Universidad de Oviedo
Universidá d'Uviéu
University of Oviedo

Departamento de Biología Funcional

Programa Oficial de Doctorado de Ingeniería Química, Ambiental y Bioalimentaria

Prevalencia e impacto de microplásticos en los componentes y especies relevantes de ecosistemas costeros.

Prevalence and impact of microplastics on the components and relevant species of coastal ecosystems.

Tesis Doctoral

Paula Masiá Lillo

Directoras: Eva García Vázquez y Alba Ardura Gutiérrez

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RESUMEN DEL CONTENIDO DE TESIS DOCTORAL

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2.- Autor	
Nombre: Paula Masiá Lillo	DNI/Pasaporte/NIE:
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RESUMEN (en español)

En la presente tesis doctoral se ha abordado la problemática de la contaminación plástica, y en especial por microplásticos, analizando el estado actual del ecosistema y algunas de las especies que lo habitan, y proponiendo recomendaciones y futuras líneas de investigación basándose en los resultados obtenidos para intentar disminuir este problema medioambiental.

Actualmente se producen más de 300 millones de toneladas de plástico anuales, y gran parte acaba en el océano. Éstos se rompen en pequeños trozos dando lugar a meso- y microplásticos (5-20mm y <5mm, respectivamente). Además, se producen directamente microesferas y fibras plásticas para distintos usos. Los plásticos y microplásticos entran en los océanos a través de los ríos, plantas de tratamiento de aguas, puertos, sector pesquero, turismo y otras fuentes.

En la costa asturiana se ha visto presencia de macroplásticos, mesoplásticos y microplásticos en todas las playas estudiadas. Los macro- y microplásticos se concentran en zonas próximas a puertos marítimos, y se relacionan con la actividad pesquera. Las playas que no disponen de servicio de limpieza están mucho más contaminadas, siendo por tanto este servicio fundamental para controlar los plásticos en origen. La concentración de microplásticos es más elevada en playas expuestas directamente al mar que en playas semi-cerradas o localizadas dentro de las rías, añadiendo una posible procedencia de aguas abiertas quizás relacionada con las actividades pesqueras.

Los plásticos, en general, y microplásticos, en particular, suponen un problema para las

especies marinas. En la presente tesis se ha comprobado la presencia de microplásticos en zonas costeras protegidas y en heces de especies con valor ecológico como son las aves migratorias, apareciendo en todas las muestras analizadas. En mejillón *Mytilus galloprovincialis* se encontraron microplásticos en todos los individuos estudiados, y se comprobó que contenían 10 veces más que en los sedimentos y en el agua de la zona de muestreo. Los microplásticos son vectores de contaminantes y contienen tóxicos presentes en el propio plástico; parte de los microplásticos encontrados en la presente tesis en mejillones son peligrosos para la salud humana debido a su composición.

En la presente tesis se ha estudiado experimentalmente el efecto de la exposición de *Mytilus galloprovincialis* a diferentes concentraciones de microesferas de poliestireno. A concentraciones elevadas disminuyó significativamente su condición física; y se evidenció un aumento de la degradación del ADN en las branquias a bajas concentraciones de poliestireno. Cuando la concentración es muy alta los mejillones reducirían su tasa de filtración, disminuyendo así la exposición directa de las branquias a este contaminante.

Evitar que los microplásticos acaben en los océanos es de vital importancia. En esta tesis se ha identificado como posible solución la utilización de organismos eucariotas como agentes de biorremediación, especialmente en plantas de tratamiento de aguas, las cuales son conocidas como una de las mayores fuentes de contaminación por microplásticos debido a su ineficiente retención. Distintas especies de algas y macrófitas parecen ser los organismos más indicados para este objetivo.

RESUMEN (en Inglés)

In the present PhD thesis, this problem has been studied by analysing the current state of the ecosystem and some of the species that we can found in the studied region. Recommendations and and future investigation lines have been proposed, based on the results obtained, in order to diminish this environmental problem.

Currently 300 million tonnes of plastic are produced every year, with a great part ending up in the oceans. Once in the ocean they break into small pieces called mesoplastics (5-20mm) and microplastics (<5mm). Moreover, microspheres and plastic fibres are directly manufactured for different uses. Plastics and microplastics enter the ocean though rivers, wastewater treatment plants, ports, fishing activities, and tourism, among others. Once in the ocean they can be transported by winds and currents.

In the asturian coast, the different types of plastics (macro-, meso- and microplastics) have been found in every beach studied. Microplastics an macroplastics are pooled nearby maritime ports and are also highly related with fishing activities, and beaches without cleaning services are more polluted, having therefore a fundamental role in controlling plastic pollution in origin. Microplastics concentration was higher in open beaches rather than semi-enclosed or estuarine beaches, adding a possible origin coming from the ocean, maybe related to fishing activities.

It is known that plastics and specially microplastics can suppose a problem for marine species. In the current thesis, microplastics have been found in protected coastal areas, and in faeces of migratory birds, species with high ecological value. Moreover, microplastic have been also found *Mytilus galloprovincialis*, in every individual analysed, being the amount found 10 times greater than the amount found in the sediments and water where they inhabit. Microplastics, due to their physicochemical properties, can act as vectors of contaminants found in the ocean, and contains chemicals in the plastic itself. In fact, some of the microplastics found in the specie studied in the current thesis, are considered hazardous for human health

Many studies are relating microplastics with cytotoxic, genotoxic, and neurotoxic problems among others, in marine organisms. In the present thesis, effects of exposure to different concentrations of polystyrene microspheres, on *M. galloprovincialis* have been studied. Under high, but ecologically realistic concentrations levels of microplastics, their physical status suffered a reduction. Moreover, DNA degradation of the gills increased when subdued to lower concentration levels of microplastics. These

results altogether suggest a possible mechanism of avoiding microplastics by reducing their filtration rates, and therefore, the direct exposition of the gills to these pollutants.

Overall, plastic and specially microplastic pollution is becoming a problem for the species inhabiting the studied region. To avoid microplastics entering the ocean is highly important. Therefore, the use of eukaryotic organisms for bioremediation has been also proposed as a possible solution for reducing microplastics entering the ocean, specially in wastewater treatment plants, as they are known to be hotspot of microplastic contamination. Different species of algae and macrophytes seem to be the best organisms for that aim.

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	SI	NO
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Acompaña memoria que incluye		
Introducción justificativa de la unidad temática y objetivos	x	
Copia completa de los trabajos *	X	
Resultados/discusión y conclusiones	X	
Informe con el factor de impacto de la publicaciones	X	
Se acompaña aceptación de todos y cada uno de los coautores a presentar el trabajo como tesis por compendio (Art. 32.4.b)	X	
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Factor de impacto	4.04

Coautor2 □ Doctora Indique nombre y apellidos	Eva García-Vázquez
Coautor3 □ Doctora Indique nombre y apellidos	Alba Ardura

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Factor de impacto	5.55

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Coautor10 □ Doctora Indique nombre y apellidos	Eva García-Vázquez

Trabajo, Artículo 3

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Coautor5 □ Doctor Indique nombre y apellidos	Fernando Rayon-Viña
Coautor6 □ Doctora Indique nombre y apellidos	Eva García-Vázquez

Trabajo, Artículo 4

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Coautor3 □ Doctora Indique nombre y apellidos	Alba Ardura

Trabajo, Artículo 5

Titulo (o título abreviado)	A threat from rock to fork: Microplastics in mussels, their environment and table salt
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Inclusión en Science Citation Index o bases relacionadas por la CNEAI (indíquese)	Q1
Factor de impacto	6.47

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Coautor3 □ Doctora Indique nombre y apellidos	Alba Ardura

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Tesis Doctoral

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Índice

Resumen/ Summary

Resumen

El conjunto de la presente tesis doctoral aborda la problemática de la contaminación plástica en ecosistemas marinos, con especial énfasis en la presencia de microplásticos en el hábitat costero asturiano y en los organismos que lo habitan. Conocer la situación actual, inferir el origen de los microplásticos y los problemas que pueden representar para algunas de estas especies, han sido los principales objetivos de la tesis.

Desde que comenzó la producción masiva de plástico a partir de 1950, ésta se ha incrementado exponencialmente llegando a producirse más de 300 millones de toneladas anuales, de los cuales gran parte acaba en los océanos. Estos macroplásticos pueden romperse en pequeños trozos de forma mecánica y/o química, dando lugar a meso- y microplásticos (5-20mm y <5mm, respectivamente). A esto se añaden las microesferas y fibras plásticas producidas para distintos usos, desde productos cosméticos y de higiene hasta materiales a nivel industrial, que entran en el océano a través de los ríos, plantas de tratamiento de aguas, puertos, sector pesquero, turismo y otras fuentes. Los microplásticos son transportados por las corrientes hasta acumularse en giros oceánicos o depositarse en el fondo marino.

En este trabajo se ha visto que los macro-, meso- y microplásticos están presentes en todas las playas asturianas estudiadas, con una concentración medio-alta de microplásticos en relación con otras costas europeas. Los macro- y microplásticos se concentran en zonas próximas a puertos marítimos, y se relacionan con la actividad pesquera; mientras que la concentración de mesoplásticos está directamente relacionada con la cantidad de macroplásticos, pudiendo producirse por su ruptura *in situ*. Las playas que no disponen de servicio de limpieza están mucho más contaminadas, siendo este servicio fundamental para controlar la contaminación en origen. La concentración de microplásticos es más elevada en playas abiertas, expuestas directamente al mar, que en playas semi-cerradas o localizadas dentro de las rías, apuntando a las actividades pesqueras y redistribución desde aguas abiertas como fuentes de contaminación.

Los plásticos, en general, y microplásticos, en particular, suponen un problema para las especies marinas. En la presente tesis se ha comprobado la presencia de microplásticos en zonas costeras protegidas y en heces de aves migratorias, de alto valor ecológico, apareciendo en todas las muestras analizadas. En mejillón *Mytilus galloprovincialis* se encontraron microplásticos en todos los individuos estudiados, en concentraciones diez veces superiores a las de sedimentos y agua de la zona de muestreo. Esta biomagnificación supone un riesgo para las especies marinas que ingieren mejillones, y también para los consumidores humanos a través de la dieta. Además, los microplásticos son vectores de otros contaminantes y su propia composición puede ser tóxica. En los microplásticos encontrados en mejillones analizados en la presente tesis se han hallado compuestos peligrosos para la salud humana, entre otros el acetaldehído, compuesto cancerígeno, y anilinas, compuestos irritantes.

Muchos estudios empiezan a relacionar los microplásticos (con otras sustancias adheridos o sin ellas) con problemas citotóxicos, genotóxicos, físicos y neurotóxicos en organismos marinos. En esta Tesis se ha estudiado experimentalmente el efecto de la exposición de *Mytilus galloprovincialis* a diferentes concentraciones de microesferas de poliestireno. A concentraciones elevadas, medioambientalmente realistas, disminuyó significativamente su condición física. Además, se evidenció un aumento de la degradación del ADN en las branquias a bajas concentraciones. En altas concentraciones los mejillones reducirían su tasa de filtración, protegiendo así las branquias de la exposición directa a este contaminante.

Viendo que la contaminación por plásticos, y especialmente por microplásticos, en la costa asturiana está suponiendo un problema para las especies marinas, evitar que los microplásticos acaben en el mar es de vital importancia. En esta tesis se ha explorado la utilización de organismos eucariotas como agentes de biorremediación en plantas de tratamiento de aguas, conocidas como una importante fuente de contaminación debido a su ineficiente retención de microplásticos por su pequeño tamaño. Distintas especies de algas y macrófitas serían los organismos más indicados para este objetivo.

Como la protección de los ecosistemas marinos y las especies que en ellos habitan es de vital importancia dada la actual situación de cambio medioambiental global, basándose en los resultados obtenidos en la presente tesis doctoral se proponen recomendaciones y futuras líneas de investigación para reducir este problema de salud medioambiental.

Summary

The overall PhD thesis encompass marine plastic pollution problems in coastal habitats and organisms of the asturian coasts. To know the current situation, infer the origin of microplastics, and understand the problems that can represent for these species have been the aims pursued.

Since the beginning of the massive plastic production in 1950, this production has been exponentially increasing, reaching 300 million tonnes of plastic production every year. A great part of this amount ends up in the oceans, polluting marine ecosystems worldwide. Due to mechanical and chemical actions, plastic in the oceans tend to break into small pieces called mesoplastics (5-20mm) and microplastics (<5mm). Moreover, microspheres and plastic fibres are directly manufactured for different uses, from cosmetic and health care products to industrial use. Plastics and microplastics enter the ocean though rivers, wastewater treatment plants, ports, fishing activities, and tourism, among others. Once in the ocean they can be transported by winds and currents, accumulating in marine ecosystems, especially in oceanic gyres or sea bottom.

In the asturian coast, the different types of plastics (macro-, meso- and microplastics) have been found in every beach studied in this thesis. Microplastic concentration is medium-high in comparison with other European beaches. Microplastics an macroplastics are pooled nearby maritime ports and are also highly related with fishing activities. Mesoplastics concentration is directly related with macroplastic, suggesting that this type of plastic can be produced by in situ breakage of bigger plastics. In addition, beaches lacking cleaning services are more polluted, having therefore a fundamental role in controlling plastic pollution in origin. Microplastics concentration was higher in open beaches rather than semi-enclosed or estuarine beaches, suggesting fishing activities and open sea input as sources of contamination.

It is known that plastics and specially microplastics can suppose a problem for marine species. In the current thesis, microplastics have been found in protected coastal areas, and in faeces of migratory birds, species with high ecological value. Moreover, microplastic have been also found in *Mytilus galloprovincialis*, in every individual analysed, being the amount found 10 times greater than the amount found in the sediments and water where they inhabit. This evidence of biomagnification represents a risk for the marine species that feed on mussels, as well as for human consumers though our diet. Microplastics, due to their physicochemical properties, can act as vectors of contaminants found in the ocean, and because of their composition can be toxic themselves. In fact, some of the microplastics found in the specie studied in the current thesis, are considered hazardous for human health, such as aldehyde, with cancerogenic properties; and aniline, which is irritant.

Many studies are relating microplastics, with or without toxic compound adhered, with cytotoxic, genotoxic, and neurotoxic problems, among others, in marine organisms. In the present thesis, effects of exposure to different concentrations of polystyrene microspheres, on *M. galloprovincialis* have been studied. Under high, but ecologically realistic concentrations levels of microplastics, their physical status suffered a reduction. Moreover, DNA degradation of the gills increased when subdued to lower concentration levels of microplastics. These results altogether suggest a possible mechanism of avoiding microplastics by reducing their filtration rates, and therefore, the direct exposition of the gills to these pollutants.

Overall, plastic and specially microplastic pollution is becoming a problem for the species inhabiting the studied region. To avoid microplastics entering the ocean is highly important. Therefore, in the present thesis, the use of eukaryotic organisms for bioremediation has been proposed as a possible solution for reducing microplastics entering the ocean in wastewater treatment plants, as they are known to be hotspot of microplastic contamination. Different species of algae and macrophytes seem to be the best organisms for that aim.

To sum up, to protect marine ecosystems and the species that inhabit, is crucial due to the global environmental situation that we are currently facing. In the present thesis, recommendations and future investigation lines have been proposed, based on the results obtained, in order to diminish this environmental problem.

Introducción

1. La Edad del Plástico: estado actual de la basura marina global

Uno de los mayores problemas medioambientales a los que nos enfrentamos hoy en día es la contaminación por plástico. Los estudios sobre de este contaminante en el océano comenzaron en 1960 (Bergmann et al., 2015), y desde entonces se ha convertido en un foco de atención principal. No sin razón, hemos entrado en una era que se empieza a denominar "Edad del Plástico" (Bergmann et al., 2015), ya que este material que comenzó a producirse a gran escala a partir de los años 1950 ha alcanzado una producción de 368 millones de toneladas anuales en 2019 (Plastics-Europe2020 https://www.plasticseurope.org/es/resources/publications/4803-plasticos-situacion-en-2020) siendo Europa el responsable del 20% de la producción total (Bonanno & Orlando-Bonaca 2018); si bien durante el año 2020 ha disminuido debido a los efectos de la pandemia del coronavirus (Plastics-Europe2020) (Figura 1). Un tercio del plástico mundial se destina a productos de un solo uso, el cual tiene una vida media en nuestros hogares mínima en comparación con los años que tardan en degradarse (Jambeck et al., 2015). Debido a su ubicuidad por todo el planeta, se ha propuesto la utilización del plástico como indicador estratigráfico del Antropoceno, acuñando el término de "tecnofósiles" (Zalasiewicz et al., 2016).

El éxito de este material se debe a sus propiedades químicas y físicas que lo hacen ligero, muy resistente y de bajo coste, y le dan una amplia versatilidad (Laist, 1987); son estas mismas características las que lo hacen tan persistente en los ecosistemas (Vegter et al., 2014). De todo el plástico producido mundialmente, el 40% está destinado a embalaje (con una gran proporción dedicada a la alimentación), seguido de los sectores de la construcción, la electrónica, la industria automotriz y la industria textil; sectores en los que la demanda sigue creciendo actualmente (Andrady & Neal 2009; UNEP 2016).

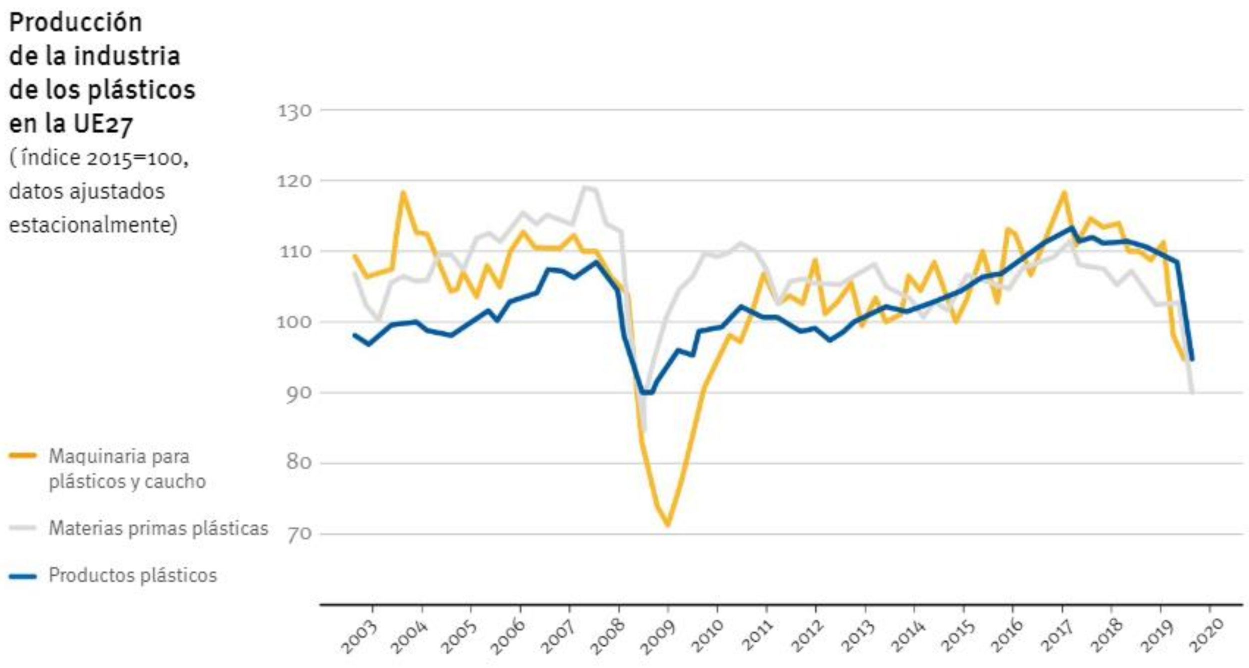


Figura 1: Tendencia de la producción de plástico en Europa. Fuente: "Plastics-the fact 2020" (www.plasticseurope.org).

El término "plástico" se utiliza para denominar a una amplia gama de polímeros sintéticos, divididos en dos grupos: termoplásticos y termoestables. Los primeros tienen la capacidad de moldearse cuando se someten a altas temperaturas, pudiendo volver a fundirse, y son por tanto adecuados para el reciclaje. Los segundos sólo pueden fundirse y moldearse en el momento de su fabricación, haciendo imposible su reutilización. Ejemplos de polímeros termoplásticos son el polietileno, polipropileno, poliestireno, policloruro de vinilo y tereftalato de polietileno; el poliuretano y las resinas son ejemplos de termoestables (UNEP, 2016).

Una gran cantidad del plástico mundial producido acaba en los océanos. Aunque la cantidad de basura plástica marina es aún desconocida, se estimó que en 2010 acabaron en el mar de 4,8 a 12,7 millones de toneladas, cifra que se espera que incremente con el crecimiento económico y de la población mundial (Jambeck et al., 2015). Se estima que hay más de 5 trillones de partículas plásticas en el océano, que constituyen el 80% de toda la basura marina (Thompson et al., 2009; Eriksen et al., 2014)

Las fuentes de contaminación por este tipo de basura son muy diversas y se agrupan según su origen terrestre como vertederos, ríos y turismo costero, entre otras; o marino, como acuicultura y actividades pesqueras y marítimas (Andrady & Neal 2009; Andrady, 2011; Cózar et al., 2014; Gourmelon 2015; Bonnano & Orlando-Bonaca, 2018). Las embarcaciones son una de las mayores fuentes de contaminación, siendo responsables al menos del 25% de la basura marina, pese a que la legislación vigente de la Unión Europea prohíbe arrojar basura desde el barco según la Directiva MARPOL73/78 (*The International Convention for the Prevention of Pollution from Ships*) (Butt, 2007; Riley et al., 2019). El plástico que acaba en el océano tiende a acumularse principalmente en el fondo marino (Angiolillo, 2019) y en giros oceánicos, donde forma las denominadas islas de plástico (Law et al., 2010; Eriksen et al., 2013).

Hoy la basura marina es un contaminante omnipresente que causa numerosos problemas. Uno de los principales es el daño directo que causa a las especies marinas, ya que cerca de 700 especies, incluidas aves, se ven afectadas por la basura plástica (Gall & Thompson, 2015), tanto por enredarse en redes de pesca abandonadas (*ghost nets*) (Figura 2) como por la ingestión de plásticos, ocasionando pérdidas en la biodiversidad marina (Moore, 2008). A nivel de ecosistema, el plástico que se queda atrapado en el fondo del océano inhibe el intercambio de gases, interfiriendo en la captación de CO_2; y el que queda acumulado en superficie destruye las zonas de cría en ecosistemas costeros (Moore, 2008). Además, los plásticos desempeñan un papel importante como vectores de dispersión de especies marinas, provocando un daño al introducir especies no nativas que pueden convertirse en invasoras alterando la biodiversidad autóctona (Rech et al., 2016). Se sabe que más de 380 taxones diferentes viajan unidos a estos plásticos (Bergmann et al., 2015), especialmente organismos sésiles (Gregory, 2009); se estima que la basura plástica es más eficaz en el transporte de especies que las incrustaciones en los cascos de los barcos o el agua de lastre (Moore, 2008).

Además de las consecuencias medioambientales, el plástico también puede tener consecuencias socioeconómicas, ya que numerosos sectores, especialmente el sector turístico y la industria pesquera, se ven afectados por este problema que impacta negativamente en los visitantes de las playas y deteriora los aparejos de pesca (Mouat et al., 2010; Jang et al., 2014; Newman et al., 2015).

Figura 2: A la izquierda, imagen de distintas especies enredadas en redes fantasma (Foto: Martin Stelfox); a la derecha, cachalote muerto por ingestión de plástico (Foto: SEA Me Sardinia). Fuente de ambas imágenes: National Geographic (www.nationalgeographic.com.es).

2. Microplásticos

a. Definición y origen

Uno de los grandes problemas asociados a la contaminación por plásticos en el océano es la formación de microplásticos, los cuales están causando una preocupación creciente entre la comunidad científica debido a su gran impacto sobre los organismos marinos (Guzzeti et al., 2018). Se denominan microplásticos a aquellos plásticos con tamaños menores de 5 milímetros, y se dividen en primarios y secundarios dependiendo de su origen. Los microplásticos secundarios son producto de la ruptura de plásticos más grandes, principalmente por acción de la radiación solar, las altas temperaturas y las mareas (Arthur et al., 2009). Los microplásticos primarios son aquellos que se producen directamente de este tamaño para utilizarlos en productos industriales o cosméticos, como limpiadores y pastas de dientes, y entran en los ecosistemas por escapes durante su fabricación, transporte o uso (Arthur et al., 2009; Duis & Coors, 2016).

Según su forma, los microplásticos se pueden clasificar en fibras, pellets, o fragmentos plásticos (Arthur et al., 2009) (Figura 3). El 90% de los microplásticos que se encuentran tanto en los ecosistemas como en el interior de los organismos marinos son fibras, que provienen principalmente de productos textiles y de redes de pesca (Browne et al., 2011). Las vías por las que estas fibras, y los microplásticos en general, entran en el océano son muy variadas. Una de las fuentes más importantes es a través de las plantas de tratamiento de aguas residuales, cuya falta de eficacia en la retención de estas micropartículas ha sido ampliamente demostrada (Sun et al., 2019). Los vertederos, las áreas urbanas y la agricultura también son grandes fuentes de contaminación de microplásticos, que acaban en el mar a través de ríos y escorrentías (Welden & Lusher, 2020). También los vientos y corrientes aéreas transportan estos microplásticos suspendidos en la atmósfera y terminan depositándolos en el mar (Enyoh et al., 2019).

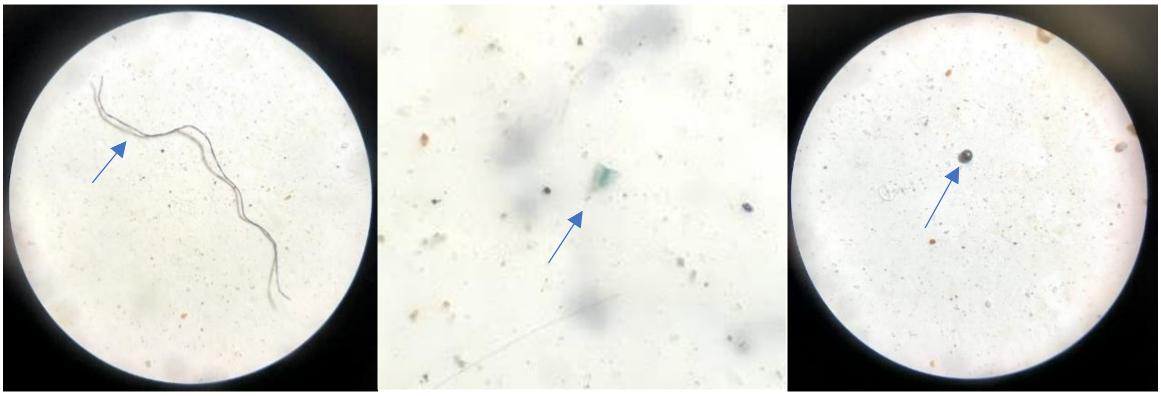


Figura 3: Imágenes de los distintos tipos de microplásticos encontrados en la presente tesis, clasificados según su forma. De izquierda a derecha: Fibra, pieza de plástico y pellet. Fuente: Paula Masiá.

b. Microplásticos en los ecosistemas marinos

Los primeros datos científicos sobre microplásticos en los océanos se recogieron en 1971 (Carpenter & Smith, 1972), y desde entonces el número de trabajos sobre estos contaminantes en distintos ecosistemas ha crecido exponencialmente hasta el día de hoy (Zhang et al., 2020). La presencia de microplásticos es ubicua en todos los ecosistemas, se encuentra desde los polos al ecuador (Shahul Hamid et al., 2018), en el fondo marino (Van Cauwenberghe et al., 2013), en la nieve de los Alpes (Bergmann et al., 2019), y en ecosistemas prístinos y remotos como corales (Huang et al., 2020) o la Antártida (Munari et al., 2017). Los microplásticos llegan a estos territorios debido a corrientes marinas, vientos y ríos. Su distribución vertical en la columna de agua depende de la acción de las olas, del viento, de la mezcla por turbulencia e incluso de los movimientos de los barcos (Welden & Lusher, 2020), y su tasa de acumulación en sedimentos es de cuatro órdenes de magnitud mayor que en el agua (Bonnano & Orlando-Bonaca, 2018).

Aunque existen numerosos estudios acerca de la cantidad de microplásticos de todo el mundo, tanto en agua como en sedimentos, la cantidad total que hay en el océano y las costas aún se desconoce. La falta de estandarización en las unidades de medida y los métodos de cuantificación hace difícil las comparaciones entre estudios (Besley et al., 2017). En un estudio realizado en playas de 13 países de Europa se estimó que la cantidad de microplásticos variaba entre 72 ± 24 a 1512 ± 187 microplásticos por kilo de sedimento (Lots et al., 2017). Incluso en áreas más restringidas, como el Golfo de Vizcaya, la cantidad de microplásticos en aguas superficiales varía enormemente, entre 0,00098 a 0,3 ítems por metro cúbico (Lusher, 2015). Pese a la incertidumbre debido a su variación, los datos apuntan a un nivel muy elevado de contaminación.

Según un informe de 2017 de la UICN (Boucher & Friot, 2017), la aportación a los océanos tan solo por microplásticos primarios es de 0.8 a 2.5 millones de toneladas al año, principalmente procedente de textiles sintéticos, ruedas de coche, y contaminación atmosférica proveniente de las ciudades. Por regiones, en Asia, África y Oriente medio, las fibras textiles son la fuente primaria de microplásticos, siendo los mayores contaminantes de este tipo de microplásticos a nivel mundial (Figura 4).

