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Effet des microplastiques sur les jeunes stades de vie des poissons marins

Hugo Jacob

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THÈSE DE DOCTORAT
DE L'UNIVERSITÉ PSL

Préparée à Ecole Pratique des Hautes Etudes (EPHE)

**Effets des microplastiques sur les jeunes stades de vie
des poissons marins**

Soutenue par

Hugo JACOB

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THÈSE DE DOCTORAT
DE L'UNIVERSITÉ PSL

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Résumé

La pollution plastique est aujourd'hui une préoccupation environnementale et sociétale majeure. Au cours des 10 dernières années, de nombreuses études expérimentales ont complété les observations de terrain dans l'espoir de mieux comprendre le devenir et l'impact des micro- et/ou nano-plastiques (MP/NP) sur divers organismes (par exemple : oiseaux, poissons et mammifères). Chez les poissons, les effets les plus significatifs rapportés après une exposition aux MP/NP ont été observés sur les fonctions comportementales et neurologiques, la perméabilité intestinale, le métabolisme et la diversité du microbiome intestinal. Cependant, la pollution plastique reste difficile à quantifier dans l'environnement et à contrôler en laboratoire, et les particules de plastique sont souvent co-contaminées de manière naturelle ou expérimentale avec divers polluants chimiques. Par conséquent, les études sur les effets comportementaux et physiologiques associés à l'ingestion des MP/NP vierges chez les jeunes stades de vie poissons marins restent peu nombreuses à l'heure actuelle. Ainsi, l'objectif de cette thèse est d'étudier les effets de deux types de plastique fréquemment rencontrés en milieu marin, à savoir le polyéthylène (PE) et le polystyrène (PS), sous la forme de microplastiques (de 10 à 100 μm), sur les jeunes stades de vie des poissons marins, connus pour être particulièrement sensibles aux stress environnementaux. Ainsi, des larves et juvéniles de différentes espèces de poissons modèles (tropicale : *Acanthurus triostegus* et tempérée : *Sparus Aurata*) ont été sélectionnés. Plusieurs paramètres physiologiques et comportementaux ont été étudiés après une exposition à des microplastiques par différentes voies (eau et nourriture) dans le cadre de diverses expériences sur ces espèces modèles. Les résultats actuels ne montrent pas d'effets marqués de ce stress à des doses environnementales, cependant certains résultats, comme une augmentation de la mortalité et des perturbations métaboliques, à des doses plus élevées, semblent montrer une toxicité à la suite de l'ingestion de ces particules. Ainsi, cette thèse révèle que la toxicité des MP/NP vierges sur les poissons devrait être évaluée plus systématiquement en utilisant des méthodes rigoureuses en laboratoire, dans le but d'acquérir une meilleure compréhension des mécanismes sous-jacents de cette toxicité chez les poissons.

Abstract

Environmental plastic pollution is a major ecological and societal concern today. Over the last 10 years, a broad range of laboratory and experimental studies have complemented field observations in the hope of achieving a better understanding of the fate and impact of micro- and/or nano-plastics (MP/NP) on diverse organisms (e.g., birds, fish and mammals). However, plastic pollution remains challenging to monitor in the environment and to control under laboratory conditions, and plastic particles are often naturally or experimentally co-contaminated with diverse chemical pollutants. In fish, the most significant effects reported after exposure to MP / NP were observed on behavioral and neurological functions, intestinal permeability, metabolism and intestinal microbiome diversity. However, plastic pollution remains difficult to quantify in the environment and to control in the laboratory, and plastic particles are often co-contaminated naturally or experimentally with various chemical pollutants. Therefore, studies on the behavioral and physiological effects associated with ingestion of virgin MP / NP in early life stages of marine fish are still few at present. Thus, the purpose of this thesis is to study the effects of two types of plastic frequently encountered in the marine environment, namely polyethylene (PE) and polystyrene (PS), in the form of microplastics (from 10 to 100 μm), in the early life stages of marine fish, known to be particularly sensitive to environmental stresses. Thus, larvae and juveniles of different model fish species (Tropical: *Acanthurus triostegus* and Temperate: *Sparus Aurata*) were selected. Several physiological and behavioral parameters were studied after exposure to MP via different pathways (water and food) in various experiments on these model species. Current results do not show any marked effects of this stressor at environmental concentrations. However some results, such as increased mortality and metabolic disturbances, at higher doses, show toxicity following ingestion of these particles. Thus, this thesis reveals that the toxicity of virgin PM / NP on fish should be evaluated more systematically using rigorous laboratory methods, in order to gain a better understanding of the underlying mechanisms of this toxicity to fish.

Contributions Scientifiques

Articles issus du doctorat

- Jacob, H.**, Gilson, A., Lanctôt, C., Besson, M., Metian, M., Lecchini, D., 2019. No Effect of Polystyrene Microplastics on Foraging Activity and Survival in a Post-larvae Coral-Reef Fish, *Acanthurus triostegus*. *Bull. Environ. Contam. Toxicol.* 102, 457–461. <https://doi.org/10.1007/s00128-019-02587-0> (**Annexe 1**)
- Jacob, H.#**, Besson, M.#, Swarzenski, P., Lecchini, D., Metian, M., 2019. Effect of virgin micro- and nano-plastics on fish: Trends, meta-analysis and perspectives. *Soumis dans Environmental Science and Technology*.
- Jacob, H.#**, Besson, M.#, Oberhaensli, F., Taylor, A., Gillet, B., Hughes, S., Melvin, S., Swarzenski, P., Lecchini, D., Metian, M., 2019. Widespread effects of virgin polyethylene microplastics on juvenile gilthead seabreams (*Sparus aurata*). *En préparation*

Articles hors doctorat

- Garnier, Y., **Jacob, H.**, Guerra, A.S., Bertucci, F., Lecchini, D., 2019. Evaluation of microplastic ingestion by tropical fish from Moorea Island, French Polynesia. *Mar. Pollut. Bull.* 140, 165–170. <https://doi.org/10.1016/j.marpolbul.2019.01.038> (**Annexe 2**)
- O'Connor, J.J., Fobert, E.K., Besson, M., **Jacob, H.**, Lecchini, D., 2019. Live fast, die young: Behavioural and physiological impacts of light pollution on a marine fish during larval recruitment. *Mar. Pollut. Bull.* 146, 908–914. <https://doi.org/10.1016/j.marpolbul.2019.05.038> (**Annexe 3**)
- Bertucci, F.#, **Jacob, H.#**, Mignucci, A., Gache, C., Roux, N., Besson, M., Berthe, C., Metian, M., Lecchini, D., 2018. Decreased retention of olfactory predator recognition in juvenile surgeon fish exposed to pesticide. *Chemosphere* 208, 469–475. <https://doi.org/10.1016/j.chemosphere.2018.06.017> (**Annexe 4**)
- Besson, M., Gache, C., Bertucci, F., Brooker, R.M., Roux, N., **Jacob, H.**, Berthe, C., Sovrano, V.A., Dixon, D.L., Lecchini, D., 2017. Exposure to agricultural pesticide impairs visual lateralization in a coral reef fish. *Sci. Rep.* 1–9. <https://doi.org/10.1038/srep11048>
- Besson, M.#, **Jacob, H.#**, Oberhaensli, F., Taylor, A., Swarzenski, P., Lecchini, D., Metian, M., 2019. Preferential adsorption of Cd, Cs and Zn onto virgin polyethylene microplastic versus sediment particles. *Soumis dans Marine Pollution Bulletin* (**Annexe 5**)
- Lanctôt, C., Bednarz, V., Melvin, S., **Jacob, H.**, Oberhaensli, F., Ferrier-pages, C., Metian, M. 2019. Physiological stress response of *Stylophora pistillata* coral exposed to polyethylene microplastics. *En préparation*

#: Co-premier auteurs

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Figure 21. Synthèse des effets observés chez les juvéniles de daurade royale (*Sparus aurata*) exposés à des MP via l’alimentation durant 35 jours.

Chapitre 1 - État de l'art

Généralités sur le plastique

Le terme « plastique » est apparu pour la première fois au début du 17^{ème} siècle, où il était utilisé pour décrire une substance pouvant être moulée ou façonnée. Le terme dérive du grec ancien *plastikos*, qui se réfère à quelque chose qui convient pour le moulage, et du latin *plasticus* qui se rapporte au moulage ou à la mise en forme. L'usage moderne du « plastique » a été remis au goût du jour par le chimiste américain L. Baekeland qui, en 1910, mit au point le premier procédé industriel de production d'un polymère synthétique, qu'il nomma la « bakélite » (Fontanille and Gnanou, 2002). Les nombreuses découvertes qui suivirent dans ce domaine furent récompensées par l'attribution de plusieurs Prix Nobel et marquèrent le début de la production en masse de polymères synthétiques, dominants au sein des plastiques (Fontanille and Gnanou, 2002). Leur faible densité, durabilité, imperméabilité, souplesse de formes et leur faible coût font des plastiques des matériaux idéaux pour un large éventail d'applications industrielles et domestiques. Ainsi, du fait du large éventail d'applications que les plastiques offrent, la production mondiale de plastique est passée de 2 millions de tonnes (Mt) en 1950 à près de 350 Mt en 2017 et devrait atteindre 1800 Mt en 2050 (Geyer et al., 2017; Kaza et al., 2018).

Bien que les avantages sociétaux du plastique soient considérables (Andrady and Neal, 2009), ce matériau fait l'objet de préoccupation environnementale croissante (Derraik, 2002; Thompson et al., 2009). En effet, la durabilité du plastique, qui en fait un matériau attrayant, le rend également très résistant à la dégradation, rendant l'élimination des déchets plastiques problématique (Barnes et al., 2009). Exacerbés par l'utilisation massive de plastiques jetables et non réutilisables, les plastiques constituent 10 % des déchets générés dans le monde (Barnes et al., 2009). Alors que certains déchets plastiques peuvent être recyclés, la plupart se retrouvent dans des sites d'enfouissement ou directement dans l'environnement, où leur décomposition peut prendre des siècles (Barnes et al., 2009; Moore, 2008). Ainsi, 60 % de tous les plastiques produits entre 1950 et 2015 (soit 4 900 Mt) ont été jetés dans des décharges et dans l'environnement naturel et 3,4 milliards de tonnes devraient subir le même sort au cours des 30 prochaines années (Geyer et al., 2017).

Une inquiétude sociétale et scientifique particulière s'est formée autour de la quantité de plastique entrant dans l'environnement marin et ses effets potentiels sur les animaux y vivant (Gregory, 2009). Ainsi, la partie visible de la pollution plastique des océans dont font partie les gros débris de plastique, appelés « macroplastiques », a attiré l'attention des médias, du grand public et des scientifiques depuis les années 1980. Du fait de cette couverture médiatique, lorsque l'on parle de pollution plastique, nous

avons tous en tête ces rivages remplis de déchets, ces tortues prisonnières d’emballages ou encore ces « continents » de sacs flottant à la surface des océans.

Les déchets plastiques, en plus de poser un problème esthétique, posent de nombreux problèmes économiques, avec des répercussions sur l’industrie du tourisme, et un risque pour de nombreuses industries comme le transport maritime ou la pêche (Barnes et al., 2009), mais affectent aussi la faune marine. Ces impacts comprennent les blessures et la mort d’oiseaux marins, de mammifères, poissons et reptiles résultants d’un enchevêtrement ou d’une ingestion (Azzarello and Van Vleet, 1987; Carr, 1987; Derraik, 2002) ou encore le transport des espèces marine « non indigènes » (Winston, 1982). Plus récemment, il a été montré qu’une fois dans le milieu aquatique, les débris de plastiques peuvent concentrer des polluants comme les polluants organiques persistants et les métaux (Mato et al., 2001; Teuten et al., 2009), représenter de potentiels vecteurs de microorganismes (Masó et al., 2003; Zettler et al., 2013) et véhiculer, dans certaines conditions, des espèces invasives susceptibles de bouleverser l’équilibre biologique des régions qu’elles colonisent (Rech et al., 2016). En outre, bien que les plastiques soient généralement considérés comme biochimiquement inertes (Teuten et al., 2009), des additifs, nommés « plastifiants », peuvent y être incorporés au cours de leur fabrication pour modifier leurs propriétés ou prolonger leur durée de vie en fournissant une résistance à la chaleur, aux dommages oxydatifs ou à la dégradation microbienne (Groh et al., 2019). Ces additifs sont également une préoccupation environnementale, car en plus de prolonger le temps de dégradation du plastique, ils peuvent aussi introduire des produits chimiques potentiellement dangereux dans l’environnement (Rochman, 2015).

La préoccupation sociétale et scientifique autour de la pollution plastique en milieu marin est à l’heure actuelle grandissante en réponse aux estimations de la quantité astronomique de ces déchets entrant dans les océans. En effet, Jambeck et al. (2015) ont estimé que 275 millions de tonnes de déchets plastiques ont été générées dans 192 pays côtiers en 2010, avec 4,8 à 12,7 millions de tonnes entrant dans l’océan. Sans amélioration des infrastructures de gestion des déchets, il est prévu que la quantité cumulée de déchets plastiques disponibles entrant dans les océans augmentera d’un ordre de grandeur d’ici 2025. Le problème des macroplastiques, visible et relativement bien documenté, fait maintenant place à une autre préoccupation liée à la pollution plastique, moins visible et par conséquent plus difficile à appréhender : celle des microplastiques (MP).

Généralités sur les Microplastiques (MP)

Définition et historique

Malgré les propriétés persistantes du plastique et les efforts importants employés par les industriels de ce secteur pour assurer leur durabilité, les gros débris de plastique se décomposent pour former des macroplastiques (définis ici comme ayant une taille > 25 mm), mésoplastiques (5-25 mm), microplastiques (MP) (< 5 mm) et nanoplastiques (NP) (< 100 nm). Il n'y a pas de consensus clair dans la littérature sur la définition des tailles de MP et de NP; dans cette thèse, je définirai les MP et les NP comme des débris de plastique avec, respectivement, des diamètres de 100 nm à 5 mm et < 100 nm comme prônés par une série d'auteurs (p. ex., Alimi et al., 2018; Arthur et al., 2008).

Les MP peuvent être également définis comme primaires ou secondaires en fonction de leurs origines (Crawford and Quinn, 2016). Les MP primaires sont intentionnellement fabriqués sous forme de particules de petite taille à des fins industrielles incluant les pastilles de préproduction, les microbilles pour abrasifs ou cosmétiques, dentifrices et sablage (Crawford and Quinn, 2016). Les MP secondaires sont les fragments dégradés de gros débris de plastique. La formation de MP secondaires à partir de débris de plastique dépend de l'action de certains facteurs environnementaux comme l'exposition aux ultraviolets, la concentration en oxygène, la température, les forces mécaniques, l'encrassement biologique, ainsi que la taille et la forme des débris (Ter Halle et al., 2016). Par exemple, il a été démontré que 1 cm^2 d'une tasse de café en polystyrène chauffée à $30 \text{ }^\circ\text{C}$ pendant une période de 24 h, et exposé à une lumière ultraviolette à 320-400 nm, est capable de produire $1,26 \times 10^8$ nanoparticules d'une taille moyenne de 224 nm après 56 jours en eau de mer (Lambert and Wagner, 2016).

La détection de MP en milieu marin remonte aux années 1970. Buchanan (1971) a montré la présence de fibres synthétiques colorées dans les eaux des côtes du Northumberland (Angleterre). De son côté, Carpenter et al. (1972) a mis en évidence la présence de sphère de plastique dans les eaux côtières de Nouvelle-Angleterre (USA), ainsi que dans le système digestif des poissons vivants dans cette région. La première utilisation du terme « microplastique » (MP) n'apparaît que longtemps après, en 1990, définissant à l'époque les particules de plastiques de tailles inférieures à 20 mm. Les activités de recherche sur le plastique ont connu une accalmie dans les années 90 (Fig. 2). C'est en 2004 que Thompson et al. (2004) dans leur article fondateur dans le domaine de la recherche sur les MP, nommé « Lost at sea: Where is all the plastic? », montrent que ces particules constituent un polluant marin omniprésent. Ainsi, associé à la publicité autour de la formation du « dépotoir du Pacifique » au niveau du gyre océanique pacifique (Moore et al., 2001) et des agrégations de MP dans d'autres gyres

océaniques (par exemple (Eriksen et al., 2014)), ces recherches ont été responsables du regain d'intérêt récent pour le problème des déchets marins et tout particulièrement des MP.

De ce fait, au cours des quinze dernières années, la connaissance des sources, du sort, et de la dynamique des MP, ainsi que leurs interactions avec le biote et les polluants, n'a cessé d'augmenter (Fig. 2). Les méthodologies ont également considérablement évolué, et le premier protocole présenté dans le document de Thompson et al. (2004) a considérablement évolué. Toutefois, nos connaissances sur l'importance relative de diverses sources, des tendances spatiales de répartition et d'abondance, des tendances temporelles ou effets sur les biotes des MP restent encore limitées.

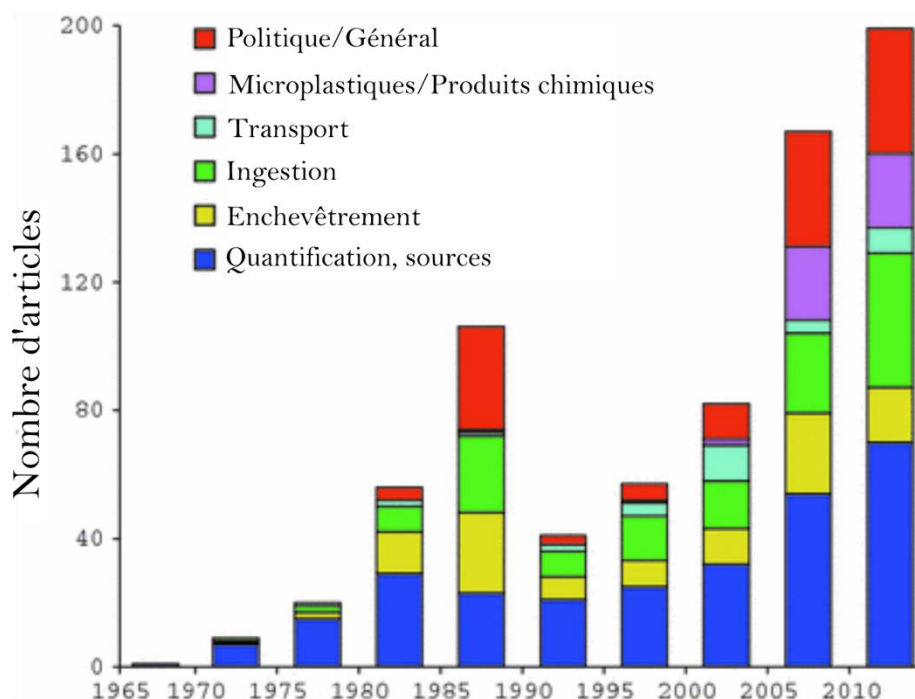


Figure 1. Nombre d'articles sur différents aspects de la question des déchets marins plastiques publiés tous les cinq ans de 1965 à 2013 (adaptée de Ryan, 2015).

Sources et voies vers l'environnement marin

Il existe un large éventail de sources entraînant l'accumulation de MP en milieu marin (Auta et al., 2017). Les sphères microplastiques présentes dans les produits cosmétiques tels que les gommages, les dentifrices, les poudres de surfaçage peuvent pénétrer le milieu aquatique via des drainages industriels ou domestiques (Auta et al., 2017). De même, les fibres synthétiques des vêtements produisent des MP qui sont transportés par les usines de traitement de l'eau ou les eaux usées sous forme d'effluents vers l'environnement marin (Murphy et al., 2016). Par exemple, une usine de traitement des eaux usées situé sur la rivière Clyde à Glasgow libère près de 65 millions de MP dans les eaux quotidiennement (Murphy et al., 2016). D'autre part, Gouin et al. (2011) ont indiqué que la population américaine émettait approximativement 263 tonnes par an de MP de polyéthylène, provenant principalement de produits de soins personnels. En outre, les pneus et les marquages routiers peuvent également causer une pollution microplastique ; le ruissellement des eaux pluviales servant de voie de transport pour le transport de particules de pneus dans les eaux de surface (Kole et al., 2017; Unice et al., 2019).

Récemment, il a été montré qu'une fraction substantielle des débris de plastique marins provenait des cours d'eau. Dix fleuves représentant de 88 à 95 % de la décharge en plastique dans l'environnement marin. Ces apports globaux de débris de plastique vers les mers ont été estimés entre 0.41 et 4×10^6 tonnes an⁻¹ (Schmidt et al., 2017). Un autre modèle global estimait qu'entre 1,15 et 2,41 millions de tonnes de déchets plastiques entre chaque année dans l'océan depuis des rivières. Les 20 fleuves les plus polluants, principalement situés en Asie, représentant 67 % du total mondial des rejets (Lebreton et al., 2017). De plus, des études récentes ont démontré que de grandes quantités de fibres avaient été transportées, en particulier dans les zones fortement urbanisées, via les retombées atmosphériques (Dris et al., 2017). Les sources possibles de microplastiques en suspension dans l'air comprennent les fibres synthétiques, le gazon artificiel, les déchets présents dans les décharges et l'incinération des déchets (Dris et al., 2017). Ces particules dans l'atmosphère peuvent être transportées par le vent vers le milieu aquatique. Les eaux de ruissellement provenant des terres agricoles et des zones urbaines sont une autre source importante de charge microplastique dans les eaux de surface marines. Des études récentes suggèrent que l'agriculture est l'une des principales activités anthropiques qui contribuent à la pollution microplastique du sol, à la fois par l'utilisation des boues d'épuration pour l'amendement des sols, et l'utilisation de plastiques agricoles, tels que les paillis de plastique, pour augmenter le rendement des cultures (Akdogan and Guven, 2019; He et al., 2018).

De manière générale, les sources secondaires de microplastiques sont considérées comme un facteur majeur de pollution microplastique, étant donné la grande quantité de déchets de macroplastiques qui

pénètrent dans l'environnement (Geyer et al., 2017). La fragmentation de ces plus gros débris de plastique résultant en MP secondaire pouvant être dispersée dans les milieux aquatiques par différents processus physiques, tels que le vent, les marées, le ruissellement de surface et les inondations (Zhang, 2017). Cependant, les taux de fragmentation du plastique sont largement inconnus et, par conséquent, peu des informations quantitatives sont disponibles sur la contribution relative des MP secondaires de manière générale (Ter Halle et al., 2016).

Composition

L'identification des formes, des tailles et des types de MP est un domaine de recherche important, car il permet de déterminer précisément leurs sources potentielles. En ce qui concerne la forme des MP retrouvés en milieu marin, les fibres sont souvent la forme dominante que ce soit dans les sédiments (Martin et al., 2017; Schwarz et al., 2019), dans les eaux de surface et dans la colonne d'eau (Aytan et al., 2016; Lusher et al., 2015), ainsi que dans le biote (Bellas et al., 2016; Güven et al., 2017). Ensuite ce sont les fragments qui sont les plus présents dans l'environnement marin (Lusher et al., 2015; Martin et al., 2017; Schwarz et al., 2019). Finalement, les sphères représentent entre 2 et 10 % des MP retrouvés dans l'environnement marin (Burns and Boxall, 2018; Schwarz et al., 2019).

De nombreux types de plastiques sont produits dans le monde entier, mais le marché est dominé par 6 types de polymères, désignés plus communément sous le nom de « Big six » regroupant le polyéthylène (PE), le polypropylène (PP), le polychlorure de vinyle (PVC), le polystyrène (PS) et le polystyrène expansé (EPS), le polyéthylène téréphtalate (PET) et le polyuréthane (PUR). Ces six polymères représentent 80 % de la production totale de plastiques en Europe (Plastics Europe, 2016) et sont les types MP les plus fréquemment trouvés dans les environnements marins (Crawford and Quinn, 2016). Une étude menée sur la côte italienne a révélé que les microplastiques les plus prédominants dans les échantillons de sédiments étaient constitués de PE (48 %) et de PP (38 % ; Vianello et al., 2013)), conformément aux polymères les plus produits (Geyer et al., 2017). De manière générale, polyéthylène (PE) et polypropylène (PP) sont actuellement les polymères principalement récupérés dans tous les compartiments environnementaux, conformément à l'échelle de leur fabrication et de leur utilisation mondiales (Geyer et al., 2017).

Distribution et abondance

La distribution spatiale et temporelle des microplastiques, basés sur des données de composition de taille, de forme et de type de polymère dans diverses matrices environnementales sont des éléments essentiels pour quantifier les niveaux d'exposition environnementale des animaux marins et des risques écologiques qui en découlent (Shim and Thomposon, 2015). Depuis leur première détection dans

l'environnement marin dans les années 1970, la présence de MP dans les océans et mers du globe est largement documentée (Geyer et al., 2017; Jambeck et al., 2015; Lusher et al., 2015). La taille des MP (<5 mm) et leur faible densité contribuent à leur transport et distribution généralisés sur de grandes distances grâce aux courants marins (Avio et al., 2017; Wang et al., 2015). Ainsi, les MP marins sont abondants et répandus dans tous les habitats aquatiques à travers le monde (Eriksen et al., 2014; Van Sebille et al., 2015). Les MP sont observés dans les sédiments (Claessens et al., 2011; Martin et al., 2017), à la surface des océans (Cózar et al., 2014; Lusher et al., 2014), dans la colonne d'eau (Lima et al., 2014; Reisser et al., 2014; Zhao et al., 2015) et dans les environnements profonds (Van Cauwenberghe et al., 2013; Woodall et al., 2014). Même les régions reculées comme l'Arctique (Obbard et al., 2014; Peeken et al., 2018) et l'Antarctique (Cincinelli et al., 2017) sont contaminées par les MP.

À l'échelle mondiale, plusieurs études ont modélisé le nombre de plastiques présents à la surface des océans en couplant les données de dénombrement des plastiques avec des modèles de circulations océaniques (Eriksen et al., 2014; Van Sebille et al., 2015). Ces différentes modélisations ont estimé la présence de $5,25 \times 10^{12}$ à 51×10^{12} MP à la surface des océans, représentant de 35 000 à 270 000 tonnes (Eriksen et al., 2014; Van Sebille et al., 2015). Néanmoins, ces estimations ne représenteraient qu'entre 0.1 et 1 % des déchets plastiques entrant dans les océans en 2010 (Jambeck et al., 2015). Cette différence peut s'expliquer par le rôle des phénomènes de photo-dégradation, de biodégradation, de sédimentation, d'ingestion par les organismes et d'échouage des particules sur le littoral qui ne sont pas pris en compte dans certains modèles. Ces modélisations mettent en évidence que les déchets plastiques s'accumulent dans les zones de convergences des courants marins : les gyres (Figure 6). Ainsi, chaque zone d'accumulation des cinq gyres océaniques présente une abondance de MP d'un ordre de grandeur plus élevé que les zones de non-accumulation (Cózar et al., 2014).

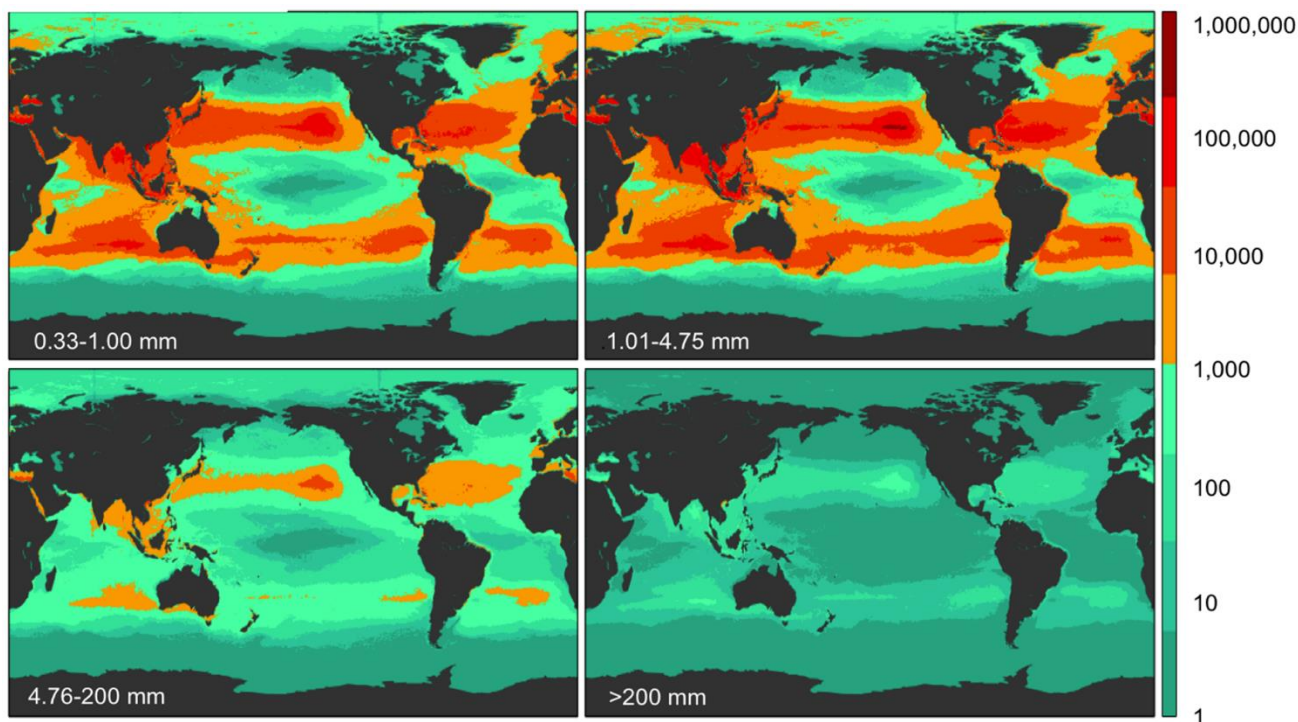


Figure 2. Résultats du modèle pour la densité du nombre de particules plastiques globale dans quatre classes de taille de Eriksen et al. (2014) (unités km^{-2} ; voir la barre de couleur) pour chacune des quatre classes de taille (MP : 0,33-1,00 mm et 1,01 -4,75 mm, Mesoplastiques et Macroplastiques : 4,76-200 mm et > 200 mm).

De manière générale, parmi les valeurs de quantification des MP en eau de mer, les plus élevées ont été trouvées dans le sud de la mer du Nord ($1\,700\,000\text{ MP m}^{-3}$, $8,5\text{ mg L}^{-1}$) pour des débris $> 80\ \mu\text{m}$ (Dubaiash and Liebezeit, 2013), en Corée du Sud ($15\,560\text{ MP m}^{-3}$; $0,0778\text{ mg L}^{-1}$) pour les débris $> 333\ \mu\text{m}$ (Song et al., 2014), et dans une baie suédoise ($2\,400\text{ items MP m}^{-3}$; $0,012\text{ mg L}^{-1}$) pour des débris $> 80\ \mu\text{m}$ (Norén, 2007). Cependant, la concentration typique de MP en milieu marin est comprise entre $0,01$ et 10 MP L^{-1} ou $0,0015$ et $500\ \mu\text{g L}^{-1}$ (Fig. 3). La détection et quantification des MP $< 50\ \mu\text{m}$ et des NP en milieu naturel reste actuellement un défi technique et peu de quantification sont actuellement disponibles sur ces classes de tailles. Néanmoins, des études prometteuses sont en cours, avec une première démonstration récente de NP (compris entre 1 et 999 nm) dans des échantillons d'eau de mer naturelle collectés dans le gyre subtropical de l'Atlantique Nord (Ter Halle et al., 2017). De même, des publications récentes ont démontré un pourcentage élevé (80 %) de petits MP (25–50 μm) dans les eaux de surface ou sédiment, comparé aux particules de grande taille (Bergmann et al., 2017; Enders et al., 2015). Finalement, une augmentation proportionnelle du nombre particules à la taille décroissante des particules de plastiques a récemment été démontrée (Erni-Cassola et al., 2017).

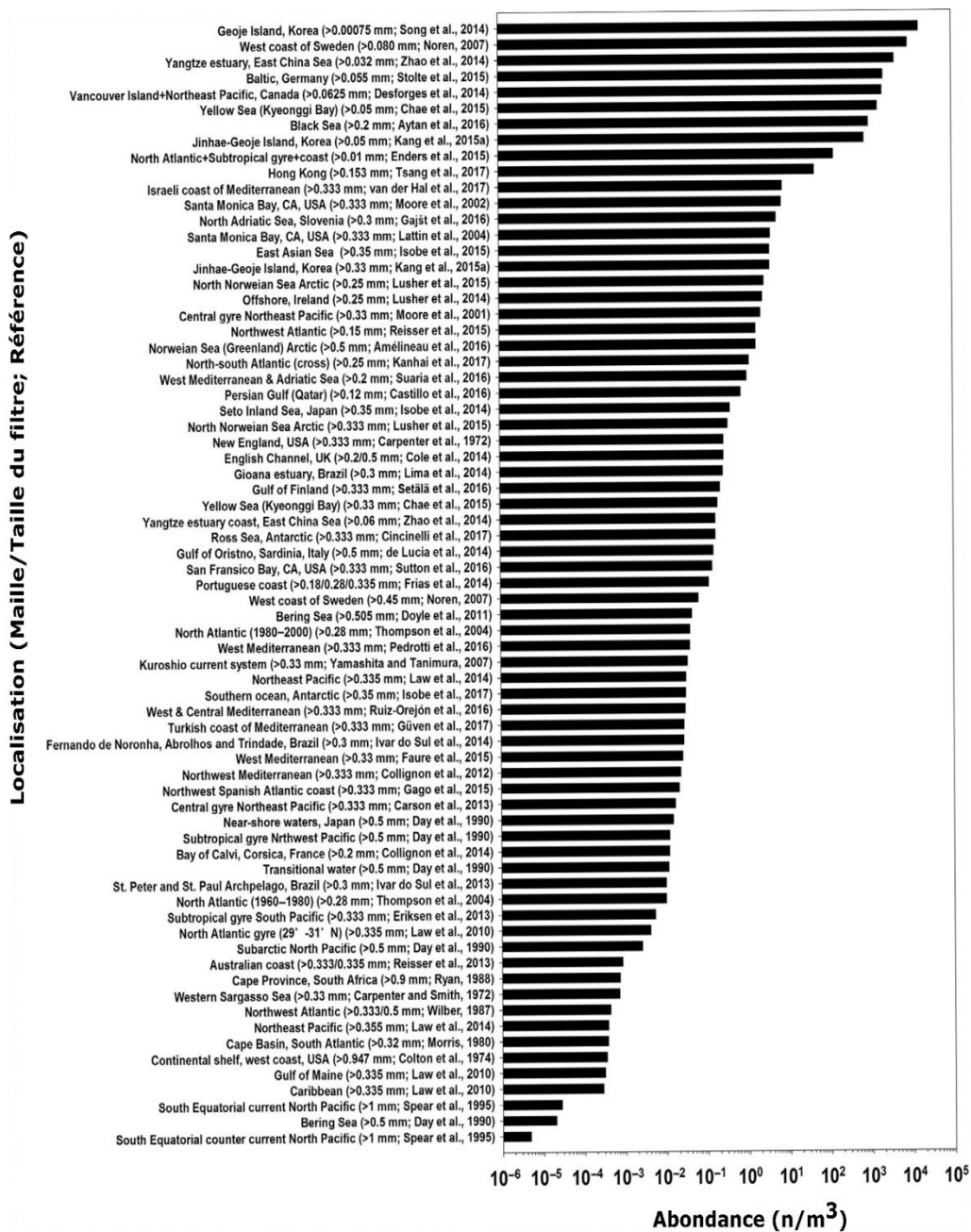


Figure 3. Abondance moyenne des microplastiques dans les océans et mers du globe (adaptée de Shim et al., 2017)

Interaction des MP avec les animaux marins

Ingestion, distribution et expulsion

Le potentiel des MP à nuire aux organismes marins est régi par la sensibilité des espèces à ingérer et/ou interagir avec eux (Kärrman et al., 2016). Ainsi, l'ingestion de MP par les organismes marins représente une préoccupation croissante en raison des risques toxicologiques associés à ces microparticules. La faible dimension des MP augmente leurs biodisponibilités pour les organismes

aquatiques et de nombreuses études ont prouvé l'ingestion de MP en détectant différents types de particules de polymère dans les intestins et l'estomac de divers organismes, tels que les zooplanctons, mollusques et crustacés, coraux, poissons et mammifères marins (Jabeen et al., 2017; Nelms et al., 2018; Reichert et al., 2018; Rintoul and Hoogenboom, 2015; Setälä et al., 2018, 2014; Tosetto et al., 2016; Vendel et al., 2017).

L'ingestion peut être directement imputable à une mauvaise identification ou à une consommation aveugle de MP via transfert trophique. La confusion avec des proies a été démontrée sur le terrain et en laboratoire chez différentes espèces (de Sá et al., 2018a, 2015; Ory et al., 2017; Santana et al., 2017). En laboratoire, par exemple, de Sá et al. (2015), ont offert 3 types de MP de polyéthylène de différentes couleurs seules et en association avec des proies (*Artemia nauplii*) à des gobies communs et ont observé une ingestion des MP seulement lors de l'association avec les proies, suggérant une confusion avec de la nourriture. Similairement, le poisson planctonivore (*Seriolella violacea*) a préférentiellement capturé des MP noirs, probablement parce qu'ils sont plus similaires aux granulés alimentaires, alors que les MP d'autres couleurs (bleu, translucide et jaune) n'ont été cocapturés que lorsqu'ils flottaient près de granulés alimentaires (Ory et al., 2017). En revanche, un exemple d'évitement de MP, probablement dû à une reconnaissance, est fourni par une étude de laboratoire dans laquelle des poissons-zèbres (*Danio rerio*) exposés à des MP ont pu les reconnaître comme « non-aliment » et les recracher (Kim et al., 2019). Des preuves d'ingestion dues à des signaux chimiques sont rapportées chez de nombreux animaux marins notamment des cnidaires (Allen et al., 2017), des mollusques bivalves (Bråte et al., 2018) et des poissons (Savoca et al., 2017). Par conséquent, les MP colonisés et longuement présents dans l'environnement marin semblent préférés aux MP vierges par les organismes aquatiques (Vroom et al., 2017).

Les microplastiques peuvent également être ingérés lorsque des prédateurs consomment des proies contaminées (Au et al., 2017). Ainsi, de nombreuses études de laboratoire et de terrain ont apporté des preuves convaincantes du transfert trophique de MP en milieu marin. Par exemple, Setälä et al., 2014 ont démontré la présence et le transfert de MP le long du réseau trophique planctonique en examinant des échantillons de zooplancton prélevés dans la mer Baltique. Nelms et al. (2018) ont observé le transfert trophique de microplastiques de maquereau (*Scomber scombrus*) capturés dans la mer Celtique vers des phoques gris (*Halichoerus grypus*). Le transfert de microplastiques dans la chaîne alimentaire a également été confirmé dans des conditions de laboratoire de la moule (*Perna perna*) au crabe (*Callinectes ornatus*) et au poisson-globe (*Spheroeroides greeleyi*) (Santana et al., 2017), ainsi que d'algues (*Fucus vesiculosus*) aux bigorneaux (*Littorina littorea*) (Gutow et al., 2016). Ainsi, les résultats rapportés suggèrent que le transfert trophique représente potentiellement une voie d'accumulation importante de MP chez les organismes marins.

De manière générale, il était assumé que les MP s'accumulent dans les organismes aquatiques uniquement au niveau des tissus en contact direct avec l'eau, tels que les tissus gastro-intestinaux et respiratoires. Cependant, au cours de ces dernières années de nombreuses preuves scientifiques s'accumulent et montrent que d'autres tissus peuvent être impactés par l'accumulation de particules, par des processus de translocation, élargissant ainsi les impacts potentiels des MP sur diverses fonctions physiologiques. Chez les mollusques par exemple, Browne et al. (2008) ont d'abord montré que chez la moule (*M. edulis*), la taille des MP influençait leurs migrations de la cavité intestinale vers l'hémolymphe, avec un pourcentage plus élevé (60 %) de MP plus petits (3 μm) dans le liquide circulatoire que de plus gros (9,6 μm). Par ailleurs, l'accumulation par adhérence et translocation de MP dans plusieurs organes de moules en milieu naturel a été récemment rapportée par Kolandhasamy et al. (2018).

Chez les poissons, bien que la recherche et quantification de MP se soit principalement focalisée sur le tube digestif (de Sá et al., 2018; Tanaka and Takada, 2016), le foie a fait l'objet d'une recherche sur la présence de MP chez poissons zooplanctivores commerciaux capturés dans la mer Méditerranée (golfe du Lion, France), notamment l'anchois européen (*Engraulis encrasicolus*), la sardine (*Sardina pilchardus*) et le hareng de l'Atlantique (*Clupea harengus*) (Collard et al., 2017). Les résultats ont révélé que les MP, principalement des PE, ont été transférés dans le foie des trois espèces. Dans l'anchois, 80 % des foies contenaient des MP de tailles relativement importantes (124 - 438 μm), montrant un niveau élevé de contamination. Contrairement, aucun MP > 20 μm n'ont été détectés dans le foie et les muscles de spécimen de bar asiatique (*Lateolabrax maculatus*) échantillonné dans des estuaires chinois, alors que leur prévalence dans les intestins et les branchies a été confirmée (Su et al., 2019). Ces études sur les populations de poissons sauvages fournissent des informations importantes quant au sort des MP une fois ingérés par les poissons, cependant certains facteurs liés à l'extraction des MP environnementaux rendent leurs résultats imprécis et difficiles à extrapoler. De ce fait, des expériences de laboratoire avec des espèces de poissons modèles et des MP marqués par fluorescence ont été mises en place et ont permis l'identification de voies et de facteurs spécifiques promouvant la translocation de MP vers certains organes.

