UNIVERSITAT POLITÈCNICA DE VALÈNCIA

Programa de Doctorado en Ingeniería y Producción Industrial

Departamento de Ingeniería Química y Nuclear

Caracterización de microplásticos en aguas naturales y residuales, y su influencia y separación en procesos biológicos de depuración

Clara Bretas Alvim

Abril 2022

Dirigido por:

Dra. Mª Amparo Bes Piá

Dr. José Antonio Mendoza Roca

Caracterización de microplásticos en aguas naturales y residuales y su influencia y separación en procesos biológicos de depuración

Agradecimientos

En primer lugar, a Dios, por haberme sostenido a través de la fe en los días difíciles. Por haber despertado en mí el amor por la ciencia y el coraje de cruzar un océano para realizar mis sueños. A mi familia y a Paco, por todo apoyo y fuerza que me han dado.

A mis directores de tesis Dra. Mª Amparo Bes-Piá y Dr. José Antonio Mendoza Roca. Gracias por todo lo que he aprendido y por la confianza depositada en mí. Con vosotros he crecido como persona y profesional. Finalizo esta etapa de mi vida con la seguridad de que he tenido los mejores tutores que alguien puede tener. Con vosotros el doctorado fue divertido, desafiante y motivador.

Al profesor Dr. José Luis Alonso Molina, responsable del Grupo de Química y Microbiología del Agua de la Universitat Politècnica de València, gracias por todo su apoyo y por todo el conocimiento que has compartido. A Inma y Julián, gracias por vuestra colaboración y ayuda.

A mis compañeros de la UPV, que me han recibido, enseñado y ayudado. Gracias por los cafés, por las conversaciones y por la paciencia para intentar entender mi "*portuñol*", ¡especialmente en los primeros años!

Gracias a Miro, del Instituto Universitario de Tecnología Nanofotónica de la UPV, un cerebro brillante que me ha ayudado a identificar centenares de microplásticos. Hemos superado muchos retos a lo largo de la tesis ¡Gracias por todo!

Caracterización de microplásticos en aguas naturales y residuales y su influencia y separación en procesos biológicos de depuración

Caracterización de microplásticos en aguas naturales y residuales y su influencia y separación en procesos biológicos de depuración

Resumen

Los microplásticos (MPs) son partículas de material plástico menores que 5 mm que están siendo identificadas en el agua, tierra y aire. La presencia de estos contaminantes emergentes en el medio ambiente supone una preocupación global actualmente, ya que no se sabe con exactitud su concentración y composición debido a la falta de protocolos de cuantificación e identificación estandarizados en los diferentes medios donde se encuentran. Igualmente, se desconoce el alcance de los efectos negativos que podrían provocar en el medio ambiente. La presente tesis estudia la presencia de microplásticos en diferentes corrientes de agua residual y natural, así como en fangos de depuración generados en las estaciones depuradoras de aguas residuales.

El objetivo principal de la tesis consiste en evaluar diferentes protocolos para la extracción de microplásticos contenidos en muestras con diferentes características, así como su cuantificación e identificación. Para ello, se propusieron metodologías para la extracción de microplásticos en matrices con elevada carga orgánica (licor mezcla, fango digerido anaeróbicamente y fango deshidratado) y también en efluentes residuales, así como en aguas naturales tratadas (potables) y sin tratar. Asimismo, se evaluaron los posibles efectos de la presencia de microplásticos en un proceso de depuración mediante un reactor biológico de fangos activos, así como la distribución de estas micropartículas en el fango y en el efluente depurado. Además, se contempló también la posible fragmentación de microplásticos en nanoplásticos (NPs) en un sistema de depuración de aguas residuales y, por lo tanto, también se evaluaron los posibles efectos de los nanoplásticos en la biomasa de los fangos activos.

Se observó que cuanto mayor era la concentración de carga orgánica en una muestra más difícil era el proceso tanto de purificación de la muestra como de identificación de microplásticos. Por lo tanto, para las muestras de fango fue necesario aplicar protocolos más complejos de extracción de microplásticos. La peroxidación, demostró ser un tratamiento eficaz de todas las muestras estudiadas, resultando en la reducción de la materia orgánica y en la mejora de la identificación visual.

En los ensayos con el reactor biológico se observó una acumulación significativa de microplásticos en el licor mezcla (fango activo) frente al efluente. Esta acumulación de microplásticos en el fango puede suponer la contaminación de los suelos agrícolas

cuando estos son aplicados como fertilizantes. Con el objetivo de mitigar la contaminación de suelos agrícolas por microplasticos presentes en fangos, se propuso el uso de la técnica de ultrasonidos para la extracción de estas micropartículas presentes en el licor de mezcla. Mediante esta técnica, cantidades significativas de microplasticos fueron separadas de la matriz orgánica de los fangos, que es la que se aplica al campo.

Finalmente, se estudió la presencia de microfibras en aguas naturales y potables mediante a técnica de tinción con Rojo Nilo con el objetivo de investigar la viabilibilidad y limitaciones de este método. Se observó que la emisión de fluorescencia, por parte de microfibras vírgenes y microfibras separadas de estas muestras de agua, puede manifestar diferentes comportamientos de fluorescencia lo cual podría estar relacionado con el grado de degradación de los materiales de las microfibras.

Resum

Els microplàstics (MPs) són partícules de material plàstic menors que 5 mm que estan siguent identificades en l'aigua, terra i aire. Actualment, la presència d'aquests contaminants emergents en el medi ambient suposa una preocupació global, ja que no se sap amb exactitud la seua concentració i composició a causa de la falta de protocols de quantificació i identificació estandarditzats en els diferents medis on es troben. Igualment, es desconeix l'abast dels efectes negatius que podrien provocar en el medi ambient. La present tesi estudia la presència de microplàstics en diferents corrents d'aigua residual i natural, així com en fangs de depuració generats en les estacions depuradores d'aigües residuals.

L'objectiu principal de la tesi consisteix a avaluar diferents protocols per a l'extracció de microplàstics continguts en mostres amb diferents característiques, així com la seua quantificació i identificació. Per a això, es van proposar metodologies per a l'extracció de microplàstics en matrius amb elevada càrrega orgànica (licor mescla, fang digerit anaeróbicamente i fang deshidratat) i també en efluents residuals, així com en aigües naturals tractades (potables) i sense tractar. Així mateix, es van avaluar els possibles efectes de la presència de microplàstics en un procés de depuració mitjançant un reactor biològic de fangs actius, així com la distribució d'aquestes micropartícules en el fang i en l'efluent depurat. A més, es va contemplar també la possible fragmentació de microplàstics en nanoplásticos (NPs) en un sistema de depuració d'aigües residuals i, per tant, també es van avaluar els possibles efectes dels nanoplásticos en la biomassa dels fangs actius.

Es va observar que com més gran era la concentració de càrrega orgànica en una mostra, més difícil era el procés tant de purificació de la mostra com d'identificació de microplàstics. Per tant, per a les mostres de fang va ser necessari aplicar protocols més complexos d'extracció de microplàstics. La peroxidació, va demostrar ser un tractament eficaç de totes les mostres estudiades, resultant en la reducció de la matèria orgànica i en la millora de la identificació visual.

En els assajos amb el reactor biològic es va observar una acumulació significativa de microplàstics en el licor mescla (fang actiu) enfront de l'efluent. Aquesta acumulació de microplàstics en el fang pot suposar la contaminació dels sòls agrícoles quan aquests

són aplicats com a fertilitzants. Amb l'objectiu de mitigar la contaminació de sòls agrícoles per microplàstics presents en fangs, es va proposar l'ús de la tècnica d'ultrasons per a l'extracció d'aquestes micropartícules presents en el licor de mescla. Mitjançant aquesta tècnica, quantitats significatives de microplàstics van ser separades de la matriu orgànica dels fangs, que és la que s'aplica al camp.

Finalment, es va estudiar la presència de microfibres en aigües naturals i potables mitjançant a tècnica de tinció amb Rojo Nil amb l'objectiu d'investigar la viabilibilitat i limitacions d'aquest mètode. Es va observar que l'emissió de fluorescència, per part de microfibres verges i microfibres separades d'aquestes mostres d'aigua, pot manifestar diferents comportaments de fluorescència la qual cosa podria estar relacionada amb el grau de degradació dels materials de les microfibres.

Abstract

Microplastics (MPs) are particles of plastic material smaller than 5 mm that are being identified in water, soil, and air. The presence of these emerging contaminants in the environment is currently a global concern since their concentration and composition are unknown exactly due to the lack of standardized quantification and identification protocols in the different media where they are found. Likewise, the extent of the negative effects that they could cause in the environment is unknown. This thesis studies the presence of microplastics in different streams of wastewater and natural water, as well as in sewage sludge generated in wastewater treatment plants.

The main objective of the thesis is to evaluate different protocols for the extraction of microplastics contained in samples with different characteristics, as well as their quantification and identification. For this, methodologies were proposed for the extraction of microplastics in matrices with a high organic load (mixed liquor, anaerobically digested sludge, and dehydrated sludge) and also in wastewater, as well as in treated (potable) and untreated natural waters. Likewise, the possible effects of the presence of microplastics in a depuration process using an activated sludge biological reactor were evaluated and the distribution of these microparticles in the sludge and in the final effluent. In addition, the possible fragmentation of microplastics into nanoplastics (NPs) in a wastewater treatment system was also considered and, therefore, the possible effects of nanoplastics on the biomass of activated sludge were also evaluated.

It was observed that the higher the concentration of organic load in a sample, the more difficult the process of both sample purification and microplastic identification. Therefore, for the sludge samples, it was necessary to apply more complex microplastic extraction protocols. Peroxidation proved to be an effective treatment for all the samples studied, resulting in the reduction of organic matter and the improvement of visual identification.

In the tests with the biological reactor, a significant accumulation of microplastics was observed in the mixed liquor (activated sludge) compared to the effluent. This accumulation of microplastics in the sludge can lead to the contamination of agricultural soils when they are applied as fertilizers. To mitigate the contamination of agricultural

soils by microplastics present in sludge, the use of the ultrasound technique was proposed for the extraction of these microparticles present in the mixed liquor. Through this technique, significant amounts of microplastics were separated from the organic matrix of the sludge, which is what is applied to the field.

Finally, the presence of microfibers in natural and drinking water was studied using the Nile Red staining technique to investigate the feasibility and limitations of this method. It was observed that the fluorescence emission by virgin microfibers and microfibers separated from these water samples can show different fluorescence behaviors which could be related to the degree of degradation of the microfiber materials.

Introducción y esquema general de la tesis

1. Microplásticos: definición y efectos sobre el medio ambiente

Los materiales plásticos se emplean en múltiples aplicaciones desde hace varias décadas. En 2016, la producción de plásticos fue superior a 330 millones de toneladas (Pastics Europe, 2017). Sin embargo, estos materiales son una fuente de contaminación importante debido a su largo tiempo de degradación. Se ha estimado que la presencia de residuos plásticos no va a disminuir en los próximos años y que en 2050 podría haber, en masa, más plásticos que peces en los océanos (Auta et al.,2017; MacArthur, E., 2017).

Se definen como microplásticos (MPs) a los polímeros sintéticos cuyas dimensiones son menores a 5 mm (Thomson et al., 2004), mientras que nanoplásticos (NPs) son aquellos plásticos con dimensiones menores a 0,1 µm (He et al., 2018, Ng et al., 2018). Basados en su origen, los MPs pueden ser clasificados en:

- MPs primarios. Son aquellos que se utilizan como tales en la formulación de productos de consumo, como las microesferas en productos cosméticos, y las microfibras presentes en aguas de lavado de ropa (Waller et al., 2017).

- MPs secundarios. Se originan a partir de residuos plásticos, principalmente de envases, tras su degradación debido a una interacción física, química y biológica (Carr et al., 2016).

La mayor preocupación causada por la presencia de MPs en el medio ambiente es su ingestión, ya que son tóxicos tanto para seres humanos como para otros seres vivos. Los MPs son contaminantes persistentes y acumulativos porque apenas son biodegradables (Li et al, 2018). Los organismos acuáticos como moluscos y peces pueden ingerirlos acarreándoles serios daños en sus funciones biológicas (Cole et al., 2014).

Por otra parte, debido a su hidrofobicidad, los MPs pueden adsorber otros contaminantes orgánicos persistentes, actuando como vectores de estos contaminantes y provocando su ingestión (Cole et al., 2013; Bouwmeester et al., 2015). Además, algunos de estos compuestos orgánicos contaminantes pueden provocar enfermedades en seres humanos o aumentar el riesgo de sufrirlas.

Aunque las estaciones depuradoras de aguas residuales (EDARs) eliminan el 90% de los MPs en el agua (pasando la mayor parte de ellos al fango), se puede considerar que son las principales fuentes de MPs en el medio ambiente (Gies et al., 2018; Li et al., 2018). Esto se debe no solo a la gran cantidad de MPs contenidos en las aguas residuales si no también al enorme volumen de agua tratada descargada por las EDARs (Talvitie et al., 2017a). Por tanto, millones de MPs son vertidos diariamente por las EDARs (Mason et al., 2016; Gündoğdu et al., 2018). Además, como se ha comentado anteriormente, la mayor parte de los MPs que llegan a las EDARs acaban en el fango, el cual se emplea normalmente en la agricultura, representando una fuente de contaminación para el suelo (Murphy et al., 2016; Talvitie et al., 2017b).

2. Las EDARs como fuentes de MPs al medio ambiente

Como se ha comentado previamente, los efluentes de las EDARs contienen aún MPs. De acuerdo con la bibliografía, el tratamiento primario de una EDAR elimina entre el 78 y el 98% de los MPs del agua residual influente, mientras que el tratamiento secundario podría eliminar entre un 5 y un 20% (Murphy et al., 2016; Talvitie et al., 2017b). En concreto, en el estudio realizado por Murphy et al. (2016) en una EDAR en Escocia (Reino Unido) que trataba el agua residual de una población de 650.000 habitantes, se informó que el 98% de MPs eran eliminados en la EDAR. Aun así, se descargaban con el efluente unos 65 millones de MPs diariamente.

Magni et al. (2019) determinaron en su estudio que una EDAR que trata el agua residual de 1.200.000 habitantes vertía alrededor de 160 millones de MPs de forma diaria.

A lo largo de esta tesis doctoral se encuentran reflejados numerosos datos de otros autores, así como las determinaciones realizadas con muestras recogidas de EDAR. Todos estos datos vienen a confirmar el grave problema medioambiental originado por los MPs y la necesidad de su control y de su eliminación, en la medida de lo posible, del ciclo integral del agua.

Las EDARs no tienen tratamientos específicos para la eliminación de MPs. Sin embargo, los procesos de separación existentes son capaces de pasar la mayor parte de los MPs del agua al fango. A este respecto, se necesitaría una modificación de los procesos para conseguir aún mayores eficiencias de eliminación en la línea de aguas de las EDARs y un nuevo enfoque al tratamiento de los fangos para evitar que lleguen los MPs a los suelos si el fango es utilizado en la agricultura.

Por otra parte, cabe comentar que es muy complicado comparar los datos bibliográficos aportados por diferentes autores. La razón es que no hay un protocolo de caracterización estandarizado. Por ello, hay autores que analizan MPs entre 150 micras y 5 mm, y otros entre 5 micras y 5 mm. Para determinar el número de MPs en muestras en las que hay que eliminar la materia orgánica para evitar interferencias, diferentes autores emplean métodos diferentes de pretratamiento. Algunos autores contabilizan como MPs las fibras naturales, mientras que otros no. En definitiva, uno de los objetivos de esta tesis doctoral es establecer protocolos que permitan realizar determinaciones fiables de los MPs de muestras de aguas residuales y aguas naturales. Estos protocolos serán diferentes en función del tipo de muestra (agua residual, fango, agua natural, etc.). En cuanto a los resultados de los análisis, se han de indicar el número de MPs por volumen de agua o unidad de masa seca de fango y su caracterización (tamaño, forma, color y material).

3. Muestreo y pretratamiento de las muestras para el análisis de MPs

Respecto al muestreo, se pueden realizar dos estrategias: toma de muestras en la EDAR de forma automatizada incluyendo bombeo y filtración de la muestra para separación de los MPs, o toma de muestra en un recipiente para realizar en laboratorio la separación. La ventaja de la primera estrategia es el elevado volumen de muestra que se puede obtener. Sin embargo, hay que prestar especial atención con la contaminación cruzada, la cual está más controlada en el laboratorio.

Como se ha comentado anteriormente, el protocolo de análisis de MPs varía mucho con el tipo de muestra a analizar. Para las muestras de menor concentración de materia orgánica (agua de salida de EDAR procedente de tratamiento terciario y aguas naturales) el pretratamiento de la muestra puede consistir únicamente en un proceso de separación mediante tamices. Estos tamices permiten separar los MPs por tamaños, y la apertura de malla se selecciona en función de los tamaños a separar. Una vez realizada la separación, los tamices son enjuagados con agua desionizada y los MPs son llevados a vidrios de reloj para su examen (Ziajahromi et al., 2017, Long et al., 2019).

Para muestras más complejas con mayor presencia de materia orgánica y de sólidos en suspensión (muestras de agua residual bruta o de fango) son necesarios otros procesos de tratamiento. Fundamentalmente son procesos de separación por densidad y de eliminación de materia orgánica mediante digestión. Estos procesos se combinan con

procesos de filtración, tal y como se detalla en las publicaciones recogidas en esta tesis doctoral.

Separación por densidad

La separación por densidad se basa en la densidad diferente que presentan los MPs (la densidad de los materiales plásticos vírgenes puede variar entre 0,9 y 1,6 g/cm^3), y los materiales inorgánicos, que pueden estar presentes en algunas muestras, de mayor densidad (Hidalgo-Ruz et al., 2012).

Además, cuando las muestras poseen elevada concentración de materia orgánica, el tamizado o filtrado directo de las mismas puede llevar a la saturación de tamices y filtros. La forma de realizar la separación por densidad consiste en mezclar la muestra con disoluciones de mayor densidad que el agua. Se suelen emplear disoluciones de NaCl, NaI o ZnCl$_2$. A continuación, el sobrenadante, que contendrá los MPs de la muestra es separado para su análisis. Normalmente, se emplean disoluciones de 1,2g/cm^3 de NaCl para extraer polietileno (PE), poliestireno (PS) y polipropileno (PP) o disoluciones de mayor densidad para extraer MPs de polietileno tereftalato (PET) o policloruro de vinilo (PVC) (Rocha-Santos and Duarte, 2015; Wang, W. and Wang, J., 2018).

Digestión

El objetivo de este proceso es la eliminación de la materia orgánica para que no interfiera en el análisis de MPs. En la bibliografía se pueden encontrar diferentes técnicas:

- Digestión por hidrólisis con disoluciones ácidas.
- Digestión por hidrólisis con disoluciones alcalinas.
- Digestión por oxidación.
- Digestión enzimática.

La elección de una técnica u otra viene condicionada por la no interacción con el MP, ya que una degradación del mismo no permitiría su correcta identificación. Por otra parte, es fundamental que la técnica sea eficiente y que, además, sea lo más rápida y económica posible.

En la bibliografía parece haber consenso en que la mejor técnica es la de oxidación. La hidrólisis ácida puede dañar a MPs de Nylon, PET y PE de alta densidad (Catarino et al., 2017) y la hidrólisis alcalina presenta menos eficiencia de eliminación de la materia

orgánica (Hurley et al., 2018). La digestión enzimática puede llegar a conseguir muy buenas eficiencias de eliminación de la materia orgánica, pero es un proceso más lento que podría llegar a durar incluso días (Mintenig et al., 2017). Por todo ello, la oxidación es la técnica seleccionada también en esta tesis doctoral.

La oxidación de la materia orgánica como pretratamiento en el análisis de MPs se puede llevar a cabo empleando peróxido de hidrógeno (peroxidación) o mediante la reacción de Fenton. Un ejemplo de aplicación de la peroxidación se puede encontrar en el trabajo de Ziajahromi et al (2017). Estos autores tamizaban las muestras para separar los MPs del agua residual, recogían la materia separada en cada tamiz con agua destilada y la peroxidaban a continuación con H_2O_2 del 30%. La proporción H_2O_2/muestra dependía del tipo de muestra, es decir, de la materia orgánica de la misma. En cuanto a las condiciones de la oxidación, ésta se realizaba a 60ºC.

La reacción de Fenton consiste en el uso de Fe^{2+} para conseguir la activación del peróxido de hidrógeno, generando radicales hidroxilo (ecuación 1).

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + H0^{\bullet} + HO^{-} \qquad \text{(Ec 1)}$$
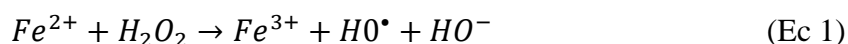
Para llevar a cabo esta reacción se necesita un pH ácido, siendo el pH usualmente empleado de 3. La ventaja de emplear este método es la mayor rapidez de la oxidación. Ejemplos de aplicación de la reacción de Fenton como pretratamiento se pueden encontrar en los trabajos de Devi et al. al., 2016 y Gies et al., 2018.

Estas técnicas de oxidación como pretratamiento en los análisis han sido estudiadas en esta tesis doctoral, constituyendo uno de los capítulos de la misma. Es muy importante conseguir un protocolo efectivo de eliminación de la materia orgánica y comprobar que los MPs no sufren degradación alguna que impida posteriormente su correcta caracterización. Por ello, se han de estudiar diferentes condiciones de operación y su influencia sobre los MPs tras la reacción de oxidación. Estas variables son la concentración del H_2O_2, el tiempo y la temperatura.

4. Técnicas de identificación

Una vez que se han separado los MPs de las muestras de agua y se han pretratado para tener las menores interferencias posibles, se ha de proceder a su análisis cualitativo y cuantitativo.

En primer lugar, se puede determinar el tamaño, la forma y el color de los MPs mediante observación en un estereomicroscopio.

Como se ha comentado anteriormente, los MPs poseen un tamaño de menos de 5 mm y de más de 1 μm, si bien se suelen medir tamaños por encima de 100 μm. Por ello, se pueden observar sin necesidad de emplear un elevado número de aumentos, siendo suficiente el empleo de un estereomicroscopio o lupa.

En cuanto a la forma, fundamentalmente se pueden clasificar los MPs en fragmentos, fibras y microesferas. En el desarrollo experimental de esta tesis doctoral apenas se han encontrado microesferas en las muestras. Ello puede ser debido a que por su forma y características se separen en el pretratamiento de las EDARs (junto a las grasas y aceites) y las muestras que se han procesado son de etapas posteriores. Otra razón para la ausencia de microesferas es el cambio de formulación de los productos que las contenían en los últimos años. Hace años era frecuente su uso en maquillaje, pasta dentífrica, etc., pero la presión social ante el problema de los MPs ha hecho que las empresas sustituyan las microesferas en sus productos. Por tanto, los MPs encontrados en las muestras han sido o bien fragmentos o bien fibras, siendo éstas las más numerosas. Sin embargo, la mayor parte de las fibras tiene un origen natural, lo cual se ha de tener en cuenta para no contabilizarlas como MPs, si bien son micropartículas que pueden acabar en el agua potable y que pueden ser vectores de contaminación a tener en cuenta. Para entender la magnitud del problema, se citan a continuación dos ejemplos. Según, Browne et al. (2011), cada lavado de prendas sintéticas genera 1900 fibras de PET y según Athey et al. (2020), el lavado de ropa vaquera genera más de 50.000 fibras.

Finalmente, en cuanto al color, cabe decir que se pueden encontrar MPs de todos los colores. De cara al análisis es muy importante que no haya problemas de subestimar el número de MPs debido a que algunos son transparentes o blancos y podrían no detectarse.

Una vez que se han clasificado los MPs por su forma, tamaño y color y, por tanto, se ha estimado el número de MPs por volumen de muestra considerada, se ha de comprobar que efectivamente son MPs y el material concreto de cada uno de ellos.

Las técnicas de identificación más empleadas son pirólisis seguida de cromatografía de gases/espectrometría de masas, espectroscopía infrarroja por transformada de Fourier y espectroscopía Raman. Es decir, se pueden clasificar en dos tipos de métodos: los termoanalíticos y los espectroscópicos, los cuales emplearán también técnicas de

microscopía para la visualización de las muestras. Por esta razón se habla de µFTIR y µRaman. Los métodos termoanalíticos no ofrecen información sobre el tamaño o el número de partículas si previamente no se ha realizado, ofreciendo los resultados en masa. Los métodos espectroscópicos, por el contrario, sí ofrecen información de la morfología, tamaño y número (Mallow et al., 2020).

5. Técnicas de tinción y epifluorescencia

Además de las técnicas de identificación que se emplean de forma extendida para la identificación de MPs, merece destacar en un capítulo aparte el papel que podrían jugar las técnicas de tinción y la identificación de MPs por fluorescencia.

En una muestra de micropartículas separadas del agua residual o natural, las técnicas de tinción nos pueden servir en primer lugar para descartar las microfibras de origen natural.

El colorante empleado para la tinción de fibras naturales es el Rosa-Bengala ("Acid Red 94"), que se conoce desde el siglo XIX. Es un reactivo de elevada solubilidad en agua, biocompatible, considerado de bajo coste y de uso frecuente como colorante. Alavian-Petrody et al. (2020) y Raju et al. (2020) trabajaron con este colorante para teñir las fibras de lana y algodón, y de esta forma, no contabilizarlas como MPs. Los MPs no se tiñen con este colorante.

Cabe comentar que las fibras podemos clasificarlas en naturales, sintéticas y artificiales. Claramente, las fibras sintéticas (por ejemplo, de PET) pertenecen al grupo de MPs mientras que las fibras naturales no, por lo que éstas pueden ser descartadas tras su tinción con Rosa Bengala. Las fibras artificiales corresponden a fibras de celulosa regenerada y la forma más común de designarla es como "rayón". Estas fibras también pueden ser teñidas con el Rosa Bengala, por lo que se separarían también para no contabilizarlas como MPs.

Cabe decir que esta separación puede ser especialmente importante cuando las medidas con µFTIR y µRaman se hacen de forma manual porque no se disponga de los elementos necesarios para realizar barridos a superficies más o menos grandes de filtros o soportes que permitan analizar gran cantidad de MPs en poco tiempo.

Las técnicas de tinción pueden también ser útiles para la identificación de MPs, tal y como se pone de manifiesto en el último artículo recogido en esta tesis doctoral. Si bien se pueden emplear diferentes colorantes, el Rojo Nilo es el más utilizado. Este reactivo

se caracteriza porque su fluorescencia está vinculada a la polaridad del disolvente empleado en su disolución (Maes et al., 2017; Martínez y Henary, 2016).

El fenómeno de fluorescencia consiste en que una molécula química se excita mediante un haz de luz incidente, siendo una fracción de la energía absorbida, lo cual conduce a un estado de mayor energía. Como los electrones han de volver al estado energético del inicio, para ello emiten luz a una longitud de onda mayor que la de la luz de excitación. Este fenómeno se denomina luminiscencia. Considerando todo ello, se podrían identificar algunos MPs o bien por su fluorescencia natural o bien por su fluorescencia tras ser excitados tras su tinción con Rojo Nilo. Esta fluorescencia se podría observar en un microscopio de epifluorescencia y con ello se podría realizar un seguimiento de los MPs más usuales, abaratando los costes de análisis y simplificando los protocolos. Esta forma de medición se ha propuesto en esta tesis doctoral para muestras de agua natural, que apenas contienen materia orgánica y por poseer, en principio, menor número de MPs.

6. Resumen de la metodología empleada

Todos los procedimientos empleados se encuentran detallados en los artículos de los que consta la tesis doctoral. Como se ha comentado anteriormente, no se ha empleado un único protocolo de separación de MPs de las muestras de agua, ya que unos de los objetivos de la presente tesis es establecer esa metodología. Además, el protocolo ha de ser necesariamente diferente según el tipo de muestra. Sin embargo, se exponen a continuación los aspectos metodológicos comunes en toda la tesis doctoral y, posteriormente, la metodología seguida que además sirve para conectar los artículos recogidos en esta tesis doctoral.

6.1. Procedimientos comunes

6.1.1 Control de la contaminación cruzada.

Las tomas de las muestras y su manipulación se ha realizado de forma que hubiera la mínima interacción entre las muestras y el ambiente. Los depósitos empleados para la recogida de muestras de aguas residuales o fangos con alto contenido en MPs son de material conocido, mientras que las de bajo contenido en MPs se han realizado en recipientes de vidrio.

Periódicamente se evaluaba la presencia de MPs en los filtros empleados, seleccionados al azar de la caja donde se encontraban. Otros filtros se dejaban en la bancada de trabajo para determinar la contaminación por fibras procedentes del aire ambiente en el laboratorio. Todos los materiales eran lavados con agua, detergente y alcohol, eran examinados con lupa y envueltos con aluminio antes de su uso.

6.1.2. Procedimientos comunes en los ensayos de tratamiento biológico.

Los objetivos de esta tesis doctoral abarcaban también estudiar la posible influencia de MPs y nanoplásticos (NPs) en procesos biológicos de depuración. Para ello era necesario trabajar con MPs y NPs conocidos y poder reproducir el proceso biológico en el laboratorio. En estos ensayos se trabajó con microesferas de 150 µm y nanoesferas de 100 nm de diámetro.

En ambos estudios se trabajó con reactores de laboratorio tipo SBR (Reactor Biológico Secuencial). En cada estudio se operaron dos reactores: un reactor que hizo de blanco, que se alimentaba con agua residual sintética, y un reactor que se alimentaba con agua residual sintética dopada con las micro o nano esferas.

El volumen de reacción era de 6 L y se mantenía una concentración de sólidos en suspensión en el licor de mezcla (SSLM) en el reactor de 2,5 g/L, de forma que se realizaban purgas periódicas para mantener constante dicha concentración. En todos los casos, el tiempo de retención hidráulico (TRH) fue de 24 horas y el número de ciclos fue de 3 al día. La Figura 1 muestra una fotografía de los reactores.

Figura 1. Reactores de laboratorio utilizados en los ensayos

Los análisis realizados durante la operación de estos reactores (el tiempo de operación en cada ensayo osciló entre 2 y 3 meses) fueron:

- Análisis de DQO, SS, nitrógeno (total, amoniacal, nitritos y nitratos), fósforo total, pH y conductividad en el agua residual efluente para poder evaluar el rendimiento de depuración.

- Análisis de la biomasa. Se midió la concentración de sólidos en suspensión volátiles en el licor de mezcla (SSVLM), el ATP total y celular y las actividades microbianas enzimáticas. Se enviaron también muestras para secuenciar y obtener el análisis de las poblaciones microbianas de los reactores, con el fin de observar si se producía alguna diferencia poblacional debido a la presencia de microplásicos.

- Se realizaron medidas de MPs y observación con el microscopio electrónico de barrido de las NPs.

Todos los procedimientos experimentales se encuentran desarrollados en los artículos que comprenden esta tesis doctoral.

6.1.3. Equipos utilizados para la visualización e identificación de MPs.

Los equipos que se emplearon para la visualización e identificación de MPs fueron:

- Lupa LEICA MZ APO del Servicio de Microscopía Electrónica de la UPV.

- μ-ATR-FTIR de Bruker. La resolución espectral de 6 cm^{-1}, posee un barrido por muestra de 128 escáneres y un rango de longitudes de onda entre 600 y 4000 cm^{-1}. Este equipo se halla en en el Instituto Universitario de Tecnología Nanofotónica, en la UPV.

- Microscopio de epifluorescencia Olympus BX50 con cámara AxioCan ICc5 (Zeiss) del Instituto de Ingeniería del Agua y del Medio Ambiente (IIAMA) de la UPV.

- Microscopio electrónico de barrido de emisión de campo (FESEM), de Zeiss, del Servicio de Microscopía Electrónica de la UPV.

- Equipo Zetasizer NanoZS para la medición del tamaño de partícula de los MPs y NPs añadidos a los reactores.

6.2. Metodología seguida y relación entre los artículos que componen la tesis
   doctoral

La presente Tesis Doctoral se ha realizado por compendio de artículos. Tras un primer

capítulo en el que se detallan los objetivos (Capítulo I), en el Capítulo II se realiza una introducción y contextualización de la problemática de los microplásticos en aguas residuales y naturales. Para ello, se lleva a cabo una extensa revisión de la bibliografía sobre las Estaciones de Depuración de Aguas Residuales (EDARs) como fuentes de microplásticos al medio ambiente. En este capítulo también se recopilan las técnicas de cuantificación e identificación comúnmente aplicadas en los estudios de microplásticos, con el fin de establecer las metodologías que se seguirán en los capítulos siguientes para el análisis de microplásticos.

En el Capítulo III, son discutidos los resultados de las metodologías aplicadas para la separación e identificación de microplásticos en los efluentes primario y secundario, y también en el fango activo del proceso biológico de una EDAR. Como principal resultado, se seleccionaron los mejores protocolos para separación e identificación de microplásticos.

En el Capítulo IV se evaluaron los efectos de microesferas de polietileno en la operación de un reactor biológico aerobio. Además, también fue estudiada la acumulación de los microplásticos en el fango activo y su presencia en el efluente secundario, con el objetivo de comprender qué ocurre con los microplásticos en el proceso y cuál es su destino tras el tratamiento secundario.

Debido a la posible fragmentación de los microplásticos en nanoplásticos, en el Capítulo V se estudió si los nanoplásticos (se seleccionaron nanoplásticos comerciales de poliestireno) podrían afectar al fango activo de una EDAR.

Dada la gran acumulación de microplásticos en el fango secundario (observado en el Capítulo IV y corroborado por otros autores), en el Capítulo VI fue propuesto el uso de ultrasonidos para eliminar microplásticos de los fangos activos. El objetivo de este capítulo era estudiar la posibilidad de minimizar la cantidad de microplásticos

en fangos que podrían ser aplicados como fertilizantes (tras su estabilización y deshidratación).

La complejidad y dificultad de cuantificar e identificar microplásticos en muestras con alto contenido orgánico, hace que en el Capítulo VII se estudiaran diferentes metodologías para la extracción de microplásticos, tanto en el fango digerido anaeróbicamente como en el fango deshidratado generado tras una etapa de centrifugación. Además, el escurrido de la centrifuga también fue estudiado.

Finalmente, en el Capítulo VIII se estudia la presencia de microplásticos en agua natural sin tratar y tratada (potable). En este capítulo se empleó una técnica de tinción con Rojo Nilo como alternativa para mejorar la identificación visual de microfibras en el agua potable. Las microfibras textiles fueron elegidas como objeto de estudio por ser la forma de micropartículas más abundante en las muestras evaluadas. La medición de la fluorescencia a diferentes longitudes de onda de excitación se propone como una forma rápida de distinguir entre microfibras naturales y artificiales. Se consideran artificiales aquellas que tienen una base de celulosa regenerada (modificada químicamente), como el rayón, mientras que las sintéticas se fabrican con materiales derivados del petróleo.

En el Capítulo IX se recogen las conclusiones de la presente Tesis Doctoral agrupadas

según los objetivos planteados en el Capítulo I, y desarrollados a través de los artículos derivados de la investigación realizada.

En la Figura 2 se muestra un resumen gráfico de los Capítulos de la presente Tesis Doctoral con los diferentes puntos de actuación y estudio realizado.
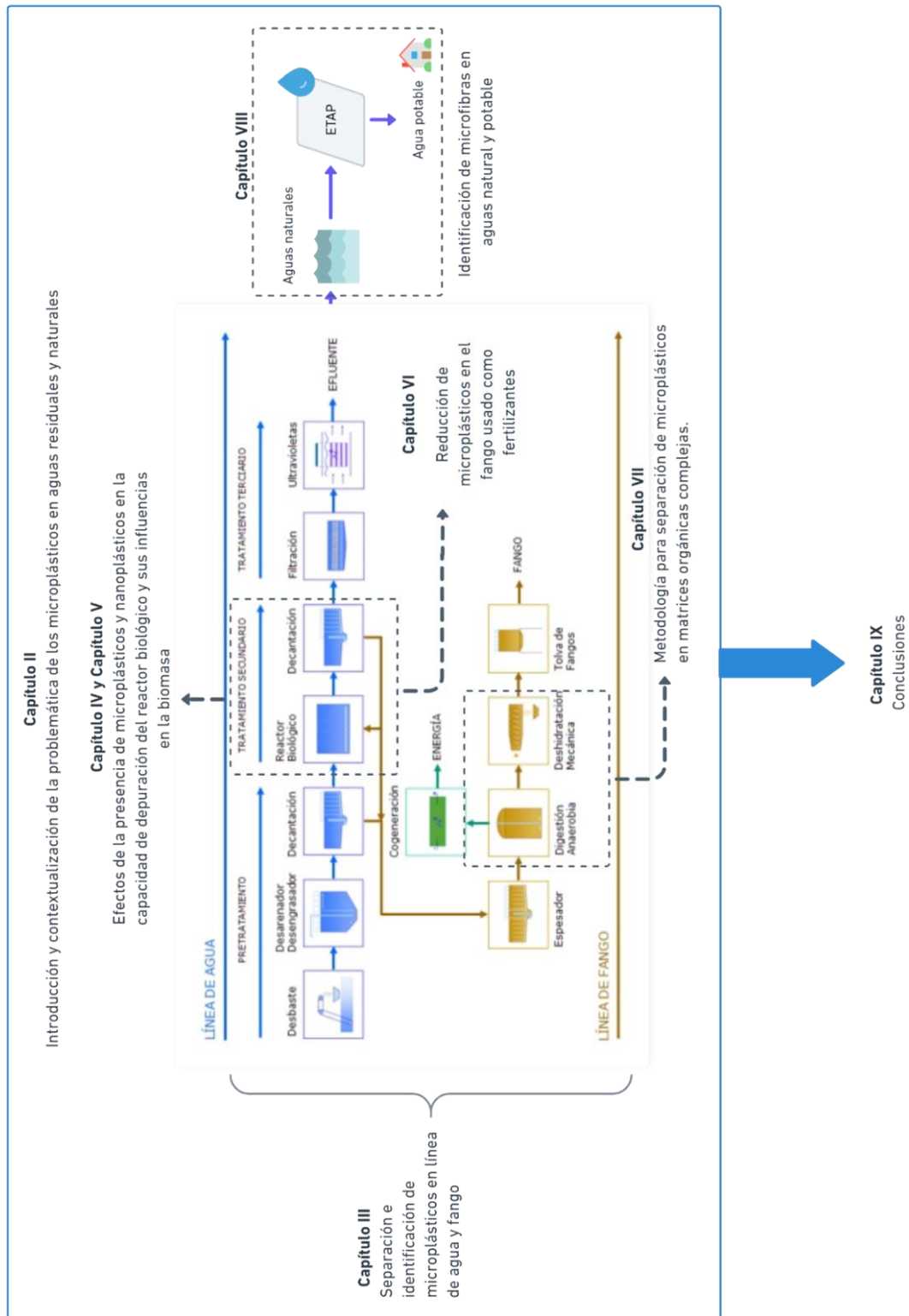
Caracterización de microplásticos en aguas naturales y residuales y su influencia y separación en procesos biológicos de depuración

Figura 2. Flujograma obtenido de la página Entidad Pública de Saneamiento de Aguas Residuales de la Comunidad Valenciana – EPSAR (https://www.epsar.gva.es/node/6220)

Bibliografía

Alavian Petroody, S.S., Hashemi, S.H., van Gestel, C.A.M., 2020. Factors affecting microplastic retention and emission by a wastewater treatment plant on the southern coast of Caspian Sea. Chemosphere 261, 128179. https://doi.org/10.1016/j.chemosphere.2020.128179

Athey, S.N., Adams, J.K., Erdle, L.M., Jantunen, L.M., Helm, P.A., Finkelstein, S.A., Diamond, M.L., 2020. The Widespread Environmental Footprint of Indigo Denim Microfibers from Blue Jeans. Environ. Sci. Technol. Lett. 7, 840–847. https://doi.org/10.1021/acs.estlett.0c00498

Auta, H.S., Emenike, C.U., Fauziah, S.H., 2017. Distribution and importance of microplastics in the marine environment A review of the sources, fate, effects, and potential solutions. Environ. Int. 102, 165–176. https://doi.org/10.1016/j.envint.2017.02.013

Browne, M.A., Crump, P., Niven, S.J., Teuten, E., Tonkin, A., Galloway, T., Thompson, R., 2011. Accumulation of microplastic on shorelines woldwide: Sources and sinks. Environ. Sci. Technol. 45, 9175–9179. https://doi.org/10.1021/es201811s.

Bouwmeester, H., Hollman, P.C.H., Peters, R.J.B., 2015. Potential Health Impact of Environmentally Released Micro- and Nanoplastics in the Human Food Production Chain: Experiences from Nanotoxicology. Environ. Sci. Technol. 49, 8932–8947. https://doi.org/10.1021/acs.est.5b01090

Carr, S.A., Liu, J., Tesoro, A.G., 2016. Transport and fate of microplastic particles in wastewater treatment plants. Water Res. 91, 174–82. https://doi.org/10.1016/j.watres.2016.01.002

Catarino, A.I., Thompson,R.,Sanderson,W.,Henry,T.B.,2017. Development and optimization of a standard method for extraction of microplastics in mussels by enzyme digestion of soft tissues.Environ.Toxicol.Chem.36,947–951. https://doi.org/10.1002/etc.3608.

Cole, M., Webb, H., Lindeque, P.K., Fileman, E.S., Halsband, C., Galloway, T.S., 2014. Isolation of microplastics in biota-rich seawater samples and marine organisms. Sci. Rep. 4, 1–8. https://doi.org/10.1038/srep04528

Devi, P., Das, U., Dalai, A.K., 2016. In-situ chemical oxidation: Principle and applications of peroxide and persulfate treatments in wastewater systems. Sci. Total Environ. 571, 643–657. https://doi.org/10.1016/j.scitotenv.2016.07.032

Gies, E.A., LeNoble, J.L., Noël, M., Etemadifar, A., Bishay, F., Hall, E.R., Ross, P.S., 2018. Retention of microplastics in a major secondary wastewater treatment plant in Vancouver, Canada. Mar. Pollut. Bull. 133, 553–561. https://doi.org/10.1016/j.marpolbul.2018.06.006

Gündoğdu, S., Çevik, C., Güzel, E., Kilercioğlu, S., 2018. Microplastics in municipal wastewater treatment plants in Turkey: a comparison of the influent and secondary effluent concentrations. Environ. Monit. Assess. 190. https://doi.org/10.1007/s10661-018-7010-y

He, D., Luo, Y., Lu, S., Liu, M., Song, Y., Lei, L., 2018. Microplastics in soils: Analytical methods, pollution characteristics and ecological risks. TrAC - Trends Anal. Chem. https://doi.org/10.1016/j.trac.2018.10.006

Hidalgo-Ruz, V., Gutow, L., Thompson, R.C., Thiel, M., 2012. Microplastics in the marine environment: A review of the methods used for identification and quantification. Environ. Sci. Technol. 46, 3060–3075. https://doi.org/10.1021/es2031505

Hurley, R.R., Lusher, A.L., Olsen, M., Nizzetto, L., 2018. Validation of a Method for Extracting Microplastics from Complex, Organic-Rich, Environmental Matrices. Environ. Sci. Technol. 52, 7409–7417. https://doi.org/10.1021/acs.est.8b01517

Li, J., Liu, H., Paul Chen, J., 2018. Microplastics in freshwater systems: A review on occurrence, environmental effects, and methods for microplastics detection. Water Res. 137, 362-374. https://doi.org/10.1016/j.watres.2017.12.056

Long, Z., Pan, Z., Wang, W., Ren, J., Yu, X., Lin, L., Lin, H., Chen, H., Jin, X., 2019. Microplastic abundance, characteristics, and removal in wastewater treatment plants in a coastal city of China. Water Res. 155, 255–265. https://doi.org/10.1016/j.watres.2019.02.028

MacArthur, E., 2017. Beyond plastic waste. Science. 358, 843-843. https://doi.org/10.1126/science.aao6749

Maes, T., Jessop, R., Wellner, N., Haupt, K., Mayes, A.G., 2017. A rapid-screening approach to detect and quantify microplastics based on fluorescent tagging with Nile Red. Nat. Publ. Gr. 1–10. https://doi.org/10.1038/srep44501

Mallow, O., Spacek, S., Schwarzböck, T., Fellner, J., Rechberger, H., 2020. A new thermoanalytical method for the quantification of microplastics in industrial wastewater. Environ. Pollut. 259, 113862. https://doi.org/10.1016/j.envpol.2019.113862

Martinez, V., Henary, M., 2016. Nile Red and Nile Blue: Applications and Syntheses of Structural Analogues. Chemistry - A European Journal, 22(39), 13764–13782. https://doi.org/10.1002/chem.201601570

Mason, S.A., Garneau, D., Sutton, R., Chu, Y., Ehmann, K., Barnes, J., Fink, P., Papazissimos, D., Rogers, D.L., 2016. Microplastic pollution is widely detected in US municipal wastewater treatment plant effluent. Environ. Pollut. 218, 1045–1054. https://doi.org/10.1016/j.envpol.2016.08.056

Mintenig, S.M., Int-Veen, I., Löder, M.G.J., Primpke, S., Gerdts, G., 2017. Identification of microplastic in effluents of waste water treatment plants using focal

plane array-based micro-Fourier-transform infrared imaging. Water Res. 108, 365–372. https://doi.org/10.1016/j.watres.2016.11.015

Murphy, F., Ewins, C., Carbonnier, F., Quinn, B., 2016. Wastewater Treatment Works (WwTW) as a Source of Microplastics in the Aquatic Environment. Environ. Sci. Technol. 50, 5800–5808. https://doi.org/10.1021/acs.est.5b05416

Ng, E.L., Huerta Lwanga, E., Eldridge, S.M., Johnston, P., Hu, H.W., Geissen, V., Chen, D., 2018. An overview of microplastic and nanoplastic pollution in agroecosystems. Sci. Total Environ. 627,1377-1388. https://doi.org/10.1016/j.scitotenv.2018.01.341

PlasticsEurope & European Association of Plastics Recycling (EPRO), 2017. Plastics – the Facts 2017, Plastics – the Facts 2017. https://www.plasticseurope.org/en/resources/publications/274-plastics-facts-2017 (accessed 19 Novembre 2018).