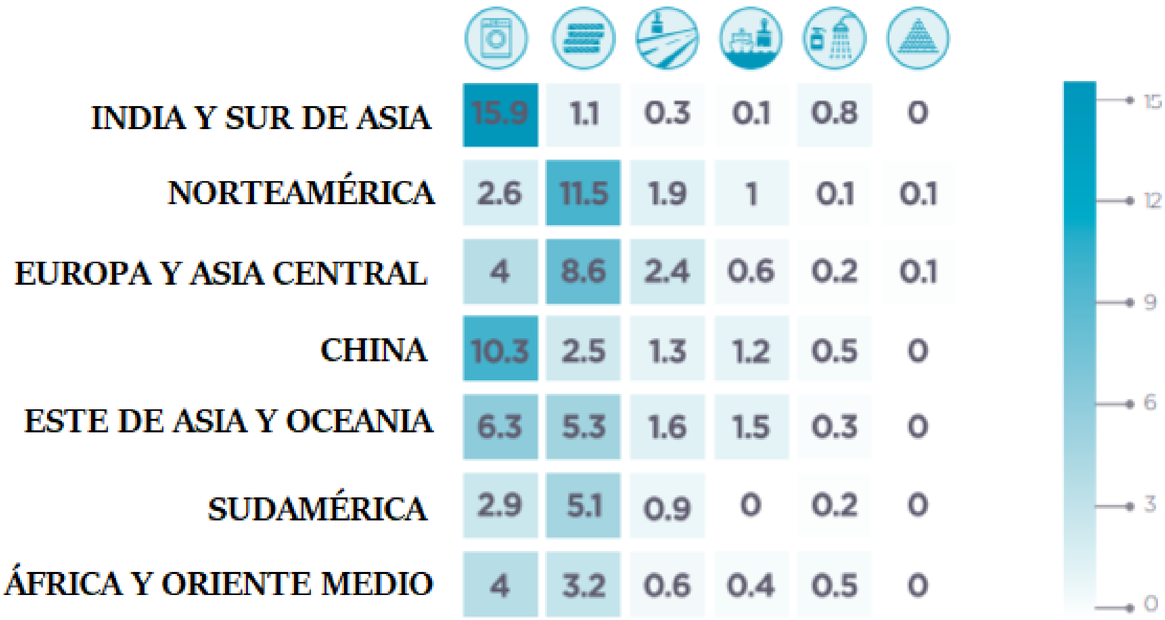


Figura 4: Porcentaje (en % de emisiones globales) de microplásticos liberados a los océanos a nivel global. Fuente: UICN (Boucher & Friot, 2017). Simbología de izquierda a derecha: Fibras textiles sintéticas, neumáticos, pinturas de carretera, revestimiento de barcos, productos personales y pellets de plástico.

c. Microplásticos como vectores de transporte

Además del transporte de especies no indígenas que pueden llegar a convertirse en invasoras con su consecuente daño al ecosistema (Moore, 2008; Gregory, 2009; Bergmann et al., 2015; Rech et al., 2016), los plásticos pueden actuar como vectores de transporte de diferentes productos químicos (Caruso, 2019). Durante su manufactura, se les añaden numerosos químicos como plastificantes, policlorobifenilos (PCBs), ftalatos o bisfenol A para darles las características que los hacen tan útiles (Tabla 1). Se ha visto que estos químicos añadidos pueden ser liberados a lo largo de su ciclo de vida, pudiendo afectar a numerosas especies debido a su toxicidad (Hamlin et al., 2015). La mayoría de estos compuestos tienen propiedades cancerígenas, de disrupción endocrina, o mutagénicos (Tabla 1) (Meyer-Rochow et al., 2015). Además, una vez en el medio ambiente estos plásticos son capaces de adsorber compuestos químicos que se encuentran en el agua, como herbicidas o pesticidas, antibióticos, y especialmente contaminantes orgánicos persistentes (POPs) (Yamashita et al., 2018). Dependiendo del tipo de plástico se han detectado distintos niveles de toxicidad debido a sus diferentes propiedades físicas, ya que los plásticos con superficie hidrofóbica tienen mayor facilidad para absorber este tipo de compuestos químicos (Hirai et al., 2011), siendo por tanto más peligrosos para la biota marina. La tasa de adsorción de compuestos químicos por parte de los microplásticos es mucho más rápida que la de otros compuestos que se encuentran en el océano, como materia particulada, lo que les hace excelentes vectores de transporte de químicos (Rochman, 2015).

Según su composición química, el polietileno, poliestireno, polipropileno y cloruro de polivinilo son los microplásticos más abundantes en ecosistemas y organismos marinos, ya que proceden de los plásticos más comúnmente utilizados, como bolsas de plástico y embalajes (Geyer et al., 2017; De Sá et al., 2018). Debido a su pequeño tamaño, los microplásticos son ingeridos por multitud de especies marinas, facilitando así la introducción de todos los compuestos químicos adheridos y su liberación dentro de los organismos. Dependiendo de su composición química el plástico tiene diferente probabilidad de ser ingerido por diferentes especies, ya que los plásticos con poca densidad, y por tanto mayor flotabilidad, alcanzarán un mayor número de especies filtradoras y planctónicas; sin embargo, los de mayor densidad tenderán a acumularse en el fondo, contaminando hábitats bentónicos y pelágicos, y alcanzando especies detritívoras (Browne et al., 2007). Una vez en el interior de los organismos, las sustancias químicas adheridas al plástico se liberan con distintas tasas de desorción, que pueden ser hasta 30 veces mayores en el interior de los organismos que en el medio ambiente (Yamashita et al., 2018).

Tabla 1: Lista de polímeros más usados con sus aditivos plásticos, su función y sus efectos sobre la salud humana. Los aditivos son comunes a todos los polímeros que aparecen en la tabla. Tabla adaptada de Hermabessiere et al. (2017).

Polímeros	Aditivos	Función	Efectos
Polipropileno (PP) **Poliestireno (PS)** **Polietileno de alta densidad (HDPE)** **Polietileno de baja densidad (LDPE)** **Policloruro de vinilo (PVC)** **Poliuretanos (PUR)**	Ignífugo bromado (BFR)	Ignífugo	Disruptor endocrino
	Ftalatos	Plastificante especialmente en PVC	Disruptor endocrino
	Nonilfenol	Antioxidante y plastificante en algunos plásticos	Disruptor endocrino, afecta a la fertilidad, quemaduras, toxico para la vida acuática
	Bisfenol A (BPA)	Monómero en policarbonatos y resinas epoxy	Disruptor endocrino, afecta a la fertilidad, irritación de piel y respiratoria, Similar al estrógeno
		Antioxidante en plásticos	
	Octilfenol	Antioxidante en plásticos	Disruptor endocrino

d. Impacto biológico de los microplásticos

Debido a su ubicuidad y pequeño tamaño, los microplásticos son ingeridos por numerosos organismos marinos (Botterell et al., 2019). Las especies en las cuales se ha observado la ingestión de microplásticos abarcan toda la cadena trófica, desde especies planctónicas, a corales, bivalvos, poliquetos, equinodermos, holoturoideos, peces, reptiles, pájaros y mamíferos, entre otros (Hall et al., 2015; Lusher et al., 2017; Miller et al., 2020). Los microplásticos pueden quedar retenidos en el organismo por un tiempo indeterminado (dependiendo del organismo), facilitando su paso a través de la cadena trófica y llegando incluso a grandes depredadores (Nelms et al., 2018), y a especies de interés comercial (Figura 5). La eficiencia de ingestión por las distintas especies marinas depende del tamaño del microplástico, de su densidad (flotabilidad), forma y color. Algunos organismos son capaces de distinguir los microplásticos de las partículas orgánicas, mientras que otros, debido a la similitud del microplástico con las partículas que normalmente ingieren, no son capaces de diferenciarlos (Franzellitti et al., 2019). Los organismos filtradores tienen mayores tasas de ingestión de microplásticos debido a su comportamiento alimenticio (Paul-Pont et al., 2018). Éstos extraen los microplásticos que se encuentran suspendidos en el agua, incorporándolos en su organismo y expulsándolos después a través de las heces, acelerando el transporte vertical en la columna de agua, y por tanto facilitando su acumulación en sedimentos. Los detritívoros que se encuentran en dichos sedimentos ingieren parte de éstos microplásticos (Piarulli & Airoldi, 2020) y la otra parte facilitan su resuspensión en la columna de agua, dejándolos de nuevo disponibles para otras especies (Bulleri et al., 2021) (Figura 5).

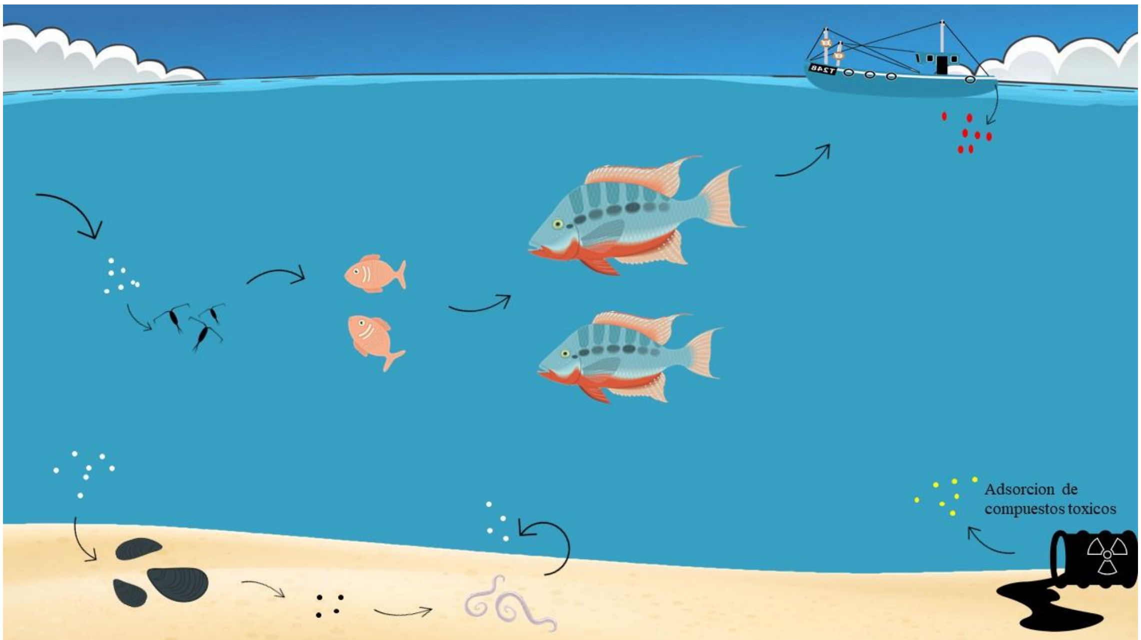
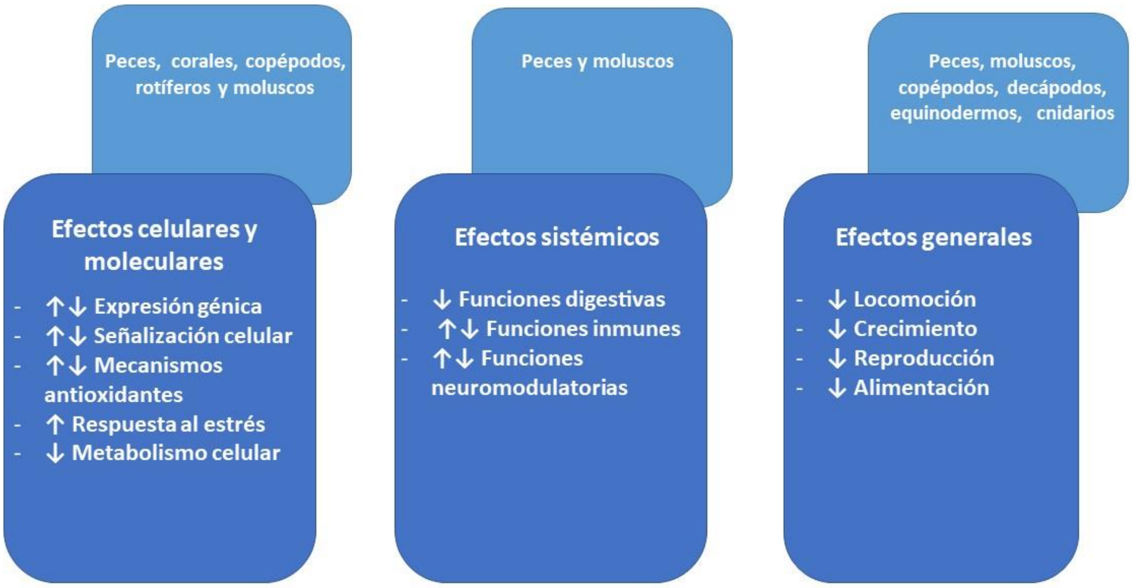


Figura 5: Esquema de las formas de transporte de los microplásticos y sus interacciones biológicas. Fuente: Paula Masiá.

Cada vez son más son los estudios realizados en condiciones de laboratorio para conocer los efectos causados por los microplásticos sobre los organismos, que varían en función del tipo, tamaño, concentración de microplástico y especie (de Sá et al., 2018). La ingestión de microplásticos puede producir daño físico debido a abrasiones internas y obstrucciones (Wright et al., 2013). En moluscos, por ejemplo, se ha visto que se empeora la forma física del organismo, ya que se reduce la formación del biso y el estatus nutricional (Yap et al., 2020). En casos extremos la obstrucción por ingestión de microplásticos puede dar lugar a efectos letales por inanición al crear falsa sensación de saciedad (Hamm & Lenz, 2021). En algunas especies planctónicas se producen cambios en la capacidad natatoria, en hábitos alimenticios, actividad enzimática y aumento del estrés oxidativo celular (Cole et al., 2015; Gambardella et al., 2017). En peces, causan problemas reproductivos, desordenes metabólicos, procesos inflamatorios, neurotoxicidad, cambios en los hábitos alimenticios y peroxidación de lípidos, entre otros (Barboza et al., 2018; Zhao et al., 2020; He et al., 2021; Rios-Fuster et al., 2021; Sheng et al., 2021). Se ha visto incluso que puede incrementar la acumulación de metales pesados como el mercurio en músculo y cerebro (Barboza et al., 2018). Además, pueden tener efecto a nivel genómico. Por ejemplo, en larvas de peces (*Dario renio*) la exposición a microplásticos induce cambios en la expresión génica a corto plazo (LeMoine et al., 2018), y en mejillones y lombrices se ha visto que la ingestión de microplásticos puede producir daños a nivel de ADN y apoptosis en células (Micic et a., 2002; Jiang et al., 2020). La Figura 6 resume los principales efectos observados en condiciones de laboratorio causados por la presencia y/o ingesta de microplásticos en diferentes organismos.

Figura 6: Resumen de los principales efectos que los microplásticos tienen sobre los organismos que los ingieren (Imagen adaptada de Franzellitti et al., 2019).



Muchas de las especies marinas afectadas por la presencia de microplásticos son especies comerciales y de consumo humano, por lo que lo que sus efectos pueden trasladarse al sector económico viéndose afectadas la pesca y la acuicultura, así como finalmente la salud humana. Aunque las consecuencias que podría tener el consumo de microplásticos por humanos aún no se conocen, se sabe que la exposición continuada de las personas a microplásticos atmosféricos produce problemas de asma y daño oxidativo en pulmones (Chen et al., 2020). Por tanto, el consumo de microplásticos a través de la ingesta de productos contaminados podría ser también un problema para la salud humana (Smith et al., 2018).

e. Eliminación de microplásticos en plantas de tratamiento de aguas residuales.

Otras de las grandes fuentes de microplásticos en el océano son las plantas de tratamiento de aguas residuales (Murphy et al., 2016). Con el fin de proteger el medio ambiente y la salud humana, el Parlamento Europeo presentó en 2019 la propuesta TA/2019/0071 para mejorar el estado de estas plantas de tratamiento mediante el monitoreo de microplásticos en el agua ya tratada (European Parliament, 2019). Y es que, aunque las plantas de tratamiento son capaces de retirar grandes cantidades de microplásticos (Carr et al., 2016), siguen siendo grandes focos de contaminación (Li et al., 2018; Sun et al., 2019). Por ejemplo, en Alemania se calculó que se pueden encontrar entre $1x10^3$ a $24x10^3$ microplásticos por kilogramo de lodo (Lassen et al., 2012); y en un estudio llevado a cabo en 28 plantas de tratamiento en China, se calculó que a través del lodo entran anualmente en el medio ambiente una media de 1.56×10^{14} microplásticos (Li et al., 2018). El mayor problema con los lodos derivados de las plantas de tratamiento es que se utilizan como fertilizante en los cultivos, y por tanto estos microplásticos retenidos acaban directamente en el medio ambiente, suponiendo un riesgo para la sostenibilidad, así como para la seguridad alimentaria. Se estima que en Europa acaban directamente en los cultivos entre 125 y 850 toneladas de microplásticos por millón de habitantes (Nizzetto et al., 2016). Esto demuestra la necesidad de mejorar las formas de tratamiento de los residuos para evitar que los microplásticos acaben en el océano.

En los últimos años se han propuesto varias alternativas, como por ejemplo la digestión anaeróbica (Mahon et al., 2017), o la eliminación de plásticos mediante biodegradación (Paço et al., 2017). Otra alternativa plausible que ya se utiliza para eliminar otros contaminantes como metales pesados (Dixit et al., 2015) es la biorremediación, que ofrece la posibilidad de eliminar estos compuestos a través de actividad biológica natural. Es un campo que se debería explorar para los microplásticos.

3. Área de estudio: Costa asturiana

La costa asturiana está localizada en el norte de la Península Ibérica, en el suroeste del Golfo de Vizcaya, donde el factor geomorfológico más importante es el Cabo Peñas situado en el centro. La corriente más importante, la Corriente del Atlántico Norte, va paralela a la costa en dirección este, la cual está fuertemente influenciada por el viento (Domínguez-Cuesta et al., 2019). Además, la costa asturiana también se ve influenciada durante los meses de invierno por la denominada Corriente Ibérica hacia el Polo (deCastro et al., 2011), también en dirección éste. La morfología de la costa (334 km) está determinada por acantilados, playas rocosas y algunas de arena, con una densa red fluvial: cinco cuencas principales y numerosos ríos menores (Cotilla-Rodríguez et al. 2005). Además, tiene múltiples irregularidades batimétricas que generan fluctuaciones en las corrientes y el viento, lo que hace que zonas próximas de la misma costa se vean afectadas diferencialmente (Domínguez-Cuesta et al., 2019).

La costa asturiana está influenciada por distintas actividades antrópicas, con mayor incidencia en la zona central donde se encuentran las ciudades costeras más importantes, Avilés y Gijón. Avilés, situada al oeste del Cabo Peñas, es la ciudad con mayor actividad industrial, además de un importante puerto comercial y pesquero. Mientras que Gijón, al este del Cabo Peñas, es la ciudad con mayor población de la región, tiene una alta influencia de turismo durante todo el año, y alberga el puerto comercial más importante del Cantábrico central y dos puertos recreativos. Respecto al resto de la costa, hay numerosos puertos pesqueros y recreativos pequeños, actividades de acuicultura en las rías del Eo y Villaviciosa, y afluencia de turismo en verano, con mayor incidencia en las últimas décadas (Mendoza et al., 2020).
Cabe destacar la presencia de numerosos espacios incluidos dentro de la red Natura 2000, muchas de ellos declaradas zonas de especial protección para las aves (ZEPA), patrimonio natural, monumento natural y/o paisaje protegido a lo largo de la costa asturiana (https://www.miteco.gob.es/es/biodiversidad/temas/espacios-protegidos/red-natura-2000/zepa_asturias.aspx).

En la presente tesis, se ha realizado por primera vez en la costa asturiana un estudio cuantitativo y cualitativo de microplásticos en elementos del hábitat (agua y sedimento) y varias especies. Las especies objeto de estudio fueron aves migratorias, registrando el contenido en heces, y el mejillón mediterráneo *(Mytilus galloprovincialis*). Esta especie es importante a nivel comercial y de amplio consumo humano. Se han analizado en poblaciones naturales, y en condiciones de laboratorio para investigar posibles daños a nivel de ADN.

Objetivos/ Objectives

Objetivos

Objetivo 1:

Cuantificar la concentración de macro, meso y microplásticos en playas de la costa asturiana e inferir las principales fuentes de contaminación.

Objetivo 2:

Determinar la concentración de microplásticos en espacios costeros protegidos de Asturias, y su posible transferencia hacia aves migratorias, como ejemplo de especies relevantes para la conservación.

Objetivo 3:

Determinar la relación entre los microplásticos presentes en el medio y su concentración en el mejillón *Mytilus galloprovincialis*, así como sus posibles implicaciones para el consumidor.

Objetivo 4

Determinar experimentalmente los daños a nivel fisiológico y de ADN causados por microplásticos en el mejillón *Mytilus galloprovincialis* como especie modelo.

Objetivo 5

Revisar la eficacia actual de las plantas de tratamientos de agua para la retención de microplásticos y explorar la posible utilidad de organismos eucariotas para su biorremediación.

Objectives

Objective 1:

To quantify the concentration of macro-, meso- and microplastics of different beaches of the asturian coast, and to determine the main sources of plastic pollution in the studied area.

Objective 2:

To determine the concentration of microplastics in protected coastal systems in Asturias, and the possible transfer to migratory birds, as an example of important species for conservation

Objective 3:

To determine the relationship between the concentration of microplastics present in abiotic environments and in the mussel *Mytilus galloprovincialis*, and their possible implications for human consumption.

Objective 4:

To determine the physiological effects and the DNA damage caused by microplastics in the mussel *Mytilus galloprovincialis*, used as model organism.

Objective 5:

To study, through literature review, the current microplastics removal efficiency in wastewater treatment plants, and to explore the use of eukaryotic organisms for bioremediation.

Material y Métodos

La metodología utilizada en la presente tesis está detallada en la sección de Material y Métodos de cada uno de los capítulos correspondientes. Brevemente, las diferentes técnicas utilizadas en cada capítulo son las siguientes:

- Cuantificación de macroplásticos y mesoplásticos en playas: Capítulo 1

Recolección de basura plástica a lo largo de tres transectos aleatorios de un metro de ancho, perpendiculares a la línea de marea, empezando en marea baja. Posterior clasificación por tamaños.

- Extracción de microplásticos de arena: Capítulos 1, 2 y 3

Recolección de cuatro muestras de 50 gramos de arena de forma aleatoria, de cinco cuadrantes situados a lo largo de un transecto de 100 metros paralelo a la línea de marea. Las muestras se secan y se separan de los microplásticos por densidad mediante una solución salina. Después, las muestras se filtran mediante una bomba de vacío, quedando los microplásticos en un filtro de 0,45 µm para su posterior cuantificación y determinación.

- Extracción de microplásticos de muestras biológicas: Capítulos 2 y 3

Las muestras biológicas se digieren con peróxido de hidrógeno al 30%, que se añade en distintas cantidades según la muestra o peso del organismo, y después se filtran mediante la bomba de filtrado al vacío con filtros de 0,45 µm.

- Extracción de microplásticos de agua: Capítulo 3

Recolección de 5 litros de agua recogidos cerca de la costa, que se llevan al laboratorio y se filtran directamente mediante la bomba al vacío con filtros de 0,45 µm.

- Extracción de microplásticos en sal: Capítulo 3

Para cada muestra, 125 gr de sal se disuelven en agua destilada prefiltrada, se agitan hasta disolverse, y se filtran con filtros de 0,45 µm

- Cuantificación y clasificación de microplásticos: Capítulos 1, 2 y 3

Los filtros se visualizan bajo un estereomicroscopio, y se contabilizan los ítems encontrados. Se separan por colores y formas. Mediante la técnica de espectroscopía infrarroja FT-IR (Fourier Transform Infrared) se determina la composición química de una submuestra de microplásticos representativa de las clases encontradas de formas y colores.

- Exposición de organismos a diferentes dosis de microplásticos: Capítulo 4

Se recogieron individuos adultos salvajes de la especie *Mytilus galloprovincialis* y se llevaron directamente al laboratorio, donde se repartieron en cuatro grupos: un control y tres grupos expuestos a tres concentraciones diferentes de microesferas de poliestireno durante 21 días.

- Determinación del índice de condición: Capítulo 4

Se utilizó el índice de condición (= peso de tejido blando/ peso total) para determinar el estatus nutricional de los individuos.

- Determinación de la integridad del ADN: Capítulo 4

Las branquias se extrajeron de los individuos al llegar al laboratorio. Se extrajo el ADN mediante un kit de extracción de ADN, se cuantificó mediante espectrofotometría, y se cuantificaron los daños visualizando la integridad de ADN mediante electroforesis en un gel de agarosa.

- Análisis de datos: Capítulos 1-4

Los análisis estadísticos se llevaron a cabo utilizando el programa PAST (Hammer et al., 2001).

Resultados

Resultados

Capítulo 1

Masiá, P., Ardura, A., Gaitán, M., Gerber, S., Rayon-Viña, F., & Garcia-Vazquez, E. (2021). Maritime ports and beach management as sources of coastal macro-, meso-, and microplastic pollution. Environmental Science and Pollution Research, 1-10, https://doi.org/10.1007/s11356-021-12821-0

Capítulo 2

Masiá, P., Ardura, A., & Garcia-Vazquez, E. (2019). Microplastics in special protected areas for migratory birds in the Bay of Biscay. Marine Pollution Bulletin, 146, 993-1001. https://doi.org/10.1016/j.marpolbul.2019.07.065

Capítulo 3

Masiá, P., Ardura, A., & Garcia-Vazquez, E. (2021). A threat from rock to fork: Microplastics in mussels, their environment, and table salt. Food Research International, en revisión.

Capítulo 4

Masiá, P., Ardura, A., & Garcia-Vazquez, E. (2021). Effects of virgin polystyrene microparticles in DNA degradation and physical condition in the mediterranean mussel Mytilus galloprovincialis. Implications for conservation. Animals, 11, 2317. https://doi.org/10.3390/ani11082317

Capítulo 5

Masiá, P., Sol, D., Ardura, A., Laca, A., Borrell, Y. J., Dopico, E., ... & Garcia-Vazquez, E. (2020). Bioremediation as a promising strategy for microplastics removal in wastewater treatment plants. Marine Pollution Bulletin, 156, 111252. https://doi.org/10.1016/j.marpolbul.2020.111252

Capítulo 1

Maritime ports and beach management as sources of coastal macro-, meso-, and microplastic pollution

Universidad de Oviedo
Universidá d'Uviéu
University of Oviedo

Masiá P, Ardura A, Gaitán M, Gerber S, Rayon-Viña F, & Garcia-Vazquez E.

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Maritime ports and beach management as sources of coastal macro-, meso-, and microplastic pollution

Paula Masia, Alba Ardura, Mario Gaitán, Steve Gerber, Fernando Rayón-Viña, & Eva Garcia-Vazquez*

Department of Functional Biology, Faculty of Medicine, University of Oviedo. C/ Julian Claveria s/n 33006-Oviedo, Spain.

ABSTRACT

Plastic pollution is a major environmental issue that affects coasts all around the world. Many studies point out the importance of a better management of this type of pollution. In this pioneering study, we have analyzed the distribution of macro-, meso-, and microplastics along the coast of Asturias (southwest Bay of Biscay, Spain). Significant correlation analysis suggests that mesoplastics are produced in situ by fragmentation of macroplastics. Differences between groups of beaches revealed the importance of maritime ports and fishing activities as sources of macroplastics and likely of microplastics as well. Another source of microplastics could be atmospheric deposition, especially for fibers. Multiple regression model allowed to confirm the utility of beach services like cleaning and trash bins to control macroplastics. These results emphasize the need of an integral treatment of marine plastic pollution involving fishers and maritime ports, as well as the importance of providing beach services.

Keywords Beach services. Marine litter. Mesoplastics. Microplastics. Ports. Wastewater treatment plant

1. INTRODUCTION

The amount of plastic produced during the last decades has reached more than 250 million tonnes per year, and continues increasing gradually (Jambeck et al. 2015). This inexpensive and durable material is the major component of marine litter worldwide (Derraik 2002). Countless amounts of plastics end up in the oceans, accumulating in different marine habitats (Thompson et al. 2004) and oceanic gyres (Moore et al. 2001), from remote beaches to tropical seabeds to Antarctica (Barnes et al. 2009, 2010). First reports of plastic occurrence in the ocean appeared in the 1970s (Bergmann et al. 2015), and the issue has been in the focus of an increasing number of studies since then.

Plastic degradation is a slow process. Mechanical and physical processes tend to break plastics into smaller pieces (Thompson et al. 2004). Plastic fragments have been classified by size into different categories: microplastics (< 5 mm), mesoplastics (5–20 mm), and macroplastics (> 20 mm) (Gago et al. 2015). However, this classification may be flex- ible as pieces of up to 25 mm have been classified as mesoplastics in other studies (e.g., Collignon et al. 2014; Jabeen et al. 2017 and references therein). Depending on the size, plastics harm marine species in different ways. Larger plastics harm marine animals by entanglement (like ghost fishing nets) and ingestion, causing drowning, injury, starvation, and death (Gregory 2009). Microplastics (< 5 mm) are able to harm more species due to their small size, acting as vectors of contaminants such as POPs (persistent organic pollutants), herbicides, or antibiotics, and causing neurotoxicity, physical malformations, and genotoxicity, among many others (De Sá et al. 2018). Indeed, plastic pollution impacts human health too. In the synthesis of plastics, additives such as plasticizers, UV stabilizers, and urethane foam are employed, which can be released during plastics' lifecycle (Bergmann et al. 2015). When plastics break down—and also with the intact object—dangerous toxic substances like nonylphenol, triclosan, and bisphenol A enter into the environment (Gatidou et al. 2010); as an example of the risk they pose to health, bisphenol A is related to cancer development in humans (Birkett and Lester 2003).

Plastic litter can also harm biodiversity acting as a vector of invasive species (Gregory 2009). It is estimated that 387 taxa are travelling on floating litter (Bergmann et al. 2015), sessile organisms being the most common species that colonize pelagic plastics (Gregory 2009). Aquaculture and fishing activities have been reported as sources of invasive species due to floating plastic objects such as ADLFG (abandoned, lost, or otherwise discarded fishing gear) that carry attached organisms into the ocean (Rech et al. 2018).

Indeed, plastics have negative socio-economic impacts. Many noxious effects of marine plastic debris on multiple sectors have been reported (Mouat et al. 2010; Newman et al. 2015). Tourism is one of the most affected sectors. A decrease in the number of tourists, daily activities, and overnight stays has been reported in relation to episodes of plastics debris increases at different beaches (McIlgorm et al. 2011; Jang et al. 2014). Shipping and yachting activities are also affected, as litter has to be removed from harbours and ports (Bergmann et al. 2015); millions of dollars in clean-ups, transportation, and disposal of marine litter are spent every year. Plastics have a direct impact on fishing, as waste can get trapped in the propeller, engines, or fishing nets, reducing the amount of catches and increasing fishing effort (Nash 1992; Mouat et al. 2010). Plastic pollution encompasses reduction in revenues, job losses, and damages in ships and yachts (ten Brink et al. 2016). Since marine animals are affected by plastic ingestion and toxins released from microplastics as explained above, the quality of fish and the harvestable catch decrease in zones affected by plastic pollution. The same problems affect the aqua- culture industry too (Bergmann et al. 2015).