Par exemple, la distribution tissulaire des MP chez le poisson-zèbre serait dépendante de leurs tailles. En quantifiant l'accumulation de MP de PS après une exposition de 7 jours à des particules de 5 μm et 20 μm (Lu et al., 2016) ont retrouvé les MP de 5 μm dans les branchies, les intestins et le foie des poissons, tandis que les MP de 20 μm ne se sont accumulés que dans les branchies et les intestins. Les auteurs ont donc suggéré la possibilité d'un transfert des plus petits MP (5 μm) vers le foie par le biais du système circulatoire. Kashiwada (2006) a par ailleurs montré que le poisson medaka (*Oryzias latipes*) accumule des NP de PS de 39,4 nm principalement dans les branchies et l'intestin, mais aussi

dans le cerveau, les testicules, le foie et le sang. L'accumulation de NP dans le cerveau a été aussi observée chez la carpe (*Carassius carassius*) (Mattsson et al., 2017) et chez le tilapia rouge (*Oreochromis niloticus*) (Ding et al., 2018). Ces études démontrent que le potentiel de bioaccumulation des MP/NP augmente alors que leur taille décroît, cependant les mécanismes sous-jacents restent à l'heure actuelle inconnus. Finalement, même si certaines tailles de MP/NP peuvent être bioaccumulées et/ou bloquées dans le système digestif, le taux d'excrétion de ces particules est un facteur prépondérant afin de déterminer le temps de résidence de ces particules dans les organismes et interpréter leurs effets écotoxicologiques chez les organismes marins. Ainsi, chez les poissons, il existe des preuves d'une excrétion efficace et rapide des MP chez le poisson rouge MP (Jabeen et al., 2018) et chez les larves du loup (*Dicentrarchus labrax*) (Mazurais et al., 2015).

Effets physiologiques des MP sur les animaux marins

Il n'y a plus aucun doute que les MP/NP sont accidentellement et/ou sélectivement ingérés par les organismes aquatiques, le champ d'investigation s'intéresse donc maintenant à élucider les effets biologiques de l'ingestion constante de ces polluants par les organismes marins. Les MP sont particulièrement préoccupants en tant que « contaminants émergents », puisque leur potentiel de bioaccumulation augmente alors que leur taille décroît, indiquant un risque d'exposition généralisé pour la santé des animaux marins (de Sá et al., 2018 ; Wang et al., 2019). Il y a relativement peu de consensus sur les impacts biologiques des MP. En effet, s'il est clair que les grosses particules de plastique (c'est-à-dire les méso- et macroplastiques) peuvent causer des effets facilement visibles au niveau de l'organisme, tel que la suffocation, enchevêtrement ou blocage de l'intestin (Gregory, 2009), les effets physiologiques directs et indirects des particules de petite taille (MP/NP) sur les animaux aquatiques restent difficiles à saisir.

En effet, les recherches écotoxicologiques sur les effets des MP restent peu nombreuses et sont confrontées à de nombreuses difficultés comme le manque de connaissances de leurs comportement, composition et quantité en milieux naturels et la complexité de reproduire une dispersion « naturelle » de ces particules en aquarium (Karami, 2017). Néanmoins, les effets écotoxicologiques des MP sur la faune aquatique marine sont rapportés dans de plus en plus d'études de laboratoire depuis quelques années, utilisant des taxons marins et d'eau douce représentant différents niveaux trophiques aquatiques (Fig. 2). Une fois que les MP/NP sont ingérés par les animaux aquatiques, les effets les plus directs sont certainement dus à l'accumulation de ces particules inertes dans le système digestif des organismes (Wright et al., 2013). Les MP/NP ingérés peuvent s'accumuler et même bloquer les voies digestives des animaux aquatiques, ce qui entraîne une diminution de l'élan alimentaire en raison d'une fausse satiété (de Sá et al., 2018). En outre, du fait de leur petite taille, la possibilité pour ces minuscules

plastiques de pénétrer dans les systèmes circulatoires et phagocytaires et cellulaires des organismes exposés augmente, ce qui peut ainsi introduire des dommages supplémentaires pour les organismes en raison de la bioaccumulation des MP/NP dans leur organisme (Lu et al., 2016). Ainsi, l'ingestion de MP/NP peut également avoir d'autres effets néfastes, de la cellule à l'organisme tout entier, qui sont plus difficiles à quantifier et dont les mécanismes sous-jacents restent compliqués à appréhender. Ces effets chez les organismes marins incluent le stress oxydatif, des altérations du comportement, du métabolisme, du système enzymatiques ou encore du microbiome intestinal (Franzellitti et al., 2019).

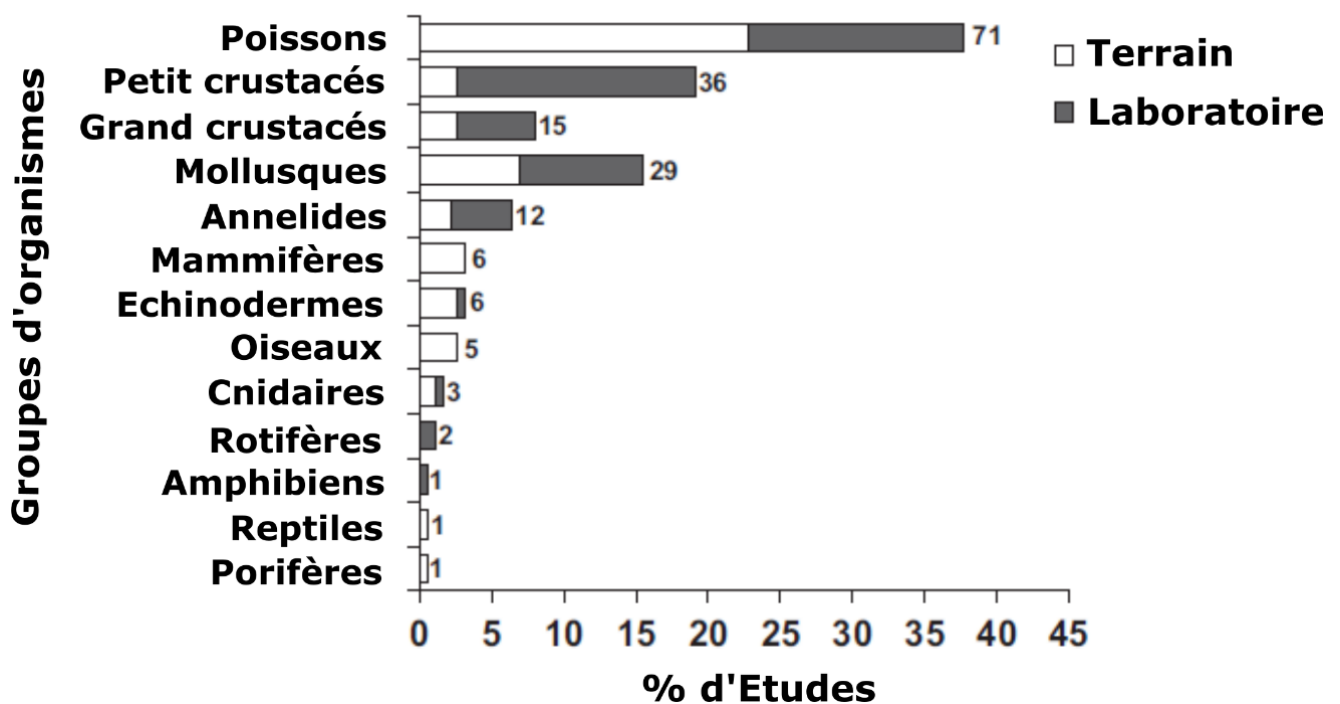


Figure 4. Nombre d'études s'intéressant à la contamination et aux effets des MP chez différents groupes d'organismes (adaptée de (de Sá et al., 2018)).

Ainsi, de nombreuses revues ont synthétisé ces effets biologiques au cours de ces dernières années (Burns and Boxall, 2018; Chae and An, 2017; de Sá et al., 2018; Foley et al., 2018; Franzellitti et al., 2019; Jovanović, 2017; Sharma and Chatterjee, 2017; Syberg et al., 2015; Stephanie L. Wright et al., 2013) qui, dans l'ensemble, montrent des effets hétérogènes, difficiles à généraliser des MP/NP et un manque général de connaissance de leurs effets sur différents taxons marins. Il n'en est pas moins clair que leurs effets potentiels sur le biote marin requièrent une investigation plus poussée. Parmi les taxons étudiés en milieux aquatiques, les poissons ont reçu une attention particulière (Fig 4.), du fait de leur abondance, et de leur valeur sociétale et économique. En effet, à l'échelle mondiale, plus de 250 millions de personnes dépendent directement de la pêche et de l'aquaculture pour leur subsistance (Thilsted et al., 2016). Malgré cela, comme pour d'autres taxon marins, les études des effets

écotoxicologiques des MP/NP sur les poissons restent dans leurs balbutiements et rapportent une variété d'effets à plusieurs niveaux d'organisation biologiques difficiles à cerner (Foley et al., 2018).

Effet des micro- et nano-plastiques vierges sur les poissons : Tendances, méta-analyses et perspectives

Avant-propos

La pollution plastique d'origine environnementale est aujourd'hui une préoccupation environnementale et sociétale majeure, en particulier dans les écosystèmes aquatiques. Au cours des 10 dernières années, de nombreuses études expérimentales et de laboratoire ont complété les observations de terrain dans l'espoir de mieux comprendre le devenir et l'impact des micro- et/ou nano-plastiques (MP / NP) sur divers organismes (par exemple : oiseaux, poissons et mammifères). Ce domaine de recherche en plein essor a également généré plusieurs revues visant à résumer les effets du MP / NP sur le biote, mais ces analyses n'ont fait aucune distinction entre MP / NP vierge et MP / NP non vierge. Par conséquent, ces examens ne permettent pas de distinguer les effets du plastique lui-même par rapport au rôle de ses additifs, biofilms et contaminants associés. De plus, aucune méta-analyse approfondie des effets du MP / NP sur les fonctions biologiques du poisson n'existe à l'heure actuelle. Ainsi, cette revue a pour but de clarifier les effets des MP / NP vierges chez les poissons marins. Cette revue présente ainsi l'ensemble de la littérature scientifique liée à mon sujet de thèse « Effet des microplastiques sur les jeunes stades de vie des poissons marins » et sert de référence au lecteur afin d'appréhender cette problématique.

Abstract

Environmental plastic pollution is a major ecological and societal concern today. Over the last 10 years, a broad range of laboratory and experimental studies have complemented field observations in the hope of achieving a better understanding of the fate and impact of micro- and/or nano-plastics (MP/NP) on diverse organisms (e.g. birds, fish and mammals). However, plastic pollution remains challenging to monitor in the environment and to control under laboratory conditions, and plastic particles are often naturally or experimentally co-contaminated with diverse chemical pollutants. Therefore, our understanding of the effects of virgin MP/NP in freshwater and marine fish is still limited. Here, we perform a systematic review of the most up-to-date literature on the effects of virgin MP/NP on fish under laboratory conditions. A total of 782 biological endpoints investigated in 46 studies were extracted. Among these endpoints, 32% were significantly affected by exposure to virgin MP/NP. Most significant effects were observed for smaller plastic particles (i.e. < 20 μm), affecting fish behavioral and neurological functions, intestinal permeability, metabolism and intestinal microbiome diversity. In addition, we propose suggestions for new research directions to lead towards innovative, robust and scientifically sound experiments in this field. This review of experimental studies reveals that the toxicity of virgin MP/NP on fish should be more systematically evaluated using rigorous laboratory-based methods, and aims towards a better understanding of the underlying mechanisms of this toxicity to fish.

Keywords: Marine litter; microplastic; nanoplastic; ecotoxicology; teleost fish

Introduction

Plastics are durable, inexpensive, lightweight and have insulation and resistance properties that offer many medical, societal, and technological benefits. For these reasons, the global production of plastics has increased from 2 Million tonnes (Mt) in 1950 to almost 350 Mt in 2017 (Plastics Europe, 2017) and is expected to reach 1800 Mt in 2050 (Ryan, 2015). In parallel, 60% of all the plastics produced from 1950 to 2015 (i.e. 4900 Mt) were discarded in landfills and in the natural environment (Geyer et al., 2017), and 3.4 Billion tonnes are expected to be disposed of in the next 30 years (Kaza et al., 2018). Given their intrinsic characteristics, these plastics rather fragment than decompose, are easily transported, and persist and accumulate in the environment. As a result, plastic pollution has been reported in all ecosystems on the planet (Bergmann et al., 2019, 2017; Chiba et al., 2018; Rochman, 2018). Aquatic ecosystems are particularly affected, with for example around 2 Mt of plastic waste that are estimated to flow from rivers to oceans every year (Lebreton et al., 2017). This increasing occurrence of plastics in the aquatic environment along with its increasing production makes plastic pollution a major ecological and societal concern today.

This concern is even higher when considering smaller plastic particles, i.e. microplastics (MP), defined typically as particles below 5 mm (Moore, 2008), and nanoplastics (NP), defined as particles below 100 nm (EFSA, 2009), as they can translocate in tissues and propagate up the food chain (Galloway et al., 2017; Kashiwada, 2006; Lu et al., 2016). The occurrence of these small plastic particles into the environment can result from (i) the release of primary MP/NP, e.g. via domestic and industrial effluents, spills, sewage discharge and run-offs of particles from the cosmetics and pharmaceutical industries, or (ii) the degradation/fragmentation (e.g. abrasion, bio-erosion, photo-oxidation) of larger plastic particles already present in the environment, and resulting in the formation of secondary MP/NP (Cole et al., 2011). MP/NP can have a wide range of shapes (e.g. fiber, film, fragment, sphere) and densities that influence their behavior in the aquatic environment, such as their dispersion in the different aquatic compartments (water surface, water column and sediment) and their availability to organisms at different trophic levels and/or occupying different habitats (Auta et al., 2017).

The number of experimental studies that examined the effects of MP/NP on aquatic organisms has sharply increased over the past few years, reporting a large variety of neutral and deleterious effects in various species (Sharma and Chatterjee, 2017; Wang et al., 2019; Stephanie L Wright et al., 2013). However, the ecotoxicology of MP/NP is still an immature area of research and subject to several experimental challenges. MP/NP usually consist of complex and dynamic mixtures of polymers and additives, to which organic material and contaminants can bind (Koelmans et al., 2016). Therefore, it is often challenging to discriminate the impacts of the plastic polymers *per se* from the impacts of their

associated-compounds. In this context, all past reviews have summarized the effects of MP/NP on aquatic organisms without discriminating between the effects of virgin MP/NP and the effects of their associated contaminants and biofilms (Chae and An, 2017; de Sá et al., 2018; Foley et al., 2018; Franzellitti et al., 2019). Therefore, it is urgent to investigate the ecotoxicology of virgin MP/NP for a comprehensive assessment of their hazard(s) on aquatic biota. Also, no review to-date has conducted an in-depth analysis of the effects of MP/NP on fish biological functions and systems (Foley et al., 2018), while fish being the most studied group of aquatic organisms in the MP/NP literature (de Sá et al., 2018).

Here, we review the literature that examined, experimentally and under laboratory-controlled conditions, the effects of virgin MP/NP in fish. From these studies, we conduct a meta-analysis investigating the importance of the exposure medium (via food *vs.* water), as well as the size and concentration of virgin MP/NP, on 33 fish biological functions and systems. Through this comprehensive analysis of the current research on the effects of virgin MP/NP on fish, we also highlight research gaps, discuss methods, identify recommendations and perspectives for future research, and provide an updated baseline for scientists working in this field.

Material and methods

Literature search

Literature search was performed to list all the available experimental studies published before March 2019, which focused on the effects of virgin micro- and/or nano-plastics (MP/NP) on fish. The following databases were used for this search: Elsevier, Google Scholar, Scopus, and Web of Science. Non-English records were excluded. Studies were selected according to the following criteria: 1) the study corresponds to experiment(s) performed under laboratory-controlled conditions, 2) the study examines at least one toxicological *in vivo* effect of direct exposure to virgin MP/NP on fish at the larval, juvenile or adult stage, 3) the study includes a “no microplastics” control treatment, and 4) the results of the study are supported by appropriate statistics. The completeness of the results obtained was considered as satisfactory based on “snowballing” (i.e. checking citations on reference lists of relevant articles until no further relevant articles could be found) (Sayers, 2007).

Main data extraction

For each study selected during the literature search, the following information was extracted: fish species, fish life stage(s), fish feeding strategy(ies), fish environment(s), MP/NP type(s), MP/NP shape(s), MP/NP size(s), MP/NP concentration(s), exposure pathway(s), exposure maximum duration(s), and presence of co-contaminant(s).

Size and concentration classes

To examine the possible effects of MP/NP size and concentration on fish, a size class and a concentration class were attributed to each study. Seven MP/NP size classes were chosen: Class 1 for sizes $\leq 0.1 \mu\text{m}$; Class 2 for $0.1 \mu\text{m} < \text{sizes} \leq 20 \mu\text{m}$; Class 3 for $20 \mu\text{m} < \text{sizes} \leq 50 \mu\text{m}$; Class 4 for $50 \mu\text{m} < \text{sizes} \leq 100 \mu\text{m}$; Class 5 for $100 \mu\text{m} < \text{sizes} \leq 300 \mu\text{m}$; Class 6 for sizes $> 300 \mu\text{m}$; and miscellaneous (MISC) for sizes encompassing at least 2 of the size classes described above. Size class 1 corresponds to NP (EFSA, 2009), size class 2 corresponds to MP that could pass through an epithelium barrier, (Deng et al., 2017; Lu et al., 2016a) and size class 6 corresponds to MP within the size range of most environmental quantifications performed during field surveys in the recent years (Paul-Pont et al., 2018). Size classes 3, 4, and 5 were then chosen arbitrarily to get homogeneous distributions of endpoints between classes (see endpoints explanations below). Similarly, different MP/NP concentration classes were chosen, depending on whether fish were exposed to the MP/NP via water or via food, and depending on whether mass or particle concentration were considered. In this regard, concentration standardization was required. When applicable, MP/NP concentration in the water was converted in $\mu\text{g L}^{-1}$ (mass concentration) and number of particles L^{-1} (particle concentration), while MP/NP concentration in the food was converted in $\text{mg g}^{-1} \text{day}^{-1}$ (i.e. mg of food per gram of fish per day, mass concentration) and particle $\text{g}^{-1} \text{day}^{-1}$ (particle concentration). Information about the MP/NP mass and particle concentrations that were used were available in only 17 out of the 46 studies. For these studies, concentration standardization was therefore trivial. For 1 out of the 46 studies, only information about the MP/NP particle concentration was available. For this study, we estimated the MP/NP mass concentration according to the size and the density of the MP/NP that were used. For 22 out of the 46 studies, only information about the MP/NP mass concentration was available. For 20 out of these 22 studies, we estimated the MP/NP particle concentration according to the size and the density of the MP/NP that were used. For the other two studies, this estimation was not possible given the absence of details concerning the MP/NP density. The particle concentration classes of these studies were therefore marked as MISC. For 6 out of the 46 studies, concentration standardization was not possible given the lack of information provided by the authors. For example, some authors exposed preys to known concentrations of MP/NP, but then they did not quantify the amount of MP/NP within the preys. As fish were exposed to MP/NP by feeding on these preys, the absence of quantification of MP/NP within the preys does not permit to establish the concentration of MP/NP to which fish were exposed. For these 6 studies, MP/NP mass and particle concentrations were both marked as MISC. When concentration standardization was possible (whether the information was provided by authors or estimated by us), we established MP/NP concentration classes arbitrarily, in order to get homogeneous distributions of endpoints between classes (see endpoints explanation below). Four classes for MP/NP mass concentration in water were chosen: Class 1 for concentrations

$\leq 50 \mu\text{g L}^{-1}$; Class 2 for $50 \mu\text{g L}^{-1} < \text{concentrations} \leq 200 \mu\text{g L}^{-1}$; Class 3 for $200 \mu\text{g L}^{-1} < \text{concentrations} \leq 700 \mu\text{g L}^{-1}$; and Class 4 for concentrations $> 700 \mu\text{g L}^{-1}$. Five classes for MP/NP particle concentration in water were chosen: Class 1 for concentrations $\leq 10^2 \text{ particle L}^{-1}$; Class 2 for $10^2 \text{ particle L}^{-1} < \text{concentrations} \leq 10^3 \text{ particle L}^{-1}$; Class 3 for $10^3 \text{ particle L}^{-1} < \text{concentrations} \leq 10^6 \text{ particle L}^{-1}$; Class 4 for $10^6 \text{ particle L}^{-1} < \text{concentrations} \leq 10^9 \text{ particle L}^{-1}$; and Class 5 for concentrations $> 10^9 \text{ particle L}^{-1}$. Three classes for MP/NP mass concentration in food were chosen: Class 1 for concentrations $\leq 0.05 \text{ mg g}^{-1} \text{ day}^{-1}$; Class 2 for $0.05 \text{ mg g}^{-1} \text{ day}^{-1} < \text{concentrations} \leq 0.25 \text{ mg g}^{-1} \text{ day}^{-1}$; and Class 3 for concentrations $> 0.25 \text{ mg g}^{-1} \text{ day}^{-1}$. Three classes for MP/NP particle concentration in food were chosen: Class 1 for concentrations $\leq 1 \text{ particle g}^{-1} \text{ day}^{-1}$; Class 2 for $1 \text{ particle g}^{-1} \text{ day}^{-1} < \text{concentrations} \leq 10^6 \text{ particle g}^{-1} \text{ day}^{-1}$; and Class 3 for concentrations $> 10^6 \text{ particle g}^{-1} \text{ day}^{-1}$. All size and concentration classes are summarized in Fig. 1.

Parameters	Exposures	Types	Units	Classes
Size	/	/	μm	
Concentration	Water	Mass	$\mu\text{g.L}^{-1}$	
		Particle	particle.L^{-1}	
	Food	Mass	$\text{mg.g}^{-1}.\text{day}^{-1}$	
		Particle	$\text{particle.g}^{-1}.\text{day}^{-1}$	

Figure 5. MP/NP size and concentration classes

This figure summarizes all the MP/NP size and concentration classes used in this review. MISC indicates miscellaneous classes, i.e. when polymer sizes encompassed two or more size classes, or when polymer concentration was not provided by the authors or not possible to standardize. Food concentrations are expressed in mg (mass concentration) or particle (particle concentration) per gram of fish per day.

Endpoints

For each study, we identified all endpoints (i.e. all the analyses that were performed) and attributed them to a category based on their biological significance. When an endpoint could have two or more biological meanings, we attributed it to a single category based on the focus adopted by the authors in their study. A total of 33 categories were established, divided into 7 main categories (see the list below). For each endpoint, we then reported the occurrence of a significant effect of MP/NP (YES if

the value for this endpoint was significantly different from the value of the associated control treatment ($p < 0.05$); NO elsewhere). The endpoint categories are:

- Alimentary and Excretory Systems: digestive enzymes (e.g. trypsin level); digestive tract histopathology (e.g. tissue damage score); hepatic and renal enzymes and products (e.g. AST level); intestinal permeability (e.g. DAO level).
- Behavioral, sensory and neuromuscular functions: activity and locomotion (e.g. swimming speed); aggressivity (e.g. number of aggressive interactions); boldness and exploration (e.g. percentage of area travelled); feeding (e.g. foraging activity); nervous system (e.g. acetylcholinesterase activity); shoaling (e.g. distance between individuals); vision (e.g. *zfrho* expression level).
- Circulatory and respiratory systems: blood components (e.g. Na^+ blood concentration); cardiac characteristics (e.g. heart rate); gills histopathology (e.g. tissue damage score); respiration (e.g. oxygen consumption).
- Fitness: body condition (e.g. Fulton's K condition factor); growth (e.g. weight change); mortality (e.g. survival rate); reproduction investment and success (e.g. fertilization success); sex hormones and endocrinology (e.g. *ER α* expression level).
- Immune system: chemokines and cytokines (e.g. *IL1 β* expression level); immune system cells (e.g. lymphocyte counting); other molecular actors (e.g. *casp3* expression level).
- Metabolism: carbohydrate metabolism (e.g. glucose blood concentration); detoxification (e.g. *cyp1a* expression level); general metabolism (e.g. metabolites abundance by NMR); lipid metabolism (e.g. cholesterol level); oxidative stress (e.g. CAT level).
- Microbiome: Actinobacteria (e.g. abundance by qPCR); Bacteroidetes (e.g. abundance by qPCR); Firmicutes (e.g. abundance by qPCR); Proteobacteria (e.g. abundance by qPCR); total diversity (e.g. Shannon diversity index).

Database construction

A bibliographic database was assembled to archive all the endpoints from all the publications selected during the literature search, along with all their associated-information, as described in the subsection 2.2. to 2.4 (Data S1).

Results and Discussion

General trends in the experimental research of the effects of virgin MP/NP in fish

Literature trend

We identified a total of 46 research articles, published before March 2019, which experimentally exposed fish species to virgin micro- and/or nano-plastics (MP/NP). Since the first study was published in 2011, this new area of research has received increasing interest with 20 articles published in 2018 (Fig. 2A). This trend will very likely continue given the growing occurrence of plastic pollution worldwide, public awareness, and scientific interest and funding (Sharma and Chatterjee, 2017).

Fish characteristics

Freshwater fish were the most studied species (53%), followed by marine (38%) and amphidromous (9%) species (Fig. 2B). This trend is partly explained by the many (30%) studies that focused on the zebrafish, *Danio rerio*, a species commonly used in ecotoxicological studies (Li and Uitto, 2014). Using the same species facilitates intercomparisons between studies, and the use of zebrafish has many advantages: they are inexpensive and easy to rear, they have rapid reproductive and growth rate, and numerous molecular and genetic tools are available for this species (Bambino and Chu, 2017). However, using other model and non-model fish species is greatly needed to broaden our understanding of the effects of virgin MP/NP on fish, as response to such stressors can be species-specific (Anttila et al., 2015; Couturier et al., 2013; Reichert et al., 2019).

Fish that were studied were mainly omnivorous (45%), followed by carnivorous (43%), herbivorous (8%) and planktivorous (4%) (Fig. 2C). Although the occurrence of plastic in fish diet is a recent ecological issue, fish are used to ingest indigestible materials on a daily basis, e.g. sediment, shell debris, fish scales and bones (Hyslop, 1980). Nevertheless, the type of diet could influence fish sensitivity to MP/NP. For example, as herbivores usually feed on softer food forms compared to carnivores and omnivores, and as they possess the highest intestinal area and longest digestion duration among all feeding strategies (Becker et al., 2010; Smith, 1980), they might be more sensitive to ingested MP/NP. Future experiments should therefore put a greater emphasis on herbivorous species, which are currently under-represented in the literature.

When looking at life-stages, we observed that adults (46%) were more investigated than juveniles (37%) and larvae (17%) (Fig. 2D). This focus on older life stages can be explained by husbandry facilities, but as that early-life stages can ingest MP/NP (Steer et al., 2017) and are particularly sensitive to environmental disturbances (Mohammed, 2013), there is a crucial need to address the effect of virgin MP/NP on early-life stages in future research.

MP/NP characteristics

The virgin MP/NP that have been used in ecotoxicological studies on fish are mainly particles of polyethylene (PE; 34.5%), polystyrene (PS; 34.5%) and Polyvinyl chloride (PVC; 8%) (Fig. 2E). Non-fiber polymers that are the most produced globally and therefore the most frequently recovered in the environment are PE, polyethylene terephthalate (PET), polypropylene (PP), PS, PVC and polyurethane (PUR) (Geyer et al., 2017). Future research should therefore put greater efforts on examining the effects of PET, PP, PUR, or mixtures of polymer types, which have received only little or no attention (Fig. 2E). Only three studies compared the effects of different types of polymers on fish (Jabeen et al., 2018; Jovanović et al., 2018; Lei et al., 2018a), but these same studies used different particles sizes and shapes, preventing a clear comparison between polymer types. This lack of diversity in terms of polymer types that were investigated in the literature is probably due to the fact that (i) most, if not all, commercially available virgin MP/NP are to date only composed of PE and PS (for example, all virgin NP commercially available are only made of PS); and (ii) certain size ranges are not always commercially available. Lastly, experimentalists should avoid MP/NP whose composition is unknown, not available or under embargo (Barboza et al., 2018d, 2018b, 2018a).

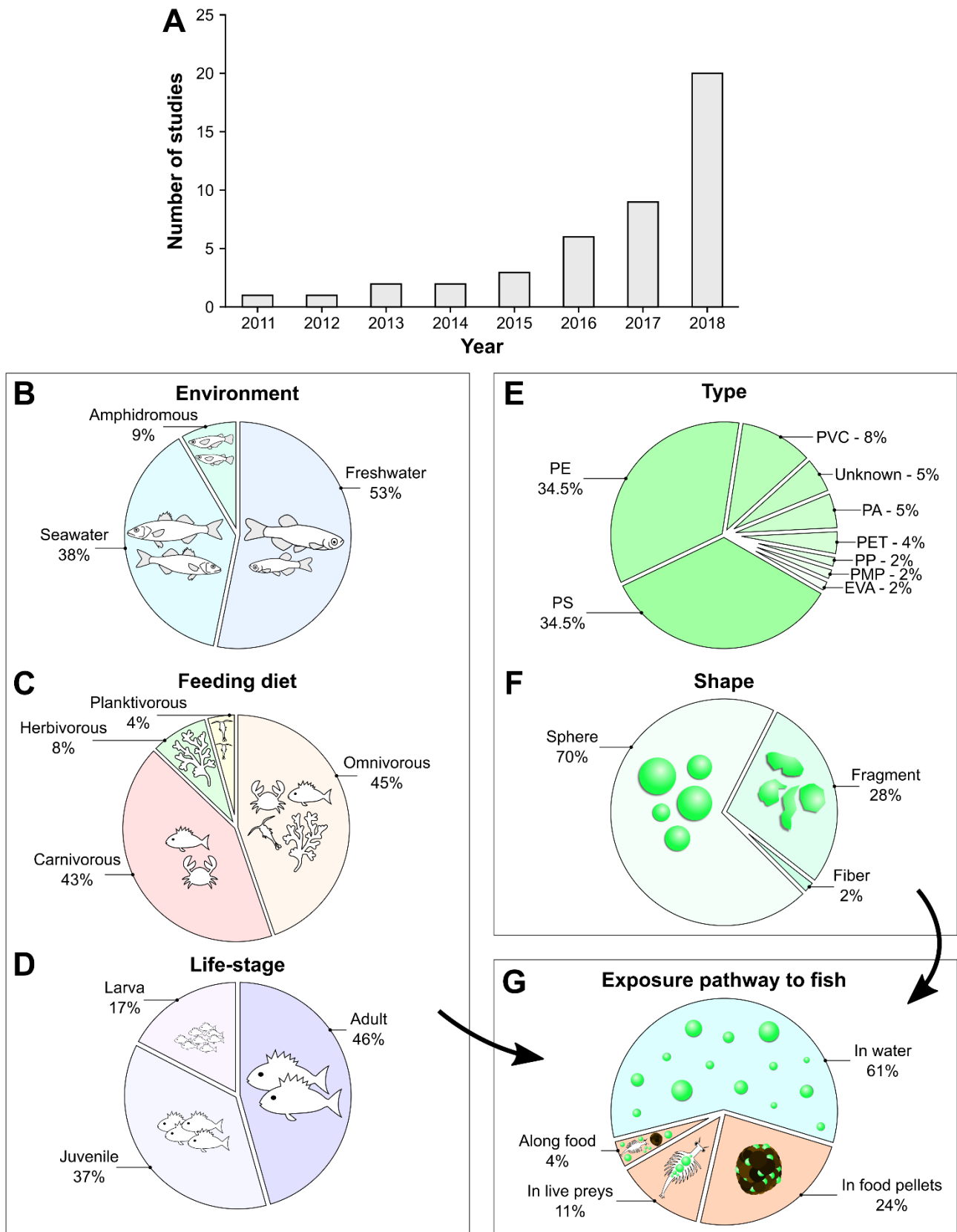


Figure 6. General trends in the experimental research on the effects of virgin MP/NP on fish
 (A) Number of studies published before 2019 that experimentally examined the effects of virgin MP/NP in fish species. (B-G) Respective proportion of fish water environments (n=47), fish feeding diets (n=47), fish life-stages (n=46), MP/NP polymer types (n=55), MP/NP shapes (n=50), and

exposure pathways of MP/NP to fish (n=46), from the studies that looked at the effects of virgin MP/NP on fish and that were published before March 2019. Some of these pie charts have a (n) superior to 46 because some of the 46 studies that were selected used more than one fish species, MP/NP polymer type, and/or MP/NP shape. EVA: ethylene-vinyl acetate; PA: polyamide; PE: polyethylene; PET: polyethylene terephthalate; PMP: polymethylpentene; PP: polypropylene; PS: polystyrene; PVC: polyvinyl chloride.

Most studies investigated the effects of spherical virgin MP/NP (70%), followed by fragments (28%) and fibers (2%) (Fig. 1F). Undoubtedly, this experimental focus limits our understanding of the effects of virgin MP/NP in fish in environmental conditions where most of the plastic particles that are encountered are fibers and fragments (Paul-Pont et al., 2018). However, again, this trend can be easily explained by commercial limitations, as the manufactured virgin MP/NP are, to date, mostly spherical. Additionally, plastic fibers are generally difficult to produce or collect, and challenging to control under laboratory conditions (e.g. because of external contamination) and their composition is often intricate to determine. For these reasons, using various environmentally relevant MP/NP polymer types and MP/NP shapes to assess their respective toxicity to fish is a clear but still challenging research avenue.

Exposure characteristics

Most studies exposed fish to virgin MP/NP via water directly (61%), while the others exposed fish to virgin MP/NP via food (via artificial feeds, 24%, or via live preys 11%) or during feeding (4%) (Fig. 2G). Artificial feeds like pellets are generally preferred over live preys because they are quicker to produce, the quantity of MP/NP they contain is easier to quantify, and they offer optimal nutritional supplement to the fish. However, live preys offer interesting exposure conditions that should be further considered. Indeed, using live preys pre-exposed to MP/NP as the exposure medium to fish implies a first digestion of the plastic particles by the preys. This digestion could potentially affect the subsequent toxicity of MP/NP to fish and is particularly environmentally-relevant when considering predator fish. However, this also implies that one needs to consider the potential toxicity of the MP/NP to the prey itself, and its subsequent effect(s) on fish. Also, this exposure medium limits the use of unrealistic MP/NP concentrations and sizes as prey have limited carrying capacities.

Regarding the concentrations of virgin MP/NP that were used in the literature, 57% of the studies exposed fish to a single concentration, while only 20% exposed fish to more than two concentrations. Future ecotoxicological studies should therefore consider exposing fish to a range of at least three concentrations, in particular to investigate potential dose or threshold response in the toxicity of virgin MP/NP to fish. In the aquatic environment, the highest concentrations of MP to date have been

recorded in the Southern North Sea, with 1700000 particles m^{-3} (i.e. 8.5 $mg L^{-1}$) for debris $\geq 80\mu m$ (Dubai and Liebezeit, 2013), but typical concentration found in the marine environment are within the wide range of 1.5 $ng L^{-1}$ to 500 $\mu g L^{-1}$ (Paul-Pont et al., 2018). Considering this concentration of 500 $\mu g L^{-1}$ as a high threshold for MP/NP found in the environment, we noted that 34% of all the analyses (i.e. endpoints) conducted in the 46 selected-studies were done after exposure to virgin MP/NP at concentrations higher than this high threshold. Since this topic is still a new area of research, the use of high concentrations that are not always environmentally relevant is still valuable to investigate the tipping points and acute toxicity of virgin MP/NP to fish. It is also true that most of the field surveys that monitored plastics in the environment still fail to recover the smallest plastic particles, especially NP, due to technical constraints, and may therefore underestimate the concentration of MP/NP in the aquatic systems (Covernton et al., 2019; Paul-Pont et al., 2018). However, the use of concentrations that are almost technically unrealistic such as 104 $\mu g L^{-1}$ of 1 μm MP (equivalent to 191.10⁹ particle L^{-1}) (Lei et al., 2018) or 690 $\mu g L^{-1}$ of 1-5 μm MP (equivalent to 127.10⁶ particle L^{-1}) (Barboza et al., 2018) should be avoided.

Concerning the sizes of MP/NP that were used in the literature, the majority (76%) of studies investigated the effects of plastic particles with a size $< 300 \mu m$. At the opposite, most field surveys that monitored MP/NP in the aquatic environments only reported plastic particles with a size $\geq 300 \mu m$ due to technical constraints.(Paul-Pont et al., 2018) Nevertheless, it is clear that such small plastic particles occur in nature(Alimi et al., 2018; Wiggin and Holland, 2019) and it is thus necessary to include a wide range of MP/NP sizes in future research.

Ecotoxicological effects of virgin MP/NP on fish

General trends

From the 46 studies that were selected, a total of 782 endpoints were identified. Overall, exposure to virgin MP/NP had significant effects (in comparison to the non-exposed fish from the control groups) on 32% of the endpoints (Fig. 3). The most studied endpoint category was metabolism (n=305 endpoints), followed by fitness (n=128), immune system (n=102), behavioral, sensory and neuromuscular functions (n=100), alimentary and excretory systems (n=72), microbiome (n=51) and circulatory and respiratory systems (n=24) (Fig. 2). Most significant differences between control fish and fish exposed to virgin MP/NP were observed for behavioral, sensory and neuromuscular functions indicators (57% of endpoints significantly affected), followed by metabolism (34%), alimentary and excretory systems (33%), microbiome (29%), immune system (24%), circulatory and respiratory systems (21%) and fitness (16%) (Fig. 3).

Effects of virgin MP/NP on fish biological functions and systems

Alimentary and excretory systems

Organs or tissues of the digestive tract (DT) are likely to get in contact and affected by MP/NP following their ingestion by fish. Impairments of the DT tissues were important but not systematic (31%), while digestive enzymes activity and intestinal permeability were highly impacted (67% and 100%, respectively). The latter point requires further attention as it has only been studied twice (Qiao et al., 2019; Romano et al., 2018). Noticeable effects on digestive enzymes were essentially increases of trypsin and chymotrypsin activities (Romano et al., 2018), and tissues damages were essentially found in the intestine (Jabeen et al., 2018; Jin et al., 2018; Pedà et al., 2016; Romano et al., 2018; Yin et al., 2018) and in the liver (Jabeen et al., 2018; Rochman et al., 2013). These damages include for example breakage and detachment of the epithelium or erosion of villi in the intestine (Jabeen et al., 2018; Pedà et al., 2016) and congestion, sinusoidal dilatation, vacuolization and glycogen-depletion in the liver (Jabeen et al., 2018; Rochman et al., 2013).

Behavioral, sensory and neuromuscular functions

Behavioral approaches are becoming increasingly popular in ecotoxicological research as they are considered to be up to 1000 times more sensitive than mortality measures, while being also non-invasive and relatively inexpensive (Hellou, 2011). The endpoints related to behavioral, sensory and neuromuscular functions were highly impacted by virgin MP/NP, especially shoaling indicators (100%), followed by feeding (67%), boldness and exploration (60%), nervous system (58%), activity and locomotion (50%), and vision (50%) (Fig. 3). At the opposite, aggressivity markers were not affected at all (Fig. 3). Interestingly, markers of the nervous system were specifically affected by small particles (from 24 nm to 45 µm in size) but our understanding of the underlying mechanisms remains for now limited. Some authors demonstrated that NP can translocate into the fish brain (Kashiwada, 2006; Mattsson et al., 2017). However, the accuracy of the methods that they used (e.g. fluorescence and brain dissection) is currently under debate given that fluorescent molecules may leach out from NP, or that NP could actually be located in the brain-surrounding conjunctive and blood-vessel tissues but not within the brain. Nevertheless, several studies demonstrated the impacts of virgin MP/NP on neurotoxicological indicators such as the acetylcholinesterase activity (Barboza et al., 2018d; Chen et al., 2017; Ding et al., 2018; Oliveira et al., 2013), and on the brain development and structure (LeMoine et al., 2018; Mattsson et al., 2015; Yin et al., 2018).

Circulatory and respiratory systems

In this endpoint category, the effects of virgin MP/NP were mostly observed on gill histopathological markers (50%) and cardiac characteristics (43%), while little to no effects found on blood components and respiration indicators (8% and 0%, respectively) (Fig. 3). In particular, virgin PE fragments (size < 60 µm) were found to cause tissue damage in the gills of the African sharptooth catfish, *Clarias gariepinus* (Karami et al., 2016), and 51 nm spherical PS NP caused increased heart rate in the zebrafish (Pitt et al., 2018).

Fitness

Fitness indicators, such as growth, mortality and reproductive success are widely used in ecotoxicological studies because (i) they convey scientifically sound cause and effect responses, (ii) they are based on usually inexpensive and easy to implement techniques, and (iii) they provide direct and rapid results for resource managers. In our analysis, fitness-related endpoints were the second most studied but actually the least affected by virgin MP/NP. Interestingly, most of the few significant effects of virgin MP/NP in this category were observed on the growth of larval and juvenile life-stages (Cedervall et al., 2012; Chen et al., 2017; LeMoine et al., 2018; Rochman et al., 2017; Yin et al., 2018) (Fig. 3). Mortality, reproduction and sex hormones were, however, slightly or even not impacted at all (6%, 0%, and 0%, respectively; Fig. 3).

Immune system

The immune system of fish is known to be very sensitive to disturbance, and can therefore serve as an early indicator of negative response to environmental stressors.(Tort, 2011) In our analysis, we referenced moderate effects of virgin MP/NP on chemokines and cytokines (19%) and on the activity of the cells of the immune system such as phagocytes (18%) (Fig. 3). Effects were higher on other indicators such as increased apoptosis biomarkers (Choi et al., 2018), and increased levels globulins and immunoglobulins (Espinosa et al., 2017; Karami et al., 2016), and therefore reached 37% in this “other molecular actors” subcategory (Fig. 3). Such inflammatory responses following MP/NP exposures has also been observed in other organisms, e.g. earthworms (Rodriguez-Seijo et al., 2017) and mussels (Von Moos et al., 2012).

Metabolism

As it encompasses broad areas of an organism biological functions, it is not surprising that this category contains most of the endpoints tackled by the 46 studies of our analysis (n=305, 39% of all endpoints; Fig. 3). Exposure to virgin MP/NP induced profound changes in fish metabolism, with effects being

the highest for general metabolism markers (56%), followed by lipid metabolism (40%), oxidative stress (34%), carbohydrate metabolism (22%) and detoxification (21%; Fig. 3). Oxidative stress endpoints were the most studied of all endpoints (n=148, 19% of the endpoints). A total of 17 studies conducted at least one analysis to compare the oxidative stress response in control fish vs. fish exposed to virgin MP/NP, and a total of 10 studies reported a significant increase in at least one of the different oxidative stress markers that they investigated. These results corroborate past studies on other organisms (e.g. crabs, copepods and mice) that showed increased oxidative stress after exposure to MP/NP (Deng et al., 2017; Jeong et al., 2017; Yu et al., 2018). The production of reactive oxygen species (ROS) in tissues can result from exposure to plastic particles <5 µm, as such small particles can be phagocytized and transported into tissues (Tabata and Ikada, 1990). For larger plastic particles, the production of ROS could result from different mechanisms such as changes in caloric intake and metabolic state (Jeong et al., 2017). Interestingly, the accumulation of ROS in fish exposed to virgin MP/NP was also associated with several other and potentially subsequent biological alterations, such as apoptosis and inflammation (Barboza et al., 2018d; Qiao et al., 2019), and changes in cholesterol levels and activity of enzymes related to the lipid metabolism (Cedervall et al., 2012; Karami et al., 2016; Wan et al., 2019).