Raju, S., Carbery, M., Kuttykattil, A., Senthirajah, K., Lundmark, A., Rogers, Z., SCB, S., Evans, G., Palanisami, T., 2020. Improved methodology to determine the fate and transport of microplastics in a secondary wastewater treatment plant. Water Res. 173, 115549. https://doi.org/10.1016/j.watres.2020.115549

Rocha-Santos, T., Duarte, A.C., 2015. A critical overview of the analytical approaches to the occurrence, the fate and the behavior of microplastics in the environment. TrAC - Trends Anal. Chem. 65, 47–53. https://doi.org/10.1016/j.trac.2014.10.011

Talvitie, J., Mikola, A., Koistinen, A., Setälä, O., 2017a. Solutions to microplastic pollution – Removal of microplastics from wastewater effluent with advanced wastewater treatment technologies. Water Res. 123, 401–407. https://doi.org/10.1016/j.watres.2017.07.005

Talvitie, J., Mikola, A., Setälä, O., Heinonen, M., Koistinen, A., 2017b. How well is microlitter purified from wastewater? – A detailed study on the stepwise removal of microlitter in a tertiary level wastewater treatment plant. Water Res. 109, 164–172. https://doi.org/10.1016/j.watres.2016.11.046

Thompson, R.C., Olsen, Y., Mitchell, R.P., Davis, V., Rowland, S.J., John, A.W.G., McGonigle, D., Russell, A.E., 2004. Lost at sea: Where is all the plastic? Science, 304 (2004), p. 838. 10.1126/science.1094559.

Wang, W., Wang, J., 2018. Investigation of microplastics in aquatic environments: An overview of the methods used, from field sampling to laboratory analysis. TrAC - Trends Anal. Chem. 108, 195-202. https://doi.org/10.1016/j.trac.2018.08.026

Waller, C.L., Griffiths, H.J., Waluda, C.M., Thorpe, S.E., Loaiza, I., Moreno, B., Pacherres, C.O., Hughes, K.A., 2017. Microplastics in the Antarctic marine system: An emerging area of research. Sci. Total Environ. 598, 220-227. https://doi.org/10.1016/j.scitotenv.2017.03.283

Ziajahromi, S., Neale, P.A., Rintoul, L., Leusch, F.D.L., 2017. Wastewater treatment plants as a pathway for microplastics: Development of a new approach to sample wastewater-based microplastics. Water Res. 112, 93–99. https://doi.org/10.1016/j.watres.2017.01.042

Índice

Capítulo I

Objetivos

Objetivo general

De acuerdo con el título de la presente tesis doctoral, el objetivo principal consiste en la caracterización de microplásticos en aguas naturales y residuales y en el estudio de su influencia y separación en procesos biológicos de depuración. Para ello, se propone estudiar la presencia de microplásticos en diferentes corrientes de aguas residuales y fangos de una depuradora de aguas residuales urbanas, así como en aguas naturales y potables, a través de la aplicación de tratamientos adecuados para la separación e identificación de estas micropartículas. El objetivo general se concreta en los siguientes objetivos específicos.

Objetivos específicos

Estudiar técnicas viables para la separación de microplásticos en aguas residuales y matrices orgánicas complejas (lodo) a través de digestiones químicas aplicadas para la reducción de la materia orgánica.

Evaluar la eficacia de la técnica de espectrometría infrarroja por transformada de Fourier (FTIR) para la identificación de los microplásticos, especialmente de las microfibras, debido a su significativa presencia en el medio ambiente y su diminuto grosor, lo que confiere mayor dificultad a su identificación.

Evaluar los efectos de los microplásticos (MPs) y nanoplásticos (NPs) tanto en el proceso de depuración por fangos activos como en las comunidades bacterianas de la biomasa de dicho proceso. El proceso de depuración se evaluará en términos de rendimiento de eliminación de la materia orgánica y de nitrificación, como etapa necesaria para la eliminación de nitrógeno. La comunidad bacteriana se determinará mediante secuenciación masiva.

Evaluar la separación de MPs de la matriz orgánica de los fangos activos mediante la aplicación de ultrasonidos, con la finalidad de reducir la cantidad de microplásticos que son añadidos a los suelos agrícolas a través de la aplicación de biosólidos.

Estudiar técnicas viables para la separación de microplásticos en aguas naturales y potables para su posterior identificación. para ello una técnica de tinción que permita la clasificación visual de polímeros sintéticos y no-sintéticos (naturales y artificiales), proporcionando igualmente información sobre los materiales de los MPs separados, será evaluada.

Capítulo II

Wastewater treatment plant as microplastics release source – quantification and identification techniques

Autores – C.B. Alvim, J.A. Mendoza-Roca, A. Bes-Piá

Abstract – The high presence of microplastics (MPs) in different sizes, materials and concentrations in the aquatic environment is a global concern due to their potential physically and chemically harm to aquatic organisms including mammals. Furthermore, the bioaccumulation of these compounds is leading to their ingestion by humans through the consumption of seafood and even through the terrestrial food chain. Even though conventional wastewater treatment plants are capable of eliminating more than 90% of the influent MPs, these systems are still the main source of MPs introduction in the environment due to the high volumes of effluents generated and returned to the environment. The amount of MPs dumped by WWTP is influenced by the configuration of the WWTP, population served and influent flow. Thus, the average of MP/L disposed vary widely depending on the region. In addition to MPs disposed in water bodies, more than 80% of these emerging contaminants, which enter the WWTP, are retained in biosolids that can be applied as fertilizers, representing a potential source of soil contamination. Due to the continuous disposal of MPs in the environment by effluent treatment systems and their polluting potential, separation and identification techniques have been assessed by several researchers, but unfortunately, there are no standard protocols for them. Aiming to provide insight about the relevance of studying the WWTP as source of MPs, this review summarizes the currently methodologies used to classify and identify them.

1. Introduction

Plastic is an important material that is widely used in multiple applications. In 2016 the world production of plastic was superior to 330 million tons. Despite its numerous applications and large participation in the economy (PlasticsEurope, 2017), these polymeric materials have been presented as a hazardous source of environmental pollution. According to the United Nations Environment Program (UNEP, 2016), more than 80% of marine litter corresponds to plastic. In addition, it is estimated that if the rate of plastic litter release in the oceans does not decrease, by 2050 the oceans could have more plastic than fishes by weight (Auta et al.,2017)

Microplastics (MPs) are defined as synthetic polymers with dimensions less than 5mm (European Food Safety Authority) and nanoplastics (NPs) are those plastics with size smaller than 0.1 μm (He et al., 2018, Ng et al., 2018). Based on the origin, the MPs can be classified as primary and secondary and both have been found in freshwater systems (Simon et al., 2018). The primary microplastics are present in the formulation of products for personal care (microbeads) and fibers from laundry (Waller et al., 2017). Secondary microplastics are originated from plastic remains (mainly of discarded consumer packaging) that suffer fragmentation by photo-degradation, physical, chemical and/or biological interaction (Auta et al., 2017; Carr et al., 2016; Li et al., 2018, Rio Mendoza et al., 2018;).

The concern for the presence of MPs in the aquatic environment occurs since the ingestion of these compounds can cause toxicity to both humans and other living organisms, in addition to their characteristic of accumulation and persistence in the environment once microbes are usually unable to mineralize and assimilate them (Li et al, 2018; Ng et al., 2018). Aquatic organisms as zooplankton, benthic vertebrates, mollusks, fishes and seabirds can ingest these MPs, leading to biological complications (reduced feeding, energetic deficiencies, injury) or even death (Cole et al., 2014).

Due to the hydrophobicity of MPs, other pollutants, such as persistent organic pollutants (POPs), can be sorbed by these particles that act as vectors for the contamination of other environments and animals, leading to biomagnification. Due to their small superficial area the amount of POPs sorbed per gram of MPs is significant, acting like a vehicle for concentration of these contaminants (Carr et al., 2016; Cole et

al., 2013; Bouwmeester et al., 2015; He et al., 2018; Rocha-Santos, 2018). In the human body, POPs (for example DDT, polychlorinated biphenyls and dioxins), may increase the risk of cancer, reproductive disorders, immune system disorders, endocrine dysregulation and increased congenital defects, even at very low concentrations. Teuten et al. (2007) developed a study to evaluate the contribution of MPs in phenanthrene distribution compared to sediments. For this purpose, polyethylene, polypropylene and PVC in dimensions of 200-250μm were used to provide a high surface area for the hydrophobic organic contaminant (HOC) sorption. The results of the work revealed the sorption preference of this compound on the plastics when compared to the sediments, although there are affinity differences for each polymer. The fact that several species mistakenly feed on plastics, the sorption of organic contaminants by MPs can make them a transport vector. On sea surface microlayer, buoyant MPs with phenanthrene sorbed can be displaced to the sediments, increasing the concentration of the latter in the benthic layer and tissues of lugworm *Arenicola marina* (a common benthic deposit feeder). The processes of sorption and desorption of organic contaminants by MPs still have many gaps. (Mato et al., 2001; Teuten et al., 2007). Little is known to what extent desorption of organic contaminants occurs within the animal organism, studies covering this aspect would provide important information regarding the actual amount of HOC that can cross the food chain.

Although conventional wastewater treatment systems can eliminate from water more than 90% of MPs, these systems are still classified as the major source of release of these emerging compounds in the aquatic environment (Gies et al., 2018; Li et al., 2018). This is due to the enormous volume of effluent discharged by the conventional treatment systems; therefore to the continuous release of these emerging contaminants into the water bodies (Talvitie et al., 2017a).

Thus, millions of MPs are disposed daily in water bodies around the world after secondary treatments (Gies et al., 2018; Ziajahromi et al., 2017; Mason et al., 2016; Gündoğdu et al., 2018). Besides the contamination of bodies of water, around 80%-90% of MPs passing through WWTP are retained in the generated biosolids that are widely applied as fertilizers, representing a substantial soil contamination (He et al., 2018; Murphy et al., 2016; Talvitie et al., 2017b). The microplastics introduced into the soil can be ingested by the terrestrial biota and transported along the food chain and finally

reach human consumption. The study by Huerta Lwanga et al. (2017) showed the possible amount of microplastics ingested by a population in a region of Mexico where chicken gizzards are usually prepared for human consumption. For this, the amount of plastics ingested by the terrestrial biota earthworms and chickens (*Gallus gallus domesticus*) and finally in the preparation of chicken gizzards was evaluated. The number of MPs found in earthworms casts, chicken feces and gizzards were respectively 14.8 ± 28.8MP/g, 129.8 ± 82.3MP / g and 10.2 ± 13.8MP/gizzard. As far as gizzard preparation is concerned, 7 out of 10 women only wash the food on the outside without cleaning it on the inside, this can be aggravating when it comes to the entry of MPs into the food chain by land, reaching the human consumption.

The current review highlights the presence and relevance of WWTP as a source of MPs in the aquatic environment and the techniques for MPs separation and identification by reviewing the current state of knowledge.

2. WWTP as a source of MPs

WWTPs are a source of MPs in both aquatic and terrestrial environment since several recent studies have demonstrated the presence of MPs in water after the secondary treatment. According to literature, microplastics are removed about 78%-98% after primary treatment (Murphy et al., 2016; Talvitie et al., 2017b), while secondary treatment is responsible for a smaller MPs decrease (7-20%) (Murphy et al., 2016; Talvitie et al., 2017b). Thus, the study conducted by Murphy et al. (2016) at a WWTP in Scotland, that serves a population equivalent to 650,000 inhabitants, showed that even with a 98% efficiency of removal of these emerging compounds, about 6.5×10^7 MPs are discharged daily at the aquatic environment after a secondary treatment (about 100MP/equivalent inhabitant).

One of Italy's largest WWTP, which serves a population equivalent to 1,200,000 people, receives approximately 1×10^9 MPs daily (2.5±0.3 MP/L) and even with a removal efficiency of 84% about 1.6×10^8 MP/day are disposed into the aquatic environment after a tertiary treatment, which corresponds to a release of 133MP/equivalent inhabitant. Focusing on the MPs size, in that study the range of 0.5-0.1mm corresponds to the mayor fraction (more than 50%) found after secondary

treatment, in the final effluent and in the sludge. In terms of the shape of these MPs, lines (according the authors this shape presents the same thickness in all length with sharp ends, differently of fibers that show frayed ends) corresponded to 41% of final effluent MPs (Magni et al., 2019). Otherwise, Talvitie et al. (2017b) showed that, in the WWTP's effluent studied by them, around 70% of the particles corresponded to the smallest size (0.100-0.020mm) and 60% were classified as fragments.

The study performed by Mason et al. (2016) in 17 wastewater treatment facilities in the United States (6 of them include advanced/tertiary treatment), showed an averaging of 0.05MP/L in the final effluent totalizing more the 4 million of MPs discharge in environment per day. About the physical characteristic of these MPs, most of them were fibers (59%) followed by fragments (33%). Concerning dimensions, the size of 57% of MPs ranged between 0.125mm and 0.355mm, whereas 43% was larger than 0.355mm.

The composition of the effluent of WWTPs in terms of MPs is singular. The shape, size and amount vary significantly, which make difficult to compare the results. As an example, in Figure 1, results obtained in different countries are represented. They are difficult to compare due to demographic and methodological differences (in this case shown in terms of the smallest considered MPs size) (Gies et al., 2018; Gündoğdu et al., 2018; Lares et al., 2018; Mason et al., 2016; Murphy et al., 2016; Ziajahromi et al., 2017). However, independent of these, the concerning topic is that the values of MPs discharged per day by WWTP around the world is alarming surpassing millions of particles per day.
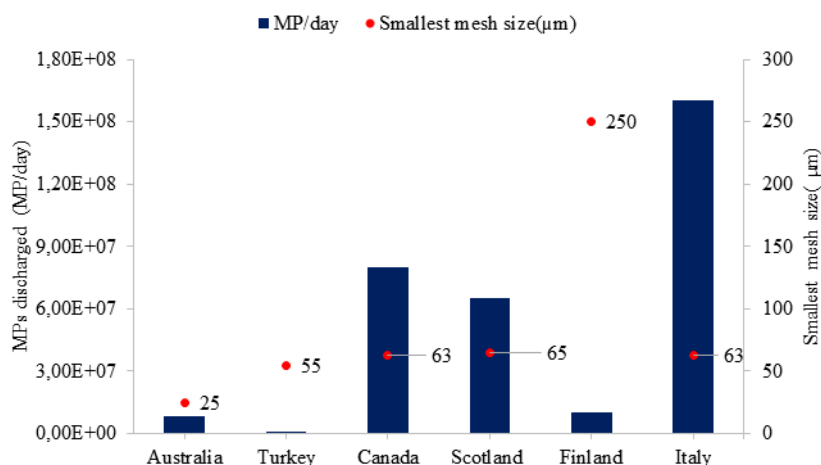

Figure 1- MPs discharged by WWTP in different countries

Source: Gies et al.(2018); Gündoğdu et al. (2018); Magni et al.(2019); Murphy et al (2016); Lares et al.(2018); Ziajahromi et al. (2017).

MPs present in domestic wastewater come from different sources. Personal care and cosmetic products (PCCPs) are a substantial source of microplastics used as exfoliating material in soaps, facial scrubs, shampoos, shaving foam and toothpaste or as beauty propose in form of plastic glitter. The microplastics added to these products can be referred as microbeads or even microspheres and are mostly made of polyethylene (Fendall and Sewell, 2009; Guerranti et al., 2019; Napper et al., 2015). Although this nomenclature refers to particles of spherical geometry, this is not always true. These MPs typically have an irregular shape, but the addition of spherical particles can be performed to enhanced consumer's visual attraction (Kalčíková et al., 2017). These authors reported that MPs incorporated in PCCPs usually are smaller than 1mm (Table 1).

Table 1 - PCCPs as source of microplastics

PCCP	Polymer	Size range (µm)	Reference
Facial cleanser A	PE	10.2–1075.0	
Facial cleanser B	PE	52.5–847.5	Fendall and Sewell, 2009
Facial cleanser C	PE	4.1–1240.0	
Facial cleanser D	PE	31.6–418.4	
Facial scrubs	PE	8-2000	Napper et al., 2015
Toothpaste	PE	90-300	Carr et al., 2016
Facial cleanser	PE	60-800	Chang, 2019
Facial cleanser	Mainly PE	7-1020	Lei et al., 2017
Shower gel	Mainly PE	110-970	
Body scrubs	Mainly PE	Mainly < 200	Kalčíková et al., 2017
Facial scrubs	Mainly PE	Mainly < 100	

Kalčíková et al. (2017) estimated that in the city of Ljubljana (Slovenia) with 300,000 inhabitants, 112,500,000 microbeads are released per day into the receiver river after the WWTP. Furthermore, according to these authors 100mL of the facial scrub can

be a source of more than 1,300,000 particles. According to Napper et al. (2015) around 4,600 – 94,500 polyethylene microbeads may get the sewage system per application of 5mL of a skin exfoliant and in each toothpaste application (1.6g of toothpaste) around 4,000 polyethylene fragments can be discharged as suggested by Carr et al. (2016).

Another commonly type of MPs found in wastewater samples is textile fibers. According to Talvitie et al. (2017b) fibers can represent around 70% of the MPs in WWTP's influent. Laundry of synthetic clothing may release into wastewater more than 1,900 polyester (polyethylene terephthalate) fibers per wash (Browne et al., 2011), one fleece garment could release approximately 110,000 fibers (Almroth et al., 2018) and 5kg of polyester fabrics can release about 6,000,000 microfibers (Falco et al., 2018). Even though the large quantities of fibers disposed in WWTPs, the amount depends on the properties of the fabric, temperature, time and speed of washing, as well as products used as detergents and softeners (Almroth et al., 2017; Falco et al., 2018).

Besides the water contamination, WWTP are also a source of MPs soil pollution once the sludge generated on WWTP is processed and reused as fertilizer (Mohapatra et al., 2016). Carr et al. (2016) estimated that 1.09×10^{9} MP/day get into the environment by biosolids pathway. Nizzetto et al. (2016) estimated that yearly 63,000−430,000 and 44,000−300,000 tons of MPs are added to farmlands in Europe and North America, respectively. In Australia about 2,800 to 19,000 tons of microplastics per year are estimated to apply to agroecosystems through biosolids (Ng et al., 2018).

Regardless where MPs are found (wastewater, biosolid, water, soil), these particles must be previously separated for their quantification and identification. Basically, the sample is subjected to separation techniques, and when necessary purifications with chemical digestions that allow a better visual sorting. Later, the possible MPs are analyzed by different instrumental techniques that allow their chemical identification and classification as polymer or not.

3. Sample processing – sampling and separation techniques

Different sampling methods have been employed for collect microplastics from WWTP effluents. Sampling procedures at wastewater treatment plants can be done in conjunction with the separation step (pumping coupled with filtration, surface filtration, and auto-sampler collection), or samples can be collected in containers and taken to laboratories for separation procedures (Table 2). In the first case, where the separation is made *in-situ* (at the sampling site) has the advantage of a high outflow at the collection points, which enables the separation corresponding to a larger sample volume. Although *in-situ* separation allows for a larger sample volume, precautions should be taken regarding cross-contamination of these samples, as separation is done in an environment with a higher exposure to contamination when compared to a closed and better controlled laboratory.

Table 2 - Sampling methods for WWTP treatment plants

Reference	Sampling	Sample	Volume	Flow rate	Material of sampler device	Range size (μm)
Long et al. (2019)	Pumping and filtration	Influent	2.98-142.98L	6-20L/min		43-355
		Secondary effluent	27.67-348.71L	10-20L/min		
Ziajahromi et al.(2017)	Pumping and sieving	Primary effluent	3-100L	10L/min	Plastic (PVC)	25-500
		Secondary effluent	27-150L			
		Tertiary effluent	200L			
Mason et al. (2016)	Pumping and sieving	Secondary effluent	500-21000L	12-18L/min		125-355
Mintenig et al. (2017)	Pumping	Primary effluent	390-1000L		Plastic	10
		Secondary effluent				

Continuación Tabla 2

Reference	Sampling	Sample	Volume	Flow rate	Material of sampler device	Range size (µm)
Talvitie et al. (2015)	Pumping and sieving	Influent	0.3L	1ml/min	Plastic	20-200
		Primary effluent	1-20L			
		Secondary effluent	1-50L			
		Tertiary effluent	2-285L			
Carr et al. (2016)	Surface filtration	Tertiary effluent				125
Simon et al. (2018)	Auto-Sampler	Influent	1L		Plastic parts	500-2000
		Secondary effluent	4.1-58.5L			10
		Tertiary effluent	81.5L		Glass bottle	10
Michielssen et al. (2016)	Container	Influent	1-2 L		Plastic	20-4750
		Preliminary effluent	1-6 L			
		Primary effluent	10-20 L			
		Secondary effluent	10-20 L			
		Final effluent	34-38 L			
Magni et al. (2019)	Container	Influent	30L		Steel bucket	63-5000
		Secondary effluent	30L			
		Final effluent	30L			
		Sludge	50ml		Glass beaker	

Continuación Tabla 2

Reference	Sampling	Sample	Volume	Flow rate	Material of sampler device	Range size (µm)
Lares et al. (2018)	Container	Influent	0.8-4L		Stainless steel bucket	250-5000
		Secondary effluent	4-30L			
		Sludge	150-200ml			
Leslie et al. (2017)	Container	Primary effluent	2L		Glass jar	0.7-5000
		Secondary effluent	2L			
		Sludge	2L			
Murphy et al. (2016)	Container	Influent	30L		Steel buckets	65
		Preliminary effluent	30L			
		Primary effluent	30L			
		Secondary effluent	50L			
Gies et al. (2018)	Container	Influent	1L		Glass jar	1-65
		Primary effluent	30L			
		Secondary effluent				
		Sludge	250ml			

The separation process is usually performed with a series of sieves of different openings through which a continuous stream of effluent is passed. The mesh sizes of the sieves are chosen according to the size range of the MPs to be collected, but they are generally in the range of 38µm to 4750µm (Hidalgo-Ruz et al., 2012; Wang, W. and Wang, J., 2018). In this range, it is possible to separate the MPs in several sizes categories by using a series of sieves of different mesh. Afterwards, the material on each sieve can be rinsed with distilled water and then stored into glass vials (Long et al., 2019; Ziajahromi et al., 2017).

Monitoring of this procedure is important since effluents constituted with high organic load tend to quickly block the sieves. The sampled volume is a function of the effluent of interest. Tertiary effluents tend to allow for a larger volume used for separation as they have less suspended solids. In addition to the drawback of sieve blockage, the microparticle morphology also influences the separation process. Microfibers, since they have a high length to thickness ratio can be retained horizontally in the sieve or pass longitudinally to a smaller aperture sieve (Michielssen et al., 2016; Ziajahromi et al., 2017). In order to obtain more homogeneous results and fewer quantification and separation errors, the sampling step should be well evaluated and established in order to provide reproducible and comparable data.

After sampling and sieving other additional separation techniques may be applied in order to separate MPs from the sample medium. Among these, the most common techniques are visual sorting, density separation, filtration. Table 3 shows separation techniques currently used. Dyachenko et al. (2017) reject the application of methods that include centrifugation and microwave, since they can cause the rupture and deformation of the MPs. Unfortunately, there is still no standard protocol for separation procedures and this fact makes difficult to compare the number of MPs reported in different sources. In this way, the establishment of standard protocols is of paramount importance for data comparisons.

Table 3 - Separation techniques for MPs in wastewater and biosolids currently used

Location	Sample	Separation	Reference
Turkey	Wastewater	Sieve; density separation (NaI).	Gündoğdu et al., 2018
Germany	Wastewater	Sieve; density separation ($ZnCl_2$).	Mintenig et al., 2017
Australia	Wastewater	Sieve; density separation (NaI).	Ziajahromi et al., 2017
Italy	Wastewater	Sieve; density separation (NaCl); 8 µm cellulose nitrate membrane filters.	Mason et al., 2016
Scotland	Wastewater	Sieve; filter paper 11 µm.	Murphy et al., 2016
Ireland	Biosolids	Sieve; elutriation; density separation ($ZnCl_2$); 1.2 µm glass fiber filter.	Mahon et al., 2017
China	Biosolids	Sieve; density separation (NaCl); glass fiber filter.	Li X. et al., 2018
Norway	Soil and biosolids	Density separation (NaI); glass fiber filter.	Hurley et al., 2018

3.1 Density separation

Each polymer, having a different chemical composition, behaves in a peculiar way in the environment. Regarding the separation of the microplastics from the sample medium, the density of the polymers is an important characteristic. The density of the virgin polymers (i.e. without additives incorporated during the manufacture of products), vary from 0.90 to 1.6 g/cm^3 (see Table 4). Since the typical density of sand and other sediments is around 2.6g/cm^3 (Hidalgo-Ruz et al., 2012; Rocha-Santos and Duarte, 2015; Wang, W. and Wang, J., 2018) the separation of MPs by density difference is a convenient technique to be applied.

Table 4 - Densities and common applications for the main plastics

Polymer Type	Material acronyms	Common application	Polymer density [g/cm^3]	Europe demand [%]
High-density polyethylene	(HDPE)	Plastic bags, plastic lumber, fuel tanks, bottle caps, milk crates	0.93-0.97	12.3
Low-density polyethylene	(LDPE)	Plastic bags and films six-pack rings, flexible snap-on lids	0.91-0.92	17.5
Polypropylene	(PP)	Rope, bottle caps, gear, strappin	0.9-0.91	19.3
Polyethyleneterephthalate	(PET)	Bottles, strapping, textile	1.37-1.45	7.4
Polystyrene	(PS)	Utensils, container	1.04-1.1	
Polyamide (nylon)	(PA)	Fishing nets, rope	1.02-1.05	
Polymethylmethacrylate	(PMMA)		1.09-1.20	
Poly-oxi-methylene	(POM)		1.41-1.61	
Polyvinylchloride	(PVC)	Film, pipe, container	1.16-1.58	
Polymethylacrylate	(PMA)		1.17-1.20	
Polyurethane	(PUR)		1.2	
Seawater			1.027	
Purewater			1	

Sources: GESAMP (2015); Hanvey et al (2017); Hidalgo-Ruz et al (2012); PlasticsEurope (2017)

When matrixes with high organic loads are evaluated, sieving and filtration processes before a previous separation by density can lead to the saturation of the sieves/filters, which makes it difficult to identify and separate the MPs (Lusher et al., 2017). In order to float all the microplastics, samples are mixed to a higher density solution like sodium chloride (NaCl), sodium iodide (NaI), zinc chloride (ZnCl$_2$) or

sodium polytungstate (SPT) solutions and stirred for a predetermined time. Subsequently, the supernatant with the plastic particles is extracted by filtration under normal pressure or a vacuum system for further processing steps (GESAMP, 2015; Hidalgo-Ruz et al., 2012; Rocha-Santos and Duarte, 2015). The saturated solution of NaCl ($1.2 g/cm^3$) is usually used to extract the low-density polymers as PE, PS and PP. The advantage of NaCl is that it is an inexpensive and eco-friendly salt. Otherwise, due to their higher density, NaI solution ($1.8 \ g/cm^3$), $ZnCl_2$ (1.5-$1.7 \ g/cm^3$) or SPT ($1.4 \ g/cm^3$) have to be applied to remove high-density MPs (such as PET and PVC) (Rocha-Santos and Duarte, 2015; Wang, W. and Wang, J., 2018;).

3.2 Filtration

Filtration systems are commonly used for the recovery of MPs from liquid samples or from the supernatant of the density separation, which is passed through paper filters of pore sizes of 1 to 2 μm (Crawford and Quinn, 2017a; Hidalgo-Ruz et al., 2012). The particles retained on the filters can be separated using tweezers for identification (Rocha-Santos and Duarte, 2015). Among the filter media used, glass fiber, nitrocellulose and polycarbonate filters can be cited. Although it is a simple process, the presence of particulate material can block the pores of the filter reducing the efficiency of the process (Wang, W. and Wang, J., 2018). To reduce this drawback, practices as reducing the volume to be filtered or adding chemicals to provide previous flocculation of solid particles can be performed (Crawford and Quinn, 2017a).

4. Sample processing – Digestions

The microplastics separated from the sample medium may contain organic particles which can interfere on the subsequent identification, requiring the removal of these materials from the MPs surface (Enders et al., 2017; GESAMP, 2015; He et al., 2018). Furthermore, the organic material in the sample can be confused with MPs leading to the overestimation of polymers (Prata et al., 2019). When a protocol for digestion is assessed, the effect of the process on the integrity of MPs is an extremely important factor. In the following sub-sections, the main types of digestion methods are reviewed.

4.1. Digestion by hydrolysis with acidic substances

Studies have shown that chemical digestion with acids, such as H_2SO_4 and HNO_3 and alkaline treatments can destroy or damage polymers (He et al., 2018). In this way, the concentration of the acidic solutions used has to be low, jeopardizing the efficacy of the digestion process, i.e. the percentage of removed organic matter. Cole et al. (2014) achieved over 80% removal of biological material using 1M HCl. Enders et al. (2017) performed different chemical digestions on 21 polymers and evaluated the resistance of plastics to the applied reagents. Among these tests, one corresponded to an acid mixture (HNO_3:$HClO_4$ (4:1)). Possible visual modifications (with assignments of different impact levels) and the Raman spectrum after digestion were observed. Digestion was performed for 5 hours at room temperature and then the samples (still immersed in the reagent) were heated at 80°C for 20 minutes. The polymers that suffered the most from chemical digestion were polyamide (PA), polyurethane (PU) and a black tire rubber elastomer, which were completely dissolved. Other polymers that were not completely dissolved showed some degree of colour loss, such as polycarbonate polymer (PC), expanded solid polystyrene (EPS, PS) and polyethylene terephthalate (PET). For polypropylene (PP), high- and low-density polyethylene (HDPE, LDPE), ethylene-vinyl acetate (EVA), and polytetrafluoroethylene (PTFE) no effect was observed. In addition to the chemical reagent, the negative effects on the polymers were also attributed to the application of heating after the digestion periods. The Raman spectra after chemical digestion, in general terms the polymers showed no severe modifications except for acrylonitrile butadiene styrene (ABS). All other polymers had a similar spectrum to the original (without digestion), some showing some signs of degradation or peak deviations, like PS and PVC, that showed fluorescence after chemical digestion, the latter also revealed weakening of the main peaks indicating degradation process. Naidoo et al. (2017) also performed acid digestion assays using HNO_3(55%), the plastics (Nylon, high density Polyethylene, Polystyrene, Poly 1,4-butylene terephthalate and Polyvinyl chloride) were immersed in acid for one month at room temperature. The polymer mass was monitored throughout the digestion period and in the first 24 hours of testing all Nylon was completely disintegrated. The other plastics were resistant to digestion. Catarino et al., 2017 also observed that the acid digestion with HNO_3(35%) affect the integrity of plastic especially Nylon, that was completely dissolved after time reaction. PET and HDPE also showed melding effects.

These studies corroborate the importance of evaluating the resistance of polymers to the digestions proposed in each study, prior to their application in the real sample to be digested. The use of acidic agents showed aggressive to some polymers what may result in the disintegration of some plastics, leading mistaken results regarding the sample count.

4.2. Digestion by hydrolysis with alkaline substances

As the acidic substances, alkaline solutions may damage the morphology of the MPs. Similar to acid treatment, sample loss in the basic treatment can lead to particle underestimation leading to erroneous results. The use of 10M NaOH at a temperature of 60ºC, for example, showed aggressive for some polymers resulting from the partial destruction of nylon fibers and the fusion of polyethylene fragments (Cole et al., 2014). Hurley et al. (2018) carried out a study using 6 different chemical digestions to find out which reagent reaches the highest removal of organic matter. These authors observed that the use of alkaline solutions (1M and 10M NaOH and 10%KOH at 60 ºC) are not appropriate for the removal of organic material once they did not reach 70% organic matter removal for sludge matrixes and soil matrixes. The other tested methods, which were based on oxidation achieved higher digestion efficacies. Nuelle et al. (2014) also reported that solutions of 30% H_2O_2 and 35% H_2O_2 promoted greater removal of organic matter in comparison with NaOH (20, 30, 40 and 50%) and HCl (20%) solutions in sediment samples.

4.3. Digestion by oxidation

Digestion with hydrogen peroxide

Peroxidation is currently being used and differences in contact time and operating temperature of the chemical digestion can be found in the bibliography (Table 5).

Table 5 - Application of peroxide and Fenton reagent for chemical digestion

Sample	Chemical digestion	Time	Temperature	Reference
Wastewater	H_2O_2, 30%	7days	RT[a]	Gies et al., 2018
Wastewater	H_2O_2, 15%	3 days	RT[a]	Magni et al., 2018
Wastewater	H_2O_2, 30%	The reaction was performed until the H_2O_2 fully evaporated.	60º	Ziajahromi et al., 2017
Wastewater	H_2O_2 30% + $FeSO_4 \cdot 7H_2O$	The reaction continued until all organic material disappeared.	75º [b]	Gündoğdu et al., 2018
Wastewater	H_2O_2 30% + $FeSO_4 \cdot 7H_2O$	~1h	75º [b]	Lares et al., 2018
Wastewater	H_2O_2 30% + $FeSO_4 \cdot 7H_2O$	~30min	70º [b]	Dyachenko et al., 2018
Sludge	H_2O_2, 30%	Performed overnight	-	Li X. et al., 2018
Sludge	H_2O_2, 30%	5h	70º	Sujathan et al., 2017
Sludge	H_2O_2, 30%	10 days	RT[a]	Gies et al., 2018
Sludge	H_2O_2, 15%	3 days	RT[a]	Magni et al., 2018
Soil and sludge	H_2O_2, 30%	6h	60º	Lusher et al., 2018
Soil and sludge	H_2O_2 30% + $FeSO_4 \cdot 7H_2O$	2h	RT[a]	Lusher et al., 2018

a RT - Room temperature

b Wet peroxide oxidation

WWTP effluents from the primary, secondary and tertiary treatments studied by Ziajahromi et al (2017) were passed through sieves of different mesh sizes and the retained material was removed with distilled water and subjected to chemical digestion with 30% hydrogen peroxide. The filtered volume (between 3 and 200 L) was stablished according to the degree of blockage of the sieves caused by each effluent. The H_2O_2 solution was added in different volumes (0, 10mL, 20mL and 50mL)

depending on the type of effluent. The digestion was performed under heat (60ºC) until the H_2O_2 fully evaporated.

Gies et al. (2018) used 30% H_2O_2 at room temperature for the chemical digestion of influent, primary and secondary effluent and sludge (biosolids). For the liquid samples the supernatant was separated from the solid organic material by decantation, the settled organic layer was then subjected to chemical digestion for 7 days with 20mL of hydrogen peroxide. For the sludge, it was concluded that a mass of 5g (wet weight) requires 10 days for chemical digestion. The sludge samples were mixed with distilled water and the settled sludge was digested at room temperature before filtration through a 1μm polycarbonate membrane filter under vacuum. The supernatant of both, liquid sample and sludge were processed with a protocol of liquid-liquid separation with canola oil to extract MPs.

In contrast to Gies et al. (2018) who performed the chemical digestion for settled organic matter, Magni et al. (2018) used 15% H_2O_2 to perform chemical digestion of the supernatant (both wastewater and sludge) obtained after separation by density with NaCl (1.2g/cm^3) and filtered through 8μm cellulose nitrate membrane filters. Li X. et al. (2018) also digested chemically the supernatant obtained after a density separation (NaCl 1.2 g/cm^3) of 20g of sludge. In this study the supernatant was passed through a 30μm sieve and the retentate was digested with 100mL of 30% H_2O_2.

Digestion with Fenton's reaction

Peroxidation to remove organic matter (Table 5) requires a high reaction time, which can reach days depending on the amount of organic material in the sample. An alternative method for reducing the need for long exposure times is the use of Fenton reagents, as stated above.

Fenton reaction consists of the use of an inorganic salt solution of Fe^{2+} which has the function of activating the peroxide (usually hydrogen peroxide, H_2O_2) acting as catalyser and leading to the formation of hydroxyl radicals (Equation 1), which has a high oxidation potential (2.80V) (Babuponnusami and Muthukumar, 2014; Bautista et al., 2008; Tagg et al., 2017). In addition to the shorter time required, the reaction does not require the addition of any external energy, i.e., the activation of peroxide occurs

under ambient conditions of temperature and pressure (Babuponnusami and Muthukumar, 2014; Bautista et al., 2008).

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + HO^{\bullet} + HO^- \tag{1}$$
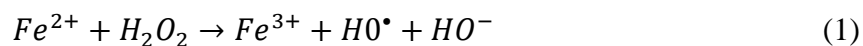
An important parameter to be controlled with respect to the Fenton reaction is pH. It has been shown that pH 3 is the ideal condition for the reaction. The pH adjustment can be performed with sulphuric acid. However, at higher pH, the precipitation of the generated Fe^{3+} as ferric hydroxide occurs and as consequently the availability of iron ions to catalyse the formation of hydroxyl radicals is lower (Babuponnusami and Muthukumar, 2014; Bautista et al., 2008).

In this way, many recent references focus on the combination of hydrogen peroxide solutions with heat and catalysts (like Fenton reagent). This represents an effective procedure to reduce the digestion time (Devi et al. al., 2016; Gies et al., 2018; Gündoğdu et al., 2018; Lares et al., 2018; Lusher et al., 2018; Magni et al., 2018; Sujathan et al., 2017; Ziajahromi et al., 2017).

Tagg et al. (2017) also reported that the polymers (PP, PVC, PE and Nylon) investigated by them showed no significant changes in the spectra generated by ATR-FTIR after chemical digestion with Fenton reaction. In terms of the use of Fenton reagents in the separation of MPs from organic materials Hurley et al., (2018) achieved more than 86% in organic matter removal of sludge sample, whereas the application of NaOH or KOH did not surpass 67%. In this context, Gündoğdu et al (2018) worked with some samples from the influent and secondary effluent. The samples were first sieved (at 55μm) and then, the retained material was subjected to a chemical digestion with Fenton's. Lares et al (2018) also carried out this technique to remove the organic matter from the material retained on the sieves before visual inspection.

Current lliterature points to that high temperatures (higher than 60º) can lead to negative results in the digestion process (Carr et al, 2016). A study realized by Munno et al. (2018) showed that the use of heat (above 60ºC) can melt microbead, which can underestimate the quantities of this MPs in samples. Napper et al. (2018) also reported that the use of heat could lead to an underrepresentation of microbeads in a typical cosmetic product. Considering the negative effects of high temperature on the MPs,

Hurley et al. (2018) proposes a chemical digestion based on the Fenton reaction where the temperature is kept below 40º.

4.4. Enzymatic digestion.

Enzymatic digestion may become an alternative for the organic matter elimination extraction from MPs samples since it is no aggressive for them. In this way, Cole et al. (2014) obtained a digestion efficacy of 88% using Proteinase-K for plankton-rich seawater samples. This efficacy was raised above 97% by increasing the incubation period, the enzyme concentration and the active temperature to 50°C. No degradation of the samples was observed. However, Proteinase-K is very expensive and the procedure is complex. In this way, other authors have proposed alternative enzymes, though the application was for bivalve tissues (Catarino et al., 2017; Courtese-Jones et al., 2017; von Friesen et al., 2019) or for plankton, sediment and biota (Löder et al. 2017). As an alternative to reducing the costs incurred in enzymatic digestion, Coustese-Jones et al. (2017) proposed the use of an enzyme considered less expensive than proteinase-K, in the study trypsin, collagenase and papain were used. Catarino et al. (2017) also used an enzyme considered most economically viable (Corolase 7089). In terms of the effects of enzymatic digestion on the subsequent identification of polymers von Friesen et al. (2019) evaluated the impact of pancreatic enzymes on ten polymers from the FTIR result matching before and after digestion and concluded that there were no significant changes in exposed polymers. As for the other digestion procedures, the enzyme still presents variability in procedures used and, besides, little is known about the enzymatic use for degradation organic matter of WWTP samples. Simon et al. (2018) used cellulase enzyme for a prior cellulose fiber degradation from wastewater samples, however, the oxidation of organic matter was performed with Fenton reaction. In a study by Mintenig et al. (2017) an enzymatic digestion was performed in WWTP effluents. Protease, lipase and cellulase enzymes were used in the treatment. Despite the satisfactory results in the removal of organic materials and MPs separation, the process took more than 10 days to be performed and involved several steps that may have led to contamination and sample loss according to the authors. In this way, studies about application of this technique to wastewater samples are needed to compare the enzymatic digestion with other techniques.

5. Identification techniques

The characterization of MPs can be divided into physics and chemistry. Physical identification is done visually using a microscope and the microparticles are categorized by size, type (fiber, film, foam, pellets or fragments) and color (Crawford and Quinn, 2017b). Visual examination is a mandatory step to identify MPs separated from the matrix and this step have been wildly used by researchers (Table 6).

Table 6 - Operational conditions for MPs identification.

Reference	Identification				
Hidayaturrahman and Lee (2019)	Visual				
Mason et al. (2016)	Visual	-	-		
Talvitie et al. (2015)	Visual	-	-		
Michielssen et al. (2016)	Visual	-	-		
Magni et al. (2019)	Visual	μ-FTIR	Attenuated total reflectance (ATR) mode 32 scans - spectra with wavelengths between 600 and 4000 cm^{-1} Spectral resolution of 4 cm^{-1}		
Lares et al. (2018)	Visual	μ-FTIR	Reflectance mode 24 scans - spectra with wavelengths between 600 and 4000 cm^{-1} Spectral resolution of 4 cm^{-1}	μ-Raman	Green laser (514.53 nm) Spectra with wavelength between 200 and 3000 cm^{-1}

Continuación Tabla 6

Reference	Identification		
Leslie et al. (2017)	Visual	FTIR	Transmission mode. 64 scans were used
			Spectral resolution of 4 cm^{-1}
Murphy et al. (2016)	Visual	FTIR	Reflection mode 16 scans - spectra with wavelengths between 600 and 4000 cm-1
			Spectral resolution of 4 cm^{-1}
Gies et al. (2018)	Visual	FTIR	Particles larger than 3 mm were analyzed with the bench-top attenuated total reflectance (ATR), whereas particles smaller than 3 mm were analyzed using the micro-ATR. For each background and sample, 16 co-added scans in the range of 3800 to 900 cm-1 were collected. Spectral resolution of 8 cm^{-1}.
Ziajahromi et al.(2017)	Visual	FTIR	Attenuated total reflection (ATR). 65 scans - spectra with wavelengths between 600 and 4000 cm^{-1} Spectral resolution of 4 cm^{-1} For smaller particles was used μ-FTIR in transmittance mode, at 8 cm^{-1} resolution and 128 scans

According to Hidalgo-Ruz et al. (2012) preliminary discrimination between plastics and non-plastics can be made from some initial observations: the samples cannot have organic matter, the fibers must have the same thickness throughout its length and the particles must show homogeneous color along the structure. Hidayaturrahman and Lee (2019) based on the observations suggested by Hidalgo-Ruz et al. (2012) to visually identify MPs. Besides the use of spectroscopy for visual identification scanning electron microscopy (SEM) is a method to study the morphology of particles. Due to its generation of high-resolution images, SEM can be applied for the

identification of impurities and possible MPs (Wang, W. and Wang, J., 2018). In addition, after a chemical digestion procedure, SEM can detect possible modifications on the MPs surface.

Despite the application of strict protocols and a very detailed visual evaluation, the number of error increases with the decrease of the particle, even so, this stage is of great importance for previous identification of MPs. In Figures 2(a) and 2(b) a great similarity between fibers is observed, one corresponds to cotton 100% (a) and the other one corresponds to a polyester fiber (b). Without an evaluation of the chemical structure of these fibers both could be erroneously classified as MP.
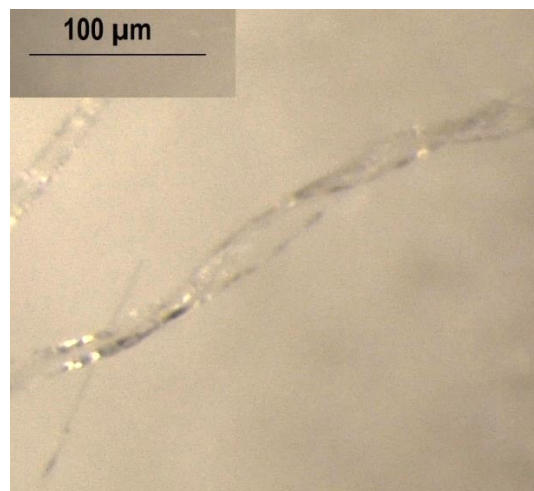
(a)
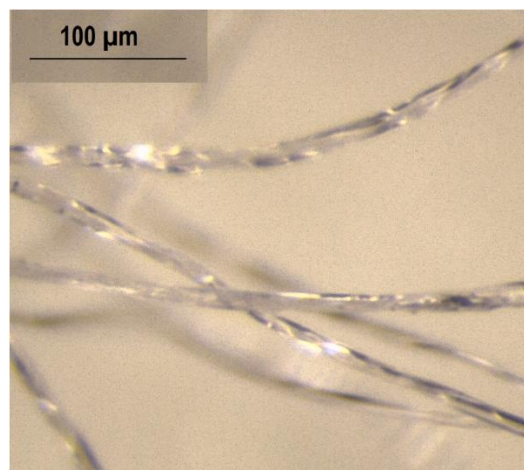
(b)

Figure 2 – Microfibers: (a) Cotton 100%; (b) Polyester 100%.

Literature reports some analytic methods as Pyrolysis gas chromatography-mass spectrometry, FTIR or Raman spectroscopy that can be applied for studying the chemical structure and to identify kinds of MPs. These techniques allow a precise identification of the chemical structure of the samples, and in addition to allowing segregation between MPs and non-MPs, it provides the polymer base and even the presence of additives. This information is important as it can be related to society's behavioural patterns and waste dispositions.

Apart from identifying the chemical structure of MPs, dyeing techniques have been employed in order to separate natural polymers and organic materials from synthetic polymers (MPs). In this context, the Rose-Bengal and Nilo Red reagents may enable this separation (Erni-Cassola et al., 2017; Maes et al.,2017; Ziajahromi et al.,2017). The first reactive acts by staining natural materials and non-MPs allowing visual separation and the second is adsorbed on the surface of plastic materials and requires the use of fluorescence microscopy. Although dyeing protocols are an alternative to rapid separation, a very effective chemical digestion process should be performed as the presence of organic materials can provide false results.

5.1 Fourier transform infrared (FTIR) spectroscopy and Raman

Among the existing techniques for MPs chemical identification Fourier transform infrared (FTIR) and Raman are the most commonly used. Both are vibrational spectroscopic techniques, which involve the molecular excitation of the sample and sequentially the generation of a characteristic spectral fingerprints. With the spectra generated is possible identify the substance by comparing with the spectra of known materials.