The deposition of plastics in different ecosystems depends on currents, wind, and anthropogenic pressure (Barnes et al. 2009). Plastic pollution sources are diverse, as they come from landfills (Gourmelon 2015), aquaculture and fishing activities (Chen et al. 2018; Cózar et al. 2014), litter produced from tourism (Andrady and Neal 2009), and others. Ships and vessels are known hotspots of marine litter (Chen and Liu 2013). Although throwing away plastics from ships is prohibited by international treaties such as MARPOL 73/78, around 20% of marine litter is estimated to come still from shipping activities (Riley et al. 2019). However, the role of ports as sources of microplastics is less clear. In Shandong (China), Zhou et al. (2018) showed that beaches nearby ports had lower quantity of microplastics than more distant beaches. Masiá et al. (2019) suggested that microplastics arrive at beaches transported by currents and tides from the open ocean, while other authors reported clear evidence of a terrestrial origin of microplastics that accumulate in river plumes and estuaries (Peng et al. 2017; Zhao et al. 2015) and near big cities (Lebreton et al. 2017). Other important sources of microplastics are wastewater treatment plants (e.g., Murphy et al. 2016; Prata 2018), where 100% of microplastics are not retained with the methodology currently employed (see a review in Masiá et al. 2020). Finally, the origin of mesoplastics is still unclear, since they are not a standardized class of marine litter; they could be simply the product of in situ fragmentation of bigger objects.

In this study, different types of marine litter (macrolitter, meso- and microplastics) were quantified from beaches located at different distances from maritime ports. Results were statistically analyzed in order to infer the role of ports as sources of the different types of marine plastics. Other stressors related to marine litter like beach services and distance to wastewater treatment plants were also considered. The southwest Bay of Biscay coast was chosen as a case study

2. MATERIALS AND METHODS

2.1. Sampling area

This study was done between January and May 2019, at eleven sandy beaches along the coast of Asturias, southwest Bay of Biscay (Northwest Spain). All of them are part of Natura 2000 network, specifically SPA (Special Protection Areas for rare or vulnerable birds). Their protection status can be consulted online on the web platform of the Spanish Ministry of Ecological Transition (https://www.miteco.gob. es/es/biodiversidad/temas/espaciosprotegidos/red-natura- 2000/zepa.aspx, accessed on May 2020). Arnao (Ar-E), Peñarronda (Pe-E), Zeluán (Ze-E), Xagó (Xa-E), Rodiles (Ro-E), and El Puntal (EP-E) are situated in/or near estuaries(E) with commercial ports (Eo, Avilés, and Villaviciosa estu- aries). Otur (Ot-F), Vega (Ve-F), and Aguilar (Ag-F) are open beaches relatively close to fishing ports (F), while Cobijeru (Co-S) and Gulpiyuri (Gu-S) are sheltered (S) beaches distant from any commercial port. Geomorphological factors have to be considered when talking about litter distribution. The greater amount of plastic litter can be found in enclosed seas and water convergences, and the lesser in seabed and ocean bottoms (Galgani et al. 2015). In Asturias coast, where this study has been carried out, the main geomorphological feature is Peñas Cape, situated in the middle of the coastline, and the main component in the coastal drift goes eastward (Dominguez-Cuesta et al. 2019). The region is located within the so-called Wet and Green Spain. The climate is typically oceanic without dry season and with temperate summers, with average annual temperature and precipitation values around 13.2–14.2 °C and 997–1122 mm year−1. The coast is geologically young, indented, with north-faced high rugged cliffs, a few beaches and many coves, and a dense fluvial network short and steeply sloped (Cotilla Rodriguez et al. 2005). Bathymetric irregularities and other accidents in the irregular coastline profile such as capes and coves generate great fluctuations in the action of wind and waves, storm impacts concentrating along cape and shallow areas and affecting coves differentially (Dominguez- Cuesta et al. 2019). There are five main rivers and many smaller rivers and streams that flow directly into the sea (Dominguez-Cuesta et al. 2019), summing a total of 34 main watersheds of first to fifth orders along the 334 km of Asturias coast, averaging roughly one per 10 km (Cotilla Rodriguez et al. 2005).

The following environmental stressors, potential sources of MP, were taken into account: distance to the closest wastewater treatment plant, WWTP (expectedly, the closer the more pollution; a map with the location of water treatment plants can be found on https://consorcioaa.com/saneamiento/, accessed in November 2020); distance to the closest river (the closer the more pollution); distance to the closest port (the closer the more pollution; all except two of the 18 fishing ports of this region are located in small villages); fishing activity in the closest port, measured from catch tonnes during the last 5 years (the more activity the more pollution; catch statistics is available online at https:// tematico.asturias.es/dgpesca/din/estalonj.php, accessed in November 2020); number of beach services including cleanings, trash bins, civic amenities, surveillance, safeguards etc., as a proxy of beach management scoring 1 to 9 (the more services, the lesser pollution)

2.2. Macroplastic and mesoplastic sampling

Litter was sampled following the protocol of Rayon-Viña et al. (2018), based on three random transects of 1-m width per beach, traced perpendicularly starting from the water line during the low tide, to the highest tide line, i.e., above which plants start growing. Transects had different lengths as every beach had different widths and shapes. All the plastic objects and fragments of plastic material bigger than 0.5 cm inside transects were collected. They were categorized by size as mesoplastics (0.5–2.5 cm following Collignon et al. 2014, Jabeen et al. 2017), and macroplastics (>2.5 cm). The variable employed was the number of items of each category per 100 m2, i.e., item density by surface for each size category.

2.3. Microplastic sampling and extraction protocol

A protocol adapted from Besley et al. (2017) was used, taking four samples of 50 g of sand randomly from five quadrants (0.5 × 0.5 m), along 100 m in parallel to the tide line during low tide, per beach. According to Besley et al. (2017), microplastics can be found regardless of the distance to the tide line. Here samples were collected from the upper half of the tidal range (at different distances depending on the beach width), crossing the transects employed to sample mesoplastics and macroplastics. Plastic fragments smaller than 5 mm were considered (Arthur et al. 2009), including fibers, pellets, and pieces of plastics. To avoid contamination, samples were stored in glass bottles. A total of 220 samples were brought to the laboratory and dried overnight in the oven at 65 °C. Microplastics were separated from sand by density using 200 mL of a hypersaline solution (358.9 g/L NaCl), stirred, and then filtered through 0.45-μm pore size polyethersulphone membranes (47 mm diameter; Supor® PES Membrane filters), using a vacuum pump. We worked under controlled conditions to prevent airborne contamination. Samples, solutions, and water were covered; all the material and the benches were carefully washed; and a laminar flow cabinet was employed when manipulating the samples. Blank controls of distilled water were employed and filters were stored in petri dishes. Microplastics were visualized under a stereomicroscope (× 40 magnification) and counted directly from the filter as explained in Masiá et al. (2019), separating fibers, pellets, and plastic fragments. The blanks contained very few microplastics: an average of 0.67 (SD 0.32) black, blue, or white fibers; thus, airborne contamination in the laboratory could be considered very low.

The variable employed for microplastic analysis was the number of microplastic items per kg of sand. Therefore, the overall value of the different beaches (4 samples of 50 g of sand in each of the five quadrants, 1 kg of sand in total) was the value used for the different statistical analyses.

Visual analysis of fibers is difficult and can be associated with uncertainties. Thus, to check the reliability of our visual classification, i.e., whether they were synthetic or natural, 10 fibers were randomly taken for their analysis by Fourier- transform infrared spectroscopy (FTIR- Varian 620-IR and Varian 670-IR).

2.4. Data analysis

Principal component analysis (PCA) was carried out to visualize the influence of different environmental variables on beached plastics. Correlation option was employed. Pairwise correlation matrix was performed in order to explore the association between the different categories of plastics and the environmental stressors considered, using Kendall's τ coefficient and significance threshold of $p < 0.05$. When multiple variables were correlated to each other, multiple regression model was run to identify significant partial correlations, i.e., independent variables explaining the variation of the dependent variable after controlling the rest. Besides correlational analysis, beaches were grouped according to the level of stress expected for each stressor (or potential plastics source), as low or high potential stress caused by the following: fishing activity in the nearest port (< or > 500 annual catch tons, because the biggest gap in the regional port catch rank was between 330.5 tons in Cudillero port, close to Aguilar beach, and 629.6 in Llanes port close to Gulpiyuri beach);

beach services (more or less than 4, being 4.5 half of the beach services in the region); distance to rivers, ports, and WWTP categorized as < or >3 km. The threshold of 3 km was arbitrarily chosen taking into account the coast roughness and indentations that make sheltered beaches and coves relatively isolated from each other, and the relatively high density of stressors: coastal rivers (average of one river mouth per 10 km of coast), fishing ports (18 in the studied coast), and WWTP (16 on the coastline or estuaries). For the three stressors, 3 km is the shortest distance between a pair in this particular coastline (Uncín - Esqueiro rivers; Candás - Luanco ports; Cudillero - Bajo Nalón WWTPs). Groups of beaches were compared regarding their plastic pollution using Kruskal-Wallis tests, after finding lack of homoscedasticity with Breusch-Pagan tests. ANOVA was employed to compare microplastic pollution among beaches, using the variable number of microplastics per samples of sand.

Statistics was performed using PAST free software v.2.17 (Hammer et al. 2001).

3. RESULTS

3.1. Plastic litter distribution

Plastics found along the different beaches did not exhibit the same profile. Microplastics occurred in all the beaches with no exception. Raw numbers of microplastics in individual sand samples are presented in Supplementary Table 1. Despite relatively large spatial variation within some beaches like Zeluán and Xagó, with means of 14.6 and 18.7 microplastics per sand sample (0.3 and 0.37 microplastics/g respectively) and the same standard deviation of 9.35, the largest in the dataset (Supplementary Table 1), the variation among beaches was much larger than the variation between samples within beach and the difference among beaches was highly significant (Table 1). Most of the microplastic items found were microfibers (97% of the total); this must be considered when interpreting the results of microplastics. Nine of the fibers analyzed were artificial (polyvinyl chloride, rayon, polyethylene, polyester, polystyrene) and one was of cellulose.As shown in Fig. 1, microplastics were the only type of plastic litter found in Ve-F and Ar-E. Mesoplastics were not found in Ar-E, Ve-F, and Co-S. They were more abundant in Ag-F and Gu-S, while Ze-E and Gu-S were the most macroplastic-pollute beaches (Fig. 1). The plastic litter found in these beaches had different compositions, being polystyrene fragments, plastic bags, and ALDFG (abandoned, lost, or discarded fishing gear) the most abundant ones (Supplementary Table 2).

3.2. Plastic litter and environmental features

Principal component analysis (PCA) helped to visualize underlying patterns of the relationships among variables and sampling sites (Fig. 2). The beaches associated with estuaries, except from Zeluán (Ze-E), appeared on quad- rants 3 and 4 of the scatter plot El Puntal (EP-E) and Rodiles (Ro-E) in a quadrant together with Xagó (Xa-E) (Fig. 2). For the variables and factors, many beaches were influenced by more than one factor, and some are located together because of some factors.

Table 1: Analysis of variance of the number of microplastics per sand sample at the eleven beaches examined

Source of variation	Sum of sqrs	Df	Mean square	F	*p*(same)
Between beaches	6155.83	10	615.58	17.15	1.98×10^{-22}
Between samples within beach	7357.65	205	35.89		
Total	13,513.5	215			

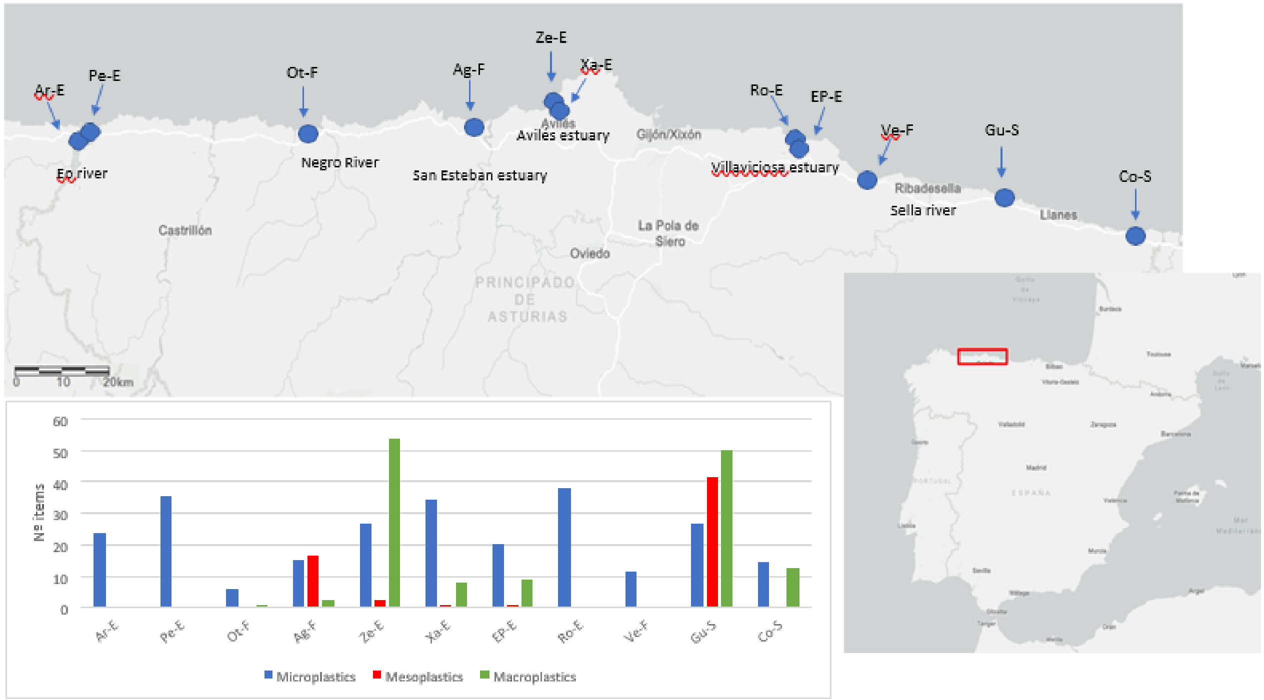

Figure 1: Plastic litter found in the 11 beaches analyzed. Units are microplastics per kg, mesoplastics and macroplastics per 100 m2. Sampling points in the graph are correlative to their position along the coast, being Ar-E (Arnao) the westernmost beach, and Co-S (Cobijeru) the easternmost one. Green figures correspond to the closest wastewater treatment plant. Letters E, F, and S next to the names correspond to Estuarine beach, Fishing port beach, and Sheltered beach, respectively.Sampling sites from left to right: Arnao (Ar-E), Peñarronda (Pe-E), Otur(Ot-F), Aguilar (Ag-F), Zeluan (Ze-E), Xagó (Xa-E), El Puntal (EP-E),Rodiles (Ro-E), Vega (Ve-F), Gulpiyuri (Gu-S), and Cobijeru (Co-S)

The diagonal representing microplastics was together with the factor fishing activity in the quadrant of Xa-E, Ro-E, and EP-E. On the other hand, meso- and macroplastics pointed to Gulpiyuri (Gu-S), which is consistent with the abundance of these plastics in the beach as shown in Fig. 1, and also to Zeluan (Ze-E), which was the beach with higher abundance of macroplastics (Fig. 1). Beach services and distance to WWTP were opposite to mesoplastics and macroplastics, and the distances to river and port were in the same quadrant of the relatively cleaner beaches Cobijeru (Co-S), and Otur (Ot-F). The stressors Port, River, and WWTP are measured from their distance to the sampling point; thus, the sampling points close to the diagonals representing these stressors are in fact far from them. The pattern visualized in the PCA showed that beaches of the same type were located more or less together in the scatter plot (F beaches in the lower part, E beaches in quadrants 3 and 4, and S beaches in the right part of the PCA).

Furthermore, the different types of plastics seemed to be associated with the different types of beaches, at least microplastics with E beaches and mesoplastics with S ones. Exploratory correlation analysis between the three types of plastics (Table 2) showed that macroplastic and mesoplastic densities were significantly and positively correlated ($p = 0.015$, significant after Bonferroni correction), which means that the higher the abundance of macroplastics, the higher the abundance of mesoplastics, while no significant correlation between microplastics and plastics of the other two size categories was found. Regarding the correlations between each type of plastics and the considered stressors, after Bonferroni correction, macroplastics were significantly correlated only with the number of beach services ($p = 0.003$).

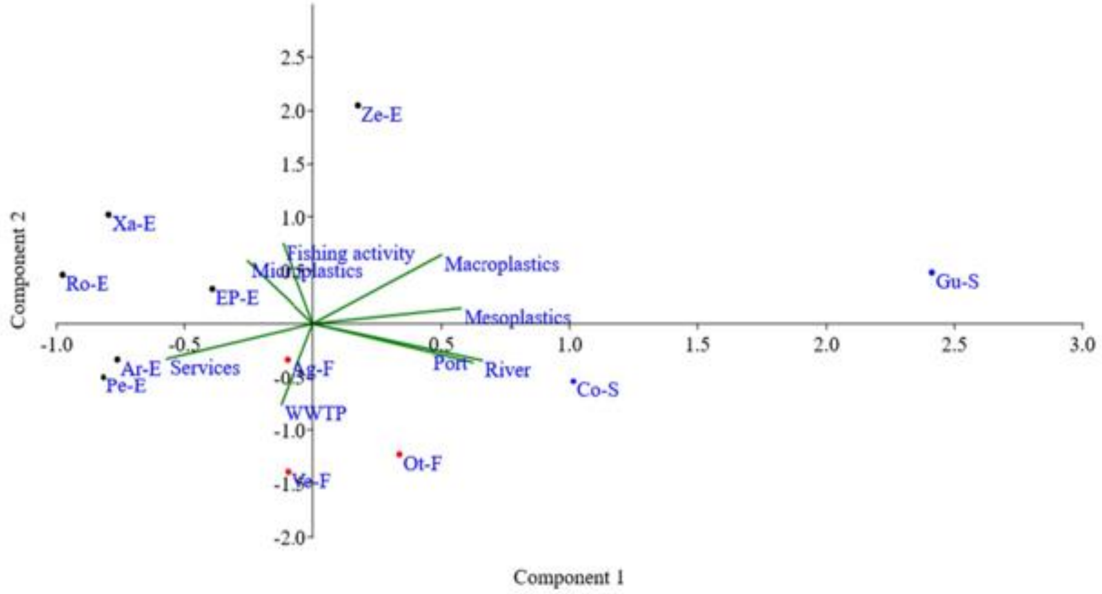

Figure. 2 PCA plot showing the correlation between the different sampling points and the variables considered. Letters E, F, and S next to the names correspond to Estuarine beach (black dot), Fishing port beach (red dot), and Sheltered beach (blue dot), respectively. Sampling sites: Arnao (Ar- E), Peñarronda (Pe-E), Otur (Ot-F), Aguilar (Ag-F), Zeluan (Ze-E), Xagó (Xa-E), El Puntal (EP-E), Rodiles (Ro-E), Vega (Ve-F), Gulpiyuri (Gu- S), and Cobijeru (Co-S). WWTP correspond to distance to the closest Wastewater treatment plant; Services correspond to Beach services; River correspond to distance to the closest river; and Port correspond to distance to the closest port.

This means that macroplastic abundance was lower at beaches with many services.

The rest of stressors, including the distance to rivers, were not significantly correlated with any type of plastic in this analysis. To check if the correlation between macroplastics and beach services was still significant after controlling the effect of the other variables, we tested multiple regression model(Supplementary Table 3). It was significant (multiple R2 adjusted = 0.623, $F(3,7) = 6.518$, $p = 0.02$) and showed that the partial correlation between the density of macroplastics and the number of beach services was significant ($p = 0.016$). This confirmed the importance of beach management (cleanings, trash bins etc.) for preventing marine plastic pollution (Rayon- Viña et al. 2018). On the other hand, some of the stressors were correlated to each other, like distance to rivers and distance to ports, and (negatively) the distance to WWTPs and fishing activity; this can be explained because most fishing ports in the region are in villages while WWTPs are generally located near urban populated areas. Kruskal-Wallis tests (Supplementary Table 4) showed significantly different pollution between groups of beaches organized by the level of their stressors (as highly or lowly affect-ed). Results indicated that plastics of different sizes were differentially influenced by the number of beach services, fishing activity, WWTP, distance to ports, and distance to rivers (Fig. 3).

Macroplastic density was significantly higher at beaches from areas with high fishing activity than in those with low activity (mean (M) = 22.2 and 0.71 respectively, Kruskal- Wallis with $p = 0.028$). Indeed, the group of beaches with more services (less impacted by lack of services) had significantly less macroplastics than the group

Table 2 Matrix with the pairwise correlations between variables, showing τ coefficients and their significance after Bonferroni correction: significant τ marked with an asterisk and p values in bold

	Microplastics	Mesoplastics	Macroplastics	Port	River	WWTP	Fishing	Beach services
Microplastics		0.748	0.875	0.209	0.15	0.116	0.17	0.617
Mesoplastics	0.075		0.015	0.467	0.680	0.063	0.134	0.265
Macroplastics	0.037	0.57*		0.692	0.686	0.032	0.034	0.003
Port	− 0.294	− 0.17	− 0.093		0.005	0.068	0.253	0.556
River	− 0.336	0.096	0.094	0.66*		0.374	0.618	0.797
WWTP	− 0.367	− 0.434	− 0.5	0.426	0.208		0.009	0.355
Fishing	0.321	0.35	0.495	− 0.267	− 0.117	− 0.61*		0.489
Beach services	0.117	− 0.26	− 0.687*	− 0.137	− 0.06	0.216	− 0.162	

of beaches with less services (M = 1.86 versus M = 25.1 macroplastics/100 m2, p = 0.017).

Microplastics were more abundant in the group of beaches located close to ports (M =27.7, and 14.7 for beaches far from ports; Kruskal-Wallis with p= 0.037), and close to a river (M = 29.8 versus 14.7; Kruskal-Wallis with p = 0.017). Finally, mesoplastics were more abundant in beaches closer to WWTP (M = 0.104 and 0.002 mesoplastics/m2 respectively, Kruskal-Wallis with p = 0.016). Figure 3 summarizes these results, representing graphically the mean of plastics (M) found in each group of beaches.

4. DISCUSSION

This study in the coast of Asturias (southwest Bay of Biscay) is the first, to the best of our knowledge, studying the relation- ships between different types of plastics (macro-, meso-, and microplastics) and a suite of land-located environmental stressors known to be associated with marine litter. Our results suggest that the stressors considered have different influences on macro-, meso-, and microplastics in the studied coast. Evidently, currents and tidal flows can have a bigger effect on litter transport along the coast than the

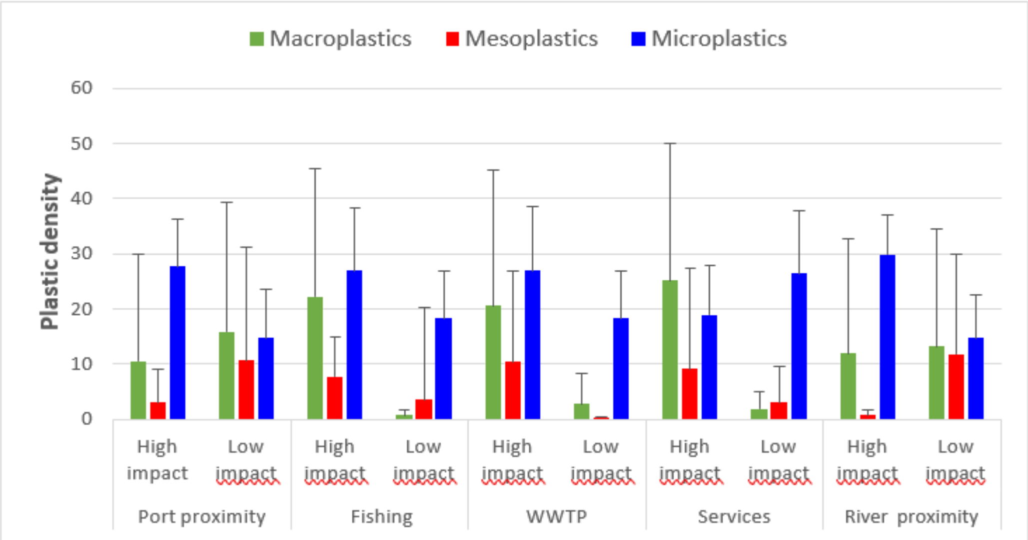

Figure 3 Mean of plastic density in groups of beaches highly or lowly impacted by stressors contributing to marine plastic pollution. Units: microplastics/kg, mesoplastics/100 m2, and macroplastics /100 m2. Standard deviation is represented as vertical bars. WWTP, wastewater treatment plants; services, beach services.

sources located in land, but from the analysis carried out, it seems that some of the stressors considered are at least partially causative of litter accumulation in the beaches studied.

Macroplastics were strongly associated with beach services, as beaches with less beach services had the higher amount of macroplastics. Beaches lacking cleaning and trash bins exhibited significantly more macroplastics, supporting earlier findings of Rayon-Viña et al. (2018).

Regarding ports and fishing, macroplastic pollution was significantly higher where the fishing activity was more intense, although it was not significantly correlated with the proximity of ports. Microplastic density was significantly higher nearby ports. These results highlight the important effect of fishing in the distribution of marine litter in the studied coast. Ports and especially vessels have been reported as main sources of some types of plastic litter such as pieces of trawl, nets, packages, ropes, and plastic bags (Horsman 1982; Neves et al. 2015; García-Rivera et al. 2017), as well as litter originated from fishing activity in general such as polymeric paints from hulls, buoys, bait buckets, baskets, pots, and bait box strapping tapes (Edyvane et al. 2004; Almeida et al. 2007). Our results on macroplastics are consistent with those publications, and microplastic concentration also suggests that maritime ports are a source of microplastics in the study region. In the analyzed fibers, we found polyvinyl chloride and polyethylene that are employed in fishing gear, and polystyrene (commonly employed in boxes to store and transport fish catch), which would be consistent with contamination from fishing activity. This contrasts with Zhou et al. (2018) results in Shandong coast, where the beaches near fishing ports had the lowest abundance of microplastics. River plumes, tourism, mariculture, and civil works like port construction and dike engineering were the most important as microplastic sources in that region, where woven plastic bags were used for flood control outside ports (Zhou et al. 2018). In our study those factors were not important (the region is not very touristic and there is no relevant mariculture, nor construction works in the coast during the study period).

Microplastics did not show a correlation with mesoplastics or macroplastics found at the beaches studied. They have probably multiple origins. Part of them may arrive on the beaches from the sea, transported by regional currents and tides (Masiá et al. 2019), a process thought to be common even in remote islands (e.g., Rech et al. 2018). In our study, most fishing ports are located in estuarine cities; perhaps microplastics come from a combination of urban waste, rivers, and ports as sources. Ports and rivers were significantly correlated in our study, $\tau = 0.66$, $p = 0.005$; thus, their relative importance in the studied area is not clear, but may be correlated because ports usually are located at the river mouths. In any case, the high microplastic density in the central part of the studied coast, characterized by a higher population density, is consistent with Browne et al. (2011) who pointed at cities as main sources of microplastic pollution.

Fibers were the main microplastics found in the study, as it happens commonly in marine environments (e.g., Gago et al. 2018). We should consider airborne contamination as a potential source because, as reported in different studies, microplastic contamination can come from atmospheric fall- out (Cai et al. 2017; Liu et al. 2019). Suaria et al. (2020) found that the majority of oceanic fibers are made of cellulose or transformed cellulose like rayon, and Stanton et al. (2019) reported a dominance of airborne textile fibers of non-plastic material. In our study, rayon and cellulose accounted for 50% of the analyzed fibers, which would support at least some contribution of atmospheric deposition for the origin of fibers. On the other hand, microplastics close to the sea may be diluted due to their transport via ocean or into the land by wind (Chen et al. 2020); thus, the results might underrepresent the real level of microplastic pollution in the region. Finally, we cannot exclude some sampling bias in our study because transects employed in the sampling protocols for microplastics and bigger plastics were different.

Although inefficient functioning of WWTP is still a problem in developed countries (Murphy et al. 2016), and WWTPs have been pointed out as sources of microplastics and other pollutants (e.g., Figuerola et al. 2012; Matamoros et al. 2016; Murphy et al. 2016), in our results we did not find any evidence of association between WWTP and microplastics. We found a higher density of mesoplastics at beaches near WWTPs, but there was no correlation between WWTP and this type of plastics of intermediate size in pairwise analysis (Table 2). Since mesoplastics are retained in WWTP, the result found in Kruskal-Wallis tests is likely spurious and could be explained from the situation of WWTPs that are negatively correlated with fishing activity—located in villages—in the study region (Table 2). WWTPs are near urban populations with anthropogenic pressure, which can be the main cause of mesoplastic release into the ocean, for example due to a bad management of street litter.

Although a specific land-based stressor could not be clearly identified here, this study added to our understanding of the origin of mesoplastics. Highly correlated with macroplastics, these small plastic pieces are probably produced at the beaches from fragmentation or degradation of bigger plastic objects. The lack of correlation between mesoplastics and beach management that was the stressor identified for macroplastics in both correlational and Kruskal-Wallis analyses, could be explained because mesoplastics are sufficiently small to be overlooked in beach cleanings. On the other hand, the cleanliness of some of the beaches studied, and therefore the relatively low number of plastic items found, together with the large variability, may lead to false positive or false negative test results. For example, at some of the beaches while doing the sampling, some pieces of plastic were not taken into account as they were out of the transects, which would lead to a false negative.

Lots et al. (2017) reported microplastics at different beaches of Europe ranging from 72 ± 24 to 1512 ± 187 microplastics kg^{-1}. In comparison, in the region studied here within Bay of Biscay, the amount of microplastics was relatively moderate (between 58 ± 1.6 microplastics kg^{-1} in Otur and 382 ± 6.5 in Rodiles, mean 230 ± 112 microplastics kg^{-1}). Regarding bigger plastic litter, the results are very different and would point to relatively clean beaches too. Williams et al. (2016) conducted a study in 20 different beaches of the south of Spain, where litter ranged from 28 to 276 items, while in our study this range was from 0 to 54 items per beach. In another study in south Spain (Asensio-Montesinos et al. 2020), a mean of 0.06 litter items m^{-2} (ranging from 0.003 to 0.26 items m^{-2}) was found, roughly comparable to the values found in our study ranging from 0 (in Arnao and Vega) to 0.5 items m^{-2}, with a mean value of 0.11 items m^{-2}. Earlier studies applied different statistical approaches for marine litter and microplastic evaluation (e.g., Tudor et al. 2002; Schulz et al. 2017, 2019); source apportionment (e.g., Kataoka et al. 2013) was also applied to check temporal trends. However, our study was limited to a single temporal point with no replicates over time. Future samplings in the same area over different years would be recommended, in order to see if there is any trend in marine litter, and if the pattern revealed in this study is maintained over time.

Improving management practices is necessary in order to reduce plastic releases from the sources identified here. As a general recommendation, WWTPs, although not identified in our study as a major contributor to beached microplastics, are a recognized source of microplastics into the ocean and need to meliorate their facilities and technology (Bui et al. 2016). In this region, actions should be focused on ports. Gaps in management procedures in ports and vessels have been identified (e.g., Gobbi et al. 2017), for example the inefficient segregation of plastic waste. Chen and Liu (2013) proposed some actions like placing trash collection facilities at port and developing recycling practices and fishers' positive views about the marine environment. Our results support these ideas and suggest the need of involving fishers' associations in the management of marine litter in general, and plastics and microplastics in particular.

5. CONCLUSION

This study in the coast in southwest Bay of Biscay shows the relationships between beached marine plastics of different sizes (macro-, meso-, and microplastics) with some anthropogenic stressors known to be associated with marine litter. From their significant correlation in the statistical test, mesoplastics are likely the product of in situ degradation of bigger plastic objects. Microplastic and macroplastic abundance was higher nearby ports, and bigger plastics were also more abundant at beaches without cleaning services. Therefore, maritime ports seem to be main sources of plastic pollution in the studied area, where WWTPs could not be associated with beached microplastics. More efforts are needed to eliminate or diminish the amount of plastic released from fishing activities, and to improve beach management, e.g., paying attention to small plastic fragments in beach cleaning, in order to protect marine ecosystems.

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

Author contribution PM wrote the original draft and developed the re- search idea, data collection, and data analysis. MG, SG, FRV, and PM did the macroplastic collection. EGV and AA developed the research super- vision, writing review and editing. EGV developed the research idea, data analysis and funding acquisition. All authors read and approved the final manuscript.

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Competing interests The authors declare no competing interests

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Capítulo 2

Microplastics in special protected areas for migratory birds in the Bay of Biscay

Universidad de Oviedo
Universidá d'Uviéu
University of Oviedo

Masiá P, Ardura A, & Garcia-Vazquez E.

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Microplastics in special protected areas for migratory birds in the Bay of Biscay

Paula Masiá, Alba Ardura, Eva Garcia-Vazquez∗

Department of Functional Biology, Faculty of Medicine, University of Oviedo. C/ Julian Claveria s/n 33006-Oviedo, Spain.

ABSTRACT

Plastic pollution is a major ecological catastrophe that endangers vulnerable species. Small plastic fragments and filaments enter the food web in the ocean threatening marine species health. Here microplastics between 0.5 and 5 mm were quantified from eight beaches of southwest Bay of Biscay (Spain) within Natura-2000 Special Protection Areas for birds. Sand samples were taken using a randomized quadrat-based protocol. Between 145 and 382 particles per kg of dry sand were found, which is relatively high in comparison with other European beaches. Microfibers were more abundant than microplastics. PERMANOVA revealed a significant effect of the beach location (inside versus outside the estuary). Open beaches contained a higher microplastic density than sheltered ones suggesting that many beached microplastics come from the ocean. Birds are at risk in the studied protected spaces as revealed from high concentrations of fibres in depositions of European shag and gulls.

Keywords: Bay of Biscay; Estuaries Seabirds; Marine protected spaces; Microplastics; Open beaches

1. INTRODUCTION

Since 1950, global plastic production has raised from five to 250 million tonnes per year, and 1/3 of this production are disposable single-use plastics (Jambeck et al., 2015). Due to their physical and chemical characteristics, such as durability, low weight and price (Laist, 1987), this material has become broadly used, and is the major component of marine litter (Derraik, 2002). It is known that almost 12.7 million tonnes of plastic end up polluting the oceans (Arthur et al., 2009). There are many sources of plastic pollution, such as landfills (Gourmelon, 2015), aquaculture and fishing activities (Cózar et al., 2014), litter deposited on beaches (Andrady, 2011), etc. These plastics tend to accumulate in oceanic gyres (Moore et al., 2001), being able to affect marine animals worldwide. Currently, as an estimation, around 260 different species are affected, whether by ingestion of these plastics or by entanglement in \`ghost nets´ especially marine mammals, birds and sea turtles (Gall and Thompson, 2015; Isangedighi et al., 2018). Ingestion of plastics can be selective depending on the species, since animals may eat plastics differentially depending on the plastic's colour, shape and size (Isangedighi et al., 2018). Eating plastics causes several damages as reduction of food consumption – leading to problems in migration, lower levels of reproduction, endocrine abnormalities, internal injuries and even death (Derraik, 2002). As common plastics are not biodegradable, they tend to accumulate in marine habitats until they break into small little pieces called microplastics, which are pieces of plastic smaller than 5 mm. They can be a product of the degradation of larger pieces of plastics, due mainly to the exposure of solar radiation and high temperatures; or they can be directly produced for use, for example, in cosmetics, or as a part of other products (Arthur et al., 2009). The first

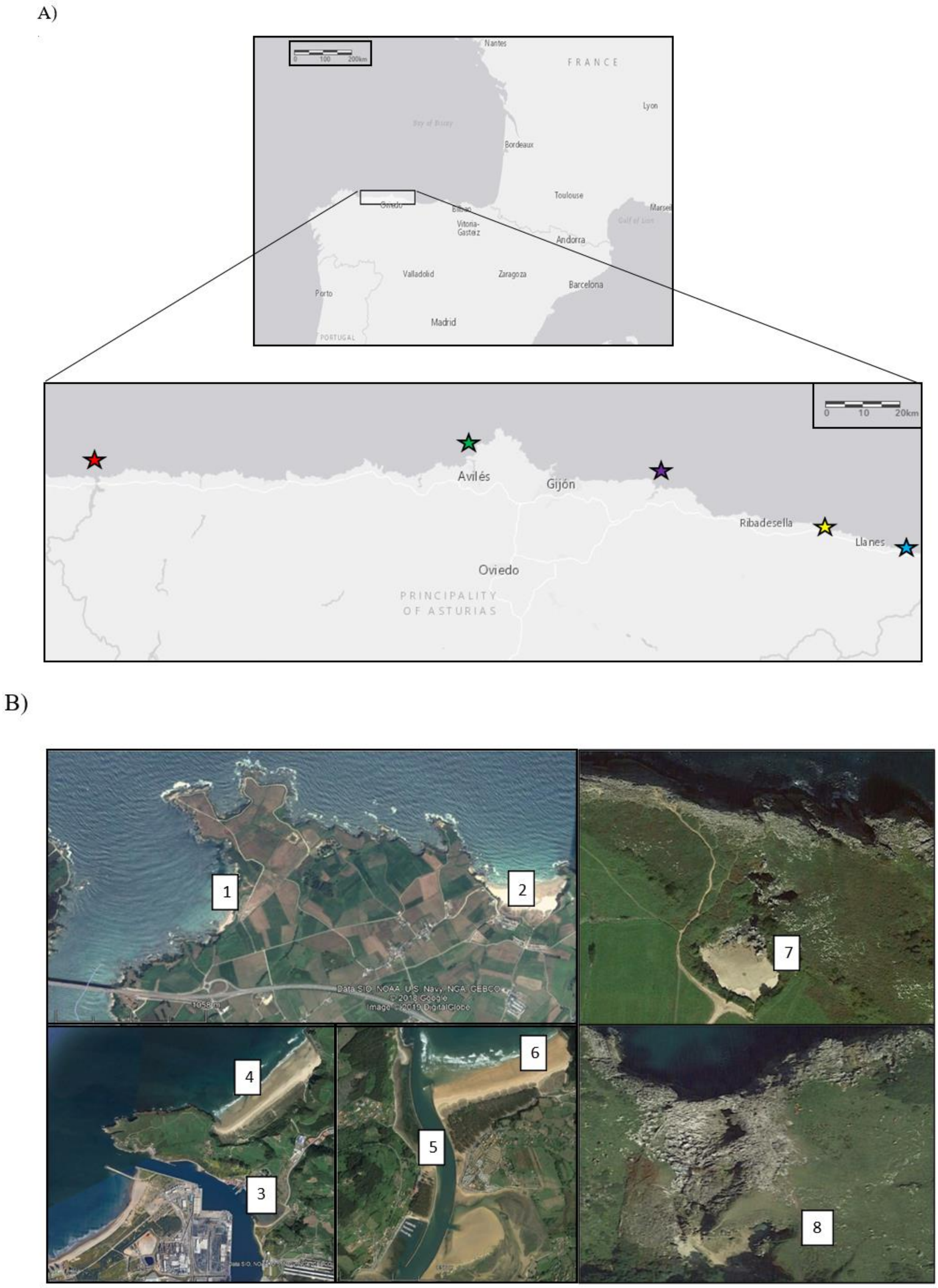

Figure 1. A) Above, map of the sampling area. The stars show the locations where the samples were taken: Eo estuary (red star), Avilés estuary (green star), Villaviciosa estuary (purple star), Gulpiyuri (yellow star) and Cobijeru (blue star). B) Below, satellite photos of the beaches in estuaries: 1 to 8 are Arnao, Peñarronda, Zeluán, Xagó, El Puntal,Rodiles, Gulpiyuri and Cobijeru respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

species than bigger plastics. Microplastics are vectors of contaminants that can be found in the water, such as POPs (Persistent Organic Pollutants), herbicides, antibiotics, metals, etc. (De Sá et al., 2018). When plastic breaks in the ocean, it releases - among other toxic substances as nonylphenol and triclosan - an endocrine disruptor called Bisphenol A (Gatidou et al., 2010). This substance has been related with estrogenic and anti-androgenic activity (Birkett and Lester, 2003). Meyer-Rochow et al. (2015) have connected microplastics with cancer development, mainly attributed to Bisphenol A. Many harmful effects of microplastics have been already proved in different organisms, such as physical effects, neurotoxicity, oxidative damage, reproduction, genotoxicity, and elevated mortality (De Sá et al., 2018).

For their implication in environmental health, there is currently a plethora of studies about the abundance of microplastics and their distribution (e.g. see reviews by Thompson et al., 2004; Lots et al., 2017). Recent studies have discovered that microplastics in the shape of synthetic fibres can be found even in the air, so human lunges are exposed between 26 and 130 microplastics daily (Gasperi et al., 2018; Prata, 2018). Also, airborne microplastics are responsible of 7% of microplastics pollution in the oceans (Boucher and Friot, 2017). They have been reported in many marine species, such as sea birds, fishes, cetaceans, molluscs and plankton among others (e.g. Li et al., 2016; Catarino et al., 2017; review in De Sá et al., 2018). Microplastics are incorporated in the food webs, being ingested first by small and low trophic level species, then by predators of consecutively higher trophic levels and finally reaching humans (e.g. De Sá et al., 2018). Moreover, animals eating microplastics expel them in faeces and when particles reach sediments, benthonic animals ingest the faeces and microplastics are incorporated also through this via into the trophic web (Wright et al., 2013).

For their varied foraging niches in the trophic chain, birds are exposed to microplastics ingestion (e.g. Gilbert et al., 2016; Terepocki et al., 2017). Basto et al. (2019) found microplastics in the stomachs of five out of 16 species collected in Portugal (31.25%). White debris is the most frequent type of microplastics found in birds' digestive tracts (Amélineau et al., 2016; Holland et al., 2016), and filaments or fibres seem to be more frequently ingested by birds than pellets (Amélineau et al., 2016; Alvarez et al., 2018), although these generalities may have exceptions. Interspecific differences for the size and colour of ingested plastics have been described for aquatic birds in Portugal (Basto et al., 2019); for example, although microplastics were the most abundant class in all the species, Yellow-legged Gulls had 70%, while Black-headed Gulls had 100% of microplastic items in their stomachs; the colour was white-clear in the first ones and preferentially green in the second ones. On the other hand, the exposure may be seasonal depending on the species; for example, Canadian Cassin's auklets are more exposed to microplastic ingestion during the winter (O'Hara et al., 2019). Coastlines worldwide are being increasingly targeted for microplastics studies because they show that the vast majority of beaches contain microplastics, and the real dimension of the problem is still unknown. Almost 90% of the microplastics found in the sand are synthetic fibres, resulting mainly from clothes (Browne et al., 2011) and fishing nets (Lots et al., 2017). Assessing the real quantity of microplastics in different components of the marine ecosystem has become a need for different reasons: in order to know their real amount and the ecosystem components where they accumulate, to predict the impact they could have in the future, and also importantly to raise awareness and prevent their impact in human health.

The microplastics dynamics in the ocean and coasts are not well known since their origin in a given location may vary depending on currents, polymer density and other factors that influence their deposition in the shore (e.g. Graca et al., 2017). Unusual events like storms may explain the appearance of microplastics in remote beaches that should otherwise be clean (Bimali Koongolla et al., 2018). Shoreline microplastics are believed to come mainly from the land, since higher quantities are found in densely populated areas, and their origin would be principally sewage contaminated with fibres (Browne et al., 2011). Other sources are possible; for example, Bimali Koongolla et al. (2018) identified fishing harbours as a main source of microplastics in Sri Lanka. Knowing where they come from in a particular area is today a priority for being able to take measures of prevention and act on the sources.

In this study we quantified microplastics in Natura 2000 beaches of the Central Cantabrian Coast (Asturias, Bay of Biscay, Spain), all included in the network of Special Protected Areas (SPAs) for the protection of vulnerable and migratory bird species in Europe. There is scarce information about microplastics in the coasts of the Iberian Peninsula, and publications show that they are more abundant in the Atlantic (Antunes et al., 2018) than in Mediterranean coast (León et al., 2019), with accumulations in river deltas (Ebro River delta, Simon- Sánchez et al., 2019). In the less studied Cantabrian coast, data from neighbouring areas suggest that the contamination may be also important. For example, in the waters of Galicia at west of the coast studied here, 95% of the samples analysed between 2013 and 2014 contained microplastics (Gago et al., 2015). Seabirds are also affected: Alvarez et al. (2018) reported microplastic fibres, principally nylon, in 63% of the regurgitations of European shag examined in the Galician coast. Our results from SPAs contributed to fill the gap of knowledge in the centre of the north Iberian coast. The comparison between beaches located inside and outside the estuaries, coupled with the type of particles, helped to inferring the land or sea origin of the microplastics and to estimate the risk posed to marine birds in special protected areas.

2. MATERIALS AND METHODS

2.1 Sampling sites

In the study region, the Central Cantabrian Coast (southwest Bay of Biscay), there is a dominant eastward current (Botas et al., 1989), except in the summer when the prevailing northeast winds generate superficial westward currents (Botas et al., 1990). Samples were taken between January and early April 2019 (winter time, thus dominant eastward currents) from sand beaches in three different estuaries of Asturias (southwest Bay of Biscay, Spain): Eo, Avilés and Villaviciosa (Fig. 1). Two sites were selected per estuary: in the outside, the closest beach at east (< 500 m from the estuary mouth), exposed to the open sea; and in the inside the closest to the mouth, under sea conditions (salinity, tidal regime) but sheltered from high waves. In addition, two beaches located far from any river were sampled to confirm the relative influence of estuaries and the sea in microplastic accumulation: Gulpiyuri and Cobijeru (Fig. 1). These two beaches are separated from the open sea by rock barriers and Gulpiyuri is closer to the open coastline than Cobijeru, thus is more exposed to the waves action (Fig. 1, Table 1). The eight beaches of this study are within the Natura 2000 network of SPA. The information about their protection status can be consulted online in the web of the Spanish Ministry of Ecological Transition at https://www.miteco.gob.es/es/biodiversidad/temas/ espacios-protegidos/red-natura-2000/zepa_asturias.aspX (accessed in July 2019). They are defined and regulated by the Law of Natural Patrimony and Biodiversity 42/2007 of 13 of December of 2007. The Eo estuary is the Natura 2000 SPA ES1200016. The inner and westernmost beach sampled was Arnao, and Peñarronda was the outer beach, considered also a Protected Natural Landmark in regional legislation. The Avilés estuary is

Table 1: Microplastics composition in each of the beaches, separated by fibres of different colours and plastic fragments + pellets (MP + Pellets). Results are given as proportion of each type of MP in each beach, and total number of particles per kg of sand (items/kg). S, E and SE are sheltered, exposed and semi-exposed beaches respectively. Beach coordinates are given. SPA, code of the Special Protected Area where the beaches are located

Location/SPA	Beach	Coordinates	Distance to open sea	F-Black	F-Blue	F-Red	F-White	F-Others	MP + Pellets	Total items/kg sand
Eo/ES1200016	S-Arnao	43°32′54.7″N 7°01′17.8″W	933	0.359	0.359	0.046	0.219	0.008	0.008	237
	E-Peñarronda	43°33′11.7″N 6°59′48.7″W	0	0.289	0.365	0.062	0.263	0.006	0.014	353
Avilés/ES1200055	S-Zeluán	43°35′15.3″N 5°55′10.1″W	1660	0.159	0.476	0.044	0.195	0.10	0.026	271
	E-Xagó	43°35′56.4″N 5°55′35.4″W	0	0.212	0.424	0.177	0.070	0.087	0.035	346
Villaviciosa/ES1200006	S-El Puntal	43°31′33.5″N 5°23′17.5″W	1020	0.332	0.401	0.025	0.198	0.039	0	201
	E-Rodiles	43°32′00.9″N 5°22′46.8″W	0	0.277	0.432	0.047	0.235	0.008	0	382
Llanes/ES0000319	SE-Gulpiyuri	43°26′51.2″N 4°53′09.8″W	55	0.171	0.313	0.287	0.196	0.007	0.025	275
	S-Cobijeru	43°23′46.2″N 4°36′36.1″W	175	0.145	0.365	0.159	0.310	0	0.021	145

located within the SPA ES1200055 Cabo Busto-Luanco. The beach inside the estuary was Zeluán, which is a Protected Natural Monument (Spain Decree 100/2002); and the outer beach sampled was Xagó that is also inside the Protected Landscape of Cape Peñas (Decree 154/2014). The Villaviciosa estuary (SPA ES1200006) was the third one. It is a Partial Natural Reserve, and within this area the outside site was Rodiles, and the inside site was El Puntal. The beaches of Gulpiyuri and Cobijeru, both Protected Natural Monuments, are located inside the Natura 2000 SPA ES0000319 Ria de Ribadesella-Ria de Tinamenor. In Spanish legislation, Protected Natural Monuments are formations of notorious singularity, rarity, or beauty with scientific, cultural and/or landscape values, and are subjects of special protection. Spatial protection in SPA implies in Spain more surveillance and limited human uses (construction, landscape modification, hunting, farming are not allowed, and only limited exploitation of fishing resources is, in the beaches here studied).

The locations have different anthropogenic activities. Avilés estuary has large industrial factories (two of steel industry, a big shipyard, and others), an international commercial port and a fishing port, and in addition, Xagó beach has a high tourism influence in summer and surfing activities in the winter. The Eo estuary has aquaculture (oyster farms) and some industrial activity (one small shipyard, small factories) although much lesser than Avilés; it has also two fishing ports, and tourism concentrated in the summer. Villaviciosa has small-scale industrial activity of cider production, small fishing ports and tourism in the summer. Gulpiyuri and Cobijeru are located in Llanes county, where there is no industry, only very small fishing ports and tourism in the summer. Population density is higher in Avilés (79,000 inhabitants in Avilés city) than in Eo (3500, 3800 and 10,000 inhabitants in Castropol, Vegadeo and Ribadeo municipalities respectively), then Villaviciosa (14,400 inhabitants), and finally Llanes (13,600 inhabitants). From the relationship between population and MP pollution (Browne et al., 2011) higher concentration of microplastics is expected in the Avilés estuary, then in Eo, Villaviciosa, and Llanes as the expectedly least polluted.

2.2. *Sand sampling and extraction protocol*

Sand samples were taken following a protocol adapted from Besley et al. (2017), with slight modifications. Firstly, a random line of 100 m was traced in the middle of the intertidal zone parallel to the water line, and five 0.5 × 0.5 m quadrates were randomly set along this transect (Fig. 2). Within each quadrat four samples of 50 g sand were also randomly taken from the first layer of 5 cm. Thus, a total of 160 sand samples were carried to the laboratory, 20 samples per site. All of them were taken during the low tide. Once in the laboratory, samples were dried in an oven at 60 °C for 24 h. Then 200 mL of a saturated NaCl solution (358.9 g/L) was added to each dry sand sample in order to separate microplastics (MP hereafter) from sand by a density separation method. The hypersaline solution was previously filtered with a 0.2 µm Millipore filter in order to remove contaminants from the distilled water or the NaCl, as fibres can be a potential contaminant. Then, each sample was stirred with a magnetic stirrer for 2 min at 900 rpm and let settle for 6 h to separate MP from sand. As MP float, the supernatant of each sample was poured into a vacuum pump and filtered three times through a 0.2 µm Millipore filter to retain as many microplastics as possible. Filters were placed in a Petri dish and visualized directly in the stereomicroscope.

A blank was performed following the same steps as the rest of the samples, but only with the saturated NaCl solution previously filtered, to discard contamination during the sample processing. All along the process of MP extraction and sample manipulation, samples, solutions and water were covered to prevent airborne contamination, and all the material was carefully washed before use.

2.3. Seabird depositions sampling and extraction of MP

Fresh individual seabird depositions (N = 10) were collected from different, separate rocks in Zeluán beach. From shape, density and colour, and the observation of the birds in situ, they likely belonged to three different species common in the beach: European shag *Phalacrocorax aristotelis*, Yellow-legged Gull *Larus michahellis*, Black- headed Gull *Chroicocephalus ridibundus*. The samples were individually transported to the laboratory in glass bottles. Once there, 100 mL of a prefiltered saturated NaCl solution (358.9 g/L) and 20 mL of 30% hydrogen peroxide solution was added to each sample. Then, samples were stirred for 1 min every hour, three times, and let settling overnight. After that, samples were filtered with a vacuum pump through filters of 0.45 µm pore. The filters were placed in a petri dish and directly visualized under the stereomicroscope.

2.4. MP identification and classification

MP between 0.5 and 5 mm were observed directly from the filter under a stereomicroscope at 40× magnification and counted. Particles larger than 5 mm were manually removed. For the identification, Hidalgo-Ruz et al. (2012) recommendations were followed: filaments found should be regular throughout their entire length, and should not present cellular or organic structures; the colour should be homogeneous, being especially careful with transparent fibres.

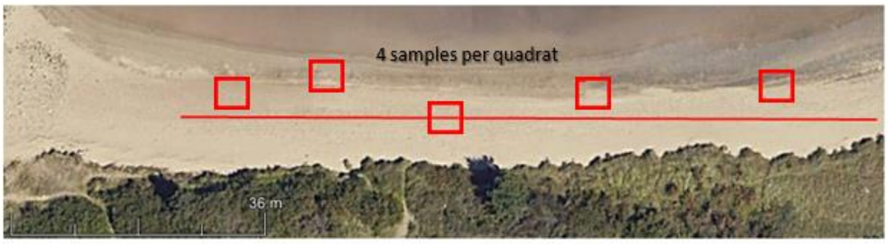

Figure 2. An example of the sand sampling scheme. Image of Zeluán beach showing the location of the 100 m transect of reference and the five 50*50 cm quadrates along the transect.

MPs were classified first by shape: synthetic fibres (= filaments), pieces of plastics (usually irregular, with sharp or semi-round shapes) and pellets (white/yellow cylindrical spheres). Foam particles were not found. Then, the particles were sorted by colours and classed in the main colour categories found black, blue, white/transparent, red and others.

2.5. Determination of MP composition

Subsamples (N = 10) of particles taken randomly from the items found from each beach were analysed by Fourier-transform infrared spectroscopy (FTIR) in order to confirm they were really plastics. Items of all the types (fibres of all the colours, fragments, pellets) were re-presented. The wavelength spectrum of each particle was compared with that of reference materials: polyethylene terephthalate (PET), high density polyethylene (PE-HD), polyvinyl chloride (PVC), polystyrene (PS), polypropylene (PP), low density polyethylene (PE-LD), for determining its composition. The analysis was done in the Scientific Technical Unit of the University of Oviedo, in a FTIR spectrophotometer attached to a microscope with an image-forming system (Varian 620-IR and Varian 670-IR) and three detection systems: one located in the spectrometer and two in the microscope (FPA for imaging and MCT for punctual measurements).

2.6. Statistical analysis

Data analysis was performed using PAST software (Hammer et al., 2001). The dataset was analysed taking into account the global MP composition, including the colour of the filaments found. For that purpose, non-metric multidimensional scaling (nMDS) was done considering the different MP categories using Bray-Curtis similarity index. Normality (Shapiro-Wilk test) and homoscedasticity were checked, and analysis of variance was conducted to test for significant differences by location for MP quantity and/or categories. Two-way ANOVA or PERMANOVA with Monte Carlo procedure (9999 permutations) if ANOVA could not be used were employed. Post-hoc pairwise tests served to identify the samples that differed significantly to each other. Standard $p < 0.05$ significance threshold was considered, applying Bonferroni correction for multiple comparisons when required.

3. RESULTS

3.1. Microplastics in sand

All of the eight beaches analysed in this study contained MP of various types (examples in Fig. 3). In Avilés estuary some metallic beads were also found, but they were not taken into account for the results because this study is focused in MP. FTIR analysis demonstrated that the

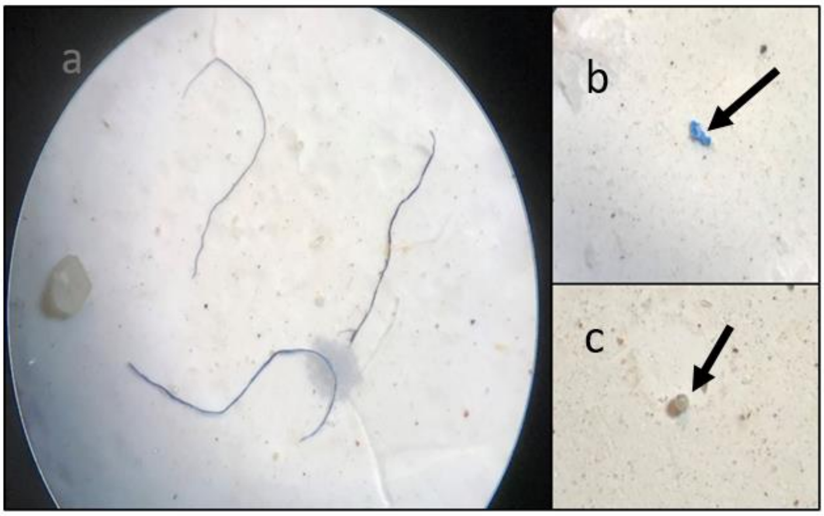

Figure 3. Stereomicroscope image showing different types of microplastics found in this study: a, b and c are blue filaments (synthetic fibres), a piece of blue microplastic and a micro-plastic pellet, respectively. (For interpretation of the refer- ences to colour in this figure legend, the reader is referred to the web version of this article.)

majority of particles identified as MP were really of plastic (all except one black fibre that was cellulose). The most abundant type of plastic was PVC (90% of the particles analysed), as indicated from a large band at a wavelength of 3300 cm−1 and two narrow peaks at 2800–2900 cm−1 wavelength.

The total number of MP particles per kg of sand varied between 145 in Cobijeru (beach sheltered in Llanes) to 382 in Rodiles, which is the exposed beach of Villaviciosa estuary (Table 1). A total of 98.4% of the MP found in this study were fibres, representing even 100% of MP particles in the two beaches of Villaviciosa estuary (Table 1). In all of the beaches the most abundant colour of the fibres was blue, then black, white (red in Xagó), and finally red (white in Xagó). The category “Others” contained principally green fibres. Avilés estuary had the highest number of non-filamentous MP (small plastic fragments), and also the highest number of MP pellets.

The four locations exhibited the same general pattern of MP contamination, with the exposed or semi-exposed beaches containing higher MP quantity than the sheltered beaches of the same location (Fig. 4).

After rejecting conditions for parametric ANOVA, two-way PERMANOVA test was performed with locations and exposure to open sea (categories of sheltered and exposed/semi-exposed) as factors for explaining the density of total MP particles. Results showed a significant effect (p-value < < 0.05) of the exposure, as observed in Fig. 4, and also of the location with p = 0.002 (Supplementary Table 1A) with the beaches of the Llanes area containing less MP than the beaches of the same level of exposure in the rest of zones (Fig. 4). Non-metric multidimensional scaling analysis considering the number of particles of each type in each beach showed differences between beaches (Fig. 5). With a Shepard plot with stress = 0.046, with r2 = 0.67 and 0.05 for axis 1 and 2 respectively and the values aligned along the diagonal, the scatter plot showed the less exposed beaches of Zeluán, Arnao, El Puntal and Cobijeru connected in the minimum spanning tree, while the exposed or semi-exposed beaches were apart in different branches. Xagó was connected with the inner beach (Zeluán) of the same estuary (Avilés, the most environmentally disturbed) in the minimum spanning tree, but clearly apart, as it was Gulpiyuri. Xagó exhibited the lowest

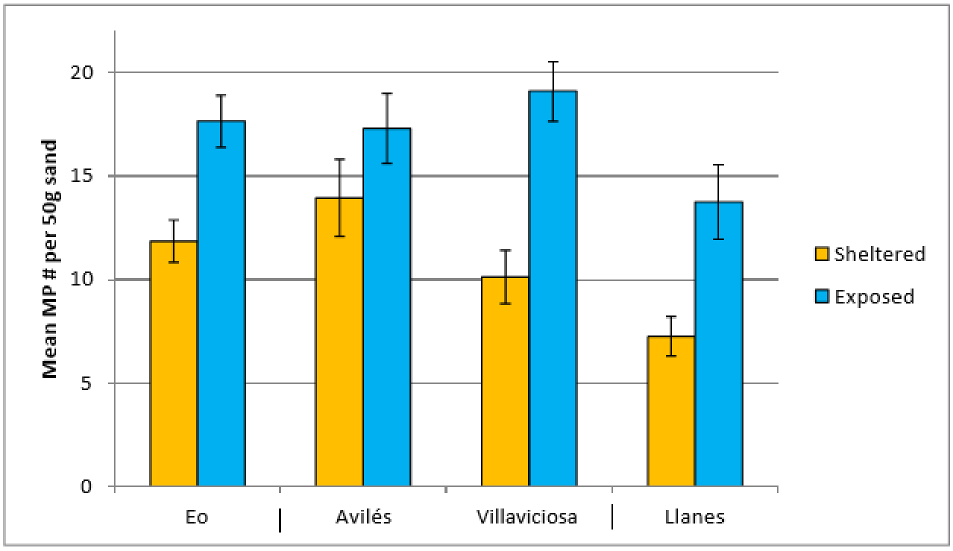

Figure 4. Density of microplastics found in the beaches located in the four Special Protected Areas considered within Bay of Biscay. Bar chart representing the mean number of MP per 50 g sand in each sheltered or exposed/semi-exposed beach (standard error as capped bars).

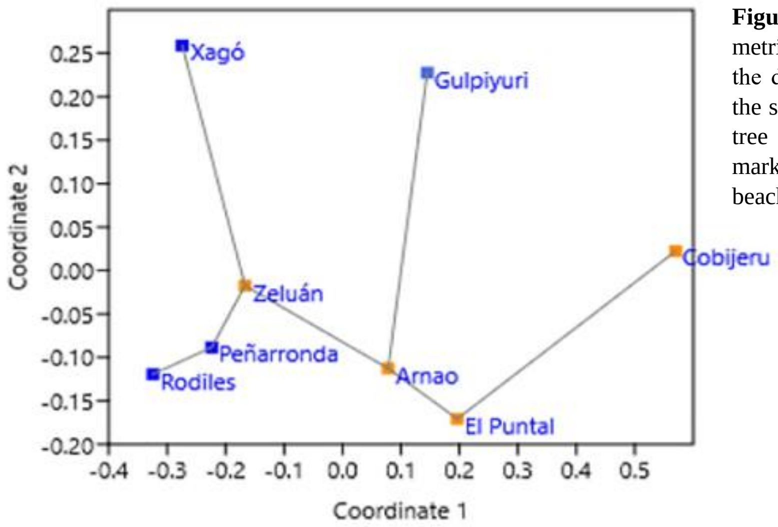

Figure 5. Scatter plot constructed from non-metric multidimensional scaling analysis of the different types of microplastics found in the six beaches analysed. Minimum spanning tree is presented. Sheltered beaches are marked in orange and exposed/semi-exposed beaches in blue.

proportion of white fibres and Gulpiyuri the highest proportion of red ones.

Given the higher abundance of white debris in birds' stomachs reported by other authors (Amélineau et al., 2016; Holland et al., 2016; Basto et al., 2019), we performed an analysis with only white fibres to check if the birds visiting all the beaches were equally exposed to MP of this colour. The two-way PERMANOVA (Bray-Curtis similarity index) revealed a statistically significant effect of the exposure but not of the location or significant interaction between exposure and location (Supplementary Table 1B). In all the locations the sand of exposed beaches contained a higher proportion of white fibres than the sand of sheltered ones except in Avilés, where it was the opposite (Table 1).

After Bonferroni correction, post-hoc pairwise analysis showed significant differences in the quantity of white fibres per sample between the exposed and sheltered beaches of Eo and Villaviciosa estuaries, consistently with the effect of the exposure on the abundance of MP (Table 2). Significant differences were found between Peñarronda, that exhibited more fibres of this colour in absolute number than any other beach, and El Puntal, Arnao, Cobijeru and Gulpiyuri (Table 2). Rodiles was significantly different from the sheltered beaches El Puntal and Cobijeru ($p < 0.05$). Xagó, with an intermediate level of white fibres, was in the middle of the two groups. For the scarce non-filamentous MP, significant effects of location or exposure were not found (Supplementary Table 1C).

3.2. Microplastics in bird depositions

The analysis of bird faeces from Zeluán demonstrated that all of them contained MP. In total 114 MP particles were found in the 10 depositions analysed. The number of MP items per sample varied between 3 and 33 per deposition (average 12.7, standard deviation 9.1). White fibres were the most abundant type in all of the samples (40.4% of the total of MP found in bird faeces), followed by blue (39.4%), then black (9.6%) and red (6.1%) fibres, in this order, and finally some fragments of microplastics (4.4%). Unless white and blue fibres that were found in all the depositions analysed, the other types of MP were found only in some samples (5, 4 and 4 depositions contained black fibres, red fibres and plastic fragments respectively).

The global profile of the microplastics was different of the profile found in the sand in Zeluán. White fibres were more abundant in faeces than in sand, and the proportion of blue

Table 2: Pairwise F and P-values (below and above diagonal respectively) between the beaches studied for the quantity of white fibres found in the sand samples analysed. Significant values after Bonferroni correction are in bold.

	Zeluán	Xagó	Rodiles	El Puntal	Peñarronda	Arnao	Gulpiy uri	Cobijeru
Zeluán		0.6693	0.0397	0.4781	0.0083	0.9941	0.9957	0.6206
Xagó	0.258		0.05	0.0961	0.0089	0.4349	0.636	0.2007
Rodiles	4.201	3.632		0.0019	0.8487	0.0103	0.0248	0.0025
El Puntal	0.65	3.115	10.44		0.0001	0.2706	0.2328	0.6509
Peñarronda	7.2	8.007	0.044	25.71		0.0005	0.0021	0.0001
Arnao	0.005	0.643	7.078	1.347	16.75		0.8065	0.4576
Gulpiyuri	0.004	0.316	5.566	1.369	11.77	0.031		0.4405
Cobijeru	0.284	1.95	9.679	0.22	21.82	0.472	0.611	

and especially black ones was clearly smaller (Fig. 6). The difference was statistically highly significant, with a contingency Chi square of 22.629, 4 d.f., P = 0.0002. Thus, the sample of faeces analysed was enriched in white MP in comparison with the sand samples of the same site.

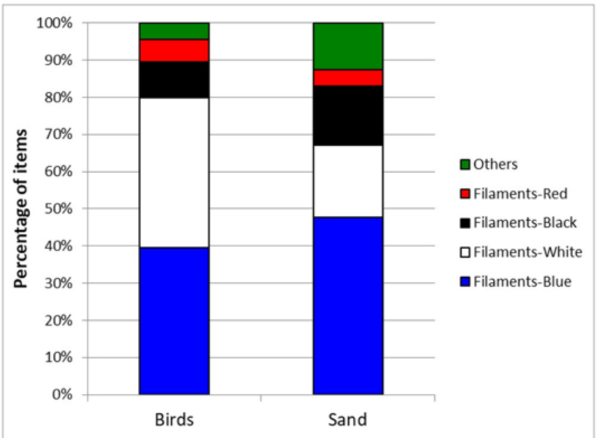

Figure 6. Percentage of different types of microplastics found in the Special Protected Area of Zeluán (Central Cantabrian Coast, Bay of Biscay): comparison between bird faeces and sand.

4. DISCUSSION

The results of the current study are, to the best of our knowledge, the first data reporting MP contamination in sandy shores and bird faeces from southwest Bay of Biscay. Taken from Special Protected Areas for migratory birds, both the MP quantity in sand and the presence of MP in bird faeces suggest that aquatic birds are at risk by in- gestion of MP in this region. In comparison with other European data (Lots et al., 2017), the level of total MP could be considered medium-high (category comprising 250–1000 MP per kg of dry sand, following Lots et al., 2017) in all the beaches except in the two beaches of Eo estuary that would fall into the category of "medium level" (150–250 in Lots et al., 2017). Our data were similar in quantity to other reports from Iberian coasts. For example, in sandy beaches of the Mediterranean Ebro River delta, the average of microplastics was 422 items/kg sand in average (Simon-Sánchez et al., 2019), a little bit higher than those of the open beaches next to the estuaries studied here. Higher abundance of microfibers than microplastic fragments was consistent with Simon-Sánchez et al. (2019) in Mediterranean Iberian coast, with 70% of microfibers, and also with the rest of studies in Europe and worldwide (e.g. Browne et al., 2011; Lots et al., 2017).

The analysis of microplastic samples with FTIR showed that the particles identified as MP were almost all composed of plastics, principally PVC. Since not all the MP and fibres were analysed, it is possible that a few of them were confounded with cellulose, roots or pieces of algae (Yu et al., 2016) and the final MP count overestimated (Song et al., 2015). However, as only one particle in our spectroscopic analysis was organic we could reasonably assume that our results are representative of MP contamination in the studied beaches and bird faeces.

The presence of MP in all the bird depositions analysed from Zeluán points out at a serious problem of plastic contamination in this SPAs in south Bay of Biscay. Other studies reported the occurrence of MP in a variable proportion of birds, ranging for example from 13% in Portugal (Basto et al., 2019) to 27% in Ireland (Acampora et al., 2016) to 63% in northwest Iberia (Alvarez et al., 2018). Although the methodology of the studies was different, some being based on stomach contents (Acampora et al., 2016; Basto et al., 2019), others on regurgitations (Alvarez et al., 2018) and ours on faeces –thus we are not 100% sure they all were produced by different birds- it seems that our results would be in the upper part of the rank, even if the number of samples analysed is far from N = 40 as recommended by Franeker van and Meijboom (2002). On the other hand, although limited in sample size our results of depositions concur with other studies in a selective microplastic uptake by birds (Amélineau et al., 2016; Holland et al., 2016), especially light-coloured fibres. This does not imply an active selective feeding of white MP by birds. Instead, it could be explained from their preys. In their review, Wright et al. (2013) reported active feeding of white MP particles by fish that feed on small zooplankton, perhaps due to resemblance with their preys (Shaw and Day, 1994). Since European shag and gulls prey upon small fish, among other animals (Arizaga et al., 2010; Howells et al., 2017), the enrichment in white MP of their depositions (compared with the MP occurring in the substrate) would be likely acquired via food web.

According to our results, a higher quantity of MP has been found in exposed beaches than in the ones sheltered inside the estuary or otherwise. This would suggest that the main source of this type of pollution in the studied shoreline was the ocean. MP, although in different densities depending on currents, flow patterns and eddies, can be considered ubiquitous in the open ocean (Pan et al., 2019), and the waters off the northwest of the Iberian Peninsula are not an exception (Gago et al., 2015). The Cantabrian coast, and therefore the studied locations, is influenced by the North Atlantic Current (Bunker, 1976), which may transport microplastics from the North Atlantic gyre into the Gulf of Biscay and leave them along the coast. As the oceanic water tends to enter slightly into the estuary, some of the microplastics found in inner beaches could also have their origin in the ocean. The influence of the sea was confirmed in Gulpiyuri and Cobijeru beaches where the main source of MP should be the ocean in absence of freshwater sources or population proximity: the beach more distant to the open sea, the sheltered Cobijeru, contained much lower density of MP than the relatively more exposed Gulpiyuri that is closer to the open sea.

In addition to the sea source, we can expect that at least a part of MP comes from inland, especially in Avilés estuary where a larger population and a bigger fishing port are predictors of MP pollution (Browne et al., 2011; Bimali Koongolla et al., 2018). Avilés estuary – especially Xagó- had the highest density of pellets and non-filamentous MP in our study, and this could be a consequence of the industry. The high numbers of fibres found in the outer beaches of the three estuaries, Eo, Avilés and Villaviciosa, were consistent with the effect of the estuaries as microplastics sinks described by Simon-Sánchez et al. (2019). A higher concentration of MP on the beaches next to the opening of the estuaries is likely deposited there by a combination of the river plume and the dominant current eastwards typical of the studied coast in winter (e.g. Botas et al., 1989, 1990; Garcia-Soto et al., 2002).

The results of this study are especially important for conservation because the beaches sampled are Special Protection Areas for birds. Sampling was done in winter that is precisely the season when marine birds use the coastal resources and are consequently more vulnerable to microplastics exposure (e.g. O'Hara et al., 2019). All of the faeces analysed, only from one beach but not the most polluted one, contained MP. Birds seem to ingest more light-coloured anthropogenic debris through the food web, especially clear and white microplastics and filaments (Amélineau et al., 2016; Holland et al., 2016; Basto et al., 2019; this study). Thus, our results with > 90 white fibres per kg of sand in other beaches like Peñarronda and Rodiles, strongly suggest that the birds that rest and eat in these protected areas are at serious risk of MP ingestion. Last but not least, our results confirm Baztan et al. (2014) alert of microplastic pollution in Atlantic coastal protected areas and expand this threat to SPA in the Central Cantabrian Sea. These facts could be added to the list of reasons for stopping urgently plastic pollution in the region, and should contribute to encourage researchers and the industry to find efficient ways of taking microplastics out of the ocean.

5. CONCLUSION

The results found in this study allow to conclude that the density of microplastics found in beaches inside Natura 2000 coastal areas in southwest Bay of Biscay is quite high in comparison with other European beaches. The amount of white fibres may put at risk the marine birds that use the resources of those areas that are catalogued under Special Protection for rare and vulnerable birds. Moreover, we have confirmed that birds in one of the sampling areas are already ingesting microplastics, probably via food web. Higher density of microplastics in exposed than in sheltered beaches suggests that a considerable part of the small debris comes from the sea. Microplastics assessments at regional scale in shores worldwide are needed in order to know the real magnitude of the problem, raise public awareness about this ecological catastrophe, and find sustainable solutions.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.marpolbul.2019.07.065.

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Capítulo 4

Virgin polystyrene microparticles exposure leads to changes in gills DNA and physical condition in the Mediterranean mussel *Mytilus galloprovincialis.*

Universidad de Oviedo
Universidá d'Uviéu
University of Oviedo

Masiá P, Ardura A, & Garcia-Vazquez E.

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Virgin polystyrene microparticles exposure leads to changes in gills DNA and physical condition in the mediterranean mussel *Mytilus galloprovincialis*

Paula Masia, Alba Ardura & Eva Garcia-Vazquez*

Department of Functional Biology, Faculty of Medicine, University of Oviedo, C/Julian Claveria s/n, 33006 Oviedo, Spain; arduraalba@uniovi.es (A.A.); egv@uniovi.es (E.G.-V.)

* Correspondence: masialillo.paula@gmail.com

Simple Summary: Microplastic pollution is damaging ecosystems and marine organisms worldwide, and, as this problem is becoming greater, the fate of these marine organisms should be studied. In this article, the physical condition and the DNA integrity of gills of Mediterranean mussels (*Mytilus galloprovincialis*) have been studied under four microplastic concentrations for 21 days. A worse physical status was shown at the end of the experiment when exposed to highest concentrations; however, DNA damage was higher when exposed to lower concentrations. These results prove that mussels can be affected by direct exposure even at a low microplastic concentration due to their filter-feeding behaviour, making them more vulnerable to this type of pollution.

ABSTRACT

The ever-growing concentration of microplastics in the marine environment is leading to a plethora of questions regarding marine organisms' present and future health status. In this article, the Mediterranean mussel (*Mytilus galloprovincialis*), a commercial species distributed worldwide, has been exposed to 21 daily doses of polystyrene microparticles (10 µm) at four different concentrations that are environmentally realistic (control: no microplastics, C1: 0.02 mg/L, C2: 0.2 mg/L, and C3: 2 mg/L). The physical status through the condition index, and damages in DNA integrity in gills, through DNA fragmentation, were determined. Results showed a minor effect on DNA integrity but a worse physical status at higher doses. Results could be interpreted as a decrease in mussel feeding activity/filtration rates when exposed to high microplastic concentrations, thus reducing the direct exposure to microplastics in gills. These effects could be happening currently and/or may happen in the near future, threatening populations inhabiting microplastics-polluted environments.

Keywords: condition index; DNA degradation; marine biota; microplastics; marine conservation

1. INTRODUCTION

Plastic pollution is one of the most abundant type of pollution worldwide, with estimates of more than 5 trillion plastics particles floating at sea [1], corresponding to ~80% of all marine litter [2]. Most of this plastic litter is constituted by small plastic fragments that come from the degradation of plastics debris, or as a consequence of their direct manufacture, especially for personal care and cosmetic products [3]. Microplastic particles have been reported in a broad range of ecosystems and organisms in the marine environment [4]. Due to currents, winds, and hydrodynamic processes, microplastic can be found in every ecosystem, even with low anthropogenic pressure, such as Antarctica [5], coral reefs [6], and deep marine environments [7]. Due to their ubiquity and small size, microplastics are bioavailable for a great number of marine organisms [8], and therefore reports of ingestion by marine animals are numerous [9], as well as their physical and ecotoxicological implications [10–13]. Animals reporting ingestion of microplastics range from planktonic species [14,15], corals [16], and cnidarians [17] to fishes [18] and top predators [19]. Filter feeders and pelagic feeders exhibit the highest rates of microplastic consumption [20].

The most abundant types of polymers reported in marine settings are polyethylene (PE), polystyrene (PS), polypropylene (PP), and polyvinyl chloride (PVC) [21], polyethylene being one of the most abundant types in most common plastic products [22], such as plastic bags, stretch film, food packaging, etc. The chemical composition is a key factor for the ability of microplastics to reach different types of marine organisms. For example, plastics exhibiting a high buoyancy, such as low density polymers, can reach a higher number of marine organisms, especially filter feeders or plankton feeders [23]. On the other hand, high-density polymers are prone to sink, therefore reaching deposit feeders [24]. Different degrees of toxicological risks have been reported depending on the physical properties of microplastics, as polymers with a large and hydrophobic surface area, such as polystyrene, can adsorb a broader range of hazardous compounds found in the marine environment [25,26]. Examples are compounds such as polychlorinated biphenyls (PCBs), organochlorine pesticides, or bisphenol A [25], which have been reported to be carcinogenic, mutagenic, and endocrine disruptors [27]. The ingestion of microplastics could act as a pathway to expose organisms to these hazardous compounds [26]. Once in the organism, microplastics can accumulate or be eliminated via fecal pellets, although the dynamics of the process are still unknown in most cases. Fernandez and Albentosa [28] showed that, in *Mytilus* mussels, 85% of the microplastics were eliminated after 6 days of depuration, having microplastics with >10 µm fastest rates of elimination. Moreover, the egestion of microplastics by fecal pellets in this species can suppose an ecological problem, as microplastics can sink and contaminate the bottom sediments, reaching detritus feeders [29,30] and sediment-dwelling organisms [31]. However, Browne et al. [32] showed that microparticles (3 µm and 9.6 µm) ingested by *Mytilus edulis* could translocate from the gut to the circulatory system within 3 days and be retained for 48 days; therefore, not all the microparticles are egested, since some are stored in mussel tissues, at least for some time. Many marine organisms have been studied regarding microplastic's physical and toxicological effects, such as crustaceans, fish, and mollusks [21], *Mytilus* genus being the most studied within the last group. Detrimental effects in terms of ecotoxicological and inflammatory responses have been reported for this genus [33,34]. As an example, effects on energy budget, enzymes, and oxidative responses have been described in *Mytilus coruscus* after two weeks of exposure to polyethylene microspheres [35], as well as an increase in hemocyte mortality and reactive oxygen species (ROS) production in *Mytilus spp*. after 96 h exposure [36]. Détrée and Gallardo-Escárate [37] found changes at transcriptome level after an acute exposure to polystyrene microplastics (24 h) in *Mytilis galloprovincialis*, and Micic´ et al. [38] demonstrated that hazardous compounds found in marine environments can cause DNA damage and apoptosis in this species. As microplastics can enhance the accumulation of toxic compounds in organisms' tissues [33], their combined effect is also being studied. Recently, Han et al. [39] proved synergistic immunotoxic effects in *M. coruscus* when microplastics (500 nm size) were combined with antibiotics after 4 weeks exposure, arriving at the same conclusion as Tang et al. [40], in which the study of immunotoxicity and neurotoxicity effects of bisphenol A were aggravated in *Tegillarca granosa* when combined with microplastics (490 11 nm size) after 2 weeks exposure. Both experiments were carried out at environmentally realistic concentrations (0.26 mg/L and 1 mg/L MPs, respectively), although most of the experiments regarding microplastics exposure use unrealistic doses that are higher than those we can find in the water [20]. Moreover, the effects of microplastics on mussels at a physiological level are reflected in changes in the physical condition of the adults, such as a reduction in byssus production [41], attachment strength [42], or a reduction of the body index [43]; however, changes in the latter are dissimilar, as some authors had not found differences, or marginally significant differences [44–46]. For these reasons, it is possible that wild *Mytilus* populations exposed to high plastic pollution are, or will be endangered in the near future. Further investigations, especially for the body index due to the variety of responses given by different authors, should be carried out.

DNA damage caused by microplastics has often been measured using the comet assay (single-cell gel electrophoresis), which measures DNA strand breaks in single cells [47]. This technique has been commonly used in human cells, but it has lately gained importance in the environmental and genetic toxicology of microplastics for different organisms [48], such as earthworms [49], mollusks [34,50,51], or fish [52]. It is not clear if microplastics alone can cause DNA damage, as some authors have opposite results when using this technique [34,53,54], and neither for microplastics with added pollutants [45,55]. Although most of the studies regarding DNA integrity and strand breaks perform the comet assay, it has certain limitations, as it cannot detect the small DNA fragments produced during apoptosis [47]. If microplastics cause cell death—not only single DNA strand breakages detected by the comet assay—then a test of general DNA degradation, such as those employed by Micic´ et al. [38], could be used instead. This technique based on agarose gel can detect DNA degradation by the visualization of smears that can appear in the agarose gel indicating, depending on the brightness of the smear, the certain degree of degradation of the extracted DNA [56]. This technique has been previously used for studying DNA degradation on frozen beef [57] or for DNA degradation caused by oxidative damage [58]. The aim of the present study is to investigate physical and DNA integrity changes derived from polystyrene microplastics. For that purpose, the commercial species *Mytilus galloprovincialis* (Mediterranean mussel) was exposed to virgin polystyrene microparticles, polystyrene being one of the most common types of plastic found in the marine environment, at different concentrations during a medium time period (21 days). No hazardous compounds were added during the experiment in order to assess if microparticles can induce these changes without biomagnification of other compounds. Studies investigating the uptake of microplastic showed a high accumulation in gills [55,59,60], through microvilli activity and endocytosis [59]; thus, we expect that this organ will experience serious DNA damage.

2. MATERIAL AND METHODS

2.1. Experimental Design and Procedures

A total of 61 adult individuals of *M. galloprovincialis* were collected in January 2020 from El Puntal beach (43◦31′33′′ N, 5◦23′17′′ W), situated on the coast of Asturias (Spain). The individuals were immediately transported to the facilities of the Aquarium of Gijón (Asturias, Spain), where the experimental part was conducted. Mussels were allocated randomly in four independent 40 L tanks, with 16 mussels in the control and 15 mussels in each concentration analyzed and allowed to acclimate for one week. Subsequently, each group was exposed daily to four different concentrations of polystyrene microparticles (size 10 µm, density 1.05 g/cm3) for two hours for 21 days. Paul-Pont et al. [20] recommend to consider realistic ecosystem scenarios when de- signing experiments to assess the effects of exposure to microplastics on marine organisms. Exposure concentrations of microplastics chosen were realistic levels (in C1), similar to those we can find in the environment [20], and higher doses to which mussels could be further exposed (C2 and C3), in accordance with experiments performed by Lu et al. [61] in zebrafish (*Danio rerio*), and Wang et al. [35] in mussel (*M. curuscus*). Experimental treatments were control or C0 (no microplastics), group 1 or C1 (0.02 mg/L of microbeads), group 2 or C2 (0.2 mg/L), and group 3 or C3 (2 mg/L). In realistic conditions, intertidal mussels living in fluctuating environments are rarely exposed constantly to the same concentration of microplastics. Microplastics coming from the ocean or from adjacent rivers are carried by tidal movements and washed by waves, thus exposure is irregular and often recurrent. Thus, we have opted for an experiment of intermittent acute exposure (for a short time repeated over days). Mussels were daily transferred from the tanks to 5 L glass chambers where microbeads were added for two hours. The time of exposure was calculated based on a mussels' filtration rate of 300 mL/min; with 15 mussels in the water volume of the experimental chambers, the totality of water was filtered in less than two hours. Mussels were then transferred back to the tanks.

Mussels were kept in an open circuit of tanks with filtered and aerated seawater. Marine phytoplankton gel in a mineral suspension was used to feed the mussels every two days, always after the exposure to microbeads, not before. After 21 days, mussels were transported, in the same glass chambers where the experiment was conducted in order to avoid stress, to the facilities of the University of Oviedo. Once in the laboratory, gills of each mussel were immediately excised to prevent possible DNA degradation due to cell death during manipulation of the specimens, and then were preserved in 1.5 mL Eppendorf tubes with ethanol for further analysis. After leaving them to settle, the precipitate found in the bottom of the tubes was taken and placed on a glass slide and visualized under the microscope for examination of microplastics in the gills. The rest of the body was employed to calculate body condition index.

2.2. Microbeads Employed

Polystyrene particles were chosen because they have a medium density, and therefore can be present not only in the water column, but also in sediments. Moreover, polystyrene particles can release toxic monomers and other chemicals used for their manufacture [24], and therefore the potential effects that can be caused in marine organisms can be greater than other polymers. In this experiment, we used microparticles based on polystyrene $(C_8H_8)_n$, 10 µm size (std dev < 0.2 µm, coeff var < 2%), in aqueous suspension, 1.05 g/cm3 density, and 10% (solids) concentration (Sigma Aldrich, Germany, ref: 72986-10ML-F). Particle size used was 10 µm diameter, as smaller particles have the ability to translocate into the circulatory system in *M. edulis* [24], and this was beyond the scope of the present investigation.

2.3. Condition Index

Condition index (CI) is broadly used to measure the nutritional status of bivalves. In our study, the formula proposed by Baird et al. [62] was used:

$$CI = \frac{Soft\ body\ wet\ weight}{Total\ weight}$$

If the calculated index has a value between 0.15 and 0.25, it indicates that the bivalve has a good nutritional status [44]. As gills were previously taken, calculated CIs were expected to be lower than the real values if all the organs were intact. Thus, results are valid for comparison between groups but should not be taken as absolute indicators of the physiological or nutritional status for each mussel.

2.4. DNA Extraction and Electrophoresis

First, ethanol-preserved gills were dried and DNA was extracted using an extraction kit designed for the recovery of genomic DNA from mollusks (E.Z.N.A.® Mollusc DNA Kit) following manufacturer's recommendations. In brief: samples were homogenized and lysed in a high salt buffer (CTAB) with 25 µL of proteinase k, incubated at 37 ◦C overnight, and extracted with chloroform to remove mucopolysaccharides. DNA purification was performed through several centrifugations with different buffers (ML buffer, BL buffer, HBC buffer (guanidinium chloride), ethanol-based DNA wash buffer, and elution buffer 10 mM Tris-HCl pH 8.5), in order to remove salts, proteins, and other contaminants. The DNA extracted was then quantified using a spectrophotometer Shimadzu UV1280 at 260 nm wavelength. For each sample, aliquots of 5 ng/µL were prepared and then, 10 µL (total mass of 50 ng) of each sample was charged into an 1.3% agarose gel and run at 80 mV for 2 h, as indicated in Micic´ et al. [38] for the detection of apoptosis from mussel gill DNA. Molecular weight marker Perfect™ 100–1000 bp (EURx) was employed as ladder. Staining was performed with 2 µL of bromophenol blue sucrose solution and DNA was visualized on agarose gels under UV illumination NuGenius (Syngene) and photographed with a camera integrated in the same transilluminator.

and therefore the sample is classified as 1. When the genomic band is almost inexistent and there is a high amount of smear, DNA is considered highly degraded, and it will be classified as 4. In between, when the genomic band is bright and the smear is light, the DNA has certain levels of degradation (category 2); when the genomic band is lighter or difficult to see and the smear has some bright, the DNA is quite degraded (category 3) (Figure 1). Apoptosis would be detected as a DNA ladder with clear, distinguishable bands. Three independent observers scored each sample, and the mean was used for the statistical analysis.

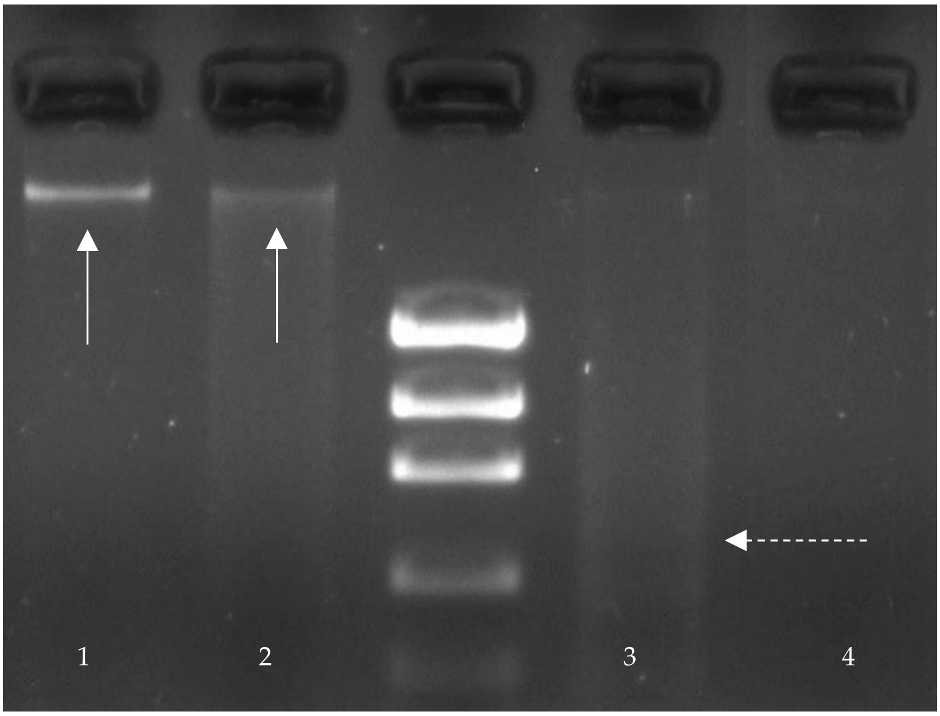

Figure 1. Samples scoring 1 to 4 for the different degrees of DNA degradation found in our study. Arrows shows genomic DNA, and broken arrow shows smear which indicates DNA degradation.

The DNA integrity was inferred from the migration pattern in the gel. Samples were classified into four different categories, depending on the level of DNA degradation, following criteria based on Quinet et al. [63]. When the genomic band is compact and completely defined with no smear or lighter bands, the DNA is considered not degraded,

2.5. Data Analysis

The software used for conducting all the statistical analysis was PAST software [64]. For the statistical analysis of the condition indexes, after checking normality with Shapiro– Wilk test, ANOVA analysis was performed in order to determine if there is any significant difference between the different groups, followed by a Dunn's multiple comparison test in order to resolve which specific means are significantly different from the others (Bonferroni corrected p values). For the DNA integrity, due to the non-normality of the data, a Kruskal– Wallis test was applied in order to determine if there were differences in DNA degradation between the four different groups, followed by post-hoc pairwise Mann–Whitney test to determine where the differences were.

3. RESULTS

3.1. Mussels' Status

No mortality was observed at the end of the experiment for any treatment, indicating that all of the effects of the experiment were at sub-lethal level. Acclimation was confirmed, as every mussel was attached to the bottom of the tanks after a week, and filter feeding was good, as every group had fecal pellets in the bottom of the tank. Microspheres were found in the precipitate of the gill samples treated, confirming that they were effectively adhered at the gill's tissue.

3.2. Condition Index

As expected, the condition index values were slightly lower than 0.15, which is the minimum value for which the nutritional status is considered optimal. Raw data are presented in Supplementary Table S1. Means ranged from 0.146 (SD 0.034) in group 2 to 0.113 (SD 0.025) in group 3 (Figure 2).

Statistical analysis showed a significant difference between the four different groups ($p = 0.0039$, $df = 3$, $F = 4.96$), and the Dunn's post hoc (Table 1) showed significant differences between group C1 and groups C2 and C3 ($p = 0.007$ and $p = 0.004$, respectively), and between C0 and C3 ($p = 0.033$), the difference between C0 and C2 being only marginally significant ($p = 0.053 < 0.10$). Thus, as visualized in Figure 3, three overlapping groups appear regarding CI: one with the best condition containing C1 and C0, another intermediate with C0 and C2, and finally the group with the worst condition containing C2 and C3.

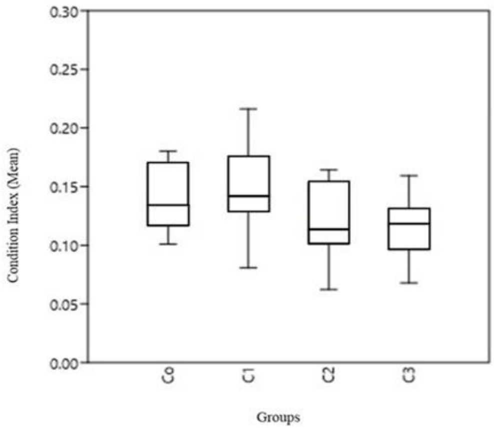

Figure 2. Boxplot for the condition indexes found in the different experimental groups.

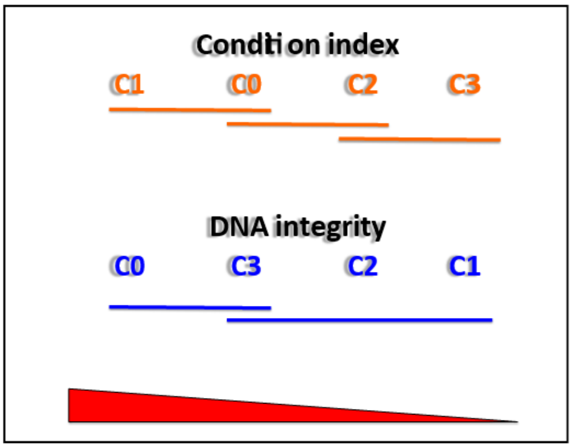

Figure 3. Graph showing the results of post-hoc tests for condition index (above) and DNA integrity (below) in the four experimental groups differences in bold).

3.3. DNA Integrity

High molecular weight DNA fragments (approximately 50–300 kpb) are seen as a band that migrates a short distance and can be interpreted as integer genomics DNA. Scores given to each individual by the three independent observers only differed in two of the 61 samples analyzed (Supplementary Table S1), for which the final score was the mean of the scores given by the three observers. DNA degradation in the C0

Table 1. Dunn's post hoc for condition index (Bonferroni corrected p values). Significant differences shown in bold.

	Control	C1	C2	C3
Control		0.423	0.053	**0.033**
C1	0.426		**0.007**	**0.004**
C2	0.053	**0.007**		0.845
C3	**0.033**	**0.004**	0.845	

(control) group was inexistent (Supplementary Figure S1), as in every sample the genomic band was perfectly defined, and in group 3 only one of the samples had a mark different from 1. Regarding C1, five out of fifteen individuals had a value different from 1, showing a certain degree of degradation, and lastly, group 2 differed from 1 in three individuals (Supplementary Table S1, Figure 4).

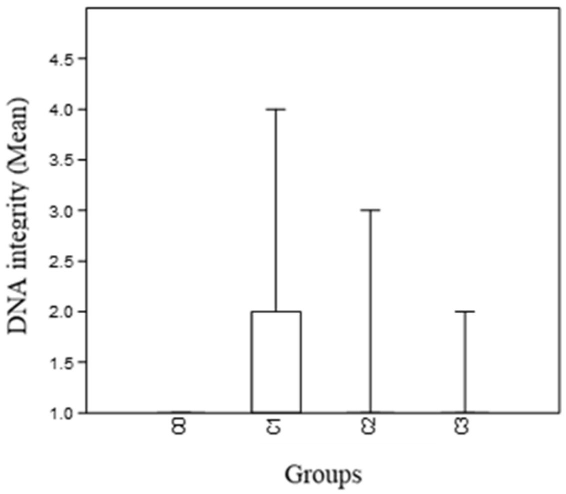

Figure 4. Boxplot for DNA integrity means in the different experimental groups.

The Kruskal–Wallis test performed showed a significant difference between sample medians ($p = 0.026$); and the Mann–Whitney test showed a significant difference between the control and group 1 ($p = 0.10$) and control and group 2 ($p = 0.045$) (Table 2). The post-hoc test exhibited a group containing C0 and C3, and another containing C3, C2, and C1The summary of the post-hoc tests displayed in Figure 4 shows that C3 and the control group (C0) are grouped together with a higher DNA integrity (low or unperceived DNA degradation), while C2 and C1 were grouped together. In Figure 5, we can see clearly that the trends of DNA integrity (in number of individuals of category 1) and CI are opposite. Clear signals of apoptosis, such as a ladder of multiple bands [38], were not detected with the method employed in this study. However, weak secondary light DNA bands were found for two treated individuals: individual 4 of C3, and individual 9 of C2 (Supplementary Figure S2). They might be early signals of apoptosis, but this cannot be ensured with this method.

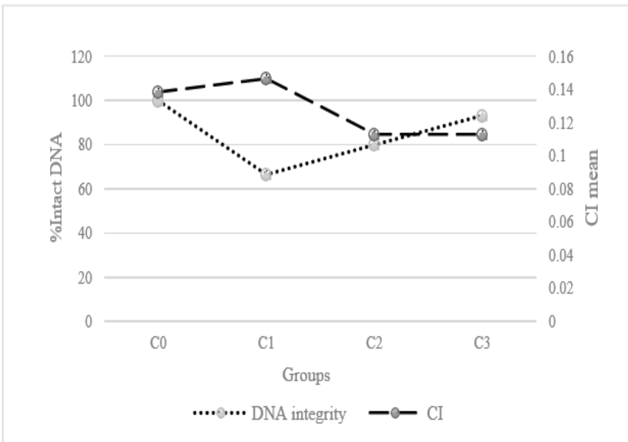

Figure 5. Diagram representing the trends of the different groups for condition index (CI), and the proportion of individuals with integer DNA (score = 1 for DNA integrity).

Table 2. Pairwise Mann–Whitney test results (p value above, U value below). Significant differences marked in bold.

	Control	C1	C2	C3
Control		**0.011**	**0.045**	0.178
C1	75		0.365	0.070
C2	90	95		0.292
C3	105	81.5	97	

4. DISCUSSION

Microplastic contamination can have numerous impacts in marine organisms. In this study, significant effects on the DNA integrity and body condition in *M. galloprovincialis* have been shown after a medium-term exposure (21 days) to microplastics. The physical status of the individuals measured though the condition index showed a clearly suboptimal status for groups C2 and C3 (mean CI = 0.14 and 0.11, respectively). This could indicate that feeding behavior or the nutritional status is altered by microplastic ingestion at not too high concentration levels (0.2 and 2 mg/L). These can be due to their filtering-feeding behavior, as bivalves present efficient rates of microplastic ingestion [65] and microplastics can accumulate at cellular and subcellular levels, with a higher concentration in gills and digestive system [59]. In fact, in our study, microspheres were detected in gills, which concur with the aforementioned studies. Moreover, microplastics can aggregate in digestive tissues and branchial epithelial cells [33], leading to a false satiated state, and decreasing fatty acid metabolism [65]. Therefore, the accumulation of microplastics in the digestive system may lead to a suboptimal health status, as shown in this study. Perhaps the effects cannot be generalized to all mussels; for example, Santana et al. [44] showed no physical impacts on the mussel *Perna perna* after 90 days of exposure to 0.1–1.0 µm PVC particles at 0.125 g/L. Studies reporting physical damage of microplastics on mussels normally use higher doses of microplastic concentration than the levels used in this study [34,44]; a novelty of our study is the confirmation that suboptimal conditions can be reached after a medium-term exposure to lower and realistic [20] levels of microplastics concentrations, especially for the lowest doses. Differences between groups in our experiment could be explained as mussels adjusting their ingestion rates by increasing their filtration, but only up to a maximum, after which they experience a decrease in the filtration: a process called a unimodal response [66]. Mussels that have a high daily dose of microplastics may have reached the maximum filtration rate for a high number of particles floating in the tank, accumulating more microplastics in their stomach; or, conversely, they may have decreased their filtration rates, leading either way to this suboptimal status. The second explanation is consistent with the study performed by Woods et al. [29] in which mussels (*M. edulis*) were shown to decrease their filtration rates at higher levels of microplastic concentrations, although results for the mentioned study did not show differences in the condition index. Wegner et al. [67] showed that mussels (*M. edulis*) exposed to nanopolystyrene beads (100 nm) were able to detect these particles, thus reducing the opening of the valve, and therefore reducing filtration rates. These explanations all together may explain the differences found between groups; in the case of C1, the particle concentration may be too low for the animals to detect the microspheres, or simply the plastic accumulation is not enough for a false satiated status.

In addition to physical damages, microplastics can cause grave problems in the DNA of filter-feeding organisms. In the present study, the DNA integrity in gills showed significant differences between groups, especially between C1 and C2 (intermediate microbeads concentrations, with more individuals exhibiting DNA degradation in gills) and the control. These results suggest that the direct physical interaction of the gills with polystyrene microparticles has altered DNA in the gill cells, perhaps increasing cell mortality, since microplastics accumulate in gills [55,60]. The physical interaction with virgin microplastics seems to trigger DNA strand breaks in hemocytes in *M. galloprovincialis* [33], and our results would extend DNA damage beyond strand breaks to higher degrees of DNA degradation, although not at the highest concentration assayed. Contrasting results of CI and DNA degradation (Figure 5) suggest a trade-off between physical and DNA damage in mussel gills. If mussels exposed to highly concentrated microplastics (such as C3) close their valves and reduce filtration rates [29,67], they will shorten the time of exposure and thus the rate of DNA damage. Mussels exposed to lower microplastic concentrations may have normal filtration rates, increasing their direct exposure of the gills to microplastics, and therefore having higher DNA damage but a better physical condition (Figure 5). This would explain the absence of dose dependence of DNA damage in mussels found in other experiments with microbeads, where individuals exposed to higher microplastics concentrations had no significantly higher DNA damage [13,53].

Regarding the experimental design, the type of exposure employed in our study does not fit with the typical models of exposure of most studies (acute or chronical), since mussels were exposed to microplastics exposure every day for only two hours. It is known that mussels have rapid ingestion and egestion rates of microplastics when treated with acute exposure to high microplastics concentrations, showing inflammatory responses due to cleaning and recycling processes occurring during digestion [68]. Acute exposure, especially the higher doses, can represent punctual microplastics spillages, as it happens during periods of heavy storms, sewer overflow, and drops in the efficiency of wastewater treatment plants [69]. On the other hand, mussels have the ability to acclimate to a chronical long-term exposure [44]. This could be the case of species that are constantly submerged in polluted waters with little or no movement, nor wave wash. However, mussels living in the intertidal, such as *Mytilus galloprovincialis*, would not fit to either of these models. Exposing mussels to acute and daily exposition, mussels are forced into a daily depuration process, without having the opportunity to acclimate to microplastics, simulating environmental conditions of the intertidal. This type of exposure is consistent with the recommendation of Paul-Pont et al. [20] of considering realistic scenarios when designing experiments to assess the effects of exposure on marine organisms.

Overall, although significant differences were found in DNA degradation, clear signs of apoptosis were not found in our study. Virgin pellets do not add the effect of toxic compounds found in the environment that can be adsorbed by microplastics and transferred into the mussel's tissue, magnifying their effects [70]. Avio et al. [33] showed that the differences between virgin pellets and contaminated pellets were not significant, independently of having pollutants adhered, which was the same conclusion as Pittura et al. [55]. Moreover, we used smooth microplastics, while microplastics with irregular surfaces or fibers can enhance the physical and DNA damage, as they can get entangled easily in the intestinal tract, prolonging retention rates, and therefore augmenting the time of damage. [14]. Genotoxic effects may vary greatly depending on the organism, the concentration and type of polymer, and even the shape [13]. Cole et al. [34] did not find DNA strand breaks in *M. edulis* after 7 days of exposure to different polymers sizes, and neither did Pittura et al. [55] after 28 days exposure to LPDE for *M. galloprovincialis.* In contrast, DNA damage has been reported for the same species in hemocytes exposed to virgin microplastics [33]: in the earthworm *Eisenia fetida* [49], in the clam *Scrobicularia plana* [50], and in fish larvae

[52]. Thus, it seems that the DNA degradation signals found in our work (with other methods) would concur with those results. Lastly, not only has DNA damage been reported in marine organisms, but also genotoxic effects derived from an exposure to polystyrene particles has been recently reported in human cells [71], possibly extending the problem of microplastics in the marine environment to human consumers in the near future.

The implications of our results are important for conservation. This species covers a wide geographical range and is a bioindicator of marine coastal microplastic pollution [72]. Their direct exposure to microplastics, due to their intense filter-feeding activity, makes this species more vulnerable to this type of pollution [73]. The ever-increasing concentration of microplastics in marine environments is leading to a broad range of physical and toxicological effects on marine animals [13], and therefore the real risk that these hazardous compounds may suppose should continue being assessed.

5. CONCLUSIONS

This study confirmed physical and DNA damage of polystyrene particles at environmental doses after a medium-term exposure of *M. galloprovincialis*. Alterations in the condition indexes were greater in mussels exposed to higher doses of microparticles; however, DNA damage in gills was lower at these higher concentrations. This may be interpreted from the active recognition of microplastics by mussels making them in order to reduce filtration rates at higher concentrations, lowering the physical condition but protecting the gills from direct physical interaction with microplastics. Overall, DNA damage was low but not negligible. Further investigations are recommended with different environmental levels of microplastic concentrations in order to understand the potential impact of this emerging contaminant in *Mytilus* mussels.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/ani11082317/s1, Figure S1: All the samples loaded in an agarose gel. From left to right: individuals 1 to 15 (16 for the control group). From up to bottom: each line represents one group (C0 to C3). Figure S2: Image of the two samples found with secondary light DNA bands (signalled with an arrow). Table S1: Condition indexes and DNA integrity scores for each individual.

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Capítulo 5

Bioremediation as a promising strategy for microplastics removal in wastewater treatment plants

Universidad de Oviedo
Universidá d'Uviéu
University of Oviedo

Masiá P, Sol D, Ardura A, Laca A, Borrell YJ, Dopico E, ... & Garcia-Vazquez E.

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Bioremediation as a promising strategy for microplastics removal in wastewater treatment plants

Paula Masiá[a,,1], Daniel Sol[b,1], Alba Ardura[a], Amanda Laca[b], Yaisel J. Borrell[a], Eduardo Dopico[c], Adriana Laca[b], Gonzalo Machado-Schiaffino[a], Mario Díaz[b], Eva Garcia-Vazquez[a]*

a Department of Functional Biology, University of Oviedo, Spain

b Department of Chemical and Environmental Engineering, University of Oviedo, Spain

c Department of Education Sciences, University of Oviedo, Spain

ABSTRACT

Microplastics (MPs) attract ever-increasing attention due to environmental concerns. Nowadays, they are ubiquitous across ecosystems, and research demonstrates that the origin is mainly terrestrial. Wastewater treatment plants (WWTPs) are a major source of MPs, especially fibres, in water masses. This review is focused on understanding the evolution and fate of microplastics during wastewater treatment processes with the aim of identifying advanced technologies to eliminate microplastics from the water stream. Among them, bioremediation has been highlighted as a promising tool, but confinement of microorganisms inside the WWTP is still a challenge. The potential for MPs bioremediation in WWTPs of higher aquatic eukaryotes, which offer the advantages of low dispersion rates and being easy to contain, is reviewed. Animals, seagrasses and macrophytes are considered, taking into account eco-ethical and biological issues. Necessary research and its challenges have been identified.

Keywords: Bioremediation Eukaryotes Microplastics Sludge Technologies Wastewater

1. FATE AND EVOLUTION OF MICROPLASTICS IN WWTPS

The aim of this review being to explore new ecologically acceptable ways to prevent MPs pollution by WWTPs, we will examine first the points where MPs can escape from WWTPs, the treatments currently employed for the capture and elimination of MPs, and then focus on bioremediation and specifically on higher eukaryotes as species that can be more easily contained inside WWTPs than unicellular organisms.

1.1. Legislation on microplastics

It has been estimated that 245 t of MPs, whose final destination is the aquatic environment, are generated every year. There is a lack of international regulation governing the production of microplastics, especially in the field of personal care and cosmetic products (Auta et al., 2017), but some countries such as Canada, Ireland, the United Kingdom and the USA are banning microbead production and products that contain them (Prata, 2018a). In the case of the USA, "The Microbead-Free Waters Act of 2015" is the legislation that bans the addition of plastic microbeads to products. This law came into force for manufacturers in

July 2017 and for retail sales in July 2018 (H. Rept. 114-371, 2015). Following the same line, in 2019, the European Chemicals Agency (ECHA) submitted a proposal to forbid the intentional addition of microplastics to different products. It has been estimated that the enforcement of this proposal would reduce the emission of these microcontaminants to the environment in the European Union by 85–95%, which means it would avoid the emission of approximately 400 thousand tonnes over 20 years. The legislation is expected to be ready by June 2020 and it would be subsequently sent to the European Commission for evaluation (ECHA, 2019). In addition, also in 2019, the European Parliament submitted a proposal (TA/2019/0071) to tackle microplastic pollution in wastewater treatment and those issues derived from sludge use as a fertilizer in agriculture (European Parliament, 2019). Proposing legislation on WWTPs is a good starting point for the implementation of measures that minimize dispersion of MPs in the environment.

1.2. Main sources of microplastics

Water is the main means by which MPs are transported (Alimi et al., 2018), so WWTPs receive millions of microplastic fragments every day (Okoffo et al., 2019). There is no linear correlation between population density and MPs concentrations in the inlet stream of WWTPs, but agricultural and industrial activities seem to be strong determinants (Eerkes-Medrano et al., 2015; Long et al., 2019). Recently, Bayo et al. (2020) demonstrated that another important factor is seasonal variability, the highest concentrations being found during hot periods, since temperature contributes to the acceleration of plastic degradation and fragmentation. Additionally, high concentrations of microplastics were also observed after rainfall events, due to urban runoff.

1.3. Evolution of microplastic at each stage of WWTP

The few studies that have analysed the evolution of microplastics in WWTPs report that, although these facilities do not completely remove these pollutants from wastewater, in some cases removal efficiency values higher than 90% are achieved (Bayo et al., 2020; Blair et al., 2019; Edo et al., 2020). MPs are defined as particles between 5 mm and 0.1 μm (Picó and Barceló, 2019), and although investigation has been carried out, completion of the task of establishing sampling, extraction and quantification protocols for microplastics smaller than 20 μm is still a huge challenge (Poerio et al., 2019).

The MPs classified as fibres and fragments are the most frequently observed types, making up, respectively, 57 and 34% of the total. In addition, fibres are the most difficult MPs to remove in WWTPs, due to their morphological characteristics (Ngo et al., 2019). Microplastics removal efficiency depends on treatment, operating conditions, sludge characteristics and microplastic buoyancy (Lusher et al., 2018; Nemerow, 2006). To understand MPs behaviour and fate during wastewater treatment processes, it is necessary to study in depth each stage of waste treatment in the processing plant. A scheme for a conventional WWTP is shown in Fig. 1. High variability of influent flow and composition makes it more difficult to obtain representative samples. This leads to underestimation of MPs concentrations because they can be retained in the sewage system by sedimentation (Lepot et al., 2017). Bigger microplastic particles in WWTP influent are removed by screening systems (screens, meshes) (Zhang and Chen, 2020). After that, the process for grit and grease removal takes place by means of sand sinking and grease floating, and during this step MPs are also separated from the wastewater stream by sinking and floating. It is noticeable that Murphy et al. (2016) reported that the highest MPs elimination efficiencies are achieved in the grit and grease removal systems. Lusher et al. (2018) indicated that 62% of microplastics extracted during WWTPs processes probably come from this specific separation phase.

Primary clarifier is used for removing settleable solids from wastewater, so during this sedimentation process, based on a pseudo-equilibrium approach (solid-liquid), some microplastics settle and others remain suspended in wastewater. The structure of suspended solids and their concentration have an important effect in solid-liquid separation (Sheng et al., 2008). A circular tank is the most frequently employed design, in which water enters centrally and radial flow towards the periphery allows sedimentation.

Sedimentation efficiency depends on different factors, such as retention time, temperature, type of flow and speed, system design, size and particle densities, etc. (Nemerow, 2006). For example, higher retention times increase the amount of settled solids (so it is also expected that the amount of settled MPs increases); high temperatures decrease the density of the medium, which also favours sedimentation.

Secondary treatment is a biological process that allows the biodegradation of organic matter. It is usually carried out under aerobic conditions, so it is necessary to supply oxygen to the microorganisms by wastewater aeration. During this aeration process, which is also pseudo equilibrium (solid-liquid-gas), some microplastics could pass to the atmosphere since it is well known that MPs can be found in air (Chen et al., 2020; Enyoh et al., 2019; Prata, 2018b).

The conventional activated sludge process (CAS), which basically involves the biological oxidation of carbonaceous organic matter, and subsequent separation of treated water from solid particles through sedimentation in a secondary clarifier, are commonly employed to treat municipal wastewaters. It has been reported that secondary treatment can reduce the MPs concentration in water by between 96% and 98% (Lares et al., 2018; Michielssen et al., 2016).Tertiary treatment is the final cleaning step that improves wastewater quality before it is reused, recycled or discharged to the environment. It usually consists of a disinfection process to kill or inactivate pathogenic organisms, and chlorination and UV irradiation are the most common processes (Zhuang et al., 2015). In general, tertiary treatment has no effect on MPs removal (Prata, 2018a; Sun et al., 2019), but in some cases microplastics can be degraded by this treatment, i.e., it has been described that chlorination can reduce MPs concentration by 7% (Liu et al., 2019). Microplastics that remain in treated water are discharged into the environment in rivers or oceans (Galafassi et al., 2019; Uurasjärvi et al., 2020; Wang et al., 2019; Waring et al., 2018) and different studies have estimated that globally WWTPs discharge millions of microplastics particles every day. For example, Edo et al. (2020) reported the release of 300 million into the Henares river (Madrid) per day, while three WWTPs located in South Carolina discharged between 500 and 1000 million per day into Charleston Harbor estuary (Conley et al., 2019), despite the fact that the MPs removal efficiencies of the plants were 93% and 85–98%, respectively. In addition, it has been found that in rivers, MPs concentration is higher downstream of WWTPs than upstream (Li et al., 2020; McCormick et al., 2014; Shruti et al., 2019; Vermaire et al., 2017). Most microplastics removed in WWTPs are accumulated in the sewage sludge produced at each stage of the treatment process, especially in the primary and secondary clarifiers (Prata, 2018a; Sun et al., 2019). This sludge is widely applied to soils, so it can be an important source of pollutants, including microplastics (Barbosa Jr et al., 2020; Gherghel et al., 2019; Lassen et al., 2015). For example, 50% of annual sludge wastes generated in Europe and North America are employed as agricultural fertilizer and it has been estimated that these wastes contain a total amount of MPs of between 44,000 and 430,000 t (Hurley and Nizzetto, 2018; Lu et al., 2019). Furthermore, stabilization processes such as lime addition or anaerobic digestion do not reduce MPs concentration in sludge (Gatidou et al., 2019; Rolsky et al., 2020).

The effects of microplastics on soils have scarcely been studied, but some studies have indicated their ability to absorb toxic contaminants such as metals, polychlorinated biphenyls (PCBs) and polycyclic aromatic hydrocarbons (PAHs) (Caruso, 2019; Rodrigues et al., 2019; Xu et al., 2019), which increases the pollutant risks of MPs (Al-Odaini et al., 2015). Today, technologies to totally avoid the presence of MPs in sewage sludge are unrealistic, but the amount of microplastics in sludge can be reduced by, for example, improving the elimination of microplastics in grit and grease removal systems (Sun et al., 2019). Anaerobic digestion can also be considered as a potential way to reduce MPs in

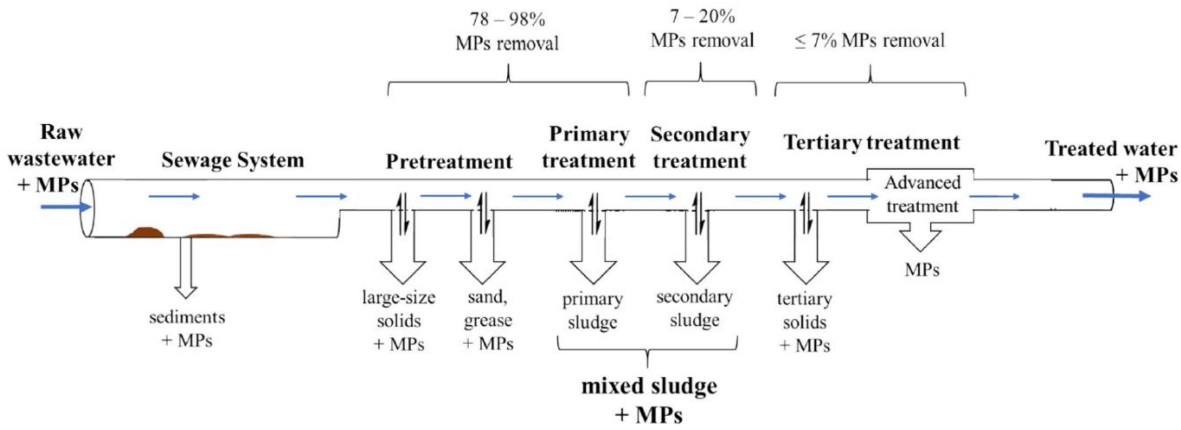

Figure 1. A schematic representation of WWTP processes and percentages of MPs removal during processing

stabilized sludge, but further research on this topic is needed, investigating areas such as bioremediation (Enfrin et al., 2019).

1.4. Advanced wastewater treatment

Although, in general, conventional WWTPs have high MPs removal efficiencies (≥90%), it is a fact that a large amount of microplastics is still discharged into the environment. For this reason, the use of advanced treatments, especially during the tertiary phase, could provide an alternative for reducing the concentration of MPs in treated water before it is discharged. A summary of different wastewater treatments that, according to the literature, have been proved to effectively remove microplastics from wastewater is seen in Table 1.

As can be seen in Table 1, dynamic membranes (DM) and membrane bioreactors (MBR) are, so far, the most efficient processes in terms of removing microplastics from wastewater, achieving MPs removal values as high as 99.9% (Li et al., 2018; Talvitie et al., 2017). The main disadvantages of MBR are membrane costs, energy demand, fouling control and low flux. In comparison, dynamic membranes offer lower costs and energy consumption, but, in this case, the filter is easily clogged (Ersahin et al., 2012; Li et al., 2018; Poerio et al., 2019). MPs removal by means of different organisms such as bacteria, fungi and algae has been recently investigated, and bioremediation is a very interesting challenge (Shahnawaz et al., 2019). Eukaryotic species have received much less attention and their efficiency is unknown, even though they can accumulate MPs.

Table 1 A list of different treatment processes used in recent studies that analysed the microplastic removal efficiency of wastewater in WWTPs.

Treatment process	Removal efficiency (%)	Reference
Conventional activated process (CAS)	96–98	Lares et al., 2018; Michielssen et al., 2016
Oxidation ditch	97	Lv et al., 2019
Chlorination disinfection	7	Liu et al., 2019
Ozone	90	Hidayaturrahman and Lee, 2019
Coagulation/flocculation	47–82	Hidayaturrahman and Lee, 2019
Rapid sand filtration (RSF)	45–97	Magni et al., 2019; Michielssen et al., 2016; Murphy et al., 2016; Talvitie et al., 2017.
Anaerobic, anoxic, aerobic (A^2O)	72–98	Lee and Kim, 2018; Yang et al., 2019
Sequencing batch reactor (SBR)	98	Lee and Kim, 2018
Discfilter	40–98	Hidayaturrahman and Lee, 2019; Simon et al., 2019; Talvitie et al., 2017
Dissolved air flotation (DAF)	95	Talvitie et al., 2017
Reverse osmosis (RO)	90	Ziajahromi et al., 2017
Dynamic membrane (DM)	99	Li et al., 2018
Membrane bioreactor (MBR)	≥ 99	Lares et al., 2018; Michielssen et al., 2016; Talvitie et al., 2017
Ultrafiltration (UF)	42	Ziajahromi et al., 2017

2. USE OF HIGHER EUKARYOTES FOR MPS BIOREMEDIATION IN WWTPS

Microplastics are present in all the oceans and in marine organisms worldwide (e.g., Andrady, 2011; Wright et al., 2013; Zhao et al., 2014; Lusher et al., 2015). As we have commented above, WWTPs are major sources of MPs pollution (e.g., Eerkes-Medrano et al., 2015; Murphy et al., 2016; Ziajahromi et al., 2017). Fibres and small fragments escape the filtering processes and are not efficiently retained in WWTPs; consequently, coastal cities are hotspots for MPs entering the ocean (Browne et al., 2011; Murphy et al., 2016). WWTPs discharging to rivers also contribute to ocean pollution because MPs transported by the current finally enter the sea; river mouths are also hotspots for MPs pollution (Leslie et al., 2017). Finding efficient and ecologically friendly ways of retaining MPs in WWTPs is urgently needed to prevent marine MPs pollution. Here we will focus on bioremediation, which is already employed for removing pollutants such as hydrocarbons or phosphates from WWTPs (Kshirsagar, 2013; Gargouri et al., 2014).

The use of living organisms for MPs bioremediation is still a challenge. Most research has been done on bacteria and lower eukaryotes (fungi). The main problem with these small organisms is their containment within WWTPs to prevent their unwanted release into the ecosystems (Nuzzo et al., 2020). Containing bigger organisms like higher eukaryotes could, in theory, be easier, but their application in MPs bioremediation is still an alternative which has received little attention. This section focusses on the potential of aquatic higher plants and animals for MPs bioremediation.

2.1. *Characteristics relevant to the use of higher eukaryotes for bioremediation*

Candidate species should possess several features (Fig. 2). First, to comply with animal welfare legislation (European Directive 2010/63/ EU http://data.europa.eu/eli/dir/2010/63/oj), species that suffer as a result of exposure to MPs cannot be employed. Under this Directive, vertebrates, decapods and cephalopods, whose capacity for suffering is recognized, should be

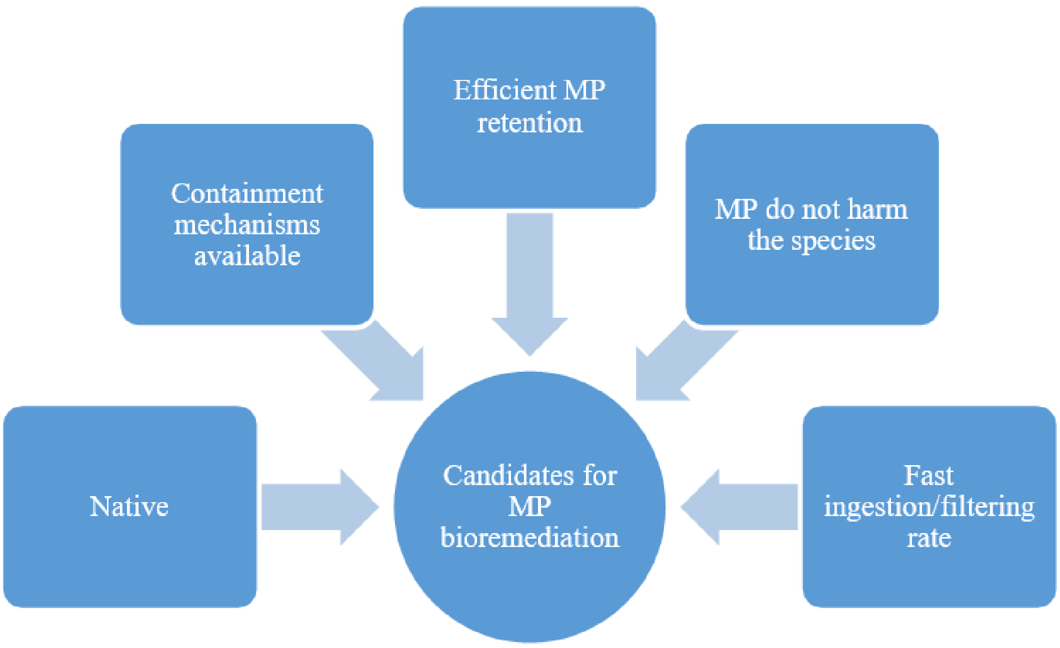

Figure 2. Characteristics required by candidate species for MPs bioremediation in WWTP.

disregarded for use in bioremediation. Secondly, the capture, retention and filtration/ingestion rates of MPs should be high, as should be their digestion/elimination, and, furthermore, they should not be returned to the environment. Species should be employed only within their native range, since geographical transfers must be absolutely disregarded for biodiversity conservation reasons (Molnar et al., 2008). Species with a broader distribution, easy control and management, would be more suitable. Finally, since the use of a species for WWTP treatments implies growing it inside or near treatment plants, containment measures for preventing releases to the environment should be efficient.

2.2. Potential of marine animals

Marine animals, from zooplankton (Frydkjær et al., 2017) to top predators (Alomar and Deudero, 2017; Masia et al., 2019; Zhu et al., 2019), ingest MPs. MPs are harmful to them (Anbumani and Kakkar, 2018), although their toxic effect in the wild is still unknown because most experiments conducted in laboratories use higher MPs densities that those observed in the environment (Lenz et al., 2016; de Sá et al., 2018). Further studies with higher ecological validity, i.e., with realistic amounts of MPs, are needed. Among the marine invertebrates (excluding Decapods and Cephalopods), many active-feeding species are not suitable for bioremediation purposes due to low retention rates. Gastropods rapidly expel MPs in faecal pellets (Gutow et al., 2015). Copepods also expel MPs in faecal pellets efficiently (Cole et al., 2013), as do amphipods (Au et al., 2015; Blarer and Burkhardt-Holm, 2016), while the Cladocera Daphnia magna expels MPs at different rates depending on their shape (Frydkjær et al., 2017). However, species that combine deposit feeding and predation, like the echinoderm *Ophiomusium lymani*, accumulate more MPs fragments and fibres (1.96 ± 0.66 to 3.43 ± 1.35 micro- plastics/g) than exclusive predators like *Hymenaster pellucidus* (0.48 ± 0 to 9.10 ± 4.21 microplastics/g) (Courtene-Jones et al., 2019). This suggests better ingestion and retention of MPs by filter- feeding or deposit-feeding organisms. Filter-feeding organisms seem to have some potential for MPs retention. *Mytilus* mussels retain pollutants, and thus serve for bioremediation in natural ecosystems (Broszeit et al., 2016). MPs fragments can be retained in their circulatory system for 48 days (Browne et al., 2008); however, most MPs fibres, which are abundant, are excreted after 24 h, reducing their eliminatory efficiency (Chae and An, 2020). Other filter-feeders like cnidarians gained interest among the scientific community because adhesion to the coral surface seems to be an effective mechanism for MPs retention (ingestion rates of 0.25×10^{-3} to 14.8×10^{-3} microplastic particles h^{-1} were observed, while adhesion to the surface was 40 times greater; Martin et al., 2019). However, although the responses to MPs vary among species (Reichert et al., 2018) it seems that MPs alter coral feeding behaviour (Hall et al., 2015; Murphy and Quinn, 2018), and cause a reduction in anti-stress capacity and the immune system activity (Tang et al., 2018). So, unfortunately, since tropical coral reefs are highly affected by climate change (Hoegh- Guldberg et al., 2007), tropical corals should not be used for bioremediation.

The sandworm *Arenicola marina* has a retention rate of 240–700 MPs over its lifetime (1.2 ± 2.8 particles/g), apparently without impacts on its metabolism (Van Cauwenberghe et al., 2015); it could be a possible candidate for bioremediation in sea and brackish waters because it tolerates salinities down to 12 ppt. More studies should be carried out into possible harm caused by MPs in this species. Another promising organism is the echinoderm sea cucumber, which has been proposed for pollution monitoring (Mohsen et al., 2019), and may be suitable for removing PCB-contaminated plastic as it selectively ingests plastic particles over other types of sediment particles (Graham and Thompson, 2009). Thus, these sediment feeders would be suitable during the bioremediation process for the solid phase in wastewater treatment plants. However, MPs affect the embryonic development of other echinoderms like sea urchins (Nobre et al., 2015) and therefore, without an analysis of the impact of MPs on holothurian health, their use for bioremediation cannot be proposed.

In summary, sandworms and holothurians are promising for MPs bioremediation, but animal welfare issues are still a concern. Further experiments should investigate the impacts of MPs on them before proposing applications for WWTP treatment.

2.3. Potential of aquatic higher plants

The greatest advantage of higher plants over animals is that there is no evidence of suffering. Algae, specifically microalgae, have been tested for bioremediation potential in water. Roccuzzo et al. (2020) described unicellular microalgae that, alone or combined with bacteria, can degrade endocrine disrupting chemicals in wastewaters. Seaweeds like *Fucus vesiculosus* can retain suspended MPs on their surface (Gutow et al., 2015). Removal of other pollutants, such as heavy metals, by means of bioremediation has already been studied. Phytoextraction is a technology proposed in 1995 by Salt et al. (1995) whereby plants that can accumulate metals and store them in harvestable parts are used to extract these pollutants from soil. Rhizofiltration is another method proposed by the same authors to eliminate heavy metals from water, instead of soils, through their roots. Therefore, the same approach could be used for MPs extraction both in the solid and in the liquid phase, by growing them in WWTPs.

Seagrasses are of interest for treating effluents near the sea because they can grow in marine and brackish waters. Soumya et al. (2015) proposed the smooth ribbon seagrass *Cymodocea rotundata* for bioremediation on textile dye effluent. Recently, attention has been paid to the relation between seagrasses and MPs. The first evidence in situ showed MPs adherence to seagrasses by encrustation, to epibionts associated with the macrophyte, and by adhesion to the polysaccharide mucus layer (Goss et al., 2018; Seng et al., 2020). Seagrasses can thus act as a trap or sink of MPs (Huang et al., 2020; Jones et al., 2020), suggesting they have potential for sludge treatment – if they can be grown on sludge. For example, the Caribbean angiosperm *Thalassia testudinum* has MPs encrusted by epibionts on its blades (average of 0.75 ± 0.25 beads/blade; and 3.69 ± 0.99 microfibres/blade) (Goss et al., 2018), and far away in Scotland, *Zostera marina* beds accumulate MPs in higher concentrations than bare sandy sites (average of 4.25 ± 0.59 MPs in blades; and 4.50 ± 0.96 MPs in seagrass-associated biota) (Jones et al., 2020). Herbivores eating these seagrasses will introduce MPs in their diet and transfer them to higher levels in the trophic chain; but perhaps growing this plant in controlled conditions on WWTP sludge could help to prevent MPs from reaching the open sea. Seagrasses are generally very sensitive to pollution, especially of nitrogen (e.g., Fernandes et al., 2019), but some species are more resistant (O'Brien et al., 2018) and could theoretically grow in disturbed areas like the outfall or the sludge of WWTPs.

Regarding other higher plants, Ali et al. (2020) proposed several freshwater Magnoliophyta for removing heavy metals in WWTPs: water hyacinth (*Eichhornia crassipes*), water lettuce (*Pistia stratiotes*) and Duck weed (*Lemna minor*), among others. It seems that at environmentally realistic concentrations, nano- and microplastics do not pose ecological risks to aquatic macrophytes. Some macrophytes like *Egeria densa* and their associated microbiome can accumulate and transform gold nanoparticles (Avellan et al., 2018); these systems could be investigated for MPs bioremediation. Since few macrophytes would grow well in brackish or seawater (Haller et al., 1974), their use for MPs retention, yet to be investigated, would be recommended for freshwater WWTPs. Summarising, seagrasses could be the optimal candidate for MPs bioremediation in marine and brackish WWTP, and aquatic macrophytes with their associated microbiota in freshwater WWTP. Further investigations should target the best methods for growing resistant seagrasses in sludge waters, the capacity of local species for MPs retention, and methods to ensure containment of species propagules. The latter objective is important for avoiding diversity disturbances outside the WWTP.

2.4. Fate of MPs in eukaryotes

As mentioned above, one of the characteristics that a species should possess in order to be a candidate for use in bioremediation is for it to efficiently digest and/or eliminate MPs, without returning them to the environment. Many species have been dismissed for this reason, as their retention rate is low (e.g., active-feeding species), and others because MPs cause the animal harm. Translocation of MPs from the digestive tract to other organs may occur in aquatic animals, as reported in fish brain and liver (Collard et al., 2017; Mattsson et al., 2017), but it is probably uncommon (Jovanović, 2017) and hardly damages the animal; if it did, removing MPs from an organ without killing the individual would be very difficult. For animals that retain MPs in the digestive tract without apparent harm, such as sandworms and echinoderms (Graham and Thompson, 2009; Van Cauwenberghe et al., 2015), the optimal accumulation time for efficient retention without harming the animal should first be studied carefully. For the elimination of MPs retained in the digestive tract, after a time in the WWTPs, individuals could be removed and placed in a clean environment where they could eliminate gut MPs by defecation; and then returned to the WWTP while the defecated MPs are disposed of. Ideally, the organisms would be grown in aquaculture facilities, transferred to WWTPs, and left there for the time considered optimal for MPs accumulation without animal harm. Then the individuals could be transported back to culture facilities for MPs disposal. The whole process should be conducted in such a way as to avoid animal suffering.

In seagrasses and higher plants, MPs retention may take place in different ways, with the particles accumulating on the blades and also their associated microbiota. In *Thalassia testudinum*, MPs are retained in the epibiont communities on the blades (Goss et al., 2018), while MPs, especially microfibres, have been found attached to blades without epibiont communities in the seaweed *Fucus vesiculosus* (Gutow et al., 2015). As no relation between epibiont communities and MPs density have been found (Seng et al., 2020), a wide range of seagrasses and algae species could be valid for bioremediation. Moreover, not only blades retain MPs. Mangrove rhizospheres have been shown to act as a sink of MPs (Li et al., 2019), and sediments of seagrasses like *Enhalus acoroides* and *Zostera marina*, can trap MPs as well (Huang et al., 2020; Jones et al., 2020). Information about patterns and efficiency of MPs accumulation in seagrasses and macroalgae is scarce, and further studies in this field should be done.

Given the diversity of retention mechanisms in higher aquatic plants, MPs elimination could be approached differently depending on the species. Generally, plants could be grown in WWTPs from the stage at which MPs retention is efficient, then the parts of plants where MPs are retained, sediments, or the whole plants, could be harvested for disposal of the MPs. Systems for preventing dispersal of small propagules (seeds, spores, others) should be designed in order not to disturb surrounding ecosystems, something that may be caused by artificial propagation even if the species are local.

3. CONCLUSIONS

WWTPs are not intentionally designed for the removal of MPs, and despite having an efficiency of retention $\geq$ 90%, millions of microplastics are still released into the environment every day, not only by treated water discharge, but also by sewage sludge use for soil improvement. Consequently, these facilities are considered to be an important source of release of MPs into aquatic environments. Some higher eukaryotes have potential for elimination of MPs from WWTPs. Animal candidates may be annelids (sandworms), echinoderms (sea cucumbers) and perhaps other groups still not investigated. Seagrasses and macrophytes seem to be good candidates, with certain precautions for containment of species propagules. The results of this review suggest that the following research and management actions could be recommended:

a) Targeting of WWTPs as priority hotspots for the avoidance of microplastics discharge into the environment.

b) The improvement and implementation of advanced processes in tertiary treatments to remove a greater amount of MPs from treated water.

c) Exploring bioremediation as a potential alternative in order to degrade or accumulate microplastics in wastewater treatment, depending on the species considered.

d) Investigation of new technologies and biotechnologies to efficiently eliminate MPs from sludges.

e) Assessment of the efficiency of candidate species for retaining MPs at realistic environmental concentrations.

f) The improvement of cultivation, manipulation and management of choice species, with special attention to containment inside WWTPs, and animal welfare if animals are employed.

Declaration of competing interest: The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Discusión

La presente tesis profundiza en un campo antes inexplorado en la costa asturiana, donde se analiza y cuantifica por primera vez la presencia de microplásticos, su distribución y posibles orígenes, así como su presencia en las especies que la habitan, profundizando en las posibles implicaciones para las mismas y el consumidor final. Además, se analiza la utilidad de las actuales plantas de tratamientos de aguas para la retención de los microplásticos como contaminantes emergentes y se estudia la biorremediación como posibles vía alternativa para mejorar esa retención.

1. Origen y distribución de la basura plástica marina en la costa asturiana

Hasta el momento de comenzar esta Tesis, la cantidad de microplásticos en la costa asturiana no había sido cuantificada. Anteriormente se había analizado la cantidad de basura visible en playas asturianas, con un máximo de 1,695 ítems/m^2, en Navia (oeste de Asturias), y un mínimo de 0,006 ítems/m^2 en Andrín (este de Asturias), donde el plástico representa el 64% de la basura encontrada, especialmente los fragmentos más pequeños (2,5- 10 cm) (Rayón-Viña et al., 2018). Los datos obtenidos para macro- y meso-plásticos en la presente Tesis son coherentes con esos datos previos, variando entre 0 y 54 ítems/m^2 por playa, lo que indica que algunas estaban muy limpias; tal como era de esperar (Rayón-Viña et al., 2018), la limpieza de playas correlacionó significativa y negativamente con los macroplásticos. En cambio, todas las playas estudiadas, con o sin servicio de limpieza, contenían microplásticos (MPs), entre 58 MPs/kg en Otur (oeste de Asturias) y 382 MPs/kg de arena en Rodiles (centro-oeste) (Tabla 2). Estas cifras, que muestran una considerable variación en una longitud de costa relativamente pequeña, sitúan a la costa de Asturias en un nivel medio-alto en comparación con las medias de microplásticos en costas europeas (150-250 MPs/Kg) (Lots et al., 2017). De todos los microplásticos encontrados en esta tesis, más del 98% fueron fibras, coincidiendo con el 98,7% encontrado en las costas europeas en ese mismo estudio. Se puede considerar que los resultados son coherentes con la literatura científica previa, y también que zonas relativamente limpias respecto a la basura visible pueden estar altamente contaminadas por microplásticos invisibles al ojo humano.

Las playas con mayor cantidad de microplásticos fueron las abiertas directamente al océano, más contaminadas que en aquellas situadas en el interior de estuarios o semicerradas (Gulpiyuri y Cobijeru). Esto indica que al menos parte de los microplásticos encontrados en la costa asturiana tienen su origen en aguas abiertas. La Corriente del Atlántico Norte, paralela a la costa asturiana, pasa a su vez por el Giro del Atlántico, los cuales tienden a acumular una gran cantidad de microplásticos (Law et al., 2010); éste, en particular, se estima que contiene de 13.000 a 174.000 piezas de pequeños microplásticos (25-1000μm) por kilómetro cuadrado, y entre 5 y 170 veces más de microplásticos grandes (1-5mm) (Poulain et al., 2018). Por tanto, es probable que esta corriente transporte microplásticos procedentes de estas zonas de alta densidad en aguas abiertas y las deposite en las costas asturianas.

Respecto a los posibles focos de contaminación, se encontró que la densidad de microplásticos y de macroplásticos está significativamente asociada a zonas cercanas a puertos, y a la actividad pesquera. Este resultado es muy coherente con otros estudios, ya que se sabe que los puertos y los barcos en general, y las actividades pesqueras y acuícolas en particular, son fuentes de contaminación de distintos fragmentos plásticos (Browne et al., 2011), siendo los mayores responsables de aparejos de pesca perdidos y redes fantasma (Richardson et al., 2021). Se pueden añadir también los microplásticos procedentes del dragado de puertos (Preston-Whyte et al., 2021), y del agua de lastre (Matiddi et al., 2017). No se puede excluir la aportación adicional de microplásticos por parte de los ríos, ya que en esta región muchos puertos están localizados dentro de estuarios o en su proximidad.

La mayor concentración de microplásticos se encontró en la zona central de Asturias, donde hay mayor concentración de población y una mayor actividad industrial (Figura 7). Estos resultados concuerdan con estudios que muestran que las zonas urbanas densamente pobladas son una fuente importante de microplásticos (Browne et al., 2011; Qiu et al., 2020).

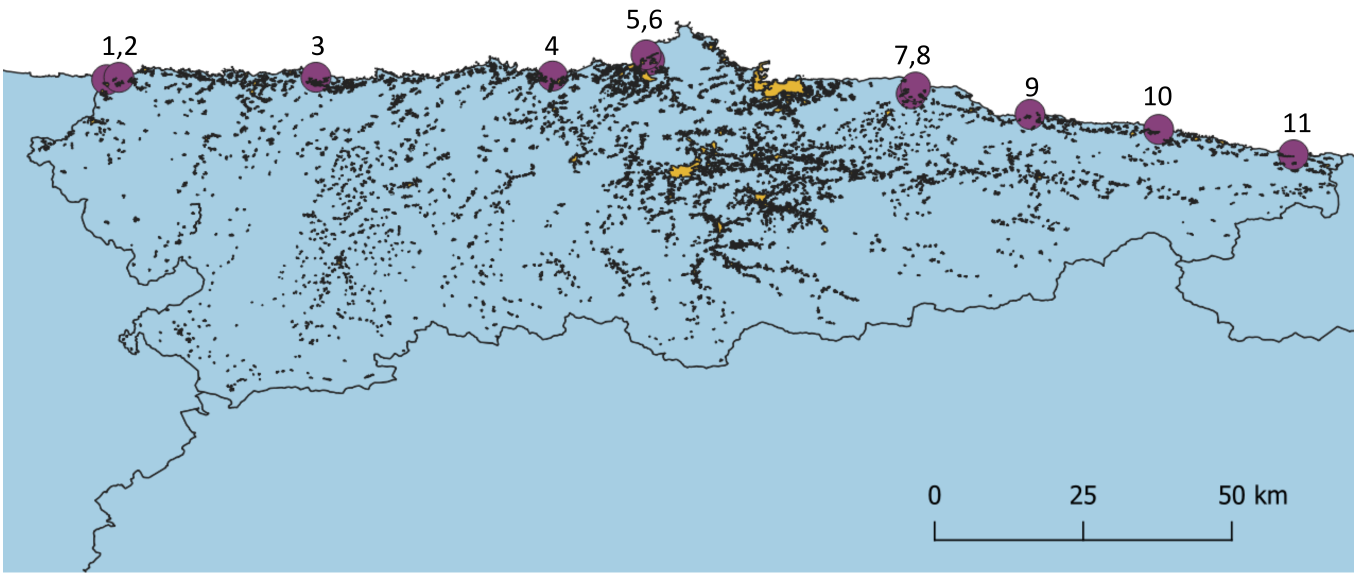

Figura 7: Mapa de Asturias con las zonas muestreados representados por los puntos morados (de izquierda a derecha: 1-Arnao, 2-Peñarronda, 3-Otur, 4-Aguilar, 5-Zeluán, 6-Xagó, 7-Rodiles, 8-El Puntal, 9-Vega, 10-Gulpiyuri y 11-Cobijeru); y la presión antropogénica representada mediante puntos negros por la cantidad de población de la región. Fuente: Paula Masiá.

Por tanto, el origen de los microplásticos en la costa asturiana sería una combinación de todos los factores comentados: desde el océano transportados por corrientes y mareas, a través de los puertos y las actividades pesqueras, y a través de los ríos como fuente de microplásticos de origen terrestre y procedentes de las ciudades costeras.

Respecto a las plantas de tratamiento de aguas, pese a ser fuente de gran contaminación por microplásticos, especialmente de fibras (Estahbanati & Fahrenfeld, 2016; Murphy et al., 2016; Magni et al., 2019), en este estudio no se encontró relación entre ellas y la densidad de microplásticos en las playas estudiadas. El origen de la contaminación por plástico en la región es por tanto complejo y depende de muchos factores. Hacen falta más estudios de este tipo para identificar el origen de los distintos tipos de plástico y mejorar la gestión de este tipo de basura tomando medidas más adecuadas para evitar cada uno de ellos.

2. Microplásticos en especies marinas de la costa asturiana

En la presente tesis se estudió por primera vez la cantidad de microplásticos en deposiciones de aves migratorias (el cormorán *Phalacrocorax aristotelis* y las gaviotas *Larus michahellis* y *Chroicocephalus ridibundus*) dentro de áreas asturianas de especial protección para aves, específicamente en el ZEPA ES1200055 Cabo Busto-Luanco. La playa de Zeluán donde fueron recogidas las muestras tiene además el estatus de Monumento Natural (Decreto español 100/2002), con una protección adicional. Se encontraron microplásticos en todas las deposiciones analizadas, con una media cercana a 13 ítems por deposición. Esta cantidad es elevada en comparación con otros estudios realizados en aves. Por ejemplo, en la especie *Fulmarus glacialis* sólo se encontraron microplásticos en un 47% de las muestras de heces analizadas, de los cuales el 97% eran fibras (Provencher et al., 2018); los resultados coinciden en la proporción de fibras, pero la proporción de heces contaminadas es mucho más baja en Provencher et al. (2018). Por otro lado, en un estudio realizado en las islas Inishkea al oeste de Irlanda (Coughlan et al., 2020) se encontró que el 78,9% de las muestras fecales de la especie *Branta leucopsis* contenían microplásticos (media de 4,7 ± 0,9). Este resultado, aunque sigue siendo más bajo, está más próximo a los encontrados en Asturias. Los datos de esta región sugieren que la alta presión antrópica (alta población y numerosas industrias próximas a Zeluán) podría ser la razón de la alta incidencia de fibras encontradas. La mayoría de microfibras, coincidente con otros estudios, puede deberse también a la contaminación atmosférica superior por microfibras en zonas con alta incidencia antrópica como la estudiada (Dris et al., 2016). Está demostrado que las aves contienen microplásticos en el sistema gastrointestinal, como por ejemplo en varias aves de presa (6,22 ± 2,46 MP/individuo), en *Fulmarus glacialis* (19,5 ± 2,1 items/individuo), *Ardenna grisea* (13,3 ± 3,5 items/individuo), *Larus michahellis* (0,8 ± 7,11) entre muchas otras (Basto et al., 2017; Terepocki et al., 2017; Carlin et al., 2020). Más aún, el género *Fulmarus* se usa como indicador de basura marina según la OSPAR (Oslo/Paris Convention for the Protection of the Marine Environment of the North-East Atlantic) y la MSFD (Marine Strategy Framework Directive) (Van Franeker & Law, 2015). Los datos relativamente elevados de microplásticos en heces de aves encontrados en esta Tesis apuntarían a una contaminación elevada en la región, ya sugerida con los datos de las playas. Al encontrarse en zonas de especial protección para aves, parece claro que hay que implementar soluciones para controlar este tipo de contaminación, prestando especial atención en una mejora de la gestión de residuos en la proximidad de espacios protegidos.

El otro organismo estudiado en la presente tesis es una especie de alto valor comercial y gran consumo humano, el mejillón mediterráneo *Mytilus galloprovincialis.* Esta especie es de gran importancia en la región, donde la pesca artesanal tiene gran importancia a nivel cultural. El consumo de pescado y marisco es alto, siendo la especie de estudio una de las más abundantes (García de la Fuente et al., 2013, 2020). Todos los individuos muestreados contenían microplásticos, con una media de 2,7 MP/individuo (1,6 MP/g).

Se observó una correlación significativa entre la cantidad de microplásticos presentes en los organismos y el sedimento, además de una evidente biomagnificación al ser la densidad en los organismos 10 veces mayor que la de los sedimentos. La correlación significativa apoya la idea de usar el género *Mytilus* como bioindicador de contaminación por microplástico, como ya se utilizan para otros contaminantes marinos como los metales pesados (Azizi et al., 2018; Li et al., 2019). La falta de correlación con los microplásticos del agua en este trabajo podría deberse a que la concentración en el agua varía según las corrientes, mareas y el viento (Isobe et al., 2014), mientras que los microplásticos permanecen en los sedimentos por periodos de tiempo más largos (Turner et al., 2019), al igual que en el interior de los mejillones (Fernández & Albentosa, 2019).

La concentración de microplásticos en esta estudio es similar a la encontrada por Reguera et al. (2019), aunque en las playas de Zeluán y Xagó es mucho mayor. Diferencias significativas entre playas situadas a pocos kilómetros concuerdan con resultados en mejillones de autores como Kazour & Amara (2020), con bastante diferencia entre zonas. Esto implica que el consumidor está expuesto a diferentes cantidades de microplásticos dependiendo de la zona local concreta de recolección o cultivo de los mejillones.

3. Riesgos y efectos del consumo de microplásticos

Los efectos adversos de los microplásticos sobre las especies que los consumen han sido demostrados en numerosos estudios (p.ej. Mallik et al., 2021), y hay una preocupación creciente acerca de los posibles efectos que puedan tener en humanos (Vethaak & Legler, 2021). En la presente Tesis se estimó la cantidad de microplásticos que se pueden ingerir a través del consumo de los mejillones y sal, y los resultados mostraron que la sal analizada aportaría cantidades reducidas, insignificantes en comparación con los que se podrían ingerir a través del consumo de mejillones (hasta 108 microplásticos dependiendo de la zona de recolección). Los datos de contaminación en sal de mesa de esta Tesis fueron relativamente bajos comparados con estudios como el de Lee et al. (2021), pero concuerdan con el de Karami et al. (2021), que concluye que el consumo de microplásticos a través de la sal es mínimo.

La composición química de los microplásticos encontrados en mejillones en esta Tesis fue diversa, incluyendo principalmente rayón, poliéster, polietileno poliestireno, y polidialil ftalato, y algunas sustancias químicas como cloroquina y acetaldehído. Siete de las sustancias encontradas están catalogadas como peligrosas para la salud humana según la ECHA (European Chemicals Agency: https://echa.europa.eu). Por ejemplo, el acetaldehído puede causar daño a los órganos e incluso cáncer, y otros como la anilina causan irritaciones en la piel y los ojos. Parece que la acumulación de microplásticos en mejillones podría tener por tanto consecuencias para el consumidor, además de para el propio organismo. Los mejillones pueden eliminar los microplásticos ingeridos en unos seis días (Fernández & Albentosa, 2019), pero algunos pueden translocarse desde el sistema gastrointestinal al circulatorio y a células epiteliales en esta especie (Browne et al., 2008; Von Moos et al., 2012). Una vez en el interior de los organismos, además de provocar daños físicos, pueden liberar los químicos adsorbidos, provocando numerosos impactos negativos tanto a nivel físico como celular (Anbumani & Kakkar, 2018). Para conocer mejor algunos de estos impactos, se estudiaron los daños físicos y a nivel de ADN de la especie *Mytilus galloprovincialis* tras la exposición a diferentes concentraciones ambientalmente realistas de microplásticos (microesferas de poliestireno de 10 µm de diámetro) en condiciones controladas de laboratorio. El factor de condición, utilizado para medir la condición física de los organismos, fue subóptimo en los grupos sometidos a mayor concentración de microplásticos (C2 y C3), probablemente por la capacidad de acumulación de microplásticos de los mejillones (Fernández & Albentosa, 2019), y de formar agregados en tejido digestivo y branquial (Avio et al., 2015), lo que da lugar a una falsa sensación de saciedad. Estos resultados difieren de los encontrados en el mejillón *Perna perna* tras exposiciones más largas a mayores concentraciones in daños físicos aparentes (Santana et al., 2019), pero hay que tener en cuenta que los daños genotóxicos varían mucho dependiendo de la especie, la concentración y el tipo de polímero al que se exponen (Prokic et al., 2019). Wegner et al. (2012) propusieron que los mejillones reconocen activamente los microplásticos y responden cerrando las valvas, reduciendo la tasa de filtración y, por tanto, su alimentación. Este sería el caso de los grupos expuestos a mayor concentración de microplásticos en este trabajo. A concentraciones menores se produce un proceso denominado respuesta unimodal, según la cual el mejillón incrementa su tasa de filtración al detectar más partículas suspendidas, pero a partir de cierto nivel la tasa de filtración disminuye (Newell et al., 2001). El grupo expuesto a baja concentración no disminuiría su tasa a filtración ni por tanto su alimentación, mostrando así un estado físico óptimo.

Otro efecto de la exposición a microplásticos fueron daños en la integridad del ADN de las branquias de los mejillones expuestos a concentraciones intermedias de microplásticos, pero no a las cantidades mayores. El daño en el ADN no fue proporcional a la concentración de microplásticos a los que se expuso al mejillón, lo que concuerda con otros estudios experimentales (p.e. Prokic et al., 2019). Parecen compensarse el daño físico y el daño en el ADN; los expuestos a concentraciones bajas tendrían mejor condición física al no cerrar las valvas, pero al mismo tiempo aumentaría la mortalidad celular (Brate et al., 2018) y por tanto degradación del ADN, mientras que a mayores concentraciones se reduce la tasa de filtración (Woods et al., 2018) disminuyendo el índice de condición, pero protegiendo a las branquias de la exposición directa y por tanto del daño a nivel celular.

El estado en el que se encuentran los mejillones de la costa asturiana podría ser similar a los que han sido estudiado en la presente tesis, ya que se han utilizado para su estudio niveles de microplásticos similares a los que se pueden encontrar en los océanos. En algunas zonas estarían expuestos a concentraciones de microplásticos que podrían reducir su condición a niveles subóptimos, y en otras quizás estarían expuestos a daños en el ADN. En este estudio no se vieron signos claros de apoptosis, pero se utilizaron microplásticos vírgenes, sin ningún químico añadido que magnifique la genotoxicidad (Wang et al., 2018). A estos daños encontrados habría que añadirle la posible interacción de estos microplásticos con otros contaminantes que se pueden encontrar en el océano. Por tanto, los microplásticos podrían poner en riesgo tanto la salud de la propia especie en la región, como de los humanos que los consumen. Se debería realizar una investigación más exhaustiva acerca del estado en el que se encuentran los mejillones de la costa asturiana.

4. Las plantas de tratamiento de aguas: alternativas de mejora

Las plantas de tratamiento de aguas, aunque son capaces de eliminar gran cantidad de microplásticos (Carr et al., 2016) y en la región no parecen suponer un factor de incremento de este contaminante, son focos significativos de contaminación (Li et al., 2018; Sun et al., 2019). Se exploró aquí la biorremediación, es decir, la utilización de organismos como mecanismo para mejorar la eficiencia de retención de microplásticos en estas plantas. Combinando la capacidad de retención de microplásticos con la capacidad de dispersión y el grado de sufrimiento de los organismos, se propusieron varias especies candidatas para su uso en este sentido.

Entre los candidatos propuestos, las algas y los macrófitos fueron los que más requerimientos cumplían. De hecho, algunas especies ya han sido propuestas como biorremediación de otros contaminantes. Por ejemplo, el alga *Cymodea rotundata* ha sido propuesta para eliminación de los efluentes de tintes textiles (Soumya et al., 2015), y la planta acuática *Pistia stratiotes* (lechuga de agua) ha sido propuesta para eliminación de metales pesados de aguas contaminadas (Ali et al., 2020). Además, estudios recientes han demostrado "in situ" la adherencia de microplásticos de ciertas macrófitas mediante diferentes mecanismos como la adhesión de éstos las capas de polisacáridos (Goss et al., 2018), y por tanto puede actuar como sumidero de microplásticos (Huang et al., 2020).

5. Recomendaciones derivadas de los resultados de la tesis y futuras líneas de investigación

En relación con todas las conclusiones de la presente tesis, una serie de recomendaciones y futuras líneas de investigación has sido propuestas a lo largo del trabajo en relación con los distintos problemas relacionados con los microplásticos:

5.a. Recomendaciones para la gestión de residuos plásticos

Se ha visto en este trabajo que los puertos son una fuente importante tanto de macro como de microplásticos. Por lo tanto, debería haber una actuación conjunta entre la administración y las cofradías de pescadores a nivel regional para implementar mejoras en la gestión de residuos plásticos. Un ejemplo serían las propuestas por Chen & Liu (2013): mejorar la regulación de la basura generada en barcos menores de 400 GT, promover el mejor uso de los contenedores de basura disponibles en los puertos, o proporcionar una mayor educación ambiental a los pescadores, entre otras.

Para controlar los macroplásticos, y por tanto los mesoplásticos, se sugiere extender la práctica de limpieza de playas a todas las de la región, no solamente a las más turísticas. Involucrar a la sociedad en esta tarea incentivando el voluntariado medioambiental sería también una forma de aumentar las conductas responsables respecto a la basura (Rayón-Viña et al., 2018).

Para mejorar las prácticas de gestión de microplásticos en las plantas de tratamiento, se propone la experimentación con distintas algas y macrófitos en dichas plantas de tratamiento a niveles medioambientalmente realísticos como línea prioritaria de investigación aplicada en este campo.

5.b. Recomendaciones para el consumidor

Dadas las diferencias entre playas en densidad de microplásticos encontrada a pequeña escala en esta región (entre playas), incluso en los sitios más cercanos entre ellos, y la elevada concentración en algunas de ellas, se propone limitar la recolección de mejillones, y marisco en general, a las zonas con una menor concentración de microplásticos. Para definir las zonas se deberían realizar muestreos regulares a lo largo del año, especialmente en zonas de recolección o de acuicultura.

Además, se propone monitorizar la cantidad de microplásticos en estas especies, e indicar la concentración en las etiquetas de los embalajes donde se distribuyen. Es decir, tratar a los microplásticos como cualquier otro contaminante de los que se encuentran en el océano que implica riesgos para la salud de los consumidores.

Conclusiones / Conclusions

Conclusiones

1) Por primera vez, se han cuantificado microplásticos, mesoplásticos y macroplásticos en playas de la costa asturiana, y se ha inferido su origen a partir de su relación con factores antropogénicos relacionados con contaminación plástica marina según estudios previos. Los servicios de limpieza en playa controlarían la cantidad de macroplásticos, e impedirían la formación de mesoplásticos in situ. Los puertos marítimos y las actividades pesqueras serían una fuente tanto de macroplásticos como de microplásticos. Se propone la implicación del sector marítimo y pesquero en el control de los plásticos marinos.

2) La cantidad de microplásticos en playas asturianas incluidas en la Red Natura 2000 y en la lista ZEPA (Zonas de especial protección para las aves) es medio-alta en comparación con otras playas europeas. Una contaminación superior en playas abiertas que dentro de las rías sugiere que al menos parte de microplásticos en esta costa tendría su origen en el mar. Por otra parte, se ha demostrado la ingestión de microplásticos en aves de estas zonas mediante el estudio de sus heces, confirmando la importancia ecológica de este contaminante.

3) La cantidad de microplásticos encontrada en poblaciones de la costa asturiana de mejillón mediterráneo (*Mytilus galloprovincialis*) está correlacionada con la cantidad de microplásticos de la arena, siendo 10 veces más alta y mostrando por tanto bioconcentración. Su consumo aportaría muchos más microplásticos en la dieta que la sal de mesa, cuya concentración es mucho menor. La composición de varios microplásticos encontrados tanto en mejillones como en sal de mesa, incluyendo aldehído y anilina, es considerada peligrosa para la salud humana. Se recomienda limitar el marisqueo a zonas con menor contaminación, para lo que es necesario un monitoreo regular de microplásticos. Sería recomendable incluir en las etiquetas comerciales la cantidad de microplásticos presentes en los productos marinos.

4) Se ha demostrado experimentalmente que la exposición intermitente a poliestireno daña al mejillón *Mytilus galloprovincialis* a varios niveles. Los mejillones expuestos a mayores concentraciones tienen un peor índice de condición, mientras que el daño producido en el ADN de las branquias es mayor a bajas concentraciones. Se interpreta como una disminución en la tasa de filtración a concentraciones mayores, que evitaría la exposición directa de las branquias a los microplásticos, y reduciría el estado nutricional.

5) El correcto funcionamiento de las plantas de tratamiento de aguas ha sido puesto en entredicho en numerosas ocasiones por la elevada cantidad de microplásticos encontrada en su proximidad. Tras la revisión realizada, se propone la utilización de algas y especies macrófitas por su eficiencia de retención de microplásticos. Se propone aumentar la investigación sobre su aplicación para retener los microplásticos en las plantas de tratamiento, así como para mejorar su cultivo y manejo.

Conclusions

1) For the first time, a quantification of the amount of microplastics, mesoplastics and macroplastics in the asturian coast has been performed, and their origin has been inferred by studying the relationship between these plastic pollutants and different anthropogenic stressors known to be sources of marine plastic pollution. Beach cleaning services are highly important in order to control macroplastics and can avoid the formation of mesoplastics that are produced *in situ* in those beaches. On the other hand, maritime ports and fishing activities are a source of both microplastics and macroplastics. There is necessary a join action from those collectives to control marine plastic litter.

2) The amount of microplastics found in beaches included on the Natura 2000 network, and in the SPA list (Special Protected Areas for migratory birds) is medium-high in comparison with other European beaches. A higher concentration found in open ocean beaches, than in estuarine beaches, suggest that microplastics will have a possible origin in the open ocean. Moreover, microplastics have been also found in bird faeces, emphasizing the ecological importance of microplastic pollution.

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3) The amount of microplastics found in populations of the mediterranean mussel (*Mytilus galloprovincialis*) in the asturian coast, is correlated with the amount of microplastics found in sediments, which is 10 times greater, showing therefore a mechanism of bioaccumulation. Their consumption could suppose an ingestion of a higher dose of microplastics than with the table salt, which was found to be minor. The composition of some of the microplastics found in both mussels and table salt were classified as hazardous for humans, such as aniline and aldehyde. Recommendations for consumers have been proposed based on these results: First, to restrict the harvesting of seafood in areas with high microplastics pollution, and consequently the monitoring of microplastics at local scale. Secondly, to include microplastics content in seafood labels.

4) Different levels of damage due to microplastic ingestion have been probed in the Mediterranean mussel, when exposed intermittently to polystyrene microspheres. Mussels subdued to highly concentration doses showed a lower body condition index, meanwhile gills DNA damage was lower at the same concentration. These results suggest a possible reduction in the filtration rates and avoiding direct exposition of gills to microplastic particles.

5) The malfunctioning of wastewater treatment plants regarding microplastic retention rates, has been considered several times due to the amount of microplastics found in their surroundings. After the review done in the current thesis, the possible use of seagrasses and algae to meliorate the retention rates have been proposed. Also, the investigation on the improvement of cultivation, manipulation, and management of these species have been proposed as well.

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Referencias

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Factores de Impacto de las publicaciones incluidas en la Tesis

Informe factores de impacto de las publicaciones incluidas en la presente tesis

(Según el Journal Citation Report ®2020)

Artículo 1: Microplastics in special protected areas for migratory birds in the Bay of Biscay

- Fecha de publicación: 31 Julio 2019
- Revista: Marine Pollution Bulletin
- SCI: Q1
- Factor de impacto: 5.55

Artículo 2: Biorremediation as a promising strategy for microplastics removal in wastewater treatment plants

- Fecha de publicación: 22 Mayo 2020
- Revista: Marine Pollution Bulletin
- SCI: Q1
- Factor de impacto: 5.55

Artículo 3: Maritime ports and beach management as sources of coastal macro-, meso-, and microplastic pollution

- Fecha publicación: 16 Febrero 2021
- Revista: Environmental Science and Pollution Research
- SCI: Q1
- Factor de impacto: 4.22

Artículo 4: Virgin polystyrene microparticles exposure leads to changes in gills DNA and physical condition in the Mediterranean Mussel Mytilus Galloprovincialis

- Fecha publicación: 5 Agosto 2021
- Revista: Animals
- SCI: Q1
- Factor de impacto: 2.75

Artículo 5: A threat from rock to fork: Microplastics in mussels, their environment and table salt

- Fecha publicación: Bajo revisión
- Revista: Food Research International
- SCI: Q1
- Factor de impacto: 6.47

Anexo I: Material Suplementario

MATERIAL SUPLEMENTARIO CAPÍTULO 1

Supplementary Table 1: Number of microplastics found in each sand sample in the eleven beaches examined. Every sampling point correspond to 50 gr of sand.

Sample	Arnao	Penarronda	Otur	Aguilar	Zeluán	Xagó	Puntal	Rodiles	Vega	Gulpiyuri	Cobijeru
#1	8	32	5	9	3	30	20	16	4	9	9
#2	8	18	4	8	10	46	5	15	6	30	11
#3	9	21	1	8	11	29	5	18	4	23	13
#4	8	15	0	13	18	30	0	26	7	17	4
#5	7	12	4	9	12	26	12	14	4	22	7
#6	9	18	2	6	13	15	11	8	4	24	12
#7	6	13	4	2	37	14	11	21	7	17	10
#8	8	14	1	5	18	13	6	23	3	12	13
#9	13	26	4	12	8	8	14	13	10	16	1
#10	9	22	2	6	38	17	9	22	7	15	9
#11	21	22	4	8	23	15	6	16	3	11	14
#12	21	22	1	4	5	14	9	13	5	24	4
#13	14	16	4	5	20	14	19	19	6	12	2
#14	10	9	2	8	14	12	14	24	5	4	0
#15	12	13	4	9	8	13	11	26	4	4	9
#16	19	21	3	5	14	9	10	14	4	4	8
#17	11	12	4	9	18	20		21	8	4	6
#18	14	12	5	6	8	11		28	8	4	7
#19	16	18	0	8	8	22		34	8	17	4
#20	14	16	4	6	9	15		11	4	6	2
Mean	11.85	17.60	2.90	7.30	14.75	18.65	10.13	19.10	5.55	13.75	7.25
SD	4.57	5.60	1.62	2.62	9.35	9.35	5.19	6.50	1.99	8.07	4.27

Supplementary Table 2: Amount of different types of plastic litter found in the eleven sampling points considered.

	Arnao	Penarronda	Otur	Aguilar	Zeluán	Xagó	Puntal	Rodiles	Vega	Gulpiyuri	Cobijeru
Microplastics/kg	23.7	35.3	5.8	14.9	27	34.4	20.1	38.2	11.5	26.8	14.7
Mesoplastics/100m^2	0	0.21	0.62	16.5	14.28	4.82	0.8	0.17	0	75	8.33
Household/ 100m^2	0	0	0	0.5	2.38	0.44	0	0	0	8.33	4.16
ALDFG/ 100m^2	0	0.42	0.62	0	20.24	0.44	6.4	0	0	0	0
Beachgoers/ 100m^2	0	0	0	0.5	10.71	2.63	1.6	0.17	0	8.33	0
Others/ 100m^2	0	0	0	1.5	16.67	1.75	3.2	0.35	0	16.67	12.5
Total macrolitter	0	0.42	0.62	2.5	50	5.26	11.2	0.52	0	33.33	16.66

Supplementary Table 3. Multiple regression model with beach services, fishing and the distance to wastewater treatment plants (WWTP) as independent variables, and macroplastics density as dependent variable. SE, standard error.

	Coefficient	**SE**	**t**	**p**	**r2**
Macroplastics	33.492	9.107	3.678	0.008	
vs beach services	3.681	1.165	3.158	**0.016**	0.602
vs fishing	8.58×10^{-7}	7.78×10^{-7}	1.103	0.306	0.215
vs WWTP	0.001	0.002	0.943	0.377	0.301

Supplementary Table 4. Kruskal-Wallis analysis testing differences on plastic pollution between beaches highly or lowly influenced by different stressors: port proximity, fishing activity, wastewater treatment plants (WWTP), beach services, river proximity. Significant *p* values are marked in bold.

		Macroplastics	**Mesoplastics**	**Microplastics**
Port proximity	H (Chi2)	0.080	0.321	4.321
	Hc (tie corrected)	0.081	0.327	4.321
	p	0.776	0.567	**0.037**
Fishing activity	H (Chi2)	4.8	0.833	1.633
	Hc (tie corrected)	4.822	0.849	1.633
	p	**0.028**	0.357	0.201
WWTP	H (Chi2)	2.7	5.633	2.133
	Hc (tie corrected)	2.712	5.738	2.133
	p	0.099	**0.017**	0.144
Beach services	H (Chi2)	5.633	0.833	1.2
	Hc (tie corrected)	5.659	0.849	1.2
	p	**0.017**	0.357	0.273
River proximity	H (Chi2)	0.075	0.033	5.633
	Hc (tie corrected)	0.075	0.034	5.633
	p	0.784	0.854	**0.017**

MATERIAL SUPLEMENTARIO CAPÍTULO 2

Supplementary Table 1. Two-way PERMANOVA analysis of the beaches considered in this study. A: One-way PERMANOVA of the number of MP per sand sample found in the analyzed beaches; B: Two-way PERMANOVA analysis of the number of white fibres per sand sample; C: number of pellets and plastic fragments per sand sample. Location groups are the three estuaries and Llanes area. Exposure levels are sheltered versus exposed/semiexposed beach.

A)

Source	Sum of squares	df	Mean square	F	p
Location	662.41	3	220.8	5.079	0.002
Exposure	1460.3	1	1460.3	33.589	0.0001
Interaction	-114.1	3	-38.032	-0.875	0.603
Residual	6434.3	148	43.75		
Total	8442.8	155			

B)

Source	Sum of squares	df	Mean square	F	p
Location	32.071	3	10.691	2.481	0.06334
Exposure	68.969	1	68.969	16	9.95E-05
Interaction	33.897	3	11.299	2.622	0.05289
Residual	637.8	148	4.309		
Total	771.077	155			

C)

Source	Sum of squares	df	Mean square	F	p
Location	0.450	3	0.150	0.888	0.456
Exposure	0.245	1	0.245	1.449	0.176
Interaction	0.420	3	0.140	0.828	0.072
Residual	25.029	148	0.169		
Total	26.145	155			

MATERIAL SUPLEMENTARIO CAPÍTULO 3

Supplementary table 1: Summary of the microplastics found at each sampling point for the different components. Units are items/gr for sand; items/gr for mussels, and items/L for water.

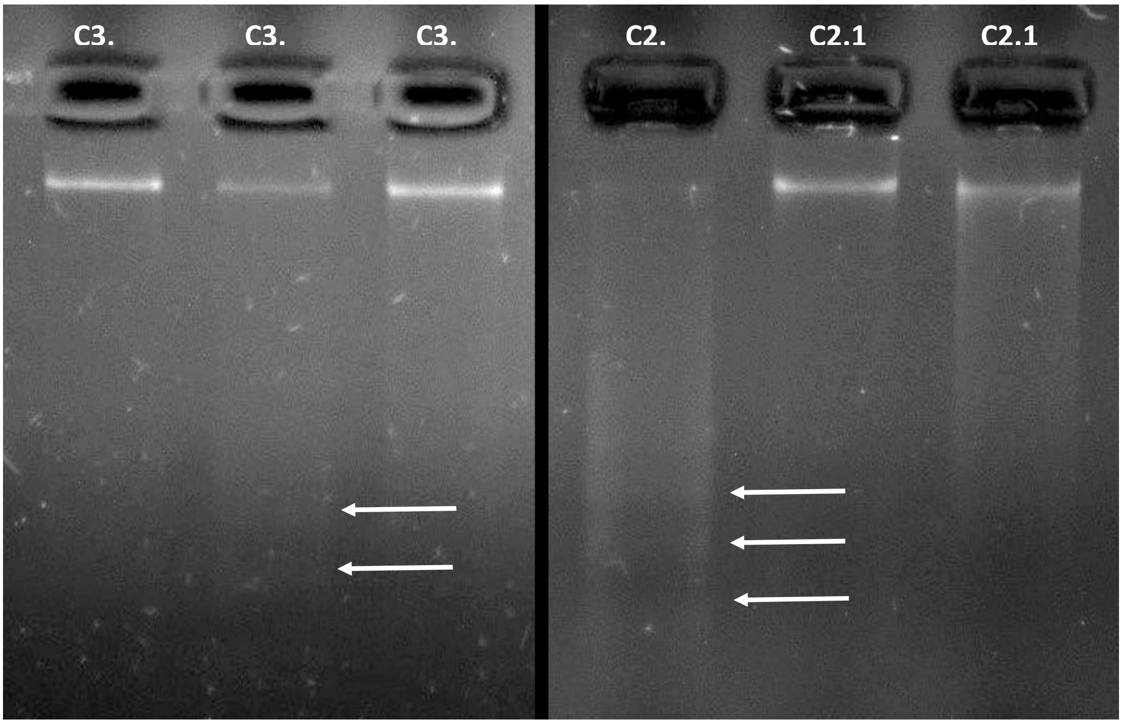
Sampling point	Component	Blue	Black	White	Red	Other colours	Fragments	Pellets
Arnao	Sand	0,085	0,085	0,052	0,011	0,002	0,002	0,000
	Water	3,667	3,333	0,333	0,000	0,333	0,000	0,000
	Mussels	0,225	0,100	0,025	0,025	0,000	0,150	0,000
Penarronda	Sand	0,129	0,102	0,093	0,022	0,002	0,004	0,001
	Water	3,667	1,000	1,667	0,000	0,333	0,000	0,000
	Mussels	0,900	0,500	0,900	0,000	0,000	0,100	0,000
Otur	Sand	0,012	0,015	0,022	0,004	0,000	0,005	0,000
	Water	1,333	0,667	0,333	0,000	0,000	0,000	0,000
	Mussels	0,200	0,125	0,200	0,000	0,000	0,025	0,000
Zeluán	Sand	0,012	0,015	0,022	0,004	0,000	0,005	0,000
	Water	2,333	1,000	1,333	0,667	0,000	0,333	0,333
	Mussels	0,310	0,212	0,310	0,047	0,000	0,000	0,285
Xagó	Sand	0,146	0,076	0,061	0,024	0,030	0,007	0,002
	Water	3,333	1,000	1,333	0,000	0,000	0,333	0,000
	Mussels	0,700	0,400	0,900	0,200	0,000	0,000	0,000
El Puntal	Sand	0,081	0,067	0,045	0,005	0,008	0,000	0,000
	Water	5,667	1,667	7,000	0,333	0,000	0,000	0,000
	Mussels	0,320	0,280	0,480	0,120	0,000	0,160	0,000
Rodiles	Sand	0,165	0,106	0,090	0,018	0,003	0,000	0,000
	Water	6,000	1,667	1,333	0,667	0,333	0,000	0,000
	Mussels	1,000	0,400	1,100	0,200	0,400	0,100	0,000

MATERIAL SUPLEMENTARIO CAPITULO 4

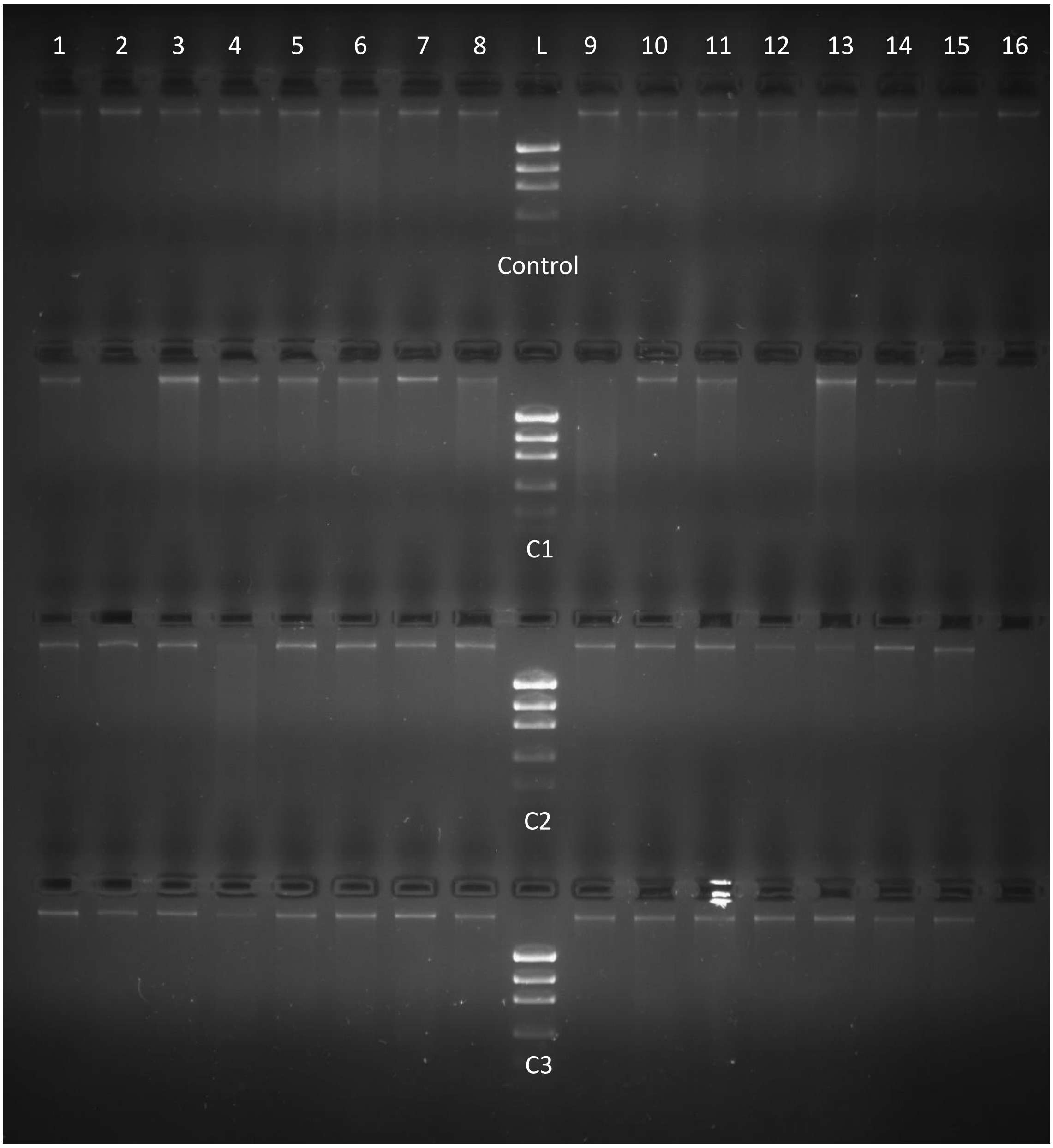
Supplementary Table S1: Condition indexes and DNA integrity scores for each individual.

Individual	Group	DNA integrity				Condition Index
		Observer 1	Observer 2	Observer 3	Mean	
1	Control	1	1	1	1	0.11
2	Control	1	1	1	1	0.16
3	Control	1	1	1	1	0.15
4	Control	1	1	1	1	0.12
5	Control	1	1	1	1	0.13
6	Control	1	1	1	1	0.16
7	Control	1	1	1	1	0.12
8	Control	1	1	1	1	0.11
9	Control	1	1	1	1	0.13
10	Control	1	1	1	1	0.14
11	Control	1	1	1	1	0.17
12	Control	1	1	1	1	0.18
13	Control	1	1	1	1	0.17
14	Control	1	1	1	1	0.10
15	Control	1	1	1	1	0.12
16	Control	1	1	1	1	0.18
1	Conc.1	1	1	1	1	0.20
2	Conc.1	4	4	4	4	0.18
3	Conc.1	1	1	1	1	0.14
4	Conc.1	1	1	1	1	0.17
5	Conc.1	1	1	1	1	0.14
6	Conc.1	1	1	1	1	0.13
7	Conc.1	1	1	1	1	0.08
8	Conc.1	2	2	2	2	0.13
9	Conc.1	3	4	3	3.3	0.22
10	Conc.1	1	1	1	1	0.15
11	Conc.1	1	1	1	1	0.12
12	Conc.1	4	4	4	4	0.13
13	Conc.1	1	1	2	1.3	0.13
14	Conc.1	1	1	1	1	0.17
15	Conc.1	1	1	1	1	0.16
1	Conc.2	1	1	1	1	0.16
2	Conc.2	1	1	1	1	0.10
3	Conc.2	1	1	1	1	0.13
4	Conc.2	3	3	3	3	0.15
5	Conc.2	1	1	1	1	0.06
6	Conc.2	1	1	1	1	0.13
7	Conc.2	1	1	1	1	0.08
8	Conc.2	1	1	1	1	0.10

9	Conc.2	1	1	1	1	0.11
10	Conc.2	1	1	1	1	0.07
11	Conc.2	1	1	1	1	0.16
12	Conc.2	2	2	2	2	0.11
13	Conc.2	2	2	2	2	0.12
14	Conc.2	1	1	1	1	0.16
15	Conc.2	1	1	1	1	0.11
1	Conc. 3	1	1	1	1	0.13
2	Conc. 3	1	1	1	1	0.16
3	Conc. 3	1	1	1	1	0.09
4	Conc. 3	2	2	2	2	0.07
5	Conc. 3	1	1	1	1	0.10
6	Conc. 3	1	1	1	1	0.15
7	Conc. 3	1	1	1	1	0.12
8	Conc. 3	1	1	1	1	0.13
9	Conc. 3	1	1	1	1	0.11
10	Conc. 3	1	1	1	1	0.12
11	Conc. 3	1	1	1	1	0.13
12	Conc. 3	1	1	1	1	0.15
13	Conc. 3	1	1	1	1	0.08
14	Conc. 3	1	1	1	1	0.10
15	Conc. 3	1	1	1	1	0.12



Supplementary Figure S2: Image of the two samples found with secondary light DNA bands (signalled with an arrow).



Supplementary Figure S1: All the samples loaded in an agarose gel. From left to right: individuals 1 to 15 (16 for the control group). From up to bottom: each line represents onegroup (C0 to C3)