Microbiome

The intestine microbiota is a complex “hidden organ” that plays an essential role in the digestion and that can affect physiology and fitness in vertebrates (Sharpton, 2018). Its role is therefore critical for the overall health maintenance and resistance to invasive pathogens (Wang et al., 2017; Yamashiro, 2018). Disturbance of the microbiota (i.e. dysbiosis) is indeed associated with an increased risk of developing inflammatory diseases and metabolic disorders (Clemente et al., 2012). Despite this, the responses of the intestinal microbiome to the exposure to virgin MP/NP has only received little attention in fish (4 out of the 46 studies). Three of these studies observed that virgin MP/NP affected at least one intestinal bacterial phylum (29% of the endpoints were affected; Fig. 3) (Jin et al., 2018; Qiao et al., 2019; Wan et al., 2019). Virgin MP/NP therefore impacted the microbiome overall diversity in these studies, but were also associated with impairments of the carbohydrate, general, and lipid metabolisms, immune system disorders, intestinal histological damages, intestinal permeability changes, and oxidative stress (Jin et al., 2018; Qiao et al., 2019; Wan et al., 2019). These results are in line with past research showing how intestinal dysbiosis can connect immune system and metabolism changes through processes such as inflammation and oxidative stress (Belizário et al., 2018). Knowing that intestinal dysbiosis can also alter the behavior of organisms (Borre et al., 2014), it is clear that studying the effects of virgin MP/NP on fish microbiome is greatly needed and may provide a better

understanding of the underlying mechanisms behind the biological impairments associated with the ingestion of MP/NP (Fackelmann and Sommer, 2019).

Importance of the exposure pathway

Given that a majority of studies exposed fish to virgin MP/NP via water rather than via food (Fig. 1G), a majority of endpoints were also investigated following exposure via water vs. food (504 vs. 278). Exposure to virgin MP/NP also affected proportionally more endpoints when fish were exposed via water (39%, 196/504 endpoints) rather than via food (20%, 55/278 endpoints). However, for some categories (e.g. microbiome) and subcategories (e.g. digestive enzymes, intestinal permeability, vision, gills histopathology and respiration), very few or no endpoints were investigated with food as a medium of exposure to virgin MP/NP (Fig. 3). When only looking at endpoint subcategories for which data is available for both exposure pathways, notable differences (i.e. > 50%) were observed for activity and locomotion (63% of endpoints affected when virgin MP/NP were exposed via water vs. 0% via food), boldness and exploration (100% vs. 0%), cardiac characteristics (50% vs. 0%), general metabolism (78% vs. 15%), growth (100% vs. 40%), and lipid metabolism (38% vs. 100%) (Fig. 3). This latter endpoint category is one of the very few more affected via food exposure than via water exposure, along with body condition, cardiac characteristics and feeding indicators (Fig. 3). These differences between water and food exposures should nevertheless be taken with caution, as this analysis ignores other exposure parameters such as the size and concentration of the virgin MP/NP that were used. The absence of endpoints in the gills histopathology subcategory after food exposure is not surprising as this is likely more affected by MP/NP suspended in water and passing through the gills. However, the microbiome, the digestive enzymes and the intestinal permeability of fish is likely to be affected by MP/NP passing through their digestive systems and it is therefore surprising to observe that such aspects have not yet been investigated. Moreover, exposure via food is advantageous from an ecotoxicological perspective as it allows to precisely quantify the dose of MP/NP ingested by fish, which is essential to determine the toxicity of these particles.

Effects of the shape of virgin MP/NP

For the reasons mentioned previously, spherical virgin MP/NP were more studied than fragments and fibers. This trend was also observed in terms of endpoints, with 536 (69%) endpoints focusing on spherical virgin MP/NP, 240 (31%) on fragments, and 6 (< 1%) on fibers. Regardless of other factors such as size and concentration, spheres had slightly more effects than fragments, with 33% (for spheres) vs. 23% (for fragments) of endpoints affected in fish exposed to these virgin MP/NP. More effects were even observed following exposure to fibers, with 67% of the endpoints affected, but only 6 endpoints were investigated by only 1 study (Jabeen et al., 2018). The very few studies (3 out of 46)

that examined, within the same experimental conditions, the effects of the shape of virgin MP/NP on fish actually used different MP/NP sizes and/or polymer types, preventing a clear understanding of any effect related to MP/NP shape (Choi et al., 2018; Jovanović et al., 2018; Lei et al., 2018a). Among them, Choi et al. (2018) demonstrated that 6-350 µm PE-fragments had greater impacts than 150-180 µm PE-spheres at a low concentration (55% vs. 9% of endpoints affected) but similar at a higher concentration (23% each). There is therefore, to date, no clear pattern of toxicity related to the shape of virgin MP/NP in fish, while some authors speculated that the ruggedness of irregular shapes imply a longer retention time in the guts of organisms, leading to potential inflammation and increasing biological impacts (Gray and Weinstein, 2017).

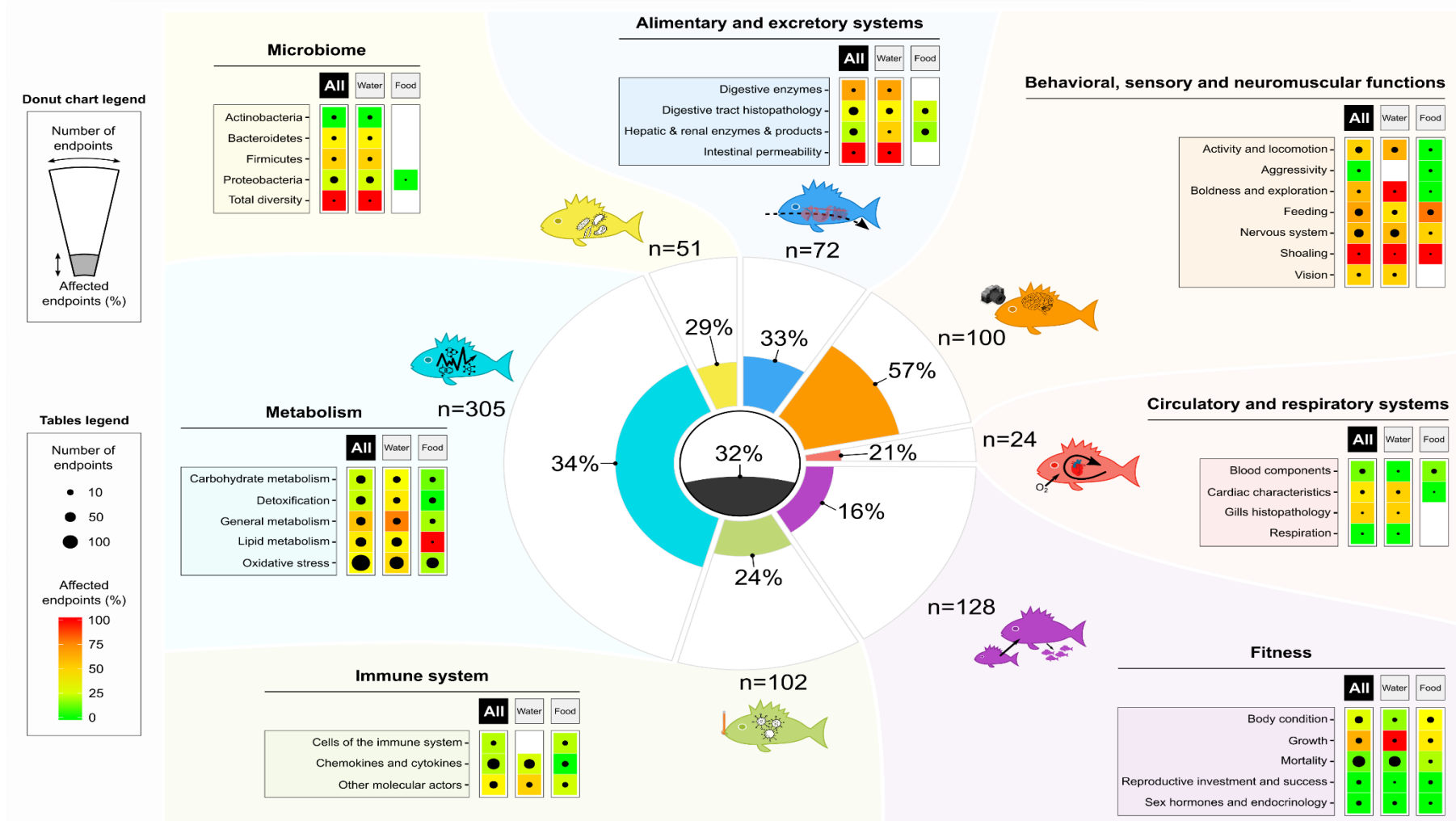


Figure 7. Ecotoxicological effects of virgin MP/NP on fish biological functions according to the exposure medium.

The white parts of the donut chart indicate the number of endpoints studied in each biological category (in the 46 studies that looked experimentally at the effects of virgin MP/NP on fish). The internal colored parts of the donut chart indicate the proportion of affected endpoints (i.e. for which significant differences were observed between control fish and fish exposed to virgin MP/NP). Heatmaps provide the same type of information, with circle size indicating the number of endpoints and color indicating the proportion of affected endpoints, but for each subcategory of the main biological functions.

Effects of the size of virgin MP/NP

As smaller particles have a larger specific surface area and are more likely absorbed and/or transported into tissues, it is therefore generally considered that NP and small MP are more toxic to living organism than larger MP (Jeong et al., 2017; Lu et al., 2016). Regardless of any other parameters, we observed that 43% (n=74/173) of endpoints were affected by exposure to virgin plastic particles of the size class 1 (i.e. NP); 40% (n=71/179) by the size class 2; 36% (n=24/66) by the size class 3; 2% (n=1/42) by the size class 4; 10% (n=4/39) by the size class 5; and 21% (n=11/52) by the size class 6 (Fig. 1, Fig. 4). For MP/NP in the size class MISC (i.e. MP/NP whose sizes cover more than one size class) 29% (n=67/231) of the endpoints were affected, a percentage comparable to the 32% when considering all size classes (Fig. 2). Size classes 1 and 2 had therefore the more impacts on fish, while also being the two most studied (45% of the endpoints). This is consistent with the idea that NP and smaller MP, which can pass the epithelium barriers (Lu et al., 2016), are the most toxic to fish (Jeong et al., 2017; Lu et al., 2016). The most studied endpoint subcategory (i.e. oxidative stress) also confirms this trend (Fig. 4). In the meantime, the percentage of effects slightly increased with plastic particle size, as size class 6 presents more affected endpoints than class 4 and 5 (Fig 4.). Interestingly, the greatest affected endpoints of this size class are related to feeding, histopathology of the digestive tract and growth markers (Fig. 4). Feeding markers investigated with virgin MP of the size class 6 actually corresponds to one study that looked at how the presence of large virgin MP in water can affect the predatory performance of the common goby, *Pomatoschistus microps* (de Sá et al., 2015). Histopathological markers of the digestive tract investigated with virgin MP of the size class 6 correspond to three studies that looked at the intestine, jaw, liver, mesentery, spleen, stomach and pancreas histological damages following large virgin MP ingestion (Jabeen et al., 2018; Jovanović et al., 2018; Rochman et al., 2013). It is therefore not surprising to observe important effects of size class 6 virgin MP on these functions, which are likely sensitive to such big particles. Growth markers on the other hand remained more impacted by smaller size classes (i.e. 1, 2 and 3) than size class 6, and were not considered for size classes 4 and 5 (Fig. 4). These results, however, do not consider other parameters such as MP/NP concentration and type and fish life-stage. Seven out of the 46 studies (Chen et al., 2017; Lu et al., 2016b; Manabe et al., 2011; Mattsson et al., 2017; Wan et al., 2019) conducted a total of 15 experiments that looked specifically at the effects of MP/NP particle size on fish (i.e. with all other parameters being controlled). Among these 15 experiments, 10 showed significant effects of virgin MP/NP on fish biological functions, with 50% of these 10

experiments showing a greater percentage of affected endpoints with small MP/NP than with large MP/NP, 10% of them showing no difference between small and large MP/NP, and 40% showing a greater percentage of affected endpoints with large MP/NP than with small MP/NP. Altogether, this suggests that under 100 μm the smaller the MP/NP the higher its toxicity to fish, while over 100 μm the bigger the MP the higher its mechanical impacts on fish (e.g. obstructions).

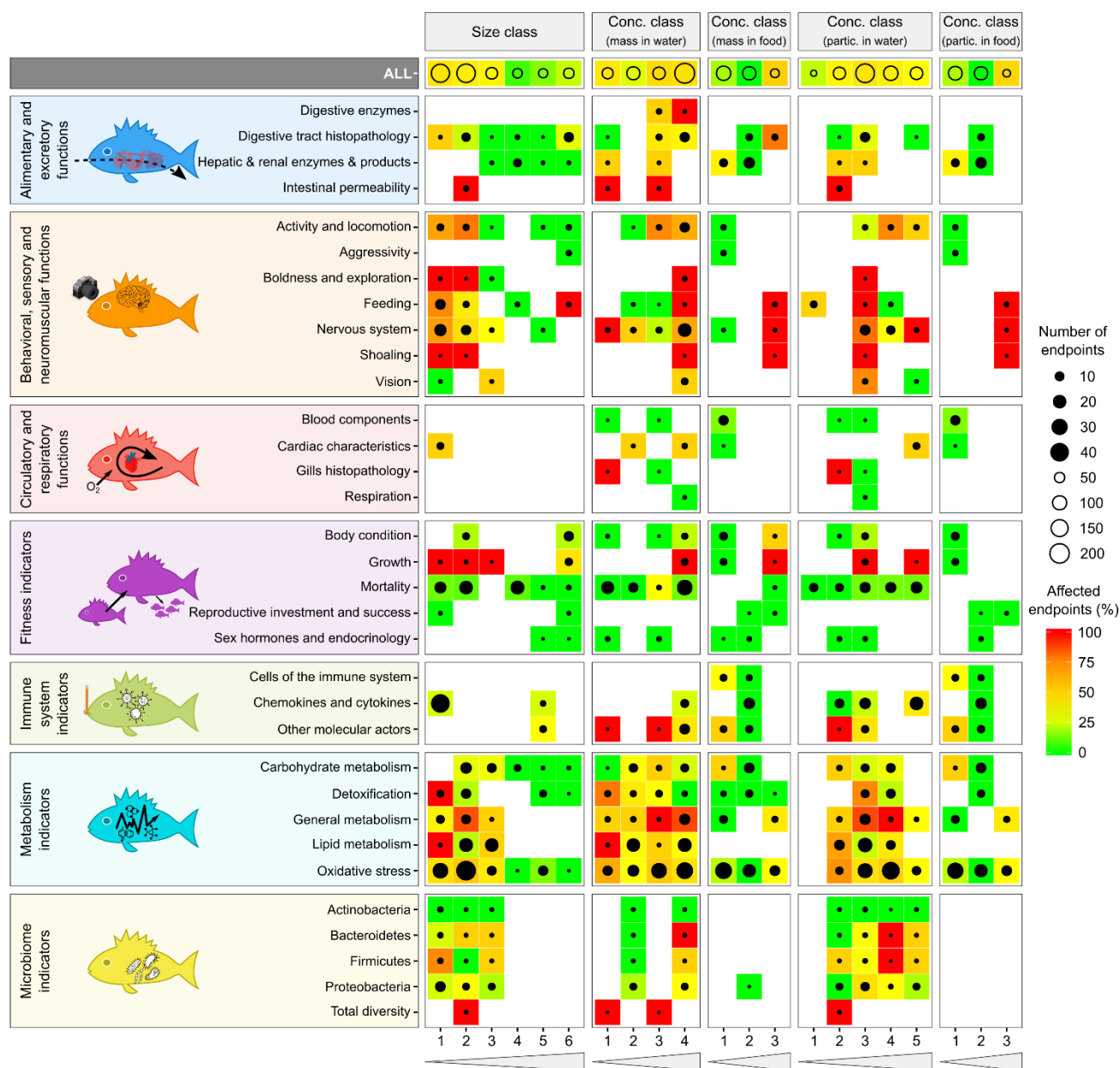


Figure 8. Ecotoxicological effects of virgin MP/NP on fish biological functions and systems according to MP/NP size and concentrations classes

Circle size indicates the number of endpoints and color indicates the proportion of affected endpoints (i.e. for which significant differences were observed between control fish and fish

exposed to virgin MP/NP) in the 46 studies that looked experimentally at the effects of virgin MP/NP on fish.

Effects of the concentration of virgin MP/NP

Large concentrations of virgin MP/NP in food appeared to have more impacts on fish biological functions and systems, with respectively 50% and 48% of all endpoints affected by virgin MP/NP at the highest mass and particle concentration classes (i.e. class 3, Fig. 4). However, this trend was not observed for fish exposed to virgin MP/NP via water, where intermediate concentration classes had the most impacts (47% and 40% of endpoints affected at classes 3 for mass and particle concentrations, respectively, Fig. 4). Neither dose nor threshold response can therefore be established following this analysis of the effect of the concentration of virgin MP/NP. No clear pattern was neither observed when looking at the effect of the concentration of virgin MP/NP on more specific biological functions. Indeed, endpoints of the “behavioral, neuromuscular and brain functions” category were more impacted by high concentrations of virgin MP/NP, while intestinal microbiome diversity, lipid metabolism and gill histopathological markers were mostly affected by lower concentration classes (Fig. 4). Regarding water exposures, it is worth noting that only a few endpoints (15 on 504; 3%) were investigated at concentrations ranging from 1 to 100 particles L⁻¹, which actually corresponds to a high range of the concentrations that can be encountered in marine waters (Paul-Pont et al., 2018). Again, these results do not account for other parameters such as particle size and type, or exposure duration. Twenty studies actually examined specifically the effect of the concentration of virgin MP/NP by conducting a total of 30 experiments that used more than one concentration with all the other exposure parameters being controlled. Among these experiments, 33% did not reveal any effects of the concentration of virgin MP/NP on fish biological functions. Among the other experiments, 65% showed that the higher the concentration the more impacts of virgin MP/NP on fish biological functions, 15% found no difference between the different concentrations that were used, and 20% found that the lower the concentration the more impacts of virgin MP/NP on fish biological functions. This latter case is particularly interesting as quite uncommon in toxicological studies, and it clearly suggests that more research is needed to clarify the importance of MP/NP concentration towards fish biological functions.

Research gaps and perspectives

Similarly to the need of standardizing sampling practices when operating field surveys of plastic pollution (Cunningham and Sigwart, 2019), there is an urgent need to standardize the exposure methods when experimentally testing the effects of virgin MP/NP in fish (Karami, 2017). In particular, the most urgent need is for researchers to report concentrations that are comparable, whether they exposed fish to MP/NP via water or food. In the field of particle toxicology, the three following units are usually used to characterize exposure in liquid mediums: particle mass per volume, particle number per volume and particle surface area per volume (Karami, 2017). When exposing fish to MP/NP via food, similar units should therefore be used, i.e. particle mass per gram of fish per day, particle number per gram of fish per day and particle surface area per gram of fish per day. Characterizing the surface of plastics fragments and fibers can be a great challenge as the shapes of such particles are all different one from another. However, several protocols exist to characterize the MP/NP mass and particle number, per liter if exposing fish via water or per gram of fish per day if exposing fish via food. It is therefore particularly striking that only 17 out of 46 studies reported both these concentrations, and that 6 out of 46 studies did not gather sufficient information to characterize the dose of MP/NP to which fish were exposed (Karami, 2017). We therefore argue that all future studies, at least for MP exposures as NP quantification is still a challenge, should report both particle mass and particle number, per liter when exposing fish via water, and per gram of fish per day when exposing fish via food. This standardization should allow easier and more meaningful comparison between studies.

Research focusing on the impacts of MP/NP on wildlife have largely considered one aspect of such particles: their potential vectors of contaminants. Indeed, 70% of the studies reviewed here also used exposure condition(s) with at least one co-contaminant in addition to the virgin MP/NP (e.g.(Barboza et al., 2018d)). In fact, exposures to virgin MP/NP were essentially conducted to serve as controls for the “MP/NP + co-contaminant” treatment where the main focus was to assess the effect of the co-contaminant. This aspect of the research on the ecotoxicology of MP/NP to fish reflects well that the toxicity of virgin MP/NP is expected to be quite low and thus generally neglected. However, the present analysis on fish revealed that virgin MP/NP affected 32% of all the endpoints tackled by the 46 studies reviewed here. We therefore argue that virgin MP/NP can be toxic *per se* to fish. This research therefore requires more in-depth attention and should not be limited to “control exposures”, with limited doses and sizes as usually done. In this regard, researchers should investigate MP/NP size classes and

concentrations classes as well as fish biological functions that have received only little or no attention yet (Fig. 4). Using various ranges of size and concentration within the same experiments would also facilitate the determination of toxicological tipping points and potential dose or threshold responses, while a particular focus on environmentally relevant exposure concentrations should also be adopted to prevent potential experimental artefacts (Cunningham and Sigwart, 2019). Even in worst-case scenarios of plastic pollution, MP/NP will likely remain a minor fraction of the other microparticles naturally present in the water and the sediment (Ogonowski et al., 2018). When using unrealistic concentrations of MP/NP, researchers should therefore consider using the same concentrations of natural particles (e.g. sediment) as controls to address the induced effects of MP/NP. Also, publishing both positive, negative and neutral effects of virgin MP/NP should be encouraged by scientific journal editors to prevent potential bias during meta-analysis (Schooler, 2011), but also to enhance our general understanding of the impacts and non-impacts of virgin MP/NP on fish.

Based on the literature review, we developed a list of actions and priorities that scientists in this field should consider (Table 1).

Table 1: List of actions and priorities for future research on the impacts of virgin MP/NP on fish

Type	Priorities and recommended actions
MP/NP characteristics	Provide comprehensive details about the size and shapes of the MP/NP that are used Evaluate the ecotoxicological effects of MP/NP fibers Use MP/NP for which the polymer composition is available
Fish characteristics	Consider multi-species approach Put a greater emphasis on early life-stages (e.g. larvae and juveniles) Investigate herbivorous and planktivorous species
Exposure characteristics	Report exposure concentrations in a standardized manner, i.e. in $\mu\text{g L}^{-1}$ and particle L^{-1} for water exposures, and in $\mu\text{g g}^{-1} \text{day}^{-1}$ and $\text{particle g}^{-1} \text{day}^{-1}$ for food exposures

	<p>Use extreme concentrations as a mean to elicit the mode of action of MP/NP on fish but not as an environmental risk assessment</p> <p>Examine the effects of natural particles (e.g. sediment) as a control to determine the effects attributable to the plastic nature of MP/NP.</p> <p>Investigate multiple concentrations within the same experiment to investigate potential dose or threshold response</p>
Biological endpoints	<p>Develop comprehensive analyzes that consider inflammation, intestinal dysbiosis, neurotoxicity and behavioral changes, and metabolic alterations (e.g. oxidative stress) to elucidate the potential mechanisms and route of action of the toxicity of virgin MP/NP to fish</p> <p>Continue the development of new ecotoxicological markers (e.g. behavioral and microbial analyses) in addition to the standard ecotoxicological markers (e.g. LC50 and LOEC) to better apprehend the sublethal effects of virgin MP/NP on fish</p>

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Présentation du Projet de thèse

Problématique et objectifs

La pollution plastique est aujourd'hui une préoccupation environnementale et sociétale majeure. Au cours des 10 dernières années, de nombreuses études expérimentales ont complété les observations de terrain dans l'espoir de mieux comprendre le devenir et l'impact des MP sur divers organismes (ex.g., oiseaux, poissons et mammifères). Chez les poissons, les effets les plus significatifs observés après une exposition aux MP/NP sont ceux affectant les fonctions comportementales et neurologiques, la perméabilité intestinale, le métabolisme et la diversité du microbiome intestinal des poissons. Cependant, la pollution plastique reste difficile à quantifier dans l'environnement et à contrôler en laboratoire, et les particules de plastique sont souvent co-contaminées de manière naturelle ou expérimentale avec divers polluants chimiques. Par conséquent, les études sur les effets comportementaux et physiologiques associés à l'ingestion des MP vierges chez les jeunes stades de vie poissons marins, connus pour être particulièrement sensibles aux stress environnementaux, restent peu nombreuses à l'heure actuelle. Ainsi cette thèse a pour objectif :

- D'approfondir les connaissances des effets écotoxicologiques des MP de polyéthylène (PE) sur un large éventail de processus biologique chez les juvéniles de poissons marin en utilisant la daurade royale (*Sparus aurata*) comme modèle (Chapitre 2).
- D'apporter de nouvelles connaissances sur les effets potentiels des MP de polystyrène (PS) sur les post-larves de poissons tropicaux (espèce modèle : *Acanthurus triostegus*), aucune étude à l'heure actuelle n'ayant quantifié l'effet de ces types de polluant chez ces organismes (Chapitre 3).

En parallèle de ces études représentant le cœur de cette thèse, j'ai eu l'occasion de m'intéresser à d'autres questionnements en rapport à la pollution microplastique comme leur présence dans le tube digestif de poissons coralliens sauvages (Annexe 2) ou leur rôle potentiel comme vecteur de métaux (Annexe 5).

Chapitre 2 - Effets des microplastiques sur les juvéniles de la daurade royale (*Sparus aurata*)

Avant-propos

Modèle et lieu d'étude

Sparus aurata est une espèce commune de Méditerranée, présente le long des côtes de l'Est de l'Atlantique. C'est une espèce rencontrée dans des environnements aussi bien marins que saumâtres tels que les lagunes côtières et les zones estuaires, en particulier durant les stades initiaux de son cycle de vie. Nés en mer ouverte durant octobre-décembre, les juvéniles migrent au début du printemps vers des eaux côtières abritées, où ils peuvent trouver des ressources trophiques abondantes et des températures plus douces. Ils retournent en mer ouverte en novembre, où les adultes se reproduisent. En mer ouverte la daurade royale est normalement trouvée sur les rochers et les herbiers marins (*Posidonia oceanica*), mais elle est aussi fréquemment capturée sur des fonds sableux. Les jeunes poissons restent dans des zones relativement superficielles (jusqu'à 30 m), alors que les adultes peuvent atteindre des eaux plus profondes, généralement pas plus que 50 m. Cette espèce est hermaphrodite protandrique. La maturité sexuelle se développe chez les mâles à l'âge de 2 ans (20–30 cm) et chez les femelles à l'âge de 2–3 ans (33–40 cm). Les femelles sont des reproducteurs en batch qui peuvent pondre 20 000–80 000 œufs chaque jour pendant une période qui peut aller jusqu'à 4 mois (FAO, 2005 ; Fig. 9). La daurade royale est la principale espèce aquacole en volume de production en Europe (FEAP, 2014). Ainsi, *Sparus aurata* a été sélectionné pour cette étude, car il s'agit d'une espèce de poisson largement produite, pêchée et consommée à l'échelle mondiale et donc importante d'un point de vue économique (Valente et al., 2011), mais aussi d'un point de vue écologique, puisqu'elle a été utilisée comme organisme modèle dans de nombreuses études écotoxicologiques ainsi que par la communauté scientifique européenne (Franch et al., 2006; Morales-Caselles et al., 2007; Rodrigues et al., 2019). Cette partie de ma thèse a été réalisée au laboratoire de l'environnement de l'Agence Internationale de L'Energie Atomique (AIEA) de Monaco.

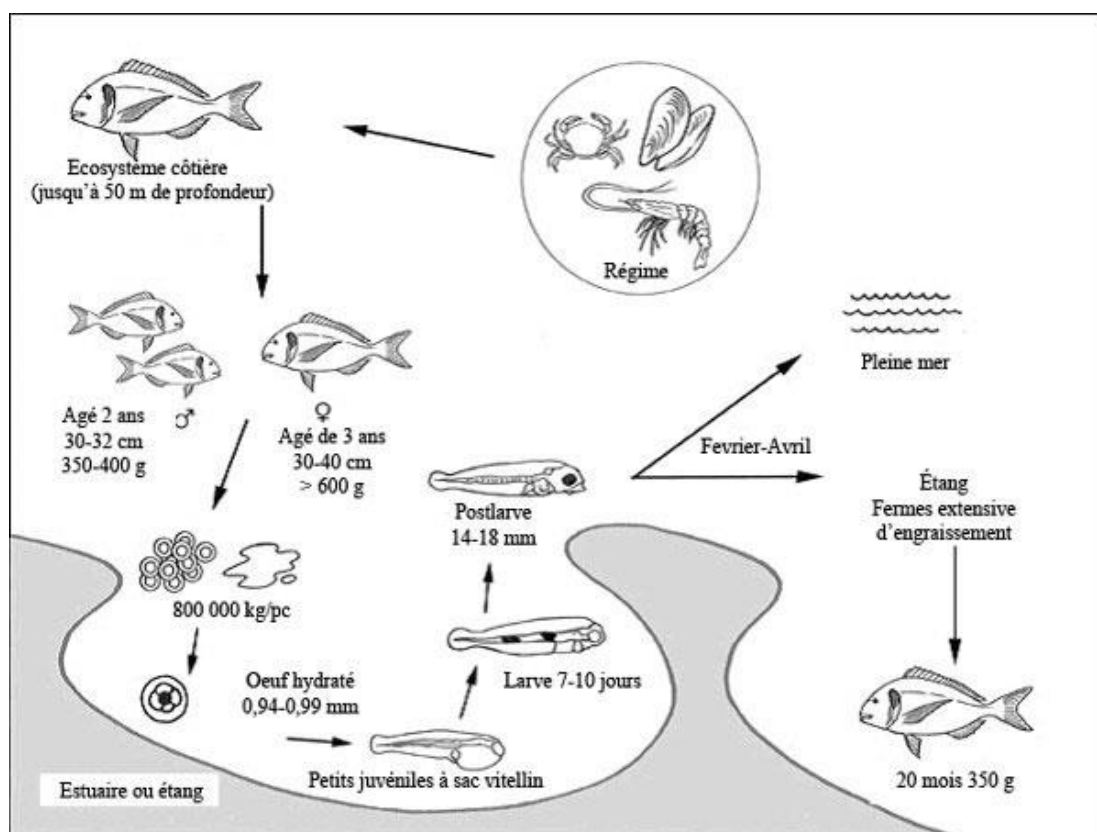


Figure 9. Cycle de vie de la daurade royale (*Sparus aurata*) adapté de FAO (2005).

Objectifs et questionnement scientifique

La littérature scientifique récente recommande l'utilisation d'une batterie de réponses biologiques dans les études écotoxicologiques, car l'utilisation de marqueurs biologiques uniques peut ne pas refléter la complexité des altérations physiologiques et/ou réactions adaptatives des organismes aux facteurs de stress environnementaux, ce qui est particulièrement pertinent au vu des effets variés et subtils des MP sur les poissons présentés dans le chapitre 1 (Jin et al., 2018; Micale et al., 2011; Wan et al., 2019). Dans cette étude, nous avons développé une approche multi-diagnostique impliquant des évaluations d'impact à plusieurs niveaux d'organisation biologique (des atomes aux organismes) afin de déterminer les impacts des MP sur un poisson marin. Plus précisément, nous avons exposé des juvéniles de daurade royale (*Sparus aurata*) (Fig. 10.B) à des microsphères de polyéthylène (PE-MP) vierges de 10 à 20 μm par voie alimentaire (*Artemia salina* exposée à 5 mg L^{-1} ; Fig. 10.A) pendant 45 jours, puis nous avons examiné leur i) histologie du foie, de l'estomac et de l'intestin; ii) microbiome

intestinal; (iii) profils de métabolites cérébraux, hépatiques et musculaires (RMN); (iv) assimilation de ^{65}Zn et ^{110}Ag ; (v) profils d'isotopes stables du carbone et de l'azote dans le muscle et (vi) croissance et mortalité. Cette variété de paramètres a été développée dans le but de réaliser un cadre complet et représentatif des impacts des MP vierges à haute dose sur une espèce de poisson. Afin de comprendre les modes d'action de ce polluant sur les poissons.

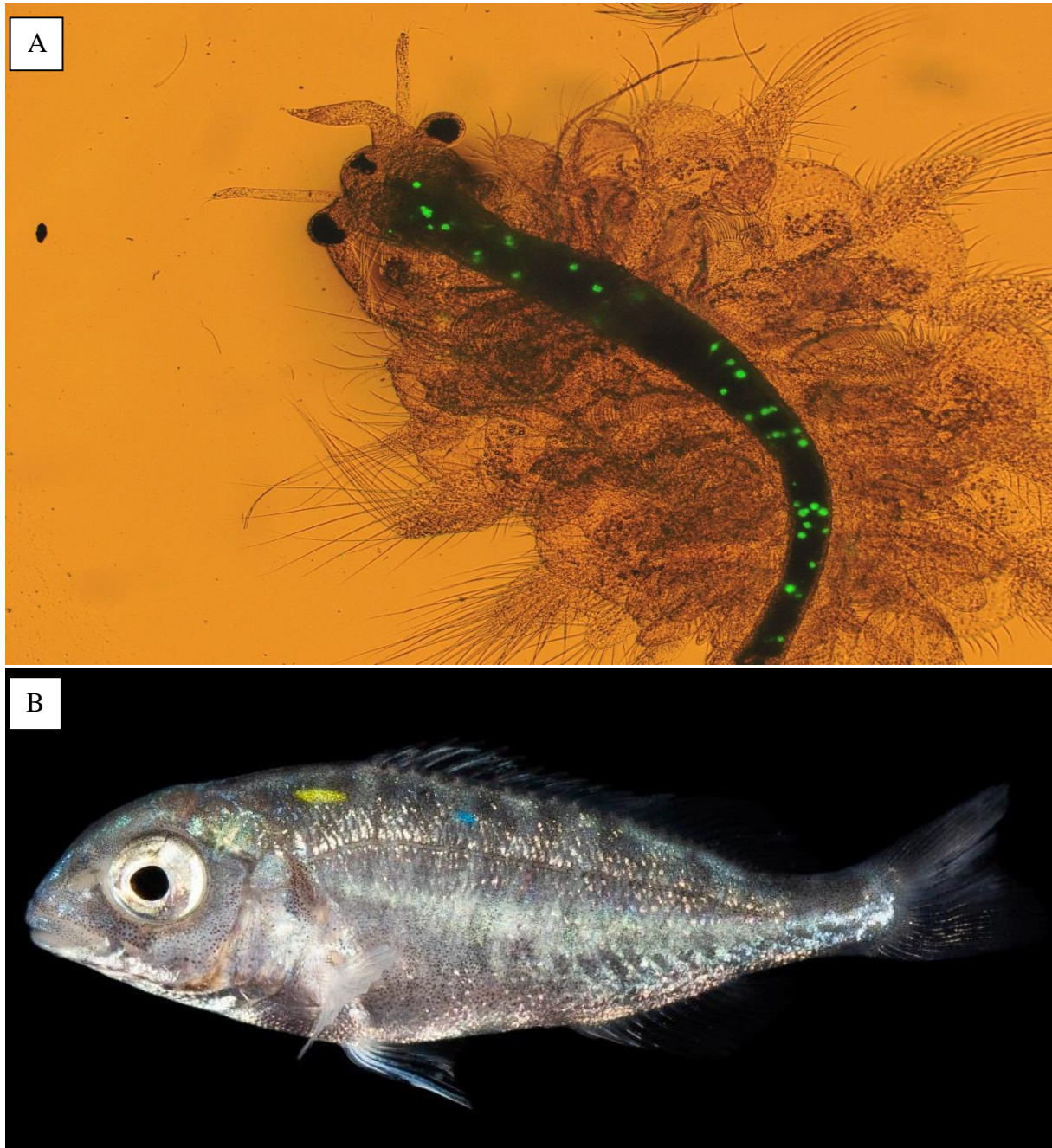


Figure 10. (A) Artémias (*Artemia salina*) exposées aux PE-MP (points verts fluorescents) et (B) Juvénile de daurade royale (*Sparus aurata*) utilisée pour l'expérience.

Titre: Widespread effects of virgin polyethylene microplastics on juvenile gilthead seabreams (*Sparus aurata*)

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Abstract

The occurrence of microplastics (MP) in the environment is a major concern globally, in particular in aquatic ecosystems where MP pollution has risen as plastic production and subsequent waste are increasing worldwide. Given their ubiquitous nature and small dimensions, MPs can be ingested by aquatic organisms, translocated in internal tissues, and transferred along the trophic chain, but these processes and their impact on biota are yet to be fully understood. Here, we developed a multi-diagnostic approach involving MP impact assessments at multiple levels of biological organization (from atoms to organisms) to determine how MP affect the biology of a marine fish, the gilthead seabream, *Sparus aurata*. Specifically, juveniles of this species were exposed to high doses of virgin 10-20 μm polyethylene MP sphere (PE-MP) through food (via pre-exposed *Artemia salina*) for 35 days. Significant effects were observed on the mortality rate, on the assimilation of an essential element (Zn), on the relative abundance primary metabolites in the brain and liver, and on liver's cell vacuolation. At the opposite, no effect was observed on growth, assimilation of a contaminant (Ag), and carbon and nitrogen stable isotopes profiles in the muscle while unclear patterns were observed for the intestinal microbiome. This thorough variety of endpoints reveals how subtle to profound the effects of virgin MP can be on fish, and opens the way towards a better understanding of the causal mechanisms and interactions between this emerging pollutant and biological processes in fish.

Keywords : Plastic ; Fish ; Marine Stressors ; Experimental approach ; Multiple endpoints

Introduction

An estimated amount of 4 to 12 million tonnes of plastic debris is reaching the marine environment per year (Jambeck et al., 2015). Accordingly, plastics are now found in all marine environments, from ocean gyres (Eriksen et al., 2014) and coastal regions (Ling et al., 2017) to polar regions (Bergmann et al., 2017; Lusher et al., 2015) and deep sea sediments (Chiba et al., 2018). Recently, microplastics (MP), defined as plastic debris < 5mm (Moore, 2008), raised a great environmental concern to marine ecosystems (Avio et al., 2017). MPs mainly come from manufactured products such as drug vectors, sunscreens, and cosmetics or degradation of large plastic debris by UV-radiation, mechanical abrasion, and biological degradation (Rochman, 2018; Ter Halle et al., 2016). Given their ubiquitous nature and small dimensions, MP can be ingested by aquatic organisms, transferred along the trophic chain, and in some case translocated in internal tissues, the hazards of which are yet to be fully understood (Chae and An, 2017; Franzellitti et al., 2019; Setälä et al., 2018; Wright et al., 2013).

Consequently, there has been a recent exponential rise in the number of toxicological studies that investigate the effects of MPs on aquatic organisms, with data reported for various taxonomic groups, from laboratory and field investigations, as well as data evaluating the role of MPs as vectors for organic contaminants (Franzellitti et al., 2019). In fish, virgin MP have been found to cause physical and physiological deteriorations (see de Sá et al., 2018; Foley et al., 2018), altering various functions among different biological level of organization of fish. For instance, it has been shown that virgin MP can cause behavioral anomalies and neurotoxicity (Barboza et al., 2018b; Choi et al., 2018; Oliveira et al., 2013; Yin et al., 2018), microbiome dysbiosis (Choi et al., 2018; Jin et al., 2018; Wan et al., 2019), oxidative stress (Barboza et al., 2018b, 2018a; Choi et al., 2018; Espinosa et al., 2017; Lu et al., 2016; Qiao et al., 2019; Wan et al., 2019), digestive organs damages (Jabeen et al., 2018; Pedà et al., 2016; Romano et al., 2018) and metabolism alterations (LeMoine et al., 2018; Lu et al., 2016; Qiao et al., 2019; Wan et al., 2019; Yin et al., 2018). The variety of MP size, type, shape and concentration, exposure route (water vs. food) and the different fish species used in those studies limit the extrapolation in the mode of action of MP on fish. Studies examining more abundant and more various ecotoxicological and physiological markers are therefore needed to enhance a more comprehensive understanding of the effects of MP on fish.

Here, we developed a multi-diagnostic approach covering different levels of biological organization to assess the impact of MPs on marine fish. More precisely, we exposed gilthead seabream juveniles (*Sparus aurata*) to virgin 10-20 µm polyethylene microspheres (PE-MP)

through food (*Artemia salina* exposed at 5 mg L⁻¹) for 35 days and looked at their (i) growth and mortality (ii) assimilation of 65Zn and 110mAg; (iii) intestinal microbiome; (iv) brain, liver and muscle metabolite profiles; (v) liver histology; (vi) carbon and nitrogen stable isotopes profiles in muscle. This variety of endpoints allowed us to develop a comprehensive and representative framework of the impact of MPs in a fish species, revealing subtle to profound biological changes.

Material and Methods

Acclimation of fish and experimental conditions

A total of 200 gilthead seabream juveniles *Sparus aurata* (average wet weight = 3 ± 0.5 g, mean \pm SD) supplied by les Poissons du Soleil (Balaruc-les-Bains, France) were acclimated at the IAEA-Environment Laboratories in 500-L aquarium for 4 weeks prior the exposure to microplastics (constantly aerated, open-circuit; the water exchange rate was set at 80 L h⁻¹; salinity = 38; temperature = 17 ± 0.1 °C; pH = 8.0 ± 0.1 ; and 12 h/12 h light/dark cycle). During the acclimation period, the fish were fed a daily ration of 3% of their biomass with 1.1-mm pellets (Le Gouessant, France). Then, two weeks before the exposure, 72 fish were randomly placed in six 20 L aquaria (n = 12 fish per aquaria; constantly aerated, open-circuit; the water exchange rate was set at 150 L h⁻¹, Temperature = 17 ± 0.1 °C). The total biomass per tank ranged between 105.48 and 108.26 g wet weight (wwt) representing a density of 5.3 kg of fish m⁻³. The mean mass of the fish in the 6 tanks was: 9.0 ± 0.8 ; 9.0 ± 0.9 ; 9.0 ± 1 ; 8.8 ± 0.8 ; 8.8 ± 0.8 and 9.0 ± 0.9 g wwt.

Experimental procedures

Diet preparation

Live adult brine shrimps (*Artemia salina*) were purchased from Grebil (Arry, France). Green fluorescent polyethylene (PE) microspheres (10-20 μ m, d = 1.025) in dry powder form were purchased from Cospheric (USA). Two diets were prepared: a control diet and PE MP diet. For the control diet, 30 g wwt of live brine shrimps were transferred to 5-L glass beakers filled with 2.5L seawater (17 °C) and 1.5-L of phytoplankton (*Isochrysis* spp.; 5.105 cell mL⁻¹), and aerated to permit the solution homogenization. For the PE MP diet, 20 mg of 10-20 μ m PE-MP were added to 2.5L seawater (17 °C) and 1.5-L of phytoplankton (*Isochrysis* spp.; 5.105 cell mL⁻¹), to reach a concentration of 5 mg L⁻¹. After 5 min of homogenization, 30 g wwt of live brine shrimps were added to the beaker. Live brine shrimps were then left 3h in their respective

treatment before being filtered in a small mesh hand-net and rinsed 3 times with 500 mL seawater to remove potential non-ingested MP. This was ensured by binocular observation, during which no MP were observed on the external parts of the body of the rinsed brine shrimps. Portion of 2.5 g wwt (control and PE-MP brine shrimps) were prepared and placed in a freezer (-20 °C) until use.

Fish exposure and MP quantification in preys

The PE-MP treatment and the control group were assigned randomly to the 6 tanks (3 tanks per condition). The experiment started on the 2nd of July 2018 and lasted for 35 days. Fish were weighted after 14 days and at the end of the exposure period. Each tank was given two times 2.5 g wwt of brine shrimps day⁻¹ representing an average of $5 \pm 0.46\%$ of fish body weight over the exposure period. As mortality occurred in some tanks, the feeding daily ration was adjusted and remained at $5 \pm 1\%$ body weight per day.

The protocol of Rist et al. (2017) was used to quantify the amount PE-MP ingested by brine shrimps. Seven subsamples of 0.1 g (22 ± 2 brine shrimps per subsample) of different batch of brine shrimp were digested. Quantification of PE-MP were done visually under inverted stereo microscope equipped with fluorescence light (Olympus, Ix73). A number of 115631 ± 24179 of PE-MP per gram of brine shrimp was measured, which corresponds 522 ± 86 PE-MP per brine shrimp (data available in Suppl. material 1). The estimated dose provided to the fish thus corresponds to 87 ± 18 µg per fish per day or 48180 ± 10074 MP per fish per day which was equivalent to 5 ± 1 µg or 2800 ± 840 MP per gram of fish per day.

Survival and growth

Mortality was assessed daily in each experimental tank. All fish were weighed at the beginning of the experiment (D0), after 14 days of exposure (D14) and at the end of the exposure period (D35) in order to determine juvenile growth in both experimental conditions and to adjust food doses.

Fish sampling for analyses and experiments

At the end of the feeding trial, 10 fish were randomly selected in each condition and were transferred to similar aquaria (2 tanks per condition; total of 4 tank) to assess the effect of MP exposure on the assimilation efficiencies of elements (see subsection 2.2.5). For identification purposes, these fish were individually tagged with a specific colour using visible implant fluorescent filament (NMT, Shaw Island, USA) injected on the dorsal muscle two days prior to

the experiment. The remaining fish ($n = 28$ for control fish and $n = 14$ for MP fish) were euthanized and dissected for other analysis (see section 2.3).

Assimilation efficiency experiment

Determination of assimilation efficiency of elements in fish was performed using gamma-emitting radiotracers (Pouil et al., 2018). More precisely, a pulse chase feeding was conducted right at the end of pre-exposure of fish to MPs. The protocol was similar to the one used in Jacob et al. (2017). Briefly, live preys (brine shrimps) were radiolabeled using waterbone ^{65}Zn and ^{110}mAg . These radiotracers (^{65}Zn as ZnCl_2 in 0.1M HCl, [$t_{1/2} = 243.9$ days] and ^{110}mAg as AgNO_3 in 0.1M HNO_3 , [$t_{1/2} = 246$ days]) were purchased from Isotope Product Lab., USA. Eighty grams of brine shrimp were placed in 10-L Nalgen bottle filled with 8-L of seawater spiked with ^{110}mAg and ^{65}Zn (activity of 15000 Bq L⁻¹ for each radioisotope) for 48 hours. The brine shrimps were then collected with a hand-net, rinsed with 3 times 500 mL of non-contaminated seawater and paper-blotted before storage at $-20\text{ }^\circ\text{C}$ until use.

The experiment consisted of a single feeding of fish (control vs. pre-exposed to MP) with radiolabelled thawed brine shrimps. Fish ate all the brine shrimps (7.5 g per tank) and 2 hours after the feeding, individual fish were whole-body γ -counted alive and then replaced into the same aquarium to follow subsequent radiotracer depuration.

The γ -counting consisted of the quantification of both tracer radioactivity using high-resolution γ -spectrometer systems; four high-purity germanium (HPGe) N or P type—detectors (EGNC 33-195-R, Canberra® and Eurysis®) were used and these detectors were controlled by a multi-channel analyser and a computer equipped with spectral analysis software (Interwinner® 6, Intertechnique). The radioactivity of each radiotracer was determined using calibrated standards of the same geometry. Measurements were corrected for background activity and radioactive decay. Live seabream specimens were placed into custom designed holding tubes filled with non-contaminated seawater for gamma spectrometric analyses. Counting time was adjusted to obtain propagated counting errors typically less than 5% (Cresswell et al., 2017); run times were typically 5–10 min for whole specimen radio-analysis (longer count times were required towards the end of long-term depuration experiment; max 20min), which was also shown to not affect the specimen's wellbeing. Water temperature was stable in counting tubes and dissolved O_2 concentrations were always $> 3\text{ mg L}^{-1}$). All the fish were regularly γ -counted to follow the ^{65}Zn and ^{110}mAg depuration kinetics over the 17 days experiment in order to assess assimilation efficiency of these elements (Pouil et al., 2018).

Observation of depuration kinetics of radiotracers were fitted using nonlinear regression routines and iterative adjustment using Statistica software 7.0. These kinetics were best fitted using a one- or two-component exponential model or a two-component model that includes an exponential component and a constant as described by Pouil et al. (2018). A comparison between the Assimilation Efficiency (AE) for the depuration experiment, weight at D14 and D35, C/N ratio and microbiome order and family abundance was made using the two-tailed Wilcoxon-Mann-Whitney test. The level of significance for statistical analyses was always set at $\alpha = 0.05$. All the statistical analyses were performed using R software (R-3.2.1).

Subsequent analyses

Stable isotope C/N ratio identification

After dissection, muscle samples from control and MP-exposed fish were freeze-dried, lyophilized and grounded. As lipids are highly depleted in ^{13}C with regards to other tissue components due to fractionation occurring during lipid synthesis (DeNiro & Epstein, 1977), isotopic analyses were performed on lipid-extracted samples. Lipid extraction was performed using 20 mL of cyclohexane on powder aliquots of about 1 g. An ultra Turax® was used to homogenize the mixture (2×15 seconds). The sample was then centrifuged for 2 min at 804 g. The supernatant containing lipids was disposed off, whereas the pellet was dried on an aluminium plate at 60 °C for 12 h. All utensils were washed with detergent, rinsed with tap water, deionized water (Milli-Q quality), followed by absolute ethanol, and dried in an oven at 60 °C before use. Stable carbon and nitrogen isotope assays were carried out on 1 ± 0.02 mg subsamples of powder loaded into tin cups. The ^{13}C , ^{12}C , ^{15}N and ^{14}N abundance in the samples were determined using continuous-flow isotope-ratio mass spectrometry (CF-IRMS). Analyses were conducted using a Europa Scientific ANCA-NT 20-20 Stable Isotope Analyzer with ANCA-NT Solid/Liquid Preparation Module (Europa Scientific Ltd., Dundee, UK). The CF-IRMS was operated in the dual isotope mode, allowing $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ to be measured on the same sample. Every 10 samples were separated by two laboratory standards (leucine), which were calibrated against “Europa flour” and IAEA standards N1 and N2.

Metabolomic analysis

Livers, muscles and brains from control and MP-exposed fish were analysed using Nuclear Magnetic Resonance (NMR) spectroscopy to explore how oxygen deprivation influences basic

metabolism. Briefly, samples were placed in Eppendorf tubes and were immediately flash-frozen in liquid nitrogen and stored at -80 °C for further processing. Samples were then lyophilised and coarsely ground with a ceramic pestle prior to extraction of polar metabolites using a modified methanol:chloroform extraction, as previously described (Melvin et al., 2018, 2017). Extracted metabolites were dried in a centrifugal vacuum concentrator and re-suspended in 200µl phosphate buffer made with deuterium oxide (D₂O) and containing 0.05% sodium-3-(tri-methylsilyl)-2,2,3,3-tetradeuteriopropionate (TSP) as an internal reference. This was then loaded into 3mm NMR tubes for analysis using and 800MHz Bruker® Avance III HDX spectrometer.

The NMR, equipped with a Triple (TCI) Resonance 5mm Cryoprobe with Z-gradient and automatic tuning and matching, was controlled with IconNMR™ automation software and included a SampleJet automatic sample changer allowing batch analysis. Spectra were acquired at 298 K using D₂O for field locking and TSP as a chemical shift reference (1H □ 0.00). Proton spectra (1H) were acquired with the zg30 pulse program, with 200 scans, 1.0 s relaxation delay, 7.83 µs pulse width and a spectral width of 16 kHz. Edited 1H-13C Heteronuclear Single Quantum Coherence (HSQC) spectra was acquired for a representative sample to assist with metabolite identification, with 310 scans, 1.0 s relaxation delay, 7.83 µs pulse width and spectral widths of 12.8 kHz (1H) and 33.1 kHz (13C).

Post processing of NMR spectra was carried out with MestReNova v11.0.3 (Mestrelab Research S.L. Spain). The 1H FIDs were Fourier transformed with a 0.3 Hz line broadening, manually phase corrected, and automatically baseline adjusted with the ablative algorithm. The HSQC spectra was processed with default Bruker parameters. All spectra were normalised and referenced to the TSP internal standard (1H □ 0.00, 13C □ 0.00), and chemical features were manually integrated and exported to excel, normalised by sample weight and imported into the MetaboAnalyst 3.0 (Xia et al., 2015; Xia and Wishart, 2016) for multivariate statistical analysis. Metabolites were identified using Chenomx NMR suite 8.2 (ChenomxInc., Edmonton, Canada) and with comparison of HSQC data to standards in the Human Metabolome Database (HMDB), as previously described (Melvin et al., 2018, 2017).

Spectral NMR data imported into the MetaboAnalyst platform was log transformed and auto-scaled, and first analysed with Principal Component Analysis (PCA) to explore broad differences in whole-body metabolite profiles of the mussels. Since moderate separation was observed, Sparse Partial Least Squares Discriminant Analysis (sPLS-DA) was applied to further highlight separation between treatment groups. Identification of significant differences

in individual metabolites was achieved using univariate ANOVAs were performed with a False Discovery Rate adjusted p-value set at $p = 0.05$ and Fisher's LSD post-hoc test.

Microbiome analysis

For 16S mass sequencing, 4 juvenile's guts of each treatment (MP vs. control diet) were dissected and stored in RNAlater at -80°C . Total DNA was extracted independently three times with an iDNesy PowerLyzer PowerSoil extraction kit (Qiagen©) following the manufacturer's recommendations. 16S Bank construction was done using Ion Xpress Plus gDNA Fragment Library Preparation Kit (ThermoFisher) following manufacturer's instructions. Sequencing of bacterial 16S was performed using a PGM Ion Torrent. A control for contamination was performed which consisted of a pool of one control and one MP empty tube filled with RNA later. The extraction control monitored the DNA extraction process. The blank control monitored the library construction. The sequencing results were then analysed using the Metagenomics w1.1 pipeline. There was no reading for MP fish which resulted in $n = 3$ for MP treatment. Relative abundance at the phylum, order and family level between control and MP-exposed fish were compared using the Wilcoxon rank-sum test.

Histopathologic analysis

Three livers of fish from each treatment were used for histological analysis. Right after the dissection, livers were fixed in PFA 4% at 4°C for 48 h before being rinsed and conserved in Phosphate Buffered Saline (PBS) 1X at 4°C . Organs were then dehydrated and included in paraffin. Sections of $5\ \mu\text{m}$ were performed using a microtome. Tissue sections were mounted on glass slides and stained with Hematoxylin and Eosin. Photography was taken on an Olympus microscope and analysed using Image J software. A total of 6 sections was performed for each liver: 3 successive sections in the middle of the liver sample (area 1), and 3 successive sections 1 mm further in the middle of the liver sample (area 2). For each area, the best (based on section and staining quality) of the 3 sections was selected, resulting in 2 sections per liver per fish: 1 for area 1 and 1 for area 2. On each of these selected sections, 5 haphazardly-selected zones were photographed at a 40X magnification. Every of these photographs was then examined by counting regions of fibrosis, hepatolysis and necrosis. This counting was therefore conducted $n = 10$ times per fish (2 areas and 5 zones per liver). Then, a grid of 20 circular subzones (each being a circle with a diameter of $40\ \mu\text{m}$ and labelled from 1 to 20) was superposed on each of the photographs. A total of 3 subzones were selected randomly using the sample function in R. In each of these selected subzones, the number of hepatocyte nuclei and

erythrocyte was counted. This counting was therefore conducted $n = 30$ times per fish (2 areas, 5 zones and 3 subzones per liver). In each of the selected subzones, 3 representative hepatocytes were selected and measured for: (i) vacuole(s) area and (ii) whole cell area. The ratio between these two areas was used as a marker of hepatocyte vacuolation. These measurements were therefore calculated on $n = 90$ hepatocytes per fish (2 areas, 5 zones, 3 subzones and 3 hepatocytes per subzone). Differences between fish and differences between treatments (control vs. microplastic) were investigated using Kruskal and Wallis tests followed by Tukey post-hoc analyses.

Results

Growth and survival

Growth of *S. aurata* juveniles was observed during the experiment (D0: 8.9 ± 0.1 g; D14: 9.3 ± 0.2 g; D35: 10.8 ± 0.8 g; data are shown as mean \pm SE), however, there was no significant effect of MP exposure on growth after 14 days ($W = 514.5$; $p = 0.34$) and 35 days ($W = 298.5$; $p = 0.06$). *S. aurata* mortality over the 35 days experiment was significantly increased by exposure to MP ($p = 0.00096$; Fig. 1). Overall, 33% of the MP exposed fish experienced mortality vs. 3% for the control fish at the end of the 35 days of experiment. Kaplan-Meier survival curves were used for data treatment (Diez, 2013).

Assimilation efficiency

Whole-body depuration kinetics of ^{65}Zn and ^{110}mAg were studied for 17 days and only ^{65}Zn was assimilated. Its depuration kinetics in control and MP fish best fitted by a two-phase model (Fig.2). Estimated AE for ^{65}Zn was higher in MP exposed fish than in control fish ($21.4 \pm 4.7\%$ vs. $17.9 \pm 4.6\%$, respectively, $W = 21$; $p = 0.03$). Activity of ^{110}mAg in both conditions at the end of the 17d experiment was lesser than 1% of initial activities in both fish groups and showed no difference between them (Wilcoxon test; $p > 0.05$) (Fig. 2).

C/N ratio

Stable isotopes profile in the muscle of juvenile seabream exposed to MP during 35 days were not significantly different from controls (Wilcoxon test; $p > 0.05$; Table. 1).

Metabolites profiles

Principle Component Analysis (PCA) of polar metabolites extracted from liver tissues identified different grouping of the control and microplastic exposed fish. Based on this outcome, it was deemed appropriate to further analyse using sparse Partial Least Squares Discriminant Analysis (sPLS-DA) to highlight the separation between treatments. Significance Analysis of Metabolites (SAM) was then performed to identify key metabolites that differed between treatment groups and were driving the observed separation. This identified 28 features in the NMR spectra that differed significantly with a false discovery rate (FDR) adjusted p-value of 0.043 (Fig. 3A), which correlated to 8 metabolites identified as occurring at significantly greater relative abundance in fish exposed to microplastics (Fig. 4). Metabolites that exhibited significantly increased relative abundance in livers of the microplastic group include: inosine, phenylalanine, tyrosine, UDP Glucose/Galactose, glucose, mannose, betaine, sarcosine, alanine and valine.

Principle Component Analysis (PCA) of polar metabolites extracted from brains again identified marginally different grouping of the control and microplastic exposed fish. Separation was less obvious in brains, however based on the level of apparent separation, further analyse using sparse Partial Least Squares Discriminant Analysis (sPLSDA) was again applied to highlight the differences between treatments. Significance Analysis of Metabolites (SAM) was also performed to identify key metabolites that differed between treatment groups and were driving the observed separation. This identified 35 features in the NMR spectra that differed significantly with a false discovery rate (FDR) adjusted p-value of 0.034, which correlated to 15 metabolites occurring at significantly greater relative abundance in fish exposed to microplastics (Fig. 3B). Metabolites that exhibited significantly greater relative abundance in brains of the MP exposure group include: 1-methylnicotinamide, phenylalanine, adenosine monophosphate, N-acetylaspartate, aspartate, myo-Inositol, creatine, glycine, taurine, GABA, glutamate, glutamine, acetamide, alanine and leucine.

Principle Component Analysis (PCA) of polar metabolites extracted from muscle tissue identified very little difference between control fish and those exposed to microplastics. With this outcome supervised multivariate analysis was not proceeded.

Intestinal microbiome

In *S. aurata* juveniles, PE-MP exposure did not change significantly the structure of microbiome, neither at the order, nor at the family level ($p > 0.05$ for all analyses; Fig. 4). At the phylum level, the two most abundant phylum were Proteobacteria (49%) and Actinobacteria (46%). The two most abundant orders of bacteria in fish intestine was Micrococcales (41.5%) and Vibrionales (34.8%). At the order level, the α -diversity ranged between 17 and 29 order overall, with 22.5 ± 4.8 and 20.7 ± 3.2 order for control and MP fish, respectively (Fig. 4A). The PCA analysis shows two groups with similarities in their intestinal microbiome order composition, the first one being composed of three control fish CT2, CT3 and CT4 (control group) and the other one composed of one control fish and two MP fish CT1, MP1 and MP2 (mixed Group) with one MP fish (MP3) with an intermediate composition but closer to the control group (Fig. 4B). Micrococcales were most abundant in MP fish ($62 \pm 20\%$) compared to control fish ($19 \pm 18\%$) while Vibrionales showed the opposite trend with a relative abundance higher in control fish ($61 \pm 20\%$) compared to MP fish ($9 \pm 4\%$) (Fig. 4C). Among the 12 most abundant families in fish, a similar pattern appears with two distinct groups composed of CT2, CT3 and CT4 (control group) and MP1, MP2, CT-1 (mixed group) and an intermediate composition (MP3) but closer to CT2, CT3 and CT4 (Fig. 4D). The control group is characterized with a higher proportion of Vibrionacea and Shewanellacea and lesser proportion of Demequinacea, Cellumonadae, Nakamurellacea, Sanguibacterae, Intrasporangiaceae, Clostridiacea compared to the mixed group (Fig. 4D)

Histopathologic analysis

Microplastic-exposed fish showed higher rates of hepatocyte vacuolation than control fish (10% higher; $\chi^2_{21} = 119.7$; p -value $< 2.2 \cdot 10^{-16}$; Fig. 5A) as well as lower densities of hepatocytes (9% lower; $\chi^2_{21} = 33.71$; p -value $= 6.4 \cdot 10^{-9}$; Fig. 5B). This is well exemplified in Fig. 6. A difference between microplastic-exposed and control fish was also observed in terms of hepatocyte area (3% higher in microplastic-exposed fish; $\chi^2_{21} = 11.988$; p -value < 0.0001), but one of the microplastic-exposed fish showed an opposite pattern (Fig. 5C). However, no difference was observed between MP-exposed and control fish in terms of vascularization

(indicated by erythrocyte density; $\chi^2_{21} = 1.621$; p-value = 0.203; Fig. 5D), fibrosis ($\chi^2_{21} = 1.906$; p-value = 0.167; Fig. 5E), hepatolysis ($\chi^2_{25} = 3.776$; p-value = 0.582; Fig. 5F) and necrosis ($\chi^2_{25} = 4.064$; p-value = 0.540; Fig. 5G).

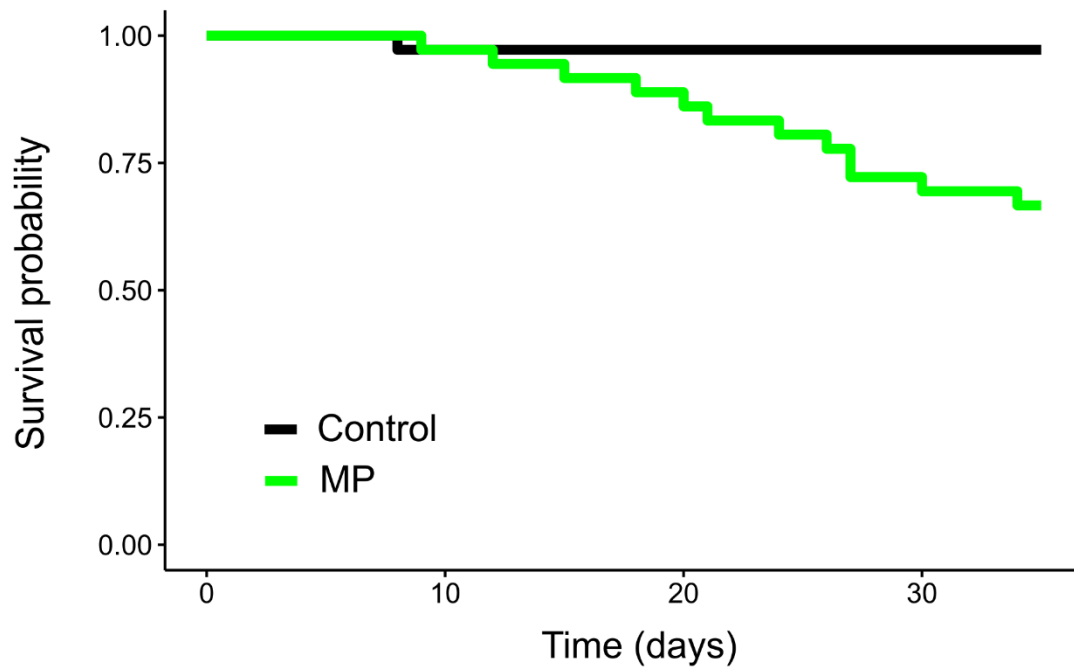


Figure 11. Kaplan-Meier survival curves for *Sparus aurata* juveniles during the experimental period (35 days) comparing exposure to MP-contaminated diet (MP) with a control diet (Control).

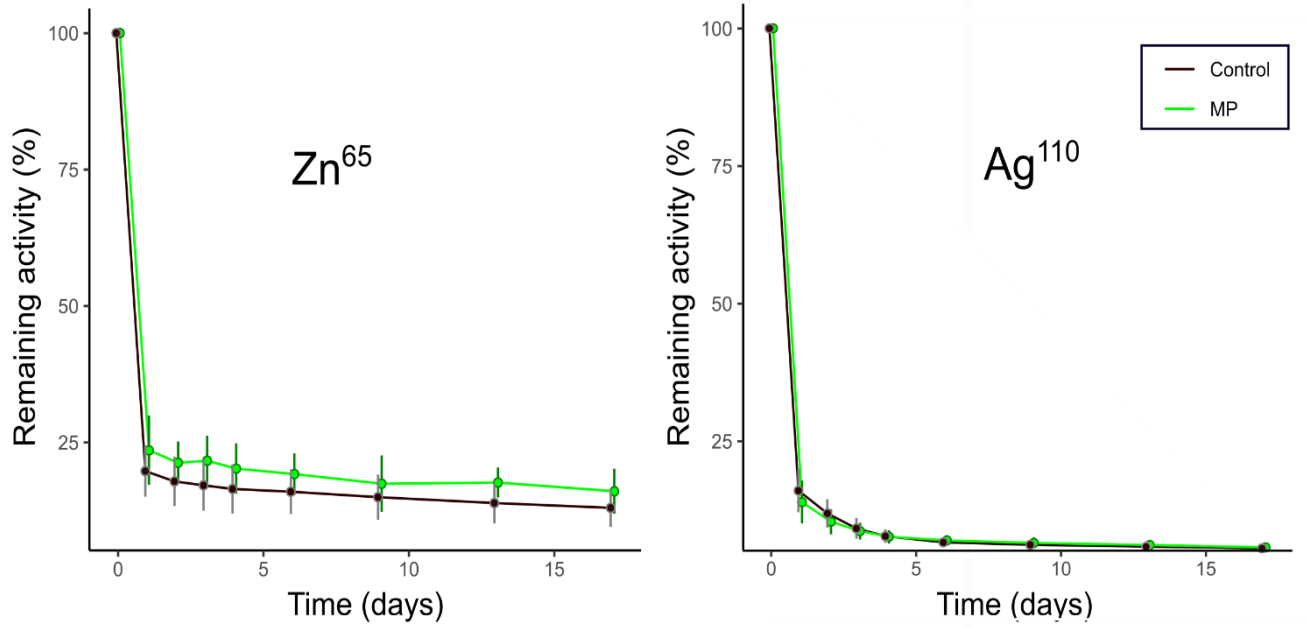


Figure 12. Depuration kinetics of Zn^{65} and Ag^{110} over 17 days of *Sparus aurata* juveniles previously exposed to microplastics-contaminated diet (MP) and control diet (Control) for 35 days

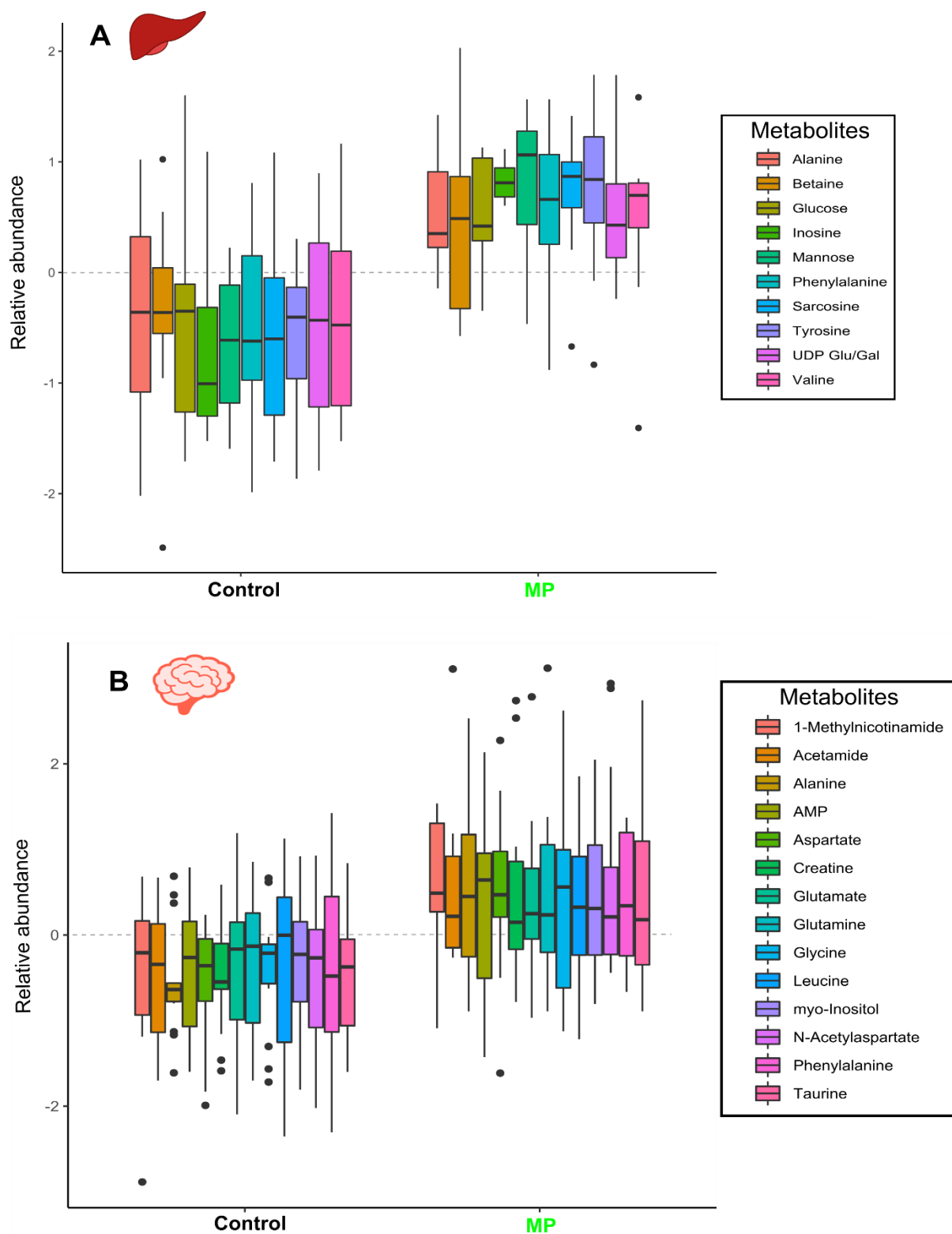


Figure 13. Hepatic (liver) polar metabolites (A) and Brain polar metabolites (B) identified as occurring at significantly different relative abundance in control fish compared to fish exposed to microplastics (MP) during 35 days, based on findings of SAM analysis.

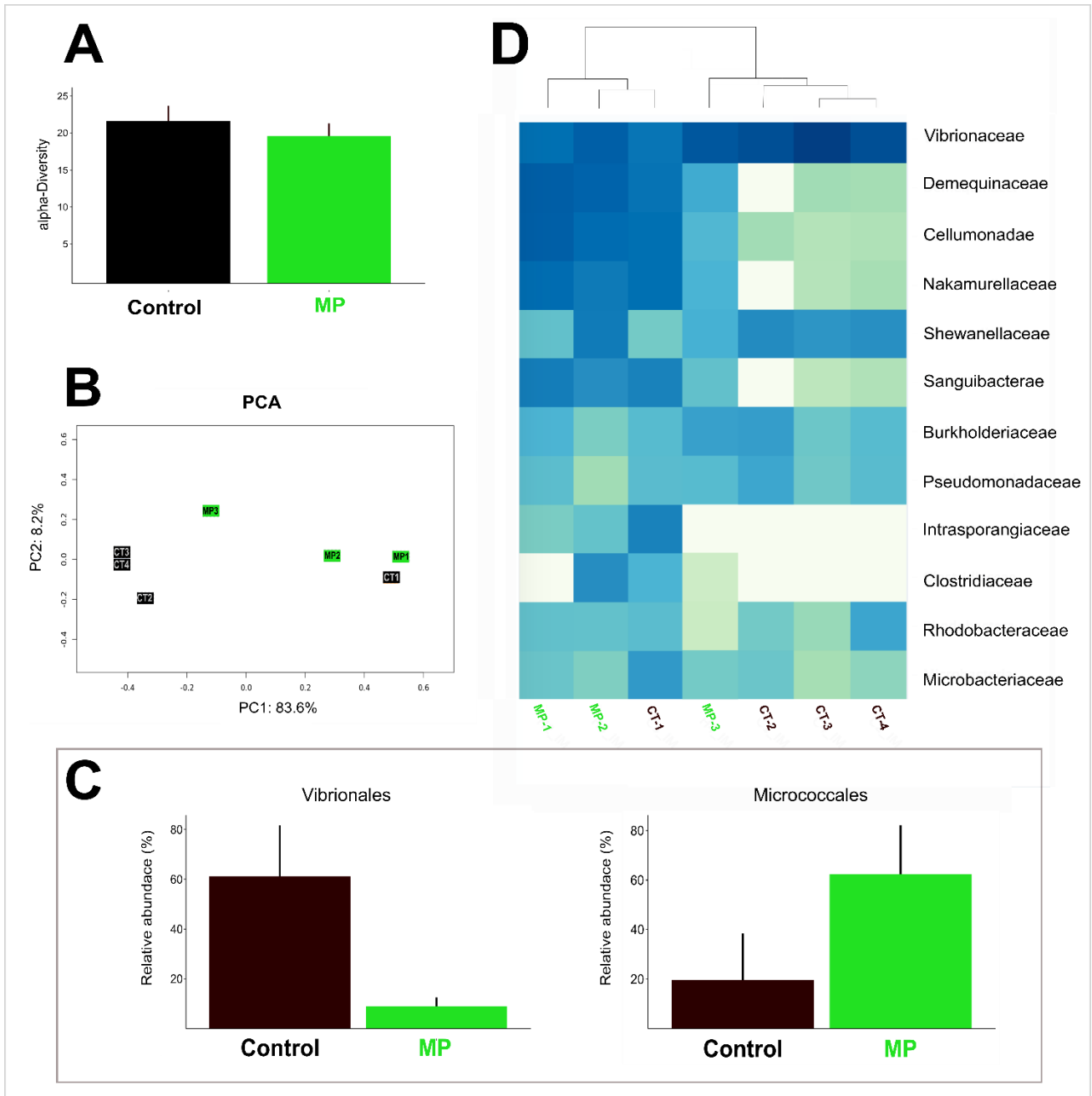


Figure 14. Effect of MP exposure on the composition of microbiome in juveniles *S. aurata*, based on 16S RNA sequencing. **(A)** Alpha diversity at the order level in control (black) vs. MP-exposed fish (green). **(B)** Principal Component Analysis (bray distance) of control fish intestinal microbiome (black) and MP-exposed fish intestinal microbiome (green) at the order level. **(C)** Relative abundance of the two most abundant order of bacteria (*Vibrionales* and *Micrococcales*) in control fish (black) vs. MP-exposed fish (green). **(D)** Heat Map showing change in the relative abundance of the 12 most abundant families of bacteria present in fish intestines (black: control fish / green: MP-exposed fish). For every analysis $n = 4$ for Control and $n = 3$ for MP treatment.

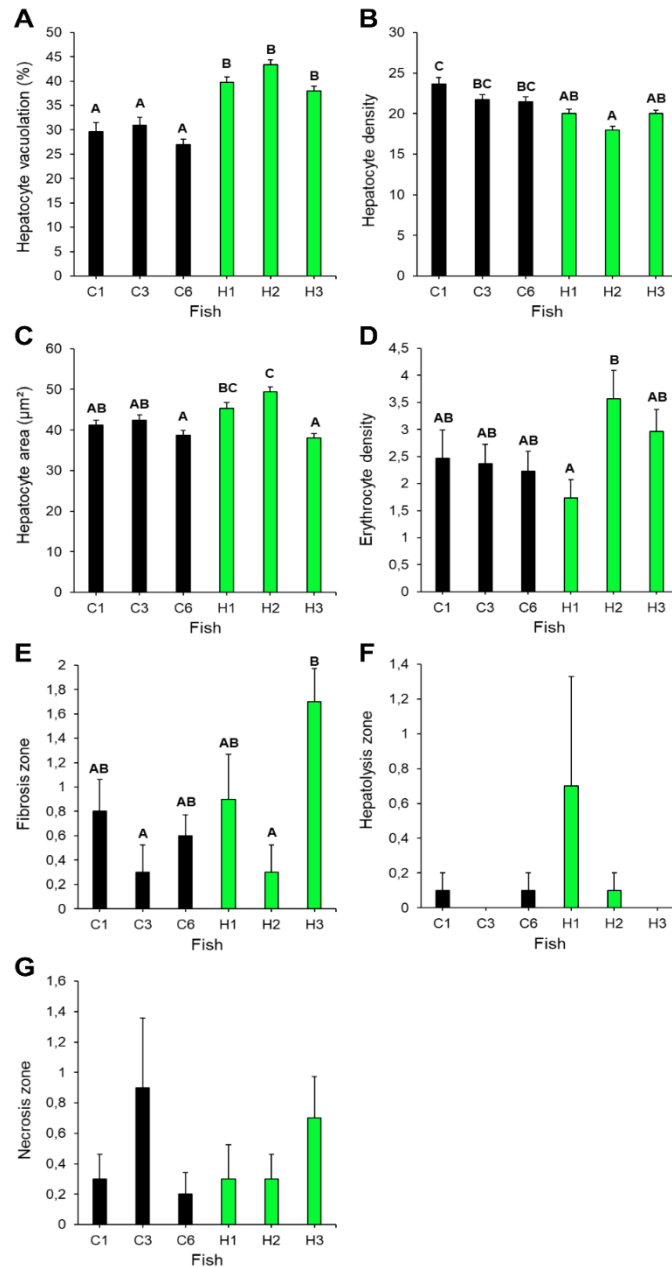


Figure 15. Liver histopathology of fish exposed to microplastics.

(A) Hepatocyte vacuolation in control and microplastic-exposed fish (n = 90 per fish; $\chi^2_5 = 132.37$; p-value < $2.2e^{-16}$; A < B (p-value < 0.001)). (B) Hepatocyte density (i.e. number of hepatocytes in $1257 \mu\text{m}^2$) in control and microplastic-exposed fish (n = 30 per fish; $\chi^2_5 = 44.576$; p-value < $1.769e^{-8}$; A < B (p-value < 0.01) and B < C (p-value < 0.01)). (C) Average hepatocyte cell surface area in control and microplastic-exposed fish (n = 90 per fish; $\chi^2_5 = 53.181$; p-value < $3.088e^{-10}$; A < B (p-value < 0.01) and B < C (p-value < 0.001)). (D) Erythrocyte density (i.e. number of erythrocytes in $1257 \mu\text{m}^2$, marker of vascularization) in control and microplastic-exposed fish (n = 30 per fish; $\chi^2_5 = 11.986$; p-value = 0.040; A < B (p-value = 0.035)). (E) Number of fibrosis zones recorded in control and microplastic-exposed fish (n = 10 per fish; $\chi^2_5 = 16.636$; p-value = 0.005; A < B (p-value = 0.016)). (F) Number of hepatolysis zones recorded in control and microplastic-exposed fish (n = 10 per fish; $\chi^2_5 = 3.776$; p-value = 0.582).

(G) Number of necrosis zones recorded in control and microplastic-exposed fish (n = 10 per fish; $\chi^2_5 = 4.064$; p-value = 0.540).

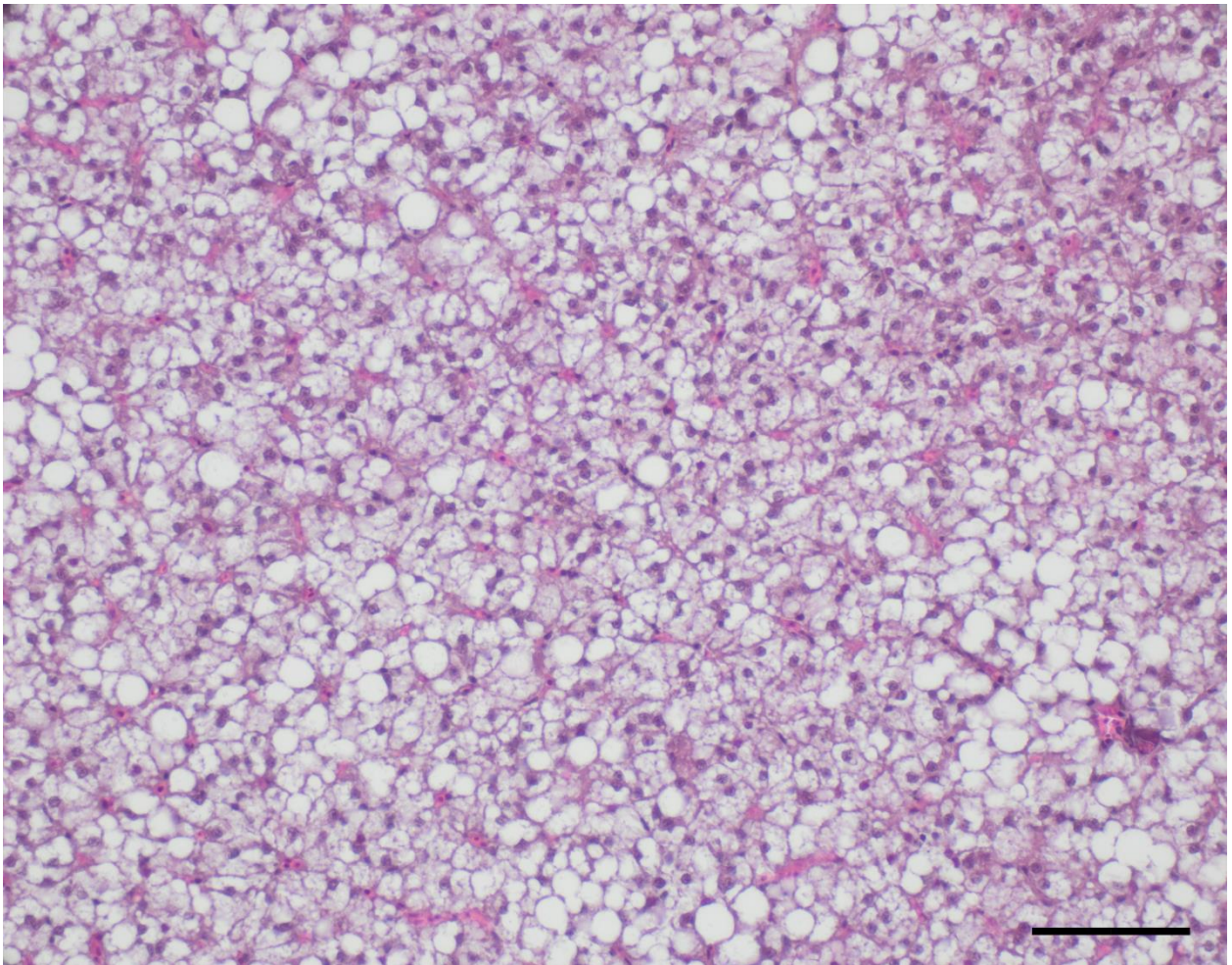


Figure 16. Vacuolated hepatocytes.

This Hematoxylin/Eosin stained 5 μm section of the liver of *Sparus aurata* is representative of the vacuolated hepatocytes observed in fish exposed to microplastics.

Table 2. Carbon (C) and Nitrogen (N) isotopic ratios in muscle of fish exposed to microplastic (MP fish), in control fish and in control brine shrimps and Polyethylene Microplastics (PE-MP).

	Δ 13C/12C (‰)		%C		Δ 15N/14N (‰)		%N	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
MP fish (n=13)	-18.4	0.4	44.6	1.3	13.1	0.2	13.3	0.8
Control fish (n=13)	-18.5	0.3	45.0	0.8	12.9	0.2	13.3	0.5
Artemia (n=3)	-20.5	0.0	40.0	0.4	13.3	0.0	8.7	0.1
PE-MP (n=1)	-29.6	-	73.1	-	-2.4	-	1.4	-

Discussion

For the last ten years, toxicity of MP towards fish physiology and biological parameters has been tested in several experimental studies (Reviewed by de Sá et al., 2018; Foley et al., 2018; Franzellitti et al., 2019). Different fish model and MP were used in these studies, which generally used a limited number of biological indicators. Therefore, no clear consensus exists on the effects and mode of action of MP on fish. Here, a series of toxicological indicators were used, from different biological level of organization (cellular to organism level) in order to understand the effect and possible mode of action of 10-20 µm PE-MP on juveniles of a common marine fish model (*Sparus aurata*). As MP exposure appears to elicit significant effects on biological functions of fish at high concentrations, which are usually several orders of magnitude higher than concentrations reported in the aquatic environment (Jacob et al. submitted), *S. aurata* were exposed to a high concentration of 48180 ± 10074 MP per fish per day in order to undermine their mode of action thanks to the various biological indicators used. This study therefore intends to initiate an understanding of the underlying mechanisms responsible for MP-induced toxicity towards fish.

Here, several biological indicators of *S. aurata* were altered by MP exposure. Firstly, assimilation of an essential elements in *S. aurata* was affected by MP exposure. Indeed, while ^{110}mAg was not assimilated in both experimental treatments, ^{65}Zn assimilation efficiency was significantly higher in MP exposed fish (Fig. 2). Numerous experimental studies have study the trophic transfer of Zn in fish (e.g. Jacob et al., 2017; Pouil et al., 2016) and, in some cases, variation of the AE of Zn can reflect changes in food source or environmental conditions (Van Campenhout et al., 2007). The fact that MP exposure affected Zn AE could be due to direct effect of MP towards mechanistic steps of the absorption and assimilation processes in juvenile *S. aurata* digestive tract. Indeed, the digestive tract is not only a major uptake site for Zn but serves also as an important sink for Zn storage (Hogstrand and Wood, 2010). Interestingly, Zn is known to be essential for the proper maintenance of intestinal tight junctions (Guthrie et al., 2015), which has been previously shown to be affected by MP exposure in fish (Qiao et al., 2019). Hence, increased Zn assimilation could be due to compensatory mechanism to restore intestinal tight junctions. As Zn is implicated in several cellular function (Hogstrand and Wood, 2010), this hypothesis could be extrapolated to several other physiological functions of fish and will require further attention.

We also observed 8 and 15 metabolites occurring at significantly greater relative abundance in liver and brain of MP fish, respectively (Fig. 3). Changes in metabolite profile have been

previously reported following MP exposure in fish, for example, in Qiao et al. (2019) most metabolites in liver of zebrafish exposed to 5 μm PS-MP were increased. Wan et al. (2019) showed more contrasted changes in their study, probably due to the type of metabolite profiling realized (viz. whole zebrafish analyzed instead of a specific organ), but hereto most metabolites were increased when fish were exposed to MP (including glucose, which was also the case in the present study). In the liver, an increase of aromatic amino-acids such as phenylalanine and tyrosine was reported in Crucian carp (Mattsson et al., 2015). Interestingly, increased tyrosine has also been observed in mice liver fed with MP (Jin et al., 2018). Hence, our results are generally consistent with the patterns being described in the literature for fish and even other organisms exposed to MP. Due to few studies done on the effect of MP exposure on metabolite profile of fish, it is difficult to undermine underlying mechanisms responsible for the observed changes. However, by highlighting similarities emerging across these limited studies it is possible to highlight patterns and possible mechanisms. Hence, after MP exposure, a collective change in metabolites is generally occurring rather than a few individual metabolites which could suggest a general disturbance of cellular function rather than specific effects due to the interaction of the MPs with a distinct cellular process, as discussed by some authors (Mattsson et al., 2015; Tang, 2017). For example, Tang, (2017) proposed that metabolic alterations have limited specific relevance and support more the notion of inflammatory responses or alterations to nutrition. Changes in the brain metabolite profile could arise from changes in the gut microbiota. Indeed, the gut microbiota of vertebrates plays critical roles in a variety of biological processes such nutrition, development, metabolism, immunity and resistance to invasive pathogens (Clemente et al., 2012; Yamashiro, 2018). But more particularly, recent studies evoked the links between the gut microbiota and brain neuromodulation (Filosa et al., 2018; Martin et al., 2018), and alterations to the gut microbiota can increase the gut permeability and the translocation of metabolites and bacteria through the intestinal barriers, potentially influencing the gut-liver and gut-brain axis (Yarandi et al., 2016). This study represents the first investigation of the effect of MP on the gut microbiome in a marine fish. Even if no significant statistical differences were observed between the gut microbiome of MP-exposed fish and control fish, some interesting trends arise from our results, with two distinct patterns in microbiome composition emerging in Control and MP-exposed fish.. Overall, the main dominant phylum in seabreams gut were Proteobacteria (49%) and Actinobacteria (46%). The prevalence of Proteobacteria in the gut microbiome of *S. aurata* is consistent with previous report on this species (Kormas et al., 2014) or in fish in general (Sullam et al., 2012). The relative abundance of these two dominant phyla differed between MP-exposed and Control fish,

with MP-exposed fish having a higher relative abundance of Actinobacteria compared to Proteobacteria, and control fish showing the opposite trend (Fig. 5). This was translated to a higher relative abundance of Micrococcales (Actinobacteria) and lower relative abundance of Vibrionales (Proteobacteria) at the order level in MP-exposed fish (Fig. 5C). Although no studies, to the best of our knowledge, looked at the effect of MP exposure through food on the gut microbiome in fish, some studies investigated the effect of PS-MP exposure in freshwater in the zebrafish *Danio rerio* (Jin et al., 2018; Qiao et al., 2019; Wan et al., 2019). While the MP type, MP exposure pathway, fish life stages and fish diet varied between these studies and the current one, we also highlighted here a systematic decrease of Proteobacteria abundance, which are known to influence fat levels in the liver in humans (Spencer et al., 2011), echoing our results on the hepatocyte vacuolation. Microbiome is also tightly connected to the metabolism in various organisms (Yamashiro, 2018), which was shown to be affected by MP in several studies (Mu et al., 2015; Qiao et al., 2019; Wan et al., 2019). This central role of the intestinal microbiome highlights how urgent it is to better examine its sensitivity to MP exposure, as this could be the starting point of all the biological impairments observed in this study and in past studies. Intestinal microbiome is therefore a promising marker when trying to understand the underlying mechanisms of the effect of MP in fish (Fackelmann and Sommer, 2019).

In this study, liver of MP-exposed fish showed higher rates of hepatocyte vacuolation (Fig. 5A), as well as lower densities of hepatocytes than control fish (Fig. 5B). The liver is one of the most intensively investigated organs in fish toxicological studies due to its vital function and its key role in detoxification processes, energy metabolism and storage, food digestion, and the synthesis of proteins of fish (Hinton et al., 2017; Van der Oost et al., 2003; Wolf and Wheeler, 2018). At the subcellular level, vacuolation may indicate energy storage in the form of glycogen or lipid, or it may reflect a degenerative change caused by fluid distension of organelles and/or an accumulation of free fluid in the cytoplasm. Such degenerative change has been related to energy depletion and inhibition of protein synthesis in response to chemical stress in liver of fish (Wolf and Wheeler, 2018). It is not the first time that histological damage are observed in the liver of fish exposed to MP. Jabeen et al. (2018) reported passive hyperaemia, dilated sinusoids, and hydrophic vacuolization in livers of goldfish (*Carassius auratus*) exposed to MP (fibers). Inflammatory responses and lipid droplets have also been reported in livers of zebrafish exposed to 5 μm , 20 μm , or 70 nm polystyrene MP (Lu et al., 2016). Finally, an elevated degree of tissue change has been reported in the livers of juvenile African catfish (*Clarias gariepinus*)

exposed to virgin PE-MP fragments (Karami et al., 2016). Given the widespread reported impairment of liver of fish exposed to MP, liver appears as a target organ of MP induced toxicity.

Finally, our study reveals that mortality was significantly higher for PE-MP exposed fish compared to control (Fig. 1), which can be viewed as the highest possible toxicological response of an organism to a stressor. Although most past studies did not report mortality changes in fish exposed to MP (e.g. Barboza et al., 2018c; Choi et al., 2018b; Jabeen et al., 2017), such increased mortality rates have been reported in a few studies. For example, zebrafish (*Danio rerio*) exposed to 10 mg L⁻¹ of 70 µm PP-MP via water during 10 days showed higher mortality rates than their control counterparts (Lei et al., 2018). The same observations were made for common gobies (*Pomatoschistus microps*) exposed to 216 µg L⁻¹ of 1-5 µm PE-MP via water during 4 days (Luís et al., 2015) and for larval seabass (*Dicentrarchus labrax*) exposed to ~200 PE-MP day⁻¹ (10-45µm) via food during 36 days (Mazurais et al., 2015). Mortality is not specific to a mode of exposure and it is often complicated to infer mortality to a particular biological alteration. It is even more complex to understand the underlying mechanisms responsible for MP-exposure induced mortality. The high biological complexity of organisms like fish, the numerous possibilities for direct and indirect effects of MP on fish biological functions, and the limited variety of biological indicators usually assessed in every single toxicological study make it highly complex to draw mechanistic conclusions about the effects of MP on fish. The use of several biological indicators in parallel might therefore enhance our understanding of reasons behind such a mortality. MP ingestion can cause intestinal blockage and damage in various marine organisms, which may ultimately result in starvation, impaired digestive processes and/or reduced nutrient uptake, known as nutrient dilution, ultimately culminating in reduced growth (Egbeocha et al., 2018; McCauley and Bjorndal, 1999; Welden and Cowie, 2016). Here, *S. aurata* juveniles exposed to PE-MP for 35 days showed no clear histological intestinal damage (data under analysis and not shown), nor altered growth, nor impaired metabolite profile and C/N ratio in muscle (Table 1). This points towards an absence of effect of spherical virgin PE-MP on the integrity of the digestive tract and subsequent assimilation of nutritive elements in *Sparus aurata*. These results are consistent with the literature showing that spherical MP are less prone to alter the integrity of the digestive tract of fish compared to MP fragments and fibers (Jabeen et al., 2017; Jovanović et al., 2018). In addition, the small dimension of the MP used (10-20 µm) would very unlikely cause intestinal blockage in juveniles seabreams. Therefore, it seems unlikely, that the increased mortality rates

of MP-exposed fish observed in this study was due to food deprivation, starvation or affected digestion resulting from MP-exposure. Hence, other mechanisms are more prone to be responsible for the observed MP-exposure induced mortality. In the present study, fish exposed to MP appeared more aggressive than controls with several attacks observed between fish over the exposure period (personal observation; behaviour not quantified). These aggressions could have been the ultimate cause of death in MP-exposed fish, even if we did not directly witness such phenomenon. Aggression has been shown to be displayed as part of the effort to cope with a social challenge or stressor in vertebrates (Verona and Kilmer, 2007) and may reflect variations in internal state of fish (Martins et al., 2012). Physiological stress caused by MP exposure may thus result in an increase of aggressive behavior by *S. aurata*. In the present study, as discussed before, several biological indicators of fish exposed to MP were significantly different than control fish (assimilation efficiency of Zn, metabolite of brain and liver, liver's cells morphology), with contrasted effects on other indicators (e.g. gut microbiome). Alteration of physiological parameters may have led to change in behavior (viz. aggressive fish) and thus indirectly to mortality. Other hypotheses connected to physiological changes of fish exposed to MP could be put forward to explain the mortality observed. For example, an aggravated health conditions of certain individuals could lead them directly to death or could make them more prone to attack of healthier fish, to diseases or to other fatal physiological impairments not measured here.

Altogether, this study shows that exposure to a high dose of 10-20 μm MP for 35 days can induce mortality and a global modification in various biological functions of a marine fish species, the seabream *Sparus aurata*. Two reasons for the mortality observed in this study are possible: either the global physiological stress of MP ingestion caused direct mortality, through immune suppression for example, or those physiological alterations resulted in an increase in the aggression between fish, which ultimately was the causality of death. However, a potential bias of these results is the absence of physiological indicator measurements on the fish that died during the exposure which could have reduced and enhanced certain differences between MP-exposed fish and controls (i.e. survivor bias, Saunders and Hoppa (1993)). Hence, further studies will be needed to solve this question, in particular ones investigating other biological functions such as the immunological and behavioural state of fish. It is worth noting that the results presented in this study are not reflecting the current environmental exposure conditions of MP encountered by fish in the wild (Mizraji et al., 2017; Nelms et al., 2018). Instead, these results have to be viewed as a proof of concept to understand the mode of action of MP in fish.

Once the underlying mechanisms responsible for the toxicity of MP towards fish will be better understood, lower MP concentrations (viz. more environmentally realistic exposure conditions) should be tested in order to assess their environmental risk.

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Chapitre 3 - Effets des microplastiques sur les post-larves du poisson corallien chirurgical bagnard (*Acanthurus triostegus*)

Avant-propos

Cycle de vie des poissons coralliens

La majorité des poissons de récifs coralliens émettent leurs gamètes (œufs et spermatozoïdes) directement dans la colonne d'eau (espèces d'œufs pélagiques), tandis qu'une minorité d'espèces déposent et fécondent leurs œufs sur un substrat de récif (espèces d'œufs benthiques, par exemple, Pomacentridae et Balistidae) (Cole, 2010). Les œufs éclosent généralement de quelques jours (œufs pélagiques) à quelques semaines (œufs benthiques) après la fécondation, et les larves émergentes sont initialement emportées des récifs par des courants océaniques (Leis and McCormick, 2002). Les larves peuvent dériver pendant une semaine (poisson clown) (Thresher et al., 1989) jusqu'à plus de 64 semaines (poisson porc-épic) (Quinn and Ogden, 1984) avant de migrer vers les récifs. Ainsi, la majorité des poissons de récif ont un cycle de vie bipartite, consistant en un stade larvaire pélagique suivi d'un stade juvénile et adulte associé à un récif essentiellement sédentaire (Figure 17) (Cole, 2010; Leis and McCormick, 2002). Une fois compétentes, généralement vers la fin de la phase pélagique, les larves sont prêtes à quitter l'océan et à rejoindre les populations de récifs démersaux (Leis and McCormick, 2002). La transition entre le stade pélagique et démersal correspond à la colonisation (figure 17).

La transition de l'environnement océanique pélagique à l'environnement récifal benthique (recrutement) représente une période critique de l'histoire de la vie des poissons coralliens, les processus comportementaux et physiologiques actuels influant directement sur la survie et l'aptitude potentielle future (Holzer et al., 2017; Lecchini et al., 2005), au cours de cette période de recrutement, souvent qualifiée de «période critique» dans le cycle de vie des poissons de récifs coralliens (Almany and Webster, 2006), où la mortalité peut atteindre 95%, les larves qui colonisent doivent compter sur de fortes aptitudes physiologiques et de nage, et tout changement dans la condition des jeunes poissons et leur capacité à trouver de la nourriture peut avoir un effet considérable sur la reconstitution de la population de poisson. En conséquence, les post-larves de poissons de récifs coralliens doivent s'appuyer sur leurs capacités sensorielles et physiologiques pour: i) trouver un habitat convenable ii) éviter les

prédateurs et iii) trouver de la nourriture afin de s'intégrer dans la population adulte et devenir sexuellement mature (Barth et al., 2015).

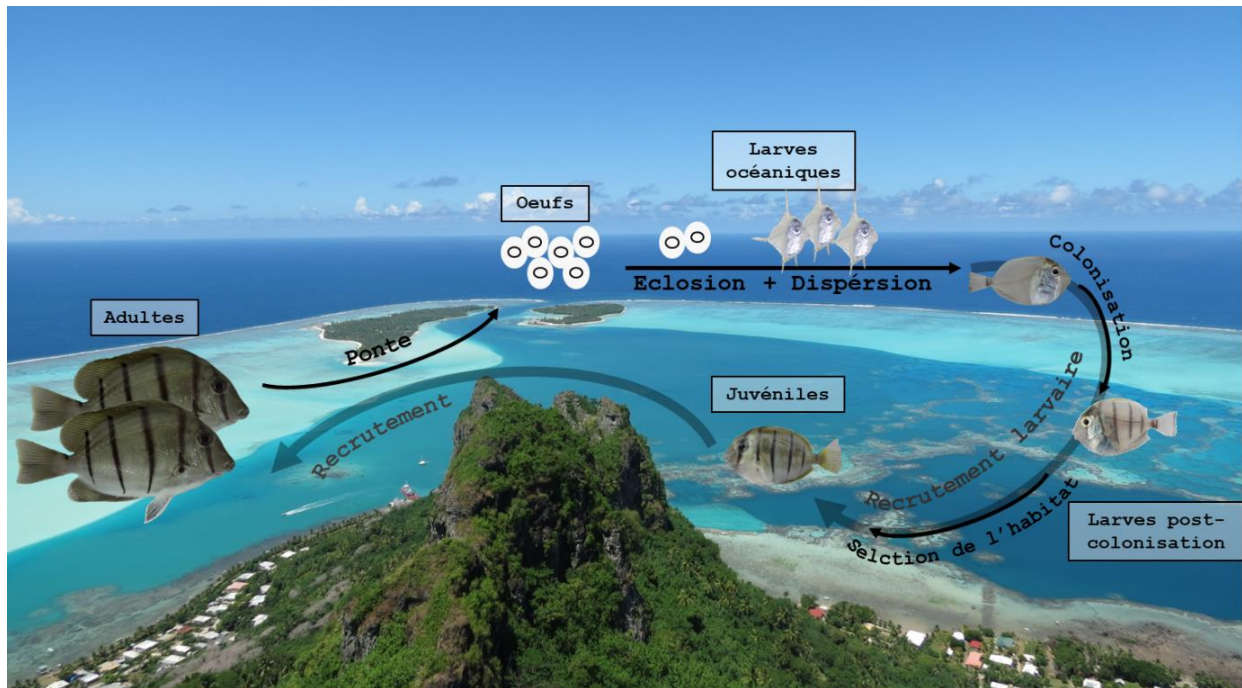


Figure 17. Cycle de vie des poissons coralliens (Crédit photo : M. Besson).

Modèle et lieu d'étude

À ce jour, *A. triostegus* est l'espèce de poisson des récifs coralliens la plus étudiée en termes de métamorphose (Figure 18) (Frédérich et al., 2012; Holzer et al., 2017; Randall, 1961). Après leur période larvaire pélagique de 44 à 60 jours (Lo-Yat et al., 2006), les larves transparentes d'*A. Triostegus* se métamorphose en juvéniles pigmentés en quelques jours (Figure 18). Au cours de cette transition, les larves se nourrissent de micro-algues; leur forme change à mesure que leur hauteur corporelle diminue, tandis que leur bouche migre vers une position plus verticale (comme chez les brouteurs) et que leurs écailles se développent (Frédérich et al., 2012). Les juvéniles et les adultes se nourrissent d'algues filamenteuses (Randall, 1961). Après la colonisation des récifs, les jeunes juvéniles sont généralement rencontrés en petits groupes de dizaines d'individus, dans des eaux calmes et peu profondes le long du littoral. À l'inverse, les adultes peuvent être solitaires, mais forment généralement de grands regroupements (jusqu'à des centaines d'individus) autour de parcelles de récif proches de la barrière de corail (Randall, 1961).



Figure 18. Représentation des différentes étapes de transitions de la larve au juvénile d'*Acanthurus triostegus*.

Acanthurus triostegus est l'une des espèces les plus abondantes et les plus représentatives de l'Indo-Pacifique (Randall, 1961) et est particulièrement abondante à Moorea (Polynésie française) (Holzer et al., 2017; Lecchini, 2004). L'île de Moorea se situe à 25 km au nord-ouest de Tahiti, à 17 ° 30 'de latitude sud et à 149 ° 50' de longitude ouest. Cette île volcanique s'est formée il y a moins de deux-millions d'années à partir d'un hotspot océanique. Elle appartient aux îles du Vent de l'archipel de la Société. Moorea a une forme triangulaire avec 61 km de côtes entourées par une lagune de 500 à 1 500 mètres de large. La lagune est délimitée par une barrière de corail qui ouvre la lagune sur l'océan en douze passes. Les fluctuations des marées sont très faibles dans cette région du monde, avec des différences de marée maximales de 40 cm. Du fait de l'accessibilité à des post-larves de poissons coralliens comme *A. triostegus*, Moorea représente un terrain de recherche propice et pour étudier l'effet des stress anthropiques sur les jeunes stades de vie des poissons coralliens. Durant ma présence à Moorea, j'ai eu l'occasion de participer et réaliser différentes études en parallèle de ma thèse sur l'effet de différents stress anthropiques sur le recrutement d'*A. triostegus* par exemple sur la pollution pesticide (Bertucci et al., 2018; co-premier auteur; Annexe 3 et Besson et al., 2017; Annexe 5;) ou la pollution lumineuse (O'Connor et al., 2019; Annexe 4).

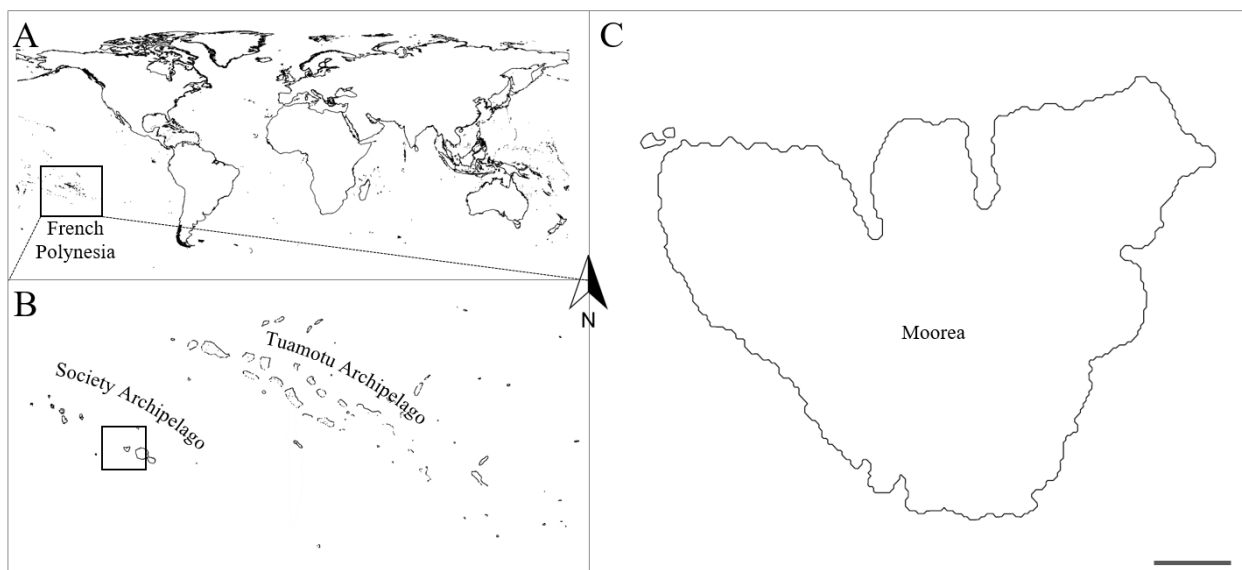


Figure 19. **A:** Localisation de la Polynésie française dans l’océan Pacifique. **B:** Localisation de l’île de Moorea en Polynésie française. **C:** île de Moorea. Barre d’échelle de 2 km. Adapté de Besson et al. (2017).

Objectifs et questionnement scientifique

Aujourd’hui, alors que les poissons marins subissent simultanément de multiples pressions anthropiques, telles que le changement climatique et la pollution sont reconnus comme les principaux facteurs affectant la population et les assemblages de poissons marins. Par conséquent, il existe une littérature abondante sur les effets potentiels de facteurs de stress anthropogéniques tels que l’acidification des océans, l’augmentation de la température des océans et de la pollution chimique sur le développement larvaire, la croissance, le comportement et la capacité de recrutement des poissons de récifs coralliens (Bertucci et al., 2018; Besson et al., 2017; Devine and Munday, 2013; Ferrari et al., 2012; Munday et al., 2011; Nowicki et al., 2012). Le domaine de la recherche sur les effets écotoxicologiques des MP est encore dans son enfance. Ainsi, à l’heure actuelle aucune étude ne s’est intéressée aux effets de ces particules sur les larves de poissons coralliens. Cependant, même si la littérature sur l’impact écologique des MP sur les poissons téléostéens est encore rare, leur potentiel d’influer sur le recrutement des poissons de récifs coralliens par différentes voies (physiologique, comportementale ou sensorielle) émerge déjà de la littérature existante (voir chapitre 1), ce qui pourrait avoir de nombreuses répercussions pour les poissons de récif corallien durant la phase critique du recrutement. En outre, une étude à laquelle j’ai pu participer durant ma présence à

Moorea a fourni la première indication de l'ingestion de MP par des poissons de récif en Polynésie française (Garnier et al., 2019; deuxième auteur; Annexe 3), confirmant ainsi l'interaction de ce type de particules avec les poissons coralliens. Par conséquent, la question demeure de savoir comment les MP peuvent affecter le processus de recrutement des poissons de récif corallien. Ce chapitre 2 présente donc la première étude sur l'effet des MP de polystyrène sur le comportement alimentaire et la survie d'une post-larve de poisson corallien, le chirurgien bagnard *Acanthurus triostegus*.

Abstract

Microplastics (MP) are ubiquitous in the marine environment and have been shown to alter the behaviour of some species due to potential neurotoxic effect. However, very little is known on the effect of this stressor on behavioural responses of early and more vulnerable life stages. This study explores the effects of polystyrene MP (90 μm diameter) on the foraging activity of newly settled surgeonfish *Acanthurus triostegus* and on their survival facing predators. Exposure to a high concentration of 5 MP particles per mL (5 MP.mL⁻¹) for 3, 5 and 8 days did not alter their foraging activity nor their susceptibility to predation. This suggests that short-term exposures to reportedly high MP concentrations have negligible effects on the behaviour of newly settled *A. triostegus*. Nevertheless, responses to microplastics can be highly variable, and further research is needed to determine potential ecological effects of MP on reef fish populations during early-life stages.

Keywords: Plastic pollution; behavior; feeding; predation; larval recruitment; *Acanthurus triostegus*.

Introduction

There is increasing evidence that exposure to microplastics (MP) leads to adverse effects on marine animals (Stephanie L Wright et al., 2013). In fish, MP have been found to cause physical and physiological defects, such as false food satiation, tissue damage and decreased swimming performance (Barboza et al., 2018c; Critchell and Hoogenboom, 2018; Lu et al., 2016b; Pedà et al., 2016). However, despite the range of sub-lethal effects reported, the effects of MP on fish behaviour remain poorly understood. For example, while MPs have been reported to cause lethargic and erratic swimming behaviours in the European seabass, *Dicentrarchus labrax* (Barboza et al., 2018d), other studies found no significant effects on fish behaviour (*e.g.* Guven et al., 2018; Tosetto et al., 2017). Discrepancies could be due to several factors including differences in MP concentrations, plastic types, species and life stages. Consequently, more research is needed to elucidate the effects of MP on the behaviour of aquatic species, such as foraging and predator avoidance. Indeed, these behaviours are not only fundamental to individuals' fitness and functional role in their ecosystem (Wolf and Weissing, 2012), but also their modifications are considered among the most sensitive indicators of environmental disturbances (Hellou, 2011; Melvin and Wilson, 2013).

In addition to the lack of understanding regarding behavioural responses in fish exposed to MP, limited information is also available on their effects on early-life stages of marine organisms that may be particularly sensitive to environmental disturbances (Besson et al., 2017; Holzer et al., 2017; Mazurais et al., 2015; van Dam et al., 2011). The vast majority of coral-reef-associated fishes have a stage-structured life history, with a relatively sedentary benthic juvenile and adult stage preceded by a pelagic larval stage (Leis and McCormick, 2002). The transition between larval and juvenile stages occurs when pelagic larvae enter and settle in the reef environment. This step is referred to as the larval recruitment phase and involves a range of physical and behavioral changes (*i.e.* metamorphosis) that are controlled by thyroid hormones (Holzer et al. 2017). This metamorphic phase is often referred to as a “critical period” in coral reef fish life cycle (Almany and Webster, 2006), where selective pressure is high and settling post-larvae must rely on strong perception abilities (Lecchini et al., 2005). Any stressors affecting their behaviour during this critical life-history transition can have significant effects on fish restocking. For example, Holzer et al. (2017) showed that a common pesticide (chlorpyrifos) affects the grazing behavior and also the metamorphosis of the tropical herbivorous fish *A. triostegus* following their recruitment to the reef. Given the fact that coastal areas are being subjected to increased plastic pollution (Avio et al., 2017), there is an increased

likelihood for reef-associated juveniles to encounter microplastic particles that could have cascading effects on coral-reef ecosystems.

The present study investigates the potential effects of MP on the behaviour of post-larvae of the convict surgeonfish *Acanthurus triostegus*. This is important since MP are hypothesized to have neurotoxic effect on fish (Barboza et al., 2018c; Oliveira et al., 2013) that can result in behavioral anomalies and increased predation-related mortalities in *A. triostegus*. More precisely, we examined how exposure to a relatively high concentration of 90- μm polystyrene MP (5 MP.mL⁻¹) influenced foraging activity and survival to predation over a period of 8 days post-settlement.

Materials and Methods

Post-larvae (*i.e.* larvae under metamorphosis) of the convict surgeonfish *Acanthurus triostegus* (n = 270) were collected in rock pools using dip nets during the overnight high tide around the new moon phase between May and August 2017 as they enter and settle to the reefs at Tema'e, Moorea Island, French Polynesia (17°29'50.7"S 149°45'15.3"W; Holzer et al., 2017).

After capture, *A. triostegus* ($0.63 \pm .019$ g wet weight for a subsample of n = 10) were immediately transferred to 20 x 15 x 15 cm aquaria (10 fish per aquaria max.) filled with 3 L of 1 μm filtered seawater. Fish were exposed to either seawater only (control) or seawater with polystyrene microplastics (MP) at a concentration of 5 MP.mL⁻¹ for 3, 5, and 8 days. MP stock solution consisted of 0.15 g of NIST Traceable colorless polystyrene beads, with a diameter of 91.26 ± 0.2 μm and density of 1.05 g/cc, in 15 mL osmosis water (6.24×10^4 MP particles mL⁻¹; Polysciences Warrington, PA, USA). MP concentrations were confirmed *a posteriori* by visual counting under a stereo microscope Leica EZ-E using a 1 mL sedgewick rafter counting chamber in both control condition (0 MP.mL⁻¹; n = 2) and the MP condition (4.77 ± 0.77 MP.mL⁻¹; n=3). Fish exposed for 3 and 5 days were not fed, while fish exposed for 8 days were fed once on the fifth day. Each aquaria was equipped with an air stone to maintain MP in suspension and ensure optimal dissolved oxygen levels. Water temperature was maintained at 28°C ($\pm 1^\circ\text{C}$) and salinity at 35 ppt. A 12-h light/dark photoperiod was maintained throughout the experiments at the CRIOBE research station. All experiments were approved by the CRIOBE-IRCP animal ethics committee (CRIOBE/IRCP-Fish-2018-04).

Foraging activity was assessed following the protocol described by Holzer et al. 2017 with minor modifications. After 3, 5 and 8 days of exposure, three randomly-selected fish from each

condition (control and MP) were placed in a 7 L glass tank containing control seawater and a coral rubble (~ 30 cm³) covered with turf algae placed at the center. Coral rubbles were collected at the post-larvae sampling site. After a 10-min acclimation period, fish foraging activity was video recorded for 20 minutes (Takara cameras set up 50 cm above the tank and configured in 720p/30fps). Feeding trials were replicated 6-10 times per condition and time point (day 3, 5 and 8; *i.e.* 18 to 30 fish per condition and time point – 108 fish in total). Each group of 3 fish was only tested once. Following each trial, seawater was completely renewed, and a new coral rubble was used. Feeding activity was analyzed by counting the total number of bites made by the 3 fish on the turf algae during 3 periods of 2 min sections that were randomly selected between 0-7 min, 7-14 min and 14-20 min (*i.e.* cumulative number of bites during a total of 6 minutes of recording). For weight measurements, after 8 days, 12 fish from each treatment (MP and Control) were euthanized by cold shock, subsequently dried in absorbent paper and weighted with a precision of 0.01 g.

To test how MP exposure affects predation risk, predation survival tests were conducted on 8-day exposed fish (control and MP treatments; Holzer et al. 2017). Survival tests were conducted in circular arenas (85 cm diameter, 40 cm depth). Ten randomly selected fish from each treatment were simultaneously placed into the test arena containing 4 lionfish *Pterois radiata* (2 adults and 2 juveniles) starved for 48 hours prior to the experiment. Trials began during the afternoon to allow *A. triostegus* post-larvae to acclimate to arena conditions before predation (lionfish are nocturnal predators). Each group of prey fish was identified via subcutaneous coloured elastomer tags (Northwest Marine Technology) injected dorsally a minimum of 12 hours before each trial. Predation trials were replicated 6 times per condition (N = 6; total of 60 post-larvae per treatment). Predator pairs were changed and colour tags were switched between each trial. Trials were ended when approximately 50% of post-larvae were predated, or after 24 hours. The percentages (number of survivors / initial number of fish) of *A. triostegus* post-larvae remaining was then assessed to measure relative (from each treatment) and overall (both treatments) survival rates. Ingestion of MP by *A. triostegus* post-larvae after 8 days of exposure was confirmed by visual inspection of the dissected digestive tract under stereo microscope (Leica EZ4 E; n = 10).

Differences in foraging activity between treatments were analyzed using a full factorial two-way ANOVA with treatments (control and MP) and time as factors. Percentages of *A. triostegus* survival were arcsine transformed and differences between treatments were analysed using an unpaired *t* test. Differences in *A. triostegus* weight between treatments were analysed using the

Mann-Whitney U test. All data were first tested for variance homogeneity and normality of distribution. The significance level was set at $\alpha = 0.05$. Analyses were performed using SPSS 22.0 (SPSS Inc., Chicago, IL).

Results and Discussion

In fish, ingestion of non-nutritive elements, such as MP, and their subsequent accumulation in the digestive tract can lead to malnutrition and intestinal damages (Boerger et al., 2010; Lei et al., 2018a). Exposure to MP has also been reported to inhibit acetylcholinesterase (AChE) activity in fish (Oliveira et al. 2013; Barboza et al. 2018a). Such inhibition of AChE activity can adversely affect the nervous and neuromuscular function, resulting in a decrease in swimming activity (Bonansea et al., 2016). Consequently, altered behavioural responses would be expected for fish exposed to MP-contaminated water. However, contrary to our hypothesis, MP exposure in the present study had no significant effect on *A. triostegus* foraging activity (Fig.1A; $F_{(1, 38)} = 0.78$; $p = 0.38$), body weight (Day 8: MP = $0.59 \pm .05$ g; Control = $0.61 \pm .03$ g; $W = 63$, $p = 0.42$) and survival facing a common reef fish predator, *P. radiata* (Fig.1B; $t = 0.52$; $df = 10$; $p = 0.62$). Ingestion of MP by *A. triostegus* post-larvae after 8 days of exposure was confirmed with 1.7 ± 1.33 MP per fish.

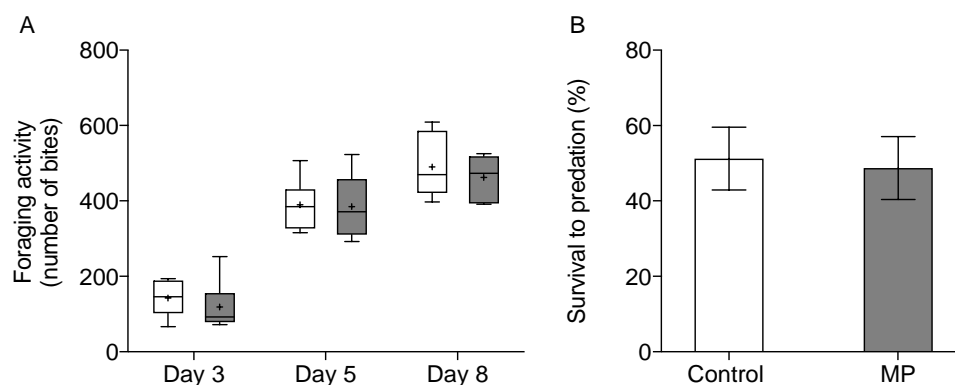


Figure 20. Effect of 90- μ m polystyrene microplastic beads on the foraging activity and survival to predation of post-larvae of the Surgeon fish *Acanthurus triostegus*. MP: microplastics, nominal concentration of 5 MP.mL⁻¹. [A] Foraging activity (cumulative number of bites on turf algae) of fish exposed to control (white) or MP (grey) during 3, 5, and 8 days. Boxplots show the interquartile range (column), median (horizontal line), minimum and maximum values (whiskers) and average (+) of 6-10 groups of 3 fish (N = 6-10). [B] Survival

to predation (%) of post-larvae by *Pterois radiata* after 8-day exposure to MP (average \pm SD; n = 10 fish per treatment; N = 6).

Regardless of MP treatment, an increase in foraging activity (number of bites on turf algae) was observed over time (Fig.1A; $F_{(2, 38)} = 88.3$; $p < 0.0001$), confirming the expected trend associated with *A. triostegus* transition from a carnivorous to an herbivorous diet (Holzer et al., 2017). As such, this confirms that post-larvae metamorphosed and behaved normally within our experimental system. The experimental design used in this study was carefully selected to be highly relevant from an ecological perspective. Fish post-larvae were caught right after their settlement to the reef coastal environment (from the open ocean) when they strongly rely on perception abilities for feeding and escaping predation, but also when they are potentially facing shift in the contamination status of their environment, coastal areas being subjected to greater plastic pollution (Avio et al., 2017). The duration of the exposure of 8 days corresponds with the critical period of *A. triostegus* larval recruitment and metamorphosis when they are the most sensitive to contaminants (Almany and Webster, 2006; Holzer et al., 2017). On the other hand, the particle concentration (5 MP.mL^{-1}) was selected to represent a worst-case scenario as it is considered to be higher than the mean concentrations encountered in the natural environment (Paul-Pont et al., 2018), but still realistic in highly contaminated areas (Dubai and Liebezeit, 2013; Peeken et al., 2018) and represent a tangible scenario in highly polluted tropical coastal waters where plastic debris accumulates (Lavers and Bond, 2017).

It is possible that the exposure duration (8 days) may not have been long enough to reach the toxicity threshold for *A. triostegus*, as the few studies that observed significant effect of plastic particles on fish behavior used longer exposure time (e.g. over 60 days: Mattsson et al. 2015, 2017). However, this is not always the case. A recent study reported that 4 days of exposure to a relatively high concentration of MP of 0.69 mg.L^{-1} , compared to 0.81 mg.L^{-1} in the present study, resulted in decreased swimming performance in juvenile fish *Dicentrarchus labrax* (Barboza et al., 2018d). This is corroborated by Oliveira et al. (2013) who observed neurotoxic effects on the common goby *Pomatoschistus microps* after 4 days of exposure to $0.184 \text{ mg MP.L}^{-1}$. Contrary to our study, Barboza et al. (2018b) and Oliveira et al. (2013) used smaller particle size (1-5 μm). It is therefore possible that smaller MP (<20 μm) or nanoplastics (NP) could have had a greater impact on *A. triostegus* post-larvae as they are generally found to have increased deleterious effect on fish compared to larger plastic particles (Chae and An, 2017; Chen et al., 2017; Mattsson et al., 2017). Importantly, the concentrations used in all the

aforementioned studies are likely to be higher than the mean concentrations encountered in the natural environment (Paul-Pont et al. 2018).

Given the lack of observed effects after 8 days of exposure to MP, it is likely that short-term exposures to relatively high MP concentrations have negligible effects on the behavior of coral-reef fish during larval recruitment. Nevertheless, as this is the first study to assess the effect of plastic pollution on the young, more vulnerable, life stages of a coral-reef fish species, more research is certainly needed to better understand the potential risks of MP pollution on these early-life stages.

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Conclusion générale

Les effets de l'exposition aux MP constituent une inquiétude grandissante pour les animaux aquatiques dans presque tous les systèmes marins de la planète, peu importe leur éloignement des sources de pollution potentielles. En effet, les MP ont été retrouvés au niveau des gyres océaniques (Eriksen et al., 2014), des régions côtières (Ling et al., 2017), des régions polaires (Bergmann et al., 2017; Lusher et al., 2015), jusqu'aux abysses des océans (Chiba et al., 2018). Parmi les taxons présent en milieu marin, les poissons sont d'une importance particulière car ils représentent la principale source de protéine de millions d'habitants des zones côtières, jouent un rôle essentiel dans le fonctionnement des écosystèmes marins et possèdent une forte valeur éco- systémique (pêche, plongée) (Dyck and Sumaila, 2010). De plus, ces organismes sont sous forte pression anthropique, que cela soit par la surpêche, ou les changements climatiques (Free et al., 2019; Rousseau et al., 2019). Ainsi, l'apparition de nouveaux polluants potentiellement toxiques envers ces organismes comme les MP ont attiré l'attention de la communauté scientifique au cours des dernières années afin de comprendre les effets potentiels de ces particules sur les poissons, jusqu'à leur potentiel ramification pour la productivité de la pêche et leur bioaccumulation dans ces espèces, qui pourraient affecter les hommes les consommant (Foley et al., 2018). Les poissons représentent donc actuellement le taxon le plus étudié au regard de la pollution microplastiques, que cela soit dans l'environnement ou en laboratoire (de Sá et al., 2018), cependant peu d'étude sont disponibles sur l'effet des MP chez les jeunes stades de vie des poissons marins. Pourtant, les jeunes stades de vie des poissons sont connus pour être particulièrement sensibles aux stress environnementaux (Mohammed, 2013). De plus, c'est durant ces jeunes stades de vie, des larves aux juvéniles, qu'une forte pression de sélection est exercée sur les poissons, en effet plus de 90% périssent durant cette période (Almany and Webster, 2006). Pour les poissons, la taille des stocks d'adultes est donc largement déterminée par la survie des individus pendant les stades larvaires et juvéniles (Shepherd and Cushing, 1980). Ainsi, au cours de cette thèse, les effets des MP sur les poissons ont été synthétisés et analysés sous la forme de méta-analyse (Chapitre 1) et leurs effets sur différents modèles de poissons marins (*Sparus aurata* et *Acanthurus triostegus*) ont été quantifiés (Chapitre 2 et 3), apportant de nouvelles informations sur la potentielle toxicité de ce genre de particules chez les jeunes stades de vie des poissons marins.

La première partie de cette thèse synthétise et analyse les études portant sur les effets des micro et nano-plastiques chez les poissons. En effet, malgré le nombre important de revues récemment publiées sur l'effet potentiel des MP/NP et leurs contaminants associés sur les organismes marins (e.g Chae and An, 2017; Foley et al., 2018; Franzellitti et al., 2019), aucune n'a synthétisé l'ensemble des études écotoxicologiques de laboratoire portant sur l'effet des MP/NP vierges au sein d'un taxon marin comme les poissons. Pourtant, il est important de pouvoir dissocier l'effet des MP/NP vierges de leurs contaminants et/ou microorganismes associés, afin de déterminer précisément le risque environnemental de ce genre de particule sur la faune marine. Ainsi, la méta-analyse des effets des MP/NP vierges sur les poissons présentée dans le chapitre 1 a révélé que les MP / NP vierges ont affecté significativement 32% des paramètres biologiques mesurés chez les poissons au sein des 46 études de laboratoire examinées, soutenant ainsi que les MP / NP vierges en soi peuvent être toxiques pour les poissons. Ces effets des MP/NP sur les poissons apparaissent divers, cependant les effets les plus significatifs ont été observés chez les petits MP (< 20 µm) affectant notamment les fonctions comportementales et neurologiques, la perméabilité intestinale, le métabolisme et la diversité du microbiome intestinal des poissons exposés (Fig. 7).

Au vu de la variété et de la subtilité des effets des MP sur la biologie des poissons présentée dans le chapitre 1, il apparaît nécessaire que les futures études écotoxicologiques prennent en compte de nombreux paramètres biologiques afin de développer un cadre complet et représentatif des impacts des MP vierges sur les poissons. Ainsi, le chapitre 2 présente une approche multi-diagnostique impliquant une évaluation d'impact à plusieurs niveaux d'organisations biologiques (de l'atome à l'organisme) afin de déterminer les effets des MP sur un poisson marin. Plus précisément, nous avons exposé des juvéniles de daurade royale (*Sparus aurata*) à des MP de polyéthylène (PE-MP) vierges de 10 à 20 µm par voie alimentaire (*Artemia salina* exposée à 5 mg L⁻¹) pendant 35 jours, puis nous avons examiné leur histologie du foie et de l'intestin; ii) microbiome intestinal; (iii) profils de métabolites cérébraux, hépatiques et musculaires (RMN); (iv) assimilation du ⁶⁵Zn et ¹¹⁰Ag; (v) profils d'isotopes stables du carbone et de l'azote dans le muscle; et (vi) croissance et mortalité. Cette étude révèle des impacts profonds des MP sur certains paramètres mesurés chez la daurade (Fig. 21). Par exemple, une forte mortalité a été observée chez les daurades exposées aux MP, qui de surcroît ont subi des altérations métaboliques au niveau du foie et du cerveau, une assimilation significativement plus élevée du Zinc ainsi que des dommages histologiques au niveau du foie. D'autre part,

certaines effets sont apparus plus subtils comme sur le microbiome intestinal (Fig. 21). Ces effets observés chez les daurades exposées aux MP semblent confirmer les résultats d'études ayant réalisées une approche « mutli-diagnostiques » similaires, ainsi que les résultats de la méta-analyse réalisée dans le chapitre 1, ou les MP, plus qu'affecter une fonction biologique précise, altèrent une variété de fonctions biologiques chez les poissons (Jin et al., 2018; Wan et al., 2019). Il est par conséquent difficile de proposer des routes d'actions des MP sur la physiologie des poissons et de manière générale les mécanismes sous-jacents aux effets biologiques observés chez les organismes exposés MP requièrent plus ample investigation. Cependant, en se basant sur la littérature actuelle (chapitre 1) et les résultats présentés dans le chapitre 2 certaines hypothèses peuvent être avancées. L'organe directement en contact avec les MP et donc potentiellement le plus impacté est le tube digestif (Jovanović, 2017; Lu et al., 2016b). Cependant, les preuves de dommages mécaniques des MP sur le tube digestif des poissons, particulièrement pour les MP sphériques, restent faibles, voire inexistantes (Jabeen et al., 2018; Jovanović, 2017). Par ailleurs, bien que certaines preuves de translocation de MP < 20 µm aient été démontrés chez les poissons, celles-ci restent faible et le potentiel des MP d'interagir avec des processus physiologiques des poissons une fois la barrière épithéliale du tube digestif passée est par conséquent peu probable (Franzellitti et al., 2019; Jovanović, 2017). Si les dommages mécaniques du tube digestif des poissons semblent être peu voir pas affecté par les MP, le microbiome intestinal des poissons est modifié (Dysbiose) de manière systématique par une exposition aux MP (Fig. 7), fournissant une hypothèse potentielle de l'éventail d'effets causés par les MP chez les poissons. En effet, la méta-analyse présentée dans le chapitre 1 montre qu'après une exposition aux MP, 29% des biomarqueurs liés au microbiome du poisson étaient atteints et dans tous les cas sauf un (Caruso et al., 2018) l'exposition à des MP a induit une dysbiose intestinale et a modifié de manière systématique la diversité totale de bactérie présente dans l'intestin des poissons (Jin et al., 2018; Qiao et al., 2019; Wan et al., 2019). Au sein de ces études, la dysbiose du microbiome était accompagnée par d'autres effets sur le métabolisme des poissons. Le microbiome intestinal est maintenant connu pour fournir des fonctions essentielles à son hôte comme la nutrition, le métabolisme, l'immunité et la résistance aux agents pathogènes invasifs, jouant ainsi un rôle majeur pour la santé de celui-ci (Martin et al., 2018; Wang et al., 2017; Yamashiro, 2018). Ainsi, étant donné que le microbiome intestinal joue un rôle essentiel dans la nutrition, le métabolisme, l'immunité et le comportement des vertébrés et que la présence d'effets sur cette catégorie après l'exposition de MP aux poissons est systématique, l'analyse du microbiome apparait comme un outil prometteur dans le but d'étudier l'effet de l'ingestion de MP chez les poissons et pourrait être profondément lié au

mécanisme sous-jacent responsable des perturbations observées dans d'autres catégories telles que le métabolisme et le comportement (Fackelmann and Sommer, 2019).

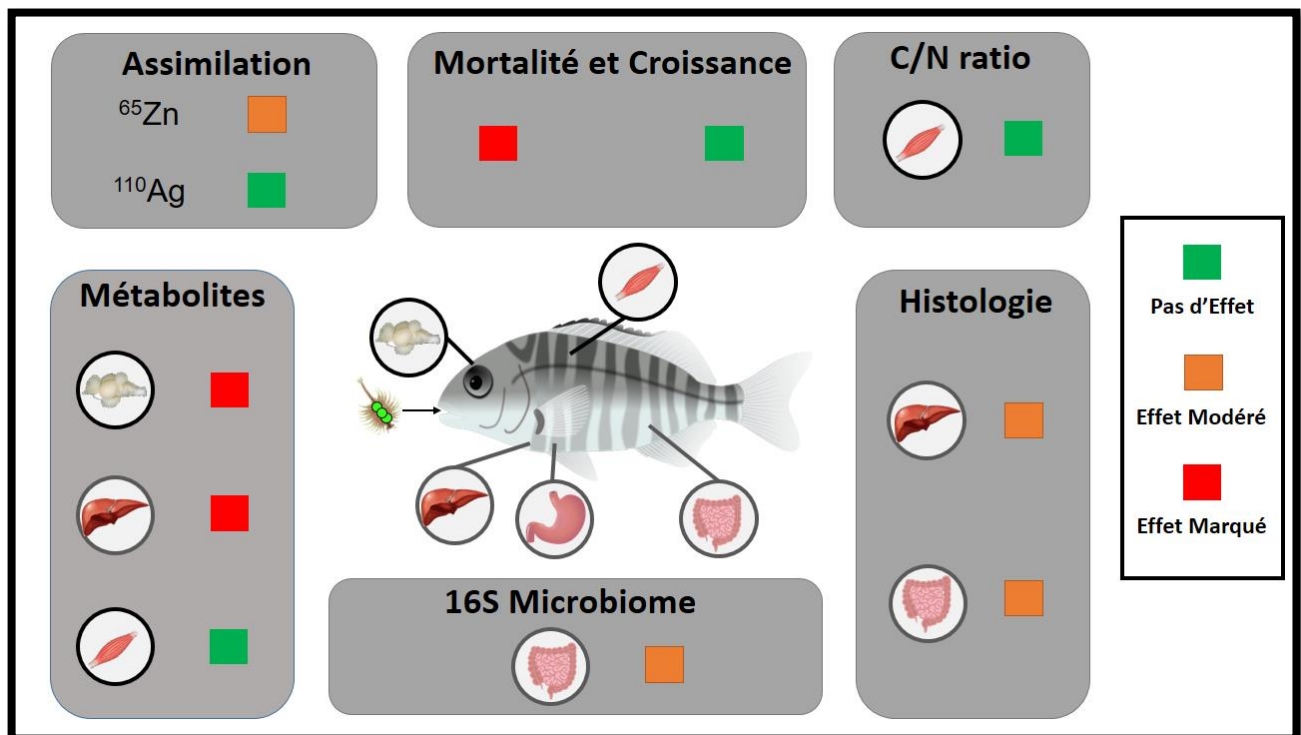


Figure 21. Synthèse des effets observés chez les juvéniles de daurade royale (*Sparus aurata*) exposés à des MP via l'alimentation durant 35 jours.

Enfin, même si la littérature sur l'impact écologique des MP sur les poissons téléostéens est encore rare, leur potentiel d'influer sur le recrutement des poissons de récifs coralliens par différentes voies (physiologique, comportementale ou sensorielle) émerge déjà de la littérature existante (voir chapitre 1), ce qui pourrait avoir de nombreuses répercussions pour les poissons de récif corallien durant la phase critique du recrutement (Fig. 17). Le chapitre 3 présente ainsi une étude sur l'effet des MP de polystyrène sur le comportement alimentaire et la survie d'une post-larve de poisson corallien, le chirurgien bagnard *Acanthurus triostegus*. Les résultats de cette étude ne montrent pas d'effets significatifs après une exposition de 8 jours à des MP de polystyrène de 90 µm (5 MP.mL⁻¹), cependant cette étude initie la recherche des effets des MP sur les jeunes stades de vie des poissons coralliens. En effet, comme présenté dans le chapitre 1 (Fig. 6), les études sur les effets des MP sur le stade larvaire des poissons marins restent minoritaires à l'heure actuelle, et l'étude présentée dans ce chapitre 3 représente la seule étude sur les larves de poissons coralliens. Connaissant la sensibilité, ainsi que l'importance de ces

stades larvaires sur les stocks de poisson adulte, il apparaît nécessaire d'approfondir la recherche sur l'effet des MP sur ce stade de vie des poissons.

En conclusion générale, cette thèse, à travers une méta-analyse et diverses expériences, montre que les MP vierges peuvent être toxiques pour les jeunes stades de vie des poissons marins (Chapitre 1 et 2), cependant ces résultats sont à prendre avec précaution. Premièrement, les expériences de laboratoire évaluant la toxicité des MP restent particulièrement difficiles à réaliser de manière réaliste du fait que les MP soient constitués d'un mélange complexe de polymères et d'additifs auxquels la matière organique et des contaminants ou des microorganismes peuvent se lier (Paul-Pont et al., 2018). Ainsi, les MP vierges utilisés au cours de cette thèse ne représentent qu'une fine fraction des MP présents dans l'environnement et leur toxicité ne peut être généralisée. De plus, la majorité des effets des MP sur les poissons marins rapportés, que ce soit dans la littérature (Chapitre 1) ou dans les expériences de cette thèse (Chapitre 2), sont observés à des doses relativement importantes par rapport aux doses environnementales quantifiées à ce jour (Paul-Pont et al., 2018; Van Sebille et al., 2015). Ainsi, l'utilisation de concentrations pertinentes de MP suscite de nombreux débats dans le milieu de la recherche en écotoxicologie des MP en milieu marin (Koelmans et al., 2017, 2016; Lenz et al., 2016). L'utilisation de doses irréalistes peut être dans un premier temps attribuable au fait que les méthodes de détections actuelles ne permettent pas, ou très rarement, de quantifier les MP < 300 µm qui sont les MP majoritairement utilisés par les études écotoxicologiques, du fait de leur potentiel accru de transfert trophique et de toxicité comparée aux MP de plus grande taille. Qui plus est, contrairement aux autres polluants chimiques, les MP ne sont pas distribués de manière homogène dans les océans, il est donc difficile de déterminer des doses représentatives de ce polluant à l'échelle globale (Koelmans et al., 2017). Ainsi, bien que les efforts de quantification environnementale des MP soient importants à l'heure actuelle, au vu de l'immense étendue et hétérogénéité des biotopes océaniques et des incertitudes quant au comportement et à la dégradation des plastiques dans les océans, évaluer les tendances issues des données de surveillances reste difficile.

Malgré cela, compte tenu des données actuelles sur les concentrations auxquels des effets néfastes sont observés chez les poissons (Fig 8), les études écotoxicologiques souhaitant utiliser des concentrations réalistes actuelles, c'est-à-dire de l'ordre de 0.01 à 10 MP.L⁻¹, aboutiront très probablement à une conclusion «sans effet». En effet, la méta-analyse du chapitre 1 montre que seulement 3% (15 sur 504) des indicateurs biologiques utilisés dans les 46 études étudiant

l'effet des MP sur les poissons ont utilisé une dose entre 1 et 100 MP.L⁻¹, démontrant le manque d'intérêt des chercheurs à étudier des doses environnementales. Qui plus est, même dans les pires scénarios de pollution plastique, les MP resteront probablement une fraction mineure des autres microparticules naturellement présentes dans l'eau et les sédiments (Ogonowski et al., 2018). Ainsi, au vu de l'essor de ce domaine de recherche, certains chercheurs émettent des doutes sur le risque environnemental véritable des MP et sur la pertinence des recherches en écotoxicologie dans ce domaine (Connors et al., 2017; Lenz et al., 2016; Lohmann, 2017). Ces doutes ne sont pas sans fondement, en effet, du fait d'une couverture scientifique et médiatique massive, parfois à tendance alarmiste, il existe de manière très certaine une dissonance entre les risques perçus et les risques réels de la pollution MP. Plusieurs mécanismes sont potentiellement responsables de cette dissonance. Premièrement un biais de publication [c.-à-d. la tendance des éditeurs de revues à préférer certains résultats que d'autres (par exemple, des résultats montrant une découverte significative (Dickersin, 1990))]. Les journaux ayant donc publié majoritairement des résultats significatifs des MP sur les organismes malgré des doses souvent irréalistes ou en tout cas, non environnementales. Ainsi, bien que dans leurs articles, les scientifiques dans ce domaine émettent quasi systématiquement des réserves, et associent les effets observés à un « stress potentiel » plutôt qu'à des risques réels des MP en utilisant des mots comme « potentiel » ou « pourrait », ces subtiles descriptions sont souvent perdues dans l'esprit des décideurs, des médias et du public ce qui engendre de nombreuses réactions excessives et exagérations involontaires. Enfin, les scientifiques ont certainement tendance à souligner le risque potentiel plutôt que le risque réel des MP afin de souligner la pertinence du sujet et assurer leur budget de recherche. Malgré cela, ces points ne doivent pas être pris comme un plaidoyer pour réduire l'attention scientifique portée aux MP dans l'environnement marin. En effet, les concentrations réalistes d'aujourd'hui ne sont pas les concentrations réalistes de demain. Dans une évaluation du risque futur, qui prend en compte l'augmentation de la production de plastique et la fragmentation progressive de particules de plastique (évaluation non existante à l'heure actuelle), des concentrations supérieures aux concentrations actuelles sont potentiellement pertinentes. De plus, les concentrations plus élevées souvent testées dans la littérature actuelle peuvent très bien être pertinentes afin d'estimer les risques spécifiques à un site fortement contaminé et restent nécessaires afin de déterminer les effets seuils et les modes d'action des MP sur les organismes marins (voir chapitre 2). Finalement, si les poissons marins adultes peuvent très certainement faire face au stress causé par l'ingestion de MP, le manque de connaissances sur l'effet des MP sur le stade de vie larvaire, le plus sensible de ces

organismes, ne permettent de conclure à une absence d'effet généralisé de ce polluant émergeant à des doses environnementales.

En conclusion, les écotoxicologues de ce domaine sont confrontés à de nombreux défis expérimentaux du fait de la nature complexe des MP en eau de mer par rapport aux composés chimiques classiques, ainsi qu'au manque de connaissances sur la composition, distribution, et quantification des MP présent dans l'environnement marin. La complexité et diversité des effets des MP sur les organismes rajoutant une complexité supplémentaire dans la compréhension des modes d'action de ce polluant. Il est ainsi certain que les avancés sur les connaissances de la distribution et quantification des MP, voir des NP, en milieu marin permettront aux écotoxicologues de ce domaine d'apporter des réponses plus précises et complètes sur leur potentiel toxicité chez les organismes marins. Malgré un manque de connaissances, basé sur les études réalisées au cours de cette thèse, les MP représentent à l'heure actuelle un risque mineur pour les populations de poissons marins.

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**ANNEXE 1 - No Effect of Polystyrene Microplastics on Foraging
Activity and Survival in a Post-larvae Coral-Reef Fish,
Acanthurus triostegus.**



No Effect of Polystyrene Microplastics on Foraging Activity and Survival in a Post-larvae Coral-Reef Fish, *Acanthurus triostegus*

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Abstract

Microplastics (MP) are ubiquitous in the marine environment and have been shown to alter the behaviour of some species due to potential neurotoxic effect. However, very little is known on the effect of this stressor on behavioural responses of early and more vulnerable life stages. This study explores the effects of polystyrene MP (90 µm diameter) on the foraging activity of newly settled surgeonfish *Acanthurus triostegus* and on their survival facing predators. Exposure to a high concentration of 5 MP particles per mL (5 MP mL⁻¹) for 3, 5 and 8 days did not alter their foraging activity nor their susceptibility to predation. This suggests that short-term exposures to reportedly high MP concentrations have negligible effects on the behaviour of newly settled *A. triostegus*. Nevertheless, responses to MP can be highly variable, and further research is needed to determine potential ecological effects of MP on reef fish populations during early-life stages.

Keywords Plastic pollution · Behaviour · Feeding · Predation · Larval recruitment · *Acanthurus triostegus*

There is increasing evidence that exposure to microplastics (MP) leads to adverse effects on marine animals (Wright et al. 2013). In fish, MP have been found to cause physical and physiological defects, such as false food satiation, tissue damage and decreased swimming performance (Lu et al. 2016; Pedà et al. 2016; Barboza et al. 2018a; Critchell and Hoogenboom 2018). However, despite the range of sub-lethal effects reported, the effects of MP on fish behaviour remain poorly understood. For example, while MPs have been reported to cause lethargic and erratic swimming behaviours in the European seabass, *Dicentrarchus labrax* (Barboza et al. 2018b), other studies found no significant effects on fish behaviour (e.g. Guven et al. 2018; Tosetto

et al. 2017). Discrepancies could be due to several factors including differences in MP concentrations, plastic types, species and life stages. Consequently, more research is needed to elucidate the effects of MP on the behaviour of aquatic species, such as foraging and predator avoidance. Indeed, these behaviours are not only fundamental to individuals' fitness and functional role in their ecosystem (Wolf and Weissing 2012), but also their modifications are considered among the most sensitive indicators of environmental disturbances (Hellou 2011; Melvin and Wilson 2013).

In addition to the lack of understanding regarding behavioural responses in fish exposed to MP, limited information is also available on their effects on early-life stages of marine organisms that may be particularly sensitive to environmental disturbances (van Dam et al. 2011; Mazurais et al. 2015; Besson et al. 2017; Holzer et al. 2017). The vast majority of coral-reef associated fishes have a stage-structured life history, with a relatively sedentary benthic juvenile and adult stage preceded by a pelagic larval stage (Leis and McCormick 2002). The transition between larval and juvenile stages occurs when pelagic larvae enter and settle in the reef environment. This step is referred to as the larval recruitment phase and involves a range of physical and behavioral changes (i.e. metamorphosis) that are controlled by thyroid hormones (Holzer et al. 2017). This metamorphic phase is often referred to as a 'critical period' in coral-reef fish life

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cycle (Almany and Webster 2006), where selective pressure is high and settling post-larvae must rely on strong perception abilities (Lecchini et al. 2005). Any stressors affecting their behaviour during this critical life-history transition can have significant effects on fish restocking. For example, Holzer et al. (2017) showed that a common pesticide (chlorpyrifos) affects the grazing behavior and also the metamorphosis of the tropical herbivorous fish *Acanthurus triostegus* following their recruitment to the reef. Given the fact that coastal areas are being subjected to increased plastic pollution (Avio et al. 2017), there is an increased likelihood for reef-associated juveniles to encounter microplastic particles that could have cascading effects on coral-reef ecosystems.

The present study investigates the potential effects of MP on the behaviour of post-larvae of the convict surgeonfish *A. triostegus*. This is important since MP are hypothesized to have neurotoxic effect on fish (Oliveira et al. 2013; Barboza et al. 2018a) that can result in behavioral anomalies and increased predation-related mortalities in *A. triostegus*. More precisely, we examined how exposure to a relatively high concentration of 90 µm polystyrene MP (5 MP mL⁻¹) influenced foraging activity and survival to predation over a period of 8 days post-settlement.

Materials and Methods

Post-larvae (i.e. larvae under metamorphosis) of the convict surgeonfish *A. triostegus* (n = 270) were collected in rock pools using dip nets during the overnight high tide around the new moon phase between May and August 2017 as they enter and settle to the reefs at Tema'e, Moorea Island, French Polynesia (17°29'50.7"S 149°45'15.3"W; Holzer et al. 2017).

After capture, *A. triostegus* (0.63 ± 0.019 g wet weight for a subsample of n = 10) were immediately transferred to 20 × 15 × 15 cm aquaria (ten fish per aquaria max.) filled with 3 L of 1 µm filtered seawater. Fish were exposed to either seawater only (control) or seawater with polystyrene MP at a concentration of 5 MP mL⁻¹ for 3, 5, and 8 days. MP stock solution consisted of 0.15 g of NIST Traceable colorless polystyrene beads, with a diameter of 91.26 ± 0.2 µm and density of 1.05 g/cc, in 15 mL osmosis water (6.24 × 10⁴ MP particles mL⁻¹; Polysciences Warrington, PA, USA). MP concentrations were confirmed a posteriori by visual counting under a stereo microscope Leica EZ-E using a 1 mL sedgewick rafter counting chamber in both control condition (0 MP mL⁻¹; n = 2) and the MP condition (4.77 ± 0.77 MP mL⁻¹; n = 3). Fish exposed for 3 and 5 days were not fed, while fish exposed for 8 days were fed once on the fifth day. Each aquaria was equipped with an air stone to maintain MP in suspension and ensure optimal dissolved oxygen levels. Water temperature was maintained at 28 °C

(± 1 °C) and salinity at 35 ppt. A 12-h light/dark photoperiod was maintained throughout the experiments at the CRIOBE research station. All experiments were approved by the CRIOBE-IRCP animal ethics committee (CRIOBE/IRCP-Fish-2018-04).

Foraging activity was assessed following the protocol described by Holzer et al. (2017) with minor modifications. After 3, 5 and 8 days of exposure, three randomly-selected fish from each condition (control and MP) were placed in a 7 L glass tank containing control seawater and a coral rubble (~30 cm³) covered with turf algae placed at the center. Coral rubbles were collected at the post-larvae sampling site. After a 10-min acclimation period, fish foraging activity was video recorded for 20 min (Takara cameras set up 50 cm above the tank and configured in 720p/30fps). Feeding trials were replicated 6–10 times per condition and time point (day 3, 5 and 8; i.e. 18–30 fish per condition and time point – 108 fish in total). Each group of three fish was only tested once. Following each trial, seawater was completely renewed, and a new coral rubble was used. Feeding activity was analyzed by counting the total number of bites made by the three fish on the turf algae during three periods of 2 min sections that were randomly selected between 0–7, 7–14 and 14–20 min (i.e. cumulative number of bites during a total of 6 min of recording). For weight measurements, after 8 days, 12 fish from each treatment (MP and Control) were euthanized by cold shock, subsequently dried in absorbent paper and weighted with a precision of 0.01 g.

To test how MP exposure affects predation risk, predation survival tests were conducted on 8-day exposed fish (control and MP treatments; Holzer et al. 2017). Survival tests were conducted in circular arenas (85 cm diameter, 40 cm depth). Ten randomly selected fish from each treatment were simultaneously placed into the test arena containing four lionfish *Pterois radiata* (two adults and two juveniles) starved for 48 h prior to the experiment. Trials began during the afternoon to allow *A. triostegus* post-larvae to acclimate to arena conditions before predation (lionfish are nocturnal predators). Each group of prey fish was identified via subcutaneous coloured elastomer tags (Northwest Marine Technology) injected dorsally a minimum of 12 h before each trial. Predation trials were replicated six times per condition (n = 6; total of 60 post-larvae per treatment). Predator pairs were changed and colour tags were switched between each trial. Trials were ended when approximately 50% of post-larvae were predated, or after 24 h. The percentages (number of survivors/initial number of fish) of *A. triostegus* post-larvae remaining was then assessed to measure relative (from each treatment) and overall (both treatments) survival rates. Ingestion of MP by *A. triostegus* post-larvae after 8 days of exposure was confirmed by visual inspection of the dissected digestive tract under stereo microscope (Leica EZ4 E; n = 10).

Differences in foraging activity between treatments were analyzed using a full factorial two-way ANOVA with treatments (control and MP) and time as factors. Percentages of *A. triostegus* survival were arcsine transformed and differences between treatments were analysed using an unpaired *t* test. Differences in *A. triostegus* weight between treatments were analysed using the Mann–Whitney U test. All data were first tested for variance homogeneity and normality of distribution. The significance level was set at $\alpha=0.05$. Analyses were performed using SPSS 22.0 (SPSS Inc., Chicago, IL).

Results and Discussion

In fish, ingestion of non-nutritive elements, such as MP, and their subsequent accumulation in the digestive tract can lead to malnutrition and intestinal damages (Boerger et al. 2010; Lei et al. 2018). Exposure to MP has also been reported to inhibit acetylcholinesterase (AChE) activity in fish (Oliveira et al. 2013; Barboza et al. 2018a). Such inhibition of AChE activity can adversely affect the nervous and neuromuscular function, resulting in a decrease in swimming activity (Bonansea et al. 2016). Consequently, altered behavioural responses would be expected for fish exposed to MP-contaminated water. However, contrary to our hypothesis, MP exposure in the present study had no significant effect on *A. triostegus* foraging activity (Fig. 1a, $F_{(1, 38)}=0.78$; $p=0.38$), body weight (Day 8: MP = 0.59 ± 0.05 g; Control = 0.61 ± 0.03 g; $W=63$, $p=0.42$) and survival facing a common reef fish predator, *P. radiata* (Fig. 1b, $t=0.52$; $df=10$; $p=0.62$). Ingestion of MP by *A. triostegus* post-larvae after 8 days of exposure was confirmed with 1.7 ± 1.33 MP per fish.

Regardless of MP treatment, an increase in foraging activity (number of bites on turf algae) was observed over time (Fig. 1A, $F_{(2, 38)}=88.3$; $p<0.0001$), confirming the expected trend associated with *A. triostegus* transition from a carnivorous to an herbivorous diet (Holzer et al. 2017). As such, this confirms that post-larvae metamorphosed and behaved normally within our experimental system. The experimental design used in this study was carefully selected to be highly relevant from an ecological perspective. Fish post-larvae were caught right after their settlement to the reef coastal environment (from the open ocean) when they strongly rely on perception abilities for feeding and escaping predation, but also when they are potentially facing shift in the contamination status of their environment, coastal areas being subjected to greater plastic pollution (Avio et al. 2017). The duration of the exposure of 8 days corresponds with the critical period of *A. triostegus* larval recruitment and metamorphosis when they are the most sensitive to contaminants (Almany and Webster 2006; Holzer et al. 2017). On the other hand, the particle concentration (5 MP mL^{-1}) was selected to represent a worst-case scenario as it is considered to be higher than the mean concentrations encountered in the natural environment (Paul-Pont et al. 2018), but still realistic in highly contaminated areas (Dubai and Liebezeit 2013; Peeken et al. 2018) and represent a tangible scenario in highly polluted tropical coastal waters where plastic debris accumulates (Lavers and Bond 2017).

It is possible that the exposure duration (8 days) may not have been long enough to reach the toxicity threshold for *A. triostegus*, as the few studies that observed significant effect of plastic particles on fish behavior used longer exposure time (e.g. over 60 days: Mattsson et al. 2015, 2017). However, this is not always the case. A recent study reported that 4 days of exposure to a relatively high concentration of MP of 0.69 mg L^{-1} , compared to 0.81 mg L^{-1} in the present

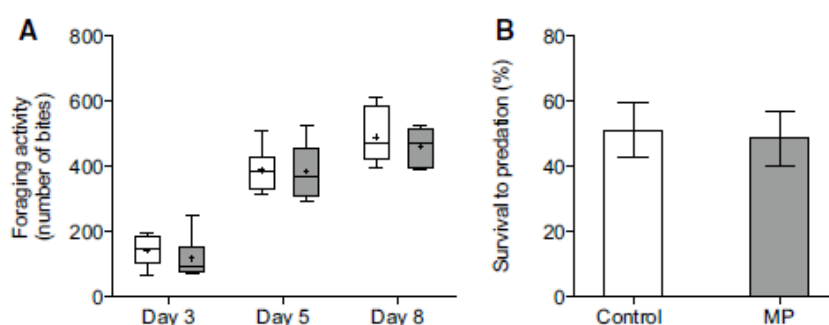


Fig. 1 Effect of 90- μm polystyrene microplastic beads on the foraging activity and survival to predation of post-larvae of the Surgeon fish *A. triostegus*. MP: microplastics, nominal concentration of 5 MP mL^{-1} . **a** Foraging activity (cumulative number of bites on turf algae) of fish exposed to control (white) or MP (grey) during 3, 5, and 8 days. Box-

plots show the interquartile range (column), median (horizontal line), minimum and maximum values (whiskers) and average (+) of 6–10 groups of 3 fish ($n=6-10$). **b** Survival to predation (%) of post-larvae by *P. radiata* after 8-day exposure to MP (average \pm SD; $n=10$ fish per treatment; $n=6$)

study, resulted in decreased swimming performance in juvenile fish *Dicentrarchus labrax* (Barboza et al. 2018b). This is corroborated by Oliveira et al. (2013) who observed neurotoxic effects on the common goby *Pomatoschistus microps* after 4 days of exposure to 0.184 mg MP L⁻¹. Contrary to our study, Barboza et al. (2018b) and Oliveira et al. (2013) used smaller particle size (1–5 µm). It is therefore possible that smaller MP (< 20 µm) or nanoplastics (NP) could have had a greater impact on *A. triostegus* post-larvae as they are generally found to have increased deleterious effect on fish compared to larger plastic particles (Chae and An 2017; Chen et al. 2017; Mattsson et al. 2017). Importantly, the concentrations used in all the aforementioned studies are likely to be higher than the mean concentrations encountered in the natural environment (Paul-Pont et al. 2018).

Given the lack of observed effects after 8 days of exposure to MP, it is likely that short-term exposures to relatively high MP concentrations have negligible effects on the behavior of coral-reef fish during larval recruitment. Nevertheless, as this is the first study to assess the effect of plastic pollution on the young, more vulnerable, life stages of a coral-reef fish species, more research is certainly needed to better understand the potential risks of MP pollution on these early-life stages.

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ANNEXE 2 - Evaluation of microplastic ingestion by tropical fish from Moorea Island, French Polynesia.



Evaluation of microplastic ingestion by tropical fish from Moorea Island, French Polynesia

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ABSTRACT

Microplastics are ubiquitous throughout the oceans, yet few studies have documented their occurrence in marine organisms associated with coral reefs. Four genera of adult fish were sampled (*Myripristis* spp., *Siganus* spp., *Epinephelus merra* and *Chelodactylus sinus*) from different trophic guilds around the tropical island of Moorea, French Polynesia. Digestive tracts from 133 adult fish were surveyed and microplastics were found in 28 tracts (21%). Abundance of ingested microplastic pieces per individual fish varied from 1 to 3 pieces, with an average of 1.25 ± 0.13 ingested microplastic pieces. Microplastics size ranged from 0.031 to 2.44 mm and 70% of microplastics did not exceed 0.3 mm in size. Overall, this study shows that the number and size of microplastic ingested per trophic groups are independent of trophic guild. Additional studies are needed to sample in other tropical regions in order to have a better assessment of microplastic occurrence in coral reefs.

1. Introduction

Global plastic production has been continuously increasing. The total amount of plastic produced globally underwent a dramatic increase from 1.5 million to 335 million tons between the onset of production in 1950 and 2016, respectively (PlasticsEurope, 2008, 2017). As a consequence of maritime activity and flow through watershed outfalls near areas of high human density, an estimated 5 to 13 million tons of plastic debris flow into the oceans on a yearly basis (Eriksen et al., 2014; Jambeck et al., 2015). A substantial proportion of plastic debris is comprised of microplastics, particles with a < 5 mm diameter (Arthur et al., 2008). In 2014, an estimated 15 to 51 trillion particles of microplastics weighing between 93 and 236 thousand metric tons entered the oceans (van Sebille et al., 2015). The highest densities of microplastics are found close to highly industrialized coastal areas and in ocean gyres (Wright et al., 2013). For example, the North Pacific Subtropical Gyre (NPSG) possesses the biggest accumulation of plastic debris observed, with an estimated exponential input of plastics in this zone that is composed of 94% microplastics (Lebreton et al., 2018). Due to their variable buoyancy, microplastics are prevalent throughout the water column (Thompson et al., 2004) and are small enough to be absorbed by a wide range of organisms. These microplastics can then be

trophically transmitted throughout the food web from contaminated prey to predators (Farrell and Nelson, 2013).

Although most raw microplastics are considered chemically inert, chemicals associated with plastics can have biological effects on organisms by disrupting endocrine system and liver function (Rochman et al., 2013, 2014). Thus, various studies have explored the ingestion of marine microplastics by fish, from the early life stage (Hoss and Settle, 1990; Mazurais et al., 2015; Steer et al., 2017) through the juvenile stage (Hoss and Settle, 1990; de Sá et al., 2015) to the adult stage (Carpenter et al., 1972; Boerger et al., 2010; Choy and Drazen, 2013; Alomar et al., 2017). However, only a few studies have focused on plastic contamination in coral reefs and coral reef fish (e.g., Richards and Beger, 2011; Forrest and Hindell, 2018; Gardon et al., 2018).

Work in the Indo-Pacific region has explored the deleterious effect of macrodebris on benthic reef communities (Richards and Beger, 2011). A recent synthesis highlighted the negative impact of marine litter on reef systems throughout the global oceans (de Carvalho-Souza et al., 2018). On shallow-waters corals, the majority of the work in the area has been conducted solely in laboratory settings (Hall et al., 2015; Allen et al., 2017), although Reichert et al. (2017) reported preliminary results on the health effects (i.e. bleaching and tissue necrosis) of microplastic exposure on corals. With regard to plastic ingestion by fish, a

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recent study explored the various effects of ingestion of microplastic particles to the health of *Acanthochromis polyacanthus*, a species commonly found in Indo-Pacific coral reefs. (Critchell and Hoogenboom, 2018).

In French Polynesia, microplastic ingestion by coral reef species remains relatively unexplored. The few existing studies have examined the effects of microplastics on the physiology of pearl oysters (Gardon et al., 2018), a harvested species of high economic importance in the country; the microplastic concentration in the water column on the fringing reefs of Moorea Island in the Society Islands archipelago (Comors, 2017); and a single study on plastic ingestion by fish (Forrest and Hindell, 2018). However, this sole study on plastic ingestion by fish in French Polynesia only assessed plastic ingestion in seven individual fish across two islands (Forrest and Hindell, 2018).

The focus on disparities in plastic ingestion among fish species with differing foraging strategies and prey items has begun to receive increased attention (Boerger et al., 2010; Choy and Drazen, 2013; Mizraji et al., 2017). Plastic particles can be trophically transmitted up the food chain by the ingestion of a prey species that had previously ingested plastic, but often fish may directly ingest plastic by mistaking it for a prey item. Thus, specific plastic characteristics (abundance, size, shape) could be a key factor influencing particle ingestion (Wright et al., 2013).

This study assesses the presence of microplastics in the digestive tract of four common tropical fish found in Moorea, French Polynesia. Here, we evaluate the variability in the microplastic contamination in coral reef fish with regard to their trophic guild (herbivore, carnivore, planktivore) and hypothesize there is a difference in the characteristics of ingested microplastic among the different trophic guilds. Specifically, we expect to find higher levels of ingested microplastic in the planktivorous species, as this feeding strategy encompasses floating or suspended prey, which could be easily confused with small plastic pieces, throughout the entirety of the water column (Tanaka and Takada, 2016). Planktivores possess two distinct capture modes, which are visual feeding and filter feeding, and both are used to feed on particulate size food at the surface and deeper in the water column. (Lazzaro, 1987). This study provides a better evaluation of the microplastics ingestion by fish in French Polynesia and an original comparison between fish of different trophic guilds.

2. Materials & methods

2.1. Fish sampling

This work was conducted on Moorea Island (French Polynesia) (17° 30'S, 149° 50'W), located in the southern tropical Pacific Ocean (Fig. 1). Fish were collected as purchases from local fishers, as well as through field collections. All collections were conducted during the dry season (January to March 2017). Four different genera (taxonomic groups) belonging to three different trophic guilds were sampled: *Siganus* spp. (herbivore, Family: Siganidae, $n = 33$), *Epinephelus merra* (carnivore, Serranidae $n = 33$), *Myripristis* spp. (zooplanktivore, Holocentridae, $n = 33$), and *Cheilopogon simus* (zooplanktivore, Exocoetidae, $n = 34$). All fish were sampled in their adult stage. As local fishermen mostly fish during the night or at dawn and selling takes place early in the morning, we specifically targeted fish that feed at night or just before dawn in order to increase the likelihood of fish having full stomachs. All four species inhabit the lagoon area except for *C. simus*, which is pelagic, but can be fished in the reef pass. As determining the species level for the genus *Myripristis* (*M. adusta*, *M. bembdi* and *M. violacea*) and *Siganus* (*S. spinus* and *S. argenteus*) is difficult due to the high physical similarity between species, these individuals were identified to genus level. However, it is commonly admitted that all *Myripristis* and *Siganus* species found in Moorea are zooplanktivorous and herbivorous, respectively (Bacchet et al., 2006).

2.2. Laboratory processing

Standard length and digestive tract wet weight were recorded for each sampled fish prior to microplastic isolation. Fish were dissected and the digestive tract of each fish was removed and desiccated at 65 °C overnight. Samples were then ground by mortar, stirred in a hypersaline solution (150 ml of NaCl, 0.4 g/cm³) in order to separate heavy material such as sediments, and allowed to settle for 10 min. Microplastics were isolated from the digestive tracts using Protocol 6 for plastic extraction as described in Avio et al. (2015). The supernatant was isolated prior to filtering under vacuum on a cellulose ester membrane (8 µm pore size). The membrane was submerged in a 15% H₂O₂ solution and subsequently dried at 50 °C overnight before microscopical observation. Microplastics in the membrane were counted, and different types of microplastics were recorded. Great care was given during the laboratory treatments in order to avoid airborne microplastic contamination: the protocol was carried out under air flow cabinet, each dissection tool was carefully cleaned with alcohol between each use, and the samples covered with glass lids at each step. Additionally, blank tests were conducted for each step to assess airborne plastic contamination. When textile fibres were found, which are recognisable from their very thin and smooth characteristics, they were excluded from the analysis (Foeckema et al., 2013; Avio et al., 2015).

2.3. Statistical analysis

Number and size of microplastic particles were compared among the four genera using a Kruskal-Wallis test. Spearman correlations were used to analyse the relationships between fish standard length and digestive tract weights of genera with microplastic characteristics (including number and size). Concerning the relationship between the weight of digestive tracts contents and microplastics characteristics, weight of digestive tract itself was considered negligible in the analysis. The data were considered significant when the p -value was < 0.05. Statistical analyses were conducted using the software R (version 3.3.0, R Core Team, 2016).

3. Results

A total of 133 fish were collected and processed: 34 *C. simus*, 33 *Myripristis* spp., 33 *Siganus* spp. and 33 *E. merra*. Dietary contents in *C. simus* and *Myripristis* spp. were mostly liquid or viscous, making diet identification difficult. *Siganus* spp. presented moderately full digestive tracts with various digested algae and sediments. In *E. merra*, three individuals contained fish in their stomach, but these prey fish were not identifiable to the species level.

In total, 35 microplastics pieces were found in 28 of the 133 surveyed fish (Table 1). The proportion of individuals in each genus that ingested microplastics was 15% for *Siganus* spp., 18% for *C. simus*, 21% for *Myripristis* spp. and 30% for *E. merra* (Table 1). The lowest number of microplastics was found in *Siganus* spp., which ingested an average of 0.15 ± 0.10 (mean \pm SD) pieces per fish, and the highest number in *E. merra*, 0.39 ± 0.14 pieces per fish (Table 1). In *Myripristis* and *C. simus*, 0.27 ± 0.13 and 0.24 ± 0.13 microplastics pieces per fish were found, respectively. There was no significant difference in the number of microplastics ingested across genera (Kruskal-Wallis test, $\chi^2 = 2.79$, p -value = 0.43). Abundance of plastic pieces per individual fish varied from 1 to 3 pieces, with an average of 1.25 ± 0.13 ingested microplastic pieces for all species. No more than one plastic piece per individual was found in *Siganus* spp. whereas up to three plastic pieces were found in the other genera. In *Siganus* spp. ($N = 5$), there was a significant relationship between standard length and microplastic size (Spearman correlation test, $R = 1$, p -value = 0.017). No significant relationships were found between standard length and microplastic size for the remaining three genera (Spearman correlation tests, $R < 0.1$, p -value > 0.15). Similarly, there was no significant relationship between

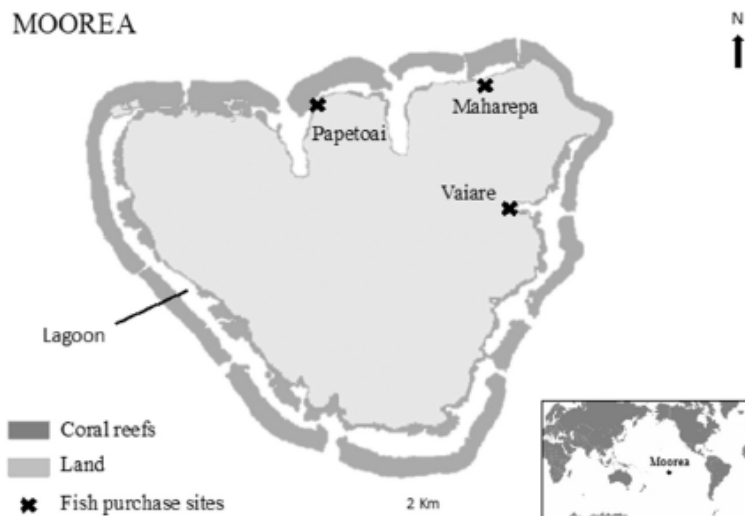


Fig. 1. Map of Moorea Island and samples location.

digestive tract fullness and the amount of microplastic in the digestive tract (Spearman correlation tests, $R < 0.1$, p -value > 0.45) for any of the genera.

Four different types of microplastics types recorded in fish digestive tracts: fibre, fragment, bead, and film (Fig. 2). The most abundant microplastic type were fragments (69%) (Fig. 3), with a maximum of ten pieces recorded across all the *E. merra* sampled (Fig. 4). A single piece of film-shape microplastic, recorded in a *C. simus* individual, was found across all fish (Fig. 4). Microplastic piece size ranged from 0.031 to 2.44 mm, with fragments comprising the smallest of these pieces (0.031–2.37 mm) and fibres comprising the largest (1.21–2.44 mm). In total, 70% of the ingested microplastics did not exceed 0.3 mm in size. No significant differences were found among the microplastics size ingested by the four genera (Kruskal-Wallis test, $\text{Chi-sq} = 2.67$, p -value = 0.45).

4. Discussion

This study provides the first assessment of microplastic ingestion by reef fish in French Polynesia. The total proportion of ingested microplastics (21%) observed was comparable to the amount observed in fish in Mediterranean coast (17% - Bellas et al., 2016), although there exists some variability in the plastic observed in this study and those observed in other regions. For example, studies conducted in the Adriatic Sea and the English Channel found 28% and 36% ingested microplastics in fish, respectively (Lusher et al., 2013; Avio et al., 2015). In urban coastal waters surrounding Tokyo (Japan), 77% of fish ingested microplastics, which can be explained by the surrounding industrial area (Tanaka and Takada, 2016). Fish sampled in the North Pacific Subtropical Gyre (NPSG), an area with some of the highest plastic accumulations, were found with a lower incidence of plastic ingestion (9%), but ingestion of larger plastic particles (up to 5 mm), relative to plastic ingestion by fish

observed in Moorea (Davison and Asch, 2011); however, the study surveyed only stomachs and not entire digestive tracts. In the same region, a different research group recorded plastic ingestion in 35% of examined fish stomachs (Boerger et al., 2010). However, as pointed out by Davison and Asch (2011), Boerger et al. (2010) have not taken into account the net feeding bias (ingestion of debris concentrating in the end of the fishing net) that might explain the high proportion of plastic ingestion.

Moorea Island is under the influence of the South Equatorial Current, which is generally weak and highly variable in this area (Kessler and Taft, 1987). At the local level, the island receives important wave exposure from the open-ocean (Hench et al., 2008) allowing the continuous flow of water through the lagoon and suggesting a low potential for plastic accumulation. Although Moorea has several villages surrounding the island, there are no high human density areas. The neighbouring island of Tahiti, however, has more industrialized areas with higher human densities, which may provide some amount of plastic input, as some studies suggest a link between local microplastic abundance with human population density (Browne et al., 2008; Gago et al., 2015). Therefore, further studies should investigate Tahiti coastline to determine the main source of marine plastic pollution.

With regard to the amount of ingested microplastics, our observed average of 1.25 ± 0.13 microplastic per fish corresponds with data collected in other studies (Avio et al., 2015; Bellas et al., 2016). Boerger et al. (2010) observed an increase in the number of plastics with the increase of fish body size; however, this was not the case in our study nor in other similar work (e.g. Foekema et al., 2013; Sanchez et al., 2014). The fish taxonomic groups we examined belong to different trophic guilds and often feed at different depths in the water column. Namely, the zooplanktivores (*Myripristis* spp. and *Cheilopogon simus*) feed along the entire water column, whereas the carnivore (*Epinephelus merra*) and herbivore (*Siganus* spp.) are bottom feeders, which can

Table 1
Descriptive data for each genus. Values are mean \pm SD.

Genera (taxa)	Number of fish examined	Standard length (cm)	Digestive tract wet weight (g)	Digestive tract with microplastics (%)	Number of microplastics pieces per fish	Number of microplastic pieces per fish that ingested microplastics
<i>Myripristis</i> spp.	33	16.7 \pm 0.2	5 \pm 0.21	7 (21)	0.27 \pm 0.13	1.29 \pm 0.12
<i>C. Simus</i>	34	25.1 \pm 0.3	4.34 \pm 0.23	6 (18)	0.24 \pm 0.13	1.33 \pm 0.16
<i>E. merra</i>	33	18 \pm 0.2	4.44 \pm 0.24	10 (30)	0.39 \pm 0.14	1.3 \pm 0.14
<i>Siganus</i> spp.	33	17 \pm 0.2	13.04 \pm 0.49	5 (15)	0.15 \pm 0.10	1 \pm 0

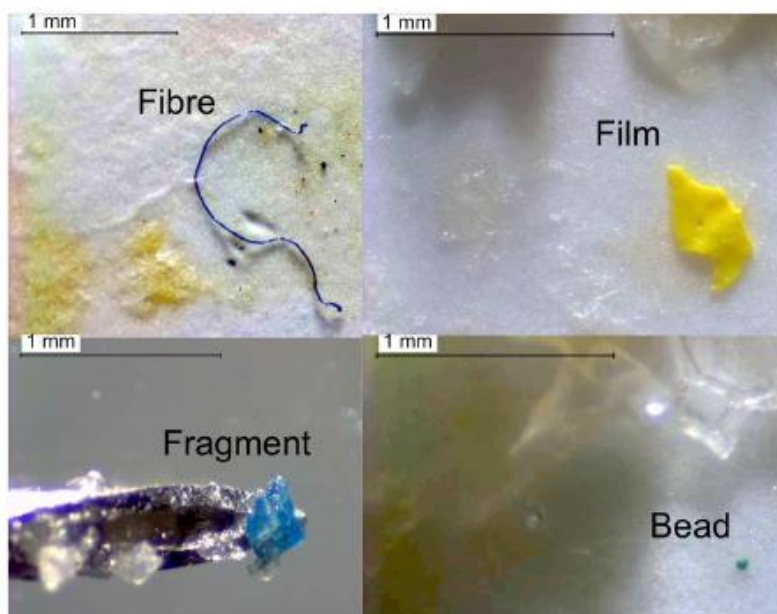


Fig. 2. Photographs of the four microplastic types identified.

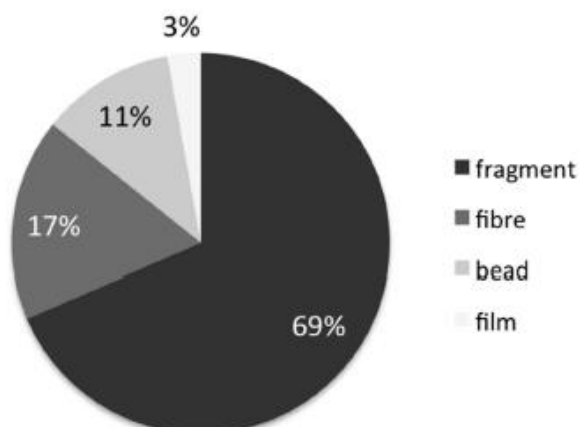


Fig. 3. Relative abundance of the plastic type identified in all four genera.

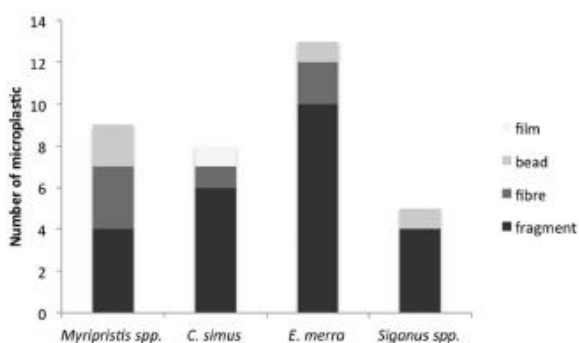


Fig. 4. Total number of each microplastic type recorded in each genus.

account for the observed differences in ingestion across the sampled taxonomic groups (Sale, 1991). For example, benthic fish have been found to ingest more plastic than pelagic fish (Neves et al., 2015), supporting the claim that fibres are more common in the sediment whereas fragments are prevalent in surface waters (Eriksen et al., 2013; Frias et al., 2016; Maes et al., 2017). However, we found more fibres in the zooplanktivores than bottom feeding fish, which could be explained by the bioavailability of microplastic in the area.

Fragments were the most common type of microplastic (69%) observed in fish at Moorea and have also been found to be the most abundant in other studies conducted in the Pacific (Davison and Asch, 2011; Tanaka and Takada, 2016). Herbivores had the lowest ingestion of microplastics; however, this might be due to difficulties regarding plastic extraction from their digestive tracts. Due to the high fat contents, the samples were not entirely dry after the first oven desiccation, which may have affected later steps in the plastic extraction protocol. Lusher et al. (2017) highlighted the numerous methods of extracting microplastics and the urgent need of the standardisation of protocols in order to have accurate results. Further studies should focus not only on the protocol design but also on checking whether the samples react to the treatments in the same way, as investigated by Karami et al. (2016).

5. Conclusion

The present study represents the most comprehensive report of microplastic contamination in fish from French Polynesia. The presence of microplastics was reported in the digestive tract of three lagoon-associated species and one pelagic species. The highest amount of ingested microplastic was found in the carnivorous species, *E. merra*, and the lowest was found in the herbivorous *Siganus* spp. This study showed that the number and size of the microplastics ingested are independent of feeding habits. Thus, further studies should examine both microplastic ingestion by additional reef fish species in addition to various semi-pelagic and pelagic species, which have different feeding strategies, as well as further quantify presence and abundance of microplastic pieces in the sediment and throughout the water column of coral reefs regions to confirm this trend. Monitoring programs should be

undertaken in order to better understand microplastics contamination in marine biota. Despite the lack of industrialized areas in Moorea and its relative remote location from the continents, plastics are also present in its coral reef habitats, highlighting the ubiquity of plastic pollution.

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Competing interest

The authors declare that they have no competing interests.

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ANNEXE 3 - Live fast, die young: Behavioural and physiological impacts of light pollution on a marine fish during larval recruitment.



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Live fast, die young: Behavioural and physiological impacts of light pollution on a marine fish during larval recruitment

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ABSTRACT

Artificial light at night (ALAN) is a recently acknowledged form of anthropogenic pollution of growing concern to the biology and ecology of exposed organisms. Though ALAN can have detrimental effects on physiology and behaviour, we have little understanding of how marine organisms in coastal areas may be impacted. Here, we investigated the effects of ALAN exposure on coral reef fish larvae during the critical recruitment stage, encompassing settlement, metamorphosis, and post-settlement survival. We found that larvae avoided illuminated settlement habitats, however those living under ALAN conditions for 10 days post-settlement experienced changes in swimming behaviour and higher susceptibility to nocturnal predation. Although ALAN-exposed fish grew faster and heavier than control fish, they also experienced significantly higher mortality rates by the end of the experimental period. This is the first study on the ecological impacts of ALAN during the early life history of marine fish.

1. Introduction

In September 1878, while Thomas Edison was devising light bulb technology for the world's first mass-produced electric lighting system, he wrote in his laboratory notes: "With the process I have just discovered, I can produce a thousand – aye, ten thousand – from one machine. Indeed, the number may be said to be infinite" (Friedel and Israel, 2010). Recent satellite data estimates over 80% of the world's human population experiences artificial light at night, with both the extent and brightness of lit areas increasing at a rate of 2.2% per year between 2012 and 2016 (Falchi et al., 2016; Kyba et al., 2017). This phenomenon of anthropogenic light transforming the natural diel light-dark cycle has been linked to widespread biological impacts in a diverse array of taxa including birds, mammals, reptiles, amphibians, insects, and fish, giving rise to the term "light pollution" (Longcore and Rich, 2004; Navara and Nelson, 2007; Riegel, 1973). In contrast to birds and mammals however, impacts on fishes have received comparatively little attention despite indications that they may be more susceptible to deleterious effects due to taxon-specific traits such as a lack of eye lids (Yokogawa et al., 2007).

Biological impacts of light pollution (e.g. metabolic disruption, oxidative stress, immunological dysfunction, sleep loss, energy expenditure and altered growth rate (Bedrosian et al., 2011; Gaston et al., 2015; Navara and Nelson, 2007; Raap et al., 2015; Wyse et al., 2011)) are linked to the disruption of endogenous rhythms driven by daily, seasonal, and lunar light cycles (Gaston et al., 2017). Artificial light at night (ALAN) also impacts species behaviour and inter-species interactions through altering the visual environment around them. Nowhere is this more apparent than in predator-prey interactions involving nocturnal species. These interactions can favour the predator (e.g. through diminishing the effectiveness of anti-predator behaviour, increasing visual acuity or attracting greater prey density (Becker et al., 2013; Bolton et al., 2017; Wakefield et al., 2015)) or the prey (e.g. by reducing foraging activity of nocturnal predators, improving predator avoidance or greater prey density providing safety in numbers (Cerri, 1983; Davies et al., 2013b)). In either scenario, these changes in interspecies dynamics have ecological implications and could lead to trophic cascades with the potential to alter entire communities (Bennie et al., 2015; Bolton et al., 2017; Davies et al., 2012; Longcore and Rich, 2004).

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Although we are becoming increasingly aware of the effects of light pollution in terrestrial systems, we still know little about the effects of light pollution in the marine environment (Davies et al., 2014; Depledge et al., 2010). Sources of artificial light at night illuminating marine areas are numerous, including direct light from point sources (e.g. recreational and commercial shipping, fishing vessels and oil platforms) as well as larger scale continuous lighting (e.g. skyglow from coastal settlements and marine infrastructure). One of our largest knowledge gaps relates to impacts in coral reef environments. While these marine biodiversity hotspots face threats from several natural and anthropogenic stressors, no study to date has investigated how light pollution might impact ecological processes. The presence of light pollution on coral reefs is of particular concern due to the reliance of coral reef fishes on natural lunar cues to regulate reproductive periodicity in adults and the timing of reef-colonization (settlement) by larvae at the end of their pelagic dispersal phase (Besson et al., 2017; Davies et al., 2013a; Naylor, 1999).

During settlement larval fish undergo the most drastic physiological and behavioural transition of their life cycle into juvenile fish more closely representing their adult form, a process known as metamorphosis. The combination of selection pressure and high energetic requirements during this brief window (often < 24 h in coral-reef species) makes it a critical interval in determining population persistence (Doherty et al., 2004; Thorisson, 1994). As replenishment from the survival of larval pulses after settlement (larval recruitment) is also critical to population persistence, any impacts on behaviours and physiological functions relating to ecological fitness during this critical life transition could have unpredictable effects at different levels of biological organisation. Here, we investigate for the first time the impacts of ALAN on 1) behaviour, 2) endocrine function, and 3) growth and survival of a coral reef fish (*Acanthurus triostegus*) during larval recruitment. With the exponential growth of the human population and continuous expansion of infrastructure into marine landscapes, increasing our understanding of how anthropogenic lighting is affecting marine habitats at night is crucial for preserving biodiversity and ecosystem function (Bulleri and Chapman, 2010; Davies et al., 2016).

2. Methods

2.1. Study species & laboratory setup

All experiments were conducted using convict surgeonfish (*Acanthurus triostegus*) collected during settlement to rock pools near Temae Beach, Moorea Island, French Polynesia (17°29'50.7"S 149°45'15.3"W). We identified newly arrived *A. triostegus* larvae as transparent with silver pigmentation over the brain case and alimentary tract (i.e. pre-metamorphosis (McCormick, 1999)). We collected larvae using dip nets during the overnight high tide around the new moon phase in May 2017. Larvae were transported to the laboratory facilities at the Centre de Recherches Insulaires et Observatoire de l'Environnement (CRIOBE) and placed in aquaria (36 cm × 46 cm × 23 cm) supplied with flow-through filtered seawater.

Immediately upon transfer into aquaria, *A. triostegus* larvae were allocated into two treatment groups: a control 12L:12D light-dark cycle, or an artificial light at night cycle (i.e. 12L:12L). Aquaria were lit from overhead with dimmable smd5050 white LED strip lights (6500 k, $\lambda_p = 450$ nm, supplementary material Fig. 1) placed 40 cm above the water's surface, supplying all tanks with a light intensity of 650–700 lx during the day (7 am–7 pm). Importantly, treatment tanks only received an ecologically relevant level of artificial light at night of 20–25 lx from 7 pm–7 am, a range used in previous studies to mimic in situ measurements taken in marine areas exposed to night-time lighting (Davies et al., 2015). We monitored light intensity in aquaria at the water's surface with an EA31 luxmeter (Extech Instruments, MA). Water temperature in the tanks was maintained at 28.7 °C (± 1.1 °C). Rock rubble covered in turf algae collected from the rock platform at Temae reef was

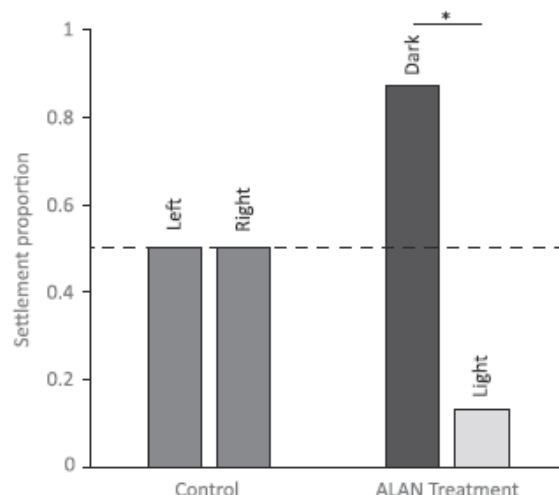


Fig. 1. Preference of *Acanthurus triostegus* larvae for rock rubble settlement habitats with and without artificial light at night compared to left and right sides of the binary choice chamber under control conditions. Dotted line shows distribution expected by chance, asterisk indicates significant difference from expected distribution ($p < 0.05$).

placed in the aquaria to provide fish with both shelter and an ad libitum food source. Rocks were replaced to replenish turf algae every two days.

2.2. Experimental procedure

2.2.1. Habitat choice experiments

Habitat choice experiments were conducted to test whether *A. triostegus* larvae preferred to settle to habitat with or without the presence of ALAN. After collection larvae were placed in holding aquaria to acclimate to laboratory conditions and recover from handling, with choice experiments commencing immediately after dusk on day 1 (d1, the day after the night-arrival of larvae). We tested larvae in a binary choice chamber (170 cm × 60 cm × 22 cm) with coral rubble placed at each end (Supplementary material Fig. S1). The chamber itself was delineated into 3 equal sections (i.e. left, centre and right) and randomly applied with either a control (i.e. dark vs. dark habitat choice) or a light treatment (i.e. a dark vs. light habitat) provided by a down-focused LED torch (250 lx) mounted 40 cm above the water's surface above one rubble habitat. To begin each trial, a single larva was introduced into a holding chamber – a vertically standing PVC pipe (12 cm diameter) – in the centre of the middle zone. The holding chamber had two mesh sides facing each end of the choice arena, so that fish could visibly assess the two habitat choices. After an acclimation period of 3 min, the holding chamber was removed allowing the fish to explore the arena. After 2 min we inspected the arena and each habitat, and the position of the larva was noted. Fish were classed as having made a decision if they were within one of the end zones or not having made a decision if they were found within the middle section of the arena. Control and treatment settlement distributions were tested against the distribution expected by chance (50:50) using a G-test for goodness of fit.

2.2.2. Endocrine response

Thyroid hormone levels were measured on fish euthanised (MS222 immersion at 0.4 mg ml⁻¹) and dry-frozen (–20 °C) at day 2 (d2) and day 5 (d5) post-settlement. Tri-iodothyronine (T₃) and thyroxine (T₄) were extracted following an extraction protocol already established for *A. triostegus* samples (Holzer et al., 2017). Thyroid hormone quantification was performed following the Roche ELICA kit on a Cobas

analysed by a medical laboratory according to the manufacturer's standardized method. Sample sizes of 8 to 10 fish per treatment were used for each sampling point.

2.2.3. Conspecific visual cue response

To test the effect of acute exposure to artificial light at night on the behavioural response of *A. triostegus* larvae to visual stimuli, choice tests were performed using a three-compartment test chamber. Clear Perspex sheets separating the compartments allowed the test fish to receive visual cues from juvenile conspecifics or heterospecifics being held in each of the end compartments. As *A. triostegus* have shown an attraction to visual cues of conspecifics during early life-history (Lecchini et al., 2014), our null hypothesis was that individuals would spend significantly more time close to conspecifics in the test chamber. A detailed description of test protocol including a diagram of the test chamber (Fig. S2) is provided in the supplementary material. Briefly, visual cue response trials were conducted on day 10 of exposure to light and control treatments in the lab. Each trial began with the introduction of a single *A. triostegus* larva from one of the two treatments into an opaque cylindrical holding chamber standing vertically in the centre of the central compartment for an acclimation period of 1 min. After the acclimation period, the pipe was removed and the position of the larva in one of three equal-sized visually-delineated zones (i.e. central, by conspecifics or by heterospecifics) was recorded every 5 s for a period of 2 min. Each larva was tested only once ($n = 40$), and after each trial the test chamber was emptied and washed with fresh water. The positions of the conspecific and heterospecific treatment fish were switched between each end of the test chamber after every 10 runs to account for a potential side bias in the experimental apparatus. Furthermore, we tested a subset of larvae using the same procedure, but with opaque screens placed between the central compartment and the cue fish compartments to eliminate stimuli and test for tank/arena effects ($n = 20$). The time spent by light (ALAN) and control treatment larvae near conspecifics and heterospecifics, and the time spent in left and right sides of the test chamber during control runs was compared with non-parametric Wilcoxon Signed Rank tests, which is a suitable test for time proportion data of this kind (O'Connor et al., 2016). We also compared the time a larva took to first move to a sub-compartment at either side of the chamber after release from the holding chamber (i.e. the time to make an initial choice) between ALAN and control treatment fish using Wilcoxon Rank Sum tests.

2.2.4. Post-settlement growth and survival

To examine the effects of artificial light at night on growth rates of fish, we measured weight and examined growth histories of *A. triostegus* larvae after 10 days (d10) of exposure to light (ALAN) or control treatments in the lab, the period in which metamorphosis is completed in this species (Holzer et al., 2017). Larvae were euthanized (as per above), and their wet weights ($n = 80$) were recorded. The sagittal otoliths were extracted from all 80 fish, and growth histories were successfully quantified for 60 of these individuals. Transverse sections through the nucleus of the otoliths were produced to expose daily growth increments across the transverse plane (Wilson and McCormick, 1997). A detailed description of otolith imaging and preparation is provided in the supplementary material. Briefly, polished samples were observed in immersion oil and a set of digital images was collected for each otolith to measure growth increments along the dorsal axis. Individual increment widths from the settlement mark to the edge of the otolith were recorded to the nearest $0.001\mu\text{m}$. This section of the otolith represented the experimental period (10 days), as fish were collected immediately upon settlement and otoliths were harvested on Day 10.

Otolith increment width was used as a proxy for daily fish growth, which is based on the generally held assumption that there is a strong relationship between somatic and otolith growth (Thorrold and Hare, 2002). The 10 daily increments closest to the otolith's edge were

summed to calculate the total post-settlement growth of individuals over the duration of the experiment. Samples were read only once by a single observer who was blind with respect to metadata associated with each sample. Where daily increments were difficult to identify, but a mark corresponding to settlement was clearly visible (Victor, 1982), a measurement from the settlement mark to the otolith edge was taken along the dorsal axis to give a measure of total post-settlement growth over 10 days. To evaluate whether exposure to artificial light at night had an effect on post-settlement growth rates and mean weight of *A. triostegus*, we used Welch two-sample *t*-tests to compare control and treatment groups.

To analyse how mortality was affected by ALAN we recorded any mortality of fish discovered during routine inspections of aquaria during the experimental period. We then used this data to generate Kaplan-Meier survival curves for each treatment and compared them to investigate differences in survival probability over time using the "survival" package in R (Diez, 2013).

2.2.5. Predator-prey interaction

To test how exposure to ALAN affects post-settlement predation risk, predation experiments on *A. triostegus* ($n = 7$) were conducted at 10 days post-settlement (d10). Circular tanks (85 cm diameter, 40 cm depth) were used as test arenas; PVC pipe sections (15 cm diameter) were placed in the arena for predator shelter, and pieces of rock rubble (4–6 cm diameter) were included for prey shelter. At least 24-h prior to being used in a predation experiment, *A. triostegus* individuals were tagged with a subcutaneous coloured elastomer tag (Northwest Marine Technology) on their dorsal side, in one of two different colours to identify the two treatment groups. On the afternoon of d10, we simultaneously released groups of fish from each treatment (ALAN vs. Control, $n = 8–10$ per group per trial) into the test arena containing a pair of nocturnal predators (clearfin lionfish *Pterois radiata*, 15–20 cm SL), starved for 48 h prior to testing. Trials began during the afternoon between 12 and 2 pm to allow *A. triostegus* acclimation time in the test arena conditions before exposure to nocturnal predation.

Predation was visually evaluated every 2 h during the afternoon by counting the number of *A. triostegus* individuals remaining in the arena. No predation was observed during these periods. Prey and predators were left overnight in the test arena and visually evaluated haphazardly to assess predation. Trials were ended when 50% of larvae were eaten, or at 24-h after introduction of fish into the arena. We then calculated the survival rate in each group (number of survivors/initial number for each group) and overall (total number of survivors/initial number for both groups) for each trial, giving us the relative survival rate for each treatment (survival rate of each treatment - overall survival rate). No trials were ended during nightly observations (i.e. at least 50% of the prey fish survived until dawn observations the next day). Predator pairs used for trials were alternated between test days to allow their appetite to regenerate.

2.3. Ethics statement

This study was carried out in accordance with the guidelines of the French Polynesia committee for animal ethics, and all experiments were approved by the CRIOBE-IRCP animal ethics committee (IRCP-2018). This study did not involve endangered or protected species.

3. Results

3.1. Settling larvae selected darker habitats

At the end of the habitat choice experiment runs, all *A. triostegus* larvae were located nestled within a settlement habitat, with exception of one individual which was found in the centre section after both habitats had been searched. As the initial position of the larva at the end of the experimental period was uncertain we excluded it from the

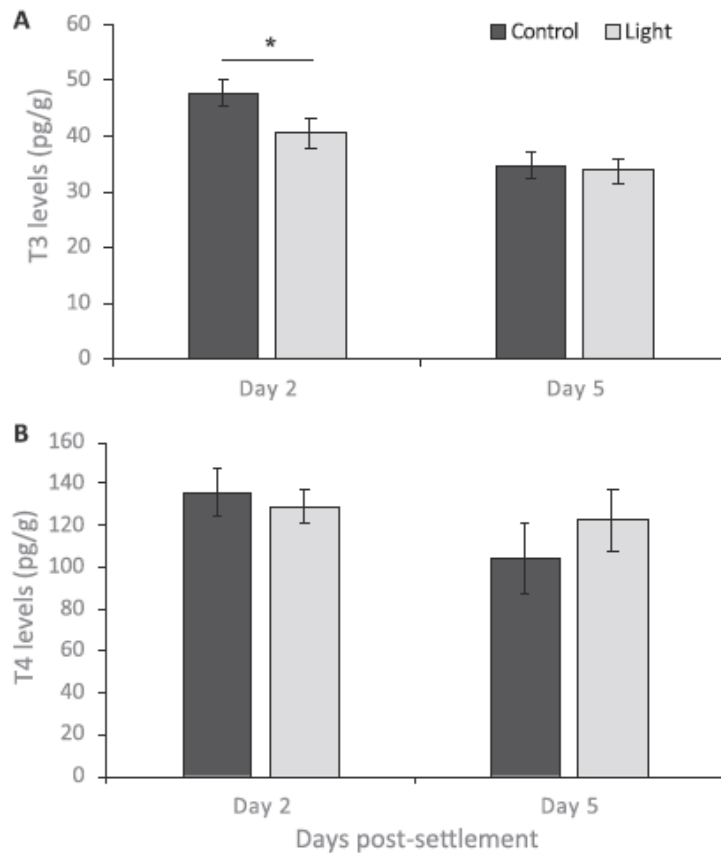


Fig. 2. Impact of artificial light at night exposure of thyroid hormone T₃ (A) and T₄ (B) levels in *Acanthurus triostegus* larvae during post-settlement metamorphosis compared to control treatments.

analysis. No larvae were observed freely swimming in the chamber at the end of the experimental period and were therefore assumed to have completed their initial settlement choice. While larval habitat preferences did not differ from that expected by chance during control trials ($G = 0.07$, $df = 1$, $p = 0.80$), larvae showed a significant preference for settlement to the dark habitat over the habitat lit with the LED light at night ($G = 9.01$, $df = 1$, $p = 0.0027$, Fig. 2).

3.2. ALAN lowered thyroid hormone levels during metamorphosis

Exposure to artificial light at night significantly reduced T₃ levels in *A. triostegus* at day 2 post-settlement compared to the control treatment fish ($W = 67$, $p = 0.039$, Fig. 3A). By day 5 post-settlement T₃ levels evened out between the two treatments ($W = 39$, p -value = 0.81). We did not find a difference in T₄ levels at day 2 ($W = 41$, p -value = 0.351) or at day 5 ($W = 20$, p -value = 0.3969).

3.3. ALAN exposed fish swam faster but did not alter visual cue response

During control runs, larvae showed no bias for one side of the choice chamber ($V = 33$, $p = 0.53$) or the middle sub-compartment ($V = 60$, $p = 0.59$). Exposure to artificial light at night did not affect choice behaviour in *A. triostegus*, with 75% of individuals from both ALAN and control treatments preferring the compartment closest to conspecific fish over heterospecific fish (i.e. fish spent > 50% of the observation period in the compartment closest to conspecifics). There was however a significant difference in the amount of time taken to make a choice.

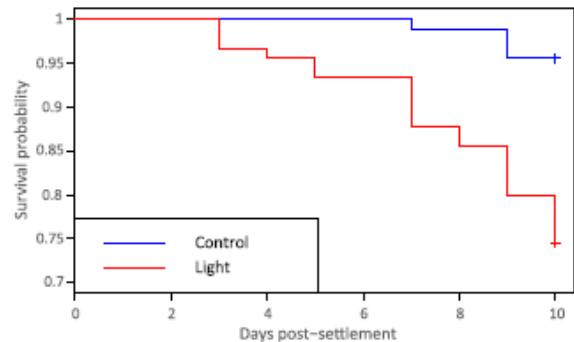


Fig. 3. Kaplan-Meier survival curves for *Acanthurus triostegus* larvae during the experimental period (10 days post-settlement) comparing exposure to artificial light at night (12L:12L cycle) with a control treatment (12L:12D). X-axis has been restricted to 0.7 probability for clarity.

Fish from the ALAN treatment that swam towards cue fish (18 of the 20 fish tested; 2 fish remained in the central compartment for the duration of the experiment) did so immediately after release with an average time of 1.2 s (± 0.89) to reach the cue fish compartment. Control fish displayed distinctly different behaviour in the choice chamber, spending significantly longer time observing the cue fish before making a choice (21.4 s, $W = 127.5$, $p = 0.035$).

3.4. ALAN exposed fish grew faster and heavier but with decreased probability of survival

Over the 10-day experimental period, *A. triostegus* grew faster when exposed to artificial light at night post-settlement, compared to control fish exposed to a normal light/dark cycle ($t = 2.12$; $df = 57.99$; $p = 0.038$). Light exposure resulted in a mean of 7.1% greater growth over the 10-day post-settlement period. Fish exposed to artificial light at night also showed significantly greater mean weight than the control group ($t = 2.35$; $df = 79.66$; $p = 0.021$). However, post-settlement mortality over 10-day period was increased by exposure to ALAN ($\chi^2 = 15.9$, $df = 1$, $p < 0.0001$). Survival was comparable for the first 3 days post-settlement in both treatments, before a significant dissociation between the survival curves becomes apparent, driven by decreasing survival probability for fish in the ALAN treatment. Overall, 4% of control fish experienced mortality by day 10, compared to 26% ($n = 90$ per treatment) in the ALAN treatment.

3.5. ALAN exposure increased probability of predation

Fish exposed to ALAN experienced higher rates of predation compared to control fish with relative mortality ratios as high as 9:1 (Fig. 4). Relative mortality was significantly different between fish which had been exposed to ALAN during metamorphosis and control fish ($W = 7$, $p = 0.98$).

4. Discussion

This study provided significant evidence that the presence of ALAN

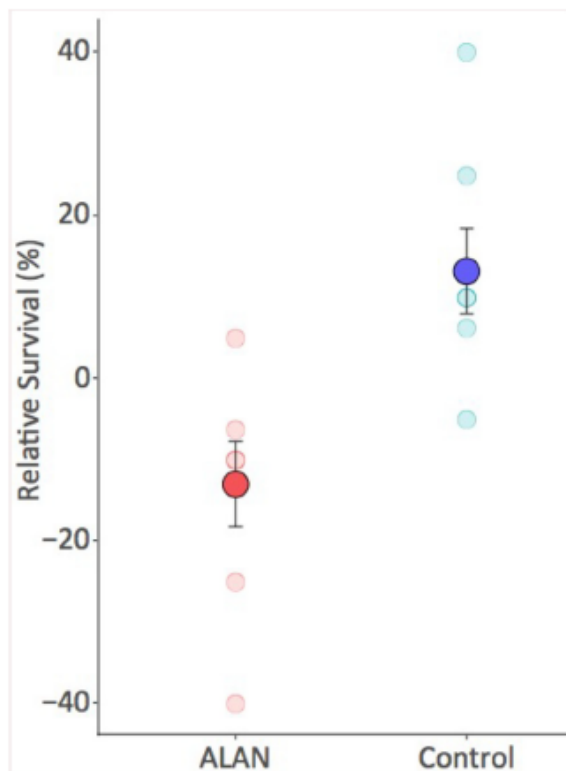


Fig. 4. Impact of exposure to artificial light at night (ALAN) on *Acanthurus triostegus* on relative survival rates when exposed to nocturnal predators at night, 10 days post-settlement.

changes the behaviour and physiology of a coral-reef fish during larval recruitment. These changes, including habitat avoidance, endocrine disruption, altered growth and increased mortality rates, may have negative implications for the fitness and post-settlement survival of fish at this critical life stage. Indeed, factors affecting survival during settlement and metamorphosis of larvae can greatly influence larval recruitment success and therefore subsequent population dynamics (Pepin and Myers, 1991; Searcy and Sponaugle, 2001). Even sub-lethal effects on fish during recruitment, where selection through competition and predation is at its highest, may indirectly affect the composition of coral reef assemblages (Hoey and McCormick, 2004; Jones, 1990). The broad range of ecological impacts we observed suggests a need for better understanding of how light pollution is affecting increasingly impacted coastal areas, which act as nursery habitats for the majority of marine fishes (Davies et al., 2014).

Fish larvae avoided settling to lit habitats when given the choice between lit and dark structures. Positive phototactic behaviour is common in marine organisms during the larval stage, encouraging vertical movement of planktonic organisms in the pelagic environment and attracting nekton into light-baited traps for collection (but see Bardounet et al., 2005; Doherty, 1987; Moore et al., 2000; Okera, 1974; Porter et al., 2008). Phototaxis often varies between species and with ontogeny, with the strength of the response generally decreasing later in the larval stage (Bulkowski and Meade, 1983; Forward and Costlow, 1974; Gehrke, 1994; Marchesan et al., 2005). This suggests the impact of ALAN on the fitness of recruiting larvae could disproportionately affect species with positive phototaxis at the settlement stage, skewing community compositions (Davies et al., 2012). Furthermore, selection on reproductive phenology has resulted in the recruitment of many fishes to coral habitat during the new moon when it is darkest in an attempt to avoid the predation gauntlet awaiting them on the reef (Almany and Webster, 2006). Indeed, there is evidence that developmental plasticity during the larval stage has evolved in some species in order to avoid settlement during bright lunar periods in favour of dark periods (Shima et al. 2018). Future studies are needed to investigate how the presence of ALAN, potentially masking lunar cues around the time of spawning and settlement, affects patterns of reproductive phenology and settlement in situ.

In fishes, the majority of knowledge on the known biological effects of artificial light intensity and periodicity come from aquaculture, where optimal photic conditions for growth during early life history are prioritised (reviewed in Villamizar et al., 2011). This research may be less ecologically relevant to most marine fishes as they are less likely to encounter chronic exposure to artificial light before they settle to coastal habitat, as dispersal during the pelagic larval phase often transports larvae hundreds of meters to thousands of kilometres offshore (Green et al., 2015). Considering the ecological effects of light pollution on fishes, the few previous studies that exist have primarily investigated impacts of ALAN on mature, freshwater species, focusing on melatonin expression (but see Barker and Cowan, 2018; Brüning et al., 2016, 2017; Brüning et al., 2015).

We found physiological evidence for the fitness benefit of light avoidance behaviour at settlement in the significant endocrine disruption experienced by *A. triostegus* exposed to ALAN during metamorphosis. Larvae experiencing ALAN upon settlement and throughout the post-settlement metamorphic stage showed depressed levels of T_3 , an important hormone for metamorphosis (Holzer et al., 2017), at day 2 post-settlement. Such an endocrine disruption at this critical life stage could impair fish development as individual turn from larvae to juveniles (Holzer et al., 2017). This is further demonstrated here with the decreased relative survival of the ALAN-exposed group compared to dark control group at day 2 post-settlement when facing predators. Mortality of coral reef fishes due to predation is estimated to be highest around the time of larval settlement and recruitment (Almany and Webster, 2006), and the further effect of ALAN exposure in increasing predation risk may even decrease recruit survival, impacting population

persistence.

We observed that *A. triostegus* exposed to ALAN swam in rapid erratic bursts during visual cue response trials, lacking the cautious assessment behaviour of control fish when presented with conspecific and heterospecific visual cues. Changes in the swimming behaviour of fishes are commonly used as indicators of sublethal toxicity in response to external pollutants (reviewed in Little and Finger, 1990). Although behavioural responses to toxins can vary between species, life stage and chemical compound used, hyperactivity and increased burst swimming are common symptoms of physiological toxicity (Jordaan et al., 2013; Saglio et al., 2003; Williams et al., 2012). As degradation of visual acuity due to ALAN was not detected during visual cue choice tests, increased vulnerability to predation may relate to changes in swimming behaviour as a result of physiological stress. For example, increased activity and boldness of coral reef fish during recruitment has been shown to elevate predation risk (Ferrari et al., 2011). In addition, as acute stress can reduce metabolic scope in fish, the physiological cost of hyperactivity may have played a role in the reduced survival rate of ALAN exposed fish during the exposure period (Barton and Schreck, 1987).

Despite the physiological impacts of ALAN exposure, we found that fish grew faster and were heavier under ALAN conditions compared to control fish. Fish larvae settling to coral reefs show natural variation in growth rate which can influence fitness and survival immediately post-settlement (Shima and Findlay, 2002). In coral reef fish ecology, growth rates are often used as a proxy for predicting recruitment success, with faster-growing individuals contributing more to population replenishment than slower-growing individuals (Bergeniuss et al., 2002; Wilson and Meekan, 2002). However, our results indicate that exposure to light pollution may override innate growth patterns of settling fish. From aquaculture studies, we know that growth rates in fish larvae can be influenced by altering regimes of light exposure, with longer photoperiods often beneficial to growth, particularly early on in the larval stage (e.g. Olivotto et al., 2003; Puvanendran and Brown, 2002). In most species however, a constant lighting regime (i.e. 24L:0D) negatively affects development and survival during early life stages (Villamizar et al., 2011). In short, despite increased growth shown by ALAN treated individuals, the net result of ALAN exposure in wild-caught larvae at the settlement stage was decreased fitness.

ALAN-exposed *A. triostegus* were also heavier than the control fish, which under natural circumstances can be an indicator of increased fitness in coral-reef fish recruits. Weight positively affects estimates of body condition (e.g. Fulton's *k* condition index), a selective trait positively correlated with post-settlement survival (Booth and Hixon, 1999; Searcy and Sponaugle, 2001). However, in some cases post-settlement predation can be selective for fish with higher standardized weight (Hoey and McCormick, 2004), indicating another potential contributor to increased predation rates in ALAN-exposed *A. triostegus*. The increased mortality we observed in settlement-stage coral-reef fish larvae exposed to ALAN, both during the exposure period and predation trials, suggests that recruitment to habitats experiencing light pollution may have negative effects on post-settlement survival, with unknown flow on consequences for coral-reef community structure.

Sleep deprivation due to ALAN exposure is another potential driver of the physiological and behavioural changes we observed in *A. triostegus* during metamorphosis. Sleep states in fish share fundamental similarities with those of mammals (Zhdanova et al., 2001). The sleep/wake regulator hypocretin/orexin (Hcr, linked to locomotive activity) is also shared, with robust locomotive sleep/wake behaviours exhibited in fish larvae as young as 5 days post-hatch (Prober et al., 2006). Sleep deprivation in humans, often caused by ALAN exposure via the disruption of circadian systems, has been shown to increase metabolic requirements and weight gain via appetite upregulation (Knutson et al., 2007; Touitou et al., 2017). As other impacts of sleep deprivation are shared between mammals and fish (e.g. disruption of cognitive performance (Pinheiro-da-Silva et al., 2017)), metabolic impairment due

to sleep deprivation may be a pathway for some of the impacts we observed here.

This is the first study to provide a multi-faceted, ecologically-relevant examination of how ALAN in coral reef habitats may affect fishes during the critical larval recruitment stage. We found significant evidence that light pollution can result in changes to behaviour, physiological function and post-settlement survival. These results raise concerns about how coastal ecosystems will fare with increasing coastal development and the loss of natural darkness in the night skies. To better understand the mechanisms behind the impacts reported here, future studies should look across a broad range of taxa and habitats to investigate: 1) the impacts of ALAN on recruitment dynamics in situ, 2) how ALAN affects sensory development across multiple modalities, 3) how ALAN affects the behaviour and physiology of predators, and perhaps most importantly 4) quantify the underwater light field in impacted areas, and determine threshold values for intensity and wavelengths related to negative impacts. This information is vital for informing mitigation measures and management of our coastal areas where human activities are significantly re-shaping marine communities (Ruppert et al., 2018).

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.marpolbul.2019.05.038>.

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ANNEXE 4 - Decreased retention of olfactory predator recognition in juvenile surgeon fish exposed to pesticide.



Decreased retention of olfactory predator recognition in juvenile surgeon fish exposed to pesticide

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HIGHLIGHTS

- Coral reefs face many threats such as ocean warming, acidification and pollution.
- No studies examined how chemicals affect the cognitive abilities of reef organisms.
- This study investigated anti-predator behavior in post-larval *Acanthurus triostegus*.
- This behavior was retained between 2 and 5 days in the absence of pesticide.
- The pesticide Chlorpyrifos induced a loss of this crucial information.

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ABSTRACT

Dory, the animated surgeonfish created by the Pixar Animation studios, famously suffered from short-term memory loss leading to many adventures. In reality, many fishes have excellent cognitive abilities and are able to learn and retain important information such as the identity of predators. However, if and how cognition can be affected by anthropogenically altered oceanic conditions is poorly understood. Here, we examine the effect of a widely used pesticide, chlorpyrifos, on the retention of acquired predator recognition in post-larval stage of the surgeonfish *Acanthurus triostegus*. Through associative learning, post-larvae of *A. triostegus* were first observed to forage significantly less in the presence of conspecific alarm cues and alarm cues associated to a predator's odor. The retention of this anti-predator behavior was estimated to last between 2 and 5 days in the absence of pesticide. However, environmentally-relevant concentrations of chlorpyrifos ($1 \mu\text{g.L}^{-1}$) induced the loss of this acquired predator recognition. This reduced ability to recognize learned predators is discussed as it may lead to more vulnerable fish communities in coastal areas subjected to organophosphate pesticide pollution.

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1. Introduction

In Disney-Pixar's *Finding Dory*, the title character short-term memory resulted in many adventures. Could fiction meet reality, especially in a world polluted by pesticides? Coral reefs are one of the most diverse ecosystem in the world, home to between 1 and 9

million species (Reaka-Kudla, 1997). In addition to their ecological and biodiversity value, many human populations rely on coral reefs for the goods and services they provide (e.g., tourism, shoreline protection, fisheries, and cultural values; Bellwood et al., 2004). However, coral reefs are threatened by numerous global and local stressors such as ocean warming, acidification, pollution, or physical disturbance (Hoegh-Guldberg, 2014). Today, 75% of world's coral reefs are directly threatened and 95% may disappear by mid-century (Hoegh-Guldberg et al., 2007). Coral reefs, even the most isolated ones, are increasingly polluted by chemicals such as pesticides (Fabricius, 2005; Lavers and Bond, 2017). Despite numerous

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studies reporting the impact of chemical pollution on the biology, ecology, ethology and evolution of coral reef organisms (Deb and Das, 2013; Besson et al., 2017a; b), no studies have examined how chemical pollution can affect the cognitive abilities of coral reef organisms, for instance the retention of learned information i.e. memory. Memory is an essential cognitive function that permits animals to acquire, retain, and retrieve different kinds of information (Kelley and Magurran, 2007). It allows individuals to take advantage of previous experience to solve a whole array of problems that their environment confronts them with, such as how to recognize conspecifics, identify predators, find foraging places, and learn the consequences of their behavior (Kelley and Magurran, 2007). For example, in fishes, remembering prior experience with predators increases the subsequent chances of survival in a future encounter (Kelley and Magurran, 2007). Thus, any reduced capacity to recall information could have dramatic fitness consequences. In vertebrates, this 'memory loss' can arise for various reasons like bilateral hippocampal lesions (Scoville and Milner, 2000), sleep deprivation (Maquet, 2001), Alzheimer disease (Shankar et al., 2008) or organophosphate pesticide exposure (Levin et al., 2002) but this has mainly been studied in humans and rats, as opposed to marine fishes where literature is very scarce (Hatfield and Johansen, 1972; Besson et al., 2017a; b). Several studies have also highlighted the negative effects of pesticides on the behavior of honeybees involved in foraging, implying that pollinator population decline could be the result of a failure of neural function of bees exposed to pesticides in agricultural landscapes (Dacher et al., 2005; Williamson and Wright, 2013). Here, we looked at how exposure to an organophosphate pesticide (chlorpyrifos) often encountered in coral reefs (Cavanagh et al., 1999; Leong et al., 2007; Botté et al., 2012) may affect the retention of memory in post-larval stage of a coral reef fish.

The life cycle of most coral reef fish species includes a planktonic larval phase which usually lasts from one to sixty weeks, followed by a sedentary reef-associated juvenile & adult phase (Leis and McCormick, 2002). After the pelagic phase, larvae return to the reef to continue their development into juveniles, and adults (i.e., recruitment phase) (Barth et al., 2015). During recruitment, post-larval fishes (i.e., larvae undergoing metamorphosis) (Holzer et al., 2017) are subjected to strong selective pressures to choose suitable reef habitat (larval fishes experience 90% mortality during recruitment due to predation) (Doherty, 2002; Lecchini et al., 2007). Many fish species show therefore specific habitat preferences based on the relative abundance of predators and competitors for food and space (Leis and McCormick, 2002; Lecchini et al., 2014; Barth et al., 2015). Hence, survival at this life stage is often mediated by anti-predator behaviors (Brown and Chivers, 2007; Lecchini et al., 2014). These anti-predator mechanisms include the ability of fish post-larvae to rapidly learn and retain the identity of predators to survive until the adult stage. However, due to anthropogenic perturbations, post-larval perception and ability to learn may be impaired (Hatfield and Johansen, 1972; Barth et al., 2015; Besson et al., 2017a; b; Lecchini et al., 2017). For example, some studies have found that ocean acidification (Chivers et al., 2014) and habitat degradation (McCormick and Lönnstedt, 2016) can reduce the capacity of early life stages of fishes to chemically learn predator odor. However, no study has yet explored the potential impact of waterborne pesticide on the olfactory-based information retention in marine fishes.

To better understand the potential effects of pesticides on the vulnerable early life stage of coral reef fishes, we examined how organophosphate pesticides affected information retention of the predator *Rhinecanthus aculeatus* in the convict surgeonfish *Acanthurus triostegus*. We used chlorpyrifos (CPF), a broad spectrum organophosphate insecticide, which is one of the most widely used

insecticide in the world (Cavanagh et al., 1999; Leong et al., 2007; Botté et al., 2012; Zhang et al., 2017; Sastre et al., 2018) and that is often encountered in tropical or coral reefs waters (Kapernick et al., 2006; Leong et al., 2007; Salvat et al., 2012). Specifically, we assessed the anti-predator response of *A. triostegus* post-larvae by video recording their foraging behavior (a reduced foraging activity is known to be a common anti-predator response in fishes) (Kelley and Magurran, 2003; Holzer et al., 2017) in the presence of different stimuli in order to identify the behavioral response to predation (Exp. 1), if this response is learnt (Exp. 2) and for how long in the presence or absence of pesticide (Exp. 3).

2. Materials and methods

2.1. Experimental design

This study was conducted at the CRIOBE Research Station, Moorea Island, French Polynesia from March to August 2017. The focal species, the convict surgeonfish, *Acanthurus triostegus*, is a common Indo-Pacific species that is highly abundant in French Polynesia and feeds on turf algae (Siu et al., 2017). Post-larvae of *A. triostegus* (2.1 ± 0.5 cm, mean \pm SE) were collected using crest nets at Moorea Island (Besson et al., 2017a; b; Holzer et al., 2017). The fish used in the different experiments were at the same developmental stage. Indeed, Holzer et al. (2017) showed the absence of variability in the ontogenetic stage of *A. triostegus* post-larvae when they recruit on the reef at Moorea. Following capture, fish were brought back to CRIOBE and transferred to 12 L aquaria filled with UV-sterilized and filtered (10 μ m filter) seawater prior to being tested. Post-larvae were tested the day of their capture (after a period of at least 8 h in order to recover from the stress of capture) and over the 5 subsequent days (Holzer et al., 2017).

For experiments, olfactory predator cues were taken from the Picasso triggerfish (*Rhinecanthus aculeatus*), an abundant species that predate on fish post-larvae. Heterospecific, non-predator cues were taken from the Manybar goatfish (*Parupeneus multifasciatus*). Several *R. aculeatus* and *P. multifasciatus* at adult stages were collected with gill nets from fringing reefs and kept individually in 250 L flow-through plastic tanks for 48 h with no food before being used to produce olfactory cues. For each replicate of each experiment, new *R. aculeatus* and *P. multifasciatus* were used.

2.2. Experiment 1: response of *A. triostegus* to chemical cues associated with predation risk

To test baseline responses to odor cues, post-larvae were exposed to five different odor treatments: 1) control seawater, consisting of UV-sterilized and filtered (10 μ m filter) seawater; 2) heterospecific odor, consisting of 30 mL of water extracted from the 1.5 L tank that had contained one goatfish for 1 h; 3) Predator odor, consisting of 30 mL of water extracted from a 1.5 L tank that had contained one triggerfish for 1 h; 4) 30 mL of alarm cue; and 5) conditioning stimuli, consisting of 15 mL of predator cue and 15 mL of alarm cue. To produce each 30 mL batch of alarm cue, one *A. triostegus* was euthanized by cold shock. Following the method of McCormick and Lönnstedt (2016) to get alarm cues in coral reef fish, five superficial cuts were made with a clean scalpel on each side of the fish. Fish with cuts were then dipped in a tube filled with 30 mL of seawater which was then collected (McCormick and Lönnstedt, 2016). For each test, 30 mL of cues were used within 10 min following its synthesis.

For each condition, 3 *A. triostegus* post-larvae were placed in a 7 L tank, filled with control seawater and with coral rubble (30 cm³) covered with turf algae (Holzer et al., 2017). The coral rubbles with turf algae were collected close to the crest net site (i.e. Temae site

rate in response to predator odor was also observed at 1 and 2 days following conditioning (Mann-Whitney test: $P = 0.04$ at 1 day, $P = 0.03$ at 2 days). However, this response was no longer seen at 5 days (Mann-Whitney test: $P = 0.39$), with foraging rates now similar to control fish (mean bite rate \pm SD: 8.9 ± 0.6 vs. 8.7 ± 0.5 respectively) (Fig. 2). These results suggest that post-larval *A. triostegus* retain olfactory cues between 2 and 5 days.

3.3. Effect of chlorpyrifos on memory retention of *A. triostegus*

Post-larval *A. triostegus* were treated with the conditioning odor for a 1 h period. Following treatment, one half of the conditioned fish were held in seawater containing $1 \mu\text{g}\cdot\text{L}^{-1}$ of CPF for 2 days, with the other half held in untreated seawater. After 2 days, all fish were transferred to fresh seawater. After a habituation period (1 h), foraging rates in response to predator odor were recorded. Foraging rates of fish exposed to CPF was significantly higher than control *A. triostegus* (Mann-Whitney test: $P = 0.03$) (mean bite rate \pm SD: 6.1 ± 0.3 for fish exposed to CPF vs. 4.1 ± 0.5 for control) (Fig. 3), suggesting that post-larvae of *A. triostegus* lost their memory of predator odor in presence of CPF. Note a difference in bites number in condition stimulated fish after 2 days between Figs. 2 and 3. This difference could be explained by the fact that predator cue water and alarm cue water were not the same between the experiments.

4. Discussion

Dory, the famous surgeonfish might not be the only coral reef fish affected by short term memory problems. Here, we show that the olfactory memory of predator cues was estimated at between two and five days in the coral reef fish *A. triostegus* (Figs. 1 and 2). Memory is a constructive process for all animals that possess a hippocampus, the part of the brain that controls the retention of

information, and fish are no exception (Laland et al., 2003). To the best of our knowledge, this is the first record of memory retention in the early life stage of a coral reef fish. Literature regarding memory retention following a single pairing of an alarm cue and predator cue in fishes is very scarce, but the length of time ranges from 2 days in the fathead minnows, up to 21 days in hatchery-reared rainbow trout (Brown and Chivers, 2007). Here, in the presence of an environmentally relevant concentration ($1 \mu\text{g}\cdot\text{L}^{-1}$) of a common pesticide (CPF), *A. triostegus* failed to retain an information usually learned in control conditions (Fig. 3). Exposure to various pesticides was proven to impair spatial memory in rats (Yan et al., 2012), impair olfactory learning and memory in honeybees (Williamson and Wright, 2013) and the ability to learn and retain a simple conditioned response in the Atlantic salmon (Hatfield and Johansen, 1972), but such an effect in an ecologically relevant context, like predator's odor memory, has never been investigated in fishes. The known inhibitory effect of CPF on acetylcholinesterase (AChE) activity in fishes and other vertebrates (Colosio et al., 2003) and the role of AChE in neurotransmission in the central nervous system could likely explain the reduced olfactory memory in *A. triostegus* (Fig. 3). Sub-toxic CPF exposure during neurulation was shown to adversely affect brain development, leading to behavioral anomalies that selectively include impairment of cholinergic circuits used in learning and memory in rat juveniles (Icenogle et al., 2004). Our study highlights that pesticides had a negative impact on fishes' ability to remember predators successfully via their effects on learning and memory. This result could be also partly attributed to the advanced or delayed metamorphosis induced by thyroid hormone disruption (Holzer et al., 2017). Yet, as coral reef fishes are particularly vulnerable to predation at their early life stages (90% of post-larvae disappeared during the recruitment by predation) (Doherty, 2002; Lecchini et al., 2007), failing to respond adequately to predation often means immediate death (Besson

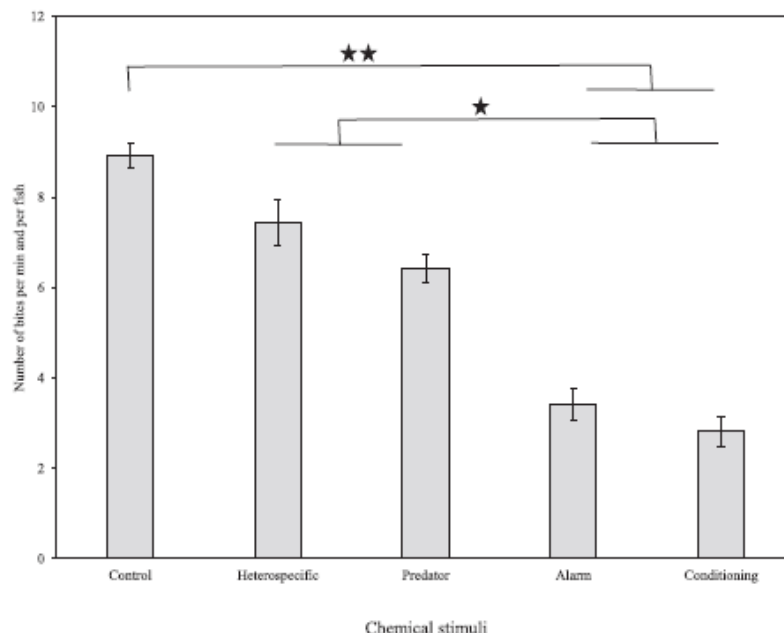


Fig. 1. Number of bites per min and per fish for *Acanthurus triostegus* post-larvae in response to chemical cues. Individuals were exposed to different chemical stimuli: Control sea water; Heterospecific odor (species: *Parupeneus multifasciatus* at adult stage); Predator odor (species: *Rhinecanthus aculeatus* at adult stage); Alarm cue (cues released from *A. triostegus* conspecific damaged skin) and Conditioning (alarm cues associated with predator odor). Values are mean \pm SD. **: $P < 0.01$; *: $P < 0.05$. Tested fish were at the same developmental stage.

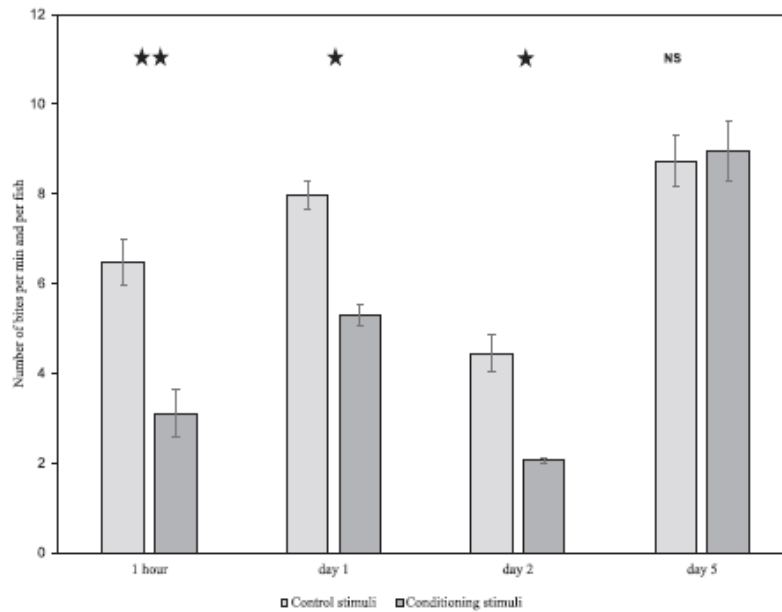


Fig. 2. Retention of olfactory memory in *Acanthurus triostegus* post-larvae. Individuals were initially conditioned either with both alarm cue and predatory cue (conditioning stimuli) or with control seawater (control stimuli), and subsequently tested at different time (1 h, 1, 2 and 5 days after) for their foraging response in the presence of the predator cue alone. Values are mean \pm SD number of bites. **: $P < 0.01$; *: $P < 0.05$; NS: Non-significant. Tested fish were at the same developmental stage.

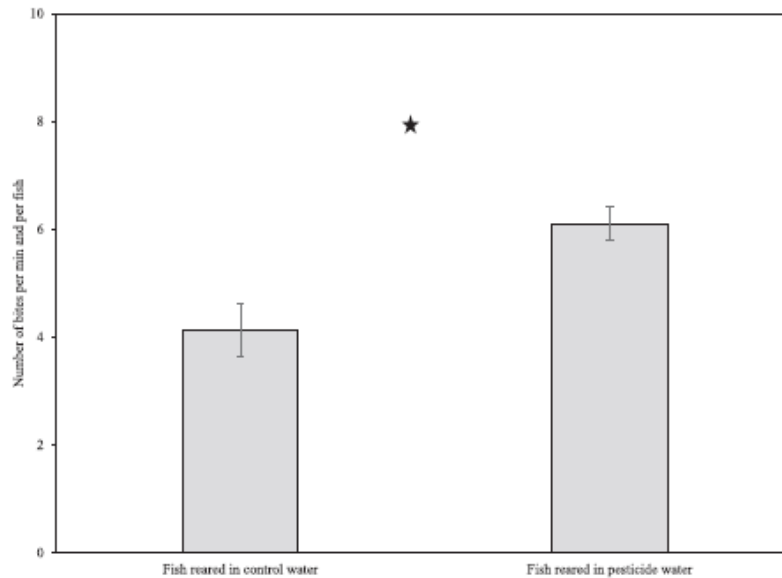


Fig. 3. Effect of chlorpyrifos on the number of bites per min and per fish for *Acanthurus triostegus* post-larvae. Individuals were initially conditioned with both alarm cue and predatory cue, and subsequently reared for 2 days either in control seawater or in seawater added with $1 \mu\text{gL}^{-1}$ of chlorpyrifos. After 2 days, their foraging behavior was recorded in clear water added with predator cues. Values are mean \pm SD. *: $P < 0.05$. Tested fish are at the same developmental stage.

et al., 2017a; b). Even if anti-predation behavior seems to have an underlying genetic basis, learning plays an important role in the development of antipredator responses (Kelley and Magurran, 2003). For example, sticklebacks reared in aquariums over several generations retained their characteristic patterns of anti-predator behavior (Foster and Endler, 1999), underlying the genetic basis of

the antipredator responses. On the other hand, fry of sticklebacks from a high-predation population that were protected by the father from approaching predators and subsequently returned to the nest went on to develop stronger avoidance responses towards a predator model than those that were orphaned (Tulley and Huntingford, 1987) showing the importance of learning on antipredator

response. Thus, since recruitment is an essential phase in a coral reef fish's life, memory is a key tool on its way to become an adult. A loss of predator-avoidance behaviors could greatly increase predator-induced mortality rates of this sensitive life stage with flow on impacts for adult populations of coral reef fishes, and the human communities that rely on them (Charlton et al., 2016).

5. Conclusions

Our study shows that, through associative learning, the retention of olfactory memory of a predator by *A. triostegus* postlarvae lasts between 2 and 5 days (Fig. 2). However, the presence of chlorpyrifos at environmentally-relevant concentrations ($1 \mu\text{g}\cdot\text{L}^{-1}$) induces the loss of acquired predator recognition (Fig. 3). This may lead to more vulnerable fishes in coastal areas subjected to organophosphate pesticide pollution. In addition, our approach illustrates the need to use neuro-behavioral indicators in order to assess the sub-lethal effect of pollutant on marine organisms. This will enable, as opposed to traditional ecotoxicological approach (LC 96h), to move from the effect of pollutant on individual level toward population or ecosystems level.

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Conflicts of interest

The authors declare that they have no competing interests.

Data and materials availability

Data will be made fully available upon request to D.L. (lecchini@univ-perp.fr).

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ANNEXE 5 - Preferential adsorption of Cd, Cs and Zn onto virgin polyethylene microplastic versus sediment particles.

Title

Preferential adsorption of Cd, Cs and Zn onto virgin polyethylene microplastic versus sediment particles

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Abstract

Plastic pollution has become a major environmental concern worldwide, and marine ecosystems have become particularly polluted with ubiquitous microplastic particles (MP). MP can contain chemical additives and scavenge pollutants from the surrounding environment, and these co-contaminants may threaten marine biota when MP become inadvertently ingested and transferred up the food chain. However, our understanding of the sorption-desorption kinetics of chemical compounds bound to MP remains limited. Moreover, whether MP are better transport vectors of co-contaminants than other natural particles (e.g. sediment) has not received much attention. Here, we used radiotracers to examine the partition coefficients (K_d) of three trace metals (^{109}Cd , ^{134}Cs , and ^{65}Zn) to virgin MP (32-75 μm polyethylene beads) and to natural sediment particles of a similar size (35-91 μm) in seawater. After 72 h, sediment particles absorbed 2.5% of ^{109}Cd , 68.0% of ^{134}Cs , and 71.0% of ^{65}Zn , while MP adsorbed less than 0.8% of the total of these elements. Results highlight that under these experimental conditions, virgin polyethylene MP may not be effective transport vectors for these trace metals. Important variations in K_d were observed between elements, inciting for further studies to decipher how chemical characteristics, MP composition, and potentially associated-biofilms, all interact in these biokinetic processes. These results demonstrate how radiotracers can allow us to address important knowledge gaps and broaden our understanding regarding the interactions between waterborne contaminants, naturally occurring particles and marine wildlife.

Introduction

Microplastic particles (MP, small particles of plastic < 5 mm in size) have been reported in all ecosystems on the planet (Rochman, 2018). Marine environments are considered to be the largest sinks of this pollution (Andrady, 2011; Cole et al., 2011), with MP accumulating in all oceanic compartments, from the surface to the deepest trenches (Thompson et al., 2004). Ingestion of MP, following inadvertent direct uptake or trophic transfer, has been reported in dozens of marine species, particularly in fish and benthic and planktonic invertebrates (Kühn et al., 2015). Evidence of the deleterious effects of MP ingestion on the biological functions and systems of these organisms are increasing (for a recent review, see Wang et al., 2019) and are often attributed to the toxicants carried and leached from MP (e.g. Browne et al., 2013). Indeed, MP often transport chemical pollutants, which were initially incorporated into plastics during manufacture (e.g. additives such as flame retardants, plasticizers and stabilizers, Koelmans et al., 2014), or which were initially waterborne and then sorbed from the surrounding aqueous environment (e.g. metals and organic pollutants, Holmes et al., 2012; Koelmans et al., 2013; Mato et al., 2001). However, the importance of MP as carriers for toxicants to marine organisms is a function of (i) the abundance of MP relative to other media such as suspended particles (e.g. sediment) and prey items, and (ii) the scavenging capability of the particles and the resulting partition coefficients (K_d) of waterborne chemical pollutants to MP in comparison to other media (Bakir et al., 2016; Koelmans et al., 2016). Since K_d are highly specific depending on the pollutant and plastic polymer characteristics (Bakir et al., 2014), and as this research is still in its infancy, our understanding of the sorption/desorption kinetics of chemical compounds to MP remains limited.

Radiotracing techniques provide unique tools for rapid, accurate, sensitive and reproducible measurements to determine the sorption/desorption kinetics of radio-labelled contaminants to MP (Johansen et al., 2018; Lanctôt et al., 2018). Here, we used these techniques to examine, under laboratory conditions, the partition coefficient (K_d) and the absorbance of three trace metal radioisotopes, ^{109}Cd , ^{134}Cs , and ^{65}Zn , to virgin polyethylene (PE) MP (size: 32-75 μm) and to natural sediment particles of a similar size (35-91 μm) in seawater and after three different exposure durations (4, 24 and 72 h). This case study therefore provides a direct comparison of trace elements scavenging ability between one of the most abundant types of MP (i.e. PE, de Haan et al., 2019; Enders et al., 2015; Gewert et al., 2017) and natural sediment particles.

Materials and Methods

Microplastic and sediment characteristics

Virgin polyethylene microplastics (PE MP) were purchased from Cospheric® (green fluorescent; density = 1.025 g cm⁻³; sizes = 32-38 µm, 38-45 µm, 45-53 µm, 53-63 µm and 63-75 µm; specific surface area = 167, 141, 119, 101 and 85 m² kg⁻¹, respectively). One gram of each of these size ranges were combined to obtain a homogeneous mixture of 5 g of 32-75 µm virgin PE MP with an average specific surface area of 123 m² kg⁻¹. Sediment was collected in Japan in May 2013 and characterized using Elementar® Analysensysteme GmbH - vario EL Element Analyzer and Mastersizer Micro from Malvern Instrument® (Carbon content = 1% (95% organic); sand = 61%; mud = 38% (34% silt and 4% clay)) and then stored at 4°C. This sediment was then rinsed repeatedly in osmotic water and manually sieved using 40 µm and 70 µm metallic sieves. The size of the sieved sediment particles was characterized using a particle size analyzer: Mastersizer Micro from Malvern Instrument® (average Dv(10) = 35 µm (n = 3); average Dv(90) = 91 µm (n = 3); average specific surface area = 195 m² kg⁻¹ (n = 3)). These sediment particles were then sterilized, dried, and stored again at 4°C.

Exposure of PE MP and sediment particles to 109Cd, 134Cs, and 65Zn radiotracers

Eighteen 100 mL glass bottles, containing 80 mL of UV-sterilized and 1 µm filtered seawater, were used to study the sorption dynamics of three radiotracers, 109Cd, 134Cs, and 65Zn, onto the PE MP and sediment particles described above. Nine bottles were inoculated with MP-PE and the nine others with the sediment particles. The targeted concentration of particles in each bottle was 103 particles mL⁻¹, which represents 66 mg mL⁻¹ for PE MP and 114 mg mL⁻¹ for the sediment. All bottles were then placed on an orbital shaker (200 rpm) for 5 min to obtain a homogeneous mixture of particles in suspension. All bottles were then removed from the orbital shaker and spiked with the 3 radiotracers, 109Cd, 134Cs, 65Zn, to reach a nominal concentration of 10 kBq L⁻¹ for each element. Solutions of radiotracers were added at microliter quantities to prevent any change in pH conditions (no change in pH was detectable after spiking the tracer). All bottles were then placed again on the orbital shaker (200 rpm) to maintain the homogeneous mix of particles in suspension during exposure to the radiotracers. During the entire duration of the experiment, the temperature was maintained at 22°C (room temperature and temperature of the water solution initially). High specific activity γ-radiotracers were used: 109CdCl₂ in 0.1M HCl (T_{1/2}: 464 d), 65ZnCl₂ in 0.5M HCl (T_{1/2}: 244 d), and 134CsCl₂ in 0.1M HCl (T_{1/2}: 754 d) purchased from Eckert & Ziegler®.

Sampling PE MP and sediment particles after exposure to ^{109}Cd , ^{134}Cs , and ^{65}Zn radiotracers five minutes after the spike of the radiotracers, 1 mL of each bottle was sampled (Water 1) to measure the initial activity in the solutions. Then, three bottles of each condition (i.e. PE MP vs. sediment particles) were sampled 4 h after the beginning of the exposure, three other bottles after 24 h, and the three last after 72 h. After sampling, the content of each bottle was filtered (vacuum filtration) on a 1 μm polycarbonate filter (Filter 1). A total of 10 mL of the filtrate seawater (Water 2) was then sampled, and the remaining filtrate seawater was filtered again on a 1 μm polycarbonate filter (Filter 2) to control for the radiotracers retained by the filter itself. Then, 10 mL of UV-sterilized and 1 μm filtered seawater were added to the bottle to collect the particles that potentially remained attached to the glass, before being filtered onto Filter 1. Potential unattached material was also removed with this rinsing. Filter 2 was also rinsed similarly with 10 mL of seawater to account for the potential loss of unattached radiotracers by this rinsing.

Measurements of ^{109}Cd , ^{134}Cs , and ^{65}Zn activity

Measurements were carried out using a γ -spectrometer system composed of 4 High purity Germanium - N or P type - detectors (EGNC 33–195-R, Canberra® and Eurysis®) connected to a multi-channel analyser, and information was treated using a computer equipped with a spectra analysis software (Interwinner 6, Intertechnique®). The Germanium detectors were calibrated with appropriate geometry to define the specific efficiency detection curves for each radionuclide. The activities of each element were measured in Water 1 (initial activity), Water 2 (activity in the water after exposure), Filter 1 (activity in the particles after exposure), and Filter 2 (activity retained in the filter). Each filter was weighted, rinsed with 10 mL of osmotic water, and weighted again before the experiment, and was dried prior to the measurements of activity, to permit the measurement of the weight of particles (PE MP or sediment) retained in the filter, and to allow the calculation of dried-particles activity in Bq g^{-1} . The activities measured in Filter 2 were subtracted from their associated Filter 1 to account for the activity retained by the filter itself.

Calculation of partition coefficients (K_d) and proportions of activity

K_d corresponds to the ratio between the activity of the radiotracers in the dry particles (in Bq g^{-1}) and the time-integrated activity of the radiotracers in the seawater (in Bq mL^{-1} , using the activities measure in Water 1 and Water 2). The proportion of activity corresponds to the ratio between the activity of the radiotracers in the dry particles and the sum of activities of the radiotracers in the seawater plus in the dry particles, at the time of sampling.

Statistics

Wilcoxon rank sum tests were conducted to examine whether the K_d values and proportions of activity were higher for sediment particles than for PE MP. All statistical analyses were conducted using R (R Core Team, 2019), and all data and detailed statistical results are available in Supplementary Data.

Results

The adsorption of ^{109}Cd was significantly higher (one order of magnitude) on sediment particles than on virgin PE MP for all durations, i.e. after 4, 24, and 72 h exposures (Fig. 1A). After 72 h of exposure, ^{109}Cd adsorption averaged at 175.17 ± 26.94 (mean \pm SE) Bq g^{-1} on sediment particles ($K_d = 18.10 \pm 3.14$ mL g^{-1}), while it averaged at 17.82 ± 14.68 Bq g^{-1} ($K_d = 1.70 \pm 1.41$ mL g^{-1}) on virgin PE MP (Fig. 1A). The same trend was observed for ^{134}Cs , whose adsorption was significantly higher (almost two orders of magnitude) on sediment particles than on virgin PE MP for all durations. After 72 h of exposure, ^{134}Cs adsorption averaged at 721.83 ± 36.40 Bq g^{-1} on sediment particles ($K_d = 96.20 \pm 3.97$ mL g^{-1}), while it averaged at 2.51 ± 0.77 Bq g^{-1} ($K_d = 0.20 \pm 0.04$ mL g^{-1}) on virgin PE MP (Fig. 1B). Again, the same trend was observed for ^{65}Zn , whose adsorption was significantly higher (almost three orders of magnitude) on sediment particles than on virgin PE MP for all durations. After 72 h of exposure, ^{65}Zn adsorption averaged at 2474.63 ± 116.27 Bq g^{-1} on sediment particles ($K_d = 2019.40 \pm 885.28$ mL g^{-1}), while it averaged at 41.16 ± 7.09 Bq g^{-1} ($K_d = 3.60 \pm 0.39$ mL g^{-1}) on virgin PE MP (Fig. 1C).

Although variations in the K_d values were observed between radiotracers, the proportion of activity was always significantly higher in sediment particles than in virgin PE MP, for all durations and for every element. After 72 h, sediment particles absorbed 2.5% of ^{109}Cd (Fig.

1D)), 68.0% of ^{134}Cs (Fig. 1E), and 71.0% of ^{65}Zn (Fig. 1F), while MP adsorbed less than 0.8% of total elements (Fig. 1D-F).

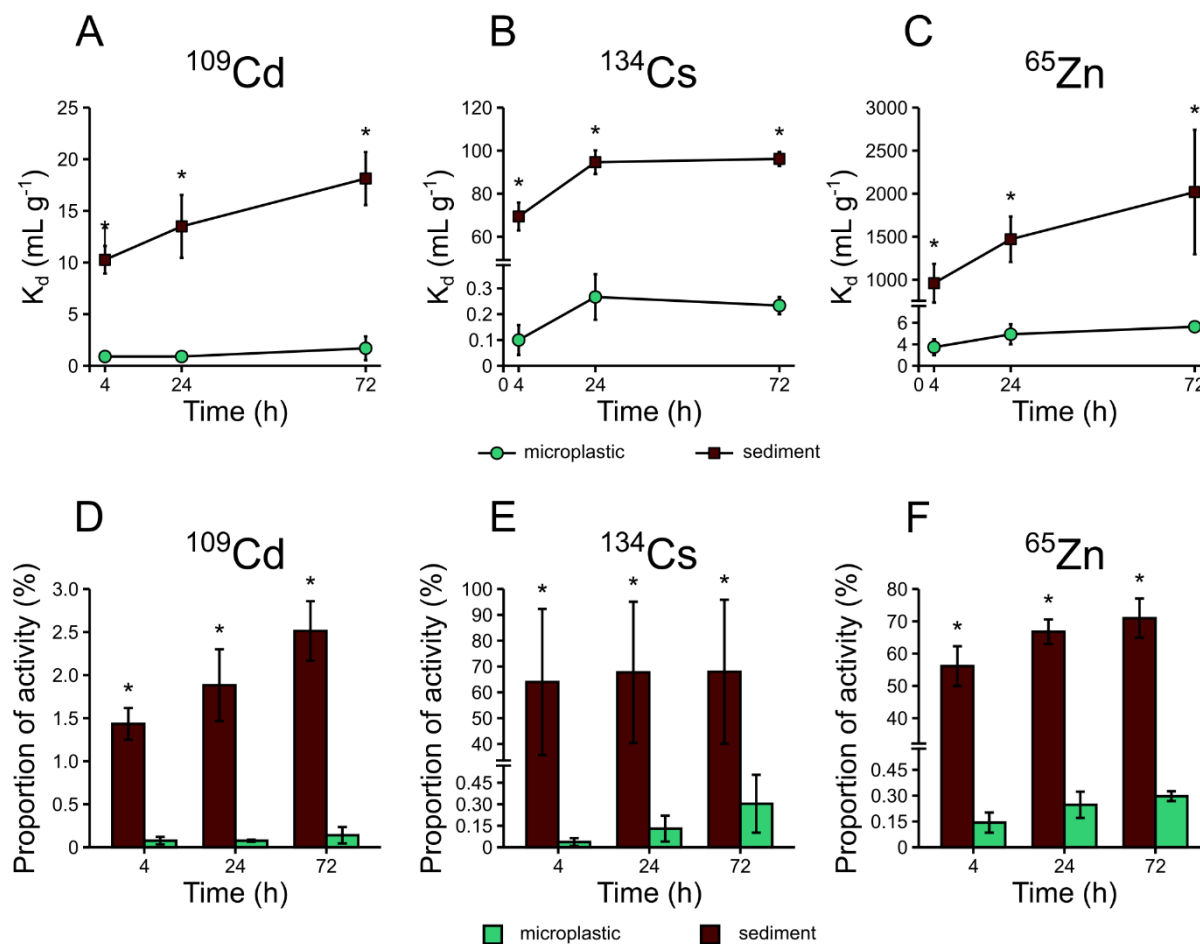


Figure 1: ^{109}Cd , ^{134}Cs and ^{65}Zn partition coefficients (K_d) and proportions of activity in polyethylene microplastics (PE MP) and sediment particles. (A) K_d of ^{109}Cd to PE MP and sediment particles. (B) K_d of ^{134}Cs to PE MP and sediment particles. (C) K_d of ^{65}Zn to PE MP and sediment particles. (D) Proportion of activity of ^{109}Cd in PE MP and sediment particles. (E) Proportion of activity of ^{134}Cs in PE MP and sediment particles. (F) Proportion of activity of ^{65}Zn in PE MP and sediment particles. All graphics indicate mean \pm SE with $n = 3$; * indicates P -value < 0.05 (all tests correspond to Wilcoxon rank sum tests, with $W = 9$ for all the comparisons between PE MP and sediment particles). Detailed statistics results are available in Supplementary Data.

Discussion

Whether MP could constitute a substantial source of toxic chemicals to marine organisms is currently a very active topic, given the growing volumes of MP observed in all marine ecosystems (Geyer et al., 2017). Our results for Cd (K_d ranging from 0.9 to 1.7 mL g⁻¹) are comparable with previous results on virgin PE MP measured in marine and freshwater after 7 days of exposure (Holmes et al., 2012; Turner and Holmes, 2015). Concerning Cs, our results (K_d ranging from 0.1 to 0.3 mL g⁻¹) are almost two orders of magnitude lower than the K_d measured in the only study that examined the adsorption of the radioisotope ¹³⁴Cs to PE MP (Johansen et al., 2018). This difference could be associated to the estuarine water used in this study, or to the fact that K_d were measured on fresh weight. This difference is also probably associated with the weathering and the biofilm associated with the PE MP used by Johansen et al. (2018). Indeed, aged MP generally absorb more metal contaminants than virgin MP because of processes such as biofilm generation, photo-oxidative weathering, and precipitation of hydrogenous chemicals that may enhance the adsorption of metal ions from the water column to the plastic surfaces (Holmes et al., 2012; Mato et al., 2001; Turner and Holmes, 2015). Numerous studies have highlighted that PE MP can contain high concentrations of Zn: from 0.3 to 23.3 µg g⁻¹ in PE MP recovered in the marine environment (Ashton et al., 2010; Holmes et al., 2012) to up to 604 µg g⁻¹ measured in recently manufactured PE plastics (Munier and Bendell, 2018). Concerning this element, our result (K_d ranging from 1.7 to 3.6 mL g⁻¹) is almost two orders of magnitude higher than reported by Turner and Holmes (2015), for virgin PE MP. However, these authors experienced highly variable recoveries for Zn (in particular among replicates) and these results should therefore be taken with caution. On virgin polystyrene microbeads, a K_d of 163 mL g⁻¹ (i.e. two orders of magnitude higher than our results on virgin PE MP) was previously recorded by Brennecke et al. (2016).

Reference values of K_d for Cd, Cs and Zn on sediment particles of various size and composition range, on average, from 102 to 105 mL g⁻¹ (IAEA, 2004), therefore one to six orders of magnitude higher than any of the K_d regarding MP measured in the studies cited above or in this study. However, most of these reference records were obtained from field data, and therefore tend to have higher apparent K_d due to dilution of the water component of the K_d equation (Periáñez et al., 2018). Indeed, the K_d values that we measured for the sediment particles are on average two orders of magnitude lower than these reference values (IAEA, 2004). As evoked by Johansen et al. (2018), it is then often misleading to compare K_d values for naturally occurring particles (e.g. sediment particles) obtained from field data with K_d

values for MP obtained under laboratory conditions. Moreover, K_d measurements regarding trace metals and MP appear highly variable across studies and exposure conditions, even for the similar polymer types, with up to two orders of magnitude in the examples cited above. We therefore suggest that direct comparisons, within the same experimental procedures, of the adsorption of chemical contaminants between MP and other naturally occurring particles are much needed.

Here, this case study reveals that the adsorption of Cd, Cs and Zn was higher in sediment particles than in virgin PE MP, with almost one to three orders of magnitude differences depending on the elements. These differences cannot be attributed to the higher specific surface area of the sediment particles vs. the PE MP that we used as it was only 1.5 times higher. Despite different raw K_d values, the relative difference between MP and sediment particles in K_d values for ^{134}Cs is comparable to the one observed by Johansen et al. (2018) for ^{137}Cs . Overall, the proportion of activity contained in virgin PE MP was less than 0.8% of the total of all elements after 72 h, while it ranged from 2.51 to 71.0% for each element in sediment particles. Knowing that transfer factors of Cd, Cs and Zn from sediment to marine organisms are generally quite low (e.g. Enuneku et al., 2018; Hédouin et al., 2010; Metian et al., 2016; Wang et al., 2016, but see also Hédouin et al., 2017), sediment particles are usually considered a minor uptake pathway for these contaminants in comparison with other media such as water and prey items. Given their lower absorbance of trace metals than sediment particles, MP may therefore constitute an even smaller uptake pathway for such contaminants, even in the case of weathered MP with associated-biofilms. In addition, the low abundance of MP relative to these other media present in the oceans further emphasize this argument. Nevertheless, the dramatic increase in oceanic plastic pollution (Geyer et al., 2017), which is expected to continue exponentially in the coming decades (Kaza et al., 2018), may impose us to regularly reconsider this assumption. In this context, radiotracer techniques offer unique opportunities to study the sorption/desorption kinetics of several waterborne pollutants to various types, sizes and shapes of MP with different weathering and biofilm characteristics, leading towards a better understanding of the importance of MP as vectors of co-contaminants to marine organisms.

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