FTIR consists in irradiating the sample by IR light. Part of the radiation is absorbed depending on the molecular structure of the sample and then it is measured in transmission or reflection mode. Since each material has different chemical bonds, the spectrum generated by each sample can be compared with a database, which makes possible the identification. FTIR has two possible measurements to identify MPs: transmittance and reflectance setting, including the attenuated total reflectance (ATR) configuration, where the crystal must be in contact with the sample. Table 6 shows the

variability of the operating conditions used in the technique for identification of microplastics whether in read mode (reflectance, transmittance), number of scans used and resolution. Fitting of reading conditions will be a function of the sample, as their size, shape and color may interfere with the analysis, requiring adjustments in equipment to provide an adequate spectrum with less noise and noticeable peaks. The ATR-FTIR proved to be efficient for the identification of larger particles (>500µm). To analyze smaller particles, ATR-FTIR coupled with a microscope (µ-ATR-FTIR) has been applied, and for these, even membrane filters can be visualized directly. Unfortunately, by this method little areas can be searched by time, which makes inviable visual sorting on the entire surface membrane area (Li et al., 2018; Käppler et al., 2018). To solve this drawback the FTIR with focal plane array (FPA) can analyze entire areas (Huppertsberg and Knepper, 2018; Li et al., 2018; Qiu et al., 2016;).

In our preliminary study, MPs were separated from the secondary effluent samples of a WWTP located in Valencia (Spain) and then identified by ATR-FTIR (Bruker) (Figure 3(a)) and µ-ATR-FTIR (Bruker) (Figure 3 (b)). Due the small thickness of the fiber (around 20µm) it was necessary the coupled microscope to place the crystal on the targeted MP.

(a)   (b)
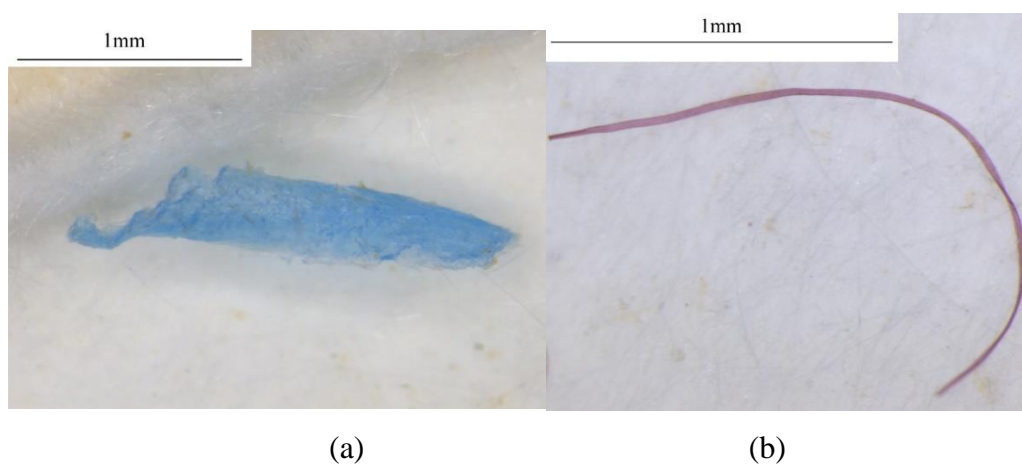
Figure 3- Examples of MPs encountered in secondary effluent:(a) PE fragment, scale bar 1mm (magnification 25x); (c) Nylon fiber, scale bar 1mm (magnification 40x).

The difference between FTIR and Raman is that the spectrum generated by the first technique depends on the change in the permanent dipole moment of the chemical

bond, while the Raman depends on the change in the polarizability of the chemical bond (Käppler et al., 2016).

Operationally, while FTIR spectroscopy uses the incidence of IR light, Raman spectroscopy applies a monochromatic laser and this energy is absorbed by the sample before generating a spectrum. Käppler et al. (2016) suggest the use of both FTIR and Raman to obtain more complete and accurate results of the analyzed particles. In this report, authors compared different range of size and composition of MPs by these advanced techniques. Once Raman can provide more information about non-polar structure, this technique improves the information about the particle. (Lenz et al., 2015)

Despite the high sensitivity of Raman analysis to identify small particles (<20 μm), the method may suffer interference from the additives present in commercial plastics, resulting in considerable modifications in the base polymer's spectrum, which makes it difficult to identify them (Araujo et al., 2018; Lenz et al., 2015; Qiu et al., 2016). These interferences include the presence of foreign band and fluorescence. In this way, the additives present in the matrix can overlay the fingerprint spectrum of the base polymer and the MPs counted can be overestimated. Another problem, in terms of Raman analysis, is the fluorescence. Dyes and pigments may strongly emit fluorescence in the presence of visible light precluding the identification of the polymer spectra (Lenz et al., 2015; Li et al.,2018; Massonnet et al., 2012, Jochem and Lehnert, 2002). Furthermore, the Raman performance depends on the equipment, laser wavelength applied and the operator. Massonnet et al. (2012) showed that a same dye fiber can provide different spectrums depending on the instrument used and mainly the excitation wavelength. Additionally, the pigment concentration and the combination of pigments affect directly on the result obtained.

5.2 Thermo-analytical methods

Apart from the application of commonly techniques, as FTIR and Raman, thermo-analytical methods also have been used on MPs characterization (Fries et al., 2017; Hermabessiere et al., 2018). Samples treated by thermo-analytical methods release gaseous compounds that are transferred to gas chromatography-mass (GC) for identification of chemical compounds (Li et al., 2018). Pyrolysis-gas chromatography-

mass spectrometry (Py-GC-MS) is a thermo-analytical method that employs pyrolyzation to identify MP (Hermabessiere et al., 2018) and its additives simultaneously by the direct introduction of the sample with minimal pre-treatment (Crawford and Quinn, 2017b). The method is capable to identify a single particle, and a small amount of sample (0.1 – 0.5mg) is suitable for one measure (Dümichen et al., 2017). The disadvantages of this method are because it is destructive, the sample must be manually placed in the instrument (Crawford and Quinn, 2017b) and compounds with high molecular weight (400 g mol^{-1}) can be condensed into the capillary from the pyrolysis to GC-MS system (Dümichen et al., 2017).

To overcome these drawbacks of Py-GC-MS another thermo-analytical method has been studied for the identification of microplastics. The TED-GC-MS combines the thermal extraction with thermogravimetric analysis (TGA) with thermal desorption gas chromatography mass spectrometry (TDS-GC-MS) which allows to identify MPs in environmental samples (Dümichen et al., 2017). The TGA provides information about the mass change of polymers during heating and when coupled to MS or FTIR is possible to identify the decomposition products of the process. (Duemichen et al., 2014). Based on this, TED-GC-MS initially uses heating of MPs sample under inert atmosphere and the use of a thermogravimetric balance. The decomposition productions are adsorbing on a solid-phase located on the air outlet of the oven and these are transferred to the thermal desorption unit. The organic compounds desorbed are separated through a chromatographic column and identified by mass spectroscopy. The generated spectrum can be compared to spectral libraries and use to create database (Elert et al., 2017).

6. **Quality assurance/Quality control (QA/QC)**

A workshop report published by the Environmental Protection Agency (EPA) highlights the importance of procedures that ensure Quality assurance/Quality control (QA/QC) in the processes of sampling, separation and identification of MPs in order to generate reliable data and reduce underestimation or overestimation of MPs. The purpose of the QA/QC procedures is to reduce any type of contamination of the sample, to establish inherent errors in the separation techniques (during the processes used, such

as filtration, sieving and density separation) as well as during the chemical digestion of MPs.

In addition, the instrumental techniques used (Raman, FTIR, thermo-analytical methods or other) must also be carefully studied for method determination, limit of detection and establishment of a reliable database (Fisher and Scholz-Bottcher 2017; Hermabessiere et al, 2018). Procedures as avoiding synthetic clothing, using glass materials instead of plastics, cleaning the work surface with alcohol and the use of blanks to evaluate sample contamination and losses are reported by some authors (Gies et al., 2018; Lares et al., 2018; Lenz et al.,2015; Li X et al., 2018; Liu et al., 2018; Magni et al, 2019). In order to assess airborne contamination petri dishes with a membrane filter can be placed on the workspace for some hours and then can be analyzed as black control (Lenz et al, 2015).

7. Conclusion and perspectives

The presence of MPs in water bodies is increasingly evident around the world. It is noticeable that in the case of effluents from WWTPs millions of MPs are released per day all over the world. Because of that, these facilities are considered as significant sources of MPs even when they have a high percentage of retention. Despite the intense efforts that have been directed towards the elaboration of methodologies of separation, quantification and identification of these emerging contaminants, no standard protocol is still applied in WWTPs. These methodological differences presented by researchers are seen even in the initial stages of sampling and in the selection of size ranges of MPs to be analysed, which makes difficult the comparison of the results among researchers. Therefore, the determination of efficient and rapid protocols for the study of MPs is extremely important, always considering steps that evaluate cross-contamination, either in the transport of samples or during analysis. In addition, the standardization of sizes (sieving, nets and filters), chemical digestion (acidic, basic, peroxidation or other), density separation (best solution to be used), visual separation (addition of staining dyes) and analytical techniques for chemical identification of the polymer, need to be optimized and applied in a standard manner.

Based on current knowledge of WWTP as sources of microplastics and the remaining gaps, the following aspects deserve attention for future research:

- Since microplastics have shown high sorption potential of organic pollutants, the better understanding of the desorption process is relevant to the understanding of how these pollutants can actually be transported in the food chain.

- Given the accumulation of MPs in biological sludge, future studies on the incorporation of these particles into terrestrial animals should be performed.

- In terms of digestion procedures, we understand that when evaluating WWTP, factors such as effluent compositions (amount of organic matter, solids, pH, among others) and the fact of treatment procedures employed in the stations differs sometimes, the standardization of a methodology can present difficulties. However, since some digestion procedures have already been shown to be extremely aggressive to MPs, a basic methodology with at least the reagent and its concentration to be used should be designed and established to facilitate reproducibility of the results.

- With the review of the literature performed in this paper, it was observed that several authors use only visual identification to distinguish microplastics from non-plastics. This step is of fundamental importance for an initial screening of possible MPs however, this may imply a wrong sample count and classification. For example, without the use of chemical identification techniques, natural fibers can be mistakenly called MPs when performing only visual separation. For better validation and comparison of results, the application of polymer identification methods should be more widely used and not just the visual method.

REFERENCES

Almroth, B.M.C, Åström, L., Roslund, S., Petersson, H., Johansson, M., Persson, N. K., 2018. Quantifying shedding of synthetic fibers from textiles; a source of microplastics released into the environment. Environ Sci Pollut Res. 25, 1191–1199. https://doi.org/10.1007/s11356-017-0528-7.

Araujo, C.F., Nolasco, M.M., Ribeiro, A.M.P., Ribeiro-Claro, P.J.A., 2018. Identification of microplastics using Raman spectroscopy: Latest developments and future prospects. Water Res. 142, 426–440. https://doi.org/10.1016/j.watres.2018.05.060

Auta, H.S., Emenike, C.U., Fauziah, S.H., 2017. Distribution and importance of microplastics in the marine environment A review of the sources, fate, effects, and potential solutions. Environ. Int. 102, 165–176. https://doi.org/10.1016/j.envint.2017.02.013

Babuponnusami, A., Muthukumar, K., 2014. Journal of Environmental Chemical Engineering A review on Fenton and improvements to the Fenton process for wastewater treatment. J. Environ. Chem. Eng. 2, 557–572. https://doi.org/10.1016/j.jece.2013.10.011

Bautista, P.A., Mohedano, F., Casas, J.A., Zazo J.A., Rodriguez, J.J., 2008. An overview of the application of Fenton oxidation to industrial wastewaters treatment. J. Chem. Technol. Biotechnol. 83,1323–1338. https://doi.org/10.1002/jctb.1988

Bouwmeester, H., Hollman, P.C.H., Peters, R.J.B., 2015. Potential Health Impact of Environmentally Released Micro- and Nanoplastics in the Human Food Production Chain: Experiences from Nanotoxicology. Environ. Sci. Technol. 49, 8932–8947. https://doi.org/10.1021/acs.est.5b01090

Browne, M.A., Crump, P., Niven, S.J., Teuten, E., Tonkin, A., Galloway, T., Thompson, R., 2011. Accumulation of microplastic on shorelines woldwide:

Sources and sinks. Environ. Sci. Technol. 45, 9175–9179. https://doi.org/10.1021/es201811s

Carr, S.A., Liu, J., Tesoro, A.G., 2016. Transport and fate of microplastic particles in wastewater treatment plants. Water Res. 91, 174–82. https://doi.org/10.1016/j.watres.2016.01.002

Catarino, A.I., Thompson,R.,Sanderson,W.,Henry,T.B.,2017. Development and optimization of a standard method for extraction of microplastics in mussels by enzyme digestion of soft tissues.Environ.Toxicol.Chem.36,947–951. https://doi.org/10. 1002/etc.3608.

Chang, M., 2015. Reducing microplastics from facial exfoliating cleansers in wastewater through treatment versus consumer product decisions. Mar. Pollut. Bull. 101, 330–333. https://doi.org/10.1016/j.marpolbul.2015.10.074

Cole, M., Webb, H., Lindeque, P.K., Fileman, E.S., Halsband, C., Galloway, T.S., 2014. Isolation of microplastics in biota-rich seawater samples and marine organisms. Sci. Rep. 4, 1–8. https://doi.org/10.1038/srep04528

Cole, M., Lindeque, P., Fileman, E., Halsband, C., Goodhead, R., Moger, J., Galloway, T., S., 2013. Microplastic Ingestion by Zooplankton. Environ. Sci. Technol. 47, 6646−6655. https://doi.org/10.1021/es400663f.

Courtene-Jones, W., Quinn, B., Murphy, F., Gary, F., 2017. Analytical Methods Optimisation of enzymatic digestion and validation of specimen preservation methods for the analysis of ingested microplastics. Anal.Methods. 9,1437–1445 https://doi.org/10.1039/c6ay02343f

Crawford, C.B., Quinn, B., 2017a. Microplastic separation techniques, in: Microplastic Pollutants. pp. 203–218. https://doi.org/10.1016/b978-0-12-809406-8.00009-8

Crawford, C.B., Quinn, B., 2017b. Microplastic identification techniques, in: Microplastic Pollutants. pp. 219–267. https://doi.org/10.1016/b978-0-12-809406-8.00010-4

Devi, P., Das, U., Dalai, A.K., 2016. In-situ chemical oxidation: Principle and applications of peroxide and persulfate treatments in wastewater systems. Sci. Total Environ. 571, 643–657. https://doi.org/10.1016/j.scitotenv.2016.07.032

Dümichen, E., Eisentraut, P., Gerhard, C., Barthel, A., Senz, R., Braun, U., 2017. Fast identification of microplastics in complex environmental samples by a thermal degradation method. Chemosphere 174, 572–584. https://doi.org/10.1016/j.chemosphere.2017.02.010

Duemichen, E., Braun, U., Senz, R., Fabian, G., Sturm, H., 2014. Assessment of a new method for the analysis of decomposition gases of polymers by a combining thermogravimetric solid-phase extraction and thermal desorption gas chromatography mass spectrometry. J. Chromatogr. A 1354, 117–128. https://doi.org/10.1016/j.chroma.2014.05.057

Dyachenko, A., Mitchell, J., Arsem, N., 2017. Extraction and identification of microplastic particles from secondary wastewater treatment plant (WWTP) effluent. Anal. Methods 9, 1412–1418. https://doi.org/10.1039/c6ay02397e

Elert, A.M., Becker, R., Duemichen, E., Eisentraut, P., Falkenhagen, J., Sturm, H., Braun, U., 2017. Comparison of different methods for MP detection: What can we learn from them, and why asking the right question before measurements matters? Environ. Pollut. 231, 1256–1264. https://doi.org/10.1016/j.envpol.2017.08.074

Enders, K., Lenz, R., Beer, S., Stedmon, C.A., 2017. Extraction of microplastic from biota : recommended acidic digestion destroys common plastic polymers. ICES Journal of Marine Science. 74, 326–331. https://doi.org/10.1093/icesjms/fsw173

Erni-Cassola, G., Gibson, M.I., Thompson, R.C., Christie-Oleza, J.A., 2017. Lost, but Found with Nile Red: A Novel Method for Detecting and Quantifying Small Microplastics (1 mm to 20 μm) in Environmental Samples. Environ. Sci. Technol. 51, 13641–13648. https://doi.org/10.1021/acs.est.7b04512

Falco, F. De, Pia, M., Gentile, G., Di, E., Escudero, R., Villalba, R., Mossotti, R., Montarsolo, A., Gavignano, S., Tonin, C., Avella, M., 2018. Evaluation of

microplastic release caused by textile washing processes of synthetic fabrics. Environ. Pollut. 236, 916–925. https://doi.org/10.1016/j.envpol.2017.10.057

Fendall, L.S., Sewell, M.A., 2009. Contributing to marine pollution by washing your face: Microplastics in facial cleansers. Mar. Pollut. Bull. 58, 1225–1228. https://doi.org/10.1016/j.marpolbul.2009.04.025

Fischer, M., Scholz-Böttcher, B.M., 2017. Simultaneous Trace Identification and Quantification of Common Types of Microplastics in Environmental Samples by Pyrolysis-Gas Chromatography-Mass Spectrometry. Environ. Sci. Technol. 51, 5052–5060. https://doi.org/10.1021/acs.est.6b06362

Fries, E., Dekiff, J.H., Willmeyer, J., Nuelle, M.T., Ebert, M., Remy, D., 2013. Identification of polymer types and additives in marine microplastic particles using pyrolysis-GC/MS and scanning electron microscopy. Environ. Sci. Process. Impacts 15, 1949–1956. https://doi.org/10.1039/c3em00214d

GESAMP, 2015. "Sources, fate and effects of microplastics in the marine environment: a global assessment" (Kershaw, P. J., ed.). (IMO/FAO/UNESCO-IOC/UNIDO/WMO/IAEA/UN/UNEP/UNDP Joint Group of Experts on the Scientific Aspects of Marine Environmental Protection). Rep. Stud. GESAMP No. 90, 96 p.

Gies, E.A., LeNoble, J.L., Noël, M., Etemadifar, A., Bishay, F., Hall, E.R., Ross, P.S., 2018. Retention of microplastics in a major secondary wastewater treatment plant in Vancouver, Canada. Mar. Pollut. Bull. 133, 553–561. https://doi.org/10.1016/j.marpolbul.2018.06.006

Guerranti, C., Martellini, T., Perra, G., Scopetani, C., Cincinelli, A., 2019. Microplastics in cosmetics: Environmental issues and needs for global bans. Environ. Toxicol. Pharmacol. 68, 75–79. https://doi.org/10.1016/j.etap.2019.03.007

Gündoğdu, S., Çevik, C., Güzel, E., Kilercioğlu, S., 2018. Microplastics in municipal wastewater treatment plants in Turkey: a comparison of the influent and secondary

effluent concentrations. Environ. Monit. Assess. 190. https://doi.org/10.1007/s10661-018-7010-y

Hanvey, J.S., Lewis, P.J., Lavers, J.L., Crosbie, N.D., Pozo, K., Clarke, B.O., 2017. A review of analytical techniques for quantifying microplastics in sediments. Anal. Methods. https://doi.org/10.1039/c6ay02707e

He, D., Luo, Y., Lu, S., Liu, M., Song, Y., Lei, L., 2018. Microplastics in soils: Analytical methods, pollution characteristics and ecological risks. TrAC - Trends Anal. Chem. https://doi.org/10.1016/j.trac.2018.10.006

Hermabessiere, L., Himber, C., Boricaud, B., Kazour, M., Amara, R., Cassone, A., Laurentie, M., Paul-pont, I., Soudant, P., Dehaut, A., Duflos, G., 2018. Optimization, performance, and application of a pyrolysis-GC / MS method for the identification of microplastics 6663–6676.

Hidalgo-Ruz, V., Gutow, L., Thompson, R.C., Thiel, M., 2012. Microplastics in the marine environment: A review of the methods used for identification and quantification. Environ. Sci. Technol. 46, 3060–3075. https://doi.org/10.1021/es2031505

Hidayaturrahman, H., Lee, T., 2019. A study on characteristics of microplastic in wastewater of South Korea : Identification , quantification , and fate of microplastics during treatment process. Mar. Pollut. Bull. 146, 696–702. https://doi.org/10.1016/j.marpolbul.2019.06.071

Huerta Lwanga, E., Mendoza Vega, J., Ku Quej, V., Chi, J. de los A., Sanchez del Cid, L., Chi, C., Escalona Segura, G., Gertsen, H., Salánki, T., van der Ploeg, M., Koelmans, A.A., Geissen, V., 2017. Field evidence for transfer of plastic debris along a terrestrial food chain. Sci. Rep. 7, 1–7. https://doi.org/10.1038/s41598-017-14588-2

Huppertsberg, S., Knepper, T.P., 2018. Instrumental analysis of microplastics—benefits and challenges. Anal. Bioanal. Chem. 410, 6343–6352. https://doi.org/10.1007/s00216-018-1210-8

Hurley, R.R., Lusher, A.L., Olsen, M., Nizzetto, L., 2018. Validation of a Method for Extracting Microplastics from Complex, Organic-Rich, Environmental Matrices. Environ. Sci. Technol. 52, 7409–7417. https://doi.org/10.1021/acs.est.8b01517

Jochem, G., Lehnert, R.J., 2002. On the potential of Raman microscopy for the forensic analysis of coloured textile fibres. Sci. Justice - J. Forensic Sci. Soc. 42, 215–221. https://doi.org/10.1016/S1355-0306(02)71831-5

Kalčíková, G., Alič, B., Skalar, T., Bundschuh, M., Gotvajn, A.Ž., 2017. Wastewater treatment plant effluents as source of cosmetic polyethylene microbeads to freshwater. Chemosphere 188, 25–31. https://doi.org/10.1016/j.chemosphere.2017.08.131

Käppler, A., Fischer, M., Scholz-Böttcher, B.M., Oberbeckamann, S., Labrenz, M., Fischer, D., Eichhorn, K., Voit, B., 2018. Comparison of µ-ATR-FTIR spectroscopy and py-GCMS as identification tools for microplastic particles and fibers isolated from river sediments. Anal. Bioanal. Chem. 410, 5313–5327. https://doi.org/10.1007/s00216-018-1185-5.

Käppler, A., Fischer, D., Oberbeckmann, S., Schernewski, G., Labrenz, M., Eichhorn, K.J., Voit, B., 2016. Analysis of environmental microplastics by vibrational microspectroscopy: FTIR, Raman or both? Anal. Bioanal. Chem. 408, 8377–8391. https://doi.org/10.1007/s00216-016-9956-3

Lei, K., Qiao, F., Liu, Q., Wei, Z., Qi, H., Cui, S., Yue, X., Deng, Y., An, L., 2017. Microplastics releasing from personal care and cosmetic products in China. Mar. Pollut. Bull. 123, 122–126. https://doi.org/10.1016/j.marpolbul.2017.09.016

Lenz, R., Enders, K., Stedmon, C.A., MacKenzie, D.M.A., Nielsen, T.G., 2015. A critical assessment of visual identification of marine microplastic using Raman spectroscopy for analysis improvement. Mar. Pollut. Bull. 100, 82–91. https://doi.org/10.1016/j.marpolbul.2015.09.026

Leslie, H.A., Brandsma, S.H., Velzen, M.J.M. Van, Vethaak, A.D., 2017. Microplastics en route: Field measurements in the Dutch river delta and Amsterdam canals ,

wastewater treatment plants , North Sea sediments and biota. Environ. Int. 101, 133–142. https://doi.org/10.1016/j.envint.2017.01.018

Li, J., Liu, H., Paul Chen, J., 2018. Microplastics in freshwater systems: A review on occurrence, environmental effects, and methods for microplastics detection. Water Res. 137, 362-374. https://doi.org/10.1016/j.watres.2017.12.056

Li, X., Chen, L., Mei, Q., Dong, B., Dai, X., Ding, G., Zeng, E.Y., 2018. Microplastics in sewage sludge from the wastewater treatment plants in China. Water Res. 142, 75–85. https://doi.org/10.1016/j.watres.2018.05.034

Liu, X., Yuan, W., Di, M., Li, Z., Wang, J., 2019. Transfer and fate of microplastics during the conventional activated sludge process in one wastewater treatment plant of China. Chem. Eng. J. 362, 176–182. https://doi.org/10.1016/j.cej.2019.01.033

Long, Z., Pan, Z., Wang, W., Ren, J., Yu, X., Lin, L., Lin, H., Chen, H., Jin, X., 2019. Microplastic abundance, characteristics, and removal in wastewater treatment plants in a coastal city of China. Water Res. 155, 255–265. https://doi.org/10.1016/j.watres.2019.02.028

Lusher, A. L.; Hurley, R. R.; Vogelsang, C.; Nizzetto, L.; Olsen, M., 2018. Mapping microplastics in sludge. Technical Report. https://doi.org/10.13140/RG.2.2.25277.56804

Maes, T., Jessop, R., Wellner, N., Haupt, K., Mayes, A.G., 2017. A rapid-screening approach to detect and quantify microplastics based on fluorescent tagging with Nile Red. Sci. Rep. 7, 44501.

Magni, S., Binelli, A., Pittura, L., Avio, C.G., Della Torre, C., Parenti, C.C., Gorbi, S., Regoli, F., 2019. The fate of microplastics in an Italian Wastewater Treatment Plant. Sci. Total Environ. 652, 602–610. https://doi.org/10.1016/j.scitotenv.2018.10.269

Mahon, A.M., O'Connell, B., Healy, M.G., O'Connor, I., Officer, R., Nash, R., Morrison, L., 2017. Microplastics in sewage sludge: Effects of treatment. Environ. Sci. Technol. 51, 810–818. https://doi.org/10.1021/acs.est.6b04048

Mason, S.A., Garneau, D., Sutton, R., Chu, Y., Ehmann, K., Barnes, J., Fink, P., Papazissimos, D., Rogers, D.L., 2016. Microplastic pollution is widely detected in US municipal wastewater treatment plant effluent. Environ. Pollut. 218, 1045–1054. https://doi.org/10.1016/j.envpol.2016.08.056

Massonnet, G., Buzzini, P., Monard, F., Jochem, G., Fido, L., Bell, S., Stauber, M., Coyle, T., Roux, C., Hemmings, J., Leijenhorst, H., Van Zanten, Z., Wiggins, K., Smith, C., Chabli, S., Sauneuf, T., Rosengarten, A., Meile, C., Ketterer, S., Blumer, A., 2012. Raman spectroscopy and microspectrophotometry of reactive dyes on cotton fibres: Analysis and detection limits. Forensic Sci. Int. 222, 200–207. https://doi.org/10.1016/j.forsciint.2012.05.025

Mato, Y., Isobe, T., 2001. Plastic Resin Pellets as a Transport Medium for Toxic Chemicals in the Marine Environment. Environ. Sci. Technol. 35, 318–324. https://doi.org/10.1021/es0010498

Michielssen, M.R., Michielssen, E.R., Ni, J., Duhaime, M.B., 2016. Fate of microplastics and other small anthropogenic litter (SAL) in wastewater treatment plants depends on unit processes employed. Environ. Sci. Water Res. Technol. 2, 1064–1073. https://doi.org/10.1039/C6EW00207B

Rios Mendoza, L.M., Karapanagioti, H., Álvarez, N.R., 2018. Micro(nanoplastics) in the marine environment: Current knowledge and gaps. Curr. Opin. Environ. Sci. Heal. 1, 47–51. https://doi.org/10.1016/j.coesh.2017.11.004

Mintenig, S.M., Int-Veen, I., Löder, M.G.J., Primpke, S., Gerdts, G., 2017. Identification of microplastic in effluents of waste water treatment plants using focal plane array-based micro-Fourier-transform infrared imaging. Water Res. 108, 365–372. https://doi.org/10.1016/j.watres.2016.11.015

Mohapatra, D.P., Cledón, M., Brar, S.K., Surampalli, R.Y., 2016. Application of Wastewater and Biosolids in Soil: Occurrence and Fate of Emerging Contaminants. Water. Air. Soil Pollut. 227, 1-14. https://doi.org/10.1007/s11270-016-2768-4

Munno, K., Helm, P.A., Jackson, D.A., Rochman, C., Sims, A., 2018. Impacts of temperature and selected chemical digestion methods on microplastic particles. Environ. Toxicol. Chem. 37, 91–98. https://doi.org/10.1002/etc.3935

Murphy, F., Ewins, C., Carbonnier, F., Quinn, B., 2016. Wastewater Treatment Works (WwTW) as a Source of Microplastics in the Aquatic Environment. Environ. Sci. Technol. 50, 5800–5808. https://doi.org/10.1021/acs.est.5b05416

Naidoo, T., Goordiyal, K., Glassom, D., 2017. Are Nitric Acid (HNO3) Digestions Efficient in Isolating Microplastics from Juvenile Fish? Water, Air, Soil Pollut. 228, 470. https://doi.org/10.1007/s11270-017-3654-4

Napper, I.E., Bakir, A., Rowland, S.J., Thompson, R.C., 2015. Characterisation, quantity and sorptive properties of microplastics extracted from cosmetics. Mar. Pollut. Bull. 99, 178–185. https://doi.org/10.1016/j.marpolbul.2015.07.029

Ng, E.L., Huerta Lwanga, E., Eldridge, S.M., Johnston, P., Hu, H.W., Geissen, V., Chen, D., 2018. An overview of microplastic and nanoplastic pollution in agroecosystems. Sci. Total Environ. 627,1377-1388. https://doi.org/10.1016/j.scitotenv.2018.01.341

Nizzetto, L., Futter, M., Langaas, S., 2016. Are Agricultural Soils Dumps for Microplastics of Urban Origin? Environ. Sci. Technol. 50, 10777-10779. https://doi.org/10.1021/acs.est.6b04140

Nuelle, M.T., Dekiff, J.H., Remy, D., Fries, E., 2014. A new analytical approach for monitoring microplastics in marine sediments. Environ. Pollut. 184, 161–169. https://doi.org/10.1016/j.envpol.2013.07.027

PlasticsEurope & European Association of Plastics Recycling (EPRO), 2017. Plastics – the Facts 2017, Plastics – the Facts 2017. https://www.plasticseurope.org/en/resources/publications/274-plastics-facts-2017 (accessed 19 Novembre 2018).

Prata, J.C., da Costa, J.P., Duarte, A.C., Rocha-Santos, T., 2019. Methods for sampling and detection of microplastics in water and sediment: A critical review. TrAC - Trends Anal. Chem. 110, 150–159. https://doi.org/10.1016/j.trac.2018.10.029

Qiu, Q., Tan, Z., Wang, J., Peng, J., Li, M., Zhan, Z., 2016. Extraction, enumeration and identification methods for monitoring microplastics in the environment. Estuar. Coast. Shelf Sci. 176.102-109. https://doi.org/10.1016/j.ecss.2016.04.012

Rocha-Santos, T.A.P., 2018. Editorial overview: Micro and nano-plastics. Curr. Opin. Environ. Sci. Heal. 1, 52–54. https://doi.org/10.1016/j.coesh.2018.01.003

Rocha-Santos, T., Duarte, A.C., 2015. A critical overview of the analytical approaches to the occurrence, the fate and the behavior of microplastics in the environment. TrAC - Trends Anal. Chem. 65, 47–53. https://doi.org/10.1016/j.trac.2014.10.011

Simon, M., van Alst, N., Vollertsen, J., 2018. Quantification of microplastic mass and removal rates at wastewater treatment plants applying Focal Plane Array (FPA)-based Fourier Transform Infrared (FT-IR) imaging. Water Res. 142, 1–9. https://doi.org/10.1016/j.watres.2018.05.019

Sujathan, S., Kniggendorf, A.K., Kumar, A., Roth, B., Rosenwinkel, K.H., Nogueira, R., 2017. Heat and Bleach: A Cost-Efficient Method for Extracting Microplastics from Return Activated Sludge. Arch. Environ. Contam. Toxicol. 73, 641–648. https://doi.org/10.1007/s00244-017-0415-8

Tagg, A.S., Harrison, J.P., Ju-Nam, Y., Sapp, M., Bradley, E.L., Sinclair, C.J., Ojeda, J.J., 2017. Fenton's reagent for the rapid and efficient isolation of microplastics from wastewater. Chem. Commun. 53, 372–375. https://doi.org/10.1039/c6cc08798a

Talvitie, J., Mikola, A., Koistinen, A., Setälä, O., 2017a. Solutions to microplastic pollution – Removal of microplastics from wastewater effluent with advanced wastewater treatment technologies. Water Res. 123, 401–407. https://doi.org/10.1016/j.watres.2017.07.005

Talvitie, J., Mikola, A., Setälä, O., Heinonen, M., Koistinen, A., 2017b. How well is microlitter purified from wastewater? – A detailed study on the stepwise removal of microlitter in a tertiary level wastewater treatment plant. Water Res. 109, 164–172. https://doi.org/10.1016/j.watres.2016.11.046

Teuten, E.L., Rowland, S.J., Galloway, T.S., Thompson, R.C., 2007. Potential for plastics to transport hydrophobic contaminants. Environ. Sci. Technol. 41, 7759-7764. https://doi.org/10.1021/es071737s

von Friesen, L.W., Granberg, M.E., Hassellöv, M., Gabrielsen, G.W., Magnusson, K., 2019. An efficient and gentle enzymatic digestion protocol for the extraction of microplastics from bivalve tissue. Mar. Pollut. Bull. 142, 129–134. https://doi.org/10.1016/j.marpolbul.2019.03.016

UNEP, 2016. Marine plastic debris and microplastics – Global lessons and research to inspire action and guide policy change. United Nations Environment Programme, Nairobi. https://doi.org/10.18356/0b228f55-en

Waller, C.L., Griffiths, H.J., Waluda, C.M., Thorpe, S.E., Loaiza, I., Moreno, B., Pacherres, C.O., Hughes, K.A., 2017. Microplastics in the Antarctic marine system: An emerging area of research. Sci. Total Environ. 598, 220-227. https://doi.org/10.1016/j.scitotenv.2017.03.283

Wang, W., Wang, J., 2018. Investigation of microplastics in aquatic environments: An overview of the methods used, from field sampling to laboratory analysis. TrAC - Trends Anal. Chem. 108, 195-202. https://doi.org/10.1016/j.trac.2018.08.026

Ziajahromi, S., Neale, P.A., Rintoul, L., Leusch, F.D.L., 2017. Wastewater treatment plants as a pathway for microplastics: Development of a new approach to sample

wastewater-based microplastics. Water Res. 112, 93–99. https://doi.org/10.1016/j.watres.2017.01.042

Capítulo III

Separation and identification of microplastics from primary and secondary effluents and activated sludge from wastewater treatment plants

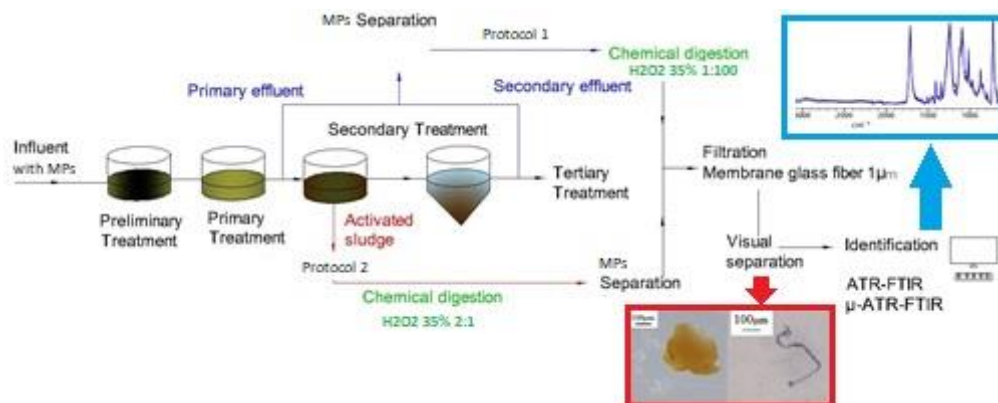Autores – C. Bretas Alvim, M.A Bes-Piá, J.A. Mendoza-Roca

Separation and identification of microplastics from primary and secondary effluents and activated sludge from wastewater treatment plants

C. Bretas Alvim, M.A. Bes-Piá, J.A. Mendoza-Roca

Show more ∨

+ Add to Mendeley   ⅋ Share   𝟱𝟱 Cite

Get rights and content

Graphical abstract

Abstract

Although wastewater treatment plants can retain a high percentage of microplastics (MP) arriving at the facilities, no method for extracting and characterizing these microparticles has been still standardized in these units. This study investigated three protocols of chemical digestion, prior to analysis of microplastics, one directed to the effluents, using peroxidation, and two for activated sludge (peroxidation and Fenton). The samples (primary effluent, secondary effluent and activated sludge) were collected from a wastewater treatment plant (WWTP) located in Valencia (Spain). In addition, four common types of polymers (Low density polyethylene-LDPE, Polypropylene-PP, Polystyrene-PS and Polyethylene terephthalate-PET) were used to assess the influence of reagent exposure on microparticle integrity. Peroxidation was effective in treating the studied effluents (primary and secondary) and was also identified as the ideal protocol for activated sludge. The analysis showed that the use of H_2O_2 does not compromise the identification of the polymers evaluated by FTIR and also significantly reduced the concentration of suspended solids, resulting in an efficient visual separation of the microparticles. After been properly separated, the microparticles were characterized according to their size, colour and shape, and a fraction submitted to identification by μ-ATR-FTIR/ATR-FTIR. In all samples, a high presence of microfibers (MF) was observed, corresponding to more than 90% of the microparticles. However, in relation to secondary effluents, only 9% of these MF were identified as plastics, the remaining ones corresponded to cotton. The fragments found in the samples were classified as secondary in origin, and were mainly PE and PP, lower than 1mm size.

Abbreviations

ATR-FTIR - Attenuated total reflectance -Fourier transform infrared

MF - Microfiber (include natural and plastic)

MP – Microplastics (include all shape)

LDPE – Low density polyethylene

PE – Polyethylene

PET - Polyethylene terephthalate

PP – Polypropylene

PS – Polystyrene

1. Introduction

Plastic materials have various applications and, due to their low cost of production, they are highly prominent across numerous industrial sectors (e.g. the construction, packaging, and automotive industries). In 2018, 359 million tons of plastics were produced globally - almost 62 million tons of which were produced in Europe alone [1]. The inevitable presence of synthetic polymers within the daily lifestyles of the world population, resulting in the uncontrolled release of significant plastic material, has implemented further pressure on environmental authorities.

Small plastic particles with dimensions smaller than 5mm can be considered as 'microplastics' (MP). In this context, MP can be classified as secondary when they are formed as a result of the fragmentation of larger plastics [2,3,4]. It is expected that these MP will be continuously generated in the future, due to several reasons: the high consumption of synthetic polymers (together with the inadequate management of subsequent residues), the insufficient disposal and recycling systems, and also the lack of public awareness regarding the issue. On the other hand, MP used as a feedstock in the formulation of various products, such as: exfoliating material in soaps, facial scrubs, shampoos, shaving foam and toothpaste, are denominated primary MP – and hence their presence in WWTP results from the consuming lifestyle habits of the modern population [5,6,7].

WWTPs are important barriers that act physically, chemically and biologically towards the control of pollutants in the environment. Despite their high performance in microplastic retention - reaching efficiencies of approximately 90% according to some authors [4,8,9] - these facilities still release high amounts of MP into the environment since the volume of effluents generated by them is so large. That is, no matter how low the concentrations of MP per litre of effluent released - by extrapolating any value to daily WWTP flows, millions of particles are still released into the environment [8,10,11,12]. Studies have shown that the high retention capacity of MP in WWTPs can be attributed to the efficiency of the primary processes and to their retention in the secondary sludge – however this may lead to an additional environmental problem, as this biological material is often reapplied as fertilizer, hence resulting in soil contamination [13,14].

To prevent the occurrence of such additional issues, several researchers have proposed techniques for separating, identifying and quantifying microparticles in WWTP. Despite the significant research that has been conducted, divergence in the stages of sampling, chemical digestion and identification has made the comparison of results difficult and unreliable. This study aims to evaluate adequate protocols for the separation, quantification and identification of MP in three streams in a WWTP (investigating effluents from the primary and secondary settling, and activated sludge).

2. Materials and methods

2.1. General definition of microparticle and microplastic

This section aims to clarify the define and differentiate microparticles and microplastics in the context of this research. Microparticles are considered particles that have dimensions smaller than 5mm. In this group, all kinds of materials were included (natural and synthetic). In this way, the reference to microparticles was attributed to unknown materials, separated and classified by visual sorting before FTIR analysis. Once carried out, the FTIR analysis properly identified the polymers, and subsequently the microparticles were classified as microplastics if they were synthetic polymers or a mixture of synthetic polymer and natural matrix.

2.2. Preliminary study with MPs samples

Preliminary experiments were carried out with known MP samples to assess the interference of chemical digestion in the MP identification process. To start the procedure with MP samples, the workbench was previously cleaned with distilled water and alcohol as well as the work materials (tweezers, scissors, petri dishes) to avoid possible contamination.

MP samples were taken from commercial plastics: garbage bags (LDPE), water bottles (PET), disposable dishes (PS) and straws (PP). These four polymers corresponded to more than 50% of Europe demand in 2018 [15]. First, the plastic samples were reduced in size by using a crusher and next, the MP were separated by stainless steel sieves in a range between 1mm and 400μm.

Before and after the chemical digestion, MP were characterized by ATR-FTIR (Bruker) spectroscopy to confirm that polymers can be correctly identified after the

chemical digestion, i.e. to check that no damage on the polymer had been produced. Four chemical digestion protocols were tested, two peroxidation processes at different concentrations (30% and 35% H_2O_2), and two Fenton's reactions. Fenton's reactions were performed at pH 3 at the same H_2O_2 concentrations used for peroxidation, plus the Fe^{+2} catalyst solution (iron sulphate heptahydrate 20mg/ml). The pH correction to 3 was performed with sulphuric acid (96%). For digestion processes, an initial mass of 20mg of plastic material was weighed and mixed with 5mL of peroxide. For the Fenton's reactions, 5mL of peroxide plus 2.5mL of $FeSO_4$ solution (catalyst solution). The chemical digestion protocols were performed separately for each polymer.

According to prior research, temperatures above 60°C may be aggressive for polymers [16]. For this reason, peroxidation were performed at temperatures of 60±2° for 4 hours and Fenton's reactions were executed at room temperature for 2 hours using ice baths, when necessary, to maintain the temperature below 40°C due the exothermic behaviour of the reaction.

After the chemical digestion, the samples were vacuum filtered on 1μm glass fiber filters and dried in a laboratory oven for two hours at 50ºC. Next, the filters were introduced in a desiccator for approximately 12 hours to complete the drying. After that, MP were carefully separated from the filters to petri dishes with tweezers. All digestion assays were triplicated, and the MP were randomly selected to finally be analysed by ATR-FTIR spectroscopy.

To assess possible mass loss due to the oxidation processes or even due to the separation process, the percentage of plastic material recovery after each chemical digestion was determined using a gravimetric method, considering the MP mass before (initial) and after (final) the digestion process. The recovery percentage was obtained by Eq.1.

$$\% Recovery = \frac{MP\ Mass_{final}}{MP\ Mass_{inicial}} \times 100 \hspace{3cm} (1)$$

2.3. Sampling and microplastics separation from WWTP

Samples were collected using plastic containers from different points at an urban WWTP located in Valencia (Spain): primary settling (primary effluent), secondary settling (secondary effluent) and aerobic biological reactor (mixed liquor). All the

samples were collected in May/2019 to avoid weather influences, and directly transported to the laboratory for processing. Firstly, the primary and secondary effluent samples were passed through a 150µm aperture stainless steel mesh screen. Next, the retained material was chemically digested. For mixed liquor, due to the high load of suspended solids, chemical digestion of the sample was performed first to avoid blocking the sieve opening. After chemical digestion, the digested sample was passed through a 150µm mesh (Fig.1).

Two different chemical digestion protocols were carried out to achieve a better MP separation from the organic matrix. The Protocol 1 was established for the primary and secondary effluents, whereas the Protocol 2 for the sludge (mixed liquor). The difference between the two protocols consists only in the order of the processes. The Protocol 1 was set up for samples with less total suspended solids (TSS), which can be subjected to physical separation through sieves before chemical digestion without causing their immediate blockage. However, in Protocol 2 chemical digestion step precedes the sieve separation to avoid the sieve blockage, which would be produced by the high organic load and TSS concentration in sludge samples.
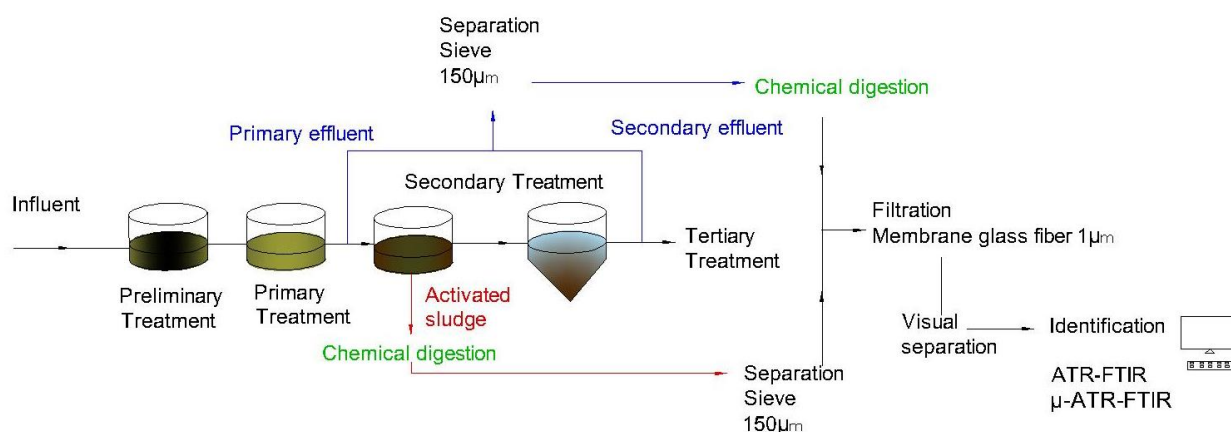
Fig.1. Flow chart of separation and identification MP from different samples

It was decided to use only a 150µm size mesh and evaluate individually the size of the retained microparticles. This methodology was thus established since the passage of microfibers through the opening sieve does not occur regularly, i.e., due to their

morphology, the microfibers can be transversely retained on the meshes or pass longitudinally through them. Ziajahromi et al. [12] used a series of sieves with different apertures (500,190,100 and 25μm) and observed that the fibre size range was larger than the size of the previous filter, fibres over 100μm in length were found in the 25μm mesh. Lee and Kim [17] also observed similar results, when using two sieves of 106 and 300μm. Fibres larger than 300μm could pass through the 300μm mesh sieve, and therefore fibres classified in the size range of 106-300μm could be underestimated. In this way, in order to avoid misclassification, it was decided to use a stainless-steel mesh of 150μm, and the material retained on the mesh was collected and characterized according to size, colour and shape one by one under a stereomicroscope.

As suggested by Lares et al. [18], density separation techniques were not used in this work to avoid the loss of high density microparticles. Polymers such as polyester (PET) ($1.37 - 1.45 g/cm^3$) could be underestimated when undergoing density separation with NaCl ($1.2 g/cm^3$). It would be necessary to use a higher density saline solution, such as NaI (1.8 g/cm3) to ensure effective separation [19,20]. However, when working with high matrix volumes, it could be expensive.

2.3.1. Protocol 1 - Primary and secondary effluent samples

The primary and secondary effluents were previously characterized in terms of TSS concentration. The protocol was adapted from Ziajahromi et al. [12]. Five litters of each sample were filtered through the stainless-steel sieve (150μm). The retained material was separated from the sieve with 100mL of distilled water and digested with 1mL of H_2O_2 35%wt for 2hours at a temperature of 60±2ºC. When necessary, higher volume of water was used without changing the aforementioned relation. After that, the digested sample was filtered through a glass fiber filter (aperture of 1μm). The filter was dried at 50ºC in a laboratory oven for 2 hours.

2.3.2. Protocol 2 –Sludge samples

To find out the proper chemical digestion for mixed liquor, two peroxidation processes and two Fenton's were applied (Fig.2). The peroxidation reactions were

performed with 10mL of sample and 20mL of peroxide 30% and 35%wt, for 4 hours at temperature of $60\pm2°C$. Fenton's reactions were also performed with 10mL of samples, 20mL of peroxide 30% and 35%wt and 10mL of catalyst solution (iron sulphate heptahydrate 20mg/ml), as suggested by Tagg et al. [21]. The temperature was maintained below 40°C, using ice baths when necessary. The chemical digestion efficiency was assessed in terms of total suspended solid (TSS) removal. Next, the digested sample was vacuum filtered on glass fiber filter (1μm) and subsequently the filter was dried at 105ºC in a laboratory oven for 1 hour and weighed for TSS determination.
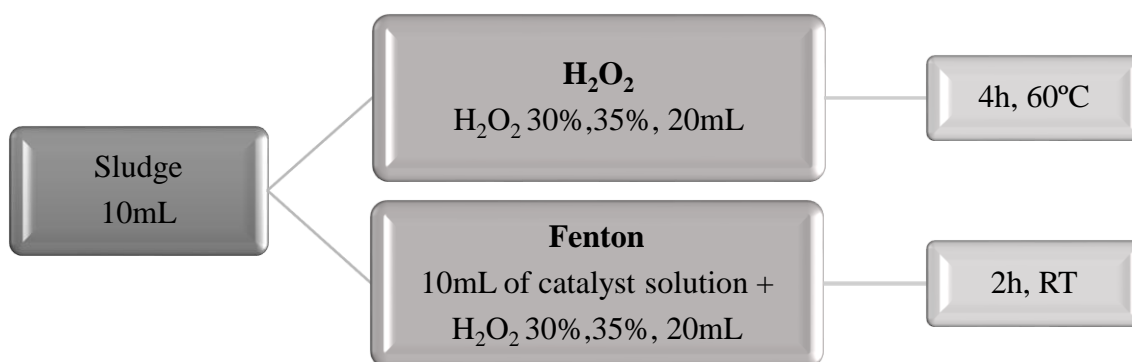
Fig. 2. Chemical digestion proposed for activated sludge

2.4. Microplastics detection

2.4.1. Visual sorting

To assist the visual inspection, a circular acetate mesh divided into octants customized by the researchers, was used to control microparticle counting, reducing the risk of microparticles over or underestimation. The materials were carefully analysed by stereomicroscope (LEICA MZ APO). The magnification was adjusted between 8X and 80X. This step allowed classification according to colour, shape and size. Three shapes were considered for the classification of the microparticles found: fibres (linear filaments with length less than 5mm), fragments (irregular shape, probably generated from the fragmentation of larger plastics) and spheres (three-dimensional circular shape).

Visual separation was carried out according to criteria addressed by Hidalgo-Ruz et al. [22]: (a) no organic materials on samples, (b) fibres should have same thickness over the entire length and (c) the colours of the samples should be evident and homogeneous. Besides that, microparticles with fragile structure, which fragmented into smaller pieces when subjected to pressure applied by the forceps were excluded from counting and classification as possible MP.

Visual identification has been used by several authors to classify microparticles [9,11,23,24]. However, an additional analysis is required to confirm the microparticle chemical structure in order to avoid misclassification, since natural fibres can be easily classified as synthetic. Consequently, microparticles identification based on polymeric analysis was key for the interpretation of results.

2.4.2. Microplastic identification

Polymeric identification was performed using attenuated total reflection (ATR) method of Fourier Transform-Infrared spectroscopy (FTIR) (Bruker) under spectral resolution of $4cm^{-1}$, sample scan 32 and spectrum with wavelengths between 400 and $4000cm^{-1}$, for microparticles larger than 400µm. For smaller fragments and fibres was necessary to use the ATR-FTIR equipment coupled to a microscope (µ-ATR-FTIR). The µ-ATR-FTIR (Bruker) was operated under spectral resolution of $6cm^{-1}$, sample scan 128 and spectrum with wavelengths between 600 and $4000cm^{-1}$. All spectra were analysed using the software Bio-Rad KnowItAll® Informatics System 2018, applying baseline correction and no-ATR correction.

From the FTIR analysis of the known MP samples (Section 2.1), an own database was set up for the subsequent polymeric characterization of samples separated from effluents and activated sludge. The spectra of known polymers, used as a reference, were directly compared with the MP extracted from the samples. To that end, the two spectra were overlaid and the analysis of each peak (starting with the characteristic peaks of each polymer) was carried out. Materials that did not match to the database spectra were compared to literature data for their classification.

Regarding the polymeric identification of fibres by μ-ATR-FTIR, Peets et al. [25] point out that the intensity of the absorbances produced by the sample depends on the pressure applied by the crystal. In addition, fibres made of more than one material, and how these materials are organized on the fibre structure can result in spectrum with different qualities and intensities depending on the point of contact with the crystal. Thus, the existence of materials made up of copolymers may make the identification more difficult, requiring careful analysis of the spectrum. In the classification of microplastic materials, both synthetic fibres and copolymers and mixtures of natural and synthetic fibres were considered.

2.5. Quality assurance/Quality control (QA/QC)

To minimize possible cross-contamination from other MP sources, glass materials were used whenever possible, avoiding the use of plastic materials as well as a cotton lab coat instead of polyester or another material made of synthetic fibres. Each new fibre glass filter was checked directly when it was extracted from the storage box with a stereomicroscope. In order to identify possible airborne contamination, a first control glass fiber filter was left exposed on the workbench for 6 hours and another filter for 7 days.

2.6. Statistical analysis

The statistical significance on the size of the microplastics counted in the primary, secondary, and activated sludge samples was assessed by Kruskal-Wallis analysis (95% confidence level) with the Statgraphics Centurion XVII. Bonferroni post hoc test was performed to identify differences between paired comparisons.

3. Results and discussion
3.1. QA/QC

No fibers were identified with the stereomicroscope when the glass fiber filters were taken out of their storage box. Regarding airborne contamination, a number of 8-

fibers were quantified on the control filter, and sorted out as 3 blue, 4 red and 1 transparent. No fragments were observed. Another test was carried out for 7 days under the same conditions and 13-fibers were counted on the filter. As the sample handling time was approximately 6 hours, the number of 8 detected fibres was discounted from the processed samples for MP quantification. To minimize contamination of the samples, precautions were taken, such as the use of aluminum foil to cover the glassware used and the use of petri dishes with a lid to avoid leaving the filters exposed after processing the samples.

3.2. Effect of chemical digestion on plastic material

Fig. 3 shows the ATR-FTIR spectra of the MP before and after chemical digestion. According to the FTIR results, after the chemical digestion protocols the FTIR spectra did not show significant deviations from the initial spectra (without digestion), making the identification of the polymer possible. For all plastic materials, the main peaks kept their intensity allowing their identification after digestion step. In the PP spectra, after being subjected to Fenton 30% and 35%, was observed the appearance of absorption bands in the wavelength between $3100\text{-}3600cm^{-1}$ and between $1670\text{-}1800cm^{-1}$, corresponding to the hydroxyl and carbonyl groups, respectively. The appearance of these bands may indicate the degradation of PP [26,27,27]. After undergoing chemical digestion with H_2O_2 30%, an increase in absorbance of peaks 1258, 1099, 1020 and $802cm^{-1}$ was observed. However, all the characteristic peaks of PP (2948; 2916; 2867; 2837; 1456; 1375; 1166; 973 cm^{-1}) were easily identified and unlike the Fenton's reaction, characteristic bands of degradation were not identified. Tagg et al. [20] also assessed the impact of H_2O_2 30% on the FTIR spectrum of microplastics and did not observe substantial changes in the spectra of PE, PP, PS, PVC and Nylon-6 polymers after being subjected to chemical digestion by 7 days. Hurley et al. [29] evaluated four protocols for chemical digestion and their influence on the identification of MP by FTIR. Among the protocols, peroxidation with H_2O_2 30% (at 60ºC) and Fenton's reaction were also evaluated, and no significant spectral modification was observed for the tested polymers (PP, LDPE, HDPE, PS, PET, PA-6.6, PC, PMMA). Based on this, it was concluded that, both peroxidation and Fenton did not compromise the chemical characterization of LDPE, PS, PP and PET via FTIR,

since they do not result in deviations from the main peaks necessary to identify the unique fingerprint of each polymer. Fenton's reaction seems more aggressive to PP, possible resulting in its degradation. Scanning electron microscopy (SEM) could be a method to confirm this degradation. It is important to highlight that these experiments were carried out with virgin polymers, it is also important to evaluate the effects of chemical digestion in MP that have already suffered weathering and degradation due to abiotic factors and biodegradations.
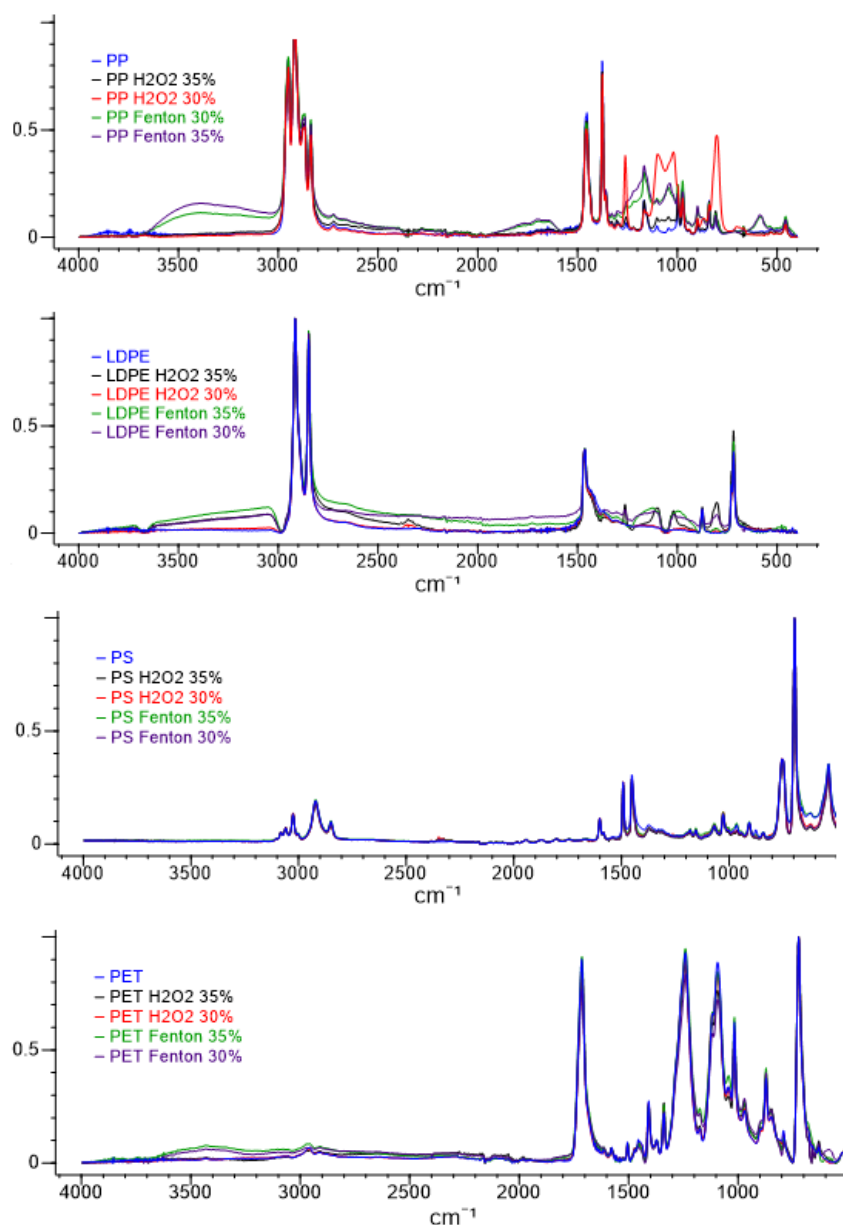
Fig. 3. MP spectra before and after the chemical digestion step

Regarding the percentage of mass recovery of the polymers after chemical digestion, PP, PET and PS reached more than 90%. Only LDPE showed a low recovery when subjected to peroxidation (Table 1). Hurley et al. [29] observed mass changes of less than 1% for PP, PET, PS and LDPE polymers when subjected to chemical digestion with H_2O_2 30%(v/v) at 60ºC and Fenton's reagent. Based on these results, a procedure with only distilled water was carried out for LDPE to ascertain possible losses during the digestion process, and we obtained a mass recovery of 78%. During the separation process, it was observed that the LDPE got stuck in the walls of the glass, making it difficult to be completely removed from the container. The lower recovery rate of this polymer was then associated with the separation process, and not with the chemical reagents used. Another important point is the percentage recovery obtained for the PP. As demonstrated by FTIR results, the PP could suffer some degradation when subjected to Fenton's reaction. The higher mass recovery of PP after Fenton's process, compared to peroxidation, could be also related to mass loss during the separation process in the peroxidation protocol. These results show that the separation process must be carried out minimizing the loss of material, since it corresponds to an important step on MP study.

Table 1 - Polymer recovery (%) after chemical digestion

| Polymer | Chemical digestion method | | | |
| | H_2O_2 | | Fenton | |
	30%	35%	30%	35%
PP	92	96	99	96
PET	95	91	100	100
PS	96	97	98	92
LDPE	71	64	93	82

3.3. Samples from WWTP

3.3.1. Primary and secondary effluents samples

For the primary effluent, it was observed that digestion was necessary to provide an effective visual characterization. For this, chemical digestion with H_2O_2 35%wt, in the proportion 1:100 (H_2O_2:water) proved to be sufficient for the reduction of suspended solids, thereby increasing visibility of the sample (Fig. 4).
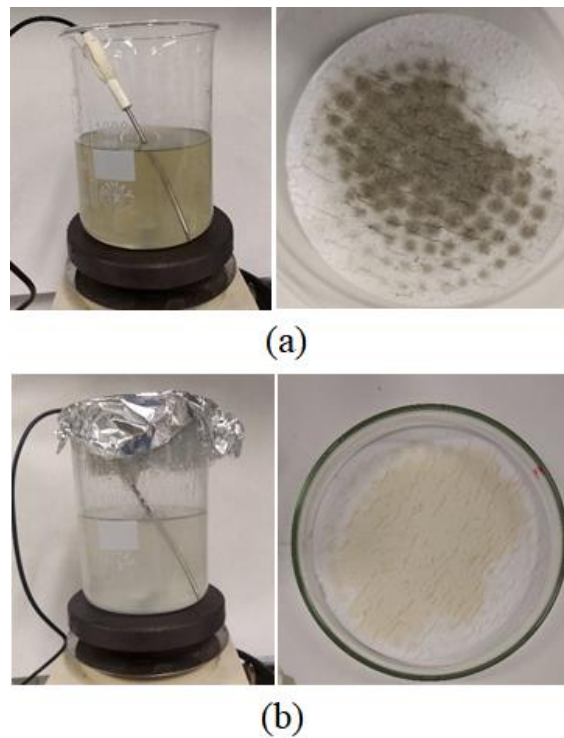
Fig. 4 . Primary effluent (250mL) before chemical digestion (a); primary effluent (250mL) after chemical digestion (b)

In the case of secondary effluent, chemical digestion will not be always necessary. Only if the sample presents a concentration of suspended solids that makes visual analysis unfeasible. In this case, digestion Protocol 1 must be properly applied. Table 2 shows the characteristics of the samples for both effluents.

Table 2 - Effluent characteristics

Sample	TSS (mg/L)	pH	Conductivity (mS/cm)
Primary effluent	42 ± 8	7.57	1.673
Secondary effluent	7 ± 3	7.91	1.757

According to the experiments carried out, it is recommended to characterize previously the samples in terms of TSS to determine if chemical digestion is necessary. Each WWTP generates effluents in different qualities, although they must be within the permitted release standards. Thus, the method developed in this work aims to guide a viable path to be applied in the separation and characterization of MP present in WWTPs.

3.3.2. Activated sludge samples

The efficiency of the chemical digestion was measured in terms of total suspended solids removal (TSS). The best results were achieved with peroxidation at both H_2O_2 concentrations (30% and 35%) and [H_2O_2:sludge] ratio of 2:1. For these conditions more than 90% of TSS removal was obtained (initial concentration 2500mg/L TSS, and volatiles corresponding 2127mg/L) (Table 3). However, for Fenton's reactions no more than 40% TSS removal was achieved. The process of advanced oxidation by Fenton is a complex process, whose efficiency depends on factors such as pH, catalyst concentration, concentration of peroxide and contaminants [30]. In non-optimized working conditions, both iron precipitation and loss of efficiency of oxidation of organic matter can occur. Furthermore, the Fenton's process, in addition to being able to oxidize organic matter, can also act as a coagulant due to the presence of ferric ions [31,32]. Thus, the value of lower percentage of TSS removal obtained by applying Fenton could also be attributed to the coagulation effect and/or to non-optimized working conditions.

Hurley et al. [29] also studied the efficiency of chemical digestion with H_2O_2 (30% at 60°C; for 6 hours) and Fenton's reagent (using 30% peroxide at room temperature; for 2 hours) and achieved 44.6%±6.76 mass removal for peroxidation and

43.8%±6.61 for Fenton. Amudha et al. [33] achieved a 22% reduction in TSS after Fenton. The efficiency of advanced oxidative processes (such as peroxidation and Fenton) regarding activated sludge digestion can be influenced not only by time, temperature and concentration of reagents, but also by sludge composition. Since extracellular polymeric substances (EPS) promote the flocculation of microbial colonies, the presence of this material in sludge can interfere on the solubilization process of organic matter. Amudha et al. [33] observed that the deflocculation of the sludge by citric acid before Fenton's reaction promotes an improvement in TSS removal, reaching 53% removal.

Table 3 – Efficiency of chemical digestion protocols for activated sludge in terms of TSS removal

Reagent	Reaction time (h)	Temperature (ºC)	[sludge mL:H_2O_2mL]	TSS Removal (%)
H_2O_2 30%	4	60 ± 2	1:2	92.35 ± 0.58
H_2O_2 35%	4	60 ± 2	1:2	93.55 ± 2.52
Fenton 30%	2	RT	1:2	35.73 ± 0.36
Fenton 35%	2	RT	1:2	45.56 ± 4.64

RT: room temperature

Some authors use extensive chemical digestion times, which can take up to days for complete sample processing [8,20,34,35].In our study, it was possible to achieve more than 90% removal of TSS in only 4 hours of digestion, which allows for efficient digestion with less time consumption.

3.4. Visual sorting

Visual sorting results are explained considering the shape (fibre, fragment, and sphere), size and colour of the microparticles.

3.4.1. Concentration and shape of the microparticles

Regarding the microparticles released daily, it is estimated that about 1.12×10^8 Microparticles/day could leave the WWTP evaluated in this work in the secondary effluent. The concentration of microparticles in the final effluent will depend on both the processes used in the purification and the population served, however concentrations of the order of magnitude of 10^8 Microparticles/day were also reported by other authors [34,35,36]. In this research, from primary effluent (11.1Microparticles/L) to secondary (2.8Microparticles/L), a reduction of 74.8% was observed. This reduction could be related to the retention of microparticles in the activated sludge (280Microparticles/L or 112.0Microparticles/g dry weight) (Table 4). Kalčíková et al. [37], in a study carried out with SBR fed with PE microbeads, also observed that part of the MP added in the reactor was transferred to the activated sludge and deposited at the bottom of the tank. According to some authors, this affinity between MP and activated sludge could be related to the formation of biofilms around MP, which would reduce the buoyancy of this material [38,39].

When applied as biosolids, the MP present in the activated sludge can also represent an environmental problem for the terrestrial environment. Liu et al. [40] reported the occurrence of microplastics in agricultural soils in twenty vegetable farmlands in Shanghai at concentrations of 78.00±12.91MP/kg and 62.50±12.97MP/ kg. The presence of these MP was related to both application of sewage sludge and the use of plastic mulching. MP can serve as vectors of pollutants either by leaching their additives or by transporting adsorbed persistent organic pollutant [41], so their monitoring and control deserves attention both in WWTP effluents and activated sludge.

Table 4 – Results of the quantification by shape of microparticle in the samples

Sample	Microfibers concentration	Fragments concentration	Total
Primary effluent	10.7 MF/L	0.4 Fragment/L	11.1 Microparticle/L
Secondary effluent	2.6 MF/L	0.2 Fragment/L	2.8 Microparticle/L
Activated sludge	264 MF/L (105.6 MF/g dry weight)	16 Fragment/L (6.4 Fragment/g dry weight)	280 Microparticles/L (112.0 Microparticle/g dry weight)

The assessment of microparticles in primary and secondary effluents and activated sludge samples showed that most of them were microfibers (>90%). Fig. 5 shows some of the fibers found in the samples. The high presence of fibers in effluents has also been observed by other authors [9,11,12,18,42,43,44]. Gündoğdu et al. [10] found that fibers also predominated over microparticles with other shapes (fragments, microspheres), constituting 44.4% of Seyhan WWTP (Turkey) secondary effluent and 86.5% of Yüreğir WWTP (Turkey) secondary effluent. Michielssen et al. [9] calculated that the microparticles in the final effluents of two WWTPs in USA included 61% and 84.7% of fibers, with estimated concentrations of 3.58 and 5.25MF/L and 1.94 and 0.8Fragment/L. In general, methodological differences in the filtration step (different size mesh) and sample preparation (digestion process) make difficult to compare results between works. However, the presence of textile fibers in the environment has proven to be an environmental issue that deserves attention. According to Browne et al. [45], more than 1,900 polyester fibers can be released from one garment per wash. Almroth et al. [46] suggests that one fleece garment can release approximately 110,000 fibers when washed.
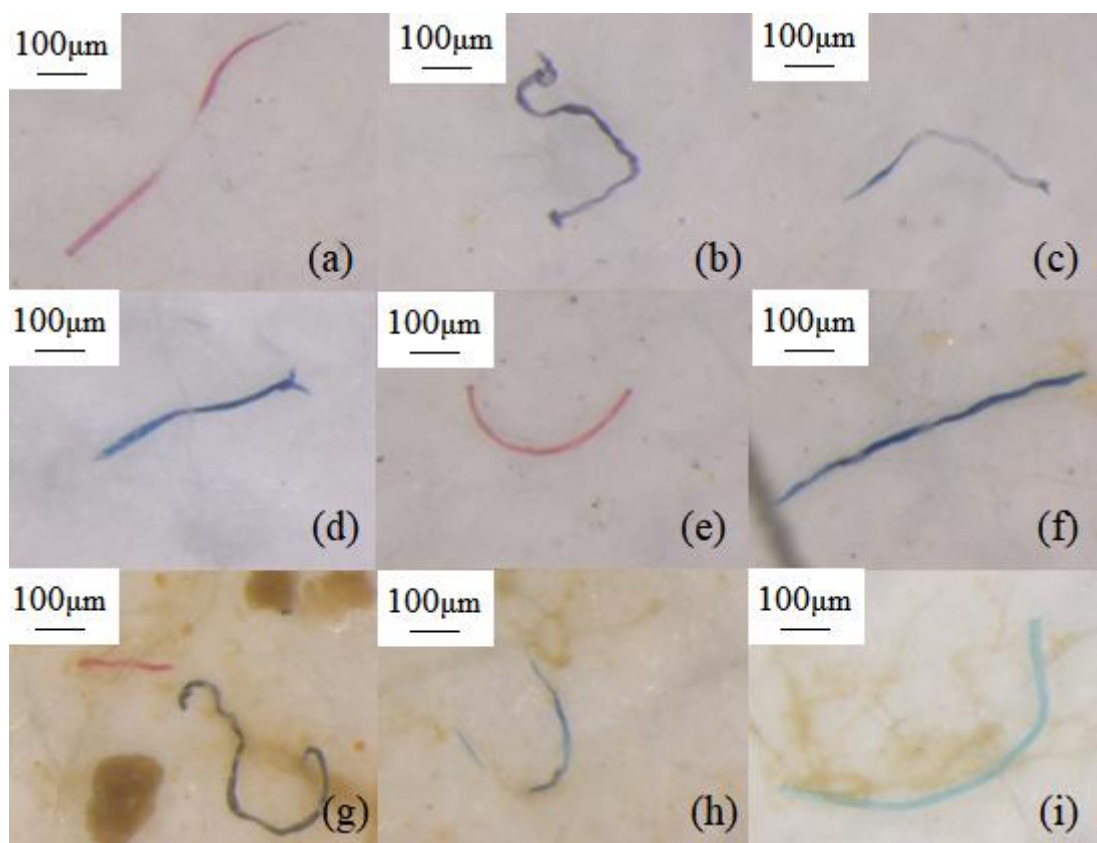
Fig. 5. Dyed microfibers: (a,b c) Primary effluent, (d,e,f) Secondary effluent, (g,h,i) Activated sludge

Fragments were also found in all samples, however in much lower concentrations compared to fibers. The identified fragments mainly had dimensions greater than 300μm and less than 1mm (Fig. 6). No fragments larger than 2mm were identified. It is worth mentioning that fragments smaller than 150μm were also visualized. The smallest fragments visualized had 20μm, but these microparticles were not counted in this study since it was not within the lower size limit established in the methodology. Similar results were obtained by Yang et al. [44]. In this work, 14.08% of the MP identified in the effluents corresponded to fragments, spheres, granules and films, which had an average size of 681.46±528.73μm, having most MP a size of around 300μm.
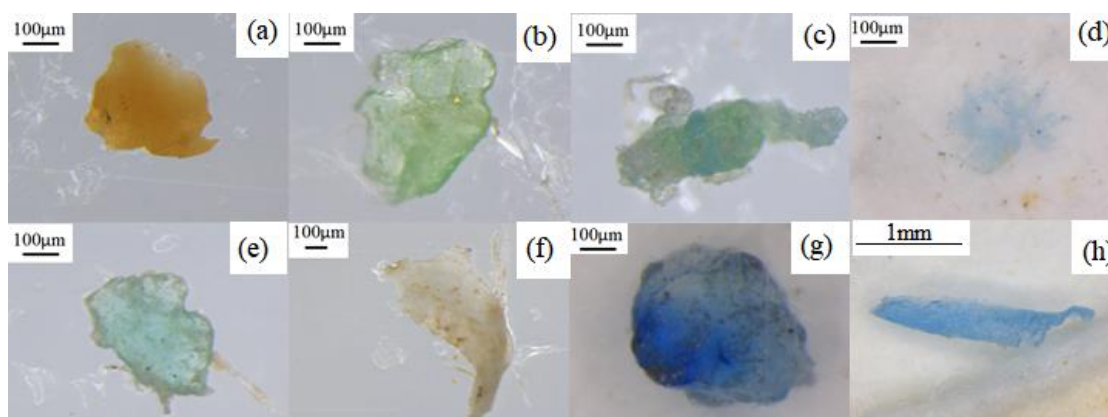
Fig. 6. MP fragments separated from activated sludge (a, b, c e, f); secondary effluent (d,h); primary effluent (g)

No plastic microspheres were found in the processed samples. This kind of microplastics is compounded mainly by PE [3,5,7,37], and its absence of in WWTP effluents may be related to its composition. Since polyethylene material has lower density than the water, these MP tend to float on the wastewater surface with fat, grease and oil materials in the preliminary treatments of the WWTP. Similar results were found by Michielssen et al. [9] and Murphy et al. [14] who also did not find microspheres in the effluents studied. In contrast, in the study by Hidayaturrahman and Lee [23] in three WWTPs located in South Korea, all the effluents mainly contained microspheres.

Factors such as WWTP treatment capacity, patterns of consumption and social behavior could have influence on the results obtained by each author. The presence of microspheres, as well as microfibers, in wastewater is the result of domestic activities. Browne et al. [45] suggests that because people wear more clothes during the winter than in the summer, and that the use of washing machines is 700% higher in winter, more fibers can enter the WWTP during winter. Similarly, considering that microspheres are present in personal care and cosmetic products (such as exfoliants, toothpaste, shampoos, among others) [7,37], their presence in the WWTP can be the result of different consumption patterns. This may be also one of the reasons why different results regarding the MP presence in WWTPs have been reported.

3.4.2. Size of the microparticles

Fig. 7 shows microparticle size distribution found in primary effluent, secondary effluent and activated sludge samples. It can be observed that in the primary effluent 73% of microparticles were larger than 500µm. Similarly, the secondary effluent mostly contained microparticles greater than 500µm and smaller than 5mm (74%). The smallest size (150-500µm) involved 27% and 26% in primary and secondary effluents, respectively. Gündoğdu et al. [10] also observed that the MP separated from the secondary effluent of WWTP (Yüreğir WWTP) mostly corresponded to the size range from 1mm to 5mm (40.5%), and a 29.7% to MP between 100µm-500µm. However, the Seyhan WWTP had a size distribution corresponding to 34.9% for both 1mm-5mm and 500µm-1mm size ranges. The final effluent of Lares et al. [18] was also composed by larger MP (between 500µm and 1mm) and a smaller part corresponded to MP between 250-500µm.

However, for activated sludge samples, the microparticle size distribution changed, and 48% were comprised between 150-500µm. This result may be due to the fact that the most part of the microparticles greater than 1mm flows out of the WWTP on the secondary effluent and the smallest microparticles keep on the sludge. Similar results were reported by other authors. Magni et al. [35] found that the 54% of the sludge of a WWTP in Italy was formed by MP between 100µm-500µm and a smaller part ranged 1mm-5mm (10%). Liu et al. [36] also observed that microplastics presented in the sludge corresponded mostly (>80%) to the smallest size range evaluated by them (20µm-300µm). The presence of smaller MP in the sludge can probably be the result of their retention on the sludge floc.
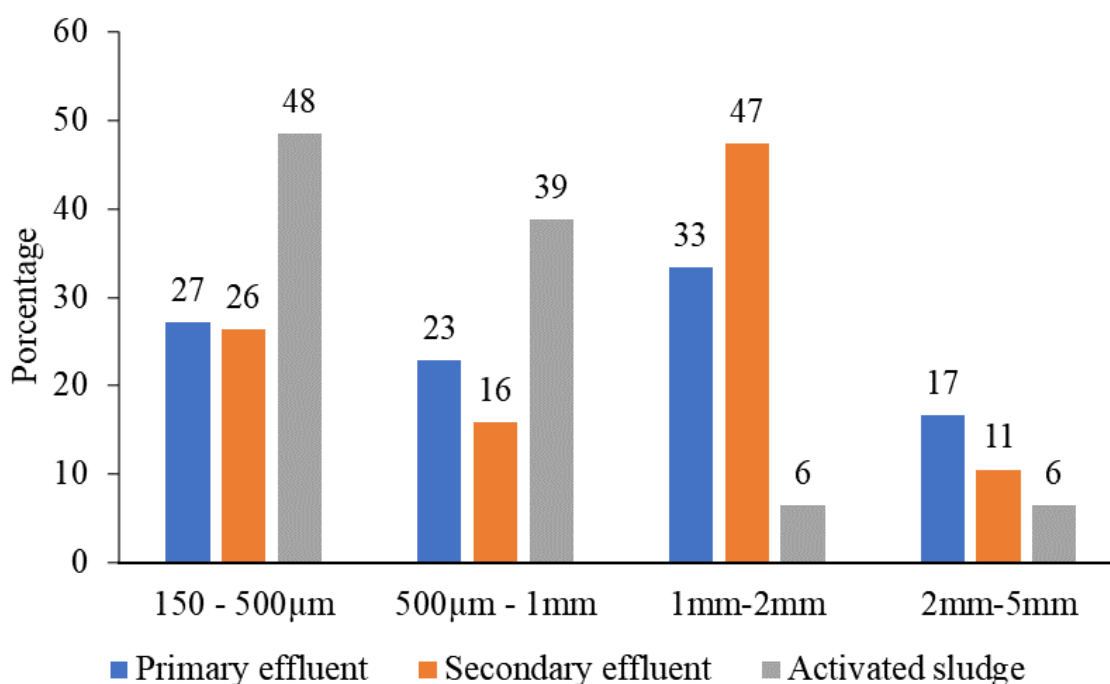
Fig. 7. Microparticle size (mm) distribution for primary effluent, secondary effluent and sludge samples

Concerning the results of the statistical analysis, a classification based on the size distribution of the microparticles (including fragments and fibers) was performed and the Kruskal-Wallis method was used (n=98). Based on the Kruskal-Wallis statistical analysis, as the P value is less than 0.05 (p-Value = 0.003) there was a statistically significant difference amongst the medians at the 95.0% confidence level. Using the Bonferroni procedure for paired comparisons, the two comparisons: 'Activated sludge-Primary Effluent' and 'Activated sludge-Secondary Effluent' were statistically significant at the 95.0% confidence level and there were no statistically significant differences between the microparticle size averages when comparing the two effluents (Table 5).

Table 5 - Kruskal-Wallis test for microparticle size by sample and Benferroni post hoc

Kruskal-Wallis			
Sample	Median (µm)	Sample Size (n)	Average Rank
Activated sludge	570	31	35.7
Primary Effluent	1050	48	54.0
Secondary Effluent	1500	19	60.5

P-Value = 0.003

Benferroni post hoc			
Contrast	*Sig.*	*Difference*	*+/- Limits*
Activated sludge – Primary Effluent	*	-18.29	15.68
Activated sludge – Secondary Effluent	*	-24.76	19.83
Primary Effluent– Secondary Effluent		-6.47	18.45

*Denotes a statistically significant difference.

3.4.3. Color of the microparticles

Related to the color of the (fibers and fragments) (n=85), the majority of them was blue (48%) and black (25%), but some red (11%) and other colors like green, orange or purple were also observed (16%). In the manufacturing process of textile fibers (both natural and synthetic) different chemical compounds are applied such as dyes, surfactants, detergents, color stabilizers, among others that can be considered hazardous substances. In this way, Remy et al. [47] identified by Raman's technique the Direct Red 28 (DR28) colorant in cellulose-based fibers ingested by macrocrustaceans. This colorant, once reduced in the human body, results in compounds classified as carcinogenic. Thus, the presence of additives, such as DR28, in natural fibers can be as worrisome as for the synthetic ones. In addition, the fact that natural fibers are more biodegradable than synthetic ones, can be an aggravating factor for the release of toxicity to the environment. [48].

3.5. Presence of MP in the samples

Numerous attempts were made to analyze the MP directly in the glass fiber filter but, a low signal quality was obtained. The presence of the filter made it difficult for the ATR crystal to apply pressure to the samples (especially to the fibers), and the crystal sometimes displaced them. To improve identification accuracy, it was necessary to manually extract each MP individually with tweezers and position them on the base of the μ-ATR-FTIR equipment. This process was very time consuming; therefore, a subset of 17% of all microparticles counted were characterized (including fibers and fragments). To overcome this problem, Tagg et al. [20] propose the use of the Focal Plane Array (FPA) technique, which enables the mapping of microparticles in entire membrane filters in a short time.

MP fragments were characterized mainly as PE and PP (63% and 25% of total MP fragments, respectively). The separated fragments were likely originated from the fragmentation of commercially used plastics such as bottles, plastic bags, etc. and therefore were classified as secondary origin. Lares et al. (2018) [18] found similar results: 65.9% of MP fragments were characterized as PE in a WWTP in Finland. According to PlasticEurope [1], PE accounted for 29.8% of European plastics demand in 2017 and PP 19.3%, being the main polymers used in packaging manufacturing. Society's behavior regarding waste disposal (recycling and waste separation processes) and solid waste management systems are relevant factors concerning the presence of these polymers in wastewater treatment plants.

Regarding the microfibers, polyester was the most prevalent synthetical polymer, corresponding to 75% of plastic MF. The results of this study are in accordance with previous studies conducted in WWTPs, which identified a large presence of polyester fibres. Browne et al. [45] also reported polyester as most frequent plastic MF in final effluent (67%). In the work carried out by Lares et al. [18], polyester MF corresponded to 79.1% of the total amount of MP collected. In our research, both polyester and cotton were found in primary effluent samples and in the activated sludge polyester, cotton, polyacrylic and polypropylene were identified. A percentage approach was performed for the secondary effluent since 52% of the fibres counted in the secondary effluent were characterized by μ-ATR-FTIR. In this sample, only 9% of MF were identified as microplastic (polyester), the remainder corresponded to natural cellulose derived MF.

Based on this, from 2.6MF/L counted, only 0.24MF/L were identified as synthetic. Talvitie et al. [49] also observed a high presence of natural MF in the analysed effluent. 44% of the fibres were characterized as cotton, followed by polyester fibres (33%). However, as mentioned above, natural MF can lead to an environmental problem due to the presence of additives and, therefore, should not be ignored.

According to the results obtained in this research, about 0.44MP/L (including MF and fragments) could be released in the environment after secondary treatment. Murphy et al. [14] and Bayo et al. [50] found similar results 0.25MP/L and 0.31±0.06MP/L, respectively, were estimated in secondary effluents. Table 6 collects the results of the MP concentrations in the WWTP effluents in different parts of the world. The discrepancy in the results obtained between authors can be attributed to the different processes of separation and identification of MP applied and the lack of standardized protocols. However, due to the high volume of effluents generated by the WWTP, millions of MPs can be released into the environment daily.

Table 6 - MP concentrations in secondary effluents of different WWTPs

Reference	Facility	Facility capacity (m³/day)	MP/L (secondary effluent)	%MF Secondary effluent	Smallest mesh size (μm)	Sample pretreatment	Identification	Country
This study	WWTP Valencia	40,000	0.44	95	150	H_2O_2 35%, 60±2º for 2h	Visual/μ-ATR-FTIR	Spain
[8]	Metro Vancouver	490,000	0.5±0.2	60	64	H_2O_2 30%, RT; 7 days	Visual/μ-ATR-FTIR	Canada
[10]	Seyhan WWTP	182.78 ± 5.97	6.999±0.764	44.4	55	H_2O_2 30% + $FeSO_4 \cdot 7H_2O$; 75º	Visual / μ-Raman	Turkey
	Yüreğir WWTP	87.49 ± 0.97	4.111±0.318	86.5	55			Turkey
[18]	Kenkaveronniemi WWTP	10,000	1 ± 0.4	53	250	H_2O_2 30% + $FeSO_4 \cdot 7H_2O$; 75º	Visual/μ-ATR-FTIR/μ-Raman	Finland
[23]	WWTP-A	26,545	710	8	1.2	30% H_2O_2	Visual	South Korea
	WWTP-B	469,249	7863	1				
	WWTP-C	20,84	433	15				
[34]		45,000	10.7±5.2		25	H_2O_2 (33% w/v) at 50ºC for 20-24 h		Spain
[35]	WWTP in Northern Italy	400,000	0.9±0.3	28	63	H_2O_2, 15%; RT; 3 days	Visual/μ-FTIR	Italy
[36]	WWTP in Wuhan City	20,000	34.1 ± 9.4	56.7	47	H_2O_2 30%, 6hours; $FeSO_4 \cdot 7H_2O$; 12hours	Visual / μ-Raman	China

Continuación Tabla 6

Reference	Facility	Facility capacity (m³/day)	MP/L (secondary effluent)	%MF Secondary effluent	Smallest mesh size (µm)	Sample pretreatment	Identification	Country
[43]	East Bay Municipal Utilities District (EBMUD)		0.071	57	125	H_2O_2 30% + Fe(II)	Visual	USA
	Central Contra Costa		0.072	59				
	East Bay Dischargers Association (EBDA)		0.022	91				
	San Francisco Airport Sanitary (SFO)		0.19	90				
[50]	WWTP Cartagena	35,000	0.31±0.06		0.45		Visual / FTIR	Spain

[a] Including all microfibers.

4. Conclusions

This study has developed a methodology for the separation and characterization of MP in different streams of a WWTP. Since there are no standardised procedures, deep discussion regarding the pre-treatment and characterisation of the samples is of paramount importance in the foreseeable future.

Concerning the chemical digestion of the samples, the use of peroxidation proved to be effective with samples of primary effluents and activated sludge - since it did not compromise the identification of polymers via ATR-FTIR, and considerably reduced the concentration of suspended solids; allowing for the effective separation and visual

classification of possible microplastics. For secondary effluent samples, its application will depend on the concentration of suspended solids. In addition to this, the spectra obtained via FTIR showed no interference from organic materials, which corroborates the efficiency of the digestion method based on peroxidation. Furthermore, the separation process is so important as chemical digestion and must be done carefully to avoid material losses.

In all analysed samples (primary and secondary effluent, and activated sludge), MF was found to be the most abundant fraction, constituting more than 90% of the microparticles. Despite the fact that the highest fraction of MF in the secondary effluent was characterized as cotton, these natural fibres can be classified as an environmental problem akin to microplastics, since they carry several additives in their composition, and their faster rate of degradation could hence release harmful compounds more quickly into the environment; affecting various forms of life. A high quantity of microparticles was detected in the activated sludge (280Microparticles/L or 112.0Microparticle/g dry weight) compared to effluents. Significant differences were observed when comparing the size of the microparticles in the effluents to those within the activated sludge - which mainly consisted of smaller particles (100-150µm). In this regard, most of the microparticles entering the WWTP accumulated in the sludge - especially the smaller microparticles. In the future, great efforts must be made towards establishing standard protocols that help make methodological unification feasible, therefore resulting in more effective comparisons across research studies, in order to better identify possible points for improvement and minimise the impact of microplastics on the environment.

Acknowledgments

REFERENCES

1] PlasticsEurope, Plastics – the Facts, Plast. – Facts 2018. (2018) 38. https://www.plasticseurope.org/application/files/6315/4510/9658/Plastics_the_facts _2018_AF_web.pdf (accessed 02 Septiembre 2019).

[2] H.S. Auta, C.U. Emenike, S.H. Fauziah, Distribution and importance of microplastics in the marine environment. A review of the sources, fate, effects, and potential solutions, Environ. Int. 102 (2017) 165–176. https://doi.org/10.1016/j.envint.2017.02.013.

 [3]S.A. Carr, J. Liu, A.G. Tesoro, Transport and fate of microplastic particles in wastewater treatment plants., Water Res. 91 (2016) 174–82. https://doi.org/10.1016/j.watres.2016.01.002.

[4] J. Li, H. Liu, J. Paul Chen, Microplastics in freshwater systems: A review on occurrence, environmental effects, and methods for microplastics detection, Water Res. 137 (2018) 362–374. https://doi.org/10.1016/j.watres.2017.12.056.

[5] L.S. Fendall, M.A. Sewell, Contributing to marine pollution by washing your face: Microplastics in facial cleansers, Mar. Pollut. Bull. 58 (2009) 1225–1228. https://doi.org/10.1016/j.marpolbul.2009.04.025.

[6] C. Guerranti, T. Martellini, G. Perra, C. Scopetani, A. Cincinelli, Microplastics in cosmetics: Environmental issues and needs for global bans, Environ. Toxicol. Pharmacol. 68 (2019) 75–79. https://doi.org/10.1016/j.etap.2019.03.007.

[7] I.E. Napper, A. Bakir, S.J. Rowland, R.C. Thompson, Characterisation, quantity and sorptive properties of microplastics extracted from cosmetics, Mar. Pollut. Bull. 99 (2015) 178–185. https://doi.org/10.1016/j.marpolbul.2015.07.029.

[8] E.A. Gies, J.L. LeNoble, M. Noël, A. Etemadifar, F. Bishay, E.R. Hall, P.S. Ross, Retention of microplastics in a major secondary wastewater treatment plant in Vancouver, Canada, Mar. Pollut. Bull. 133 (2018) 553–561. https://doi.org/10.1016/j.marpolbul.2018.06.006.

[9] M.R. Michielssen, E.R. Michielssen, J. Ni, M.B. Duhaime, Fate of microplastics and other small anthropogenic litter (SAL) in wastewater treatment plants depends on unit processes employed, Environ. Sci. Water Res. Technol. 2 (2016) 1064–1073. https://doi.org/10.1039/c6ew00207b.

[10] S. Gündoğdu, C. Çevik, E. Güzel, S. Kilercioğlu, Microplastics in municipal wastewater treatment plants in Turkey: a comparison of the influent and secondary effluent concentrations, Environ. Monit. Assess. 190 (2018). https://doi.org/10.1007/s10661-018-7010-y.

[11] S.A. Mason, D. Garneau, R. Sutton, Y. Chu, K. Ehmann, J. Barnes, P. Fink, D. Papazissimos, D.L. Rogers, Microplastic pollution is widely detected in US municipal wastewater treatment plant effluent, Environ. Pollut. 218 (2016) 1045–1054. https://doi.org/10.1016/j.envpol.2016.08.056.

[12] S. Ziajahromi, P.A. Neale, L. Rintoul, F.D.L.L. Leusch, Wastewater treatment plants as a pathway for microplastics : Development of a new approach to sample wastewater-based microplastics, Water Res. 112 (2017) 93–99. https://doi.org/10.1016/j.watres.2017.01.042.

[13] D. He, Y. Luo, S. Lu, M. Liu, Y. Song, L. Lei, Microplastics in soils: Analytical methods, pollution characteristics and ecological risks, TrAC - Trends Anal. Chem. 109 (2018) 163–172. https://doi.org/10.1016/j.trac.2018.10.006.

[14] F. Murphy, C. Ewins, F. Carbonnier, B. Quinn, Wastewater Treatment Works (WwTW) as a Source of Microplastics in the Aquatic Environment, Environ. Sci. Technol. 50 (2016) 5800–5808. https://doi.org/10.1021/acs.est.5b05416.

[15]Plastics - the Facts 2019, (2019). https://www.plasticseurope.org/en/resources/market-data. (accessed 30 January 2020).

[16] K. Munno, P.A. Helm, D.A. Jackson, C. Rochman, A. Sims, Impacts of temperature and selected chemical digestion methods on microplastic particles, Environ. Toxicol. Chem. 37 (2018) 91–98. https://doi.org/10.1002/etc.3935.

[17] H. Lee, Y. Kim, Treatment characteristics of microplastics at biological sewage treatment facilities in Korea, Mar. Pollut. Bull. 137 (2018) 1–8. https://doi.org/10.1016/j.marpolbul.2018.09.050.

[18] M. Lares, M.C. Ncibi, M. Sillanpää, M. Sillanpää, Occurrence, identification and removal of microplastic particles and fibers in conventional activated sludge process and advanced MBR technology, Water Res. 133 (2018) 236–246. https://doi.org/10.1016/j.watres.2018.01.049.

[19] C. Bretas Alvim, J.A. Mendoza-Roca, A. Bes-Piá, Wastewater treatment plant as microplastics release source – Quantification and identification techniques, J. Environ. Manage. 255 (2020). https://doi.org/10.1016/j.jenvman.2019.109739.

[20] A.S. Tagg, M. Sapp, J.P. Harrison, J.J. Ojeda, Identification and Quantification of Microplastics in Wastewater Using Focal Plane Array-Based Reflectance Micro-FT-IR Imaging, Anal. Chem. 87 (2015) 6032–6040. https://doi.org/10.1021/acs.analchem.5b00495.

[21] A.S. Tagg, J.P. Harrison, Y. Ju-Nam, M. Sapp, E.L. Bradley, C.J. Sinclair, J.J. Ojeda, Fenton's reagent for the rapid and efficient isolation of microplastics from wastewater, Chem. Commun. 53 (2017) 372–375. https://doi.org/10.1039/c6cc08798a

[22] V. Hidalgo-Ruz, L. Gutow, R.C. Thompson, M. Thiel, Microplastics in the marine environment: A review of the methods used for identification and quantification, Environ. Sci. Technol. 46 (2012) 3060–3075. https://doi.org/10.1021/es2031505.

[23] H. Hidayaturrahman, T.G. Lee, A study on characteristics of microplastic in wastewater of South Korea: Identification, quantification, and fate of microplastics during treatment process, Mar. Pollut. Bull. 146 (2019) 696–702. https://doi.org/10.1016/j.marpolbul.2019.06.071.

[24] J. Talvitie, M. Heinonen, J.P. Pääkkönen, E. Vahtera, A. Mikola, O. Setälä, R. Vahala, Do wastewater treatment plants act as a potential point source of

microplastics? Preliminary study in the coastal Gulf of Finland, Baltic Sea, Water Sci. Technol. 72 (2015) 1495–1504. https://doi.org/10.2166/wst.2015.360.

[25] P. Peets, I. Leito, J. Pelt, S. Vahur, Identification and classification of textile fibres using ATR-FT-IR spectroscopy with chemometric methods, Spectrochim. Acta - Part A Mol. Biomol. Spectrosc. 173 (2017) 175–181. https://doi.org/10.1016/j.saa.2016.09.007.

[26] E. Andreassen, Infrared and Raman spectroscopy of polypropylene, (1999) 320–328. https://doi.org/10.1007/978-94-011-4421-6_46.

[27] C.C. Tang, H.I. Chen, P. Brimblecombe, C.L. Lee, Morphology and chemical properties of polypropylene pellets degraded in simulated terrestrial and marine environments, Mar. Pollut. Bull. 149 (2019) 110626. https://doi.org/10.1016/j.marpolbul.2019.110626.

[28] J. Xiong, X. Liao, J. Zhu, Z. An, Q. Yang, Y. Huang, G. Li, Natural weathering mechanism of isotatic polypropylene under different outdoor climates in China, Polym. Degrad. Stab. 146 (2017) 212–222. https://doi.org/10.1016/j.polymdegradstab.2017.10.012.

[29] R.R. Hurley, A.L. Lusher, M. Olsen, L. Nizzetto, Validation of a Method for Extracting Microplastics from Complex, Organic-Rich, Environmental Matrices, Environ. Sci. Technol. 52 (2018) 7409–7417. https://doi.org/10.1021/acs.est.8b01517.

[30] M. hui Zhang, H. Dong, L. Zhao, D. xi Wang, D. Meng, A review on Fenton process for organic wastewater treatment based on optimization perspective, Sci. Total Environ. 670 (2019) 110–121. https://doi.org/10.1016/j.scitotenv.2019.03.180.

[31] M.I. Badawy, M.E.M. Ali, Fenton's peroxidation and coagulation processes for the treatment of combined industrial and domestic wastewater, J. Hazard. Mater. 136 (2006) 961–966. https://doi.org/10.1016/j.jhazmat.2006.01.042.

[32] S.F. Kang, C.H. Liao, M.C. Chen, Pre-oxidation and coagulation of textile wastewater by the Fenton process, Chemosphere. 46 (2002) 923–928. https://doi.org/10.1016/S0045-6535(01)00159-X

[33] V. Amudha, S. Kavitha, C. Fernandez, S. Adishkumar, J.R. Banu, Effect of deflocculation on the efficiency of sludge reduction by Fenton process, Environ. Sci. Pollut. Res. 23 (2016) 19281–19291. https://doi.org/10.1007/s11356-016-7118-y.

 [34] C. Edo, M. González-Pleiter, F. Leganés, F. Fernández-Piñas, R. Rosal, Fate of microplastics in wastewater treatment plants and their environmental dispersion with effluent and sludge, Environ. Pollut. 259 (2020). https://doi.org/10.1016/j.envpol.2019.113837.

[35] S. Magni, A. Binelli, L. Pittura, C.G. Avio, C. Della Torre, C.C. Parenti, S. Gorbi, F. Regoli, The fate of microplastics in an Italian Wastewater Treatment Plant, Sci. Total Environ. 652 (2019) 602–610. https://doi.org/10.1016/j.scitotenv.2018.10.269.

[36] X. Liu, W. Yuan, M. Di, Z. Li, J. Wang, Transfer and fate of microplastics during the conventional activated sludge process in one wastewater treatment plant of China, Chem. Eng. J. 362 (2019) 176–182. https://doi.org/10.1016/j.cej.2019.01.033.

[37] G. Kalčíková, B. Alič, T. Skalar, M. Bundschuh, A.Ž. Gotvajn, Wastewater treatment plant effluents as source of cosmetic polyethylene microbeads to freshwater, Chemosphere. 188 (2017) 25–31. https://doi.org/10.1016/j.chemosphere.2017.08.131.

[38] A.L. Andrady, Microplastics in the marine environment, Mar. Pollut. Bull. 62 (2011) 1596–1605. https://doi.org/10.1016/j.marpolbul.2011.05.030.

[39] E.R. Zettler, T.J. Mincer, L.A. Amaral-Zettler, Life in the "plastisphere": Microbial communities on plastic marine debris, Environ. Sci. Technol. 47 (2013) 7137–7146. https://doi.org/10.1021/es401288x.

[40] M. Liu, S. Lu, Y. Song, L. Lei, J. Hu, W. Lv, W. Zhou, C. Cao, H. Shi, X. Yang, D. He, Microplastic and mesoplastic pollution in farmland soils in suburbs of Shanghai, China, Environ. Pollut. 242 (2018) 855–862. https://doi.org/10.1016/j.envpol.2018.07.051.

[41] H. Bouwmeester, P.C.H. Hollman, R.J.B. Peters, Potential Health Impact of Environmentally Released Micro- and Nanoplastics in the Human Food Production Chain: Experiences from Nanotoxicology, Environ. Sci. Technol. 49 (2015) 8932–8947. https://doi.org/10.1021/acs.est.5b01090.

[42] H.A. Leslie, S.H. Brandsma, M.J.M. van Velzen, A.D. Vethaak, Microplastics en route: Field measurements in the Dutch river delta and Amsterdam canals, wastewater treatment plants, North Sea sediments and biota, Environ. Int. 101 (2017) 133–142. https://doi.org/10.1016/j.envint.2017.01.018.

[43] R. Sutton, S.A. Mason, S.K. Stanek, E. Willis-Norton, I.F. Wren, C. Box, Microplastic contamination in the San Francisco Bay, California, USA, Mar. Pollut. Bull. 109 (2016) 230–235. https://doi.org/10.1016/j.marpolbul.2016.05.077.

[44] L. Yang, K. Li, S. Cui, Y. Kang, L. An, K. Lei, Removal of microplastics in municipal sewage from China's largest water reclamation plant, Water Res. 155 (2019) 175–181. https://doi.org/10.1016/j.watres.2019.02.046.

[45] M.A. Browne, P. Crump, S.J. Niven, E. Teuten, A. Tonkin, T. Galloway, R. Thompson, Accumulation of microplastic on shorelines woldwide: Sources and sinks, Environ. Sci. Technol. 45 (2011) 9175–9179. https://doi.org/10.1021/es201811s.

[46] B.M. Carney Almroth, L. Åström, S. Roslund, H. Petersson, M. Johansson, N.K. Persson, Quantifying shedding of synthetic fibers from textiles; a source of microplastics released into the environment, Environ. Sci. Pollut. Res. 25 (2018) 1191–1199. https://doi.org/10.1007/s11356-017-0528-7.

[47] F. Remy, F. Collard, B. Gilbert, P. Compère, G. Eppe, G. Lepoint, When Microplastic Is Not Plastic: The Ingestion of Artificial Cellulose Fibers by

Macrofauna Living in Seagrass Macrophytodetritus, Environ. Sci. Technol. 49 (2015) 11158–11166. https://doi.org/10.1021/acs.est.5b02005.

[48] S.M. Ladewig, S. Bao, A.T. Chow, Natural Fibers: A Missing Link to Chemical Pollution Dispersion in Aquatic Environments, Environ. Sci. Technol. 49 (2015) 12609–12610. https://doi.org/10.1021/acs.est.5b04754.

[49] J. Talvitie, A. Mikola, O. Setälä, M. Heinonen, A. Koistinen, How well is microlitter purified from wastewater? – A detailed study on the stepwise removal of microlitter in a tertiary level wastewater treatment plant, Water Res. 109 (2017) 164–172. https://doi.org/10.1016/j.watres.2016.11.046.

[50] J. Bayo, S. Olmos, J. López-Castellanos, Microplastics in an urban wastewater treatment plant: The influence of physicochemical parameters and environmental factors, Chemosphere. 238 (2020).
https://doi.org/10.1016/j.chemosphere.2019.124593.

Capítulo IV

Effect of polyethylene microplastics on activated sludge process - accumulation in the sludge and influence on the process and on biomass characteristics

Autores – C.Bretas Alvim, S. Castelluccio, E. Ferrer-Polonio, M.A Bes-Piá, J.A. Mendoza-Roca, J. Fernández-Navarro, J.L. Alonso, I. Amorós

Process Safety and Environmental Protection
Volume 148, April 2021, Pages 536-547

ELSEVIER

Effect of polyethylene microplastics on activated sludge process - Accumulation in the sludge and influence on the process and on biomass characteristics

C. Bretas Alvim [a] ✉, S. Castelluccio [a], E. Ferrer-Polonio [a], M.A Bes-Piá [a], J.A. Mendoza-Roca [a], J. Fernández-Navarro [b], J.L. Alonso [b], I. Amorós [b]

Show more ∨

+ Add to Mendeley   ⚹ Share   📖 Cite

Get rights and content

Abstract

According to previous research, it has been proved that wastewater treatment plants (WWTPs) can retain more than 90% of the MPs contained in wastewater. However, a significant fraction of the MPs removed in WWTPs is retained in the sludge floc and this may lead to an environmental issue since biosolids can be used as fertilizers. The purpose of this research was to evaluate how the presence of polyethylene (PE) could interfere with the activated sludge performance. For this, a sequencing batch reactor (SBR) was continuously fed during 93 days with synthetic sewage and PE microbeads. It was observed that 98±2% of the total amount of MPs that entered SBR was accumulated in the activated sludge. Despite the high accumulation of MPs in the sludge, the depuration performance of the reactor was not compromised. However, the presence of MPs decreased the richness (Chao1) and abundance-based coverage estimators (ACE) and diversity (Shannon) of the bacterial community on day 93. Based on the analysis of the diversity indices and the relative abundances of microbial taxa, it was concluded that MPs had selective effects on activated sludge microbial community. However, MPs did not affect the abundance of nitrifying and denitrifying bacteria in the sludge.

1. Introduction

Municipal wastewater treatment plants (WWTPs) treat daily thousands of liters of wastewater generated from human activity, and among other pollutants, it has been proven that microplastics (MPs) reach the WWTPs and a large part (more than 90%) is retained during the depuration process (Carr et al., 2016; Edo et al., 2020; Gies et al., 2018; Li et al., 2018; Murphy et al., 2016; Simon et al., 2018; Talvitie et al., 2017). However, since a complete removal of MPs is not achieved, these particles are also present in the final effluent (Sun et al., 2019). The variation in the quantity reported by previous studies may be related to the treatment units involved in the WWTPs, the size of the population served, consumption patterns and also to the lack of standardization of MPs separation, quantification and identification protocols. In the WWTPs, although preliminary and primary treatments are responsible for the greatest removal of MPs (Talvitie et al.,2017, Hou et al., 2021; Jiang et al., 2020; Murphy et al., 2016, Ziajahromi et al., 2021) the secondary treatment is drawing the attention of researchers due to the accumulation of these particles in the sludge (Hou et al., 2021; Kalčíková et al., 2017; Lares et al., 2018; Talvitie et al., 2017). Activated sludge from WWTPs can be treated and applied as biosolids, which has been causing terrestrial contamination by MPs in agricultural soils. For instance, Corradini et al. (2019) measured concentrations of 1.1-3.5 particles/g of dry soil in agricultural soils in Chile that received different applications of wastewater sludge. Liu et al. (2018) reported concentrations of 78.00±12.91MP/kg and 62.50±12.97MP/kg in agricultural soils in Shanghai (China) and the authors correlated the presence of MPs to both application of sewage sludge and the use of plastic mulching.

When the MPs are surrounded by an organic matrix their surface can serve as a substrate for the growth of bacterial communities, for nutrient cycles and biofilm formation. These environments have been called 'plastisphere' (Delacuvellerie et al., 2019, Eckert et al., 2018; Zettler et al., 2013). Despite this symbiotic relation, the presence of microplastics in organic matrices can disturb microbiological activities and communities and can have toxic effects on some cultures (Sun et al., 2019; Huang et al., 2019; Wei et al., 2019a; Zhang et al., 2020). Zhang et al. (2020) investigated the influence of the presence of PET microplastics (0.2mm of size) in anaerobic granular sludge and reported that when the UASB systems were fed with PET-MPs

concentrations of 75, 150 and 300MP/L, a reduction in the efficiency of COD removal was observed. In addition, when subjected to a concentration of 300MP/L, a reduction of 28.4% in the methane production compared to the control was also recorded. Bacterial community analyzes showed that populations of key acidogens and methanogens decreased after the exposure to PET with concentration of 300MP/L. Although being considered emerging contaminants in aquatic and terrestrial environments, the consequences of the presence of MPs in the microbial communities of activated sludge systems still have many gaps and require a lot of investigation.

Information of biomass characteristics allows to assess how the presence of MPs impacts the biomass, whether by compromising cell viability, bacterial growth and/or metabolic activities. Based on the study of biological parameters, it is possible to identify whether the quality of the effluent or whether the nitrification process suffers some type of inhibition and allows strategic action to improve WWTPs performance. Therefore, this study was carried out in order to evaluate the effect of PE microplastics on the biological process performed in a sequencing batch reactor (SBR). PE was selected because it is a plastic widely used commercially (PlasticsEurope, 2019) and commonly found in WWTPs (Simon et al., 2018; Lares et al., 2018; Bayo et al., 2020; Ziajahromi et al., 2017). Thus, the effects of MPs on the biomass characteristics and its capacity of depuration were assessed. In addition, since few studies have been carried out to characterize the effects of MPs on activated sludge microorganisms (Tang et al., 2021), next-generation amplicon sequencing has been applied to better understand the ecological effect of MPs contamination on bacterial communities in activated sludge.

2. Materials and methods
2.1. Activated sludge

The activated sludge used in this study was collected from the aeration basin of a WWTP located in Comunitat Valenciana (Spain). The facility was designed to treat 40,000 m^3/day and consists of pre-treatment (coarse and fine screens, and basins for removal of grease and sand), primary settling followed by activated sludge system, including secondary settling (secondary treatment), and tertiary treatment.

2.2. Sequencing Batch Reactor (SBR) operation

To evaluate the influence of the presence of polyethylene MPs on activated sludge process, two SBRs were fed with simulated urban wastewater and they were operated for 93 days. One SBR was used as control (SBR-Control), i.e. without PE microspheres addition in the feed, and the other one was doped with PE microspheres (SBR-MP). Table 1 details the operational conditions established for the reactors. The reactors consisted of cylindrical tanks measuring 30x20 cm of height and diameter, respectively. The oxygen was supplied by air diffusers positioned at the bottom of the tanks, and the concentration of dissolved oxygen was 1.8-2.2mg/L. In addition, the equalization of the systems was provided by mechanical stirrers. The SBR feeding and the effluent drawing were performed by peristaltic pumps.

Table 1 - Operational SBR condition

Cycles/day	3
Hydraulic retention time (HRT)	24h
Filling and aerobic reaction	6h
Sedimentation	90min
Draw and idle	30min
Operating days	93

As mentioned above, the reactors were fed with synthetic wastewater to minimize external interference. The synthetic wastewater was prepared with peptone, meat extract and K_2HPO_4 (supplied by Panreac) diluted in tap water, in proportions that guaranteed a feed of 500mg/L of COD, as reported by Ferrer-Polonio et al. (2019) and a supply of nutrients following the COD:N:P of 100:5:1 ratio. To maintain a food/microorganisms (F/M) ratio of 0.2gCOD/(gSS·d) (Eq.1), the reaction volume (V_R) was 6L, the daily feeding volume ($V_{feed|draw}$) was 6L and the concentration of mixed liquor suspended solids (MLSS) was maintained around 2.5g/L. Periodic sludge withdrawals were performed to maintain the MLSS concentration around that value.

$$F/M = \frac{COD \times V_{Feed|draw}}{V_R \times MLSS} \tag{1}$$

2.3. Microspheres monitorization in the SBR

Green PE microspheres, with a diameter between 150μm-180μm and density around 1.00g/cc, were purchased from the Cospheric provider. The microspheres were dosed directly into the SBR feed tank and the equalization of the systems was provided by mechanical stirrers, providing a feed concentration of 50MP/L (0.2mg/L). The SBR was operated for 70 days with this concentration. To assess the eventual effects of an abrupt increase in MPs concentration on the SBR performance, on day 70 the concentration of MPs in the feed was increased to 253MP/L (1mg/L). The SBR was operated under that concentration for 23 days.

To assist the separation of MPs a filtration system consisting of a 150μm stainless steel mesh placed inside a 50mm diameter PVC tube was coupled to the peristaltic effluent drain pump (Fig.S1). Once a week the mesh was removed and the retained material was rinsed with distilled water and stored in a beaker, which was covered with aluminum foil to avoid possible contamination. When the presence of suspended solids made the identification of the MPs unfeasible, a chemical digestion procedure based on peroxidation (H_2O_2 35%wt) was applied. In this case, the retained material on the mesh was rinsed with distilled water and treated for 2 hours at 60±2ºC, using a volumetric ratio (mL) of 1:100 [H_2O_2: distilled water]. Afterwards, the material was filtered through a glass fiber membrane (1μm) and dried in an oven at 50ºC for 2 hours for the visual identification of the MPs.

The mixed liquor was also collected and pretreated by a chemical digestion with H_2O_2 35%wt once a week. For the pretreatment, a volumetric ratio[mL] of 2:1 [H_2O_2: sludge] was used and the chemical digestion was performed at 60±2ºC for 4 hours. This chemical digestion protocol proved to be sufficient to remove more than 90% of suspend solids of the mixed liquor with an initial concentration of 2.5g/L. After digested, the sample was passed through a 150μm mesh and the retained material was removed with distilled water. Finally, the material was filtered through a glass fiber membrane (1μm) and dried in an oven at 50ºC for 2 hours for visual identification of the MPs. The methods of chemical digestion applied in this study, for both effluent and activated sludge, were thoroughly described in our previous research (Bretas Alvim et al., 2020). The material retained on the filter was carefully analyzed by

stereomicroscope (LEICA MZ APO), with the magnification adjusted between 8X and 80X, and the MPs were counted. This previous sample characterization was also important to ascertain the presence of microspheres in the initial sludge, which could influence later the monitoring of the SBR-MP.

In order to control possible losses of microspheres in the feed doping, in the separation and in the identification process, the theoretical concentration of microspheres in the sludge was estimated based on the amount of MPs that entered in the SBR via feed, and amount that was removed from the SBR during the sludge withdrawals and the MPs output through the effluent. The number of MPs was expressed as a function of the volume for the effluent (MP/L) and as a function of dry matter (d.w) for the sludge (MP/d.w).

2.4. Analysis

2.4.1. SBR performance

Throughout the three months of monitoring, physical and chemical characterizations were carried out both in the effluent and in the sludge. The effluent was characterized in terms of pH, turbidity, conductivity and soluble COD three times a week, and TN, NH_4-N, NO_3-N, NO_2-N, TP and PO_4-P once a week. The sludge was monitored in terms of MLSS (three times a week), mixed liquor volatile suspended solids (MLVSS) and soluble microbial products ($SMP_{proteins}$, $SMP_{carbohydrates}$) once a week. The samples for SMP analysis were obtained from the MLSS centrifuging at 12,000rpm for 15min and filtering the supernatant through 0.45µm cellulose acetate filters. $SMP_{proteins}$ were measured by BCA method (Zuriaga-Agustí et al., 2013) and carbohydrates by anthrone method (Frølund et al., 1996). Sludge samples were immediately observed after sampling with Carl Zeiss phase contrast microscope, Axiostar Plus model.

2.4.2. Adenosine triphosphate (ATP) and Cellular viability

During the SBRs operation, nine samples of the mixed liquor were collected periodically from the SBRs, at the end of the reaction time, to determine ATP and cell viability. ATP measurements were performed using PhotonMaster ™ Luminometer from Luminultra® (Ferrer-Polonio et al., 2019). The ATP detection method is based on the production of light when reacting a sample containing ATP with the enzyme

luciferase. The light produced is detected in a luminometer and is proportional to the amount of ATP in the sample. The detected light response is given in units of relative light (RLU). The results obtained were converted to ng ATP/mL using the Standard ATP solution UltraCheck ™ and LumiCaptureTMLite software.

From ATP measurements, the bio stress index (BSI), Eq.2, has been calculated. This parameter indicates eventual toxic effects on the microbial community.

$$BSI\ (\%) = {dATP}/{tATP} \times 100\% \tag{2}$$

Cellular viability was performed by Film Tracer™LIVE/DEAD™Biofilm viability kit (Molecular Probes Eugene, OR, USA) using the methodology reported by Ferrer-Polonio et al. (2019). The BX50F microscope (Olympus, Tokyo, Japan) equipped with a 100-W high-pressure mercury lamp was used to evaluate the samples.

2.4.3. Microbial hydrolytic enzymatic activities (MHEA)

Six microbial hydrolytic enzymatic activities were determined: Lipase, Acid phosphatase, Alkaline phosphatase, α-D-Glucosidase, Dehydrogenase and Protease in the SBR-MP and SBR-Control. They were analyzed using the same samples taken for ATP and cell viability analyses. For this purpose, the methodology reported by Ferrer-Polonio et al. (2019) was used.

2.4.4. Statistical analysis

Statistical significance was evaluated by *t*-test analysis (confidence level of 95%) with Statgraphics Centurion XVII. The following parameters were assessed in both reactors: %removal of COD, MHEA, ATP, BSI.

2.4.5. Microbial community analysis

DNA from SBR samples was extracted in duplicate, as previously described (Luján-Facundo et al., 2018) using a FastDNA® SPIN kit for soil (MP Biomedicals, OH, USA), according to the manufacturer's protocol. DNA quality was measured using a NanoDrop ND-1000 UV/Vis spectrophotometer (NanoDrop Technologies, DE, USA). DNA concentration was measured using Qubit® dsDNA BR Assay Kit (Molecular

probes, Eugene,OR, USA). DNA samples were sent to Fundación FISABIO sequencing service (Valencia, Spain) for V3-V4 16S rRNA gene amplification using the primers PRO341F and PRO805R. The subsequent amplicon sequencing on the Illumina Miseq platform was also performed by Fundación FISABIO sequencing service (Valencia, Spain) using a 2×300 nucleotide paired-end reads protocol.

2.4.6. Bioinformatics analysis of Illumina-generated amplicons

Sequence data processing and operational taxonomic units (OTU) picking were described in an earlier work (Luján-Facundo et al., 2018). Briefly, the Microbiome Helper standard operating procedure was used to process and analyse the sequencing data (Comeau et al., 2017). Raw Illumina sequences were analyzed using QIIME™ 1.8.0 (Caporaso et al. 2010). The most abundant sequence of each OTU was picked as its representative and it was used for taxonomic assignment against MiDAS v3.6 (Nierychlo et al., 2020) at 97% identity (3% cutoff level) using default parameters. Alpha-diversity (Chao1, ACE and Jacniffe indicators of species richness, Shannon and Phylogenetic diversity) were generated using CL_OPEN_REF_UCLUST_MC2 method (OTU-picking method) against EzBioCloud PKSSU4.0 database (Yoon et al., 2017).

3. Results
3.1. Behavior of polyethylene MPs in the SBR
The microspheres used in SBR-MP had a spherical shape and a very evident green color (Fig.1), which facilitated their separation and quantification.
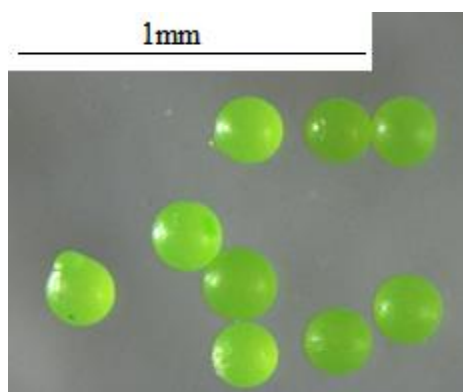
Fig.1 – Polyethylene Microspheres

During the operation of the SBR-MP reactor, a continuous accumulation of MPs was observed in the mixed sludge, reaching a final concentration of 6260MP/L (2607MP/g d.w) (Fig.2). This trend was observed throughout the monitoring period of the SBR-MP and $98\pm2\%$ (n=13) of the MPs were accumulated in the activated sludge. MPs could serve as a substrate for the formation of biofilms by bacterial communities, which would decrease their buoyancy (Zettler et al., 2013; Andrady, 2011, Lobelle and Cunliffe, 2011), and this could be the reason why MPs were found in the activated sludge rather than in the secondary effluent.
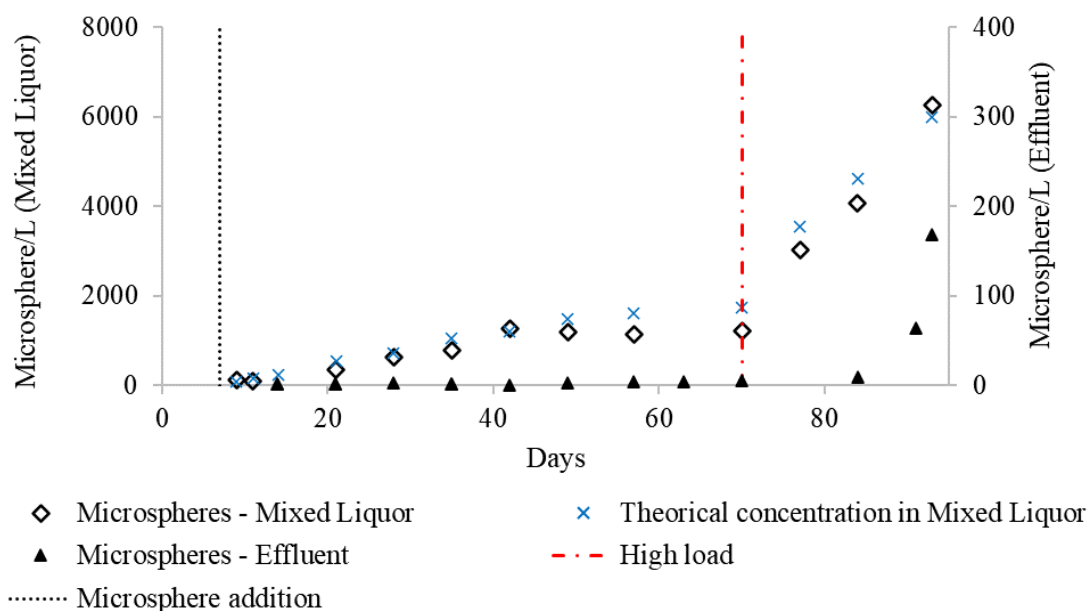
Fig.2 – Temporal evolution of the disposal of microspheres in mixed liquor and effluent

Some authors also describe similar results, where a huge portion of MPs was removed during secondary treatment due the affinity between MPs and sludge. Edo et al. (2020) reported 94% removal efficiency in the secondary settler in a WWTP in Spain preceded by an Anaerobic-Anoxic-Oxic (A2O) biological process. During the secondary treatment in a WWTP in Finland, around 87% of microparticles were removed in the activated sludge process (Talvitie et al., 2017). Kalčíková et al. (2017), although

reported lower removal percentage (of 56±16% and 47±17%) of PE microbeads in two SBRs, also observed that the MPs were visibly retained in mixed liquor.

During the feeding of the SBR-MP with low MPs load (50MP/L), concentrations of 3±2MP/L in the SBR-MP effluent were counted. When the MPs concentration in the feed was raised (253MP/L), an increase in the MPs concentration in the effluent was also observed (64±81MP/L), but in terms of percentage the activated sludge still accumulated around 98% of the MPs. Since biosolids can be used as fertilizer, MPs retained on this biological material can lead to contamination of the terrestrial environment (Corradini et al., 2019; Liu et al., 2018). Talvitie et al. (2017) estimated that 80% of microparticles, including MP, removed by a WWTP (Finland) are contained in the dried sludge. That WWTP annually produced about 60,000 tons of dry sludge. Thus, considerable amounts of microparticles are allocated to the environment by the application of the sludge as biosolids. Van den Berg et al. (2020) reported that in Spain agricultural soils receiving biosolids from sewage sludge, presented on average 256% more MPs than soils without application.

3.2. Effluents characterization (SBR-MP and SBR-Control)

In general terms, the effluents from both reactors SBR-Control and SBR-MP were very similar in characteristics. A percentage of COD removal of 97.35±0.81% (n=33) and 96.35±1.56 (n=33) was obtained in SBR-MP and SBR-Control, respectively, representing a high purification capacity in the two reactors. The results of the microbial community, which will be discussed in the section 3.3.1, demonstrated that the presence of nitrifying and denitrifying bacteria increased in the SBR-MP during the reactor performance. Thus, the high ammonium concentration reported in the Fig.3 (days 9-16 and 37-45) could be attributed to a malfunction in the aeration system. It explains the high standard deviation of the values of the parameters NH_4-N and NO_3-N. When the aeration was corrected, the ammonium nitrogen concentration decreased due to its oxidation and consequently the nitrate concentration increased. Furthermore, the aeration problems associated with the MPs did not compromise the COD removal efficiency and even implied nitrogen removal by biological denitrification due to short anoxic periods in the reactor, which is proved by the sum of the concentrations of the nitrogen species in the effluent. Table 2 shows the characteristics of the effluents of

both SBRs. Results are expressed in average values and their respective standard deviations (±SD).

Table 2 – Average values of the physical-chemical parameters of the effluents

Parameter	SBR-MP	SBR-Control
pH	7.45±0.31	7.31±0.20
Conductivity (mS/cm)	1.16±0.12	1.10±0.07
Turbidity (NTU)	0.82±0.96	1.50±1.27
COD (mg O_2/L)	13.25±4.06	18.24±7.78
COD removal efficiency (%)	97.35±0.81	96.35±1.56
NH_4-N (mg/L)	11.61±12.00	4.00±0.00
NO_3-N (mg/L)	20.20±15.19	38.38±5.05
NO_2-N(mg/L)	0.15±0.12	0.11±0.11
TP (mg/L)	5.66±1.42	5.52±1.34
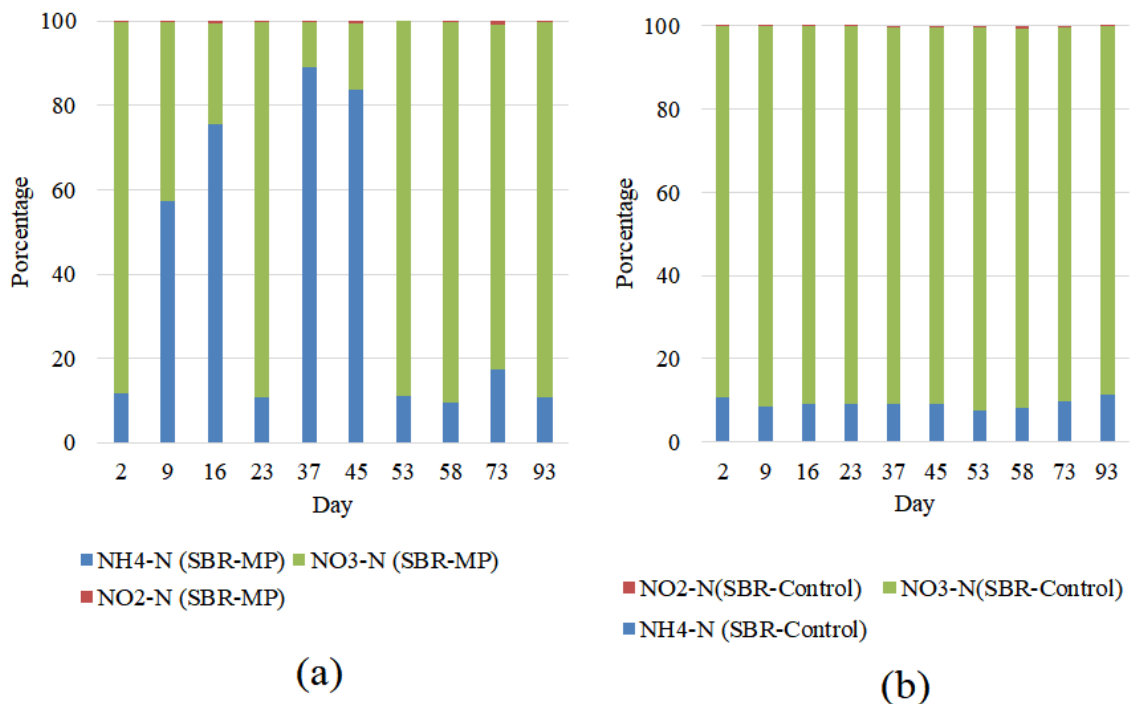
Fig.3- Nitrification in the SBR-MP (a) SBR-Control (b)

In the depuration process based on biological treatments, the microbial soluble products (SMP) correspond to the mayor fraction of the COD in the effluents and may be related

to cell lysis, substrate metabolism and biomass growth (Laspidou and Rittmann, 2002; Potvin and Zhou, 2011; Xie et al., 2013). These substances mainly correspond to proteins (SMPp) and carbohydrates (SMPc). Despite the discrepancy in the average values of SMPp (6.76±0.81mg/L SBR-Control and 5.86±1.35mg/L SBR-MP) and SMPc (16.60±4.10mg/L SBR-Control and 4.59±2.12mg/L SBR-MP) between the reactors, the SBR-MP showed an average concentration of SMP lower than the SMP-Control. It suggests that the presence of microspheres did not promote biomass disruption, which could release more SMP in the system.

With all these results, the presence of PE does not seem to be a stress factor to the biological process, as the depuration capacity of SBR-MP was high and it was very similar to that measured for the SBR-Control. Kalčíková et al. (2017) also evaluated the possible impacts of the presence of PE microbeads (0.5g/L), extracted from cosmetics, on the performance of the SBR, reporting that the nitrification process was not inhibited with the presence of PE. Removals of 96±8% (n=6), 95±7% (n=6), and 93±7% (n=6) of dissolved organic carbon were achieved by these authors in the SBRs with microbeads (MP1 and MP2) and in the control, respectively. Li et al. (2020) evaluated the effect of microplastics (PP, PVC, PE, PS and polyester - PES) with concentrations of 1000, 5000 and 10000MP/L in the nitrification process in bench tests with activated sludge and reported that the presence of MPs did not stop nitrification, but impacted in different ways this process. For instance, 1000MP/L of PP and PVC slightly improved the ammonia oxidation rate compared with the control (without adding MPs), but concentrations of 5000 and 10000MP/L negatively affected the nitrification process. The presence of PE, PS and PES also decreased the ammonia oxidation rate, but regarding the denitrification it was concluded that 5000MP/L of PVC and PES increased expressively the denitrification rate compared with the control. Liu, et al. (2019) also assessed the impacts of PE, PVC and PES MPs on the nitrification and denitrification process and the results demonstrated that at concentrations of 50 – 10,000particles/L, both ammonium-oxidizing bacteria activity and nitrite oxidizing bacteria activity and also the denitrifiers activity were not significantly affected by the presence of MPs. However, Tang et al. (2021) observed controversial results when studied the influence of PVC microplastics (PVC-MPs) on an ANAMMOX process. The nitrification process was negatively impacted by the presence of 0.1g/L and 0.5g/L PVC-MPs, the average ammonia nitrogen removal rates decreased by 3.8 % and 6.2 %,

respectively, compared with the control. All these results reported in the bibliography suggest that the MPs may have different impacts in the nitrification process and this may be due to the polymer type, the concentration of the MPs, the presence of additives in the formulation of the polymer and the structure of the microbial community. Therefore, the possibility of the inhibition of the nitrification and denitrification must not be neglected especially considering the continuous production of MPs and the high amount of MPs reaching the WWTPs and, consequently, accumulating in the activated sludge.

3.3. Biomass characterization

Due to the high retention of MPs in the activated sludge, parameters to assess MPs toxicity on the biomass were evaluated. Firstly, cell viability based on the images obtained by the epifluorescence microscope (Fig.4) -which shows green and red zones corresponding to the viable and damaged cells, respectively– was measured. It can be observed that the presence of 50MP/L (days 31, 52, 65) and 253MP/L (day 93) did not increase the percentage of dead cells in the SBR-MPs. Hence, the cell viability in SBR-MPs was not affected by the MPs presence (p-Value = 0.9494), representing 75±7% and 75±8% in the SBR-Control and SBR-MP, respectively during the monitoring period.
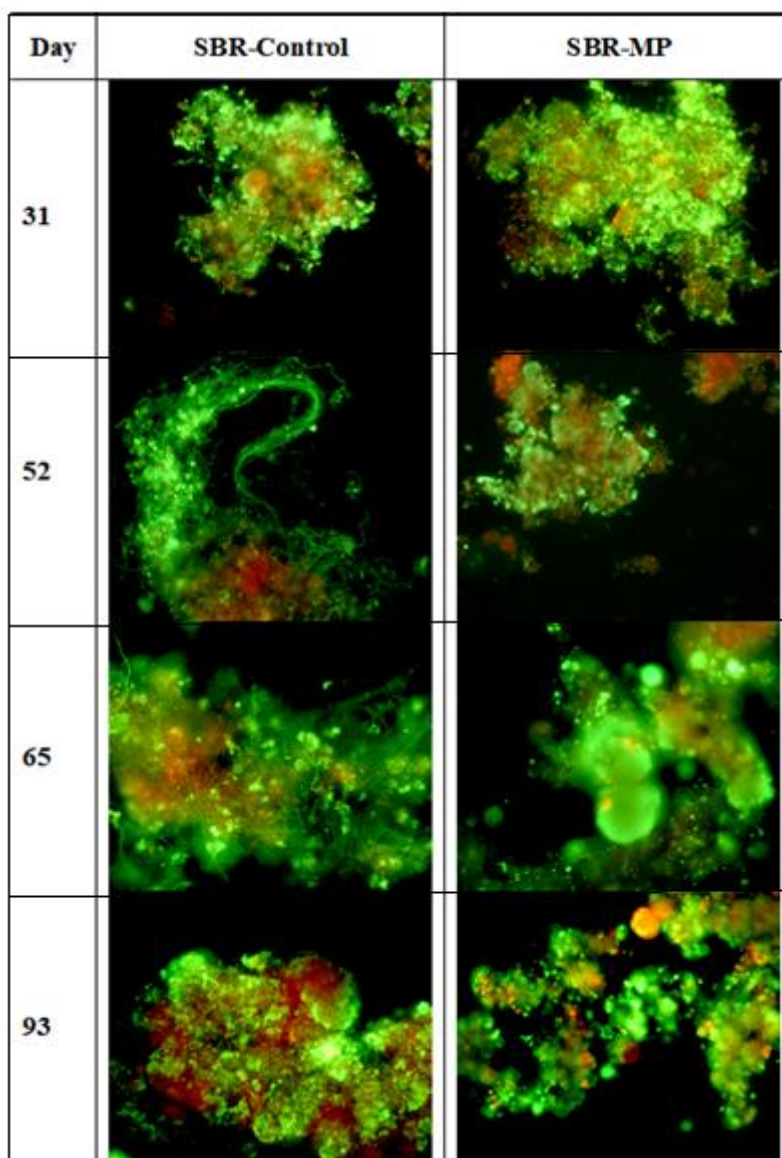
Fig.4 – Epifluorescence microscope images (200x magnification) of SBR - Control and SBR-MP

In addition to the cell viability, c-ATP and BSI were also measured to ascertain the impacts of the PE-MPs on the SBR biomass. The amount of ATP in biological systems is related to the metabolic activity of viable organisms. Therefore, the introduction of a new component in the SBR (in this case PE) may affect the bacterial metabolism, resulting in changes in cellular ATP (c-ATP) (Bäckman and Gytel, 2015; Dalzell and Christofi, 2002). However, according to the c-ATP values measured in this work, the addition of MPs in the SBR did not result in significant differences in the microbiological metabolic activity over the days of monitoring, comparing to the SBR-

Control (p-Value=0.1083). Furthermore, the values of BSI also showed that the presence of MPs did not result in toxic impacts on the microbial community, with values of 13.88±3.98% and 14.00±5.18% (SBR-Control and SBR-MP, respectively) without statistically significant differences, at 95% confidence level, between the reactors (p-Value=0.9576).

Despite no toxic impacts on the microbial community was assessed, in the period from day 70 to day 93 (period in which the SBR-MP was operating with a high load of MPs, 253MP/L), it was observed that the biomass growth was impacted (Fig.5). In the Fig.5, the initial period (from day 0 to 7) is related to the acclimation of biomass in the reactors. From day 7 it is observed that both SBR-Control and SBR-MP maintained their growth rate achieving the steady state. When SBR-MP was subjected to an increase in the load of microspheres (from day 70), it suffered an abrupt growth rate reduction (from 0.30gSS/d to 0.14gSS/d).
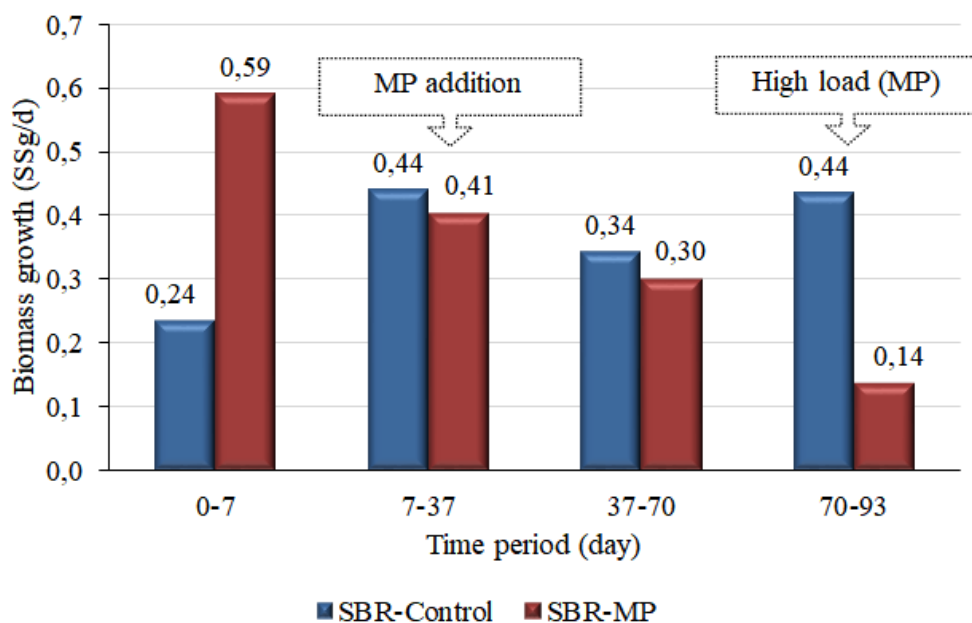
Fig.5 – Biomass growth in SBR-Control and SBR-MP

To explain this growth rate reduction, it is necessary to consider that the microorganisms used the surface of the MPs for the development of microbial communities (biofilms). Once the formation of biofilms around MPs had started, it was

expected an increase of the production of bound EPS to keep these structures well established. Therefore, the external substrate, in addition to being used for the cell growth, was also required for the bound EPS formation (Laspidou and Rittmann, 2002). Once the external substrate was depleted, the active sludge reached the endogenous phase, and the oxidation of the biomass started in order to obtain nutrients for maintaining the microbial community. In brief, on day 93, due to the high accumulation of MPs in the sludge, it was assumed that the microorganisms were able to use the polymer surface extensively for the establishment of biofilms, and for this reason the endogenous phase was more quickly reached.

The lower growth rate of biomass in the SBR-MP, and consequently greater solid retention time (SRT), was corroborated by the microscopic analysis of the sludge structure at the beginning (a, b), middle (c,d) and end (e,f) of the experiments (Fig.6). In the SBR-MP the presence of protozoa at a higher extent was observed, mainly at the end of the monitoring (Fig.6f). These organisms are commonly found in activated sludge systems with longer SRT (Ghyoot and Verstraete, 1999; Hao et al., 2010; Madoni, 2011).
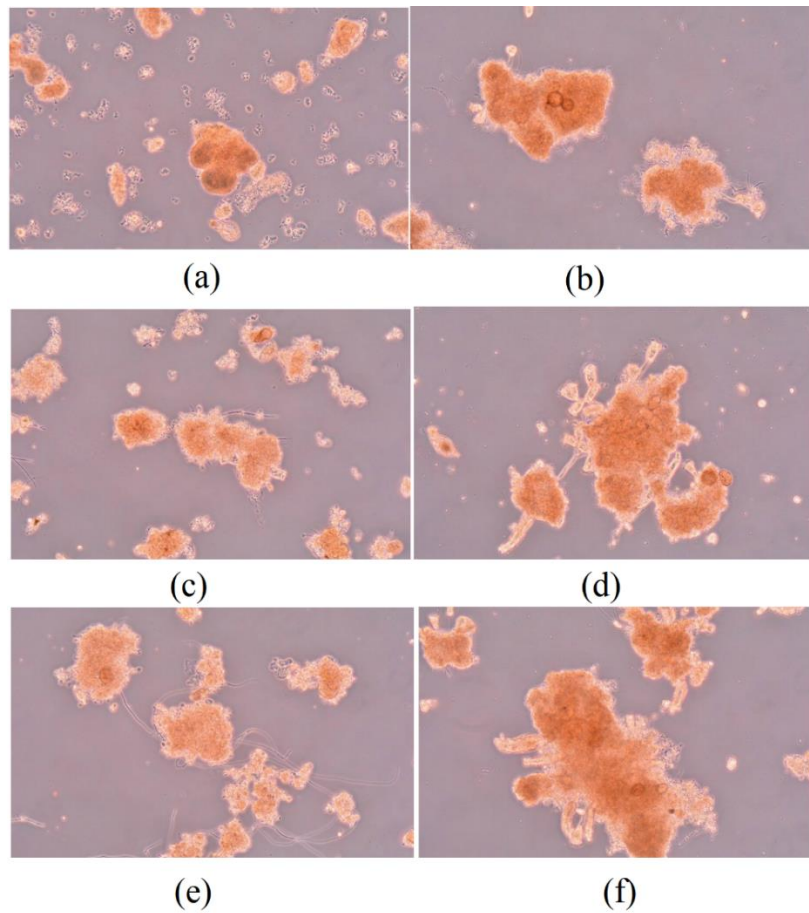
Fig.6 – Biomass images 100x from SBR-Control (Left) and SBR-MP (Right)

The SBR-Control started with a high protozoa diversity but with a low population density of individual microorganisms. At the end of the study, in the SBR-Control, the protist diversity was almost completely declined with occasional observation of *Opercularia articulata* and *Arcella vulgaris*. *Opercularia articulata* is usually associated with discharges of high Food/Microorganisms ratio. On the contrary, at the end of the study, the SBR-MP presented a low protozoa diversity, but a high population density. In this last stage was also observed in the SBR-MP the coexistence of *Rotaria* sp. and *Zoothamnium* spp. species, and occasionally *Arcella vulgaris*. The *Zoothamnium* spp. is usually associated with high COD removal, high quality of the wastewater effluent and is an excellent indicator of low Food/Microorganisms ratio in biological reactors. Microscopically, the activated sludge of the SBR-MP presented a more compact floc structure with larger flocs and slightly more mineralized than SBR-Control. These results confirmed that the SRT in SBR-MP was higher than in SBR-

Control, which showed a slightly open and less mineralized floccular structure. Another revealing aspect observed was the absence of filaments in SBR-MP, while in SBR-Control their presence was quite evident (Fig. 6e). This result might suggest that, in the SBR-MP, the structures of filamentous organisms were destroyed by mechanical shocks caused by microspheres.

3.3.1. Microbial community analysis

The performance of the activated sludge during the wastewater treatment is related to the sludge microbiome; therefore Illumina sequencing was performed in both reactors SBR-Control and SBR-MP to assess the impacts of the presence of PE-MPs on the microbial community. The MIDAS 3 database was used to analyse the microbiome in 8 SBR sludge samples. The 8 Illumina libraries of bacterial 16S rRNA gene yielded 703,359 reads after quality filtering and removal of chimeric sequences from 1,008,864 raw reads. The species richness, library coverage and diversity estimation were calculated for each library and shown in Table 3. The Chao1 demonstrated that the richness of bacteria in SBR-MP samples on day 93 was lower than that in SBR-Control. Furthermore, the bacterial biodiversity in SBR-MP was also lower than in SBR-Control on day 93, according to the ACE and Jackniffe indices. Based on these results, our findings suggest that MPs can have selective effects on activated sludge microbial community.

Table 3 – Index of microbial community diversity of 16S rRNA gene amplicon analysis for SBR sludges

Sample	Day	Clean reads	OTU	ACE	Chao1	Jackniffe	Shannon	Phylogenetic diversity	Coverage
SBR-Control	23	92,980	1958	2139	2090	2281	5.466	2755	0.930
SBR-Control	52	93,049	1415	2221	2166	2355	5.790	2782	0.930
SBR-Control	65	63,348	1425	1838	1791	1962	5.604	2425	0.921
SBR-Control	93	93,196	2423	1999	1945	2128	5.562	2629	0.932
SBR-MP	23	93,494	1682	2165	2068	2245	5.545	2735	0.935

SBR-MP	52	93,007	1270	1808	1755	1935	5.222	2413	0.930
SBR-MP	65	93,419	1604	1697	1639	1820	5.195	2289	0.934
SBR-MP	93	76,284	2084	1608	1567	1727	5.330	2180	0.924

The predominant phyla in both reactors SBR-Control and SBR-MP belonged to *Proteobacteria*, *Bacteroidetes*, *Actinobacteria, Chloroflexi* and *Patescibacteria* (Fig.7a). The dominant presence of these phyla is usually reported in the microbial community of activated sludge (Guo et al., 2017; Nguyen et al., 2019). During the performance of the SBR-MP was observed an expressive reduction of the relative abundance of *Chloroflexi* from 9.6% (Day 23) to 4.4% (Day 93). Once these microorganisms correspond to filamentous bacteria, this decrease could be linked to the bacteria disruption by the mechanical shocks caused by the MPs, as explained in the section 3.2. Unlike *Chloroflexi* phylum, the relative abundance of *Proteobacteria* raised from 26.4% (Day 23) to 39.3% (Day 93) in the SBR-MP. Delacuvellerie, et al. (2019) studied the formation of 'plastispheres' on LDPE, PET and PS macroplastics (>5cm) and observed that the microbial community on the plastic surface was mainly formed by *Proteobacteria* (especially *Gammaproteobacteria* and *Alphaproteobacteria*), which is considered the primary colonizers in a plastic biofilm in the marine environment (De Tender et al., 2015). In our study we did not evaluate the biofilm on the PE-MPs surface, but results suggest that these bacteria can play an important role in the biofilm formation on PE-MPs since the activated sludge is the only source of bacterial colonization and it is mainly compound by *Proteobacteria*.

Another important aspect about the presence of Proteobacteria is that this phylum includes bacteria groups that are responsible for ammonia (*Nitrosomonas*, AOB) and nitrite oxidation (*Nitrospira*, NOB) and for the denitrification process (denitrifying bacteria, *Thauera*). Their presence in the activated sludge contributes to the good depuration performance of the SBR. In this context, *Nitrosomonas*, *Nitrospira* and *Thauera* were detected during all the studied period of SBR-Control and SBR-MP (Fig.7b) and their relative abundance were not affected by the presence of MP. In addition, an increase of these groups in the SBR-MP during the experimental time

suggests that these bacteria can play an important role in the biofilm formation on PE-MPs. Therefore, these results indicate that the nitrification and denitrification process in the SBR-MP were not inhibited by the presence of MPs, even under higher PE-MPs concentration (Day 93). More detailed information is included in Fig.S2.
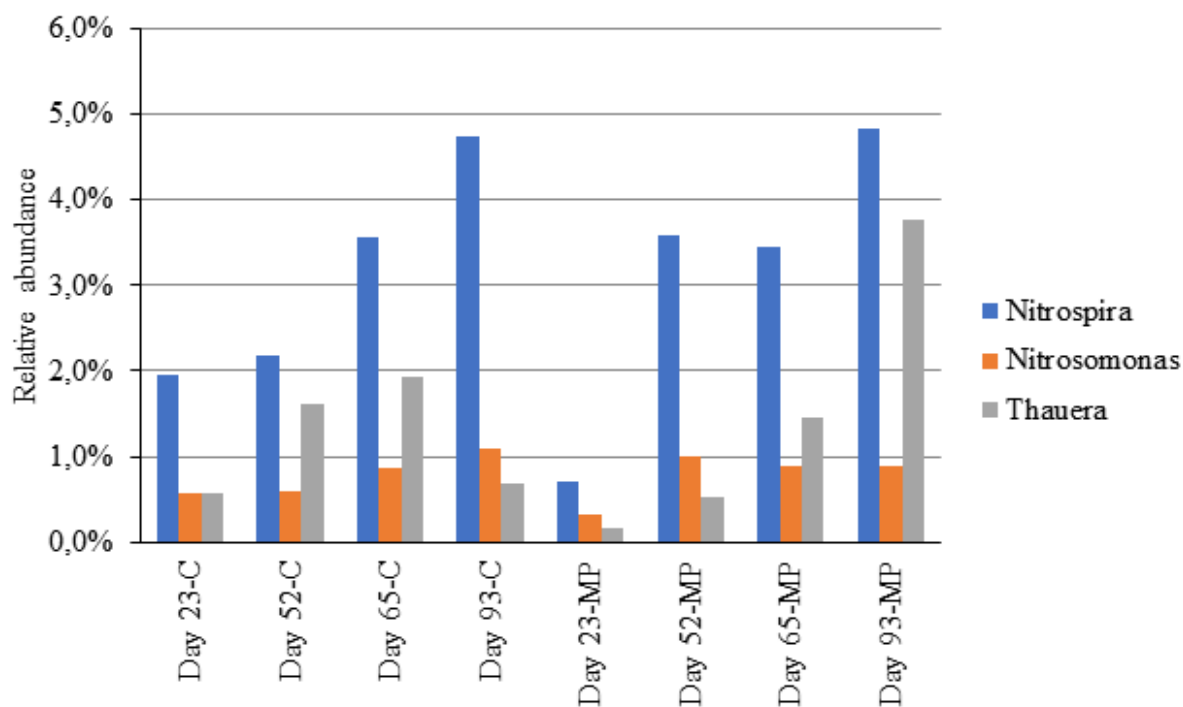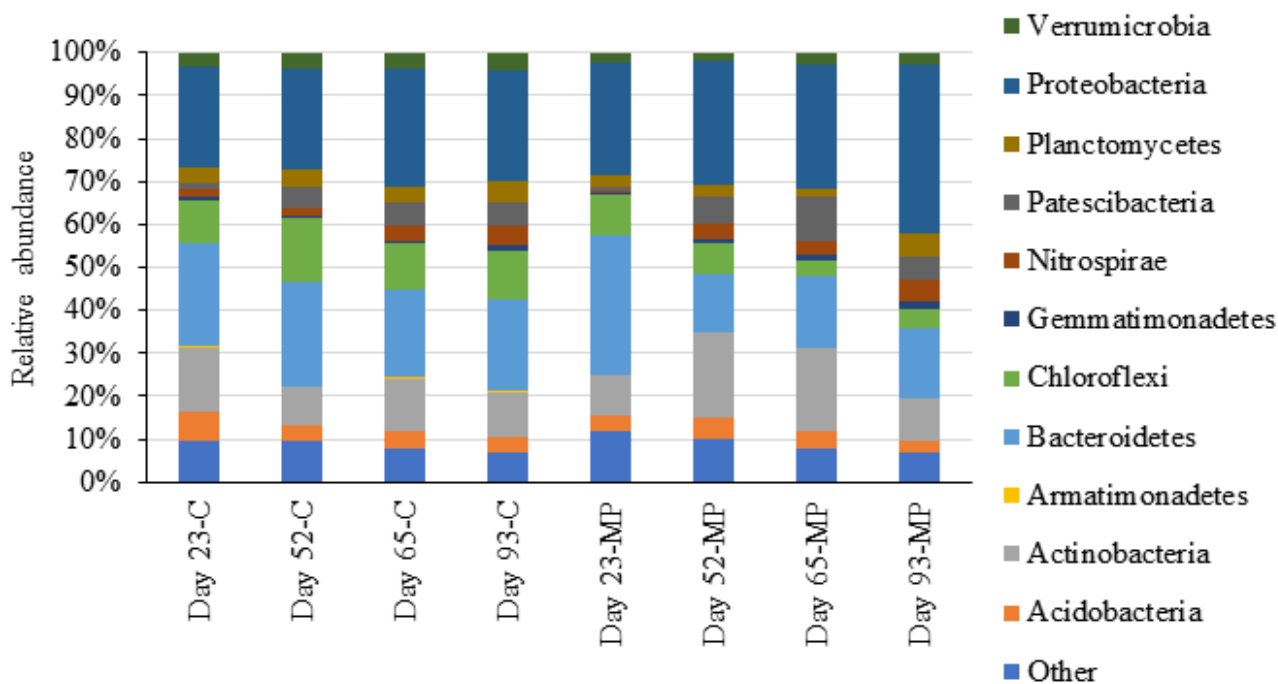

(a)

(b)

Fig.7 – (a) Bacterial community at phylum level by MiSeq sequencing (C: SBR-Control and MP: SBR-MP samples); (b) Relative abundances of nitrifying and denitrifying bacteria by MiSeq sequencing (C: SBR-Control and MP: SBR-MP samples)

Across all 8 libraries, 57 and 51 different bacterial classes were found in SBR-Control and SBR-MP, respectively. The dominant class was *Bacteroidia* with relative abundance of 23.0-19.7% in SBR-Control and 31.2-16.4% in SBR-MP. *Actinobacteria*, *Gammaproteobacteria* and *Alphaproteobacteria* accounted for 14.2-10,0%, 11.7-12.1%, 8.9-9.5%, respectively, in the SBR-control. The other three dominant classes in SBR-MP were also *Actinobacteria*, *Gammaproteobacteria* and *Alphaproteobacteria* accounting for 8.6-19.3%, 14.4-11.0%, 11.9-12.0%, respectively. Relative abundances at genus level can be observed in supplementary materials (Fig.S3)

Until now, there is a lack of knowledge about how and why the MPs can impact the microbial population in activated sludge. However, it has been reported that the presence of MPs in organic matrices can indeed alter their microbial community. For instance, Wei et al. (2019a) observed that the exposure of the waste activated sludge (which was submitted to anaerobic digestion) to PE-MPs (200MPs/g-TS) reduced the total microbial population, without affecting the microbial diversity. In another study Zhao et al. (2020) assessed the effects of polyamide 66 (PA66) on the aerobic granular sludge and observed that 0.1g/L PA66 leaded to a reduction of the diversity of the bacterial communities and an increase of microbial richness. The authors also observed that 0.2g/L of PA66 contributed to increase the abundance of *Proteobacteria*, however, 0.5g/L of PA66 negatively impacted the abundance of this phyla. Tang et al. (2021) reported that 0.5 g/L of polybutylene succinate (PBS) stimulated the enrichment of the *Proteobacteria* on 10.23 % on the anaerobic ammonium oxidation (ANAMMOX) sludge. However, the authors also observed that PVC microplastics inhibited the growth of anaerobic ammonia-oxidizing bacteria on the anammox process. Since the biosolids from WWTPs can be applied in the farmlands, the presence of MPs in this organic matrix can also impact on the microbiome of soils, an in this context Wang et al. (2020) observed that the presence of LDPE-MP altered the soil microbial community and selectively enriched specific members of bacteria. After 90 days exposure, it was noticed that the phyla *Acidobacteria*, *Armatimonadetes*, *Bacteroidetes*,

Gemmatimonadetes, and *Proteobacteria* were significantly abundant in the microplastic amended soils.

3.4. Enzymatic activities

The results of MHEA (Fig.8) show that SBR-MP had slightly lower phosphatase activity and equal dehydrogenase and α-D-glucosidase enzyme activity compare with the SBR-Control, and almost all activities remained constant throughout the monitoring period. However, the increase in protease activity over time is noteworthy. Few studies have reported how the presence of MPs can affect microbiology communities and their enzymatic activities; thereby conclusions that justify the increase in certain enzyme activities are not clear. Wei et al. (2019b) demonstrated that the presence of PVC microplastics (60particles/g Total Solid) can reduce the enzyme activities Protease, AK and F420, which are considered key enzymes for the anaerobic digestion process. In addition, they reported that the additive bisphenol-A (BPA), when leached from PVC microplastics inhibits methane production. In another study, Wei et al. (2019a) observed that during the waste activated sludge anaerobic digestion, fed with PE-MPs with concentrations of 200particles/g of Total Solids, methane production could be reduced by more than 20%. A study carried out by Huang et al. (2019) showed that the presence of MPs of PE in soils (200fragments per 100g of soil dw = 0.076 g/kg) stimulated an increase in urease and catalase activity (175% and 139%, respectively) after being exposed to the plastic for 90 days. Unfortunately, we cannot perform a direct comparison between the results obtained in our work and those presented by the authors previously cited, because different conditions and different types of enzymatic activities have been measured. In this way, further research about how the presence of MPs in activated sludge would affect microbial communities and their enzymatic activities should be developed. Thus, due to the lack of knowledge of the impacts of MPs on enzymatic activities of activated sludge, two hypotheses were stipulated to justify the increase in protease in the SBR-MP. The first hypothesis is based on the EPS hydrolysis and the second one on the PE biodegradation.
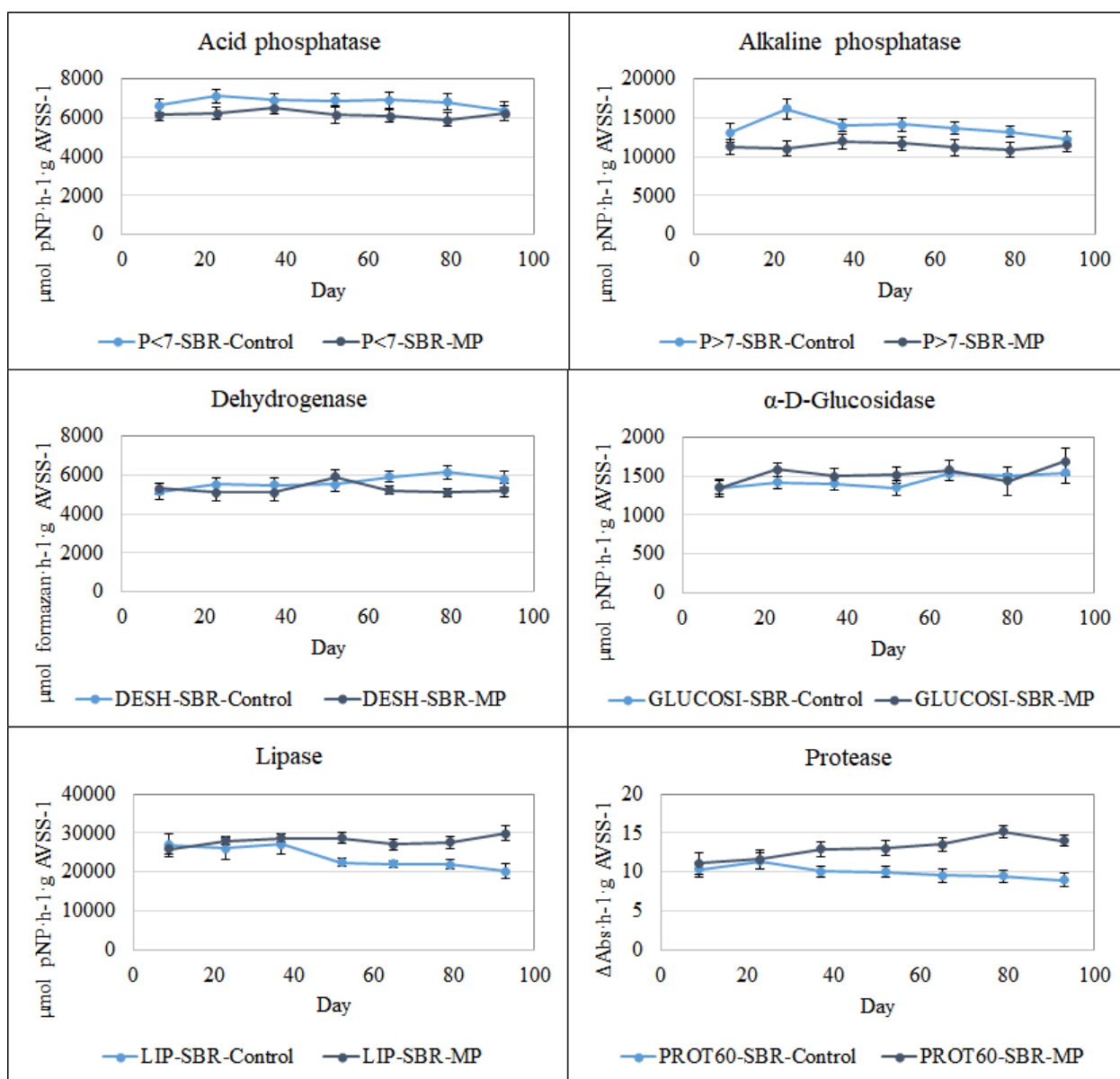
Fig. 8- Microbial hydrolytic enzymatic activities in SBRs

The first hypothesis was initially explained by the biomass growth decline in the SBR-MP, which was attributed to the biofilm formation on the MPs surface, as explained in the section 3.3. The faster substrate consumption makes that the SBR-MP reaches the endogenous phase earlier. In this phase, to guarantee the maintenance of active biomass, hydrolysis of bound EPS was enhanced (Laspidou and Rittmann, 2002). Therefore, in the SBR-MP the hydrolysis of EPS would lead to an increase in protease enzymatic activities. However, EPS are mainly formed by carbohydrates and proteins (Sheng et

al., 2010); thereby an increase of the enzymatic activity for carbohydrates depletion would be also expected. It cannot be discarded because other enzymes in addition to glucosidase may play a role in carbohydrates degradation.

Therefore, a second hypothesis was elaborated considering the possible biodegradation of polyethylene after reach the endogenous phase as an attempted to use the PE as carbon source. The biodegradation process could be started with the formation of colonies of microorganisms on the polymer surface, followed by the secretion of extracellular enzymes in order to produce low molecular weight products which could be used as a carbon source (Arutchelvi et al., 2008; Das and Kumar, 2015). The enzymes proteases, ureases and esterases could be involved in the biodegradation process (Kumar et al., 2020).

Tribedi and Sil (2013) reported the biodegradation of polyethylene film by *Pseudomonas* sp. In this process, besides the presence of biofilm on LDPE surface, the authors also suggested that *Pseudomonas* sp. have enzymatic activities which lead to polyethylene biodegradation. Park and Ki (2019) evaluated the biodegradation of PE microspheres (40µm-600µm) as sole carbon source, by mixed bacterial strains mainly composed of *Bacillus sp.* and *Paenibacillus sp.* isolated from a municipal solid waste disposal site in Korea. In this study it was observed that the microorganisms, besides colonizing the surface of the PE, promoted a weight loss of 14.7% of the microplastic after 60 days of incubation. In another work, where *Bacillus amyloliquefaciens* isolated from municipal solid soil (India) were used to assess the biodegradation of LDPE film microplastic, showed that these bacteria were capable of colonizing the microplastic surface and degrading it, achieving 16% of its weight loss (Das and Kumar, 2015). The degree of biodegradation may vary according to the type of polymer, experimental protocols and microorganisms present in the process. The two hypotheses proposed were based on the results reported in previous studies, nevertheless, more specific investigations regarding the exposure of activated sludge to MPs must be performed to validate them. The measure of EPS in biological systems fed with microplastics can provide relevant information about the microbial structure, and analyses such as scanning electron microscopy can validate the formation of microbial colonies on the microplastic surface. Moreover, spectroscopy analyses, FTIR for example, on the MPs surface can assess their biodegradation and their use as carbon source.

4. Conclusions

MPs entering the biological process of WWTPs are accumulated in the activated sludge reactors. In this work, during the operation of a laboratory SBR, it was observed that $98\pm2\%$ of the PE-MPs entering the system were accounted in the mixed liquor, and the remaining fraction was withdrawn in the effluent. This fact makes interesting to assess the eventual effects of MPs on the biomass.

Depuration capacity of both operated reactors (SBR-Control and SBR-MP) was practically the same, which means that MPs did have not influence of organic matter removal and nitrification process. However, the results of the microbial community analysis revealed that the presence of MPs may have selective effects on activated sludge microbiome, reducing the bacterial diversity.

Other biomass characterization parameters were also measured to compare the biomass in SBR-Control and SBR-MPs. It can be concluded that parameters describing toxicity as cell viability and BSI showed similar results. However, the MHEA revealed an increase of protease activity in the SBR-MP, which may be connected to an attempt to degrade the PE-MPs.

Acknowledgments

REFERENCES

Andrady, A.L., 2011. Microplastics in the marine environment. Mar. Pollut. Bull. 62, 1596–1605. https://doi.org/10.1016/j.marpolbul.2011.05.030

Arutchelvi, J., Sudhakar, M., Arkatkar, A., Doble, M., Bhaduri, S., Uppara, P.V., 2008. Biodegradation of polyethylene and polypropylene. Indian J. Biotechnol. 7, 9–22.

Bäckman, G., Gytel, U., 2015. Activated sludge optimization using ATP in pulp and paper industry. Water Sci. Technol. 71, 1173–1179. https://doi.org/10.2166/wst.2015.081

Bayo, J., Olmos, S., López-Castellanos, J., 2020. Microplastics in an urban wastewater treatment plant: The influence of physicochemical parameters and environmental factors. Chemosphere 238. https://doi.org/10.1016/j.chemosphere.2019.124593

Bretas Alvim, C., Bes-Piá, M.A., Mendoza-Roca, J.A., 2020. Separation and identification of microplastics from primary and secondary effluents and activated sludge from wastewater treatment plants. Chem. Eng. J. 402, 126293. https://doi.org/10.1016/j.cej.2020.126293

Caporaso, J.G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F.D., Costello, E.K., Fierer, N., Peña, A.G., Goodrich, J.K., Gordon, J.I., Huttley, G.A., Kelley, S.T., Knights, D., Koenig, J.E., Ley, R.E., Lozupone, C.A., McDonald, D., Muegge, B.D., Pirrung, M., Reeder, J., Sevinsky, J.R., Turnbaugh, P.J., Walters, W.A., Widmann, J., Yatsunenko, T., Zaneveld, J., Knight, R., 2010. QIIME allows analysis of high-throughput community sequencing data. Nat. Methods 7, 335–336. https://doi.org/10.1038/nmeth.f.303

Carr, S.A., Liu, J., Tesoro, A.G., 2016. Transport and fate of microplastic particles in wastewater treatment plants. Water Res. 91, 174–82. https://doi.org/10.1016/j.watres.2016.01.002

Comeau, A.M., Douglas, G.M., Langille, M.G.I., 2017. Microbiome helper: a custom and streamlined workflow for microbiome research. mSystems 2, 1-11. https://doi.org/10.1128/mSystems.00127-16 e00127e16.

Corradini, F., Meza, P., Eguiluz, R., Casado, F., Huerta-Lwanga, E., Geissen, V., 2019. Evidence of microplastic accumulation in agricultural soils from sewage sludge disposal. Sci. Total Environ. 671, 411–420. https://doi.org/10.1016/j.scitotenv.2019.03.368

Dalzell, D.J.B., Christofi, N., 2002. An ATP luminescence method for direct toxicity assessment of pollutants impacting on the activated sewage sludge process. Water Res. 36, 1493–1502. https://doi.org/10.1016/S0043-1354(01)00346-3

Das, M.P., Kumar, S., 2015. An approach to low-density polyethylene biodegradation by Bacillus amyloliquefaciens. 3 Biotech 5, 81–86. https://doi.org/10.1007/s13205-014-0205-1

Delacuvellerie, A., Cyriaque, V., Gobert, S., Benali, S., Wattiez, R., 2019. The plastisphere in marine ecosystem hosts potential specific microbial degraders including Alcanivorax borkumensis as a key player for the low-density polyethylene degradation. J. Hazard. Mater. 380, 120899. https://doi.org/10.1016/j.jhazmat.2019.120899

De Tender, C.A., Devriese, L.I., Haegeman, A., Maes, S., Ruttink, T., Dawyndt, P., 2015. Bacterial Community Profiling of Plastic Litter in the Belgian Part of the North Sea. Environ. Sci. Technol. 49, 9629–9638. https://doi.org/10.1021/acs.est.5b01093

Eckert, E.M., Di Cesare, A., Kettner, M.T., Arias-Andres, M., Fontaneto, D., Grossart, H.P., Corno, G., 2018. Microplastics increase impact of treated wastewater on freshwater microbial community. Environ. Pollut. 234, 495–502. https://doi.org/10.1016/j.envpol.2017.11.070

Edo, C., González-Pleiter, M., Leganés, F., Fernández-Piñas, F., Rosal, R., 2020. Fate of microplastics in wastewater treatment plants and their environmental dispersion

with effluent and sludge. Environ. Pollut. 259. https://doi.org/10.1016/j.envpol.2019.113837

Ferrer-Polonio, E., Fernández-Navarro, J., Alonso-Molina, J.L., Mendoza-Roca, J.A., Bes-Piá, A., Amorós, I., 2019. Towards a cleaner wastewater treatment: Influence of folic acid addition on sludge reduction and biomass characteristics. J. Clean. Prod. 232, 858–866. https://doi.org/10.1016/j.jclepro.2019.06.021

Frølund, B., Palmgren, R., Keiding, K., Nielsen, P.H., 1996. Extraction of extracellular polymers from activated sludge using a cation exchange resin. Water Res. 30, 1749e1758. https://doi.org/10.1016/0043-1354(95)00323-1.

Ghyoot, W., Verstraete, W., 2000. Reduced sludge production in a two-stage membrane-assisted bioreactor. Water Res. 34, 205–215. https://doi.org/10.1016/S0043-1354(99)00138-4

Gies, E.A., LeNoble, J.L., Noël, M., Etemadifar, A., Bishay, F., Hall, E.R., Ross, P.S., 2018. Retention of microplastics in a major secondary wastewater treatment plant in Vancouver, Canada. Mar. Pollut. Bull. 133, 553–561. https://doi.org/10.1016/j.marpolbul.2018.06.006

Guo, J., Ni, B.J., Han, X., Chen, X., Bond, P., Peng, Y., Yuan, Z., 2017. Unraveling microbial structure and diversity of activated sludge in a full-scale simultaneous nitrogen and phosphorus removal plant using metagenomic sequencing. Enzyme Microb. Technol. 102, 16–25. https://doi.org/10.1016/j.enzmictec.2017.03.009

Hao, X., Wang, Q., Cao, Y., van Loosdrecht, M.C.M., 2010. Measuring the activities of higher organisms in activated sludge by means of mechanical shearing pretreatment and oxygen uptake rate. Water Res. 44, 3993–4001. https://doi.org/10.1016/j.watres.2010.05.013

Hou, L., Kumar, D., Yoo, C.G., Gitsov, I., Majumder, E.L.-W., 2021. Conversion and removal strategies for microplastics in wastewater treatment plants and landfills. Chem. Eng. J. 406, 126715. https://doi.org/10.1016/j.cej.2020.126715

Huang, Y., Zhao, Y., Wang, J., Zhang, M., Jia, W., Qin, X., 2019. LDPE microplastic films alter microbial community composition and enzymatic activities in soil. Environ. Pollut. 254, 112983. https://doi.org/10.1016/j.envpol.2019.112983

Jiang, J., Wang, X., Ren, H., Cao, G., Xie, G., Xing, D., Liu, B., 2020. Investigation and fate of microplastics in wastewater and sludge filter cake from a wastewater treatment plant in China. Sci. Total Environ. 746, 141378. https://doi.org/10.1016/j.scitotenv.2020.141378

Kalčíková, G., Alič, B., Skalar, T., Bundschuh, M., Gotvajn, A.Ž., 2017. Wastewater treatment plant effluents as source of cosmetic polyethylene microbeads to freshwater. Chemosphere 188, 25–31. https://doi.org/10.1016/j.chemosphere.2017.08.131

Kumar, G.A., Anjana, K., Hinduja, M., Sujitha, K., Dharani G.,2020. Review on plastic wastes in marine environment – Biodegradation and biotechnological solutions. Mar. Pollut. Bull. 150, 110733. https://doi.org/10.1016/j.marpolbul.2019.110733

Lares, M., Ncibi, M.C., Sillanpää, Markus, Sillanpää, Mika, 2018. Occurrence, identification and removal of microplastic particles and fibers in conventional activated sludge process and advanced MBR technology. Water Res. 133, 236–246. https://doi.org/10.1016/j.watres.2018.01.049

Laspidou, C.S., Rittmann, B.E., 2002. A unified theory for extracellular polymeric substances, soluble microbial products, and active and inert biomass. Water Res. 36, 2711–2720. https://doi.org/10.1016/S0043-1354(01)00413-4

Li, J., Liu, H., Paul Chen, J., 2018. Microplastics in freshwater systems: A review on occurrence, environmental effects, and methods for microplastics detection. Water Res. 137, 362–374. https://doi.org/10.1016/j.watres.2017.12.056

Li, L., Song, K., Yeerken, S., Geng, S., Liu, D., Dai, Z., Xie, F., Zhou, X., Wang, Q., 2020. Effect evaluation of microplastics on activated sludge nitrification and denitrification. Sci. Total Environ. 707, 1–6. https://doi.org/10.1016/j.scitotenv.2019.135953

Liu, H., Zhou, X., Ding, W., Zhang, Z., Nghiem, L.D., Sun, J., Wang, Q., 2019. Do Microplastics Affect Biological Wastewater Treatment Performance? Implications from Bacterial Activity Experiments. ACS Sustain. Chem. Eng. 7, 20097–20101. https://doi.org/10.1021/acssuschemeng.9b05960

Liu, M., Lu, S., Song, Y., Lei, L., Hu, J., Lv, W., Zhou, W., Cao, C., Shi, H., Yang, X., He, D., 2018. Microplastic and mesoplastic pollution in farmland soils in suburbs of Shanghai, China. Environ. Pollut. 242, 855–862. https://doi.org/10.1016/j.envpol.2018.07.051

Lobelle, D., Cunliffe, M., 2011. Early microbial biofilm formation on marine plastic debris. Mar. Pollut. Bull. 62, 197–200. https://doi.org/10.1016/j.marpolbul.2010.10.013

Luján-Facundo, M.J., Fernández-Navarro, J., Alonso-Molina, J.L., Amorós-Muñoz, I., Moreno, Y., Mendoza-Roca, J.A., Pastor-Alcañiz, L., 2018. The role of salinity on the changes of the biomass characteristics and on the performance of an OMBR treating tannery wastewater. Water Res. 142, 129–137. https://doi.org/10.1016/j.watres.2018.05.046

Madoni, P., 2011. Protozoa in wastewater treatment processes: A minireview. Ital. J. Zool. 78, 3–11. https://doi.org/10.1080/11250000903373797

Murphy, F., Ewins, C., Carbonnier, F., Quinn, B., 2016. Wastewater Treatment Works (WwTW) as a Source of Microplastics in the Aquatic Environment. Environ. Sci. Technol. 50, 5800–5808. https://doi.org/10.1021/acs.est.5b05416

Nguyen, L.N., Commault, A.S., Johir, M.A.H., Bustamante, H., Aurisch, R., Lowrie, R., Nghiem, L.D., 2019. Application of a novel molecular technique to characterise the effect of settling on microbial community composition of activated sludge. J. Environ. Manage. 251. https://doi.org/10.1016/j.jenvman.2019.109594

Nierychlo, M., Andersen, K.S., Xu, Y., Green, N., Jiang, C., Albertsen, M., Dueholm, M.S., Nielsen, P.H., 2020. MiDAS 3: an ecosystem-specific reference database, taxonomy and knowledge platform for activated sludge and anaerobic digesters

reveals species-level microbiome composition of activated sludge. Water Res. e115955. https://doi.org/10.1016/j.watres.2020.115955.

Park, S.Y., Kim, C.G., 2019. Biodegradation of micro-polyethylene particles by bacterial colonization of a mixed microbial consortium isolated from a landfill site. Chemosphere 222, 527–533. https://doi.org/10.1016/j.chemosphere.2019.01.159

PlasticsEurope - the Facts 2019, (2019). https://www.plasticseurope.org/en/resources/market-data. (accessed 14 April 2020).

Potvin, C.M., Zhou, H., 2011. Interference by the activated sludge matrix on the analysis of soluble microbial products in wastewater. Chemosphere 85, 1139–1145. https://doi.org/10.1016/j.chemosphere.2011.07.057

Sheng, G.P., Yu, H.Q., Li, X.Y., 2010. Extracellular polymeric substances (EPS) of microbial aggregates in biological wastewater treatment systems: A review. Biotechnol. Adv. 28, 882–894. https://doi.org/10.1016/j.biotechadv.2010.08.001

Simon, M., van Alst, N., Vollertsen, J., 2018. Quantification of microplastic mass and removal rates at wastewater treatment plants applying Focal Plane Array (FPA)-based Fourier Transform Infrared (FT-IR) imaging. Water Res. 142, 1–9. https://doi.org/10.1016/j.watres.2018.05.019

Sun, J., Dai, X., Wang, Q., van Loosdrecht, M.C.M., Ni, B.J., 2019. Microplastics in wastewater treatment plants: Detection, occurrence and removal. Water Res. 152, 21–37. https://doi.org/10.1016/j.watres.2018.12.050

Talvitie, J., Mikola, A., Setälä, O., Heinonen, M., Koistinen, A., 2017. How well is microlitter purified from wastewater? – A detailed study on the stepwise removal of microlitter in a tertiary level wastewater treatment plant. Water Res. 109, 164–172. https://doi.org/10.1016/j.watres.2016.11.046

Tang, L., Su, C., Chen, Y., Xian, Y., Hui, X., Ye, Z., Chen, M., Zhu, F., Zhong, H., 2021. Influence of biodegradable polybutylene succinate and non-biodegradable

polyvinyl chloride microplastics on anammox sludge: Performance evaluation,
suppression effect and metagenomic analysis. J. Hazard. Mater. 401, 123337.
https://doi.org/10.1016/j.jhazmat.2020.123337

Tribedi, P., Sil, A.K., 2013. Low-density polyethylene degradation by Pseudomonas sp.
AKS2 biofilm. Environ. Sci. Pollut. Res. 20, 4146–4153.
https://doi.org/10.1007/s11356-012-1378-y

van den Berg, P., Huerta-Lwanga, E., Corradini, F., Geissen, V., 2020. Sewage sludge
application as a vehicle for microplastics in eastern Spanish agricultural soils.
Environ. Pollut. 261, 114198. https://doi.org/10.1016/j.envpol.2020.114198

Wang, J., Huang, M., Wang, Q., Sun, Y., Zhao, Y., Huang, Y., 2020. LDPE
microplastics significantly alter the temporal turnover of soil microbial
communities. Sci. Total Environ. 726, 138682.
https://doi.org/10.1016/j.scitotenv.2020.138682

Wei, W., Huang, Q.S., Sun, J., Dai, X., Ni, B.J., 2019a. Revealing the Mechanisms of
Polyethylene Microplastics Affecting Anaerobic Digestion of Waste Activated
Sludge. Environ. Sci. Technol. 53, 9604–9613.
https://doi.org/10.1021/acs.est.9b02971

Wei, W., Huang, Q.S., Sun, J., Wang, J.Y., Wu, S.L., Ni, B.J., 2019b. Polyvinyl
Chloride Microplastics Affect Methane Production from the Anaerobic Digestion
of Waste Activated Sludge through Leaching Toxic Bisphenol-A. Environ. Sci.
Technol. 53, 2509–2517. https://doi.org/10.1021/acs.est.8b07069

Xie, W.M., Ni, B.J., Seviour, T., Yu, H.Q., 2013. Evaluating the impact of operational
parameters on the formation of soluble microbial products (SMP) by activated
sludge. Water Res. 47, 1073–1079. https://doi.org/10.1016/j.watres.2012.11.022

Yoon, S. H., Ha, S. M., Kwon, S., Lim, J., Kim, Y., Seo, H. and Chun,
J.,2017. Introducing EzBioCloud: A taxonomically united database of 16S rRNA
and whole genome assemblies. Int. J. Syst. Evol. Microbiol. 67, 1613-1617.
https://doi.org/ 10.1099/ijsem.0.001755.

Zettler, E.R., Mincer, T.J., Amaral-Zettler, L.A., 2013. Life in the "plastisphere": Microbial communities on plastic marine debris. Environ. Sci. Technol. 47, 7137–7146. https://doi.org/10.1021/es401288x

Zhang, Y.-T., Wei, W., Huang, Q.-S., Wang, C., Wang, Y., Ni, B.-J., 2020. Insights into the microbial response of anaerobic granular sludge during long-term exposure to polyethylene terephthalate microplastics. Water Res. 179, 115898. https://doi.org/10.1016/j.watres.2020.115898

Zhao, L., Su, C., Liu, W., Qin, R., Tang, L., Deng, X., Wu, S., Chen, M., 2020. Exposure to polyamide 66 microplastic leads to effects performance and microbial community structure of aerobic granular sludge. Ecotoxicol. Environ. Saf. 190, 110070. https://doi.org/10.1016/j.ecoenv.2019.110070

Ziajahromi, S., Neale, P.A., Rintoul, L., Leusch, F.D.L.L., 2017. Wastewater treatment plants as a pathway for microplastics : Development of a new approach to sample wastewater-based microplastics. Water Res. 112, 93–99. https://doi.org/10.1016/j.watres.2017.01.042

Ziajahromi, S., Neale, P.A., Telles Silveira, I., Chua, A., Leusch, F.D.L., 2021. An audit of microplastic abundance throughout three Australian wastewater treatment plants. Chemosphere 263, 128294. https://doi.org/10.1016/j.chemosphere.2020.128294

Zuriaga-Agustí, E., Bes-Piá, A., Mendoza-Roca, J.A., Alonso-Molina, J.L., 2013. Influence of extraction methods on proteins and carbohydrates analysis from MBR activated sludge flocs in view of improving EPS determination. Sep. Purif. Technol. 112, 1–10. https://doi.org/10.1016/j.seppur.2013.03

Supplementary Material

Effect of polyethylene microplastics on activated sludge process - accumulation in the sludge and influence on the process and on biomass characteristics

Autores – C.Bretas Alvim[1], S. Castelluccio[1], E. Ferrer-Polonio[1], M.A Bes-Piá[1], J.A. Mendoza-Roca[1], J. Fernández-Navarro[2], J.L. Alonso[2], I. Amorós[2]
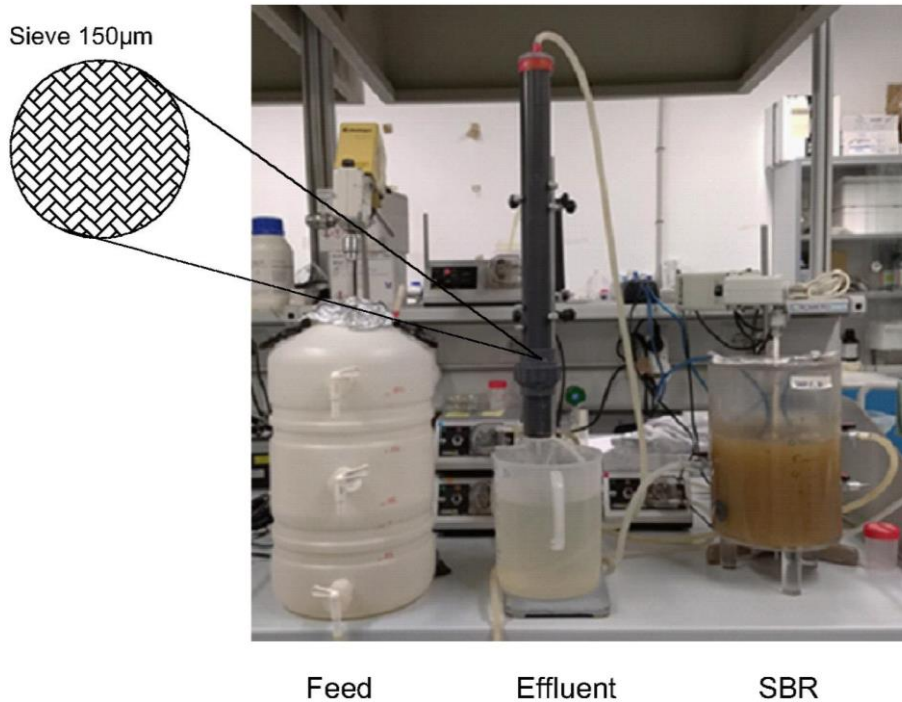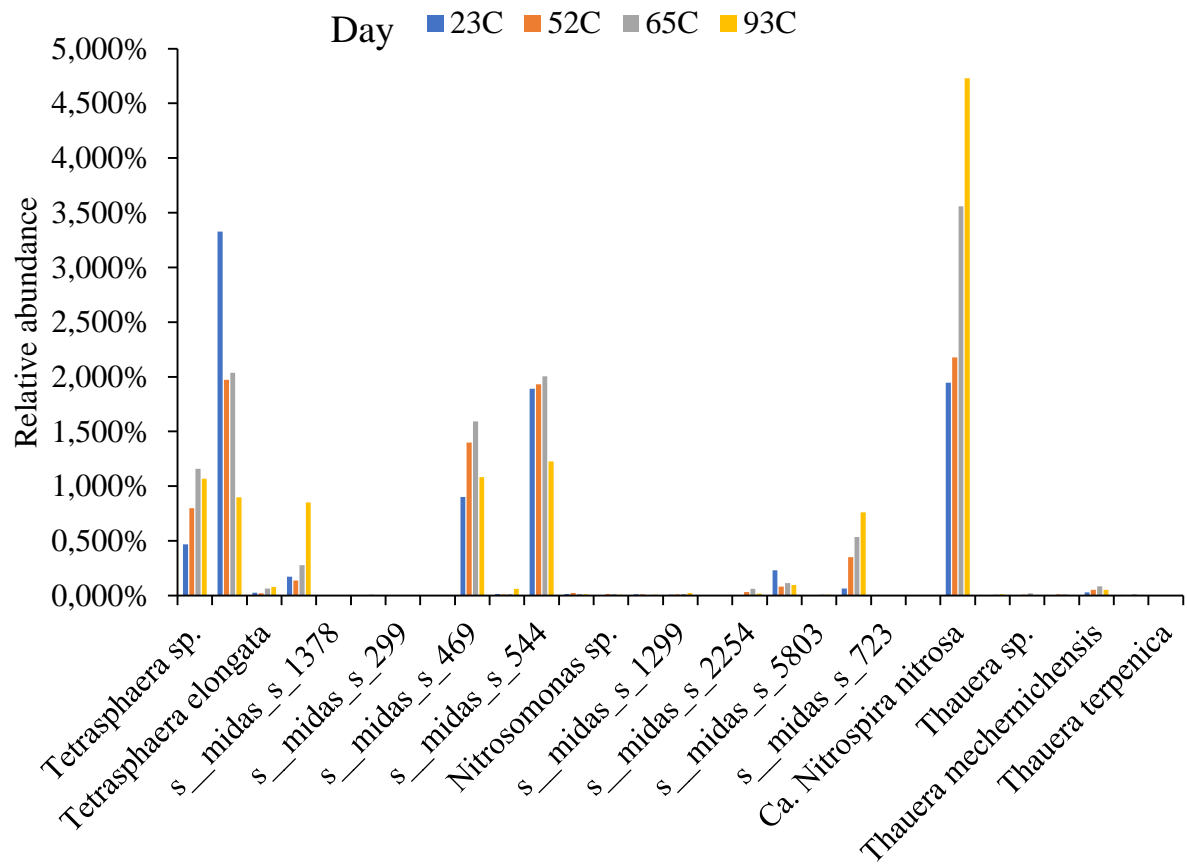
Fig.S1 – Filtration system coupled to the SBR

Fig. S2a- Relative abundance of *Tetrasphaera*, *Nitrosomonas*, *Nitrospira* and *Thauera* species in SBR-Control during the studied period
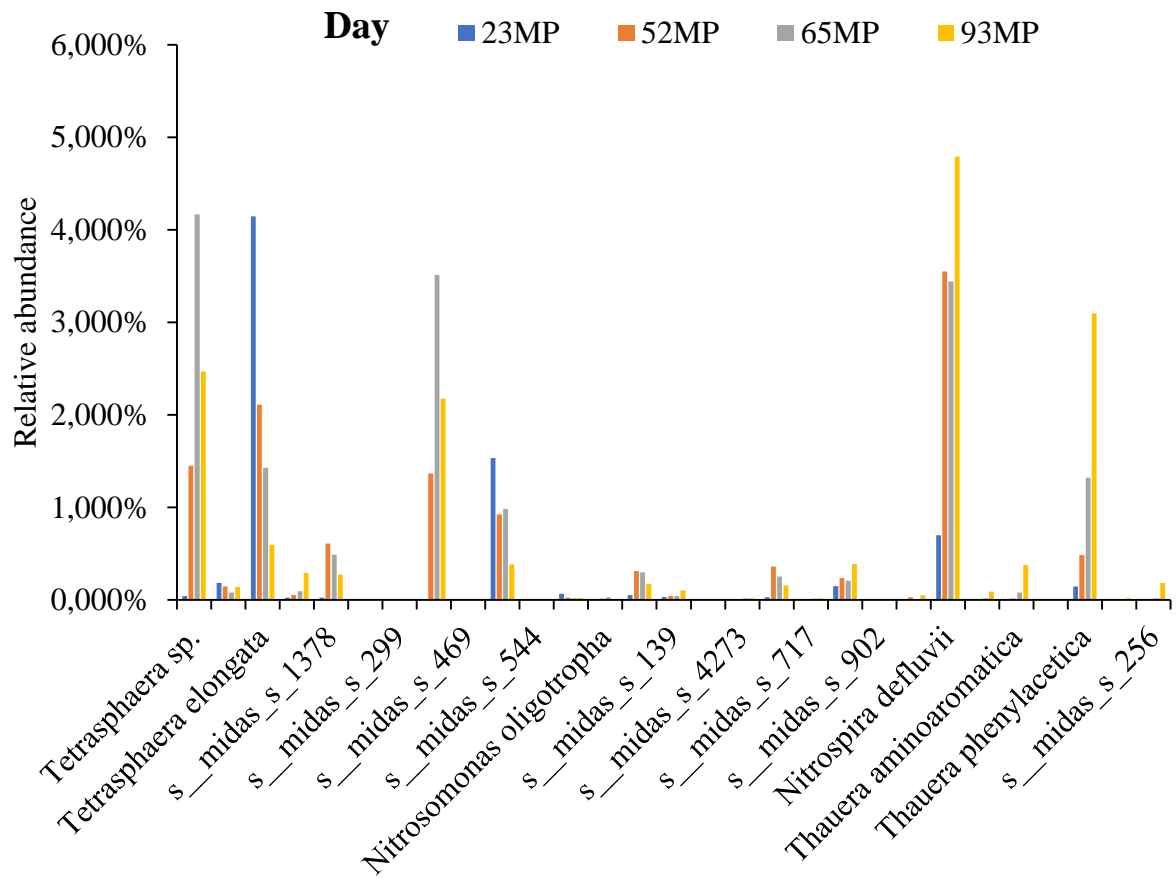
Fig.S2b - Relative abundance of *Tetrasphaera*, *Nitrosomonas*, *Nitrospira* and *Thauera* species in SBR-MP during the studied period
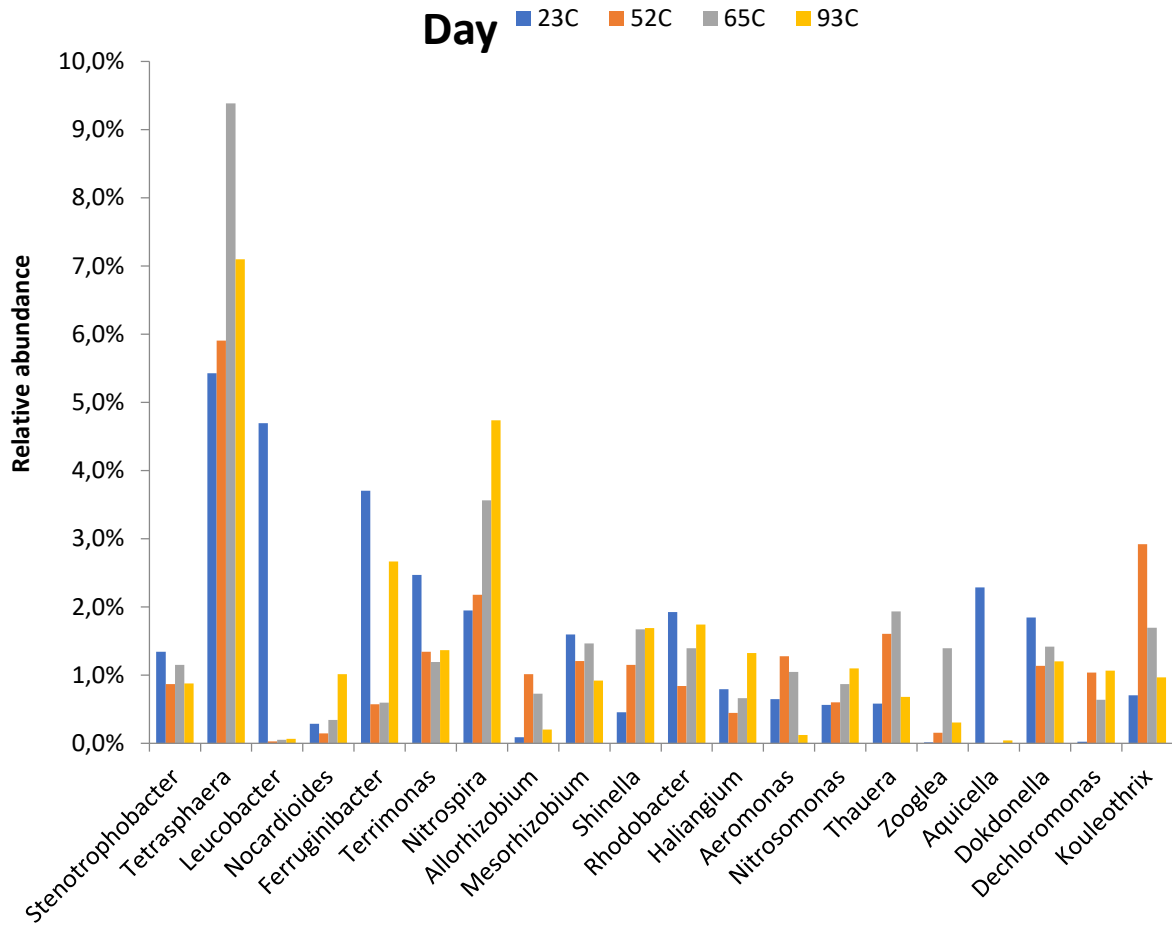
Fig.S3a - Relative abundance of the most abundant genera (>1%) in at least one of sludge samples) in SBR-Control during the studied period

Fig.S3b - Relative abundance of the most abundant genera (>1%) in at least one of sludge samples) in SBR-MP during the studied period

Capítulo V

Do polystyrene nanoplastics affect the activated sludge process? A laboratory study with a sequencing batch reactor

Autores – C.Bretas Alvim[1], E. Ferrer-Polonio[1], M.A Bes-Piá[1], J.A. Mendoza-Roca[1], J. Fernández-Navarro[2], J.L. Alonso[2], I. Amorós[2]

[1]Instituto de Seguridad Industrial, Radiofísica y Medioambiental, Universitat Politècnica de València, Camino de Vera, s/n, Valencia 46022, Spain

[2] Instituto de Ingeniería del Agua y Medio Ambiente, Universitat Politècnica de Valencia, Valencia 46022, Spain

Abstract

The fate and presence of nanoplastics in wastewater treatment systems is a topic of increasing interest. Furthermore, challenges related to their quantification and identification have made it difficult to set up experimental conditions and compare results between studies. Herein, the effect of 100 nm polystyrene nanoplastics on activated sludge was evaluated. For that, a concentration of 2 µg/L was used to continuously feed a sequencing batch reactor (SBR-NPs). Under the experimental conditions used in this study, no changes were observed in the process performance of the SBR-NPs compared to the reactor used as a control. Neither nitrification nor organic matter removal was affected by the presence of 100 nm polystyrene nanoplastics, which suggested that the polymer type, size, and concentration of nanoplastics were not sufficiently toxic to the biomass. Although no significant differences in the relative abundances of predominant phyla between SBR-Control and SBR-NPs were observed, a slight shift in the relative abundance of *Patescibacteria* occurred. The higher abundance of this phylum in SBR-NPs compared to SBR-Control may suggest that these bacteria have some sensibility to the presence of 100 nm PS-NPs. Furthermore, even with the absence of nitrification inhibition, was observed stagnation of the growth of *Nitrotoga* bacteria in SBR-NPs, which also suggests that the PS-NPs could have an inhibitory effect on these cells and an impact on nitrification in the long term.

1. Introduction

Nanoparticles are defined as particles with at least one dimension with a size between 1 to 100 nm (or even between 1 nm to 1000 nm, according to some authors) (Borm et al., 2006; Gigault et al., 2018; Zhang et al., 2009). Nanoplastics (NPs) are plastic-based nanoparticles formed from the fragmentation of larger plastics or man-made plastics in nano dimensions. Due to their nano dimensions, more atoms are found on nanoparticle's surfaces, which make these materials more reactive and prone to aggregate on microbial surfaces and to interact with bacterial cells (Borm et al., 2006; Morones et al., 2005). The mechanism related to the microorganism's interaction with nanoparticles involves several parameters (e.g., ionic strength of the bulk solution, zeta potential, and bacterial cell properties) that the degree of their interaction is very difficult to predict (Borm et al., 2006; Lee et al., 2022, Li et al., 2020).

Activated sludge systems are complex microbial communities, responsible for the removal of organic pollutants in wastewater treatment plants (WWTPs). In these systems, the nitrification process and the removal of organic biodegradable compounds occur by metabolic routes which can be inhibited by the presence of foreign compounds (e.g., NPs). Unfortunately, there is a gap in methodologies regarding the identification and quantification of NPs in WWTPs and determining the fate of NPs in activated sludge is unfeasible nowadays. To overcome this lack in protocols and to provide information regarding the fate of NPs in sewage treatments, Mitrano et al. (2019) synthesized metal-doped-NPs, which made achievable the track of NPs in a batch study simulating the secondary treatment of a WWTP. These authors stated that more than 99% of NPs were retained in the activated sludge and the remaining fraction was in the final effluent. In another research, Li et al. (2020) studied the fate of white PS-NPs in activated sludge and observed that the color of the biomass changed from dark brown to light yellow. This observation was related to the adsorption of NPs on the biomass. Moreover, it was also reported that after 10 h, the mixed liquor suspended solids (MLSS) concentration increased from 7.96 g/L to 10.92 g/L, and this increase was attributed to the retention of NPs in the activated sludge. The authors also observed an increment of the average particle size of the sludge (6.5 %, 7.2 %, 9.4 %, and 11.2% compared with the control) with the increase of PS-NPs concentration (0.1 g/L, 0.5 g/L,

1 g/L, and 5 g/L). These results were also justified by the large number of NPs retained in the sludge.

After adsorption of nanoparticles on microorganisms' cell surface, nanoparticles (e.g., NPs) can be uptake into the cells through diffusion or facilitated transport. Transport of nanoparticles into the cells depends on the size of the nanoparticles and some studies have reported that nanoparticles smaller than 10 nm can enter bacterial cells and damage cellular constituents (Choi and Hu., 2008; Kiser et al., 2010; Morones et al., 2005). In this context, Lee et al. (2022) evaluated the effects of 50 nm and 500 nm PS on nitrification and stated that the impact of PS on the nitrifying process depends on polymer size and exposure time. It was shown that 50 nm PS-NPs interacted easier with nitrifying bacteria than 500 nm PS, and an inhibition of nitrite utilization rate by exposure to 50 nm PS-NPs was also stated by the authors. Nevertheless, since the presence of PS-NPs inside bacterial cells was not observed, the impact of 50 nm PS-NPs on nitrification would be related to physical damage of bacterial membrane and not to the uptake of PS-NPs by microorganisms.

The primary objective of this study was to investigate the influence of 100 nm PS-NPs on microbial community composition and activity of activated sludge and the quality of the final effluent. For that, a sequencing batch reactor was continuously fed with 2 μg/L of NPs and inorganic nitrogen content and chemical oxygen demand removal were monitored to assess eventual inhibition on biomass leading to a decrease of the wastewater depuration performance. The shift of the microbial community in the presence of NPs has also been assessed. Finally, a discussion regarding the parameters that may directly affect the interaction between NPs and bacterial cells is proposed to comprehend the results obtained in this study and in previous investigations.

2. Materials and methods

2.1. Polystyrene nanoplastics

The polystyrene nanoplastics with nominal diameters of 100 nm (10% in water) used in this study were purchased from Sigma-Aldrich. A 10 mg/L stock solution was prepared by dilution with deionized water and stored at 4°C. The hydrodynamic diameter and zeta potential of PS-NPs in deionized water were measured three times

using a particle size analyzer (Nano ZS Malvern Instruments, USA) at 25 ℃. Field Emission Scanning Electron Microscopy (FESEM) (Zeiss) was used to characterize the morphology of PS-NPs and to investigate the adsorption of NP on activated sludge and for that, a small aliquot of mixed liquor (around 25 mL) was allowed to settle for 2 hours, and the solid settled phase was collected for analysis. Samples were imaged without coating under 1 kV EHT with a working distance of 3.0 mm, and an SE2 detector was used to display the image.

The number of NPs in the stock solutions was estimated considering their hydrodynamic diameter and the density of the PS (1.05 g/cm^3). For that, initially, the volume of one PS-NP was calculated considering the hydrodynamic diameter (Eq.1). With the volume and density of PS, the mass of one PS-NP was determined by Eq.2. Finally, considering a mass of 10 mg of PS-NPs in the stock solution, the number of microbeads related to this concentration was achieved.

$$Volume\ NP\ = \frac{4}{3} \cdot \pi \cdot rNP^3 \qquad\qquad\qquad (1)$$

$$Mass\ NP = Volume\ NP \cdot \rho PS \qquad\qquad\qquad (2)$$

Some authors justify the use of high concentrations of PS-NPs (such as 100 mg/L and 300 mg/L) because these conditions seem to be high enough to assess the toxic or inhibitory effect of NPs on biomass (Lee et al., 2022; Qian et al., 2021). Although high concentrations can be used to investigate the risk related to the presence of NPs in activated sludge, they may not be realistic conditions. Therefore, to try to simulate a realistic environment, the concentration of NPs used in this study was stipulated according to the previous research performed by Enfrin et al. (2020). These authors suggested that the concentration of NPs can be 10 to 10^{14} times higher than the concentration of MPs found in WWTPs due to the fragmentation of MPs into small pieces. In this way, it was considered that the concentration of NPs in primary effluent was 10^{10} higher (0.4 x 10^{10} PS-NPs/mL; around 2 µg/L) than MPs' concentration. This was based on the above-mentioned consideration and on previous results regarding the concentration of MPs in primary effluent in the WWTP where the activated sludge was collected (0.4 MPs/L fragment shape) (Bretas Alvim et al., 2020),

2.2. Sequencing Batch Reactor (SBR) operation

The activated sludge used in this study was collected from the aeration basin of a WWTP located in *Comunitat Valenciana* (Spain). To evaluate the influence of PS-NPs on the activated sludge process, a sequencing batch reactor (named SBR-NPs) was continuously fed with simulated urban wastewater and 2 μg/L of PS-NPs. The performance of SBR-NPs was compared to a control reactor (SBR-Control), which was fed with the same simulated urban wastewater but without NPs addition. Both reactors had a working volume of 6 L and were operated for 63 days. The initial 7 days corresponded to the acclimation period before NPs addition.

The synthetic wastewater was prepared with peptone, meat extract and K_2HPO_4 (supplied by Panreac) diluted in tap water, in proportions that guaranteed a feed of 750 mg/L of COD and nutrients (nitrogen and phosphorus) following the proportion of 100:5:1 (COD: N:P). Periodic sludge withdrawals were necessary to keep the MLSS concentration around 2.5 g/L. The oxygen was supplied by air diffusers positioned at the bottom of the tanks to provide a concentration of dissolved oxygen (DO) of around 3 mg O_2/L. The reactors were operated under three cycles per day. Each cycle included 6 hours of filling and aerobic reaction, 90 min of sedimentation, and 30 min of the draw and idle.

2.3. Analysis

2.3.1. Microbial community composition and depuration performance

SBRs effluents were characterized in terms of turbidity, conductivity, and soluble chemical oxygen demand (COD) three times a week. Ammonia (NH_4-N), nitrite (NO_2-N), and nitrate (NO_3-N) were measured once a week. The sludge was monitored in terms of MLSS, temperature, and pH three times a week, meanwhile, mixed liquor volatile suspended solids were measured once a week. The microbial community analysis was investigated through the extraction of DNA from SBRs using a FastDNA® SPIN kit for soil (MP Biomedicals, OH, USA) and the samples DNA samples were sent to Fundación FISABIO sequencing service (Valencia, Spain) for V3-V4 16S rRNA gene amplification using the primers PRO341 Fand PRO805R. The subsequent amplicon sequencing on the Illumina Miseq platform was also performed by Fundación

FISABIO using a 2×300 nucleotide paired-end reads protocol as previously described (Bretas Alvim et al., 2021; Luján-Facundo et al., 2018). Statistical analysis was determined by one-way analysis of variance (ANOVA).

2.3.2. Microbial hydrolytic enzymatic activities (MHEA) and Adenosine triphosphate (ATP) analysis

The microbial hydrolytic enzymatic activities (Lipase, Acid phosphatase, Alkaline phosphatase, α-D-Glucosidase, Dehydrogenase, and Protease) were determined in the SBR-NPs and SBR-Control to assess the influence of NPs in biomass activity (Table 1). Activated sludge samples were collected every two weeks and processed for the determination of MHEAs. The mixed liquor was previously centrifuged, and the pellet obtained was resuspended in Tris-Hcl buffer (0.2 mol·L^{-1}, pH = 7.6) to eliminate possible chemical interferences. Afterward, the activated sludge was put in contact with the reaction substrates at concentrations of 0.1% (w/v) for phosphatases, lipase, and α-D-glucosidase, 0.3% for dehydrogenase and 0.5% for protease enzymatic activity following the basic procedures of Goel et al. (1998) and Gessesse et al. (2003) with some modifications described in Ferrer-Polonio et al. (2017) and Ferrer-Polonio et al. (2019). A Thermo Scientific ™ 9423UVG1002E spectrophotometer was used to measure the absorbance values of reaction products. All the reaction substrates were from Sigma-Aldrich.

Table 1 – Experimental conditions for analysis of microbial hydrolytic enzymatic activities

MHEA	Substrate	Reaction product	Maximum wavelength absorbance (nm)	Units
Acid phosphatase	4-Nitrophenyl phosphate bis(tris) salt	p-Nitrophenol	410	$\mu mol\ pNP \cdot h^{-1} \cdot g\ VSS^{-1}$
Alkaline phosphatase	4-Nitrophenyl phosphate bis(tris) salt	p-Nitrophenol	410	$\mu mol\ pNP \cdot h^{-1} \cdot g\ VSS^{-1}$
Lipase	4-Nitrophenyl palmitate	p-Nitrophenol	410	$\mu mol\ pNP \cdot h^{-1} \cdot g\ VSS^{-1}$
α-D-glucosidase	4-Nitrophenyl a-D-glucopyranoside	p-Nitrophenol	410	$\mu mol\ pNP \cdot h^{-1} \cdot g\ VSS^{-1}$
Dehydrogenase	Iodonitrotetrazolium chloride	1,3,5-Triphenyltetrazolium formazan	490	$\mu mol\ formazan \cdot h^{-1} \cdot g\ VSS^{-1}$
Protease	Azocasein	Colored Unknown peptides	340	$\Delta Abs \cdot h^{-1} \cdot g\ VSS^{-1}$

Adenosine triphosphate (ATP) was analyzed every two weeks according to the method proposed by Ferrer-Polonio et al. (2019). The cellular ATP (cATP) can be used to estimate the amount of living and active microorganisms in activated sludge. Thus, it can be an indicator of biomass resistance to foreign compounds (Whalen et al., 2014).

3. Results
3.1. Characterization of PS-NPs

The hydrodynamic diameter obtained from DLS and the zeta potential of PS-NPs in deionized water (10 mg/L) were 109.4 ± 0.9 nm and -7.4 ± 0.7 mV, respectively. The hydrodynamic diameter matched the nominal size of NPs informed by the provider (100 nm), which indicates that NPs did not aggregate in the stock solution. The zeta potential of activated sludge before the addition of PS-NPs was also measured and it was -10.6 ± 1.3 mV.

3.2. Microbial community composition and depuration performance

The analyses of nitrogen revealed that the presence of PS-NPs did not inhibit either ammonia oxidation or nitrite oxidation, since the highest fraction of total nitrogen was NO_3-N (15.1 ± 4.5 and 8.8 ± 2.0, in SBR-NPs and SBR-Control, respectively), and lower concentrations of NH_4-N (4.0 ± 0.0 and 4.5 ± 1.3, in SBR-NPs and SBR-Control, respectively) and NO_2-N (0.7 ± 0.8 and 0.3 ± 0.4, in SBR-NPs and SBR-Control, respectively) were quantified. A slightly better nitrification performance was observed in SBR-NPs, compared to SBR-Control. Thus, although it was noticed a lower concentration of nitrite-oxidizing bacteria in SBR-NPs than in SBR-Control, it was not significant enough to reduce nitrification. Besides nitrification, the performance of the SBR-NPs regarding the removal of organic carbon (expressed in terms of COD) was not affected by the presence of the NPs along all the monitored time. The COD removal rate (%) in the SBR-NPs and in the SBR-Control was 96 % $\pm$ 3 and 96 % $\pm$ 2, respectively, with no significant differences (p-Value > 0.8).

Yang et al. (2020) investigated the impact of the presence of PS-NPs (particle size approximately 60 – 70 nm and concentrations of 10 µg/L and 1000 µg/L) on nitrogen removal in a constructed wetland for 180 days and observed high removal efficiencies (between 70 – 75%) from day 1 to 45. However, from day 45 removal efficiencies started to decrease, reaching values between 53 – 68 % from day 45 to 160, and 45 – 50 % from day 160 to 180, which indicates that PS-NPs inhibited the nitrogen removal under the conditions tested. Moreover, regarding the abundance of the genus *Nitrosomonas* and *Nitrospira*, it was reported a decrease by 36.1 – 47.0 % and 15.8 – 56.7 % after exposure to 10 and 1000 µg/L PS-NPs, respectively. The differences between results achieved in this study and the stated by Yang et al. (2020) could be related to the different sizes and concentrations of PS-NPs used. The interaction between NPs and biomass can be influenced by the size of NPs. Therefore, the smaller the size, the higher the interaction with microorganisms may probably be (He et al., 2018; Lee et al., 2022). In addition, it would be also expected that the higher the concentration of NPs, the higher the interaction with bacterial cells. (Li et al., 2020). Yang et al. (2020) used smaller PS-NPs and a high concentration of NPs compared to the one used herein, which could lead to a more intense response of microbial community after contact with NPs.

16S rRNA gene sequencing showed that the predominant phyla in both reactors were *Proteobacteria, Bacteroidetes, Chloroflexi, and Actinobacteria* (Fig. 1), and no significant differences in the relative abundances of predominant phyla were observed between reactors. This result is in accordance with previous studies that also observed the predominance of these phyla in activated sludge (Al Ali et al., 2020; Rehman et al., 2020; Tang et al., 2021). However, minor changes were observed in the relative abundance of *Patescibacteria.* From day 15 – when reactors were in steady-state – to day 63, an average relative abundance of *Patescibacteria* around 1.5 ± 0.6 % and 3.7 ± 0.8 % in SBR-Control and SBR-NPs, respectively, were found. In a recent study, Rüthi et al. (2020) observed that the relative abundance of *Patescibacteria* strongly increased in the 'plastisphere' (microbial community on microplastics' surface) of polylactic acid microplastic (a plastic with high biodegradability) after days of incubation in soil. Sun et al. (2022) also stated that the greater the concentration of PP microplastics in soil, the greater the relative abundance of *Patescibacteria*. In another study, the relative abundance of *Patescibacteria* in an activated sludge system was also enriched by the presence of 40–48 μm PET microplastics (Tang et al., 2021). A direct relation between the abundance of some bacterial communities and the presence of NPs is not straightforward, since the influence of micro and nanoplastic on bacteria may depend on size, shape, concentration, and polymer type. However, since the relative abundance of the phylum *Patescibacteria* has shown shifts in different studies with different polymers, these bacteria could behave as bacterial biomarkers for plastic contamination and further studies are necessary to endorse this hypothesis.
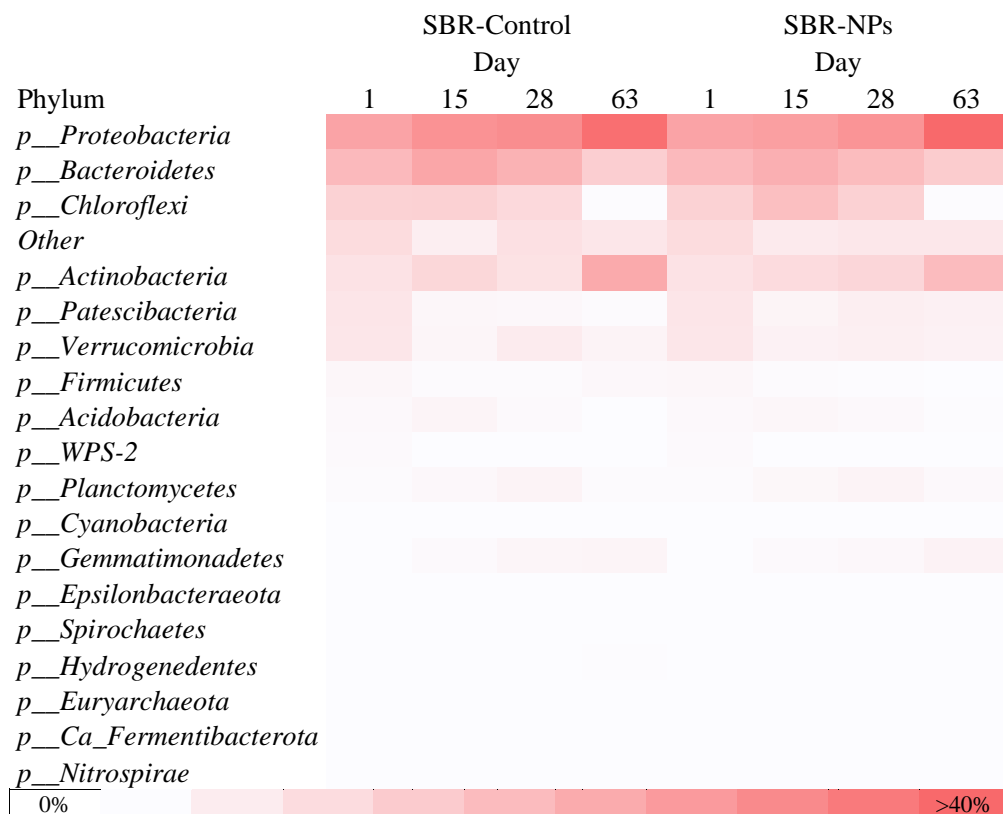
	SBR-Control				SBR-NPs			
	Day				Day			
Phylum	1	15	28	63	1	15	28	63
p__Proteobacteria								
p__Bacteroidetes								
p__Chloroflexi								
Other								
p__Actinobacteria								
p__Patescibacteria								
p__Verrucomicrobia								
p__Firmicutes								
p__Acidobacteria								
p__WPS-2								
p__Planctomycetes								
p__Cyanobacteria								
p__Gemmatimonadetes								
p__Epsilonbacteraeota								
p__Spirochaetes								
p__Hydrogenedentes								
p__Euryarchaeota								
p__Ca_Fermentibacterota								
p__Nitrospirae								

0%　　　　　　　　　　　　　　　　　　>40%

Fig.1. Microbial community composition at phylum level in SBR-Control (a); and in SBR-NPs (b) sampled in the initial (day 1), on day 15, 28 and 63

Ammonia oxidizing bacteria (AOB) (*Nitrosomona*s) and nitrite-oxidizing bacteria (NOB) are classified within the *Proteobacteria* phylum. They are responsible for the nitrification process. *Nitrotoga* is a newly recognized genus belonging to NOB, which may contribute significantly for nitrification, along with the *Nitrospira* genus, from *Nitrospirae* phylum (Lücker et al., 2015; Saunders et al., 2016). AOB and NOB coexist for the success of the nitrification process in activated sludge. Fig.2 illustrates the relative abundance of the bacterial genera involved in nitrification in both SBRs in the analyzed samples. It can be observed that the main difference in both reactors was the relative abundance of the *Nitrotoga* genus. *Nitrotoga* bacteria increased in SBR-Control until reaching the highest relative abundance on day 63 (around 0.9%). It has to be commented that on the first operating day the temperature of the reactors was around 21ºC and under this condition, the genus *Nitrotoga* was practically absent (relative abundance of 0.002%) in both SBRs. On day 15, at a temperature of 18 ºC, the relative abundance of *Nitrotoga* increased to 0.34% and 0.43% in the SBR-Control and SBR-

NPs reactors, respectively. The relative abundance of *Nitrotoga* in the SBR-Control continued to increase in the subsequent days, reaching a maximum value of 0.89% on day 63, when the temperature of the system dropped to 15 ºC. Previous studies observed that *Nitrotoga* bacteria can be a competitive nitrite oxidize in activated sludge at low temperatures (Kim et al.,2021; Liu et al., 2021), therefore the enrichment of this genus with the reduction of temperature was expected. In SBR-NPs the relative abundance of *Nitrotoga* remained practically constant (around 0.4 %) even when the temperature of the reactor dropped. Little is known about the influence of NPs on the *Nitrotoga* community, however, since both reactors were operated under the same conditions, the stagnation of relative abundance of *Nitrotoga* in SBR-NPs could be related to the presence of PS-NPs. Concerning the relative abundance of *Nitrosomonas* (AOB), the reactors showed a similar composition during the study (Fig.2). A reduction of *Nitrosomonas* was observed in both reactors from day 1 to day 15. After that, the relative abundance of *Nitrosomonas* in SBR-Control and in SBR-NPs started to increase until reaching a steady state.
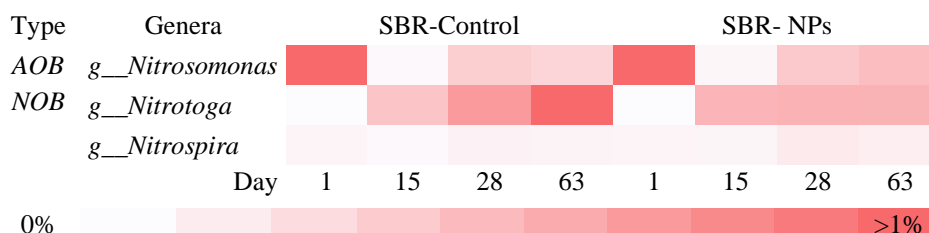
Fig.2. Changes in the relative abundance of ammonia-oxidizing bacteria (*Nitrosomonas*) in and in nitrite-oxidizing bacteria - *Nitrotoga* and *Nitrospira* - in SBR-Control and SBR-NPs

3.3. Effects of PS-NPs in activated sludge activity

The parameter cATP was used as an indicator of sludge activity in SBRs. It was observed that SBR-NPs and SBR-Control showed a similar amount of cATP (Fig.3), which indicates that the presence of 2 µg/L of 100 nm PS-NPs was not sufficient to induce a stress condition in the SBR.
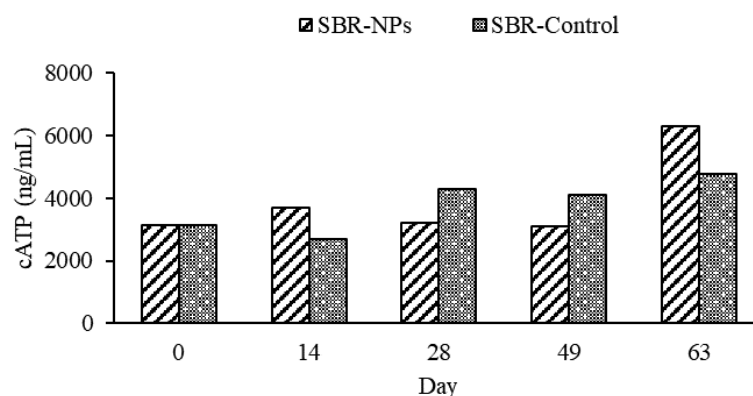
Fig.3. Effects of PS-NPs on the cATP

The results of Protease and Lipase activities suggest that the PS-NPs did not negatively affect the microorganism, since a similar trend in SBR-NPs and in SBR-Control was observed (Fig.4). If the PS-NPs had a negative influence on biomass promoting the cell lysis, an increase in these two activities would be expected. Concerning the phosphatase activities, both acid and alkaline remained very similar in both SBRs; thereby the presence of PS-NPs did not affect the bioavailability of inorganic phosphorus. The α-D-glucosidase enzymatic activity suggests that polystyrene under operating conditions is not degraded by microorganisms. Regarding the dehydrogenase enzymatic activity, the presence of PS-NPs did not influence the oxidative capacity of the microorganisms.

Summarizing, the results obtained from MHEA analysis corroborated that the presence of PS-NPs did not affect the activity of the biomass, which is in accordance with the maintenance of the depuration performance. It suggests that the experimental conditions used (aerobic digestion, concentration of NPs, NPs' size, and polymer type) did not result in global inhibitory effects or dysregulation on biomass.
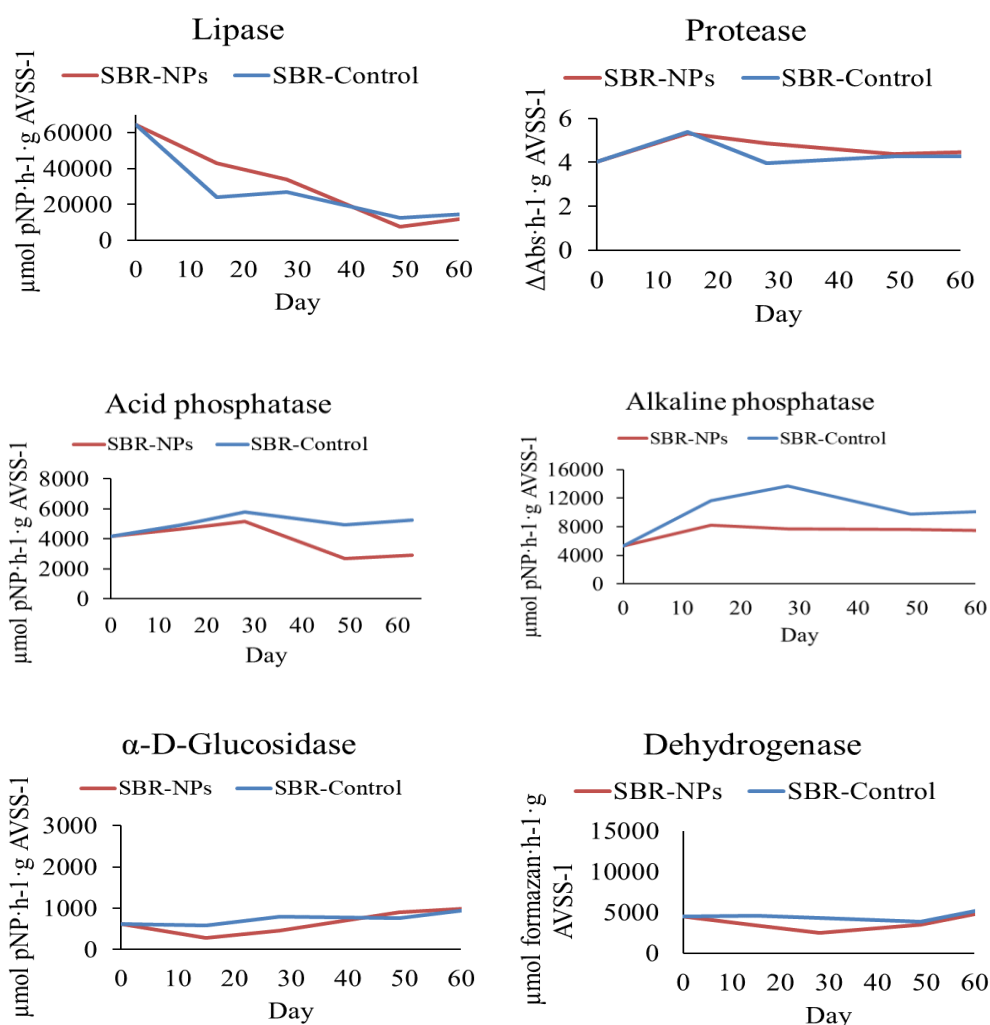
Fig.4. Microbial hydrolytic enzymatic activities in SBR-Control and SBR-NPs during long-term and short-term PS-NPs exposure

3.4. Parameters related to the interaction between NPs and bacterial cell

On the nanometric scale, the interaction between the surface of nanomaterials and their surroundings is an important factor to be considered. Due to the small size of these particles, more atoms are found on their surface, which makes nanomaterials more reactive. Therefore, nanomaterials have high surface energy, which makes them susceptible to create aggregate structures between particles of the same type (homoaggregation) and/or between particles of different types, sizes, and properties (heteroaggregation) (Wang et al., 2015). In the secondary treatment of WWTPs, nanoparticles can interact with microorganisms creating heteroaggregation which

contributes to the removal of nanoparticles from the water stream (Li et al., 2020; Mitrano et al., 2019; Xu et al., 2021). However, the extent of the interaction between nanoparticles and biocolloids (e.g., bacteria, virus, protozoa) will depend on several factors, including particles surface properties, such as zeta potential, and ionic strength of bulk solution (Fig.5) (Ustabasi and Baysal, 2020).
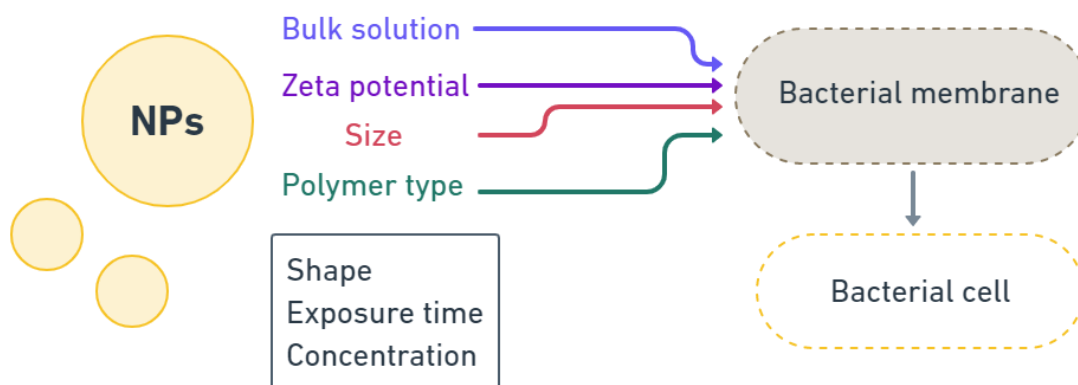
Fig.5. Parameters related to the interaction between NPs and bacterial cell

In this study, images acquired with FESEM showed the formation of agglomerations between PS-NPs and activated sludge. Since the characterization of NPs was not possible, the identification of PS-NPs was based on the shape and size of nanoparticles found in the activated sludge (Fig.6). It can be observed that a moderate heteroaggregation was found in the activated sludge images.

Park et al. (2013) observed that TiO_2 and SiO_2 nanoparticles with -21 mV zeta potential were more quickly removed from activated sludge than Ag nanoparticles with -53 mV zeta potential. This may be due to more electronegative nanoparticles are more stable and less prone to aggregate (Salopek et al., 1992). According to Salopek et al. (1992) from -10 to -15 mV the particles' stability is low enough to start agglomeration. Zeta potential lower than this is prone to induce strong (+5 mV to – 5 mV) and maximal agglomeration (0 mV to + 3 mV). In this study, the low electronegativity of PS-NPs (-7.4 ± 0.7) and activated sludge (-10.6 ± 1.3) could induce the aggregation of NPs on biomass as commented above and observed in Figure 8. Although the aggregation of 100 nm PS-NPs on biomass was observed, the PS-NPs did not behave as a toxic compound decreasing the performance of bacteria during depuration of wastewater

effluent. This may be due to the low or null interaction of PS-NPs with the bacteria membrane since PS-NPs used could not cross the bacterial cell due to their size and the concentration of PS-NPs was not sufficient to induce negative effects on biomass activity.
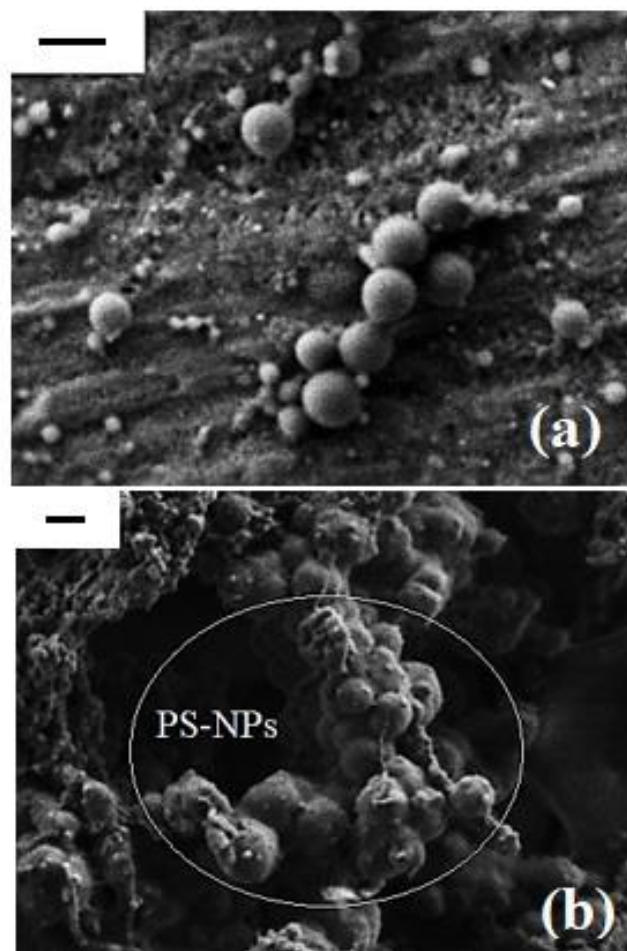

Fig.6 FESEM images of PS-NPs - magnification 100 Kx (scale bar 100 nm) (a); Aggregation of PS-NPs on activated sludge - magnification 39.63 Kx (scale bar 100 nm) (b)

The aggregation of microorganisms on surfaces can be explained by the Derjaguin – Landau – Verwey – Overbeek (DLVO) theory. Briefly, according to DLVO theory, the interaction between microorganisms and substratum will depend on van der Walls' attractive force and the electrical double layer (repulsive force). Therefore, the positive or negative interaction between particles will be a result of the sum of these two forces (Hermansson et al., 1999; Pajerski et al., 2019; Zhang et al., 2009). The ionic

strength of the bulk solution is an important parameter, which can affect the aggregation of the bacterial cell on the nanoparticle's surface. The increase of electrolyte concentration could increase bacterial adhesion on substratum due to the compressing of electrostatic double layer (reducing repulsive forces) (Hermansson et al., 1999; Wu et al., 2019). In activated sludge systems, Kiser et al. (2010) observed that the increase of biomass concentrations led to the raise of ionic strength of the sample. The higher the ionic strength, the greater the percentage of nanoparticles removal, which may be attributed to the compression of the electrical double layer. Chen et al. (2020) also found that an increase in MLSS concentrations improved the removal of AgNPs (10 – 30 nm) from the liquid phase and reached a maximum removal of 98% under 1000 mg/L of MLSS. No significant changes in removal were observed between 1000 – 5000 mg/L MLSS. Since activated sludge systems are usually operated under 2500 – 4500 mg/L MLSS, this condition could be suitable for removing nanoparticles (including nanoplastics) from wastewater and to promote their accumulation in sludge.

Besides the ionic strength of the bulk solution, the zeta potential of NPs and bacteria plays a very important role in the interaction between NPs and bacteria (Pajerski et al., 2019; Ustabasi and Baysal, 2020). Zeta potential corresponds to the surface charge of nanoparticles and biocolloids and it is related to the electrostatic repulsion/attraction between them (Wang et al., 2015). Bacteria surface is generally negatively charged, and the zeta potential value can diversify between bacteria strains (Deschênes and Ells, 2020; Pajerski et al., 2019; Wilson et al., 2001; Xie et al., 2010). Regarding, PS-NPs, different results of zeta potential have been reported and can vary significantly (Feng et al., 2018; Lee et al., 2022). Hence, according to DLVO theory, if bacteria surface and NPs have similar charges, repulsive electrostatic forces may be predominant, which will promote a low interaction between NPs and bacterial membrane. However, if NPs and bacterial membrane have a difference in zeta potential sufficient to provide the interaction between them, NPs can be in contact with bacterial membrane and in the worst case, alter its properties such as permeability and selectivity, which facilitates the entry of NPs after membrane disruption (Neal, 2008).

Another relevant parameter is the size of NPs. Some authors reported that the interaction between NPs and bacteria cells is size-dependent. He et al. (2018) stated that smaller particles (20 nm) were adsorbed on bacterial cell surfaces whereas 2 μm particles were not. Lee et al. (2022) showed that 50 nm PS interacted better with

bacterial cells than 500 nm PS. Besides the interaction of NPs with bacterial membrane, these nanoparticles can also be uptake into the cell. In this case, since the cell wall has small pore sizes (around 10 nm), it is expected that smaller nanoparticles can cross intact bacterial membranes (Deschênes and Ells, 2020; Kiser et al., 2010; Morones et al., 2005; Turner et al., 2013). In addition, other factors, e.g. exposure time, the presence of additives, and shape and concentration of NPs, can also contribute to the toxicity of NPs on microorganisms (Lee et al., 2022; Li et al., 2020; Sun et al., 2022; Ustabasi and Baysal, 2020). Furthermore, the degree of toxicity of NPs to microorganisms may be related to the type of monomer, presence of additives, and polymerization solvents (Lithner et al., 2011).

Conclusions

Particles of 100 nm PS-NPs (2 μg/L) did not represent a toxic compound to the activated sludge process during the experiment (63 days) conducted under the proposed experimental conditions. That means that NPs did not modify the global process, i.e. neither organic matter removal nor nitrification was affected. In accordance with these results, activity sludge activities were also not influenced by the PS-NP. However, some changes in the microbial population were observed, which could imply long-term changes in the process. The lower relative abundance of *Nitrotoga* bacteria in the SBR-NPs compared to SBR-Control suggests that the PS-NPs could inhibit the development of this genus and in the long-term, the nitrification process could be jeopardized. Moreover, a slightly higher relative abundance of *Patescibacteria* was observed on SBR-NPs compared to SBR-Control, which suggests that this phylum may have some sensibility to the presence of 100 nm PS-NPs. As a result, this phylum could be considered a bioindicator for plastic contamination.

The impact of NPs on activated sludge may depend on several factors, such as the size of NPs (the smaller the NPs the higher their interaction with bacteria cells), the polymer type (some monomers are more hazardous than other one), the time of exposure and the concentration of NPs. The lack of a standard method to quantify and identify NPs in activated sludge systems makes the understanding of the impact of these nanoparticles on bacteria cells not obvious. In this way, more research about it will be necessary to go deeper in the complex interactions between NPs and activated sludge microbiota.

Acknowledgments

REFERENCES

Al Ali, A.A., Naddeo, V., Hasan, S.W., Yousef, A.F., 2020. Correlation between bacterial community structure and performance efficiency of a full-scale wastewater treatment plant. J. Water Process Eng. 37, 101472. https://doi.org/10.1016/j.jwpe.2020.101472

Bretas Alvim, C., Bes-Piá, M.A., Mendoza-Roca, J.A., 2020. Separation and identification of microplastics from primary and secondary effluents and activated sludge from wastewater treatment plants. Chem. Eng. J. 402, 126293. https://doi.org/10.1016/j.cej.2020.126293

Bretas Alvim, C., Castelluccio, S., Ferrer-Polonio, E., Bes-Piá, M.A., Mendoza-Roca, J.A., Fernández-Navarro, J., Alonso, J.L., Amorós, I., 2021. Effect of polyethylene microplastics on activated sludge process - Accumulation in the sludge and influence on the process and on biomass characteristics. Process Saf. Environ. Prot. 148, 536–547. https://doi.org/10.1016/j.psep.2020.10.014

Borm, P.J.A., Robbins, D., Haubold, S., Kuhlbusch, T., Fissan, H., Donaldson, K., Schins, R., Stone, V., Kreyling, W., Lademann, J., Krutmann, J., Warheit, D.B., Oberdorster, E., 2006. The potential risks of nanomaterials: A review carried out for ECETOC, Particle and Fibre Toxicology. https://doi.org/10.1186/1743-8977-3-11

Chen, L., Feng, W., Fan, J., Zhang, K., Gu, Z., 2020. Removal of silver nanoparticles in aqueous solution by activated sludge: Mechanism and characteristics. Sci. Total Environ. 711, 135155. https://doi.org/10.1016/j.scitotenv.2019.135155

Choi, O., Hu, Z., 2008. Size dependent and reactive oxygen species related nanosilver toxicity to nitrifying bacteria. Environ. Sci. Technol. 42, 4583–4588. https://doi.org/10.1021/es703238h

Deschênes, L., Ells, T., 2020. Bacteria-nanoparticle interactions in the context of nanofouling. Adv. Colloid Interface Sci. 277, 102106. https://doi.org/10.1016/j.cis.2020.102106

Enfrin, M., Lee, J., Le-Clech, P., Dumée, L.F., 2020. Kinetic and mechanistic aspects of ultrafiltration membrane fouling by nano- and microplastics. J. Memb. Sci. 601. https://doi.org/10.1016/j.memsci.2020.117890

Feng, L.J., Wang, J.J., Liu, S.C., Sun, X.D., Yuan, X.Z., Wang, S.G., 2018. Role of extracellular polymeric substances in the acute inhibition of activated sludge by polystyrene nanoparticles. Environ. Pollut. 238, 859–865. https://doi.org/10.1016/j.envpol.2018.03.101

Ferrer-Polonio, E., Fernández-Navarro, J., Alonso-Molina, J.L., Amorós-Muñoz, I., Bes-Piá, A., Mendoza-Roca, J.A., 2017. Changes in the process performance, sludge production and microbial activity in an activated sludge reactor with addition of a metabolic uncoupler under different operating conditions. Journal of Environmental Management. 203, 349-357. https://doi.org/10.1016/j.jenvman.2017.08.009

Ferrer-Polonio, E., Fernández-Navarro, J., Alonso-Molina, J.L., Mendoza-Roca, J.A., Bes-Piá, A., Amorós, I., 2019. Towards a cleaner wastewater treatment: Influence of folic acid addition on sludge reduction and biomass characteristics. Journal of Cleaner Production. 232, 858-866. https://doi.org/10.1016/j.jclepro.2019.06.021

Gessesse, A., Dueholm, T., Petersen, S.B., Nielsen, P.H., 2003. Lipase and protease extraction from activated sludge. Water Res. 37, 3652-3657. http://dx.doi.org/10.1016/S0043-1354(03)00241-0.

Gigault, J., Halle, A. ter, Baudrimont, M., Pascal, P.-Y., Gauffre, F., Phi, T.-L., El Hadri, H., Grassl, B., Reynaud, S., 2018. Current opinion: What is a nanoplastic? Environ. Pollut. 235, 1030–1034. https://doi.org/10.1016/j.envpol.2018.01.024

Goel, R., Mino, T., Satoh, H., Matsuo, T., 1998. Enzyme activities under anaerobic and aerobic conditions in activated sludge sequencing batch reactor. Water Res. 32, 2081-2088. http://dx.doi.org/10.1016/S0043-1354(97)00425-9.

He, L., Wu, D., Rong, H., Li, M., Tong, M., Kim, H., 2018. Influence of Nano- and Microplastic Particles on the Transport and Deposition Behaviors of Bacteria in

Quartz Sand. Environ. Sci. Technol. 52, 11555–11563. https://doi.org/10.1021/acs.est.8b01673

Hermansson, M., 1999. The DLVO theory in microbial adhesion. Colloids Surfaces B Biointerfaces 14, 105–119. https://doi.org/10.1016/S0927-7765(99)00029-6

Kim, T., Hite, M., Rogacki, L., Sealock, A.W., Sprouse, G., Novak, P.J., LaPara, T.M., 2021. Dissolved oxygen concentrations affect the function but not the relative abundance of nitrifying bacterial populations in full-scale municipal wastewater treatment bioreactors during cold weather. Sci. Total Environ. 781, 146719. https://doi.org/10.1016/j.scitotenv.2021.146719

Kiser, M.A., Ryu, H., Jang, H., Hristovski, K., Westerhoff, P., 2010. Biosorption of nanoparticles to heterotrophic wastewater biomass. Water Res. 44, 4105–4114. https://doi.org/10.1016/j.watres.2010.05.036

Lee, J., Jeong, S., Long, C., Chandran, K., 2022. Size dependent impacts of a model microplastic on nitrification induced by interaction with nitrifying bacteria. J. Hazard. Mater. 424, 127363. https://doi.org/10.1016/j.jhazmat.2021.127363

Lithner, D., Larsson, A., Dave, G., 2011. Environmental and health hazard ranking and assessment of plastic polymers based on chemical composition. Sci. Total Environ. 409, 3309–3324. https://doi.org/10.1016/j.scitotenv.2011.04.038

Li, H., Xu, S., Wang, S., Yang, J., Yan, P., Chen, Y., Guo, J., Fang, F., 2020. New insight into the effect of short-term exposure to polystyrene nanoparticles on activated sludge performance. J. Water Process Eng. 38, 101559. https://doi.org/10.1016/j.jwpe.2020.101559

Liu, Y., Li, S., Ni, G., Duan, H., Huang, X., Yuan, Z., Zheng, M., 2021. Temperature Variations Shape Niche Occupation of Nitrotoga -like Bacteria in Activated Sludge. ACS ES&T Water 1, 167–174. https://doi.org/10.1021/acsestwater.0c00060

Lücker, S., Schwarz, J., Gruber-Dorninger, C., Spieck, E., Wagner, M., Daims, H., 2015. Nitrotoga-like bacteria are previously unrecognized key nitrite oxidizers in full-scale wastewater treatment plants. ISME J. 9, 708–720. https://doi.org/10.1038/ismej.2014.158

Luján-Facundo, M.J., Fernández-Navarro, J., Alonso-Molina, J.L., Amorós-Muñoz, I.,Moreno, Y., Mendoza-Roca, J.A., Pastor-Alcañiz, L., 2018. The role of salinity onthe changes of the biomass characteristics and on the performance of an OMBRtreating tannery wastewater. Water Res. 142, 129–137, http://dx.doi.org/10.1016/j.watres.2018.05.046.

Morones, J.R., Elechiguerra, J.L., Camacho, A., Holt, K., Kouri, J.B., Ramírez, J.T., Yacaman, M.J., 2005. The bactericidal effect of silver nanoparticles. Nanotechnology 16, 2346–2353. https://doi.org/10.1088/0957-4484/16/10/059

Mitrano, D.M., Beltzung, A., Frehland, S., Schmiedgruber, M., Cingolani, A., Schmidt, F., 2019. Synthesis of metal-doped nanoplastics and their utility to investigate fate and behaviour in complex environmental systems. Nat. Nanotechnol. 14, 362–368. https://doi.org/10.1038/s41565-018-0360-3

Neal, A.L., 2008. What can be inferred from bacterium-nanoparticle interactions about the potential consequences of environmental exposure to nanoparticles? Ecotoxicology 17, 362–371. https://doi.org/10.1007/s10646-008-0217-x

Pajerski, W., Ochonska, D., Brzychczy-Wloch, M., Indyka, P., Jarosz, M., Golda-Cepa, M., Sojka, Z., Kotarba, A., 2019. Attachment efficiency of gold nanoparticles by Gram-positive and Gram-negative bacterial strains governed by surface charges. J. Nanoparticle Res. 21. https://doi.org/10.1007/s11051-019-4617-z

Park, H.J., Kim, H.Y., Cha, S., Ahn, C.H., Roh, J., Park, S., Kim, S., Choi, K., Yi, J., Kim, Y., Yoon, J., 2013. Removal characteristics of engineered nanoparticles by activated sludge. Chemosphere 92, 524–528. https://doi.org/10.1016/j.chemosphere.2013.03.020

Qian, J., He, X., Wang, P., Xu, B., Li, K., Lu, B., Jin, W., Tang, S., 2021. Effects of polystyrene nanoplastics on extracellular polymeric substance composition of activated sludge: The role of surface functional groups. Environ. Pollut. 279, 116904. https://doi.org/10.1016/j.envpol.2021.116904

Rehman, Z.U., Fortunato, L., Cheng, T., Leiknes, T., 2020. Metagenomic analysis of sludge and early-stage biofilm communities of a submerged membrane bioreactor. Sci. Total Environ. 701, 134682. https://doi.org/10.1016/j.scitotenv.2019.134682

Rüthi, J., Bölsterli, D., Pardi-Comensoli, L., Brunner, I., Frey, B., 2020. The "Plastisphere" of Biodegradable Plastics Is Characterized by Specific Microbial Taxa of Alpine and Arctic Soils. Front. Environ. Sci. 8. https://doi.org/10.3389/fenvs.2020.562263

Salopek, B., Krasic, D., Filipovic, S., 1992. Measurement and application of zeta-potential. Rud. Zb. 4, 147–151.

Saunders, A.M., Albertsen, M., Vollertsen, J., Nielsen, P.H., 2016. The activated sludge ecosystem contains a core community of abundant organisms. ISME J. 10, 11–20. https://doi.org/10.1038/ismej.2015.117

Sun, Y., Duan, C., Cao, N., Li, Xinfei, Li, Xiaomin, Chen, Y., Huang, Y., Wang, J., 2022. Effects of microplastics on soil microbiome: The impacts of polymer type, shape, and concentration. Sci. Total Environ. 806, 150516. https://doi.org/10.1016/j.scitotenv.2021.150516

Tang, Q., Wu, M., Zhang, Y., Li, J., Liang, J., Zhou, H., Qu, Y., Zhang, X., 2021. Performance and bacterial community profiles of sequencing batch reactors during long-term exposure to polyethylene terephthalate and polyethylene microplastics. Bioresour. Technol. 126393. https://doi.org/10.1016/j.biortech.2021.126393

Turner, R.D., Hurd, A.F., Cadby, A., Hobbs, J.K., Foster, S.J., 2013. Cell wall elongation mode in Gram-negative bacteria is determined by peptidoglycan architecture. Nat. Commun. 4, 1–8. https://doi.org/10.1038/ncomms2503

Ustabasi, G.S., Baysal, A., 2020. Bacterial interactions of microplastics extracted from toothpaste under controlled conditions and the influence of seawater. Sci. Total Environ. 703, 135024. https://doi.org/10.1016/j.scitotenv.2019.135024

Xie, B., Gu, J., Lu, J., 2010. Surface properties of bacteria from activated sludge in relation to bioflocculation. J. Environ. Sci. 22, 1840–1845. https://doi.org/10.1016/S1001-0742(09)60329-6

Wang, H., Adeleye, A.S., Huang, Y., Li, F., Keller, A.A., 2015. Heteroaggregation of nanoparticles with biocolloids and geocolloids. Adv. Colloid Interface Sci. 226, 24–36. https://doi.org/10.1016/j.cis.2015.07.002

Whalen, P.A., Whalen, P.J., Tracey, D.R., 2014. Cellular ATP – A Superior Measure of Active Biomass for Biological Wastewater Treatment Processes. Proc. Water Environ. Fed. 2006, 3025–3037. https://doi.org/10.2175/193864706783751177

Wilson, W.W., Wade, M.M., Holman, S.C., Champlin, F.R., 2001. Status of methods for assessing bacterial cell surface charge properties based on zeta potential measurements. J. Microbiol. Methods 43, 153–164. https://doi.org/10.1016/S0167-7012(00)00224-4

Wu, J., Jiang, R., Lin, W., Ouyang, G., 2019. Effect of salinity and humic acid on the aggregation and toxicity of polystyrene nanoplastics with different functional groups and charges. Environ. Pollut. 245, 836–843. https://doi.org/10.1016/j.envpol.2018.11.055

Xu, J., Wang, X., Zhang, Z., Yan, Z., Zhang, Y., 2021. Effects of chronic exposure to different sizes and polymers of microplastics on the characteristics of activated sludge. Sci. Total Environ. 783, 146954. https://doi.org/10.1016/j.scitotenv.2021.146954

Yang, X., He, Q., Guo, F., Sun, X., Zhang, J., Chen, M., Vymazal, J., Chen, Y., 2020. Nanoplastics Disturb Nitrogen Removal in Constructed Wetlands: Responses of Microbes and Macrophytes. Environ. Sci. Technol. 54, 14007–14016. https://doi.org/10.1021/acs.est.0c03324

Zhang, Y., Chen, Y., Westerhoff, P., Crittenden, J., 2009. Impact of natural organic matter and divalent cations on the stability of aqueous nanoparticles. Water Res. 43, 4249–4257. https://doi.org/10.1016/j.watres.2009.06.005

Capítulo VI

An innovative approach to the application of ultrasounds to remove polyethylene microspheres from activated sludge

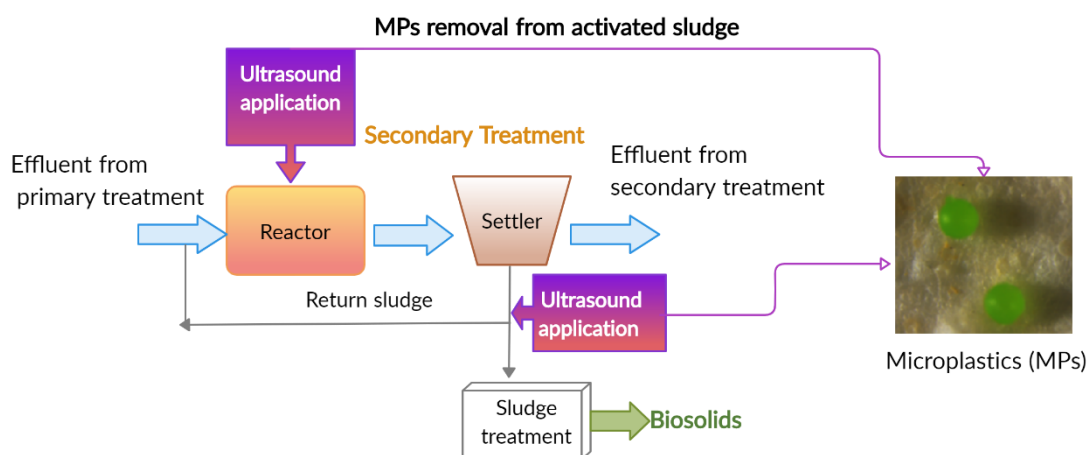Autores – C.Bretas Alvim, M.A Bes-Piá, J.A. Mendoza-Roca

Graphical Abstract

MPs removal from activated sludge

Ultrasound application

Secondary Treatment

Effluent from primary treatment

Reactor

Settler

Effluent from secondary treatment

Return sludge

Ultrasound application

Sludge treatment

Biosolids

Microplastics (MPs)

Abstract

During the secondary treatment of Wastewater Treatment Plants (WWTP) previous studies have reported that microplastics (MPs), which correspond to plastic fragments smaller than 5mm, are mostly retained in the activated sludge. Since the sludge can be applied as a fertilizer in agricultural land, the presence of MPs in this matrix can lead to soil contamination. In this study, the application of ultrasounds at a frequency of 80 kHz was tested to remove polyethylene (PE) microspheres from activated sludge samples in order to reduce their concentration in this organic matrix. The application of ultrasounds at a power of 50 W and 8 min 45 sec promoted the removal of $48 \pm 4\%$ of MPs from the mixed liquor ($2,820 \pm 51$ mg/L of TSS). However, under the same operating conditions, $12 \pm 4\%$ of MPs were recovery from the concentrated mixed liquor ($9,395 \pm 164$ mg/L of TSS). For the concentrated sludge, a higher power and a longer sonication time (80 kHz; 90 W; 30 min) was necessary to improve the MP recovery. Under these conditions $38 \pm 4\%$ of MPs were removed from the sludge. This is the first study applying ultrasounds to remove MPs from activated sludge and the results indicate that ultrasonication can be a suitable instrument in minimizing the concentration of PE microspheres in activated sludge, and thereby minimizing soil contamination when biosolids are applied to agriculture.

1. Introduction

A great interest has been shown in the applications of ultrasounds (US) in wastewater treatment plants (WWTPs) since the beginning of this century. Focusing on municipal wastewater management, the US irradiation has been studied for four main purposes: elimination of compounds that are not biodegraded in the biological treatment, disinfection, reduction of sludge production and enhancement of methane production in anaerobic digesters [1-12]. Cavitation generated by US can concentrate energy in microreactors leading to the removal of a wide range of organic compounds like surfactants [1] and polycyclic aromatic hydrocarbons, among others [2]. The removal mechanism is based on the collapse of the bubbles created by the cavitation phenomenon, which causes changes in the liquid phase leading to oxidation or to breaking of the chemical bonds and pyrolysis [3]. US can be applied to assist other techniques such as electrochemical treatment [4] or oxidation processes with ozone or hydrogen peroxide [5]. US can be also used for water disinfection as reported by [6], also in combination with conventional oxidation techniques like chlorination [7]. In addition, the combination of US and chlorine reduces the concentration of disinfection by-products as trichloromethane and trichloroacetic acid, which are compounds of great concern for the environment [8].

Concerning to the applications focused on the sludge, US has been applied to produce cell lysis in activated sludge reactors. In this way, the sludge production is reduced, with the consequent saving in the sludge management. In this way, Lin et al. [9] combined oxidation with chlorine dioxide and US irradiation to achieve the reduction of the sludge production and Romero-Pareja et al. [10] assisted the OSA (Oxic-settling-anaerobic) process with US for optimize the reduction of the sludge production. Finally, the application of US for the enhancement of methane production is a well-known application that is based on the effect of US on dissolving organic matter of the waste sludge, which means that the hydrolysis phase of the anaerobic digestion is accelerated, and methane production is enhanced [11,12].

The aforementioned applications have been hardly implemented in WWTPs due to the investment and operating costs. However, the increasingly concern about microplastics (MPs) and emerging compounds can modify this scenario. MPs are plastic particles smaller than 5mm, that can be classified as primary (microfibers and those intentionally

added in the formulation of products) and secondary (originated from the fragmentation of larger plastics) [13,14]. Despite the small dimensions of MPs, their numerous harmful effects to living organisms have been reported. For instance, animals are not able to distinguish between a plastic fragment and suitable food, and its ingestion can lead to blockage of the animals' trachea, causing starvation and even death [15]. The harmful effects of MPs on the human body are still unclear. However, due to their hydrophobic surface and high surface area, MPs can act as a vector for large quantities of persistent organic contaminants [16,17,18] which can increase the risk of cancer in humans [18] and can also result in endocrine dysregulation [17].

Previous studies have also reported the high retention of MPs in the activated sludge during the secondary treatment [19,20,21]. During the secondary treatment, the microorganisms can use the MPs' surface as support for their growth and the formation of complex structures of microorganisms on the MPs can lead to the reduction of the MPs' buoyancy and thereby promote their accumulation in the sludge [22-25]. Since this biological material, also named 'biosolid', can be applied as a fertilizer on agricultural land, the retention of MPs in this organic matrix can lead to soil contamination [26,27]. Nizzetto et al. [28] estimated that between 63,000-430,000 and 44,000-300,000 tons per year of MPs are added to farmlands in Europe and North-America, respectively. In Australia it was estimated that 2,800-19,000 tons per year of MPs enter agroecosystems due to the application of biosolids [29]. The impacts of the presence of MPs in soils still have many gaps, but recent research has proved that these small particles can alter the germination and growth processes by preventing the uptake of water by plants due to physical blockage [30]. Furthermore, plants can also uptake these particles [31,32]. A recent study by Oliveri Conti et al. [32] reports the presence of MPs in edible fruits and vegetables. Therefore, MPs can be ingested by humans when contaminated plant matter is consumed. Due to their capacity to adsorb and leach toxic substances, such as Bisphenol A (BPA) and polycyclic aromatic hydrocarbons (PAHs) [18,33], the ingestion of MPs can lead to health issues for humans and animals.

There is still no process to remove the MPs from the activated sludge to minimize terrestrial contamination. Therefore, this study is focused on using the US process to remove PE microspheres retained in the activated sludge flocs. With the use of ultrasounds, the sludge floc could be disrupted, releasing the MPs to the liquid phase.

Once the MPs were displaced to the liquid phase, these particles could be collected and removed from the sludge. As a result, the concentration of MPs in biosolids and the terrestrial contamination would be reduced. Furthermore, to determine the capacity of the US process to remove MPs from sludge with different concentrations of suspended solids, samples from the aeration basin (mixed liquor) and from the secondary settling (concentrated mixed liquor also named secondary sludge), were studied.

2. Materials and methods

2.1. PE Microspheres and mixed liquor

In this study, the mixed liquor was collected from the aeration basin of a WWTP located in Comunitat Valenciana (Spain). The mixed liquor (also named activated sludge) was used in a sequencing batch reactor (SBR), which was monitored for five days. Sludge characteristics were presented in Table 3. The SBR was operated with a hydraulic retention time of 24 h, and 3 cycles per day. In each cycle 2 L of feed entered the system and 2 L of effluent (supernatant) were withdrawn (Table 1). During the test, the SBR was fed with a synthetic feed. The synthetic feed was prepared with peptone, meat extract and K_2HPO_4 (supplied by Panreac) diluted in tap water, in proportions that guaranteed 500 mg/L of COD and nutrients (nitrogen and phosphorous) following the ratio of COD:N:P of 100:5:1, as reported by Ferrer-Polonio et al. [34]. Regarding to the PE microsphere dosage, a concentration of 253MP per liter of feed was prepared. The MPs used in this research was polyethylene microspheres (from COSPHERIC) with a diameter of 150-180 µm. PE microspheres concentration was calculated considering the diameter of the microspheres and the density of the polymer (around 1 g/cm^3). The volume of one sphere was estimated from the diameter value and the mass from the estimated volume and the density. Based on these calculations, the mass required to provide a concentration of 253 MP per liter was added in the feed. This method was already applied in a previous study [24].

Table 1 - SBR operational conditions and parameters

Cycles/day	3
Hydraulic retention time (HRT)	24h
Filling and aerobic reaction	6h
Sedimentation	90min
Draw and idle	30min

To measure the concentration of the MPs in the mixed liquor, 50mL of sample were chemically digested with 100 mL of hydrogen peroxide (35%wt) (AcrosOrganics) for 4 hours at $60 \pm 2°C$. The efficiency of this method was reported in a previous study [35]. After that, the sample was passed through a 150µm aperture sieve, and the retained material was rinsed with distilled water and filtered on glass fiber filter (1 µm). The number of microspheres was counted visually in a stereomicroscope (LEICA MZ APO).

2.2. Mechanism of extraction process via US

When MPs are dispersed in the activated sludge, these particles may have their surface colonized by microbial communities, which increases their density and promotes their settlement in the sludge (Fig. 1.a) [19,23,24]. When the sludge is submitted to US, the cavitation process promotes the rupture of the sludge floc [36], and consequently MPs are released to the liquid phase (supernatant). Therefore, this study aims to evaluate different operational conditions to remove via US the PE microspheres retained in the activated sludge floc. As colloids are generated from the disruption of the sludge floc, they will remain in suspension due to their low density, which increases the supernatant turbidity (Fig. 1.b) [36,37].
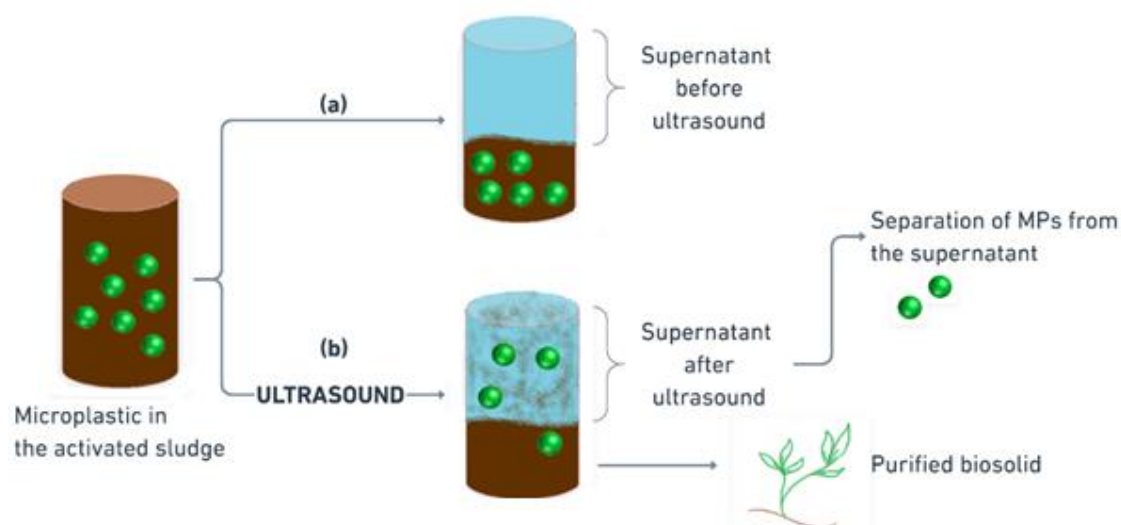
Fig. 1. Extraction of PE microspheres from activated sludge via US

2.3. Mixed liquor – Extraction of MPs by ultrasound

The ultrasound tests for the mixed liquor were performed at a frequency of 80kHz, and varying the power input (30 W, 60 W and 90 W) and sonication time (5, 10 and 15 minutes). For each test, 100mL of sample were collected and poured into a beaker (250mL) for the extraction process, including an initial sample that was not submitted to US. The US tests were operated under sweep mode controlling the temperature of the mixed liquor. Since the cavitation could result in a heat increase, it was key to maintain the temperature at 25 ± 5ºC. At the end of the US, the samples were settled for 90 min and 25 mL of supernatant was extracted and filtered through a 1 μm glass fiber filter. The experiments were replicated to check their reproducibility. The MPs that were attached on the beaker wall were carefully collected using high precision tweezers. The mixed liquor after ultrasonication was centrifuged at 12,000 rpm during 15 min at 4 ºC to extract the soluble microbial products (SMP). The supernatant was filtered through a 0.45 μm cellulose acetate filter for SCOD, SMP (SMPp - proteins and SMPc - carbohydrates) analysis. SMPp were measured by BCA method [38] and SMPc by anthrone method [39]. The SCOD was measured with a kit of the provider Merck Milipore.

1.1.1. Experimental Design

An experimental design was carried out with the software Statgraphics Centurion XVII to predict the optimum point of maximum recovery of MPs and minimum turbidity of the supernatant when the mixed liquor was submitted to US. Specifically, a three-level factorial design with response surface method (RSM) was performed. For this, the independent variables were power input (W) and sonication time (min) (Table 2), whereas the response variables were the percentage of microspheres recovered from the mixed liquor and turbidity. In this work, 18 experimental runs were performed and executed in two blocs. In addition, Statgraphics Centurion XVII was used for data analysis. Thus, based on the results of RSM it was possible to better visualize the behavior of a single variable response under the operational conditions proposed, and the interaction of the variables in the optimum condition to obtain the highest percentual removal of MPs from the mixed liquor. The analysis of variance (ANOVA) was applied in the experimental models to assess if the independent variables had significance, and to determinate the degree of the correlation between the predicted and real responses.

Table 2 – Values of variables for the experimental design.

Independent variables	Coded values		
	-1	0	1
(Factor_A) Time (min)	5	10	15
(Factor_B) Power (W)	30	60	90

2.4. Concentrated mixed liquor

Concentrated mixed liquor (simulating sludge from secondary settling) was achieved concentrating the mixed liquor 3 times to reach a concentration of around 9,000mg/L. US tests for this sample were performed using a frequency of 80 kHz and a power of 90 W. Longer US times, compared to the tests with mixed liquor, were applied (30 min and 60 min). The temperature was also controlled and maintained at 25 ± 5ºC. 100 mL of concentrated mixed liquor were used for each experiment, which were replicated to evaluate the reproducibility of the method.

Unlike the procedure applied for the mixed liquor (Section 2.3), the supernatant of the concentrated mixed liquor was not drained due to the small column of water in this

sample. Therefore, after US, the sample was left to stand for 90 min and then the MPs were carefully removed from the sample surface with high precision tweezers and placed in other beaker with distilled water. This final sample was filtered through a glass fiber filter, similarly to the process adopted in section 2.3.

2.5. Sonochemical effects

The sonochemical effects, which correspond to the production of hydroxyl radicals due to the abrupt collapse of the microbubbles, were determined by KI dosimetry method (Weissler reaction). This method is based on the oxidation of I^- when free radicals are generated during the ultrasonication, and the final product is the ion I_3^-, which can be measured at wavelength of 355 nm. A solution of KI 1% was prepared for the tests. Quantification of sonochemical effects was carry out applying the same conditions used for the activated sludge. 100 mL of KI solution were placed in a 250 mL beaker and exposed to ultrasonication. The power values used were 30 W, 60 W and 90 W. An aliquot of KI solution was collected at the beginning of the test (initial sample) and after 5 min, 10 min, 15 min and 20 min of reaction, and its absorbance was measure at λ=355 nm by DR6000 (Hach) spectrophotometer. As suggested by Wang et al. [40], the concentration of free radicals (HO•) in the system is related to the absorption of the KI solution after US treatment and can be estimated by Eq.1.

$$C(\text{HO} \bullet) = \frac{2A}{\varepsilon b} \times 10^6 \times 2$$

(1)

Where the concentration of hydroxyl radical C(HO•) is obtained in μmol/L, A is the absorption of the KI solution after US, ε is the molar absorptivity of I_3^- (26,303 $dm^3 mol^{-1} cm^{-1}$) and b is the thickness of quartz cuvette (10 mm).

2.6. Particle size distribution of MPs after ultrasonication

Since MPs could be downsized to nanoplastics (NPs) due to water shear forces [41] the particle size distribution of the supernatant was determined by Dynamic Light Scattering (Zetasizer Nano ZS90) to assess the eventual fragmentation of PE microspheres into smaller fragments. The experiments were performed with distilled

water because the high presence of suspended solids from the activated sludge after US could make the analysis unfeasible. After the US, the sample was passed through a 150 µm aperture sieve to remove the initial MPs size. The removal of the initial MPs of 150 µm was required since the equipment identified particles in a range size of 0.3 nm and 5 µm and bigger particles could interfere in the results. Moreover, the analysis of the distilled water blank was also required to minimize the influence of contamination.

3. Results

3.1. Microspheres concentration in the mixed liquor

When the supernatant of the initial mixed liquor (without US application) was collected, only $2 \pm 0.5\%$ of the microspheres were recovered, i.e. $98 \pm 0.5\%$ were accumulated in the sludge (Fig. 2). Due to the high deposition of MPs in the biomass the final concentration of microspheres in the mixed liquor was 1,400 MP/L (Table 3). The concentrated mixed liquor had a TSS concentration of $9,395 \pm 164$mg/L and a concentration of microspheres approximately 3 times higher than the mixed liquor (4,480 MP/L). Likewise, for this sample $2 \pm 0.5\%$ of MPs were recovered from the supernatant before the US.

Table 3 – Sludge samples characterization (mean±S.D)

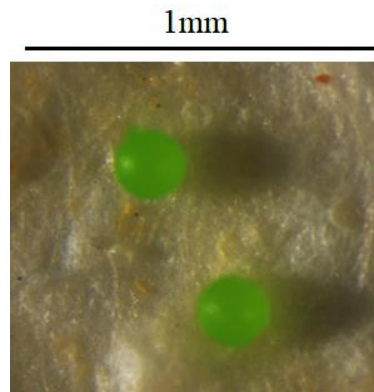| Parameter	Sample	
	Mixed liquor	**Concentrated mixed liquor**
TSS (mg/L)	$2,820 \pm 51$	$9,395 \pm 164$
VSS/TSS	0.86	0.81
MPs/L	1,400	4,480

Fig.2. PE microspheres removed from the activated sludge

3.2. Influence of power and time on MP extraction by ultrasounds

### 3.2.1. Results of the experiment design for mixed liquor

In the Fig.3.a, it was observed that the increase in the power and the exposure time contributed to the increase of the supernatant turbidity, reaching a maximum value of 332 NTU when the sludge was exposed to 90 W and 15 min. The increase of the turbidity indicates that the sludge flocs were disrupted by the hydro-mechanical effects of the US, leading to a higher concentration of suspended solids in the supernatant. The contribution of the power and the sonication time to the disintegration of the sludge floc and therefore the rise of the turbidity, is clearly noted with the surface response (Fig.3.b). The following equation (Eq.2) corresponds to the regression model established according to the results of turbidity response in the experimental design, where A is the time and B the power.

$$Turbidity(NTU) = -34,44 + 1.78A + 1.70B - 0.23A^2 + 0.17AB - 0.00092B^2$$
(Eq.2)

The analysis of variance for the regression model also corroborated that the variables A and B and the interactive model terms (AB) are significant (p-Value < 0.05), at the 95% confidence level, for the increment in the turbidity value. Moreover, a high squared regression coefficient (R^2) and adjusted squared regression coefficient (R^2_{adj}) were

achieved (0.95 and 0.93, respectively), indicating a great correlation between the predicted and real responses.
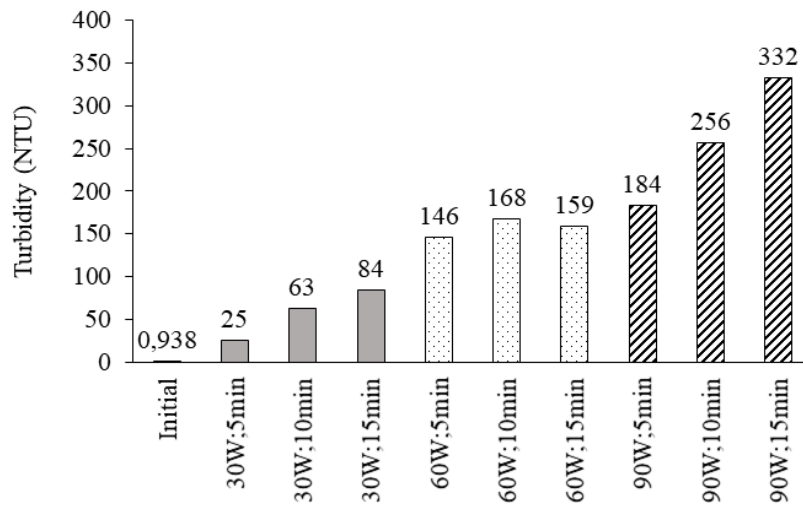

(a)
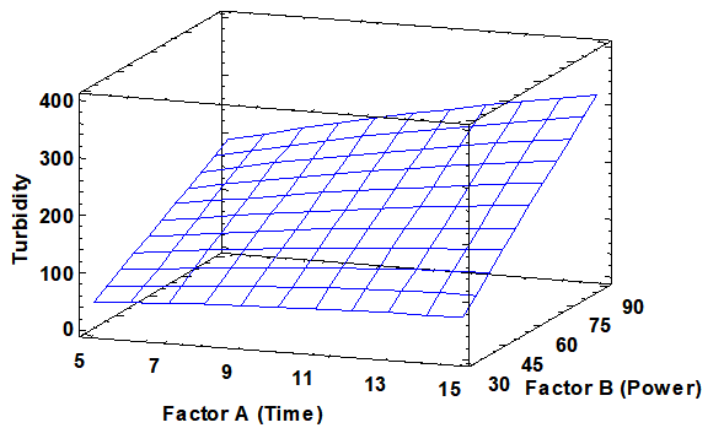
(b)

Fig.3. Turbidity of the supernatant after US tests at different operating conditions (a); Response surface plot for turbidity (b)

The percentage of recovery of MPs as a function of the power and time is represented in Fig.4.a. In general terms, the higher time and power, the more MPs were recovered from the mixed liquor supernatant. For 90 W and 15 min the turbidity was so high that it made impossible the separation of the supernatant and the MPs. Because of that, the

value for this operational condition of MPs recovery was registered as null. Appling 60 W and 15 min, the MPs recovery decreased compared to 10 min, which does not follow the aforementioned trend. In order to better understand the relation between the recovery of PE microspheres and the sludge disintegration, the Fig.4.b shows that the percentage of microspheres recovered from the mixed liquor can be directly related to the degree of sludge floc disintegration (express in terms of turbidity). As the rupture of the sludge is accompanied by increased turbidity in the supernatant, the lower recovery of PE microspheres under 60 W and 15 min compared with the recovery under 60W and 10 min can be justified due to the lower sludge disintegration in the first operational condition (lower turbidity in the supernatant).
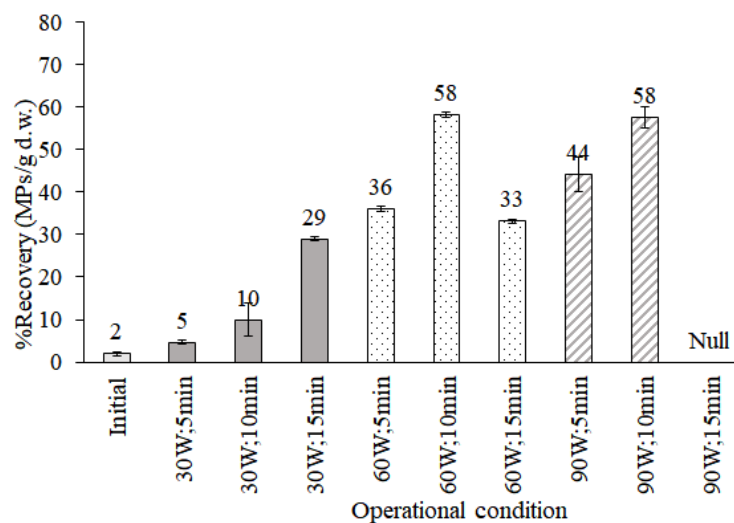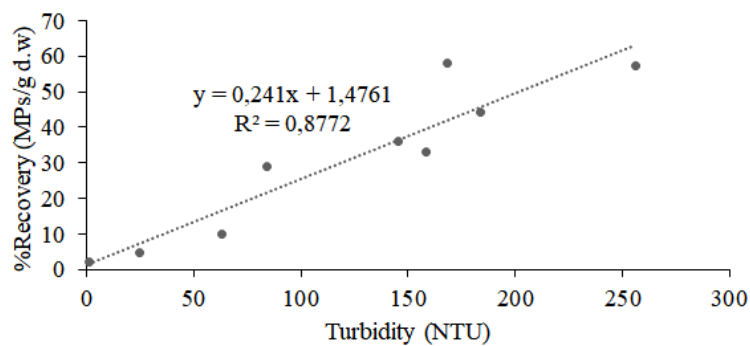
Fig.4. Recovery of MPs after US tests at different operating conditions (a); Recovery of MPs in turbidity function (b)

The following equation (Eq.3) corresponds to the regression model established according to the results of percentual MPs recovery response in the experimental design. The analysis of variance conduced for the regression model, showed that the factor B, the quadratic models (A^2 and B^2), and the interactive model terms (AB), were all significative (p-Value< 0.05), at the 95% confidence level.

$$\%Recovery\ (MP) = -164.22 + 19.35A + 3.82B - 0.66A^2 - 0.11AB - 0.019B^2$$
(Eq.3)

The squared regression coefficient (R^2) and the adjusted squared regression coefficient (R^2_{adj}) (0.82 and 0.73, respectively) indicate that there is a correlation between the predicted and real responses. The recovery of MPs from mixed liquor was influenced by power and time (Fig.5), but the extent of this dependence should be further evaluated in future studies to improve the extraction process and reach a higher correlation between the model and real responses. The contribution of the frequency can also be an important parameter to understand better the role of US in the recovery of MPs from activated sludge.
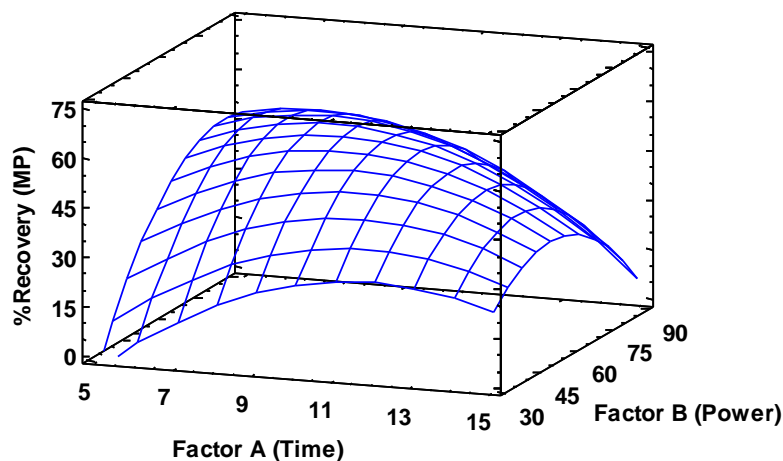

Fig.5. Response surface plot for %Recovery (MP)

3.2.2. Mixed liquor disintegration by ultrasonication

As stated above, the sonication power and time are parameters that played an important role in the degree of sludge disintegration. Thus, the higher power input, the more effective the sound pressure was, and therefore, a greater disintegration of the sludge was obtained. