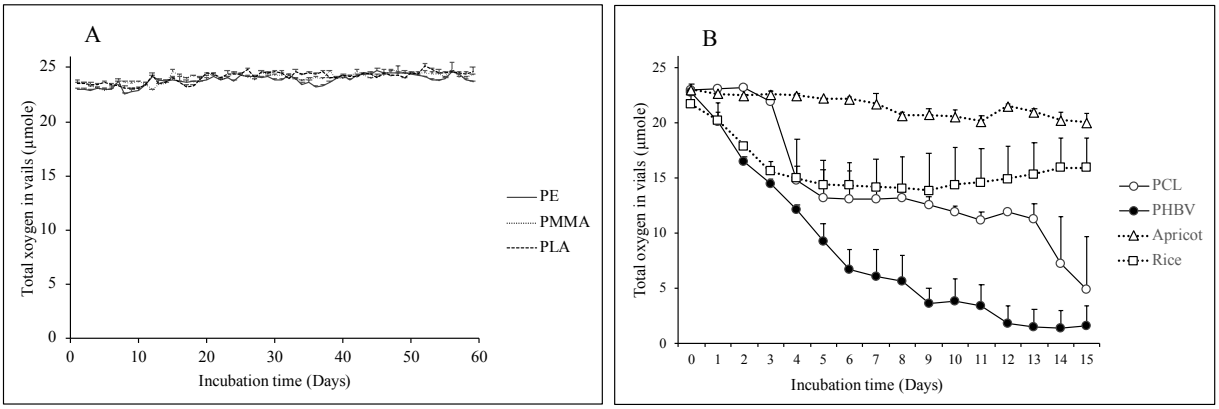



Figure 3. Oxygen consumption (in µmole) during the first 10 days after incubation in minimum medium with microbeads made of the seven material types (PE, PMMA, PLA, PCL, PHBV, apricot, rice) and their respective mature biofilm (with standard deviation). (A) Daily changes of oxygen in vials (in µmole) are given for (A) non-degradable polymers and (B) degradable polymers.

### *3.3. Weight loss*

Weight loss measurement between the beginning and the end of the incubation in minimum medium separated the polymers in 2 groups similar to those obtained by oxygen measurement (Table 1). No weight loss was found on polymer for PE, PLA and PMMA. Apricot showed 5.5±8.8% of weight loss, when PCL, PHBV showing about 17.8±7.2% and 17.0±6.1%. Rice had the highest weight loss with 80.1±4.8%.

*3.4. Changes in the molecular weight*

Significant signs of degradation were only observed for PCL that showed a decrease by 30% of average molecular weight (Mn) (from 33 000 to 23 000 g $mol^{-1}$) and an increase of their polydispersity index (1.7 to 2.0) that indicates a higher molecular weight dispersion (Table 1). No significant change was found for PE, PMMA and PLA microbeads. No sign of degradation was found by the molecular weight assay for PE, PMMA, PLA. Measurement of the molecular weight was not applicable for the, PHBV, rice and apricot.

*3.5. Modification of the chemical properties*

The composition of microbeads incubated under biotic conditions was controlled by FTIR after 2 months of incubation in MM. No biodegradability could be found for PE, PMMA and PLA (Figure S2-4). Moreover, the absence of characteristic carbonyl band at 1720 $cm^{-1}$ indicate that PE microbeads did not undergo photodegradation.

The FTIR spectra of PCL microbeads showed a decrease of carbonyl index and a concomitant increase of crystallinity index (Table1, Figure S5). Such pattern is typical of ester bonds cleavage in amorphous region of the polymer chains and confirms the reduction of the molecular weight of the polymer. Rice microbeads presented a spectrum characteristic of starch with specific monosaccharides and polysaccharides signals (Figure S6). The strong modification of the spectra over time revealed a transformation of starch constituent amylopectin, amylose into simple monomer of glucose (Figure S7). The FTIR analysis of Apricot microbeads presented a typical spectrum of lignocellulosic material. The spectra of these sample did not change over time, thus indicating a low transformation of the material as observed by weight loss measurements (Figure S8). An increase of the crystallinity index was observed for PHBV samples under biotic condition, suggesting the possible cleavage of ester bonds in amorphous region of the polymer chains (Table 1, Figure S9).

*3.6. Products release during the biodegradation*

Under abiotic conditions, $^{1}H$ NMR signal was detected only in the supernatant of PCL, apricot and rice samples incubated in minimum media while no signal was recorded for the other materials (Figure 4A). This indicate the release of small molecular weight compounds from these polymer matrixes in the aqueous medium when no biological process occurs. The extraction of oligomers quickly started on PCL after one day of incubation. The integration of

the total NMR signal clearly showed a gradual release of oligomers during the 60 days of incubation (Figure 4A). By comparison with the literature data, the signal attribution confirms the identification of short chains of PCL oligomers and typical signal of carbohydrates such as glucose was observed for the oligomers extracted from rice and apricot samples (Figure S10 and Table S9). It is worth to notice that oligomers from PCL, apricot and rice were not detected under biotic conditions, suggesting an assimilation concomitant to their production. On the contrary, the PHBV microbeads did not release any measurable oligomers under abiotic conditions but a signal was recorded in presence of microorganisms, thus confirming the biotic degradation of polymers (Figure 4B). The release of oligomers (3-hydroxyvalerate, 3-hydroxybutyrate repeating unit) started slowly after one day of incubation with microorganisms and increased up to 30 days. The signal then decreased until the end of experiment which is typical of microbial consumption.

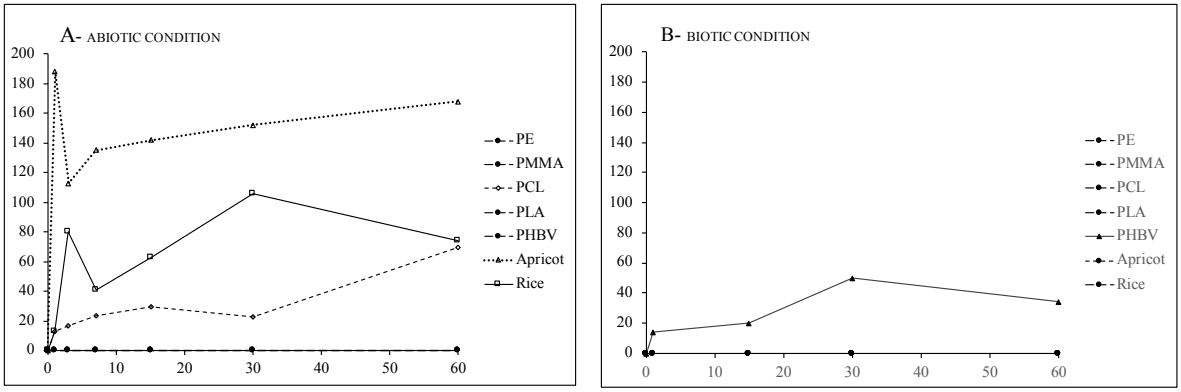


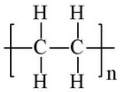
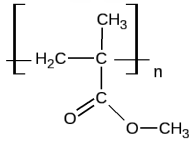
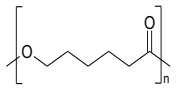
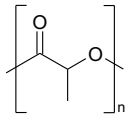
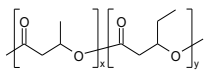
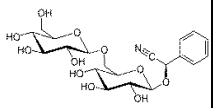
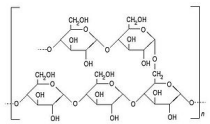

Figure 4. Production of oligomers over 60 days of incubation in minimum medium with with microbeads made of the seven material types (PE, PMMA, PLA, PCL, PHBV, apricot, rice) under (A) abiotic (without biofilm) and (B) biotic (with biofilm) conditions. The results represent the total integration of $^{1}$H NMR spectra expressed in relative intensity of $TSPD_4$ internal standard.

### *3.7. LC Orbitrap TM Mass Spectrometry*

The oligomers detected in the supernatant were analyzed by mass spectrometry to identify their molecular composition. Analysis of PCL, apricot and rice supernatants confirmed the presence of small molecular weight compounds corresponding to the abiotic degradation of the polymer matrix into various size of oligomers (Figure S11 and Table S10). Under abiotic conditions, the ester hydrolysis of PCL generated caprolactone oligomers that were identified as monomer,

dimer and trimer of polycaproic acid oligomers. As well, the mass spectra obtained from rice and apricot supernatant provided a complex profile of low molecular weight compounds. Carbohydrates and amino acids were detected in rice samples while degradation products from lignin and fatty acids were identified in apricot kernel samples, resulting from the transformation of the raw material by abiotic processes. Finally, the PHBV microbeads incubated with microorganisms released various dimer/trimer units of hydroxy-butyrate and hydroxy-valerate. In our conditions, PHBV microbeads were more sensitive to enzymatic cleavage than to abiotic degradation processes.

Table 1. Synthesis of the microbeads composition, size and characterization before colonization and after biodegradation assessment.

| Type | Polymer | Monomer | Mean diameter (µm) | | Biodegra-dation rate % | Weight loss % (S.D.) | Molecular weight | | | | Carbonyl */ Crystallinity index | | $^1$H NMR Signal | Mass Id |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | Mn | | Mw/Mn | | | | Biotic/ abiotic | |
| | | | *b.c.* | *D60* | *D10* | | *b.c.* | *D60* | *b.c.* | *D60* | *b.c.* | *D60* | *D60* | *D60* |
| Conventional | Low Density Polyethylene (PE) |  | 278 | 282 | n.d. | 0% | 6 000 | 6000 | 8.9 | 9.1 | 0* | 0* | n.d./ n.d. | n.d. |
| | Poly(methyl methacrylate) (PMMA) |  | 57 | 56 | n.d. | 0% (25.5%) | 125 000 | 118 000 | 2.6 | 3.0 | n.a. | n.a. | n.d./ n.d. | n.d. |
| | Polycaprolactone (PCL) |  | 88 | 61 | 1.0% (0.1%) | 17.0% (6.1%) | 33 000 | 23 000 | 1.7 | 2.0 | 7.57 | 4.24 | n.d./ ++ | Caprolactone (and its dimer and trimer) |
| Biosourced | Poly(lactic acid) (PLA) |  | 115 | 119 | n.d. | -0.3% (18.6%) | 65 000 | 65 000 | 1.8 | 1.7 | n.a. | n.a. | n.d./ n.d. | n.d. |
| | Poly (3-hydroxybutyrate-co-3-valerate) (3 mol % HV) PHBV |  | 126 | 106 | 4.1% (0.4%) | 17.8% (8.2%) | 34 0000 | | n.a. | | 0.74 | 0.83 | +/ n.d. | 3-hydroxybutyric acid<br>3-hydroxyvaleric acid<br>3-hydroxybutyrate dimer<br>3-hydroxybutyrate-3-hydroxyvalerate dimer<br>3-hydroxyvalerate dimer<br>(and its Heterotrimers) |
| Natural polymer | Crushed Apricot kernel (APRICOT) |  | 158 | 120 | 0.5% (0.2%) | 5.5% (8.8%) | n.a. | | n.a. | | n.a. | | n.d./ ++ | Glucose. Hydroxyhydroquinone. Benzoic acid. 2-lauroleic acid. cis-9-palmitoleic acid. Myristoleic acid. hydrocinnamic acid. 4-Methoxycinnamic acid. Syringic acid. m-Coumaric acid. Cinnamic acid |
| | Crushed rice seed (RICE) |  | 120 | 277 | 3.8% (1.9%) | 80.1% (4.8%) | n.a. | | n.a. | | n.a. | | n.d./ ++ | Glucose.<br>L-Valine. L-Carnitine<br>Isobutyrylglycine<br>L-Proline |

*b.c.* before colonization*; D60* after 60 days in minimum medium*;* n.a. non applicable; n.d. not detected in the condition; + level of signals

## 4. Discussion

### *4.1. An original multidisciplinary approach for the estimation of polymer biodegradability in marine environment*

Standard test procedures for estimating polymer biodegradability are commonly based on measurements of biological $O_2$ demand or $CO_2$ consumption, which are giving indication on the complete biomineralization of the material by microorganisms (Harrison et al. 2018). In this study, we combined the standard test for the "determination of the ultimate aerobic biodegradability of plastic materials in an aqueous medium" (ISO 14851, 2004) together with other methodologies in order (i) to confirm the standard test results based on biomineralization processes and (ii) to give more insights on the other steps involved in biodegradation that include also the biodeterioration, biofragmentation and the bioassimilation of the polymers (Dussud and Ghiglione, 2014). Physico-chemical and structural modification of the microbeads and evidence of biodeterioration were assessed by granulometry, gravimetry and FTIR spectroscopy and size exclusion chromatography, $^1$H NMR and high-resolution mass spectrometry were performed to identify some degradation products (monomers and oligomers) in biotic and abiotic conditions and then give information on the biofragmentation. Finally, $^1$H NMR analysis has also been showing the bioassimilation of the polymers. To our knowledge, this is the first time that molecular analysis was used to identify the biofragmentation products (i.e. oligomers) and their bioassimilation in polymer degradation by complex natural microbial communities. Such approach has been previously used to assess the transformation and biodegradation of other contaminants such as pesticides or persistent organic pollutants ( Medana et al., 2005; Biache et al., 2017), but never in polymer biodegradation studies. While the study of contaminants is generally facilitated by the focus on single or few components (represented by a unique molecular weight), the detection and identification of oligomers from polymer fragmentation deals with thousands of compounds. Here, we propose that NMR spectroscopy and mass spectrometry provide new and highly informative results on the molecular fragments of the polymers that can be generated during its biodegradation. Moreover, it brings a complementary information on direct assimilation of polymers by microorganisms, that are missing parameter in the classic methodological approaches which focus either on the growth of microorganisms or on the loss of material properties, thus addressing the biodeterioration with indirect processes.

While there is no harmonized indication today for the preparation of the microbial inoculum to test its polymer biodegradation capabilities, convergent views indicated that incubation with pre-selected isolated strains, as it is specified for regional standard tests (ASTM D6691-09, 2009), do not mimic the large diversity of microorganisms living on plastics in the natural conditions, the so-called 'plastisphere' (Zettler et al., 2013; Dussud et al., 2018). Complex natural marine inocula were adopted by two international standards (ISO 18830, 2016; ISO 19679, 2016), where it is unfortunately indicated that the inocula could be stored for a month at 4 °C, which drastically increase the possibility of a strong modification of the plastic-degrading community (Stenberg et al., 1998). Another novelty of our study is to propose an original two-steps protocol to test the polymer biodegradability by a natural mature biofilm. The first step of formation of the mature biofilm was performed by incubating the polymer types in natural seawater. Our experimental procedure used aquarium with direct circulation to the sea, which was previously shown (i) to mimic the environmental conditions, with similar communities found during several months in the aquarium compared to the *in situ* conditions and (ii) to allow the formation of a mature biofilm after 15 to 30 days, depending on polymer types (Dussud et al., 2018a). The second step is the biodegradability test, where the polymers with its mature biofilm were transferred into a minimal medium with no other carbon source and energy. This procedure is classically used to test the capability of microorganisms to degrade various organic compounds or pollutants ( Rodríguez et al., 2010; De Wilde, 2012), but this is the first time that it has been used for polymer degradation by complex community under marine condition.

Finally, another originality of our study is that no other feasibility study exists ranging from the bio-based and biodegradable microparticle design to the evaluation of its fate at sea. The recent legislation implementation of the United States and some European countries on the rinse-off microbeads progressively prohibit the use and marketing of synthetic polymers in personal care products for exfoliation or cleansing, except for biobased and biodegradable particles. Most of the biodegradation study so far were using commercially plastics, mainly in the form of film (Harrison et al. 2018; Dilkes-Hoffman et al., 2019) and to a less extent in powder (Deroiné et al., 2015; Sashiwa et al., 2018), rarely in the form of microbeads, our biodegradation test also enrich the biodegradability results in the form of microbeads. The successful use of cryogrinding and solvent emulsion-evaporation techniques showed that such approaches may be useful to conduct further studies on other polymer types or for the production of

biodegradable substitutes to replace conventional microbeads (Coombs Obrien et al., 2017; King et al., 2017).

We are aware that the specific surface area (surface area in a given amount of mass) could impact the degradation results, especially the degradation rate for the one degradable, while the impact could be limited and also cannot be evaluated, Firstly, all the material in our study were in the same size range. Besides, several studies showed that the impact of specific surface area only emerged at the initial degradation phase (e.g. first several days) (Yang et al., 2005; Chinaglia et al., 2018). Considering that the plastic used in this study was already immerged in seawater for 2 months, thus, the impact on degradation rate in this study was not conducted any normalization.

### *4.2. Successive steps of biodegradability by complex marine bacterial communities for bio-based and petroleum-based polymers*

Our multidisciplinary approach provided the first evidence of the successive steps involved in polymer biodegradation in seawater. These steps have been theoretically proposed by several authors (Jacquin et al., 2019), but never evidenced before by a complementary set of techniques on the same samples and by involving a complex mature biofilm grown under natural seawater conditions. Here, we have highlighted a group of polymers including PCL, PHBV, apricot and rice showing congruent signs of biodegradation, and another group made of PE, PMMA and PLA that gave no sign of biodegradation under our conditions. We prove here that PCL, PHBV, apricot and rice microbeads undergo a succession of physico-chemical transformation (as revealed by FTIR and granulometry) that lead to the biofragmentation of the material (weight loss, molecular weight) resulting in the release of short length oligomers (mass spectrometry;$^{1}$H NMR). Then, these hydrosoluble compounds can be readily assimilated by the microorganisms ($^{1}$H NMR) and support the respiratory activity of the biofilm (BOD). This sequential process does not occur for PE, PMMA and PLA microbeads as no or slight oxidation can be initiated in our experimental time frame.

Our results are congruent with other studies describing PHBV, PCL and in a lesser extend PLA as biodegradable polymers under natural conditions in soil (Hoshino et al., 2001; Tokiwa and Calabia, 2007). Other studies found no sign of biodegradation for PLA in freshwater (Bagheri et al., 2017). PCL films were also found to present less biodegradability potential in freshwater

than under marine condition, probably due to very different bacterial communities inhabiting these ecosystems (Heimowska et al., 2017).

Interestingly, we found that one of the bio-based polymers (PLA) did not fall into the group of biodegradable polymers and contrariwise, one of the petroleum-based polymers (PCL) showed clear signs of biodegradation. These counterintuitive results underline again that bio-based polymers does not necessary mean environmental degradable polymers, and inversely petroleum-based polymer does not necessary mean non-biodegradable products, as already mentioned elsewhere (Krzan et al., 2006).

### *4.3. A set of congruent results for each polymer type*

*4.3.1. PCL biodegradability.* We found that the biodegradability of PCL was 0.1% per day under our experimental conditions (BOD assay). This is lower than another study with static BOD assay for PCL films in seawater (0.9 % per day) (Nakayama et al., 2019). Other studies based on weight loss assays found 0.3% per day (Lu et al., 2018) or 0.2-0.6% per day (Rutkowska et al., 1998). The release of oligomers in abiotic condition proved here that a slight hydrolysis of PCL occurred in seawater without any other biological attack, which could not be detected by other techniques (Bagheri et al., 2017). There was no signal captured by $^{1}$H NMR for PCL in biotic condition, indicating the rapid utilization of the oligomers released by abiotic processes and the presence of bioassimilation activity, which is consistent with other evidence of biotic degradation of PCL in seawater by others (Heimowska et al., 2017; Suzuki et al., 2018). Finally, the increase of crystallinity index from the FTIR results together with the reduction of molecular weight confirmed the degradability of PCL in seawater.

*4.3.2. PHBV biodegradability.* In our conditions, PHBV showed a biodegradability of 0.4% per day (BOD assay), which is comparable (0.2-0.8%) to the only one study conducted in seawater ($CO_2$ production assay) (Deroiné et al., 2015), while much lower compared to another study under composting conditions (4%) (Iggui et al., 2015). $^{1}$H NMR assay showed oligomer release only in biotic but not in abiotic condition, thus making evidence of an efficient biofragmentation of PHBV by the marine bacteria which was ready or beyond their capability to assimilate the large amount of oligomer produced. Abiotic degradation has been observed under other seawater conditions but only after 6 months incubation at 25°C (Deroiné et al., 2014), suggesting that abiotic degradation may be considered if the experiment was prolonged for more than 2 months, the biotic degradation during this study could also promote the abiotic

degradtion, and forming the synergistical degradation scenario. Negligible abiotic degradation was also found after 21 days under composting conditions, where PHBV was described as quite resistant to moisture or abiotic attack (Eldsäter et al., 1997). We also found an increase of crystallinity index that showed the preference of the biofilm to attack amorphous region of the polymer, while other studies claimed that the preferential degradation is only happened in the initial 2-3 days, and this preference disappears at later stages of the degradation procedure, when amorphous and crystalline regions are degraded indiscriminately (Spyros et al., 1997; Hakkarainen, 2002; Iggui et al., 2015).

*4.3.3. Apricot and Rice biodegradability.* The two natural products showed similar biodegradability as other biodegradable polymers, with biodegradation rate (BOD assays) of 0.1% per day for apricot kernels microbeads (similar to PCL) and 0.4% per day for rice seeds (similar to PHBV). Lignocellulosic part of apricot kernel was gradually degraded and the released soluble carbohydrate fraction was readily assimilated by the microorganisms. Previous studies on rice straw also showed a high biodegradation rate in the soil and freshwater environments (Bilo et al., 2018; Sain, 1984).

*4.3.4. Other polymers.* No sign of biodegradation was detected by our set of complementary techniques for PE, PMMA and PLA. Beside their chemical structures and properties (carbon-carbon bonds and/or crystallinity), the environmental conditions (relatively low temperature, salinity, low nutrients and others) may also be a cause of limited degradation in seawater (Agarwal et al., 1998; Min et al., 2020). The lack of obvious oxidation of these polymers limit their fragmentation, the production of intermediate products (< 1500 Da) and thus their assimilation by microbes in marine environment (Andrady, 2011; Eyheraguibel et al., 2017; Jacquin et al., 2019).

**Concluding remarks:**

An increasing number of countries have implemented laws to restrict the production of microbeads over the world. We present here that microbeads made of PHBV or rice and in a lesser extent PCL and apricot are good candidates for substitution of conventional microplastics classically made of PE or PMMA. Our study presented several novelties regarding its experimental design but also by the various and complementary technologies used. We advise here that not only respirometry tests classically used in standard tests, but rather a multidisciplinary approach should be considered for further studies dealing with the biodegradability of polymers in the environment, which include other banned single-used

plastics such as plastic bags, straws, disposable dishes, cotton swabs (such as Q-tips) and coffee stirrers. A recent study underlined our capability to reduce 78% of plastic pollution by 2040 using current knowledge in a coordinated global scenario, including the use of bio-based and biodegradable polymers (Lau et al., 2020). Here, we underlined the fact that clear distinction should be made with bio-based polymers that may not be biodegradable (such as PLA), when less sustainable substitute materials showed clear signs of biodegradation at sea (such as the petroleum-based PCL), some study recently proposed using the PLA microbeads as the cosmetic substitutes could be unadoptable (Nam and Park, 2020). Finally, further studies are needed to evaluate the toxicity of the polymers and their degradation products, as well as the release of additives generally supplemented by manufacturers in the microplastic composition.

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***Conflict of interest.*** *All of the reported work is original, and authors have seen and approved the final version submitted. The material has not been submitted for publication elsewhere while under consideration for Environmental Science & Policy journal. The authors declare that there are no conflicts of interest. Likewise, consent is given for publication in the Environmental Science & Policy journal, if accepted.*

# Chapter 4 Supporting information

**Table of content**

Figure S 2 : Size of the microbeads granulometry of an example of PHBV on D0 and D60

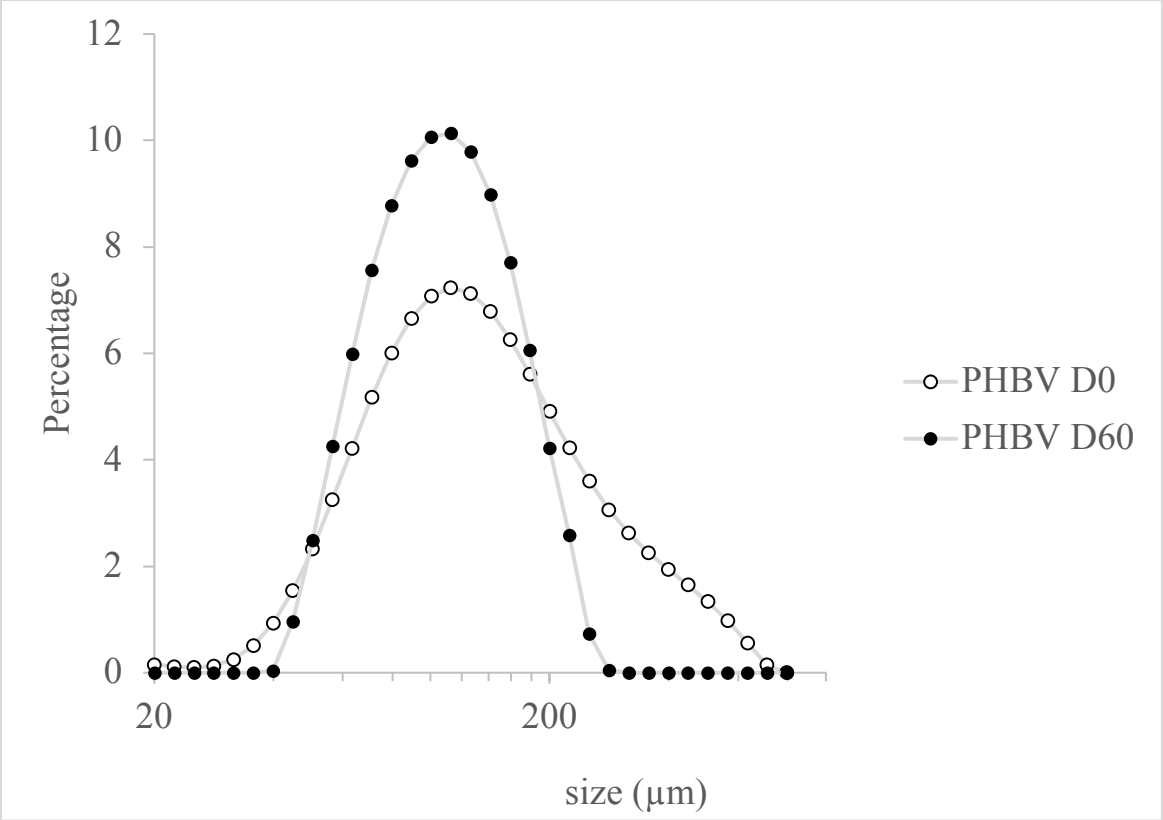



*Table S 4* : Quantities of biopolymers and aqueous solutions used for microbeads elaboration

| *Polymers* | $m_{polymer}$ *(g)* | $V_{PVA2\%}$ *(mL)* |
|---|---|---|
| *PCL* | 45 | 600 |
| *PLA* | 30 | 600 |
| *PHBV (3% HV)* | 10 | 900 |

**Fourier Transform Infrared (FTIR) spectroscopy**

1-PE

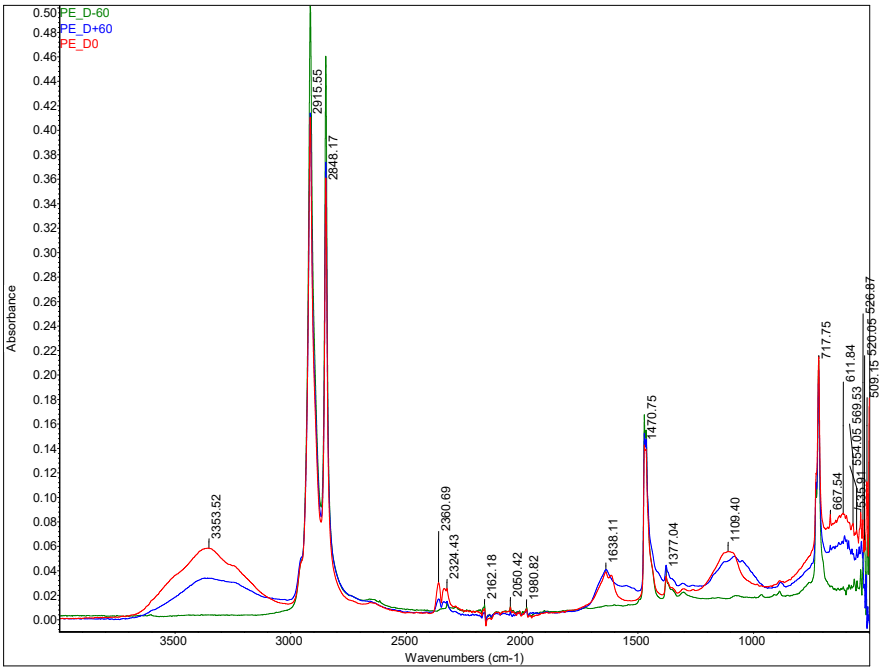



*Figure S 3 : FTIR spectrum of PE microbeads in the range 500-4000 cm$^{-1}$, PE_D-60 indicates polymers before bacterial colonization, PE_D0 indicates after 60 days colonization in aquaria, PE_D+60 indicates 60 days after in minimum medium.*

| Position | Functional group | Vibrational mode | Band intensity | | |
|---|---|---|---|---|---|
| cm$^{-1}$ | | | PE RAW | Colonization-D0 | Biotic-D60 |
| 3353 | O-H | | 0 | 0.058 | 0.034 |
| 2915 | $CH_2$ | Asymmetric stretching | 0.506 | 0.412 | 0.417 |
| 2848 | CH2 | Symmetric stretching | 0.465 | 0.364 | 0.377 |
| 1720 | C=O | Carbonyl stretching | - | - | - |
| 1638 | C=C or C-N; N-H | stretching | 0 | 0.038 | 0.041 |
| 1470 | C–O and C–C | stretching in the crystalline phase | 0.168 | 0.145 | 0.153 |
| 717 | $CH_2$ | rocking | 0.214 | 0.216 | 0.205 |
| | | | Carbonyl Index 1720/1470 | | |
| | | | 0 | 0 | 0 |

*Table S 5 : Characteristic infrared bands of PE and Carbonyl index calculation* (Verleye et al., 2001; Noda et al., 2007; Da Costa et al., 2018)

The infrared spectra of PE after exposition does not present any signal corresponding to ketones (peak at 1720 cm$^{-1}$) nor carboxylic acids (1713 cm$^{-1}$), esters (1735 cm$^{-1}$) and lactones (1780 cm$^{-1}$). The carbonyl index (ratio between the absorbance peak of the bands at 1720 and 1470 cm$^{-1}$ ) cannot be calculated. This indicate that there is no oxidation of PE during exposition. The formation of the band at 1638cm-1 could either attributed to amid bound and explained by the presence of the biofilm. The absorption band at 1641 cm$^{-1}$ is attributed to the formation of double bonds, we have no explanation for the formation of unsaturation during exposure conditions, some author consider this band as the oxidation, while are under debate for us (Da Costa et al., 2018).

2-PMMA

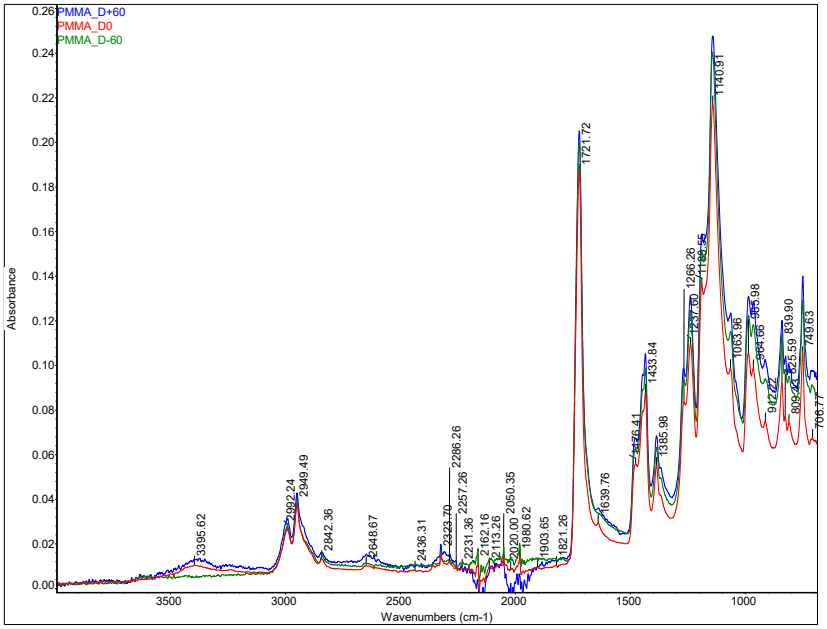



*Figure S 4 : FTIR spectrum of PMMA in the range 500-4000 cm$^{-1}$*

| Position | Functional group | Vibrational mode | Band intensity | | |
|---|---|---|---|---|---|
| cm$^{-1}$ | | | PMMA RAW | Colonization-D0 | Biotic-D60 |
| 2996 | $CH_3$ | Asymmetric stretch | 0.0185 | 0.0263 | 0.004 |
| 2952 | $CH_2$ | Symmetric stretch | 0.0244 | 0.0371 | 0.0078 |
| 1720 | C=O | Carbonyl stretching | 0.111 | 0.187 | 0.0611 |
| 1434 | $CH_2$ | Bending | 0.0567 | 0.0881 | 0.0283 |
| 1386 | $CH_2$ | Symmetric wagging | 0.0376 | 0.0551 | 0.0161 |
| 1240 | C-O-C | Asymmetric stretch | 0.0701 | 0.11 | 0.0366 |
| 1140 | C-O-C | Symmetric stretch | 0.133 | 0.217 | 0.075 |
| 1063 | C-O | stretching | 0.0654 | 0.0988 | 0.0342 |
| 985 | C-O-$CH_3$ | rocking | 0.0693 | 0.106 | 0.0366 |
| | | | Carbonyl index 1720/2952 | | |
| | | | 4.55 | 5.04 | 7.83 |

*Table S 6 : Characteristic infrared bands of PMMA* (Alshehry and Ismail, 2008; Szilasi et al., 2011; Galhardo et al., 2018; Huszank et al., 2019)

The carbonyl index of PMMA microbeads was calculated as the ratio between the absorbance peak of the bands at 1720 and 2952 cm$^{-1}$ (Galhardo et al., 2018)

3- PLA

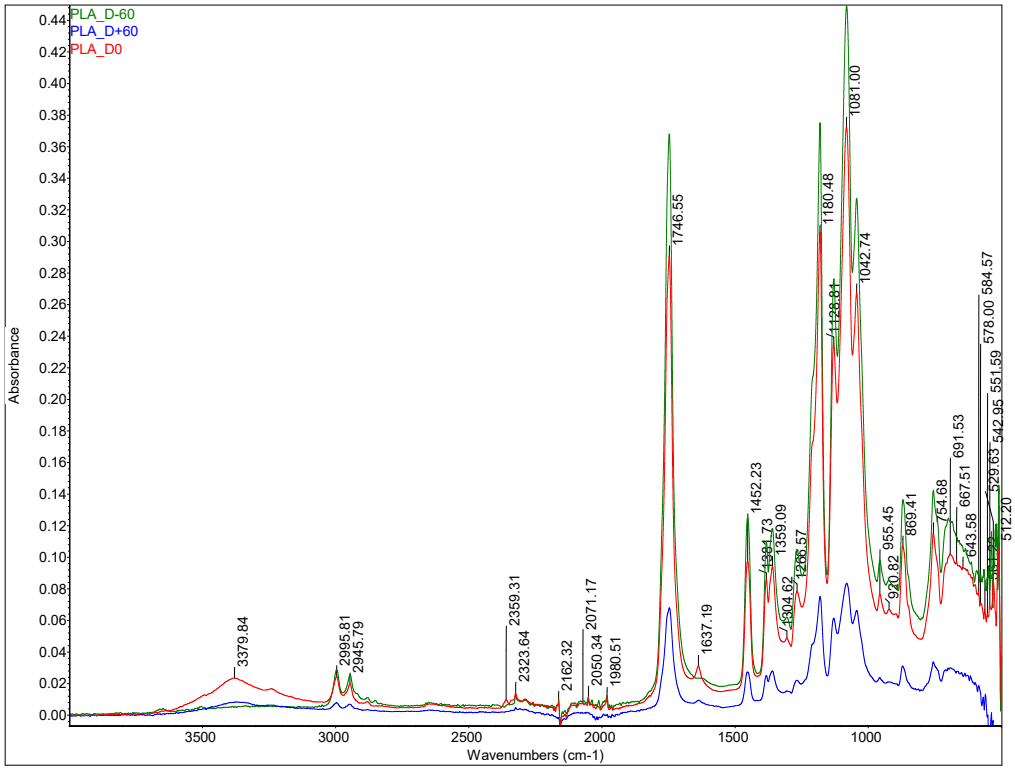



*Figure S 5 : FTIR spectrum of PLA in the range 500-4000 cm$^{-1}$*

| Position | Functional group | Vibrational mode | Band intensity | | |
|---|---|---|---|---|---|
| cm$^{-1}$ | | | PLA RAW | Colonization-D0 | Biotic-D60 |
| 1746 | C=O | Carbonyl stretching | 0.256 | 0.291 | 0.055 |
| 1452 | $CH_3$ | asymmetric stretching | 0.088 | 0.098 | 0.018 |
| 1381 | $CH_3$ | Symmetric wagging | 0.078 | 0.087 | 0.016 |
| 1359 | CH | | 0.083 | 0.094 | 0.018 |
| 1180 | C-O-C | Symmetric Stretching | 0.260 | 0.307 | 0.070 |
| 1128 | C-O | Stretching | 0.192 | 0.236 | 0.054 |
| 1089 | C-O-C | Stretching | 0.311 | 0.373 | 0.021 |
| | | | Carbonyl Index 1746/1452 | | |
| | | | 2.91 | 2.98 | 3.05 |

*Table S 7 : Characteristic infrared bands of PLA and Carbonyl index calculation* (Kister et al., 1998; Meaurio et al., 2006; Ndazi and Karlsson, 2011; Da Silva Gois et al., 2017)

The carbonyl index of PLA microbeads was calculated as the ratio between the absorbance peak of the bands at 1746 and 1452 cm$^{-1}$ (Coleman et al., 20104); no significant change was observed.

4- PCL

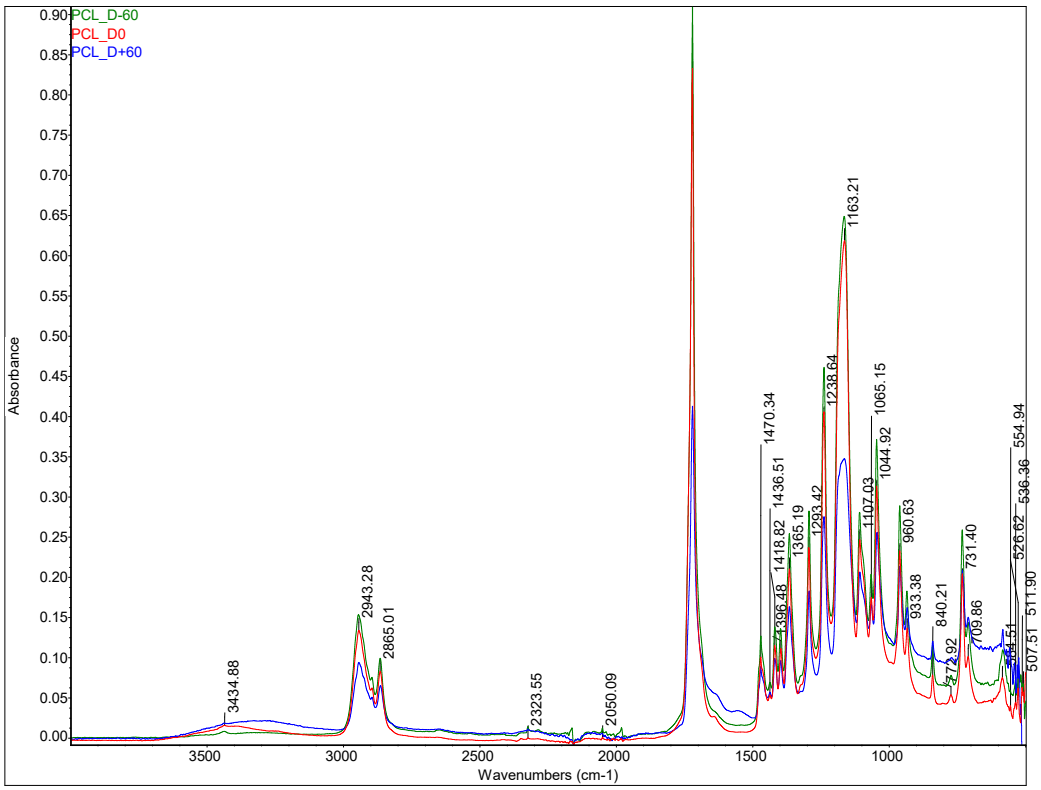



*Figure S 6 : FTIR spectrum of PCL in the range 500-4000 cm$^{-1}$*

| Position | Functional group | Vibrational mode | Band intensity | | |
|---|---|---|---|---|---|
| cm$^{-1}$ | | | PCL RAW | Colonization-D0 | Biotic-D60 |
| 2943 | $CH_2$ | Asymmetric stretching | 0.145 | 0.134 | 0.047 |
| 2865 | $CH_2$ | Symmetric stretching | 0.095 | 0.083 | 0.033 |
| 1720 | C–O | Carbonyl stretching | 0.840 | 0.840 | 0.205 |
| 1397 | $CH_2$ | bending | 0.130 | 0.111 | 0.076 |
| 1293 | C–O and C–C | stretching in the crystalline phase | 0.267 | 0.240 | 0.092 |
| 1238 | COC | Asymmetric stretching | 0.428 | 0.407 | 0.137 |
| 1163 | C–O and C–C | stretching in the amorphous phase | 0.620 | 0.619 | 0.172 |
| | | | Crystallinity Index 1293/1163 | | |
| | | | 43% | 38% | 53% |
| | | | Carbonyl Index 1720/1397 | | |
| | | | 6.4 | 7.57 | 4.24 |

*Table S 8 : Characteristic infrared bands of PCL , carbonyl and crystallinity index calculation from* (Coleman and Varnell, 1980; Elzein et al., 2004; Khatiwala et al., 2008)

The carbonyl index of PCL microbeads was calculated as the ratio between the absorbance peak of the bands at 1720 and 1397 cm$^{-1}$ and the crystallinity index was calculated as ratio between the absorbance peak of the bands at 1294 and 1167 cm$^{-1}$(Coleman and Varnell, 1980; He and Inoue, 2000; Khatiwala et al., 2008).

5- Rice

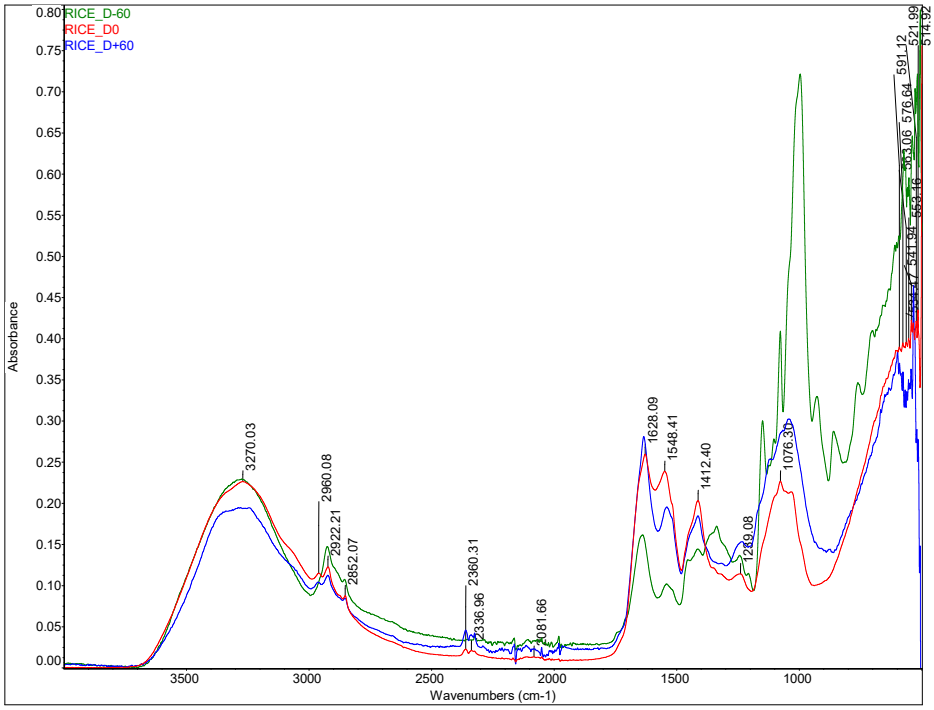



*Figure S 7 : FTIR spectrum of Rice in the range 500-4000 cm$^{-1}$*

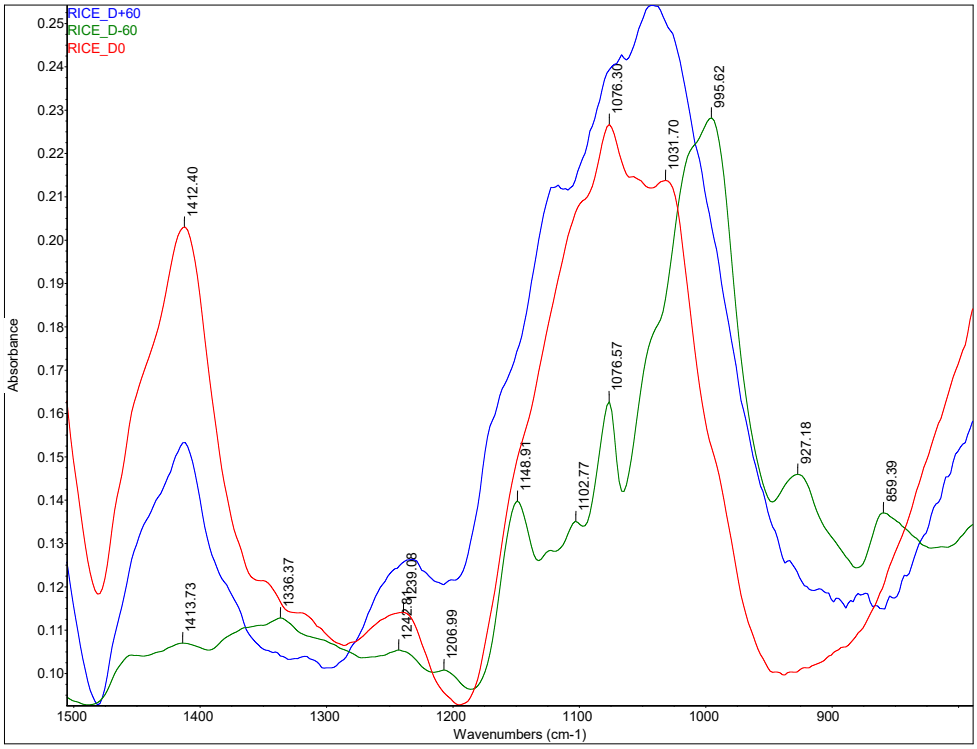



*Figure S 8 : FTIR spectrum of Rice in the range 900-1200 cm$^{-1}$*

| Position | Functional group | Vibrational mode | Band intensity | | |
|---|---|---|---|---|---|
| $cm^{-1}$ | | | Rice RAW | Colonization-D0 | Biotic-D60 |
| 3275 | O-H | Stretching | 0.0864 | 0.226 | 0.0696 |
| 2925 | C-H | Asymmetric Stretching | 0.0585 | 0.123 | 0.0410 |
| 2854 | C-H | Symmetric Stretching | 0.045 | 0.0875 | 0.0313 |
| 1638 | O-H | Bend (water) | 0.0633 | 0.259 | 0.0997 |
| 1413 | CH | Bending | 0.0574 | 0.203 | 0.0662 |
| 1336 | COH, CH | Bending | 0.0669 | nd | nd |
| 1149 | C-O-C | Stretching | 0.111 | nd | nd |
| 1076 | COH | Bending | 0.148 | 0.227 | 0.1 |
| 995 | C-O-C | Glycosidic linkage | 0.0864 | 0.226 | 0.0696 |
| 928 | C-O-C | Glycosidic linkage | 0.121 | n.a. | n.a. |

*Table S 9 : Characteristic infrared bands of Rice* (Goheen and Wool, 1991; Nybacka, 2016; Pacia et al., 2017)

Starch is main component of rice grain ( Juliano, 1985; Amagliani et al., 2016). The FTIR analysis of rice microbeads present a spectrum characteristic of carbohydrates with specific absorption bands at 995, 1076, 1149, 2925, 3275 $cm^{-1}$ identified as a mix of monosaccharides (glucose) and polysaccharides signals (Pacia et al., 2017) (figure S5). The strong modification of the spectra over time reveal the transformation of the material. The attenuation of absorption bands at 928, 995, 1076, 1149 $cm^{-1}$, characteristic of amylopectin, amylose indicate the transformation of starch into glucose as reveal by the increase of specific band at 1042 and 1121 $cm^{-1}$ (figure S6).

6-Apricot

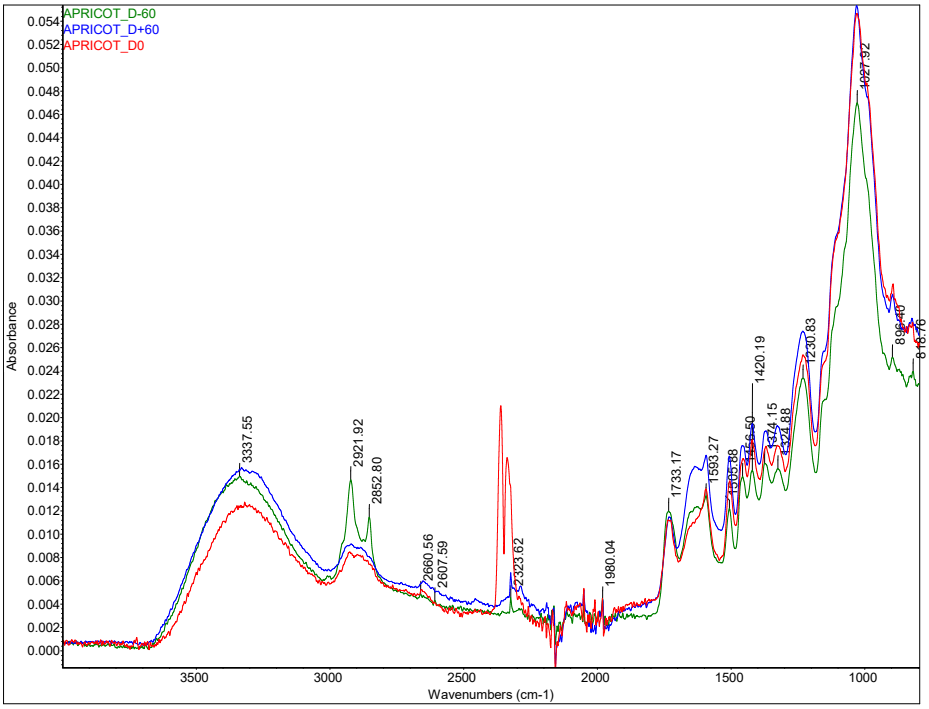



*Figure S 9 : FTIR spectrum of Apricot in the range 500-4000 cm$^{-1}$*

| Position | Functional group | Vibrational mode | Band intensity | | |
|---|---|---|---|---|---|
| cm$^{-1}$ | | | Apricot RAW | Colonization-D0 | Biotic-D60 |
| 3337 | O-H | Stretching | 0.031 | 0.013 | 0.029 |
| 2922 | C-H | Asymmetric Stretching | 0.031 | 0.009 | 0.015 |
| 2852 | C-H | Symmetric Stretching | 0.026 | 0.011 | 0.020 |
| 1733 | C=O, (hc) | Carbonyl stretching | 0.028 | 0.014 | 0.031 |
| 1627 | | | 0.026 | 0.015 | 0.031 |
| 1593 | | | 0.031 | 0.017 | 0.033 |
| 1505 | C=C aromatic ring (l) | Stretching | 0.032 | 0.018 | 0.037 |
| 1456 | $CH_3$ | Asymmetrical angle | 0.033 | 0.018 | 0.036 |
| | $CH_2$ | Bending | 0.031 | 0.013 | 0.029 |
| 1420 | $CH_2$ (c, hc) | Scisor | 0.031 | 0.009 | 0.015 |
| 1374 | C-H (c, hc) | Deformation | 0.026 | 0.011 | 0.020 |

*Table S 10 : Characteristic infrared bands of Apricot* (Corbett et al., 2015; Li et al., 2018) *c=cellulosis; hc= hemicellulosis; l=lignin*

Apricot pit shell is mainly constituted by lignocellulosic fibers and contain a low amount of water-soluble carbohydrates, such as free sugars, glycosides, and starch (Cañellas et al., 1992; Corbett et al., 2015). The FTIR analysis of Apricot microbeads present a typical spectrum lignocellulosic material with specific absorption band of lignin, cellulose and hemicellulose (Corbett et al., 2015; Li et al., 2018). The spectra of these sample do not change over time indicating a low transformation of the material as observed with weight loss measurement.

7-PHBV

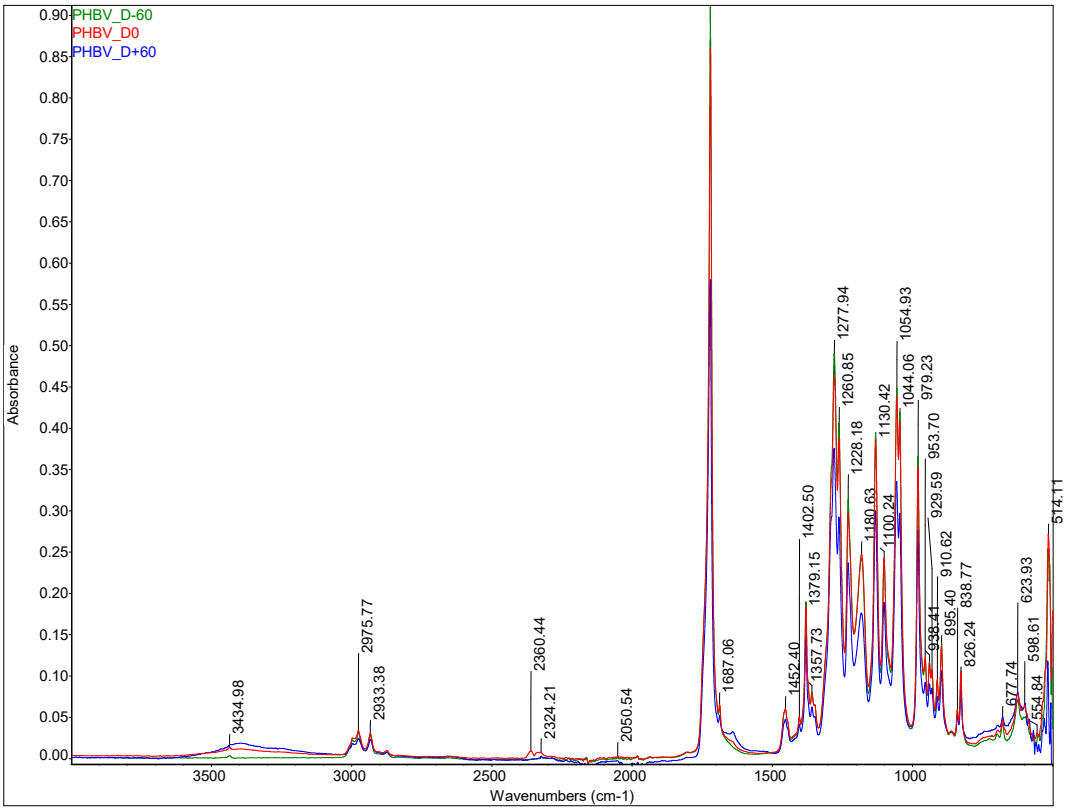



*Figure S 10 : FTIR spectrum of PHBV in the range 500-4000 cm$^{-1}$*

| Position | Functional group | Vibrational mode | Band intensity | | |
|---|---|---|---|---|---|
| cm$^{-1}$ | | | PHBV RAW | Colonization-D0 | Biotic-D60 |
| 2975 | $CH_3$ (c) | Asymmetric stretching | 0.903 | 0.862 | 0.360 |
| 2933 | $CH_3$ (c) | Symmetric stretching | 0.064 | 0.061 | 0.029 |
| 1720 | C=O (c) | Carbonyl stretching | 0.192 | 0.184 | 0.090 |
| 1452 | $CH_3$ | bending | 0.488 | 0.466 | 0.060 |
| 1379 | $CH_3$ | Symmetric wagging | 0.405 | 0.388 | 0.179 |
| 1277 | CH (c) | bending | 0.313 | 0.298 | 0.145 |
| 1260 | C-O-C | stretching | 0.243 | 0.248 | 0.108 |
| 1228 | C-O-C | stretching | 0.395 | 0.387 | 0.185 |
| 1181 | C-O-C | Asymmetric stretching | 0.367 | 0.356 | 0.169 |
| 1130 | C-O-C | Symmetric stretching | 0.903 | 0.862 | 0.360 |
| 979 | C-C (c) | stretching | 0.064 | 0.061 | 0.029 |
| | | | Cristallinity Index 1379/1181 | | |
| | | | 0.79 | 0.74 | 0.83 |
| | | | Cristallinity Index 1228/1452 | | |
| | | | 0.89 | 0.90 | 0.93 |

*Table S 11 : Characteristic infrared bands of PHBV and Crystallinity index calculation* (Xu et al., 2002; Conti et al., 2006; Samantaray and Mallick, 2012)

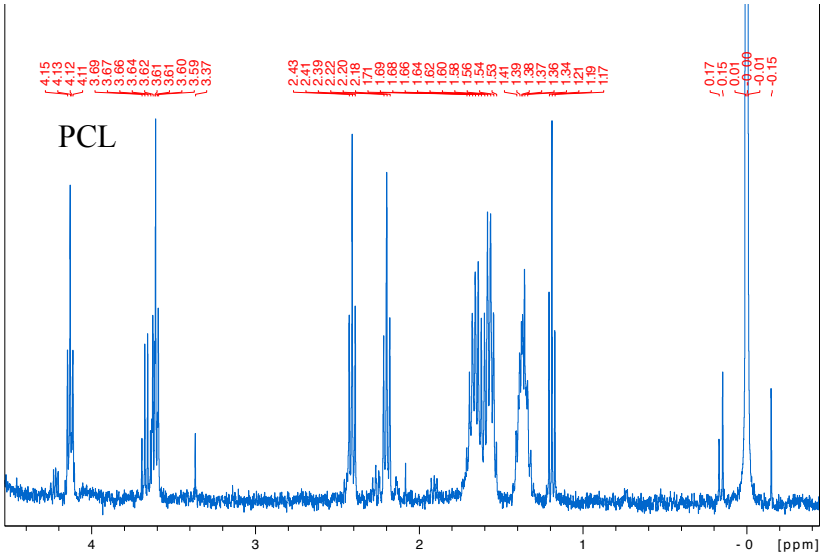
The FTIR spectra of PHBV microbeads show a slight increase of the crystallinity index (ratio between the absorbance peak of the bands at 1379 and 1181 cm$^{-1}$) for PHBV samples. Using the calculation of Xu et al (Xu et al., 2002) (ratio between the absorbance peak of the bands at 1228 and 1452 cm$^{-1}$), the crystallinity index systematically slightly increase under biotic incubation suggesting possible cleavage of ester bonds in amorphous region of the polymer chains.

**Characterization of the degradation products by $^{1}$H NMR spectroscopy**

$^{1}$H NMR analysis were performed on culture supernatant to detect the presence of degradation products. For each material, the $^{1}$H NMR oligomers signal consist in several peaks, located within the proton chemical shift range of 1 to 4.5 ppm (figure S11). The $^{1}$H NMR signal of oligomers was attributed by comparison with spectra from the literature. The signal attribution confirms the identification of short chains PCL oligomers with the presence of characteristic peaks of methylene ends groups at 2.4, 3,6 ppm and methylene repeating unit at 1.4, 1.55, and 4.3 ppm (Báez et al., 2005). Typical signal of carbohydrates was observed for the oligomers extracted from rice and apricot samples with a large resonance band between 3.2 and 4.2 ppm. The anomeric protons of α and β-glucose were noticed at 5.24 and 4.65 ppm respectively, in both spectra (Bubb, 2003; Corbett et al., 2015; Pomin, 2012). For PHBV oligomers, the signal present at 0.9 and 1.2 ppm correspond to $CH_3$ of 3-hydroxyvalerate, 3-hydroxybutyrate repeating unit, while $CH_2$ signal were observed between 2.0 and 2.5 ppm (Kwiecień et al., 2017; Zhao et al., 2015).

| Microbeads | chemical shift (ppm) | assignment | reference |
|---|---|---|---|
| PCL | 1.44 ; 1.55 ; 4.3 | $CH_2$ | (Báez et al., 2005) |
| PCL | 2.4 ; 3.6 | $CH_3$ | (Báez et al., 2005) |
| Rice/Apricot | 3.2 ; 4.2 | CH carbohydrates | (Bubb, 2003; Pomin, 2012; Corbett et al., 2015) |
| Rice/ Apricot | 5.24 ; 4.65 | α and β-glucose anomeric protons | (Bubb, 2003; Pomin, 2012; Corbett et al., 2015) |
| PHBV | 0.9 ;1.2 | $CH_3$ | (Zhao et al., 2015; Kwiecień et al., 2017) |
| PHBV | 2.0 ; 2.5 | $CH_2$ | ( Zhao et al., 2015; Kwiecień et al., 2017) |

*Table S 12 : $^{1}$H NMR attribution signal*



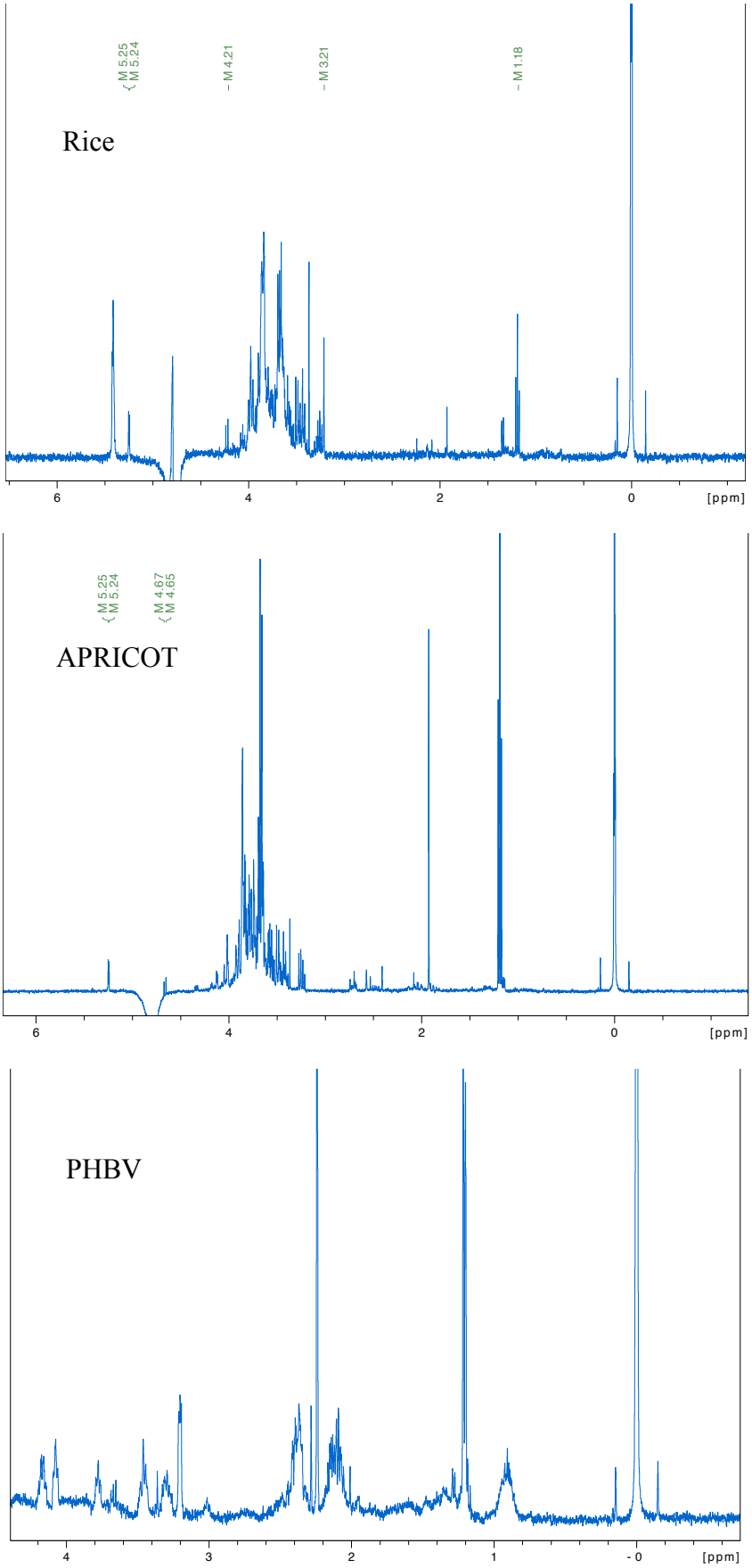


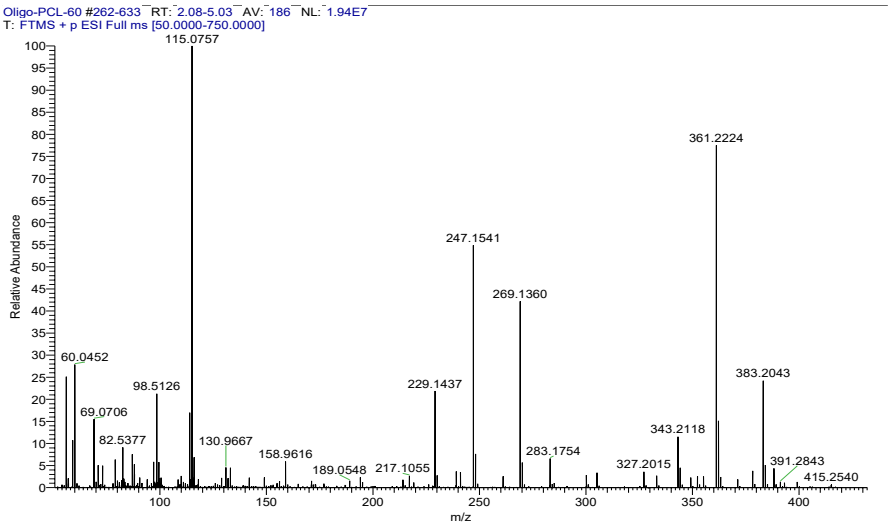

*Figure S 11 : [1]H NMR spectra of PCL, Rice, Apricot Pit Shell and PHBV oligomers.*

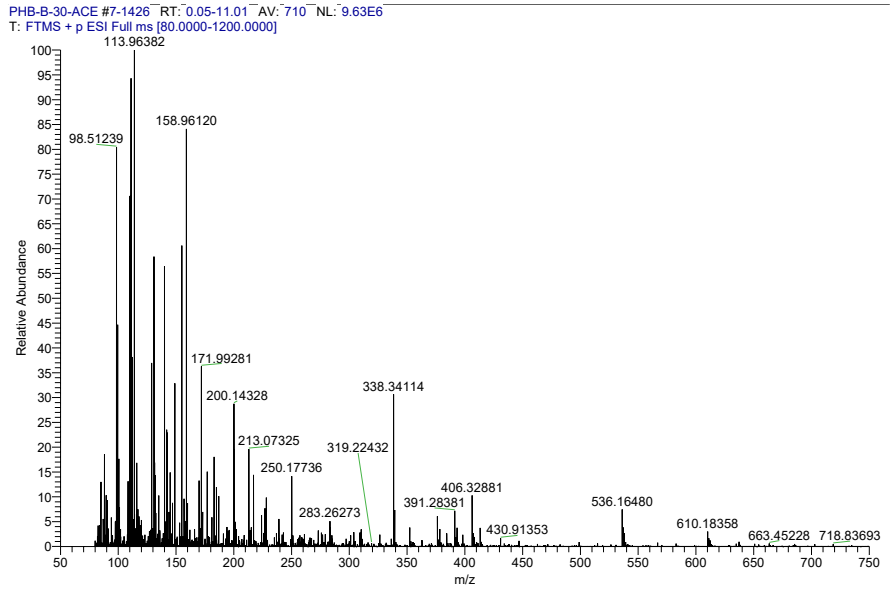
**Liquid Chromatography- High Resolution Mass Spectrometry**

Under abiotic condition, the ester hydrolysis of PCL generate caprolactone oligomers that can be identified as polycaproic acid oligomers (Figure 4). The different chemical forms represent monomer (m/z 115.07), dimer (m/z 229. 14 ; 247.15) and trimer units(m/z 343.21; 361.22). The difference between the polymer series was the repeating unit of m/z 114.0681 (Caprolactone, $C_6H_{12}O_2$)(Rivas et al., 2017) (table S9).
As observed with NMR analysis, the PHBV microbeads did not release measurable oligomers under abiotic conditions but a signal was recorded when the polymers were incubated with microorganisms. Several peaks were identified as hydroxy-butyrate (m/z 88.05) and hydroxy-valerate (m/z 102.06) units and their dimer/trimer with various composition (figure S10 table S9).
Various compounds derived from the degradation of carbohydrates, amino acids, lignin and fatty acids can be detected in rice and apricot kernel samples.

| Polymer | m/z H+ | Units | formula | Compound |
|---|---|---|---|---|
| PCL | 69.0706 | | $C_5H_8$ | |
| | 97.0652 | | $C_6H_8O$ | |
| | 115.0757 | monomer | $C_6H_{10}O_2$ | caprolactone |
| | 229.1436 | dimer | $(C_6H_{10}O_2)_2$ | |
| | 247.1541 | dimer +$H_2O$ | $(C_6H_{10}O_2)_2$+$H_2O$ | |
| | 343.2118 | trimer | $(C_6H_{10}O_2)_3$ | |
| | 361.2223 | trimer +$H_2O$ | $(C_6H_{10}O_2)_3$ +$H_2O$ | |
| | 133.086 | | $C_6H_{12}O_3$ | 6-hydroxyhexanoic acid |
| | | | | |
| PHBV | 87.044 | | $C_4H_6O_2$ | Crotonic acid |
| | 101.060 | | $C_5H_8O_2$ | Pentenoic acid |
| | 89.060 | monomer | $C_4H_8O_2$ | Butyric acid - HB |
| | 173.081 | dimer | $(C_4H_8O_2)_2$ | $HB_2$ |
| | 259.115 | trimer | $(C_4H_8O_2)_3$ | $HB_3$ |
| | 103.075 | monomer | $C_5H_{10}O_2$ | Valeric acid HV |
| | 201.143 | dimer | $(C_5H_{10}O_2)_2$ | $HV_2$ |
| | 301.165 | trimer | $(C_5H_{10}O_2)_3$ | $HV_3$ |
| | 187.097 | Heterodimer | | HBHV |
| | 287.147 | Heteo trimer | | $HBHV_2$ |
| | 273.134 | Heterotrimer | | $HB_2HV$ |
| | | | | |
| RICE | 203.061 | | $C_6H_{12}O_6$ | Glucose |
| | 117.0790 | | $C_5H_{11}NO_2$ | L-Valine |
| | 161.1052 | | $C_7H_{15}NO_3$ | L-Carnitine |
| | 145.0739 | | $C_6H_{11}NO_3$ | Isobutyrylglycine |
| | 115.0633 | | $C_5H_9NO_2$ | L-Proline |
| | | | | |
| APRICOT | | | $C_6H_{12}O_6$ | |
| | 126.0317 | | $C_6H_6O3$ | Hydroxyhydroquinone |
| | 122.0368 | | $C_7H_6O_2$ | Benzoic acid |
| | 198.1620 | | $C_{12}H_{22}O_2$ | 2-lauroleic acid |
| | 254.2246 | | $C_{16}H_{30}O_2$ | cis-9-palmitoleic acid |
| | 226.1933 | | $C_{14}H_{26}O_2$ | Myristoleic acid |
| | 150.0681 | | $C_9H_{10}O_2$ | hydrocinnamic acid |
| | 178.0630 | | $C_{10}H_{10}O_3$ | 4-Methoxycinnamic acid |
| | 198.0528 | | $C_9H_{10}O_5$ | Syringic acid |
| | 164.0473 | | $C_9H_8O_3$ | m-Coumaric acid |
| | 148.0524 | | $C_9H_8O_2$ | Cinnamic acid |
| | | | | |

*Table S 13 : List of monomers and chemical compounds identified in oligomers of PCL, PHBV, Rice and Apricot microbeads.*


Oligo-PCL-60 #262-633 RT: 2.08-5.03 AV: 186 NL: 1.94E7
T: FTMS + p ESI Full ms [50.0000-750.0000]
Relative Abundance
m/z
115.0757
361.2224
247.1541
269.1360
60.0452
98.5126
229.1437
383.2043
69.0706
82.5377
130.9667
158.9616
189.0548
217.1055
283.1754
343.2118
327.2015
391.2843
415.2540


PHB-B-30-ACE #7-1426 RT: 0.05-11.01 AV: 710 NL: 9.63E6
T: FTMS + p ESI Full ms [80.0000-1200.0000]
Relative Abundance
m/z
113.96382
158.96120
98.51239
171.99281
338.34114
200.14328
213.07325
319.22432
250.17736
406.32881
391.28381
536.16480
283.26273
610.18358
430.91353
663.45228
718.83693

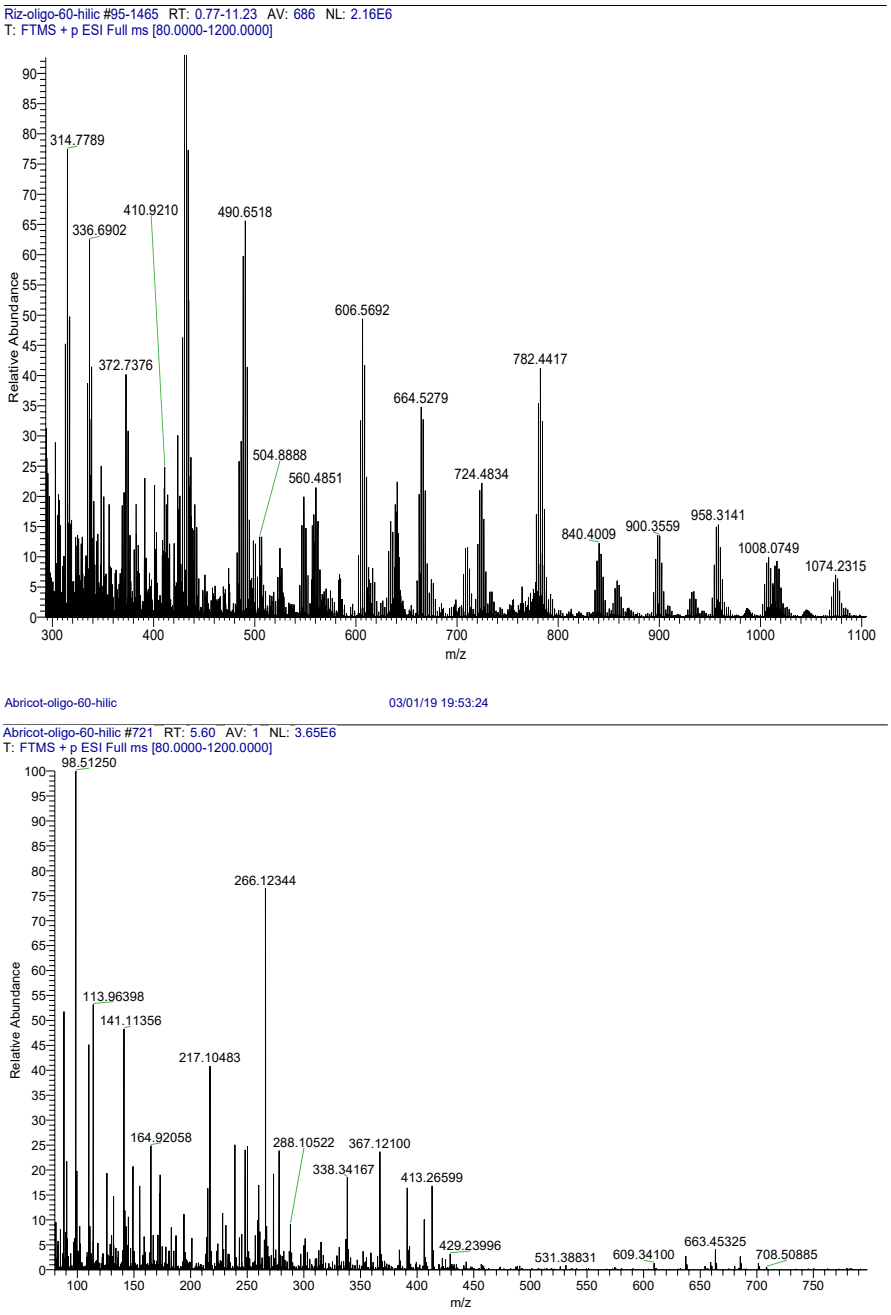



*Figure S 12 : Positive ion ESI-MS mass spectra of mass spectra of PCL , Rice, Apricot Pit Shell and PHBV oligomers*

# *CHAPTER 5: General discussions and perspectives*

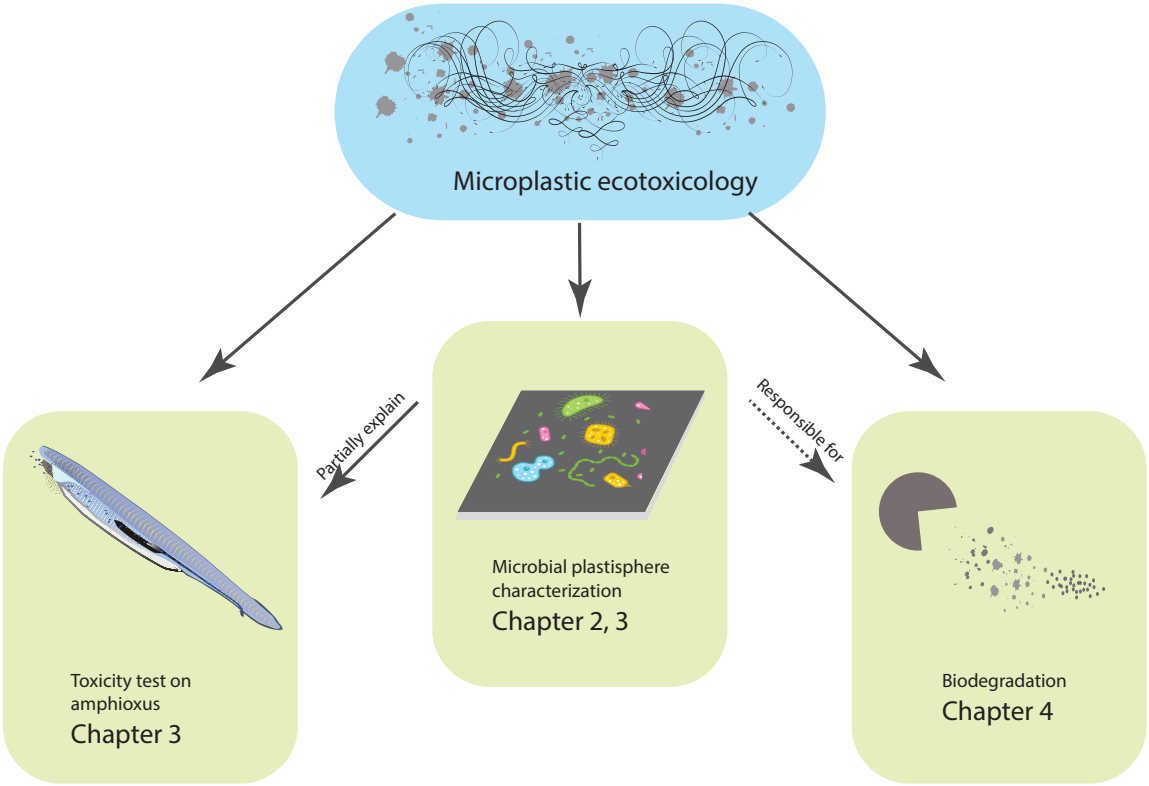



Figure 1. Schematic overview of the three chapters of this PhD, modified from the concept of "microbial ecotoxicology" (Jacquin et al., 2019)

## 1. Main results and general discussions

### *1.1 Main thesis results*

This thesis investigated the microplastics ecotoxicity in the marine environment, mainly in a biological perspective. Chapter 2 focused on the impact of microplastic to the microbial community, factors (plastic size and shape, composition and temporal evolution) driving bacterial assemblage on 'plastisphere' were also investigated. Chapter 3 investigated the toxicity of polystyrene microplastics on amphioxus *Branchiostoma lanceolatum.* Chapter 4 aimed to find out a strategy to reduce the microbeads pollution, different material's

degradability in the marine environment were tested, the substitutes of conventional microbeads used in cosmetic and personal care products were proposed. The bacterial communities living on plastisphere is central in this thesis, since we described its composition and also in seawater on chapter 2, their role in the holobiont when performing toxicity test in chapter 3, and their capability to biodegrade plastic with distinct composition in chapter 4 (Figure 1).

In Chapter 2, the study was conducted at the aquaria closed to the Banyuls Bay (France), during the two-month colonization of PE, PLA and glass. Our main contribution was to show that the bacterial community from seawater was distinct to that of plastisphere. The colonization process (3 successive phases of biofilm formation) and chemical composition were more important than the plastic size and shape in term of bacterial abundance, bacterial activity and bacterial diversity.

In chapter 3, polystyrene microplastic (~60 µm) together with their mature biofilms were used to evaluate their toxicity on amphioxus. No oxidative stress, apoptosis, immune system modification and gut microbiota were observed, while several ASVs were transferred from the plastisphere to the gut microbiota. The microplastic could also increase the goblet cell differentiation. Finally, we suggest that microplastic is not toxic for amphioxus and rather used by amphioxus to harvest nutrients from the biofilm.

In chapter 4, we underlined the importance of using plastic together with their mature biofilm to evaluate their biodegradability. We tested seven material types (PE, PMMA, PCL, PLA, PHBV, rice seeds and apricot kernel) that were first immersed in the aquaria with direct circulation to the sea for 2 months, and then transferred into minimum medium with no other source of carbon for another 2 months. We found that PE, PMMA and PLA did not show sign of biodegradability under our experimental conditions. On the other hand, we were able to classify the biodegradability of the tested materials in rice seeds > PHBV > PCL > apricot kernel.

## *1.2 General discussion*

### 1.2.1 Description and role of the plastisphere

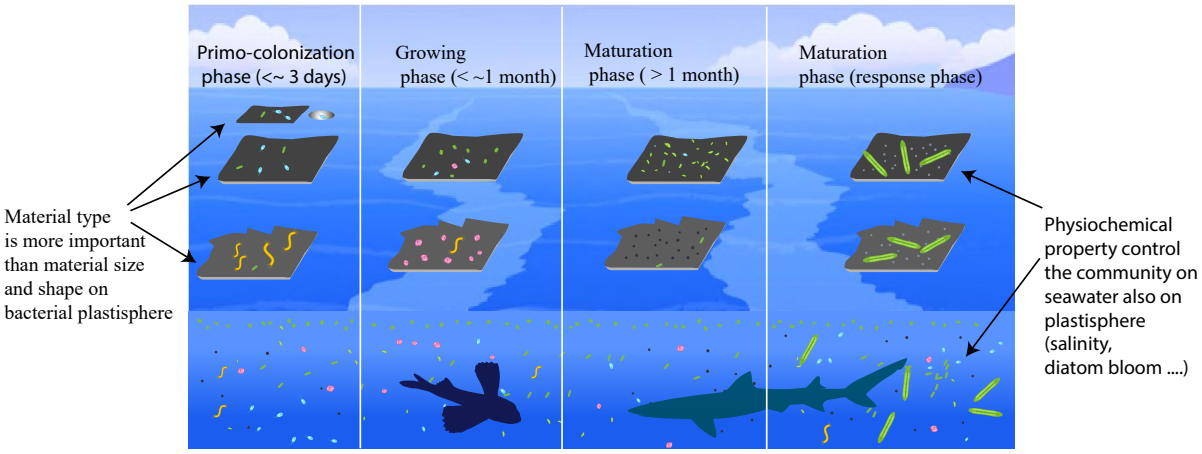



Figure 2. Schematic view of the successive phases of biofilm formation on plastics and the response of the plastisphere to environmental changes.

*Bacterial colonization on plastic in the ocean:*

For the experiment of **chapter 2**, we observed 3 distinct colonization phases of primo-colonization phase, growing phase and maturation phase in term of bacterial abundance, diversity and activity (Figure 2). We followed the dynamic of bacterial colonization process, i.e. the continuous increase in bacterial abundance from the primo-colonization to the maturation phase. We have shown that the bacterial abundance reached $3.10^3$ cell $mm^{-2}$ (for PE) on D3 and increased to a mean of $2.5 \times 10^4$ cell $mm^{-2}$ after one month. One month was enough to reach a 'mature 'biofilm', as the bacterial abundance was comparable to what has been found in the natural environments ($4.4 \times 10^4$ cells $mm^{-2}$ in the Mediterranean Sea by Dussud et al., 2018).

During this study, we also observed a rapid modification of the bacterial community structure and diversity. We identified Roseobacter and Alteromonas as marine primary colonizer, which were consistent to previous study (Dang and Lovellc, 2015). Alteromonas were particularly abundant on the glass samples, which could be due to the high affinity of some taxa to the hard surface. Bacteroides and Planctomycetes are prone to be the secondary colonizers.

*Possible scenario on factors driving the bacteria plastisphere*

Both the bacterial plastisphere and seawater community were shaped by physiochemical factors in our specific condition, which were suggested on study from the brackish water of the North Sea and the China's estuary (Oberbeckmann et al., 2018; Li et al., 2019). The bacterial

plastisphere could respond rapidly to the environmental changes, as previous study that showed an average residence time of bacteria on marine sinking aggregates of about 3 h (Kiørboe et al., 2002). It could be also similar on plastisphere, but never tested, the canonical model for the bacterial colonization process could be not totally applied to the marine environments (as mentioned in the introduction). As we revealed that the share ASV kept almost constant even though the modification of bacterial community from seawater, thus, the bacterial community from the biofilm and seawater could shift in the same direction (Figure 2).

During our study, we propose that the plastic size and shape are not the main factors impacting the bacterial plastisphere, even it has been suggested in previous studies (De Tender et al., 2017; Frère et al., 2018). It suggests that future works on functional analysis of metagenomic and metatranscriptomic on the meso- or macroplastics may be transposable on microplastic.

*Heterotrophic bacterial production related to the carbon biogeochemical cycle in the oceans*

The heterotrophic bacterial production is classically used by marine microbial ecologists to evaluate the role of bacteria in organic carbon remineralization and its corresponding impact on the carbon biogeochemical cycle. Because of the large and increasing amount of plastic on the surface oceans, the role of the plastisphere on biogeochemical cycles is thought-provoking.

Table 1. Plastic surface area estimation in the ocean

| Size[#] | Model[*] | Plastic counts in Mediterranean Sea[#] | Plastic counts in open ocean[#] | Total surface area in Mediterranean Sea ($mm^{-2}$) | Total surface in open ocean ($mm^{-2}$) |
|---|---|---|---|---|---|
| 0.33-1 mm | round cubic (0.665 mm in diameter) | $8.5 \times 10^{10}$ | $174.5 \times 10^{10}$ | $15 \times 10^{10}$ | $309 \times 10^{10}$ |
| 1.01-4.75 mm | film (2.88 mm in length) | $14.6 \times 10^{10}$ | $287.4 \times 10^{10}$ | $121 \times 10^{10}$ | $2384 \times 10^{10}$ |
| 4.76-200 mm | film (100 mm in length) | $1.6 \times 10^{10}$ | $36.4 \times 10^{10}$ | $16000 \times 10^{10}$ | $364000 \times 10^{10}$ |
| > 200 mm | film (100 mm in length) | $0.04 \times 10^{10}$ | $0.86 \times 10^{10}$ | $400 \times 10^{10}$ | $8600 \times 10^{10}$ |
| Total | | | | $16536 \times 10^{10}$ | $375292 \times 10^{10}$ |

#: Raw data of plastic count in ocean were taken from Eriksen *et al.* (2014)
*: Model considering the small size as cube, larger size as film (Ter Halle et al., 2016).

Table 2. Heterotrophic bacterial activity estimation in the surface ocean

| Heterotrophic production | Total surface area ($mm^{-2}$) | Cell counts ($mm^{-2}$) | Total cell abundance on plastisphere | Specific activity ($fgC\ cell^{-1}\ day^{-1}$) | Total incorporated carbon ($Kg\ day^{-1}$) |
|---|---|---|---|---|---|
| Mediterranean Sea | $16536 \times 10^{10}$ | 44000# | $7.3 \times 10^{18}$ | 10.8★ | 78.8 |
| Open ocean | $375292 \times 10^{10}$ | 1644* | $6.2 \times 10^{18}$ | 10.8 | 67.0 |

# data taken from Dussud *et al.* ( 2018)
* data taken from Carson *et al.* ( 2013)
★ data of PE from this study on day 30.

By setting the bacterial production between 0.45 fg $cell^{-1}$. $hour^{-1}$, we calculated here the first estimation the total carbon transformed into the bacteria on plastisphere in the surface ocean by coupling the total surface from the plastic in the surface ocean to the specific activity and bacterial abundance (Table 1). The results turn out that 78.8 and 67 kg of organic carbon could be transformed into bacterial biomass from the Mediterranean Sea and also the open ocean, respectively. It is unexpected to observe such similar results between the Mediterranean Sea and all the open ocean, but the reason actually came from the relatively high bacterial abundance from the Mediterranean Sea. Assuming that the ocean total surface area as 362 $Km^2$ (https://en.wikipedia.org/wiki/Ocean) and the bacterial abundance as $10^4$-$10^6$ $mL^{-1}$ (Mével et al., 2008), it could be inferred that all the bacterial abundance from plastisphere could be equivalent to the 0.037-3.7 nm depth of total surface ocean. The results could be far more less evaluated, since the surface ocean represent for less than 1% of total plastic dumped into the ocean. Others studies are needed to generalize these data. For instance, bacterial abundance from the plastisphere, the accuracy of total surface area, the bacterial specific activity in different region of the ocean, would need other observations in order to better estimate the influence of the plastisphere on the biogeochemical carbon cycle in the ocean.

*Reconsideration of experiment setup*

During this experiment, the bacterial abundance from seawater was considered not to be accurately measured, making it impossible to compare the bacterial activity between plastisphere and seawater. In future, if there are similar experiment to be carried out, I suggest to use different methods to measure the bacterial abundance from the seawater, such as the flow cytometry and epifluorescence microscopy.

1.2.2 Toxicity of plastic on amphioxus and the role of plastisphere as nutrient source for the host

*Effect of biofilm on microplastic behavior in seawater and toxicity tests*

The experiments performed during this PhD showed that it is important not to use pristine plastic but rather use plastic together with its natural complex mature biofilm in order to better fit with the environmental conditions. This underlined the importance of taking into account the change of buoyancy that may influence the microplastic suspension in seawater during the experiment. In our study, we observed that the microplastic of 50-100 µm diameter together with its mature biofilm presented a higher percentage of suspended microplastic percentage in seawater compared to pristine microplastics (Figure 3).

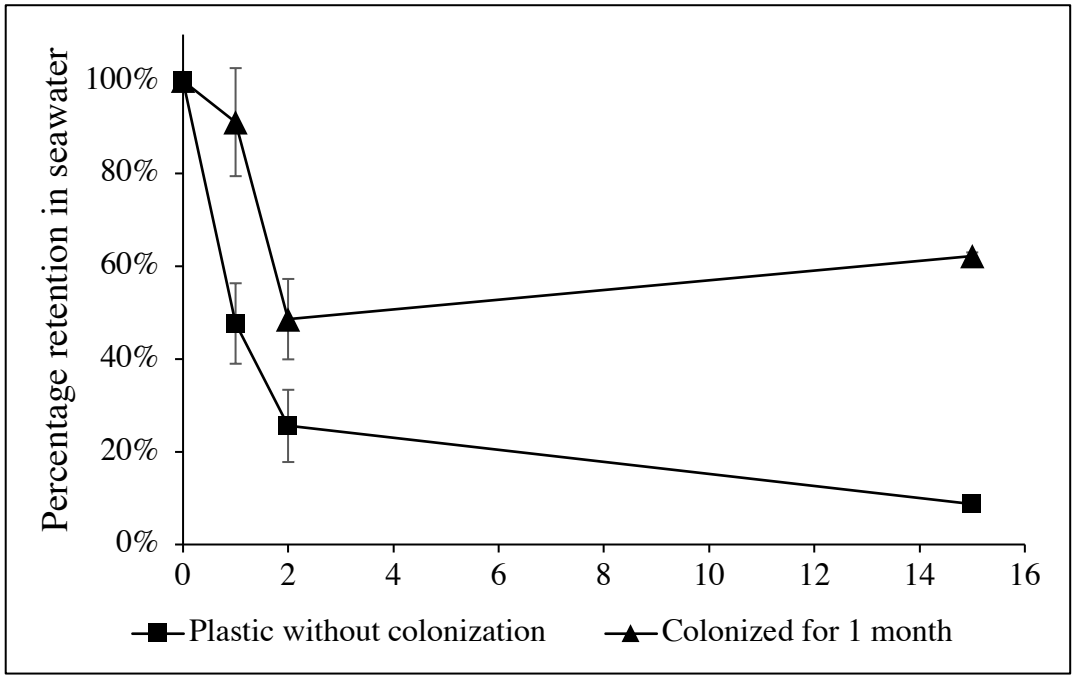



Figure 3. Percentage of retention in seawater for polystyrene particles of 50-100 µm diameter without or with their mature biofilm (1 month incubation in natural seawater) during the first 15 hours of incubation in 2L aquariums with a bubbling flow of 35 ml $sec^{-1}$.

The number of suspended microplastic in seawater became stable after 2 hours (~ 52%) for the microplastic together with their mature biofilm, the bigger size of microplastic going to the bottom of the container. In the same conditions, the suspended pristine microplastic continuously decreasing until the 15 hours experiment (~ 9%) and later (data not shown). As the density of polystyrene (1.06 g $cm^{-3}$) is higher than seawater (1.02 g $cm^{-3}$), what we suspected

is that the biofilm could decrease the polystyrene microplastic density (= increase its buoyancy) in seawater. The impact of biofilm on microplastic behavior were also suggested by Rummel *et al* (2017). By this test, there are several implications. Firstly, almost all the current studies use the pristine microplastic in the toxicity test. Because we found that the biofilm changes the density of microplastic, we underline here that care should be taken and pre-tests should be done in order to adjust the experimental design to the number of particles that may be in contact with the tested animals. For example, smaller microplastic could increase the number of suspended particles in seawater but have less representability of the diversity of plastic sizes found in the natural environment. Secondly, there is also an implication for the modelisation of the quantity of microplastic in the sea surface, in the water column and in the sediment, where the impact of the biofilm on plastic buoyancy should be better considered.

Moreover, we observed that the mature biofilm may serve as a source of nutrient for amphioxus, a parameter that has never been taken into consideration by previous studies. Other studies underlined the importance also of the metal or organic pollutants that may have a role on the toxicity of plastics to marine organisms after ingestion (Browne et al., 2013; Hodson et al., 2017; Dilkes-Hoffman et al., 2019). But none of them take into consideration the nutrient input by the mature biofilm that colonize the plastics.

Finally, we observed a transfer between the bacteria growing on plastic to the microbiome of amphioxus. This transfer may have positive or negative effect depending on the composition of the plastisphere. It has been noted for example that until 24% of the plastisphere may be composed of *Vibrio sp.*, which may be potential pathogenic species (Zettler et al. 2013, Dussud et al. 2018). Such results underline again the importance of taking into account the plastisphere when running toxicity tests on plastic pollution.

*Polystyrene microplastics are not toxic to amphioxus?*

During the toxicity test, no obvious toxic effects were found on polystyrene microplastics (~ 63 µm), after testing a broad set of tests on the immune system, oxidative stress and apoptosis. Previous study has shown that the toxicity effect is size dependent for rotifer *Brachionus koreanus* (Jeong et al., 2016), and nematode *Caenorhabditis elegans* (Lei et al., 2018), in which could be also applicable on amphioxus. We are not sure if microplastic smaller than 50 µm may have a toxic effect on amphioxus.

Previous studies have shown that the pristine polystyrene microplastic could induce the microbiota dysbiosis from the gut contents from the zebrafish and mice (Jin et al., 2018; 2019). As far as we know, this is the first test on the mucosal gut microbiota, but without any significant modification. One consideration is the bacterial taxa transfer from microplastics to the amphioxus, It might be not the abundance-dependent of taxa from plastisphere. For instance, the one taxon of *Sphingobium sp.* was abundant on plastisphere with the average abundance of 4.27%, while this taxon was not found on the gut microbiota after microplastic exposure on amphioxus. On the other hand, the taxon of *Staphylococcus sp.* (~0.02% on plastisphere) was found to be transferred to the gut microbiota of amphioxus. The bacterial transfer could be strongly selected by the host and its health condition. Even though we observed the bacterial transfer, we cannot answer the question "Are microplastics a vector of pathogens for the marine organism?". We could speculate the possibilities in different situation: it could be more harmful if the host is injured on the intestine, which were observed in previous study (Pedà et al., 2016). On the other hand, if the host is healthy, and the microplastic could be egested efficiently, it would be less harmful for the host.

*Reconsideration of experiment setup*

In future, if there are similar experiment to be carried out, I will suggest to take microplastic around 30 μm to make all the microplastics suspended in the seawater. In the meantime, the microplastic size is big enough to form and investigate the effect of the biofilm. Generally, the primers were good in this study, it provided the information both for prokaryotes and also on eukaryotes, while in future, I would suggest to take the prokaryotes and eukaryotes specific primers to get more sequencing depth to have more convinced results for the bacterial transfer.

### 1.2.3 The role of plastisphere on plastic biodegradation

*A multidiscipline approach for the estimate the degradation behavior in marine environments*

To provide the natural inocula, the microbeads were immersed in the natural circulating seawater for two months to ensure the formation of a stable and mature biofilm, which overcome the shortcomings underlined by Harrison, such as preselected strain and prolonged inocula storage time (Harrison et al., 2018a). Traditional standard test generally utilizes the respirometry method only to follow plastic biodegradation (ISO 18830, 2016; ISO 19679, 2016). To better understand the 4 biodegradation steps (biodeterioration, biofragmentation, assimilation and mineralization), a multidisciplinary approach is indispensable. The

granulometry and scanning electron microscopy and weight loss could give the insights for the biodeterioration step, molecular modification and Fourier transform infrared (FTIR) spectroscopy and mass spectrometry give the insight for the biofragmentation step, in terms on the functional group change and oligomers production. The 1H NMR spectroscopy give the insights for the bioassimilation step, and oxygen consumption finally measure the mineralization step.

Congruent results showed the PCL, PHBV, rice seeds and apricot kernel were experienced the 4 mentioned biodegradation processes. While for PE, PMMA and PLA, the biodegradation processes could not be detected.

The PE material could be not representative for the one found from natural environment, where the surface 100 µm were generally oxidized (ter Halle et al., 2017). Here, we did not employ artificial ageing that increase the oxidative process of PE to be closer to the environmental conditions, a parameter that may be considered for future studies.

Conventionally, the measurement of respirometry was conducted by the principle of atmosphere pression reduction due to the oxygen consumption (Sashiwa et al., 2018), or by the titrations on the produced $CO_2$ (Deroiné et al., 2015). These classical techniques are time consuming and are not suitable for large-scale experiment. Here, we underlined the high potential of using oxygen sensor that permit the continuous measurement of $O_2$ concentration during short or long-term experiment. Relative short time is required for the measurement, it is easy to handle, so that it could be easily applied for future biodegradation tests, especially for big experiment setup.

*Reconsideration of experiment setup*

During this assay, the oxygen sensors were placed in the liquid phase in the vials, in the future, I will suggest to put the sensors in the air phase to have more accurate measurement.

## 2. Perspective

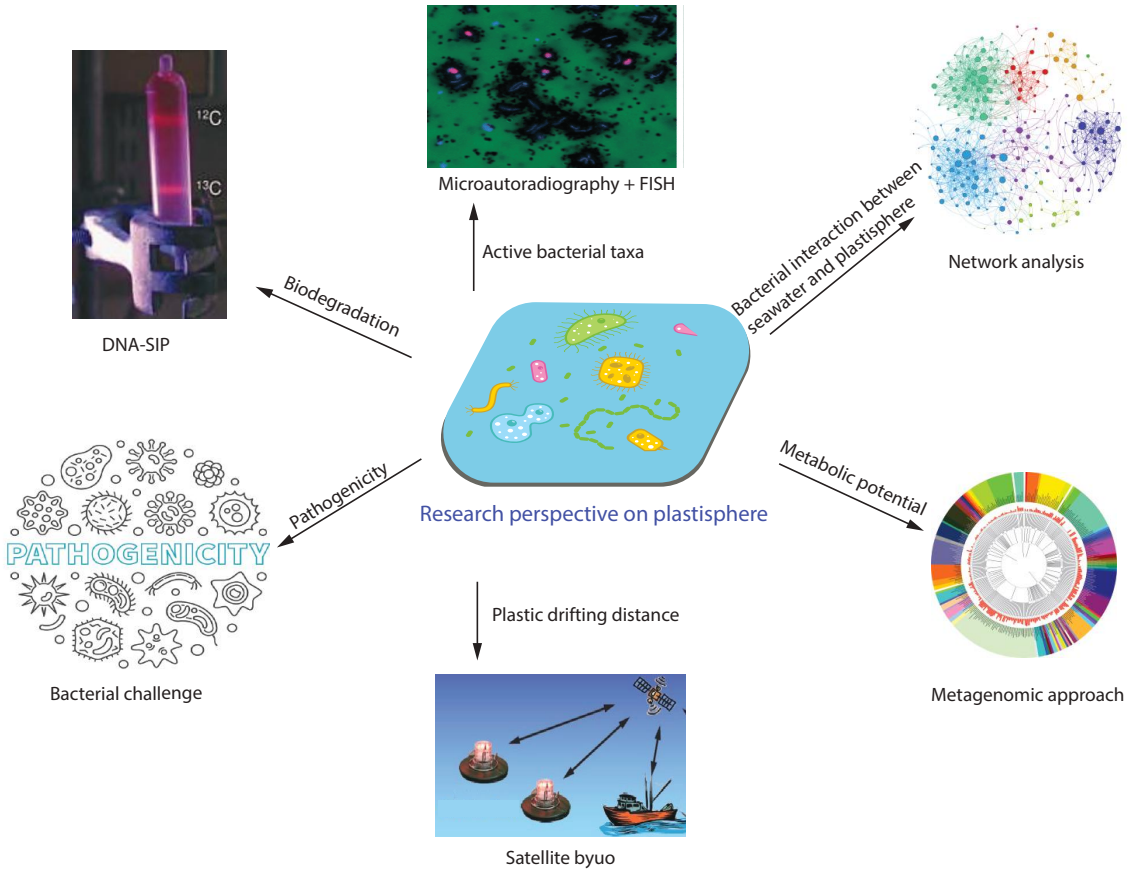



Figure 4. Proposed future research questions and solutions on plastisphere.

During this thesis, microplastic ecotoxicity was investigated on different aspects. The bacterial plastisphere were characterized for two distinct polymers (PE and PLA), and the factors driving the formation of bacterial plastisphere (mainly environmental change and polymer type) were explored and improved (Chapter 2). Limited toxic effect was found on polystyrene microplastics (~63 µm) on amphioxus, with positive effect of the plastisphere shown as nutrient supplier for the host (Chapter 3). Biodegradation on different polymers were compared, and potential substitutes (PHBV, PCL, rice seeds and apricot kernel) for the conventional microbeads were proposed (Chapter 4).

As the new emerged concerns on microplastics, there are still more works to answer the question mentioned in the introduction. Here, I pointed some aspects to be considered for future studies in these research fields (Figure 4).

First of all, to better understand the contribution of plastic in the biogeochemical cycle, it would be necessary to perform measurements of primary production together with heterotrophic bacterial activity and respirometry measurements in order to test the net carbon increase or decrease on plastisphere, and make the comparison to the surrounding seawater. A complex community with large diversity has been observed on plastic, including classical autotrophs (such as Cyanobacteria) and heterotrophs (Gammaproteobacteria, Alteromonas). Combined with fluorescence in situ hybridization (FISH), the current MAR-FISH approach could also provide excellent information on activity and identity at the single-cell level in complex environment (Hesselsoe et al., 2005). A large fraction of bacteria in the ocean are inactive, so it is interesting to detect the active bacterial taxa and bacterial growth efficiency on plastisphere.

Secondly, for now, only one metagenomic study was conducted on plastisphere (Bryant et al., 2016). The functional role of the plastisphere remained poorly investigated, as mentioned by the rare use of activity measurements of the plastisphere (Dussud et al. 2018a and the studies presented here). The metagenomic and metatranscriptomic together with activity tests would need to be applied on the future studies to investigated the metabolic potential and also the metabolic process on plastisphere. It will provide important information on different elemental cycle process, such as carbon, sulphur, phosphate, iron etc.

Thirdly, the observation of plastisphere by microscopy showed more scattered distribution that previously shown by culture-based approaches (Chapter 2). The formation of microcolony and macrocolony on plastics could be different from the one proposed in the canonical way (details explained in the introduction). Even if several studies underlined the difference between the plastisphere to the surrounding free-living or organic particle-attached bacteria, there are no studies on the interaction between these fractions that appeared to be distinct but not interacting. Previous study has showed that residence time of particle-attached bacteria is 3 hours, and attachment and detachment on plastic may play a crucial role in the interaction between the plastisphere and its environment, that appeared in our study in shaping the plastisphere composition and activity. Network analysis on 16S rRNA- or metagenomic-based data would be one option to handle this question.

Fourthly, it has been hypothesized that plastic could be the vector for transporting microorganisms for a long distance in the ocean. While by now, no test was conducted on how long distance the bacterial plastisphere could be traveled in the ocean. It could be tested in future in field or laboratory simulated condition. For instance, the drifting buoys with satellite positioning could be one of the options.

Fifthly, the potential pathogenicity from plastisphere could be further tested, during this PhD thesis, several months from the first year were used to verify the virulence of four bacterial inocula isolated from moribund amphioxus (*Vibrio splendidus, Vibrio harveyi, Vibrio alginolyticus* and *Amphritea ceti*) under different stress treatments on the hosts (heat shock and starvation), while the experiments cannot be reproduced. There might be the methodology constrain, further tests are required to conclude that pathogenicity of pathogen from plastisphere.

Sixthly, no direct proof of biodegradation is given by classical tests. Our laboratory has developed the capability to use $^{13}C$-labeled polymers to evaluate (i) the part of the plastisphere that is directly involved in the biodegradation and (ii) the rate of biodegradation in natural conditions. This implies the use of new technologies with the combination of organic chemistry (done in collaboration with ICCF laboratory) and DNA-stable isotope probing (DNA-SIP) coupled with metagenomic analysis. Such approach is time consuming and expensive, but the labeling of the polymers constitute the only option so far to be able to give direct answer to 'who is doing what?' and to better understand the biodegradation processes performed by the complex natural plastisphere.

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# *Annexes :*

**Detail information of annex 1-3 were documented in the PhD thesis of *"Ecotoxicologie microbienne des plastiques en mer : Colonisation et biodégradation par la plastisphère" from Justine Jacquin in the supervison of Jean-François Ghiglione***

## *Annex 1: A new strain capable of synthesizing and degrading PHBV using atypical metabolic pathways*

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***Abstract***

Plastics from petrochemicals represent a major problem, due to their low biodegradability and their accumulation in many environments. PHAs presented as biodegradable seems to be a good alternative for this issue environmental. PHBV is a PHA with strong commercial appeal for its good ability to replace conventional plastic with conventional plastic in many areas. In this study we have identified a strain belonging to *Alteromonas* sp. capable of synthesizing and degrading. This strain was isolated from a consortium of bacteria that developed on PHBV as only carbon source. Different parameters such as degradation halo on an agar plate, oxygen consumption, bacterial production, weight loss and scanning electron microscopy were performed to confirm the ability of this strain to degrade PHBV.. Analysis of the complete genome of the strain revealed three external depolymerases explaining this ability to degrade PHBV. In addition, genome analysis revealed two pathways of PHA synthesis, allowing this genome to synthetize $PHA_{-SCL}$ and $PHA_{-MCL}$. Contrary to what is usually observed, here the genes involved in the biosynthesis of $PHA\_{SCL}$ are not organized in operon. Experimental validations have to be carried out to confirm that *Alteromonas* sp. possess atypical pathways of degradation and synthesis of PHAs.

## ***Annex 2: Marine plastisphere activity and diversity during successive colonization and biodegradation phases of various composition of plastic sticks***

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***Abstract:***

The European parliament recently approved a new law banning single-use plastic items in 2021 such as plates, cutlery, straws, cotton buds sticks and plastic balloon sticks. Transition to bioeconomy include the substitution of these banned products by biodegradable polymers. Several polymers such as PLA, PBAT, PBS, PHBV, Bioplast, mater-Bi could be good candidates to substitute for conventional plastics sticks. However, the biodegradability of this polymers need to be test following a multidisciplinary approach in marine condition. First, we followed a 40 days colonisation of sticks made of the 6 putative biodegradable polymer types compared to controls made of non-biodegradable PP or biodegradable cellulose. Clear changes in bacterial diversity (16S rRNA Illumina sequencing) and heterotrophic activity ($^3$H-Leucine incorporation) showed classical succession of primo-colonisation, growth and maturation of the biofilm. Second, biodegradability of the polymers was tested by transferring the sticks along with their mature biofilm for another 94 days in strict diet condition with the polymers as sole carbon source. The drastic decrease of bacterial activity on PP, PLA and PBS suggested no bacterial attack of these polymers under our experimental conditions, whereas bacterial activity in PBAT, Bioplast, mater-Bi and PHBV presented similar activities as cellulose. Different trends were observed in term of bacterial diversity for biodegradable *versus* non-biodegradable polymers. For example, rapid changes were observed for the biodegradable PHA, with *Marinobacter* sp.*, Lewinella* sp. and *Alteromonas sp.* becoming abundant and remaining stable during the three months of incubation in minimum medium, whereas the mature biofilm on PP remained stable during the same period. This original study underlined the importance of microbial ecotoxicology when looking for biodegradable substitutes of conventional plastic products.

## ***Annex 3*: Global diversity and core microbiome of the plastisphere compared to organic-particle attached and free-living planktonic lifestyles from the Tara Oceans expeditions in the Mediterranean Sea and in the North Pacific gyre.**

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***Abstract:***

The North Pacific and the Mediterranean are two areas known to be heavily polluted by plastic debris. Plastics thus provide a habitat distinct from the surrounding marine environment. A previous study carried out in the Mediterranean showed a clear partitioning between free life (FL), attached organic particles (PA) and plastic marine debris (PMD). This study made it possible to compare the diversity of the plastisphere found on the different fractions (PMD, PA and FL) between the Pacific and the Mediterranean. The plastics communities at the two sites are different from those found in the surrounding seawater (PA and FL). Microbial communities were mainly separated by region and not by polymer, suggesting that environmental factors are more important than polymer type for the composition of the plastisphere. Cyanobacteria are overrepresented in all PMDs. The PMD fractions were not enriched in hydrocarbonoclasts but potentially in pathogenic microorganisms found in abundance. However, the study of the V4-V5 region of microorganisms does not make it possible to conclude on the effective pathogenicity of OTUs, this deserves further study by another molecular method.

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Annex 4

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# Colonization of Non-biodegradable and Biodegradable Plastics by Marine Microorganisms

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Plastics are ubiquitous in the oceans and constitute suitable matrices for bacterial attachment and growth. Understanding biofouling mechanisms is a key issue to assessing the ecological impacts and fate of plastics in marine environment. In this study, we investigated the different steps of plastic colonization of polyolefin-based plastics, on the first one hand, including conventional low-density polyethylene (PE), additivated PE with pro-oxidant (OXO), and artificially aged OXO (AA-OXO); and of a polyester, poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV), on the other hand. We combined measurements of physical surface properties of polymers (hydrophobicity and roughness) with microbiological characterization of the biofilm (cell counts, taxonomic composition, and heterotrophic activity) using a wide range of techniques, with some of them used for the first time on plastics. Our experimental setup using aquariums with natural circulating seawater during 6 weeks allowed us to characterize the successive phases of primo-colonization, growing, and maturation of the biofilms. We highlighted different trends between polymer types with distinct surface properties and composition, the biodegradable AA-OXO and PHBV presenting higher colonization by active and specific bacteria compared to non-biodegradable polymers (PE and OXO). Succession of bacterial population occurred during the three colonization phases, with hydrocarbonoclastic bacteria being highly abundant on all plastic types. This study brings original data that provide new insights on the colonization of non-biodegradable and biodegradable polymers by marine microorganisms.

**Keywords: plastic pollution, biofouling, microbial ecotoxicology, plastisphere, biodegradable plastics**

## INTRODUCTION

Within a few decades, plastic has become the biggest form of pollution in the world's oceans (80% of marine litter consists of plastic) due to its very slow degradability and the growing accumulation of human waste products (Gewert et al., 2015). When released into the environment, plastic litter is fragmented by both physical and chemical processes into small pieces (<5 mm), commonly

referred to as "microplastics" (MPs) (Barnes et al., 2009). MPs represent more than 90% of the total counts of plastic debris at the sea surface (Eriksen et al., 2014).

At sea, plastics are almost immediately coated by inorganic and organic matter (so called the "conditioning film"), which is then rapidly colonized by microorganisms that form a biofilm on their surfaces (Loeb and Neihof, 1975; Cooksey and Wigglesworth-Cooksey, 1995). Bacterial biofilms are defined as surface-associated bacterial communities which are embedded within an exopolymeric substance matrix (EPS) (Costerton et al., 1995). These natural assemblages act as a form of protection, nutritive resource, offer metabolic cooperativity, and an increase in the possibility of gene transfer among cells (Davey and O'toole, 2000). The successive phases of biofilm formations are well described within marine waters on artificial (glass, acryl, and steel) or natural surfaces (rocks and algae) (Dang and Lovell, 2000; Salta et al., 2013). First, the "primo-colonization" describes the occupation of the surface by pioneer bacteria through reversible attachment, where they interact with the conditioning film and form the first layer of the initial biofilm. Second, the "growth phase" promotes irreversible attachment by active mechanisms such as the formation of pili, adhesion proteins and EPS produced by secondary species, which induce modifications in the properties of the substratum. Third, the "maturation phase" occurs through diverse, competitive or synergistic interactions between cells, with either further recruitment or loss of species (Lorite et al., 2011).

Very few studies have so far described the formation of biofilms on plastics in marine environments. Early stage processes were followed on polyethylene (PE)-based plastic bags or MPs during 3 weeks in seawater (Lobelle and Cunliffe, 2011) and in sediments (Harrison et al., 2014). Two studies are available on longer-term biofilm formation on the surface of PE or PE terephthalate (PET) in marine environment, which were carried out over a 6-month period (Webb et al., 2009; De Tender et al., 2017). Only one study has so far compared biofilm formation on PE with that observed on so-called "biodegradable" plastics made of starch-based biopolymer-PET blend (Mater-Bi N°014), conducted during 1 month in marine environment (Eich et al., 2015). These studies were mostly based on scanning electron microscopy (SEM) observations and taxonomic identification, but none of them focused on bacterial abundance and activity, meaning that populations and community dynamics in these biofilms remains largely unknown. Moreover, the formation of a biofilm was depicted as strongly dependent on substrate properties including hydrophobicity/hydrophilicity, structure, and roughness (Lorite et al., 2011), which were never taken into account in studies exploring marine environment.

Polyethylene dominates the composition of plastic waste at sea surface, followed by polypropylene (PP) and polystyrene (PS) (Auta et al., 2017). The stable aliphatic chains in PE make it a very recalcitrant material (Tokiwa et al., 2009). Within the frame of sustainable development, a wide range of potentially biodegradable plastics were developed and classified into two major groups depending on the mode of biodegradation pathway: "OXO-biodegradable" and "hydro-biodegradable" (Vázquez-Morillas et al., 2016). The former are polyolefin-based polymers (generally PE) with pro-oxidant additives (OXO; for OXO-degradable polymer). In case of release in the environment, the additive accelerates abiotic oxidation process by heat and/or UV light, a phenomenon that can be simulated by artificial aging of the OXO (AA-OXO, for artificially aged OXO). If the initial formulation of OXO is recalcitrant to biodegradation, the oxidized AA-OXO can be further biodegraded by oxidative mechanisms (Koutny et al., 2006; Eyheraguibel et al., 2017). Several studies on OXO pre-oxidized films showed 50 to 80% mineralization under half to one and a half year of incubation (Jakubowicz, 2003; Chiellini et al., 2007) or between 12 and 24% mineralization after 90 days of incubation (Ojeda et al., 2009 and Yashchuk et al., 2012). Hydro-biodegradable plastics are based on polymers that can be biodegraded by hydrolytic mechanisms (Nampoothiri et al., 2010). They include cellulose, starch and more generally polyesters, such as polyhydroxyalkanoates (PHA). Because PHA are polyesters made by bacteria for intracellular storage of carbon and energy, they received considerable attention as promising biodegradable polymers to substitute for traditional plastics, with mechanical properties similar to various synthetic thermoplastics (Corre et al., 2012; Elain et al., 2015, 2016). Various bacteria were shown to degrade AA-OXO or PHA in different conditions (Tokiwa and Calabia, 2004; Sudhakar et al., 2008; Ammala et al., 2011).

In this study, we characterized the biofilm colonization phases on PE, OXO-degradable polymer with (AA-OXO) or without (OXO) artificial-aging, and poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) as PHA representative. Each polymer type was separately incubated and its evolution monitored during 6 weeks in natural seawater from Banyuls Bay (NW Mediterranean Sea). The dynamics of bacterial biofilms was described in terms of changes in abundance, diversity and heterotrophic activity, together with changes in polymer surface physical properties (contact angle and roughness).

## MATERIALS AND METHODS

### Polymer Samples Preparation and Design of the Incubation Experiments

In this study, we used four types of polymer: PE corresponded to commercially available commodity film grade low-density PE resin Borealis FA6224, which had the following characteristics: density = 0.922 g $cm^{-1}$, average molecular weight $\overline{M}_W \approx 97{,}000$ kg $mol^{-1}$, with a melt-flow index (MFI) = 2.1 g/10 min (190°C, 2.16 kg). OXO was made of the same PE formulation but additivated with $D_2W$ OXO based on manganese and iron (provided by Symphony Environmental Ltd., United Kingdom). AA-OXO was made of same OXO formulation but thermally aged for 180 days in an aerated oven at 70°C, which resulted in fragmentation, loss in mechanical properties and increase in oxidation level as depicted by absorbance increase at 1,712 $cm^{-1}$ determined by micro-FTIR spectroscopy reaching more than x/100 (where x was the film thickness). The level of x/100 was previously demonstrated as a prerequisite for biodegradability of OXO, as already demonstrated for *Rhodococcus rhodochrous* and described in

the French Agreement Association Française de Normalisation (AFNOR) (2012) PE, OXO, and AA-OXO were extruded at 180°C using a laboratory scale Rondol linear 18 mm blown film line.

Poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (provided by University of South Brittany, France) had the following characteristics: density = 1.25 g $cm^{-1}$, average molecular weight $\overline{M}_w \approx 400$ kg $mol^{-1}$, with a MFI = 3.6 g/10 min (210°C, 2.16 kg). This grade has been comprehensively characterized in a previous paper (Corre et al., 2012). Prior to compression molding, the PHBV pellets were dried over 12 h under vacuum at 60°C to minimize the hydrolytic PHBV degradation during processing and compression molded in a Carver® hydraulic press at 180°C under a pressure of 10 metric tons for 3 min.

The thickness of polymer films was 200 μm for PHBV and 100 μm for PE, OXO and AA-OXO. Each sample was a circular piece of 9 mm diameter (area = 63.6 $mm^2$), except for AA-OXO that was constituted of irregular fragments of mean area of 13.9 ± 4.8 $mm^2$ after artificial aging. Each polymer sample was cleaned with 70% ethanol and washed with sterile seawater (SSW) before incubation.

We used five identical aquariums consisting in trays with a 1.8 L capacity (Sodispan, Spain), in which 1.5 L seawater was continually renewed by direct pumping at 4 m depth in Banyuls bay, close to the SOLA observatory station (NW Mediterranean Sea, France). A flow rate of 50 mL $min^{-1}$ was chosen to ensure a sufficient renewal of natural bacteria (every 30 min) and an homogeneous distribution of the plastic pieces in the aquariums during the entire experiment. Each aquarium contained polymer pieces of one of the composition (PE, OXO, AA-OXO, and PHBV), except one aquarium used as control, containing only circulating seawater (hereafter called "control aquarium"). Pieces of each polymer type were put in the 18th of January, 2016 and sampled after 7, 15, 22, 30, and 45 days. Aquariums were kept in the dark to avoid UV-driven degradation of the polymers. Throughout the experiment, seawater temperature (between 12.5 and 13.5°C) and salinity (38.5) in the aquariums were similar to seawater from Banyuls bay.

## Atomic Force Microscopy

Atomic force microscopy (AFM) was performed on each sample to get resolved picture of the colonization and accurate insight of the surface state of the polymer. At each sampling time, one piece of each polymer was rinsed with SSW and fixed for at least 1 h at 4°C with 1% (v/v) glutaraldehyde (final concentration) before freezing. At least three 40 × 40 $\mu m^2$ areas images were acquired for each sample using a Nanoscope V (Bruker instruments, Madisson, WI, United States) in dynamic mode (Binnig et al., 1986) and standard silicon probes (Bruker, TESP-V2). Root mean square (RMS) roughness of the polymer surface were measured on height images of 40 × 40 $\mu m^2$ with Gwyddion software, using masks to remove remaining bacterial cells and other organic deposits from the measurements. Boxes of gradual sizes (10, 20, 30, and 40 μm) were used to estimate RMS standard deviation and to check the dependence of the RMS on the lateral size of the picture. On every sample, a plateau was reached at 30 μm, which validates the use of RMS measured on 40 μm size to characterize the surface state of the sample.

Since surface state characterization is likely to be affected by the development of a biofilm and the deposit of EPS, pretreatments by sonication and rinsing with SSW were performed on some samples to ensure the access to polymer surface. Experiments performed on the same area before and after sonication showed no change in roughness values (data not shown). Comparison with masking method described above showed that no difference was measured within the experimental uncertainties.

## Contact Angle Measurement

Contact angles (SSW/air/polymer) were measured on each polymer in its initial state (before incubation in seawater) and after 7 days of immersion in SSW, using a profile analysis tensiometer (PAT1M, Sinterface Technologies, Berlin, Germany). We did not measured contact angles after 7 days since surface hydrophobicity was too modified by the conditioning film, as previously observed (Lorite et al., 2011). A series of profiles was acquired for three different droplets of millimetric diameters on each sample during successive advancing and receding stages. All series were analyzed using ImageJ software (version 1.46r, Wayne Rasband, National Institutes of Health, United States) to get the receding and advancing angle in each sample.

## Epifluorescence Microscopy

At each sampling time, one piece of each polymer was rinsed with SSW and fixed for at least 1 h at 4°C with 1% (v/v) glutaraldehyde (final concentration) before freezing. Epifluorescence microscopy observations were done using an Olympus AX-70 PROVIS after 4′,6-diamidino-2-phenylindole (DAPI) staining according to Clays-Josserand et al. (1999). Pictures were taken on 10 fields of each polymer type (Microbe Counter software). The surface areas covered by only bacterial cells and by biofilm (cells + EPS) were determined using the ImageJ software (version 1.46r, Wayne Rasband, National Institutes of Health, United States).

## Flow Cytometry

Three pieces of each polymer were sampled at each sampling time with sterilized forceps and rinsed with SSW. A cell detachment pre-treatment was applied using 1 mmol $L^{-1}$ pyrophosphate during 30 min at room temperature in the dark, followed by a sonication step (3 × 5 s, 40 kHz, 30% amplitude, sterilized probe Branson SLPe). The efficiency of cell-detachment was verified by epifluorescence microscopy before and after cell-detachment, as well as comparison between flow cytometry and epifluorescence microscopy cell counts. The cell detachment pre-treatment was optimized by a set of tests on each polymer substrates. Various mechanical or chemical pre-treatments were tested alone or combined: tetrasodium pyrophosphate (1 and 10 mM); sonication step including a combination of vortex and sonication bath or the use of a sonication probe alone (Branson SLPe, see above); or addition of enzymes mix (Lipase 48 units, beta-galactosidase 10 units, and alpha-glucosidase 0.8 units; Sigma Aldrich). A total of 12 conditions were tested. The chosen protocol was based on a combination of tetrasodium

pyrophosphate (1 mM) and sonication probe, which showed the best correspondence between cell counts obtained by flow cytometry and epifluorescence microscopy for the same sample, the latest being 1- to 5-fold higher values than the first. After cell detachment, samples were fixed for at least 1 h at 4°C with 1% (v/v) glutaraldehyde (final concentration) and frozen before further analysis. In parallel, 3×1 mL of seawater (polycarbonate, 47 mm diameter, Whatman) from the control aquarium were also fixed using the same procedure. A 500-μL subsample of the detached cells from plastic or from control seawater was mixed with the nucleic acid dye SYBR Green I (final concentration 0.05% [v/v], Sigma Aldrich) for 15 min, at room temperature and in the dark. Cell counts were performed with a FACSCanto II flow cytometer (BD Bioscience, San Jose, CA, United States) equipped with a blue laser (488-nm, air-cooled, 20-mW solid state), as previously described (Severin et al., 2014).

## Heterotrophic Bacterial Production

Bacterial production (BP) was measured on each polymer type at each sampling time by $^{3}$H-leucine incorporation into proteins, using a modified protocol from Van Wambeke et al. (2009). Briefly, the same cell detachment pre-treatment protocol as for flow cytometry (see above) was used, based on pyrophosphate together with sonication procedure. This pre-treatment improved the BP signal by a factor from 1.0 to 5.7 compared to control condition with no pre-treatment. This pre-treatment gave also the best results when compared to the other conditions tested (including the combination of vortex and sonication bath and the addition of mix of enzymes, together or alone with the other treatments, see above in the flow cytometry section). Immediately after cell-detachment, $^{3}$H-leucine (specific activity 112 Ci mmol$^{-1}$; Perkin Elmer) was added at a final concentration of 1 nmol L$^{-1}$ (completed with cold leucine to 150 nmol L$^{-1}$) in triplicate for each sample, which consisted of 1.5 mL of seawater sterilized water containing the piece of plastic together with the detached cells. For seawater samples from the control aquarium, $^{3}$H-leucine was added at a final concentration of 4.3 nmol L$^{-1}$ to 1.5 mL of control seawater. All samples were incubated in the dark at *in situ* temperature for 3 h. We used the empirical conversion factor of 1.55 ng C pmol$^{-1}$ of incorporated leucine to calculate BP (Simon and Azam, 1989). Cell-specific activities (CSA) were calculated as the ratio between BP and cell counts obtained by flow cytometry.

## DNA Extraction, PCR, and Sequencing

Four replicates of each polymer type were sampled at all sampling times, except for day 15, and stored at −80°C until analysis. In parallel, 1 L seawater was sampled in the control aquarium, successively filtered onto 3 and 0.2 μm pore size polycarbonate filters (47 mm diameter, Nucleopore) and filters were stored at −80°C until analysis. DNA extraction was performed on polymers and filters using a classical phenol-chloroform method for seawater samples (Ghiglione et al., 1999) and a slight modification of the method for polymer samples (Debeljak et al., 2017).

Briefly, the same cell detachment pre-treatment was used as for flow cytometry and BP (see above) before chemical and enzymatic cell lysis (1 mg mL$^{-1}$ lysozyme at 37°C for 45 min followed by 0.2 mg mL$^{-1}$ proteinase K and 1% SDS at 50°C for 1 h). The pre-treatment improved cell lysis since no cells were visible by epifluorescence microscopy after this stage. The molecular size and the purity of the DNA extracts were analyzed using agarose gel electrophoresis (1%) and DNA was quantified by spectrophotometry (GeneQuant II, Pharmacia Biotech).

PCR amplification of the 16S V3–V5 region was done using 515F-Y and 926R primers, particularly well-suited for marine samples according to Parada et al. (2016). Sequencing was performed on Illumina MiSeq by Research and Testing Laboratories (Lubbock, TX, United States). Raw FASTA files were deposited at GenBank under the accession number SRP116996. Sequences were analyzed using Mothur pipeline (Schloss et al., 2009). Paired raw reads were assembled, sequences with homopolymers (>8) and ambiguities were removed and the remaining sequences were aligned using SILVA database. Sequences were trimmed to a same length and a chimera were removed (uchime command). Sequences were classified and operational taxonomic units (OTUs) were defined as clusters sharing 97% sequence identity. Only bacteria were treated in this study, due to the small number of archaeal reads. Chloroplast, mitochondrial and eukaryotic sequences were removed. Bacterial sequences were randomly resampled in the OTU file to enable comparison between samples, by normalizing the number of sequences between samples to the sample with the fewest sequences ($n$ = 6,186) using Mothur v.1.38.1. All further analyses were performed on randomly resampled OTU table.

## Statistical Analysis

Alpha-diversity was estimated using the non-parametric, Chao1 species richness estimator from the SPADE software. Simpson, Shannon, and Pielou diversity indexes were obtained using the PRIMER 6 software (PRIMER-E, United Kingdom). Differences between polymers and seawater richness and diversity indexes were tested using a *post-hoc* LSD test after an ANOVA test (Statistica 8.0, Statsoft).

An unweighted-pair group method with arithmetic mean (UPGMA) dendrogram based on Bray–Curtis similarities was used for visualization of beta-diversity. A similarity profile test (SIMPROF, PRIMER 6) was performed on a null hypothesis that a specific sub-cluster can be recreated by permuting the entry species and samples. The significant branch (SIMPROF, $p < 0.05$) was used as a prerequisite for defining bacterial clusters. One-way analysis of similarity (ANOSIM, PRIMER 6) was performed on the same distance matrix to test the null hypothesis that was no difference between bacterial communities of different clusters (Berdjeb et al., 2011). Significant correlations between environmental variables were tracked using Spearman rank pairwise correlations.

# RESULTS

## Polymer Surface Properties

Surface properties of the polymers were derived from AFM data (**Figure 1**). Before incubation in SW, PE, OXO, and AA-OXO

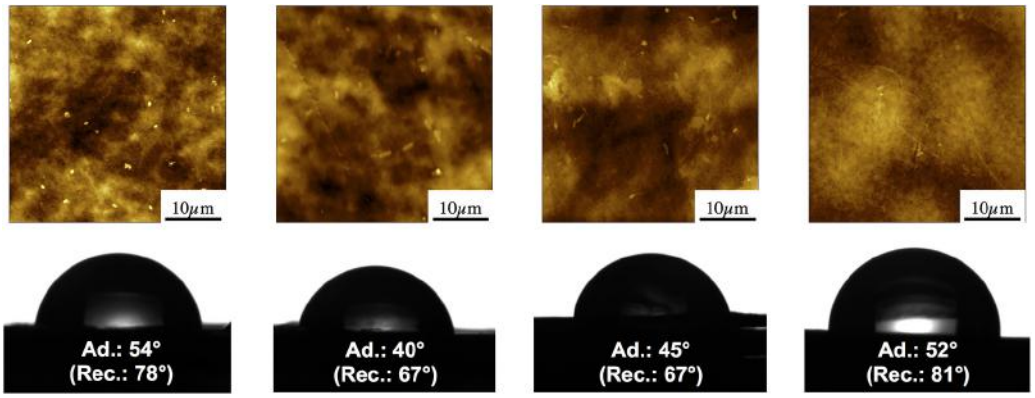


**FIGURE 1 |** Atomic force microscopy images of PE, OXO, AA-OXO, and PHBV polymer surfaces after a 7-day immersion in seawater, showing different amounts of bacterial cells at the end of the primo-colonization stage (*upper image*). Contact angle drops at advancing point of the four polymers after 7 days of immersion in seawater (receding angle in *brackets*) showing different levels of surface hydrophobicity (*lower image*).

presented a rather smooth surface. On the contrary, PHBV showed a rough surface, due to the presence of a spherulitic structure of about 20 μm in diameter. Whereas the first three polymers did not present significant surface modifications with increasing immersion times up to 45 days, the PHBV spherulitic structure went through observable morphological alteration, with clear evidences of swelling and erosion (Supplementary Figure S1).

Root mean square roughness measured on 40 × 40 $\mu m^2$ pictures provided quantitative assessments of surface alterations (**Table 1**). PE, which initially showed the lowest roughness (56 ± 7 nm) at ambient air, did not change significantly for the first 22 days of immersion and slightly increased after 45 days (RMS = 84 ± 9 nm). OXO roughness presented a similar evolution with slightly higher values. AA-OXO roughness remained in the same range as the previous polymers, fluctuating between 63 and 110 nm in the first 22 days. It should be noted that AA-OXO roughness could not be measured at D45 due to a strong bacterial attachment that resisted the washing protocol. PHBV showed the highest initial roughness with 208 ± 21 nm at ambient air. During the incubation period, its value presented large fluctuations over time, with a global increasing trend following important alteration of the initial spherulite structure. The maximum value of RMS was reached after 45 days, where surface erosion (induced most probably by water itself) was clearly visible in AFM micrographs and was then four times higher than that of PE.

Advancing and receding contact angles (SW/air/polymer) were measured on initial dry samples and after 7 days of immersion in SW (**Figure 1** and **Table 1**). Initially, all polymers presented a rather hydrophobic surface with receding and advancing contact angle close to 90°, with PHBV and PE being the most hydrophobic. The addition of polar groups from PE to OXO and AA-OXO explains their lower hydrophobicity. The contact angle hysteresis (difference between receding and advancing contact angle), which is directly related to the roughness or the chemical heterogeneity of a surface, showed higher values for OXO and AA-OXO compared to PE, in agreement with the more homogeneous chemical composition of the latter. PHBV showed a large hysteresis, probably reflecting its structuration in big spherulites, in agreement with AFM observation and roughness measurements (Supplementary Figure S1). After immersion, the decrease in hydrophobicity for all polymers can be connected to surface reconstruction for OXO and AA-OXO, surface reconstruction and water swelling for PHBV and probably adsorption of polar molecules on the surface in the case of PE.

**TABLE 1 |** Physical data for the four plastic types (PE, OXO, AA-OXO, and PHBV) according to immersion time in days (D), including roughness (RMS, in nm), contact angle (CA, receding – advancing, in degree) and carbonyl index (CI).

| | | DO | D7 | D15 | D22 | D30 | D45 |
|---|---|---|---|---|---|---|---|
| PE | RMS | 56 | 49 | | 46 | | 84 |
| | CA | 85–94 | 54–78 | | | | |
| | CI | | 0.74 | 0.48 | 0.39 | 0.56 | 0.57 |
| OXO | RMS | 87 | 122 | | 106 | | 112 |
| | CA | 61–79 | 40–67 | | | | |
| | CI | | 0.49 | 0.5 | 0.39 | 0.47 | 0.85 |
| AA-OXO | RMS | 110 | 63 | | 64 | | ND |
| | CA | 52-76 | 45–67 | | | | |
| PHBV | RMS | 208 | 129 | 322 | 240 | | 358 |
| | CA | 78–99 | 52–81 | | | | |

## Dynamics of Bacterial Cell Counts on Polymers and in Seawater

Epifluorescence microscopy observations were not possible for AA-OXO and PHBV samples, because of strong auto-fluorescence background under UV light for these two polymers. Because our cell detachment pre-treatment showed that flow cytometry approach was possible for all polymer and it slightly underestimated cell counts as compared to epifluorescence microscopy by a factor of 1 to 5, we decided to use the flow cytometry cell counts to provide comparable data obtained with the same technique. Then, epifluorescence microscopy was used

only to confirm the results obtained by flow cytometry and to estimate plastic surface area covered by bacterial cells, when available (only for PE and OXO).

Flow cytometry data highlighted three distinct phases of biofilm formation for all polymer types: primo-colonization, growth, and maturation (**Table 2**). Primo-colonization lasted for the first 7 days following immersion, with cell counts being, respectively, 1.5, 1.6, and 1.3 $\times$ $10^5$ cells $cm^{-2}$ for PE, OXO, and PHBV and 9.3$\times10^5$ cells $cm^{-2}$ for AA-OXO. Cell counts increased on all polymers during the growing phase, but at different rates: after 15 and 22 days, cell counts on PHBV and AA-OXO biofilms were about fivefold more than that on PE and OXO. The stabilization phase was visible after 22 days for PE, OXO, and PHBV, reaching, respectively, 3.7, 6.9, and 16.3 $\times$ $10^5$ cells $cm^{-2}$ at the end of the experiment, whereas cell counts continued to increase for AA-OXO to finally reach 34.1 $\times$ $10^5$ cells $cm^{-2}$.

The three phases were also visually observed by epifluorescence microscopy (**Figure 2**). Primo-colonization was characterized by single cells spreading out homogenously on the surface resulting in cell coverage of 1 and 3% of the PE and OXO surface at day 7, respectively (**Table 2**). Cell abundance increased unevenly during the growing phase, leading to a patchy distribution of cell aggregates on both PE and OXO films, representing, respectively, 6.5 and 10.1% coverage at day 22. Together with an increase in exuded EPS clearly visible on micrographs after day 22, the biofilm coverage on the surface reached 29.2 % and 18.1% after 45 days for PE and OXO, respectively (**Table 2**).

**TABLE 2 |** Biological data for the four plastic types (PE, OXO, AA-OXO, and PHBV) compared to seawater (SW) according to immersion time in days (D), including bacterial cell count (BC, $\times10^5$ cell $mL^{-1}$ for SW or $\times10^5$ cell $cm^{-2}$ for plastic samples), bacterial production (BP, in ngC $L^{-1}$ $h^{-1}$ for SW or ngC $dm^{-2}$ $h^{-1}$ for plastic samples), and bacterial specific activity (SA, $\times10^{-3}$ fgC $cell^{-1}$ $h^{-1}$).

| | | D7 | D15 | D22 | D30 | D45 |
|---|---|---|---|---|---|---|
| SW | BC | 1.16 | 0.89 | 1.71 | 1.15 | 3.07 |
| | | *(0.03)* | *(0.04)* | *(0.11)* | *(0.001)* | *(0.14)* |
| | BP | 9.09 | 10.5 | 16.8 | 21.3 | 41 |
| | | *(0.8)* | *(1.1)* | *(0.7)* | *(2.0)* | *(1.8)* |
| | SA | 0.079 | 0.118 | 0.098 | 0.185 | 0.133 |
| PE | BC | 1.53 | 3.4 | 6.76 | 9.05 | 3.7 |
| | | *(0.34)* | *(0.51)* | *(1.45)* | *(1.23)* | *(1.33)* |
| | BP | 38.5 | 352.3 | 426.7 | 29 | 55.6 |
| | | *(20.4)* | *(114.9)* | *(241.5)* | *(13.5)* | *(36.7)* |
| | SA | 2.52 | 10.37 | 6.33 | 0.32 | 1.50 |
| | cov | 1.00% | 5.1% | 6.5 % | 15.1% | 29.2 % |
| OXO | BC | 1.57 | 3.75 | 5.65 | 4.92 | 6.89 |
| | | *(0.34)* | *(1.29)* | *(1.27)* | *(1.35)* | *(1.40)* |
| | BP | 105.5 | 178.7 | 217.2 | 60.3 | 55.3 |
| | | *(36.2)* | *(4.0)* | *(10.2)* | *(21.4)* | *(12.2)* |
| | SA | 6.74 | 5.02 | 3.85 | 1.23 | 0.80 |
| | cov | 3.4% | 3.4 | 10.1% | 12.3% | 18.1% |
| AA-OXO | BC | 9.25 | 16.1 | 28.4 | | 34.1 |
| | | *(3.50)* | *(4.47)* | *(3.86)* | | *(8.47)* |
| | BP | 145.9 | 1396.9 | 1369.7 | | 131.4 |
| | | *(20.1)* | *(90.0)* | *(193.6)* | | *(7.25)* |
| | SA | 1.58 | 8.67 | 4.82 | | 0.39 |
| PHBV | BC | 1.25 | 15.2 | 15.3 | | 16.3 |
| | | *(0.37)* | *(2.92)* | *(3.94)* | | *(3.61)* |
| | BP | 67 | 1090.4 | 259.4 | | 240.9 |
| | | *(58.4)* | *(513.5)* | *(125.9)* | | *(100.4)* |
| | SA | 5.38 | 7.19 | 1.70 | | 1.47 |

## Dynamics of Bacterial Community Structure and Diversity on Polymers and in Seawater

Next-generation DNA sequencing resulted in 265,998 tags falling into 823 bacterial OTUs at 97% similarity level, after randomly resampling to 6,186 sequences per sample to provide statistical robustness when comparing diversity between samples. The cluster analysis showed a clear dissimilarity (>70%) between seawater controls and polymer samples during the course of the experiment (**Figure 3**). Overall, bacterial community structure on all polymer types showed spectacular changes, first in the diversity of bacteria that colonized the polymers compared with the surrounding seawater, and second in the growing and maturation phases compared to the primo-colonization phase. All polymer types sampled at day 7 clustered together in a group showing low similarity (<25%) with other samples (**Figure 3**). Within this cluster, the PHBV community structure significantly differed (SIMPROF test) from the other polymer types. The temporal dynamics of the bacterial assemblages during the growing and maturation phases differed with the polymer type. PE and OXO biofilms formed distinct, yet close sub-clusters and showed few changes from days 22 to 45. Conversely, both communities from AA-OXO and PHBV presented strong changes during this period (<40% similarity from days 22 to 45 between samples from the same polymer type).

Overall, the observed changes in the diversity indexes (Shannon, Pielou, Chao1, and Simpson; Supplementary Table S1) were related to the polymer type (ANOVA test, $p < 0.05$), but not to incubation time: we could not find any relation between the changes in diversity indexes and the different stages of biofilm formation. The equitability (Pielou) on PE was significantly higher than on AA-OXO, PHBV and seawater (LSD test, $p$-value $< 0.05$) (Supplementary Table S1). The Shannon diversity index was also higher on PE compared to AA-OXO (LSD test, $p$-value $< 0.05$). The Chao1 index ranged from 113 (OXO at day 7) to 322 (PHBV at day 45).

Taxonomic analyses confirmed the specificity of the community structures formed on the polymers compared to seawater, the latter being dominated by Alphaproteobacteria, Flavobacteria, Cyanobacteria, and Actinobacteria throughout the experimentation (**Figure 3**). On all four polymers type, the primo-colonizers belonged to Gammaproteobacteria, which represented between 45 and 75% of the total OTU in each community (**Figure 3**). On PE, OXO and AA-OXO, this group was mainly dominated by *Alcanivorax* sp., *Aestuariicella hydrocarbonica, Alteromonas* sp., and *Thalassolituus* sp. followed by *Marinobacter* sp. and *Maricurvus* (**Figure 4**). On PHBV,

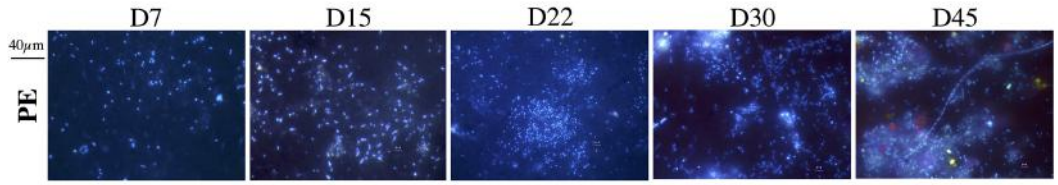


**FIGURE 2 |** Epifluorescence micrographs of DAPI-stained PE plastics after 7, 15, 22, 30, and 45 days of immersion in seawater. Bar: 40 μm.

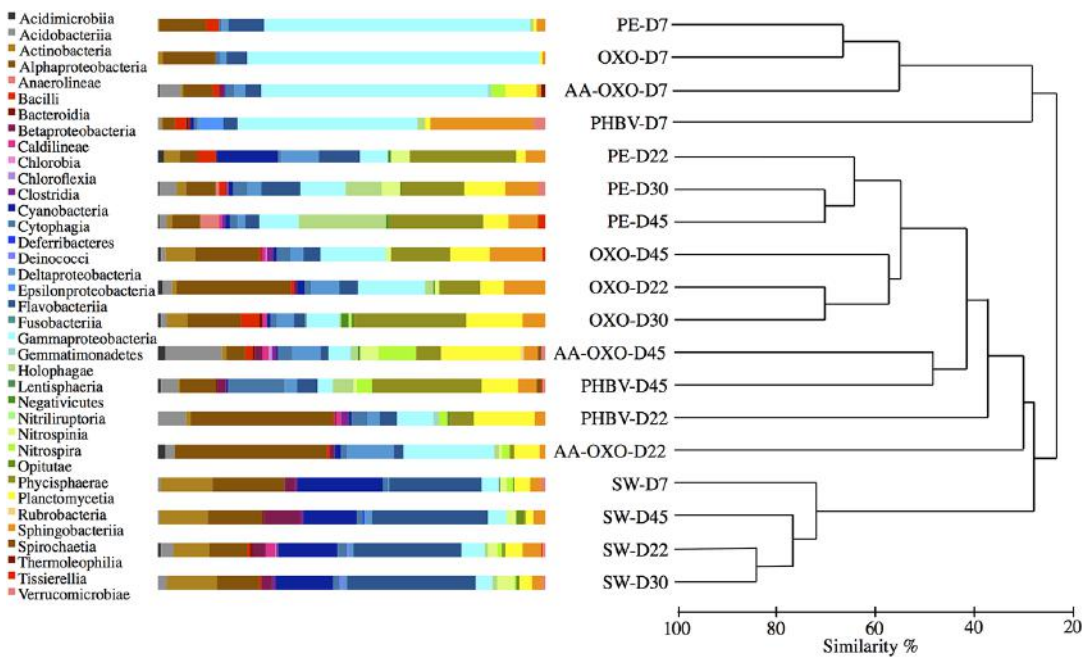


**FIGURE 3 |** Comparison of taxonomic abundances and community structure of bacteria in seawater (SW) and attached in the four plastics (PE, OXO, AA-OXO, and PHBV) according to immersion time in days (D), by cumulative bar charts comparing relative class abundances (*left*) and by UPGMA dendrogram based on Bray–Curtis similarities between sequencing profiles (*right*).

*Neptiniibacter* sp. made up for more than 30% of the community, while this OTU remained undetected on all other polymers.

The growing and maturation phases were characterized by few changes on PE and OXO samples, where *Croceibacter* sp. was the dominant OTU on PE, whereas *Sneathiella glossodoripedis* dominated on OXO (**Figure 4**). Only a significant increase of *Solimonas* sp. occurred during the stabilization phase on OXO. More changes were observed on AA-OXO and PHBV during the growing and maturation phases, with large dissimilarities between sampling time. The OTUs *Lutibacterium anuloederans* and *Pseudospirillum* sp. were found in high amounts on AA-OXO during the growth stage, whereas *Phaeobacter* sp. stand out on PHBV. The majority of OTUs identified at day 22 on AA-OXO and PHBV decreased at day 45, giving way to a higher abundance of unclassified OTUs. During the maturation phase, Gammaproteobacteria decreased and Alphaproteobacteria increased proportionally, with Phycisphaerae, Planctomycetia, and Sphingobacteriia classes in particular whatever the plastic type (**Figure 3**).

## Presence of Putative Hydrocarbonoclastic Bacteria

We identified 34.4% of the total sequences on polymer samples as being putative hydrocarbonoclastic bacteria (HCB), compared to 4.1% in control seawater. Among the most abundant OTUs per polymer sample (>5% of the total OTUs in one sample), we found the HCB *Alcanivorax* sp., *Aestuariicella hydrocarbonica, Marinobacter* sp., *Lutibacterium anuloederans,* and *Neptuniibacter* sp. (**Figure 4**). SIMPER analysis showed that these 5 OTUs explained more than 13% of the dissimilarity between polymers and seawater communities. Overall, HCB were particularly abundant in bacterial communities during the primo-colonization phase on all polymer types (1.7 to 3-fold more HCB were identified on polymers compared to seawater) and generally decreased afterward. *Aestuariicella hydrocarbonica* was found in higher abundance on all polymer types, reaching up to 20 and 24% of sequences in OXO and AA-OXO, respectively. *Alcanivorax* sp. reached similar relative abundances, but was not detected on PHBV, where HCB were instead dominated by

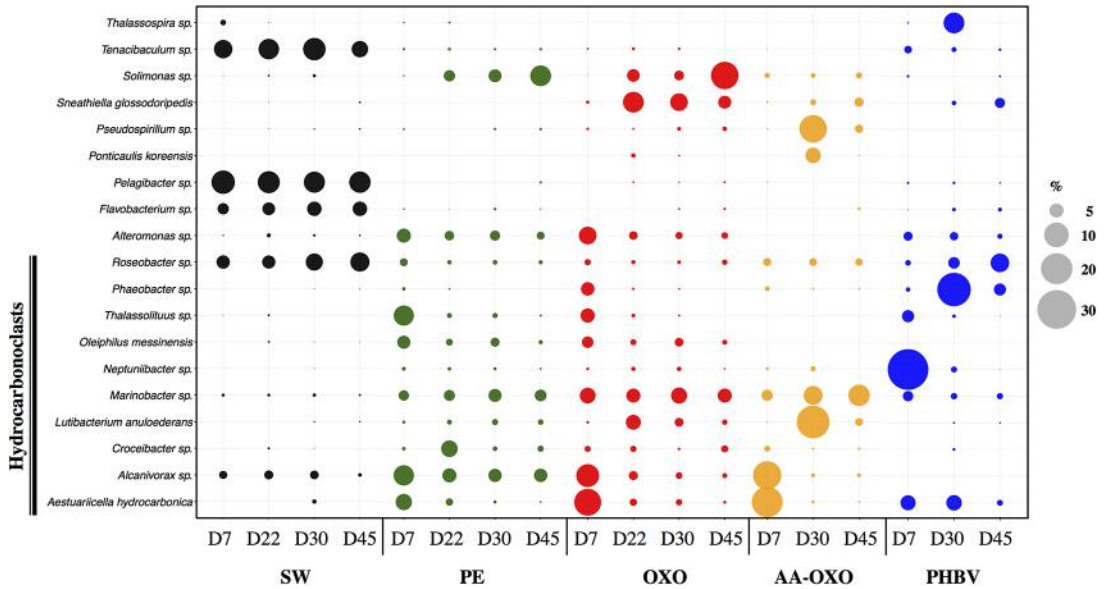


**FIGURE 4 |** Bubble plot showing the relative abundance (%) of the majority OTUs (>5%) in each compared sample between immersion times in days (D) in seawater (SW) and in the four plastic types (PE, OXO, AA-OXO, and PHBV). Putative hydrocarbonoclastic OTUs were *highlighted*.

*Neptuniibacter*. These three OTUs decreased after day 7 and were replaced by another HCB, such as *Marinobacter* on PE, OXO and AA-OXO, and *Lutibacterium anuloederans* on AA-OXO.

## Presence of Putative Pathogenic Bacteria

We identified 23 putative pathogen OTUs in all our samples, which represented <3% (3,817 sequences) of the total sequences (plastic and seawater samples). A 80% of putative pathogen OTUs were found in seawater samples (mainly *Tenacibaculum* sp.). On plastic samples, half of the putative pathogen OTUs belonged to *Vibrio* sp., 20% being identified as *Tenacibaculum* sp. and 11% as *Staphylococcus aureus*. Overall, the abundance of putative pathogenic OTUs remained steady during the different biofilm stages, except for PHBV showing two times more putative pathogen OTUs during primo-colonization.

## Heterotrophic BP and CSA on Polymers and in Seawater

During the primo-colonization stage, BP were in the same order of magnitude between the four polymers (from 38.5 ngC $dm^{-2}$ $h^{-1}$ on PE to 145.9 ngC $dm^{-2}$ $h^{-1}$ on AA-OXO) (**Table 1**). The temporal dynamics of BP on PE and OXO were comparable, peaking during the growing phase (426.7 and 217.2 ngC $dm^{-2}$ $h^{-1}$ at day 22, respectively) and decreasing during the maturation phase (55.6 to 55.3 ngC $dm^{-2}$ $h^{-1}$ at day 45, respectively). AA-OXO and PHBV biofilms presented different trends. BP peaked at day 15 for both AA-OXO and PHBV (1396.9 and 1090.4 ngC $dm^{-2}$ $h^{-1}$, respectively), being until eightfold higher than PE and OXO. PHBV biofilm became less active at day 22, reaching a plateau around 250 ngC $dm^{-2}$ $h^{-1}$ until day 45. AA-OXO kept a high activity until day 22 (1396.7 ngC $dm^{-2}$ $h^{-1}$) and decreased drastically at day 45 (131.4 ngC $dm^{-2}$ $h^{-1}$). Seawater BP remained lower than polymers BP throughout the experiment rising from 9.09 ngC $L^{-1}$ $h^{-1}$ at D7 to 41 ngC $L^{-1}$ $h^{-1}$ at D45.

Cell-specific activity was very high on plastic compared to free-living bacteria (maximum of 10.37 and $0.13 \times 10^{-3}$ fgC $cell^{-1}$ $h^{-1}$, respectively) and especially during the growing phase of the biofilm on plastics (from 43- to 88-fold higher than in seawater). Indeed, cell-specific activity peaked at day 15 but decreased generally after 22 days on plastic, whereas it changed more randomly in seawater.

# DISCUSSION

In the present study, we show that plastic polymers with different composition, when immersed under identical marine conditions, are first colonized by similar bacterial communities to constitute support matrices for the formation of contrasted biofilms with dissimilar diversities and activities, growth efficiency, and maturation properties. We also investigated the possible relation between surface properties and bacterial cell counts on plastics, speculated to be a key factor controlling biofilm formation (Pasmore et al., 2002).

## Succession of Biofilm Colonization Phases on Polymers

In this study, we observed three typical successive phases of biofilm formation on artificial surfaces: initial, growth and maturation phases. The initial phase lasted for the first week of immersion and was characterized by an abundant and homogeneous bacterial colonization on all polymers within

the first 7 days of incubation, with a cell density ranging from $1.25 \times 10^5$ to $9.25 \times 10^5$ cell $cm^{-2}$. The growing phase (after day 7 to 22) significantly differed between non-biodegradable (PE and OXO) and biodegradable (AA-OXO and PHBV) polymers, with a higher biomass increase on the latter. At this stage, cells formed aggregates and biofilms became more patchy, as also observed on plastic marine debris in the North Pacific Gyre (Webb et al., 2009; Carson et al., 2013) and in the Mediterranean Sea (Dussud et al., 2018). The stabilization phase generally occurred after 3 weeks (from day 22 to 45) with the highest cell abundance reached on AA-OXO and PHBV, being more than five times higher than that accumulated on the non-biodegradable PE and OXO. These results are in accordance with Lobelle and Cunliffe (2011) reporting stabilization phase within a month on PE-based food bags, even if their results were based on cultivable bacteria that greatly underestimate cell counts of the entire biofilm (Ferguson et al., 1984). Other studies evaluated cell abundance using SEM, AFM, or epifluorescence microscopy (Harrison et al., 2014; Bryant et al., 2016), but none of them provided direct cell counts. As far as we know, this study presents the first results of direct cell counts on polymers using flow cytometry coupled with epifluorescence microscopy. It should be emphasized that epifluorescence microcopy was not usable for some polymers due to strong auto-fluorescence background (i.e., AA-OXO and PHBV), whereas our cell detachment pre-treatment permits to use flow cytometry as accurate technique to estimate cell counts in all polymers. When possible, the comparison of the two techniques showed systematic underestimation of cell counts for flow cytometry by a factor of 1 to 5, which is consistent with previous studies on organic particle-attached bacteria (Worm et al., 2001; Mével et al., 2008).

We also explored the possible relation between polymer surface characteristics and microbial colonization. This is a complex question, since several effects need to be considered at once: the chemical nature (Lorite et al., 2011; Siddiqa et al., 2015), roughness (Riedewald, 2006), and heterogeneity (Morra and Cassinelli, 1997) of the polymer surface on the one hand, and the potential hindrance of these properties by the microbial conditioning film (Lorite et al., 2011), on the other hand. Moreover, polymers are known to alter their properties when immersed in water, due to water diffusion or reconstruction of their surface in order to minimize the interfacial energy. Indeed, we observed here a decrease in hydrophobicity for all polymers after 7 days of immersion in seawater. This complexity might explain why there is still no consensus today, as to whether, for instance, a hydrophobic surface will increase or not bacterial adhesion (Morra and Cassinelli, 1997). Several articles on biofouling nevertheless acknowledge that high-energy surfaces ("hydrophilic surfaces") tend to favor biofilm growth (Callow and Fletcher, 1994; Artham et al., 2009). Our study presents the first results combining the observation of successive biofilm colonization phases on plastics together with the evolution of their surface roughness, contact angles and hysteresis before and after immersion in seawater. When comparing the three types of PE-based polymers, we clearly observed that colonization increased with increasing polarity (AA-OXO > OXO > PE) for similar roughness. In the same way, colonization was higher for PHBV than for PE, probably because PHBV is more polar, even though its roughness was larger than that of PE. A clear conclusion that can be drawn from these results is that the surface polarity has definitely an impact on colonization at sea, whether through the adsorption of a more abundant or different conditioning film, or directly through attracting more bacteria. Finally, one should keep in mind that cells numbers reflect not only their rate of adhesion but also the multiplication/disappearance rate of the different species, which can be affected in the case of biodegradable substrates where plastic is not only a physical support matrix but also a potential source of nutrients for bacteria. A hint into these rates is given by the measured activity and diversity of the bacterial colonies which are discussed thereafter.

## Bacterial Community Succession on Polymers

The bacterial communities accumulated on the polymer surfaces differed from those in the seawater during the entire course of the experiment. This assessment is in line with previous studies revealing a clear niche partitioning between bacteria living on plastics versus surrounding seawaters (Zettler et al., 2013; Amaral-Zettler et al., 2015; Dussud et al., 2018). Our experimental conditions did not disrupt the natural assemblages of seawater bacteria circulating in the aquarium during the course of the experiment, as observed in the control aquarium that did not contain plastic. Together with the slight changes observed in bacterial abundance in the control aquarium which are in line with values commonly found in the Mediterranean Sea (Pulido-Villena et al., 2011), these results validated our capability to maintain natural conditions for 45 days in an experimental setup renewed with natural seawater every 30 min.

Primo-colonizers of the plastics represented <0.1% of the bacterial diversity found in the water, corresponding to the less abundant or rare taxa that make up a substantial portion of bacterial communities in the oceans and constitute the so called "rare biosphere" (Sogin et al., 2006). These results demonstrate that the "seed bank" theory (Pedrós-Alió, 2012; Sauret et al., 2014) applies particularly well to the early colonizers and to the plastisphere in general. Members of the bacterial communities living on plastics, although rare in the seawater, prove here to be opportunistic species able to grow and to become the "core species" living on plastics. Overall, we found that Gammaproteobacteria dominated primo-colonizers on all polymer types, as already reported for the early colonization of PE (Harrison et al., 2014; De Tender et al., 2017). This taxonomic group was also identified as a family of primo-colonizers on other artificial surfaces in coastal waters such as acryl, glass, steel, or filtration membranes from drinking water treatment plants (Hörsch et al., 2005; Lee et al., 2008). The bacterial community structures of primo-colonizers were similar between all polymer types, except for PHBV, for which bacteria belonged to the same cluster but presented much less similarity and were largely dominated by *Neptuniibacter* sp.

In the next phase of biofilm growth and during the maturation phase, we observed a clear distinction between bacterial communities growing on non-biodegradable and biodegradable polymers. While PE and OXO eventually hosted a homogeneous cluster, the community structures on AA-OXO and PHBV continued to change over time. Previous studies also underlined rapid shifts in bacterial communities between the initial and successive colonization phases on other artificial surfaces, such as polyurethane painted plastics (Dang and Lovell, 2000), desalination plant system (Elifantz et al., 2013) or on acryl, glass and still coupons (Lee et al., 2008). With time, we observed that members of the class Alphaproteobacteria became increasingly abundant whatever the polymer type and remained distinct from the communities living in the control seawater.

Our study compared for the first time the dynamics of marine bacterial communities on polymers of similar chemical basic formulation (i.e., PE-based) but with d2w additives (Symphony Environmental Technology) with or without pre-aging. The cluster analysis showed that similar communities dominated the non-biodegradable PE and OXO during the growing and maturation phases, but differed drastically from the biodegradable AA-OXO. Difference in bacterial community structure may be explained by surface properties, since AA-OXO present higher oxidation state, lower hydrophobicity compared to PE and OXO.

The two biodegradable polymers AA-OXO and PHBV continued to change over the growing and maturation phases of the biofilm. Polymer degradation is considered to proceed through several stages (i.e., biodeterioration, biofragmentation, assimilation, and mineralization), which result from complex synergetic interactions between bacterial communities that also change over the biodegradation process (Lucas et al., 2008; Dussud and Ghiglione, 2014). Even if biodegradation processes occurring in both AA-OXO and PHBV are becoming better understood for bacteria cultured in the laboratory (Deroiné et al., 2015; Eyheraguibel et al., 2017), further studies are needed to describe the complex interactions between bacterial communities in the biofilm and their role in plastic biodegradation in natural conditions.

## Potential Bacterial Degradation of Complex Carbon Molecules in Plastics

The SIMPER analysis revealed a clear dominance of putative HCB on plastic compared to seawater. Their presence on the plastic surface has been observed in various neustonic debris (mainly of PE and PP composition) in the North Pacific Gyre (Zettler et al., 2013; Debroas et al., 2017) and in the Mediterranean Sea (Dussud et al., 2018), or on 5- to 6-weeks immersed PET drinking water bottles (Oberbeckmann et al., 2016). All these authors postulated that these plastic-dwelling microbes possessed the metabolic potential to degrade plastics and/or plastic-bound organic pollutants. Such hypothesis was recently supported by metagenomic analyses highlighting an overexpression of xenobiotic degradation functions by plastisphere communities in the North Pacific Gyre (Bryant et al., 2016).

Another hypothesis is the capability of HCB to overcome the poor accessibility of hydrophobic substrates, which may play a crucial role in the early colonization phase on hydrocarbon-based plastics (Lobelle and Cunliffe, 2011). Biofilm formation at the hydrocarbon–water interface has been observed with various alkane-degrading strains including *Oleiphilus messinensis* (Golyshin et al., 2002) and *Marinobacter* sp. (Vaysse et al., 2009), which dominated the early colonization phase on PE, PE-OXO, and AA-OXO in our study, together with other known alkane-degraders *Alcanivorax* sp. (Yakimov et al., 2007) and *Aestuariicella hydrocarbonica* (Lo et al., 2015). Biofilm formation has been shown to promote growth at the hydrocarbon–water interface by facilitating interfacial access, thus constituting an efficient adaptive strategy for assimilating hydrocarbon (Bouchez-Naïtali et al., 2001).

If putative HCB dominated on hydrocarbon-based plastics (PE, OXO, and AA-OXO), PHBV showed instead a succession of PHA-degraders. Indeed, members of *Neptuniibacter* sp. (Chen et al., 2012), *Phaeobacter* sp. (Frank et al., 2014), and *Roseobacter* sp. (Xiao and Jiao, 2011) previously shown to present the capability to accumulate or metabolize PHA, were dominant in the early colonization, growth and maturation phases, respectively. Further biodegradation studies in natural environment are needed to further describe the role of these species in PHA polymers degradation.

## A High and Variable Heterotrophic BP on Polymers

Our study provides the first BP data on polymers. A high temporal variability of BP was found during the successive phases of biofilm formation on each polymer. Overall, BP peaked after 2 weeks during the growing phase in all polymer types (from day 15 to 22), where CSA were the highest, and both parameters decreasing in the maturation phase.

In our seawater circulation system, BP reached 41 ngC $L^{-1}$ $h^{-1}$, a value similar to what is generally reported *in situ* in the NW Mediterranean Sea (Lemée et al., 2002), thus making extrapolation of our results to natural seawater possible.

Comparing BA and BP data between polymer films (in $cm^{-2}$) and seawater (in $mL^{-1}$) was irrelevant because one is counted in a volume and the other one on a surface, but using cell-specific activity (in ngC $cell^{-1}$ $h^{-1}$ for both plastic and seawater) made this comparison possible. We then found that bacteria attached on polymer were particularly active compared to the free-living bacteria, the cell-specific activity being from 43- to 88-fold higher especially during the growing phase in the polymers. Such difference may be explained by the presence of labile inorganic and organic matter on the plastic, as on any solid surface immerged in seawater (Cooksey and Wigglesworth-Cooksey, 1995). Another explanation could be that biodegradation has started on some polymers, since they can theoretically be used as carbon source by bacteria (Dussud and Ghiglione, 2014). The BP observed on the two biodegradable polymers (AA-OXO and PHBV) proved until 30 times higher than that measured on non-biodegradable polymers support this hypothesis. Unfortunately, no specific biodegradation assays on

organic matter or plastic were performed in this study, which may help to test these hypotheses and their complementarity. Further studies are needed to differentiate organic matter utilization from polymer biodegradation when measuring BP on plastics.

In this paper, we did not evaluate the biodegradability of the polymers tested during our experiment. Nethertheless, a better understanding of the biofilm forming on plastic in natural conditions is necessary to develop realistic tests of biodegradation. A very recent review pointed that current standards and test methods are insufficient in their ability to realistically predict the biodegradability of plastics in aquatic environment (Harrison et al., 2018). In particular, the type of inoculum and the presence of organic matter are potential sources of uncertainties on the biodegradability tests, generally based on respirometric measurements (Sharabi and Bartha, 1993). For example, a study on PHBV aged film demonstrated a large loss of weight after 180 days in natural seawater and a biodegradation by respirometry (Deroiné et al., 2015). To complete this study a characterization of the microorganisms diversity would have been important to better understand the mechanisms of PHBV biodegradation in seawater. Differences in the oxidation degree of the polymers, in the environmental conditions or in the methodologies used are also important factors that may explain controversary results showing ever no significant proof of mineralization of pre-oxidized OXO in marine water (Alvarez-Zeferino et al., 2015) or clear biodegradation in other environments (Jakubowicz, 2003; Chiellini et al., 2007; Ojeda et al., 2009; Yashchuk et al., 2012; Eyheraguibel et al., 2018). Giving the fact that relatively few studies focused on colonization of plastic at sea, this study should help further researches on biodegradability of plastics in marine habitats.

## AUTHOR CONTRIBUTIONS

CD and CH have conceived and designed the study. CD, CH, MG, and PF acquired the data. CD, CH, MG, PF, and J-FG analyzed and interpreted the data. J-FG, PH, SB, PF, and MG provided the equipment. CD and J-FG drafted the manuscript. PF, MG, and A-MD critically revised the manuscript for important intellectual content. BE, A-LM, JJ, JC, NC, CO, and SR approved the version of the manuscript to be published.

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## SUPPLEMENTARY MATERIAL

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Annex 5

REVIEW
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# Microbial Ecotoxicology of Marine Plastic Debris: A Review on Colonization and Biodegradation by the "Plastisphere"

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Over the last decades, it has become clear that plastic pollution presents a global societal and environmental challenge given its increasing presence in the oceans. A growing literature has focused on the microbial life growing on the surfaces of these pollutants called the "plastisphere," but the general concepts of microbial ecotoxicology have only rarely been integrated. Microbial ecotoxicology deals with (i) the impact of pollutants on microbial communities and inversely (ii) how much microbes can influence their biodegradation. The goal of this review is to enlighten the growing literature of the last 15 years on microbial ecotoxicology related to plastic pollution in the oceans. First, we focus on the impact of plastic on marine microbial life and on the various functions it ensures in the ecosystems. In this part, we also discuss the driving factors influencing biofilm development on plastic surfaces and the potential role of plastic debris as vector for dispersal of harmful pathogen species. Second, we give a critical view of the extent to which marine microorganisms can participate in the decomposition of plastic in the oceans and of the relevance of current standard tests for plastic biodegradability at sea. We highlight some examples of metabolic pathways of polymer biodegradation. We conclude with several questions regarding gaps in current knowledge of plastic biodegradation by marine microorganisms and the identification of possible directions for future research.

**Keywords: bacteria, marine plastics debris, colonization, biodegradation, metabolic pathways**

## INTRODUCTION

The amount of land-based plastic debris entering the ocean is estimated at 4.8 to 12.7 million tons per years (Jambeck et al., 2015). It is so important that plastic is regarded as a marker of the Anthropocene (Duis and Coors, 2016; Zalasiewicz et al., 2016). A growing body of research has investigated plastic distribution (Willis et al., 2017; Worm et al., 2017) and toxicity for marine fauna (Bakir et al., 2014; Gewert et al., 2015). A comparatively smaller but growing literature has been devoted to the microbial ecotoxicology of marine plastic debris, i.e. (1) the impact of plastic on marine microbial life together with the various ecosystem services that marine microbial life

ensures and inversely, (2) the role of microorganisms in the degradation of ocean plastic (Ghiglione et al., 2014, 2016). Both aspects will be successively explored by this review, which covers the last 15 years of literature.

The investigation of microorganisms colonizing plastic surfaces using modern techniques of massive DNA sequencing (Zettler et al., 2013) was introduced only recently. The authors introduced the world "plastisphere" to describe the microbial life growing on these surfaces. They also detected members of the potentially pathogenic genus *Vibrio*, which may be dispersed over long distances by floating persistent plastics. Since then, several studies investigated various marine environments, such as the North Pacific Gyre (Debroas et al., 2017) or the Mediterranean Sea (Dussud et al., 2018a). In parallel, a growing literature described the first steps of colonization of new plastic until the formation of a mature biofilm (Lobelle and Cunliffe, 2011; Oberbeckmann et al., 2015; Dussud et al., 2018a).

Such knowledge is of great interest to better understand the impact of plastic on marine microbial life and ecosystem functions. Only one study so far used shotgun metagenomics, showing that plastic-inhabiting microbes present an enriched gene repertoire compared to microbes living in the surrounding waters (Bryant et al., 2016). In this review, we argue that current knowledge is insufficient to draw a clear picture of the impact of plastic on marine microbial life and ecosystem functions, and we propose several directions for further studies in this field (see section "Microorganisms Colonizing Plastic at Sea").

The role of microbes on plastic degradation in the ocean is a second subject of concern. Very recently, an excellent comprehensive review concluded that "current international standards and regional test methods are insufficient in their ability to realistically predict the biodegradability of carrier bags in marine environment, due to several shortcomings in experimental procedures and a paucity of information in the scientific literature" (Harrison et al., 2018). The capability of microorganisms to biodegrade plastic was reported for numerous bacterial strains (Krueger et al., 2015). Fungi also have the capability to biodegrade plastics, but most of the studies were conducted in terrestrial conditions (Cosgrove et al., 2007; Koitabashi et al., 2012; Gajendiran et al., 2016; Magnin et al., 2018) whereas very few studies so far exist in marine conditions (Gonda et al., 2000; Pramila and Ramesh, 2011). Moreover, most of these studies were based on the selection and testing of single strains in laboratory conditions, which is very far from environmental conditions. In this review, we underscore the knowledge gaps on plastic biodegradation by marine microorganisms and we attempt to identify possible directions for future research in this area (see section "How Much Can Microorganisms Participate in Plastic Degradation at Sea?").

# MICROORGANISMS COLONIZING PLASTIC AT SEA

## A New Niche for Marine Microorganisms

It was not until recently that the first work using modern techniques of massive DNA sequencing provided a detailed picture of the microbial life on plastic and introduced the term "plastisphere" (Zettler et al., 2013). Bacteria, Archaea, Fungi and microbial Eukaryotes were detected in several studies, starting from plastics sampled at sea or from new plastics experimentally incubated in marine conditions (**Table 1**). Plastic debris are mainly composed of polyethylene (PE) at sea surface, followed by polypropylene (PP) and polystyrene (PS) (Auta et al., 2017). Whatever the polymer type, recent studies emphasized the difference between the bacteria living on plastics and the bacteria living in free-living state (Debroas et al., 2017) or on organic particles in the surrounding seawater (Dussud et al., 2018a; Oberbeckmann et al., 2018). Similar observations have been made for fungal communities (Kettner et al., 2017).

Another aspect that received much less attention is the plastisphere living in the water column other than the surface layer. Because of methodological constraints, most of the studies so far have been limited to sampling surface seawater using manta trawls, which represents less than 1% of the global load of plastic in the open ocean (Cózar et al., 2014). Only certain types of plastics made of PE and PP with high surface-to-volume ratios, such as rigid plastics and bundled fishing nets and ropes, have the capability to remain for a very long time at the surface of the oceans (Lebreton et al., 2018). Most other buoyant plastic such as films or smaller pieces, tend to sink to the sediment owing to biofouling (Fazey and Ryan, 2016; Kalogerakis et al., 2017). Very limited information is available concerning the composition of microbial communities on plastic items sampled from the seafloor (De Tender et al., 2015). If photoautotrophic bacteria such as the cyanobacteria of the genera *Phormidium* and *Rivularia* dominate the sub-surface plastisphere communities (Zettler et al., 2013; Bryant et al., 2016; Dussud et al., 2018a), the core microbiome of the seafloor and sub-surface plastisphere seems to share some taxa: Bacteroidetes (*Flavobacteriaceae*) and Proteobacteria (*Rhodobacteraceae* and *Alcanivoracaceae*) (Zettler et al., 2013; Bryant et al., 2016; De Tender et al., 2017; Dussud et al., 2018a).

## Successive Colonization Stages of New Plastics Incubated in Marine Conditions

In parallel to studies on plastic directly sampled at sea, other studies focused on the successive colonization steps of new plastics incubated in marine conditions (**Table 1**). At sea, plastics are rapidly covered by the "conditioning film" made of inorganic and organic matter, which is then rapidly colonized by bacteria (mainly *Gammaproteobacteria* and *Alphaproteobacteria*) (Oberbeckmann et al., 2015). With time, members of Bacteroidetes become increasingly abundant (Lee et al., 2008). Hydrophobicity and other substratum properties (crystallinity and crystal structure, roughness, glass transition temperature, melting temperature, modulus of elasticity) may play a role in the selection of bacterial community in the early stages of colonization (Pompilio et al., 2008), but probably in a lesser extent when the biofilm becomes mature (Dussud et al., 2018a). The successive growing and maturation phases of biofilm formation, already described for other surfaces such as glass, acryl, steel or rocks and algae (Salta et al., 2013), were also

**TABLE 1 |** List of recent studies using molecular techniques to evaluate the biodiversity of the plastisphere in different geographic regions, for plastic samples taken at sea or incubated in seawater conditions for the purpose of the studies.

| Studied area | Sample type | Method | Gene target | Target | References |
|---|---|---|---|---|---|
| North Pacific subtropical Gyre | Sampling at sea surface | Metagenomic sequencing | | Bacteria and Eukaryote | Bryant et al., 2016 |
| Baltic Sea | Incubation in seawater | V4 18S rRNA sequencing | 565-981 | Microbial Eukaryote, Fungi | Kettner et al., 2017 |
| Estuary, Baltic Sea | Incubation in seawater | V4 16S rRNA sequencing | 515-806 | Bacteria and Archaea | Oberbeckmann et al., 2018 |
| North Sea | Incubation in seawater | V4 16S rRNA sequencing | 515-806 | Bacteria and Archaea | Oberbeckmann et al., 2016 |
| | | V9 18S rRNA sequencing | 1391-1795 | Microbial Eukaryote, Fungi | |
| North Sea | Sampling at sea surface- Incubation in seawater | DGGE 16S rRNA and sequencing | 341-534 | Bacteria and Archaea | Oberbeckmann et al., 2014 |
| North Sea | Incubation in seawater and sediment | V3-V4 16S rRNA sequencing | 341-785 | Bacteria and Archaea | De Tender et al., 2017 |
| | | rDNA-ITS2 sequencing | | Fungi | |
| North Atlantic subtropical gyre | Sampling at sea surface | V4 16S rRNA sequencing | 515-806 | Bacteria and Archaea | Debroas et al., 2017 |
| | | V7 18S rRNA sequencing | 960-1438 | Eukaryote | |
| North Atlantic | Sampling at sea surface | V4-V6 16S rRNA sequencing | 518-1046 | Bacteria | Zettler et al., 2013 |
| | | V9 16S rRNA sequencing | 1380-1510 | Microbial Eukaryote | |
| Mediterranean Sea | Sampling at sea surface | V3-V5 16S rRNA sequencing | 515-926 | Bacteria and Archaea | Dussud et al., 2018a |
| Mediterranean Sea | Incubation in seawater | V3-V5 16S rRNA sequencing | 515-926 | Bacteria and Archaea | Dussud et al., 2018b |
| Mediterranean Sea | Incubation in seawater | V3-V5 16S rRNA sequencing | 515-926 | Bacteria and Archaea | Briand et al., 2012 |
| Arabian Sea | Incubation in seawater | V4 16S rRNA sequencing | ND | Bacteria | Muthukrishnan et al., 2018 |
| Estuary, North Sea | Incubation in marine sediment | 16S rRNA cloning and sequencing | 27-1492 | Bacteria | Harrison et al., 2014 |
| Estuary, East China Sea | Sampling at sediment surface | V3-V4 16S rRNA sequencing | 319-806 | Bacteria | Jiang et al., 2018 |

*The PCR-amplified regions and the corresponding targeted organisms are indicated. ND, Non-described in the publication.*

observed for plastics of different compositions (Oberbeckmann et al., 2015). Biofilm developments were followed during several weeks in seawater on PE-based plastic bags (Lobelle and Cunliffe, 2011), polyethylene terephthalate (PET)-based plastic bottles (Oberbeckmann et al., 2014), polyvinyl chloride (PVC) (Dang et al., 2008), or polystyrene (PS) coupons (Briand et al., 2012). PE-based plastics were also rapidly colonized by microorganisms in marine sediments (Harrison et al., 2014). Clear differences in bacterial abundance, diversity and activity were found between non-biodegradable and biodegradable plastics (Eich et al., 2015; Dussud et al., 2018b). Higher colonization by active and specific bacteria were found after six weeks on poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) and pre-oxidized PE-based oxodegradable polymers (OXO) in comparison to non-biodegradable PE polymers (Eich et al., 2015; Dussud et al., 2018b). Longer-term studies carried out over a 6-month to one year period also showed differences in biofilm formation and maturation according to the polymer type, i.e. PE, PP, PET, or polycarbonate (PC) (Webb et al., 2009; De Tender et al., 2017). Not only bacteria but also fungi were shown to form biofilms on plastic surfaces (Pramila and Ramesh, 2011), mainly dominated by Chytridiomycota, Cryptomycota (Kettner et al., 2017) and Ascomycota (Oberbeckmann et al., 2016; De Tender et al., 2017; Kettner et al., 2017).

## Potential Impact of Plastic on the Microbial Role in Regulation of Biogeochemical Cycles

The quantity of plastic in the oceans can no longer be considered as a limited ecological problem, since small pieces of plastic called "microplastics" (<5 mm) found at sea could cover 4.2 million $km^2$ of the sea surface (Charette and Smith, 2010; Hidalgo-Ruz et al., 2012; Eriksen et al., 2014). Marine microorganisms that compose the plastisphere are known to play a key role in the biogeochemical cycles in the oceans (Pomeroy et al., 2007). One-half of oceanic primary production on average is channeled *via* heterotrophic bacterioplankton into the microbial loop, thus contributing significantly to food web structure and carbon biogeochemical cycling in the ocean (Fenchel, 2008; **Figure 1**). Only one recent study compared the heterotrophic production of bacteria living on plastic and in seawater. Heterotrophic bacteria living on plastics were

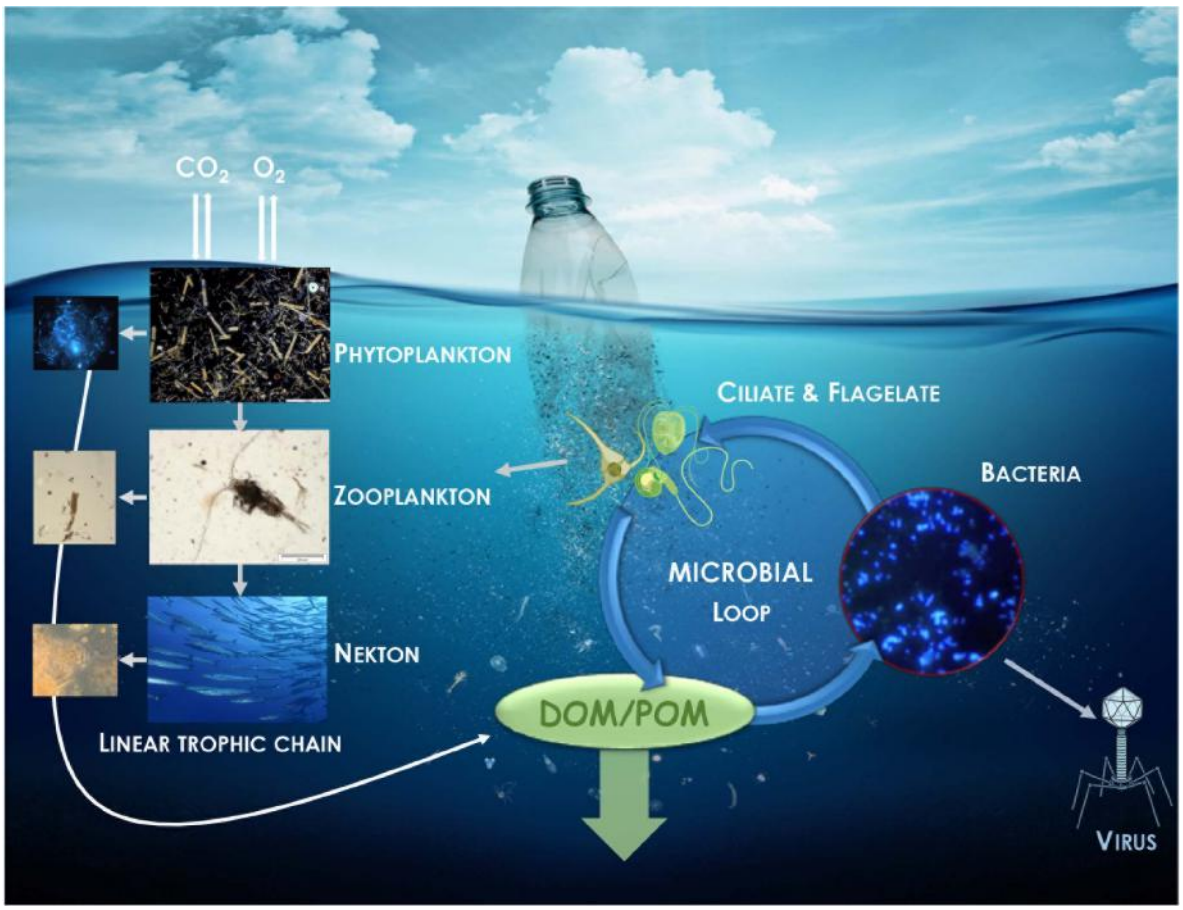


**FIGURE 1 |** Illustration of the potential impact of plastic in the regulatory role of carbon and nutrient cycles played by bacteria *via* the microbial loop. Dissolve (DOM) and particulate (POM) organic matter originated from the linear trophic chain is returned to higher trophic levels *via* its incorporation in bacterial biomass.

particularly active, the cell-specific activity measured by $^{3}$H-leucine incorporation into proteins being 43- to 88-fold higher than that of the free-living fraction (Dussud et al., 2018a). Unfortunately, these results were obtained in the frame of a study on colonization of new plastics incubated at sea for a relatively short period (45 days). Similar methodologies applied to plastics that had spent several years at sea would be necessary to evaluate how much the large amount of plastic and the accompanying plastisphere influence the biogeochemical carbon cycle in the oceans.

Interestingly, most of the studies aiming to characterize the plastisphere mentioned that Cyanobacteria were overrepresented on plastics compared to the surrounding free-living and organic particle-attached fractions. The relative importance of photosynthetic activities that Cyanobacteria living on plastic have on global pelagic primary production is still unknown.

Coupling primary production and heterotrophic production measurements over large temporal and spatial scales will be necessary to obtain a better view of the role of the plastisphere on carbon cycling in the oceans. Microorganisms are not only involved in the carbon cycle, but basically in all other biogeochemical cycles including nitrogen, sulfur, iron, manganese, chromium, phosphorus, calcium and silicate cycles, which may also be impacted by the presence of plastic at sea (Hutchins and Fu, 2017).

## Potential Dispersion of Pathogen Species

Interest has been raised about opportunist pathogen dispersal on plastics, such as animal or human pathogenic *Vibrio* sp. (Zettler et al., 2013). Marine plastic debris as vector of harmful species was first suggested by Masó et al. (2003), who identified potential harmful dinoflagellates such as *Ostreopsis* sp. and *Coolia* sp. Putative pathogens of fish (*Tenacibaculum* sp.) and of invertebrates (*Phormidium* sp. and *Leptolyngbya* sp.) were found to be more common on plastic compared to surrounding seawater (Dussud et al., 2018a). Some bacterial taxa considered as putative pathogens for human, coral and fish were also found in the intertidal zone of the Yangtze Estuary, at relatively low abundance (<1.6%) (Jiang et al., 2018). A putative pathogen for coral *Halofolliculina* spp. was found to be abundant on some western Pacific plastic debris (Goldstein et al., 2014). Some toxic eukaryotic species were also mentioned by Debroas et al. (2017) at low abundance (<0.04%), but might be regarded as

hitchhiker organisms. Nevertheless, caution should be taken since the 16S rRNA metabarcoding approach used in all these studies was not an appropriate method for describing bacterial virulence. The recent coupling of the 16S rRNA metabarcoding technique with the detection of virulence-associated genes may be an interesting option to address this question (Kirstein et al., 2016). Pathogenicity evidence on marine animals in relation to the plastisphere has never been proven, and further research will be required before publicizing alarmist conclusions on the possible responsibility of plastic debris as vector for the spread of disease-causing organisms. Apart from those results, microplastics colonized by pathogens may also pose threats to humans who are exposed to contaminated beach and bathing environments (Keswani et al., 2016). Evidence is still missing to determine whether plastic debris could lead to the spread and prolonged persistence of pathogenic species in the oceans.

### Factors Driving the Plastisphere Composition and Activities

Factors driving the plastisphere composition are complex, mainly spatial and seasonal, but are also influenced by the polymer type, surface properties and size. Plastisphere communities studied in different polymer types floating in the North Pacific and North Atlantic reflected first their biogeographic origins, and to a lesser extent the plastic type (Amaral-Zettler et al., 2015). Similar conclusions were found for bacterial communities colonizing plastics along an environmental gradient. These communities are shaped firstly by the freshwater to marine environmental conditions and secondarily by the plastic type (PS and PE) (Oberbeckmann et al., 2018). Inversely, another study based on a large number of microplastics sampled in the western Mediterranean Sea showed no effect of geographical location (including coastal and open ocean samples) or plastic type (mainly PE, PP, and PS) on the bacterial community composition. The growing number of studies on the plastisphere are giving a better view of the microbial biofilm community on plastics in the oceans, but the complex network of influences is still the subject of ongoing debate. A clearer picture will hopefully emerge from more extensive investigations with widespread and numerous samples, together with better descriptions of the physical and chemical properties of the polymers.

The physical properties of plastic offer a unique habitat that contribute to the long-distance transport of diverse microbial hitchhikers attached to its surface (Harrison et al., 2011; Zettler et al., 2013). A vast range of other phyla, including Arthropoda, Annelida, Mollusca, Bryozoa, and Cnidaria have conferred on plastics the role of vector for the transfer of organisms, some of them being cataloged as invasive alien species (Oberbeckmann et al., 2015). For instance, plastic debris with tropical biota including corals was detected in the Netherlands (Hoeksema, 2012), and Southern Ocean bryozoans were observed in Antarctica (Barnes and Fraser, 2003). Interactions between micro- and macro-organisms, their substratum and their surroundings are needed to better predict the ecological consequences of microplastics transported through the global oceans.

## HOW MUCH CAN MICROORGANISMS PARTICIPATE IN PLASTIC DEGRADATION AT SEA?

### Definition and Main Processes Involved in Plastic Biodegradation

Biodegradation of plastic is a process that results in total or partial conversion of organic carbon into biogas and biomass associated with the activity of a community of microorganisms (bacteria, fungi, and actinomycetes) capable of using plastic as a carbon source (Shah et al., 2008). Depending on the respiratory conditions (aerobic / anaerobic) and the microorganisms involved, the biogas will be different ($CO_2$, $CH_4$, $H_2S$, $NH_4$, and $H_2$) (Mohee et al., 2008).

Microorganisms, including bacteria and fungi, present the capabilities to degrade or deteriorate plastics and several review papers updated the list of plastic-degraders (Shah et al., 2008; Bhardwaj et al., 2013; Kale et al., 2015; Pathak, 2017). *Arthrobacter*, *Corynebacterium*, *Micrococcus*, *Pseudomonas*, *Rhodococcus*, and *Streptomyces* were the prominent microbial taxa able to use plastic as sole carbon source and energy in laboratory conditions. **Table 2** proposes an update of the current list of microorganisms proven to present biodegradation capabilities under laboratory conditions.

Biodegradation is considered to occur after or concomitant with physical and chemical degradation (abiotic degradation), which weakens the structure of polymers as revealed by roughness, cracks and molecular changes (İpekoglu et al., 2007). Alteration of plastic properties due to abiotic degradation is called "aging" and in nature depends on several factors such as temperature, solar light and chemicals that enhance the rate of degradation by oxidizing or disrupting the length of the polymer chain.

Biodegradation can be summarized in four essential steps, which have been described in detail in a review by Dussud and Ghiglione (2014):

- Bio-deterioration relates to the biofilm growing on the surface and inside the plastic, which increases the pore size and provokes cracks that weaken the physical properties of the plastic (physical deterioration) or releases acid compounds that modify the pH inside the pores and results in changes in the microstructure of the plastic matrix (chemical deterioration).
- Bio-fragmentation corresponds to the action of extracellular enzymes (oxygenases, lipases, esterases, depolymerases and other enzymes that may be as diverse as the large spectrum of polymer types) released by bacteria colonizing the polymer surface. These enzymes will reduce the molecular weight of polymers and release oligomers and then monomers that can be assimilated by cells.

**TABLE 2 |** List of microbial strains able to biodegrade various types of polymers.

| Type of polymer | Strains | Reference |
|---|---|---|
| PE | *Brevibacillus borstelensis* | Hadad et al., 2005; Mohanrasu et al., 2018 |
| | *Bacillus weihenstephanensis* | Ingavale and Raut, 2018 |
| | *Comamonas* sp. | Peixoto et al., 2017 |
| | *Delftia* sp. | Peixoto et al., 2017 |
| | *Stenotrophomonas* sp. | Peixoto et al., 2017 |
| | *Achromobacter xylosoxidans* | Kowalczyk et al., 2016 |
| | *Bacillus* sp. YP1 | Yang et al., 2014 |
| | *Enterobacter asburiae* YT1 | Yang et al., 2014 |
| | *Bacillus amyloliquefaciens* | Das and Kumar, 2015 |
| | *Bacillus pumilus* M27 | Harshvardhan and Jha, 2013 |
| | *Kocuria palustris* M16 | Harshvardhan and Jha, 2013 |
| | *Lysinibacillus xylanilyticus* | Esmaeili et al., 2013 |
| | *Bacillus mycoides* | Ibiene et al., 2013 |
| | *Bacillus subtilis* | Ibiene et al., 2013 |
| | *Pseudomonas aeruginosa* PAO1 (ATCC 15729) | Kyaw et al., 2012 |
| | *Pseudomonas aeruginosa* (ATCC 15692) | Kyaw et al., 2012 |
| | *Pseudomonas putida* KT2440 (ATCC 47054) | Kyaw et al., 2012 |
| | *Pseudomonas syringae* DC3000 (ATCC 10862) | Kyaw et al., 2012 |
| | *Brevibacillus parabrevis* | Pramila, 2012 |
| | *Acinetobacter baumannii* | Pramila, 2012 |
| | *Pseudomonas citronellolis* | Pramila, 2012 |
| | *Bacillus sphaericus* | Sudhakar et al., 2008 |
| | *Rhodococcus ruber* | Gilan and Sivan, 2013 |
| | *Aspergillus versicolor* | Pramila and Ramesh, 2011 |
| | *Aspergillus* sp. | Pramila and Ramesh, 2011; Sheik et al., 2015 |
| | *Chaetomium* sp. | Sowmya et al., 2012 |
| | *Aspergillus flavus* | Sowmya et al., 2012 |
| | *Penicillium simplicissimum* | Yamada-Onodera et al., 2001; Sowmya et al., 2014 |
| | *Lasiodiplodia theobromae* | Sheik et al., 2015 |
| | *Paecilomyces lilacinus* | Sheik et al., 2015 |
| | *P. pinophilum*, *A. niger*, *Gliocladium virens*, and *P. chrysosporium* | Manzur et al., 2004 |
| | *Aspergillus glaucus* and *A. niger* | Kathiresan, 2003 |
| PET | *Bacillus amyloliquefaciens* | Novotný et al., 2018 |
| | *Nocardia* sp. | Sharon and Sharon, 2017 |
| | *Ideonella sakaiensis* | Yoshida et al., 2016 |
| | *Humicola insolens* | Ronkvist et al., 2009 |
| | *Pseudomonas mendocina* | Ronkvist et al., 2009 |
| | *Thermobifida fusca* (DSM 43793) | Müller et al., 2005 |
| | *Penicillium citrinum* | Liebminger et al., 2007 |
| | *Thermomonospora fusca* | Alisch et al., 2004 |
| | *Fusarium oxysporum* | Nimchua et al., 2007 |
| | *Fusarium solani* | Alisch et al., 2004; Nimchua et al., 2007 |
| PHB | *Crupriavidus* sp. | Martínez-Tobón et al., 2018 |
| | *Marinobacter algicola* | Martínez-Tobón et al., 2018 |
| | Mixed cultures | Ansari and Fatma, 2016 |
| | *Schlegella thermodepolymerans* | Romen et al., 2004 |
| | *Caenibacterium thermophilum* | Romen et al., 2004 |
| | *Acidovorax* sp. TP4 | Kobayashi et al., 1999 |
| | *Pseudomonas stutzeri* | Uefuji et al., 1997; Martínez-Tobón et al., 2018 |
| | *Leptothrix discophora* | Takeda et al., 1998 |

*(Contiuned)*

**TABLE 2 |** Contiuned

| Type of polymer | Strains | Reference |
|---|---|---|
| | *Alcaligenes faecalis* | Tanio et al., 1982; Kita et al., 1995 |
| | *Comamonas acidovorans* YM1609 | Kasuya et al., 1997 |
| | *Comamonas testosteroni* | Kasuya et al., 1997; Martínez-Tobón et al., 2018 |
| | *Pseudomonas lemoignei* | Uefuji et al., 1997; Martínez-Tobón et al., 2018 |
| | *Ralstonia pickettii* | Yamada et al., 1993; Martínez-Tobón et al., 2018 |
| | *Pseudomonas fluorescens* YM1415 and nine Gram- | Mukai et al., 1994 |
| | *Aspergillus niger* | Kumaravel et al., 2010 |
| PHBV | *Clostridium botulinum* | Abou-Zeid et al., 2001 |
| | *Clostridium acetobutylicum* | Abou-Zeid et al., 2001 |
| | *Streptomyces* sp. SNG9 | Mabrouk and Sabry, 2001 |
| | *Pseudomonas lemoignei* | Jendrossek et al., 1993 |
| | *Paecilomyces lilacinus* | Sang et al., 2001 |
| PS | Strain TM1 and ZM1 | Tang et al., 2017 |
| | *Bacillus subtilis* | Asmita et al., 2015 |
| | *Staphylococcus aureus* | Asmita et al., 2015 |
| | *Streptococcus pyogenes* | Asmita et al., 2015 |
| | *Exiguobacterium* sp. | Yang et al., 2015 |
| | *Bacillus* sp NB6, *Pseudomonas aeruginosa* NB26, *Exiguobacterium* sp., *Microbacterium* sp. NA23, *Paenibacillus urinalis* NA26 | Atiq et al., 2010 |
| | *Rhodococcus ruber* | Mor and Sivan, 2008 |
| | *Pseudomonas putida* CA-3 (NCIMB 41162) | Ward et al., 2006 |
| | *Bacillus* sp. STR-Y-O | Oikawa et al., 2003 |
| | Mixed microbial communities | Kaplan et al., 1979 |
| | Mixed microbial communities (*Bacillus*, *Pseudomonas*, *Micrococcus*, and *Nocordia*) | Sielicki et al., 1978 |

*polyethylene (PE), polyethylene terephthalate (PET), polyhydroxybutyrate (PHB), and Poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV), and polystyrene (PS). Detailed information on the origin of the strains and the methods used to prove biodegradation are available in the* ***Supplementary Table S1****.*

- Assimilation allows oligomers of less than 600 Daltons to be integrated inside the cells to be used as a carbon source, thus increasing the microbial biomass.
- Mineralization is the ultimate step in the biodegradation of a plastic polymer and results in the excretion of completely oxidized metabolites ($CO_2$, $N_2$, $CH_4$, and $H_2O$).

## Rates of Plastic Degradation

Rates of degradation of conventional plastics by microorganisms are extremely low, even in optimized laboratory conditions (Krueger et al., 2015). Most of the conventional plastics are recalcitrant to biodegradation in marine and terrestrial environments, resulting in lifetimes of decades or even centuries (Krueger et al., 2015). Plastics present low bioavailability since they are generally solid and made of densely cross-linked polymers that provide low accessibility for microbes and enzymes circumscribed to the outermost layer of the items. In the pelagic ecosystem, plastics are biodegraded by the aerobic metabolism of microorganisms, i.e., the end product of the reaction will be microbial biomass, $CO_2$ and $H_2O$. The anaerobic biodegradation pathway would be more frequently encountered in sediment and is supposed to be even slower than in the pelagic zone (Ishigaki et al., 2004). Unfavorable C/N ratio is a key factor for biodegradation of other hydrocarbon-based products in the oceans (Sauret et al., 2016) and may potentially also limit plastic biodegration.

Data currently available rely heavily on culture-based approaches in laboratory conditions, although bacteria that can be cultured represent less than 1% of the number of bacteria in nature (the so-called "great plate count anomaly") and a very small proportion of its very large diversity (Hugenholtz et al., 2009). To date, data on the rate of plastic mineralization in the oceans are still virtually non-existent. Congruent descriptions of the plastisphere that forms an abundant biofilm characterized by very diverse bacteria with active plastic-specific characteristics are available (Debroas et al., 2017; Dussud et al., 2018b). Evidence of pits visualized in the plastic debris that conform to bacterial shapes directly found in the marine environment (Zettler et al., 2013) together with a number of putative xenobiotic degradation genes likely involved in plastic degradation that were found to be significantly more abundant in the plastic-specific communities (Bryant et al., 2016; Debroas et al., 2017; Dussud et al., 2018b) are thus of great interest. A recent study underlined the need of cometabolic pathways on PE biodegradation, thus confirming that complex microbial communities rather than single species are necessary to degrade recalcitrant plastic (Syranidou et al., 2017). So far, the timescales of degradation and the characterization and the fate of the degradation products, are fundamental, yet still unanswered questions.

## Standard Tests for Plastic Biodegradability at Sea

The current standards for marine environments propose tests based on respirometry measurements, susceptible to describe the mineralization step of plastic biodegradation in aerobic conditions (see **Figure 2**). They impose a minimum percentage of conversion from plastic to $CO_2$ ranging from 60 to 70% over a period of 3 months (ASTM D6691-09), 6 months (ASTM D7473-12), or 24 months (ISO 18830, ISO 19679, ASTM D7991-15) under aerobic conditions (see **Figure 3**). Anaerobic biodegradation is characterized by specific standards (see for example ASTM D5511-18), but to our knowledge none of these standards applies to the marine environment. Biodegradation of a plastic is characterized by the time required to achieve mineralization under controlled conditions. These tests cannot be considered as a proof of ready biodegradability (total conversion of plastic into biomass and $CO_2$), but rather an indication about a potential for biodegradation in the oceans.

Recently, these standards were considered insufficient in their ability to realistically predict the biodegradability in marine environment (Harrison et al., 2018). These tests can significantly underestimate the time required for polymer biodegradation within natural ecosystems. First, the authors underlined "biases associated with the preparation of experimental inocula and the test conditions themselves, including the use of preselected and/or pre-conditioned strains, artificially modified inocula, powdered test materials, nutrient-rich synthetic media and test temperatures that are frequently higher than those encountered within the environment." The authors also pointed out "the lack of clear guidelines for the analysis of different polymer types, including composite materials and plastics that contain additives," which can considerably influence the rates of biodegradation. "There is also a paucity of guidelines for materials of varying shapes and sizes and, in certain cases, the test procedures lack a sufficient level of statistical replication." Another concern, not raised by Harrison et al. (2018), is the biases associated with the common method for determining biodegradability, i.e., measurements of $CO_2$ evolution. This method may lead to either underestimation or overestimation of the plastic biodegradation due to other processes. It is noteworthy that plastic generally presents high sorption capability of organic matter (especially hydrophobic organic chemicals including pollutants) that can be biodegraded by the plastisphere biofilm,

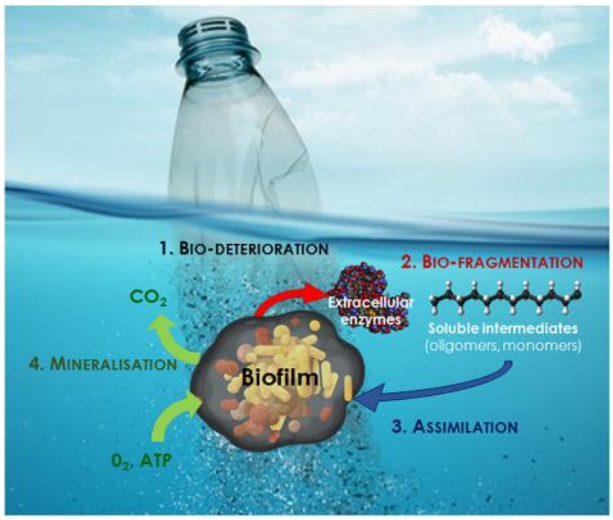


**FIGURE 2 |** The different steps of plastic biodegradation at sea (modified from Dussud and Ghiglione, 2014).

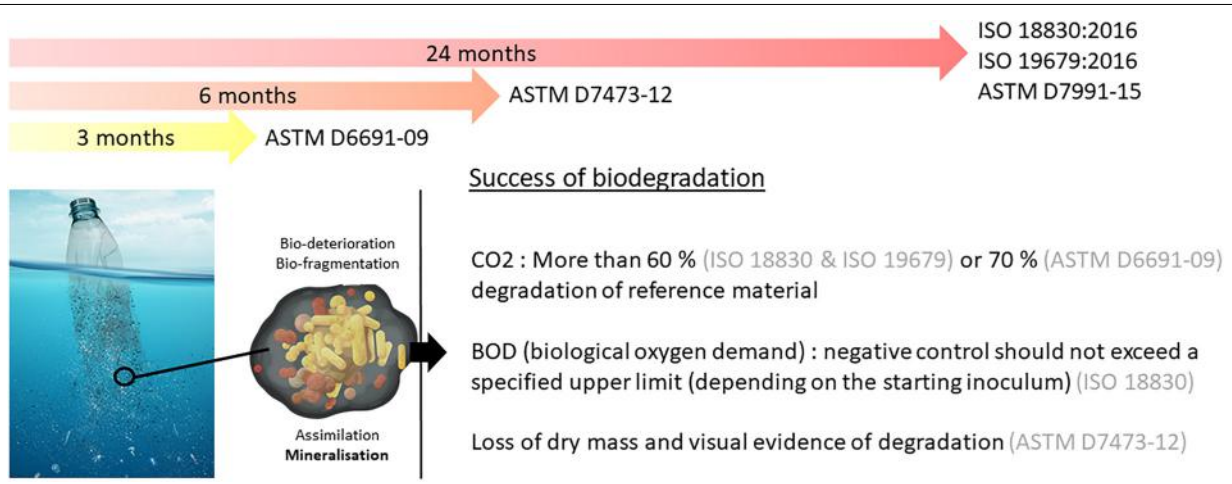


**FIGURE 3 |** Current standards on biodegradability of plastics at sea. ISO 18830: Plastics-determination of aerobic biodegradation of non-floating plastic materials in a seawater/ sandy sediment interface – Method by measuring the oxygen demand in closed respirometer. ISO 19679: Determination of aerobic biodegradation of non-floating plastic materials in a seawater/sediment interface – method by analysis of evolved carbon dioxide respirometer. ASTMD7991: Determining aerobic biodegradation of plastics buried in sandy marine sediment under controlled laboratory conditions. ASTM D7473: Standard test method for weight attrition of plastic materials in the marine environment by open system aquarium incubations. ASTM: Standard test method for determining aerobic biodegradation of plastic materials in the marine environment by a defined microbial consortium or natural sea water inoculum.

thus resulting in a $CO_2$ production that has nothing to do with plastic biodegradation (Lee et al., 2014). Inversely, several papers reported the importance of photosynthetic microorganisms growing on plastics, which consume $CO_2$ regardless of plastic biodegradation (Zettler et al., 2013; Bryant et al., 2016; Dussud et al., 2018b). Further studies are needed to evaluate the relative degree of $CO_2$ consumption by photosynthesis, $CO_2$ production by organic matter degradation by the plastisphere as compared to $CO_2$ production due to plastic biodegradation.

The limitations of the respiratory methods described above can be overcome by other additional analytical techniques and approaches to confirm changes in the physical properties and the chemical structure of polymers during biodegradation. Alterations in the visual appearance and in the mass or changes in mechanical properties are relatively easy and low-cost methods for the evaluation of physical changes during biodegradation. Other methods could be combined to confirm changes in the molecular structure of polymers, such as measurements of surface hydrolysis and other chromatographic (gas chromatography with or without flame ionization detection, liquid chromatography, gel-permeation chromatography) measurements coupled or not with spectrometric techniques (mass spectrometry, nuclear magnetic resonance spectroscopy, Fourier-transform infrared spectroscopy). Optical, atomic force and scanning electron microscopy can also be used to assess the biodeterioration of the surface due to microbial activity or biofilm formation. Any of these techniques are enough to prove biodegradation by its own, and each of them has limitations that have been previously detailed for example in the excellent reviews of (Koutny et al., 2006; Harrison et al., 2018; Ho et al., 2018). The current standards sometimes propose to use such techniques to corroborate the main test based on respirometry measurement, but no clear guidelines on how to use these tests is provided.

## Examples of Metabolic Pathways of Polymer Biodegradation

There are currently more than 5,300 grades of synthetic polymers for plastics in commerce (Wagner and Lambert, 2018). They are generally produced with a range of chemical additives such as plasticizers, flame retardants, antioxidants and other stabilizers, pro-oxidants, surfactants, inorganic fillers or pigments (Wagner and Lambert, 2018). Their heterogeneous physical-chemical properties will likely result in very heterogeneous metabolic pathways of biodegradation, especially when considering the large variety of microorganisms that may interact for the degradation of a single piece of plastic, together with the environmental factors of very dynamic oceanic conditions. We are aware that treating plastic as a single compound does not make sense and providing details on the metabolic pathways of plastic biodegradation would necessarily be unrepresentative of the complexity of the various processes that occur in the environment. We have chosen to focus on the metabolic pathways associated with the biodegradation of model compounds used in the formulation of conventional (PE, PET, and PS) and so called "biodegradable" plastics (PHA) that are the most popular and the most extensively studied in the literature. Moreover, it should be noted that because of the difficulty of dealing with long-term experiments and complex communities under natural conditions, all the following studies describing the metabolic pathways of plastic biodegradation were done using a culture-based approach.

### Metabolic Pathways of Polyethylene (PE) Biodegradation

High- and low-density polyethylene is a long linear carbon chain ($CH_2$) belonging to the family of polyolefins. Polyethylene is derived from petroleum sources and its large use in our daily life made it the first plastic waste found at sea surface. PE is considered difficult to biodegrade because the long chains of carbons and hydrogens are very stable and contain very balanced charges. Microorganisms generally need imbalance of electric charge to perform biodegradation. To destabilize the local electric charge, bacteria use oxygenases: enzymes able to add oxygen to a long carbon chain (Krueger et al., 2015). For instance, mono-oxygenases and di-oxygenases incorporate, respectively, one and two oxygen atoms, forming alcohol or peroxyl groups that are less recalcitrant for biodegradation. Oxidation may also be processed by abiotic reactions associated with UV radiation or temperature (for more details, see the review by Singh and Sharma, 2008). Oxidation of PE results in the formation of carboxylic groups, alcohols, ketones, and aldehydes by a radical reaction (Vasile, 1993; Gewert et al., 2015). The oxidation and fragmentation of PE make the polymer more hydrophilic and facilitates access to other extracellular enzymes, such as lipases and esterases after the formation of carboxylic groups, or endopeptidases for amide groups (Gewert et al., 2015). Other enzymes such as laccase in *Rhodococcus ruber* are excreted and can facilitate the biodegradation of PE (Santo et al., 2013). Interestingly, a recent study focused on soluble oxidized oligomers showed that 95% of these compounds were assimilated by a strain of *Rhodococcus rhodochrous* after 240 days of incubation (Eyheraguibel et al., 2017). The polymer is broken down into small oligomers of 600 Da incorporated in the cells by carriers belonging to the Major Facilitor Superfamily (MFS) or harboring ATP binding cassettes (ABC) (Gravouil et al., 2017). β-oxidation transforms oxidized carboxylic molecules (having an even number of carbon atoms) into acetyl coA or propionyl coA (if odd number of carbons). Carboxylation of propionyl coA into succinyl coA is performed by propionyl-coA carboxylase. Gravouil et al. (2017), propose identification of an overexpressed enzyme, when the bacteria find PE in the medium (Gravouil et al., 2017). Acetyl coA and succinyl coA enter the tricarboxylic acid (TCA) cycle (**Figure 4**). This cycle produces chemical energy in the form of a reducing power (NADH, H + and $CoQ_{10}H_2$) used in the respiratory chain to produce ATP, which is necessary to create new microbial biomass *via* replication processes. It also produces $CO_2$ and $H_2O$ that sign the complete mineralisation of PE.

For 20 years now, scientists have been interested in the biodegradation of polyethylene by the microbial community. Bacterial and fungal strains presenting biodegradation capabilities of PE are listed in **Table 2** and **Supplementary Table S1**.

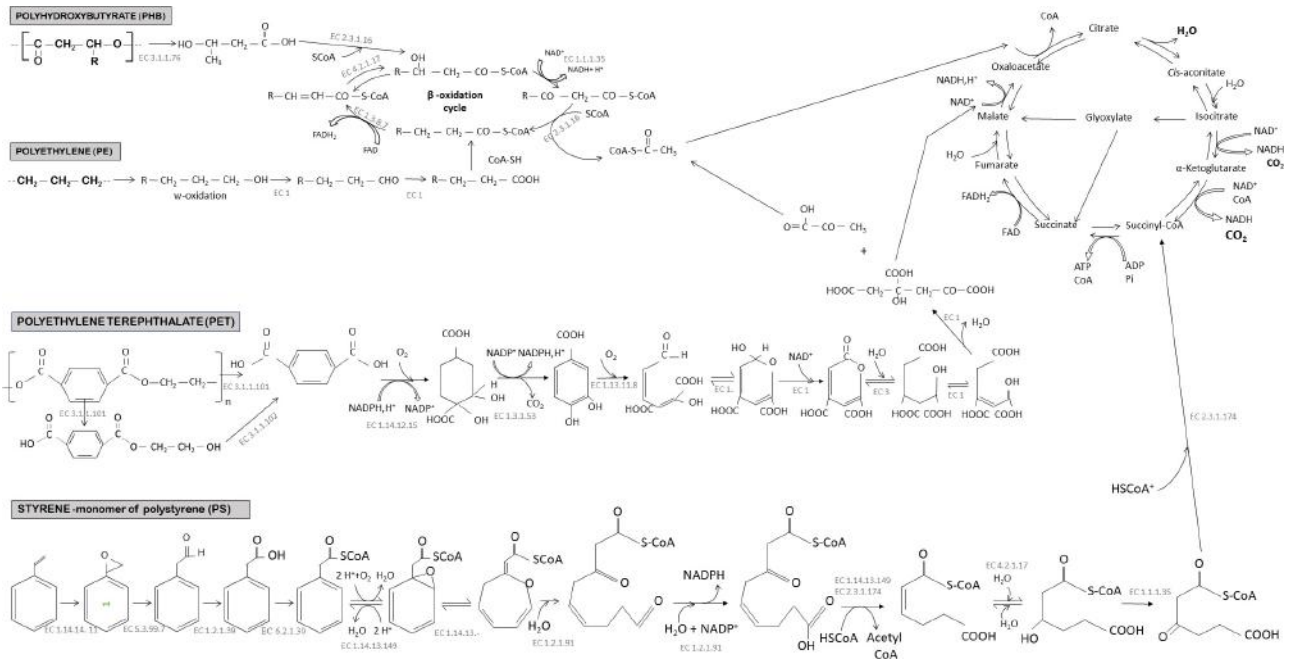


**FIGURE 4 |** Biodegradation pathways under aerobic conditions of three conventional plastics (polyethylene, polyethylene terephthalate, and polystyrene) and one biodegradable plastic (polyhydroxybutyrate). See explanation in the text indicating that degradation rates may be very different between polymer types. Complete mineralisation into $CO_2$ and $H_2O$ occurred after several steps of transformation of the initial molecule involving several microbial enzymes. The common stage of transformation through the TCA cycle produce also ATP, which is a key component for bacterial growth and biomass production. Enzyme commission numbers (EC numbers) were given for each enzyme-catalyzed reactions. EC 3.1.1.76, poly(3-hydroxyoctanoate) depolymerase; EC 2.3.1.16, acetyl-CoA C-acyltransferase; EC 1.1.1.35, 3-hydroxyacyl-CoA dehydrogenase; EC 1.3.8.7, medium-chain acyl-CoA dehydrogenase; EC 4.2.1.17, enoyl-CoA hydratase; EC 3.1.1.101, poly(ethylene terephthalate) hydrolase; EC 3.1.1.102, Mono(2-hydroxyethyl) terephthalate hydrolase; EC 1.14.12.15, terephthalate 1,2-dioxygenase; EC 1.3.1.53, 3,4-dihydroxycyclohexa-1,5-diene-1,4-dicarboxylate dehydrogenase; EC 1.13.11.8, protocatechuate 4,5-dioxygenase; EC 1, Oxidoreductase; EC 3, Hydrolase; EC 1.14.14, 11 styrene monooxygenase; EC 5.3.99.7, styrene-oxide isomerase; EC 1.2.1.39, phenylacetaldehyde dehydrogenase; EC 6.2.1.30, phenylacetylCoA ligase; EC 1.14.13.149, phenylacetyl-CoA 1,2-epoxidase; EC 1.14.13, ring 1,2-epoxyphenylacetyl-CoA isomerase; EC 1.2.1.91, 3-oxo-5,6-dehydrosuberyl-CoA semialdehyde dehydrogenase; EC 2.3.1.174, 3-oxoadipyl-CoA/3-oxo-5,6-dehydrosuberyl-CoA thiolase; EC 4.2.1.17, 2,3-dehydroadipyl-CoA hydratase; EC 1.1.1.35, 3-hydroxyadipyl-CoA dehydrogenase.

Genetic evidence of PE biodegradation remains scarce in the literature, but preliminary work highlighted enzymes, transporters or genes that may be involved in this process (Gravouil et al., 2017). Alkane hydroxylase genes were found to play a central role in PE biodegradation for *Pseudomonas* sp. E4 strain, which was capable of mineralizing 28.6% of the organic carbon of the polymer in 80 days. The alkB gene was then introduced in *Escherichia coli* BL21 strain, which was then able to mineralize 19.3% of the organic carbon of the polymer (Yoon et al., 2012). Only two other studies used genetic analysis to provide evidence for the importance of laccase in PE biodegradation by *R. ruber* (Sivan, 2011; Santo et al., 2013; Gravouil et al., 2017).

## Metabolic Pathways of Polyethylene Terephthalate (PET) Biodegradation

Polyethylene terephthalate is part of the polyester family and it is widely used in the design of bottles and synthetic fibers. It is considered as persistent plastic in the environment because of its long carbon chains containing aromatic rings that are difficult to biodegrade (Marten et al., 2005). In recent years, studies have shown that some bacterial strains were able to degrade PET as sole carbon source and energy, such as *Ideonella sakaiensis* (Yoshida et al., 2016), *Nocardia* sp. (Sharon and Sharon, 2017) *Pseudomonas mendocina* (Ronkvist et al., 2009), *Thermobifida fusca* (Müller et al., 2005). Some fungal communities are also known to biodegrade PET, such as *Humicola insolens*, several *Fusarium* species, and *Penicillium citrinum* (Silva et al., 2005; Liebminger et al., 2007; Nimchua et al., 2007; Ronkvist et al., 2009). Cutinases or hydrolases play key roles in PET biodegradation (Danso et al., 2018). For example, *I. sakaiensis* 201-F6 adhered to the PET surface and first secreted two enzymes involved in the biodegradation process of PET: PETase (hydrolase) and MHETase. PETase is an extracellular enzyme capable of hydrolysing PET to mono-(2-hydroxyethyl) terephthalate (MHET), terephthalic acid (TPA), and bis (2-hydroxyethyl) terephthalate (BHET). Fungi seem to have the same biodegradation strategy and are able to degrade PET into BHET and MHET (Liebminger et al., 2007). The MHETase hydrolyzes MHET to TPA and ethylene glycol (EG). The terephthalic acid molecule is then internalized in the bacterial cells by the TPA transporter (Hosaka et al., 2013) and then

catabolized by TPA 1,2-dioxygenase (TPADO) and 1,2-dihydroxy-3,5-cyclohexadiene-1,4-dicarboxylate dehydrogenase (DCDDH) to give protocatechuic acid (PCA) as the final molecule (Yoshida et al., 2016). This PCA is cleaved by PCA 3,4 dioxygenase (PCA34) to give the hemiacetal form of 4-carboxy-2-hydroxymuconic. The latter becomes the substrate of a dehydrogenase to form 2-pyrone-4,6-dicarboxylic acid that enters the TCA cycle and initially transformed into pyruvate and oxaloacetate, then assimilated as $CO_2$ and $H_2O$ (**Figure 4**).

## Metabolic Pathways of Polystyrene (PS) Biodegradation

Polystyrene is a polymer composed of styrene monomers ($CH_2$ = $CH_2$-Ph). The polymer is highly hydrophobic and presents a high molecular weight. Like other conventional plastics, partial biodegradation in the laboratory has been observed while it continues to accumulate in the oceans (Auta et al., 2017) thus inciting increasing interest in PS biodegradation (see **Supplementary Table S1**; Oikawa et al., 2003; Mor and Sivan, 2008; Atiq et al., 2010; Asmita et al., 2015; Yang et al., 2015; Tang et al., 2017).

Several biodegradation pathways may be considered, depending on the microorganism involved. The predominant pathway is the oxidation pathway of the styrene side chain presented in **Figure 4**. The styrene is directly oxidized with a styrene monooxygenase to form a styrene epoxide which will then be oxidized to phenylacetaldehyde by styrene oxide. This molecule is then catabolized into phenylacetic acid. This conversion of styrene to phenylacetic acid is called the upper pathway of styrene metabolism. Phenylacetic acid is converted to phenylacetyl-CoA (acetyl coenzyme A) by the so-called lower pathway (Luu et al., 2013) then subjected to several enzymatic reactions (**Figure 4**) to finally enter the tricarboxylic acid (TCA) cycle. The biodegradation products enter the TCA cycle through the final formation of acetyl-Co A and succinyl-CoA (succinyl-CoenzymeA) (Luu et al., 2013).

Interestingly, *Pseudomonas putida* CA-3 can accumulate polyhydroxyalkanoates (PHA at medium chain length) when growing on styrene, thus using an original biodegradation pathway. A catabolic operon has been identified as responsible for this bioconversion; this path is called the PACoA (Phenylacetyl-CoA) catabolon. It involves oxidation of the aromatic ring, followed by entry into the β-oxidation cycle and the conversion to acetyl-CoA (O'Leary et al., 2005). This acetyl-CoA can follow different metabolic pathways, either entering the TCA cycle or following the *de novo* fatty acid biosynthesis path which will give as final product medium-chain-length polyhydroxyalkanoates (mcl-PHAs) (O'Leary et al., 2005). This study shows the complexity of studying the biodegradation pathways of these polymers and indicates the great range of possibilities when considering the large diversity of microorganisms found in the plastisphere.

## Metabolic Pathways of Polyhydroxyalkanoate (PHA) Biodegradation

The current global production of PHA is increasing, reaching 49,200 tons per year that represents 2.4% of the production of bioplastics[1]. PHAs are biopolymers of hydroxylated fatty acids produced within a bacteria in granular form. Each PHA monomer ($[CO\text{-}CH_2\text{-}CHR\text{-}O]_n$) consists of hydroxyalkanoates linked together by ester bonds. The alkyl group (R) varies from a methyl group to a tetradecyl group. When bacteria are placed in a medium with an excess carbon source and low nutrient content, they accumulate storage granules. Over 300 bacterial species are capable of producing 80 different hydroxyalkanoate monomers, and some bacteria can accumulate up to 90% of their total weight of polymer in very specific conditions (Peña et al., 2014). One of the most commonly used PHA for plastic production is polyhydroxybutyrate (PHB), which has a methyl as an alkyl group (R) ($[CO\text{-}CH2\text{-}CHCH3\text{-}O]_n$). PHB is one of the homopolymers with high commercial power because it has thermoplastic, hydrophobic, low oxygen permeability and is considered biodegradable (Mothes et al., 2004; Chang et al., 2012). It is not very deformable, because of its high crystallinity (Gorke et al., 2007) and it has a high melting point close to its thermal degradation temperature (Reis et al., 2003). A copolymer made of Poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) that reduces the melting point of PHB is seen to emerge in PHA production. The advantage of using PHA is that it is stable over time, as long as the conditions governing its biodegradation are not met (Jaffredo et al., 2013).

Due to their microbial origin, PHAs were found to be biodegradable in many environments such as soil, marine ecosystems or sewage sludge (Eubeler et al., 2010). Biodegradation of Poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) has been proven with comparable rates to that of cellulose, with faster degradation found under aerobic (85 days) compared to anaerobic (6 months) conditions (Wang et al., 2018). The biodegradation scheme in **Figure 4** shows the different steps of PHB biodegradation. When the biodegradation is not carried out inside the cells by bacteria that produce their own PHB, other bacteria initiate the biodegradation of PHB in the medium by external hydrolysis using ectoenzymes that convert the polymers into hydroxylated acid monomers of hydroxybutyrate (HB) (Peña et al., 2014). This molecule is water soluble and small enough to passively diffuse across the bacterial membrane and enter the β-oxidation cycle. The resulting acetyl-CoA will be oxidized in the TCA cycle until final mineralisation (Alshehrei, 2017). PHA-degradation has been proven in the laboratory under aerobic or anaerobic conditions (see a non-exhaustive list in **Supplementary Table S1**). The dominant bacteria in aerobic marine conditions belong to *Clostriales*, *Gemmatales*, *Phycisphaerales*, and *Chlamydiales*, whereas *Cloacamonales* and *Thermotogales* dominate in anaerobic sludge (Wang et al., 2018).

[1]https://www.european-bioplastics.org

## CONCLUDING REMARKS

In this review, we have presented both aspects of microbial ecotoxicology on marine plastic debris, namely the impact of plastic on marine microbial life and inversely how microbes can play a role in plastic biodegradation. An increasing number of studies either describe the different steps of biofilm formation under marine conditions, or give new insights on bacteria colonizing the aged plastics directly sampled at sea. The very diverse and active bacteria living on plastics as compared to the surrounding waters suggest a potential impact on the global biogeochemical cycles associated with the relatively recent introduction of plastic in the oceans, impact that remains to be determined. Plastic released in the oceans is also accused to be a raft for invasive species including pathogenic bacteria, but no proof of pathogenicity on marine animals or humans in relation to plastic ingestion has emerged so far.

A better knowledge of the plastisphere is also a critical issue in understanding the role played by bacteria in plastic biodegradation. Several studies have underlined that current standards are failing to prove biodegradability at sea for several reasons that have been highlighted in this review. Biodegradation of a polymer at sea depends on many factors related to its own composition, but also on the various ecosystems and environmental conditions encountered during its very long lifetime. It is for these reasons that plastic polymers continue to accumulate at sea and that biodegradation rates reported in the laboratory are never reached in the environment. Thus, a complete study of the biodegradation of a polymer at sea must combine several monitoring parameters, and especially be confirmed in the field with experiments *in situ*. Given the complexity of the plastic problem, research network initiatives such as "Polymers & Oceans" that bring together physicists, chemists and biologists are required to answer the wishes and needs of many scientists to face this environmental problem and its resonance in the society.

[2] https://po2018.wixsite.com/po2018

## AUTHOR CONTRIBUTIONS

J-FG designed the general plan of the review. JJ, A-LM, JC, and J-FG made the figures. JJ, JC, CO, CP, PC, MP-P, VB, A-LM, and J-FG wrote the manuscript and approved the final version.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2019.00865/full#supplementary-material

**TABLE S1 |** Detailed information on the origin and methodology used to prove the biodegradation of various polymer types (PE, PET, PHB, PHBV, and PS) by microorganisms cited in **Table 1**.

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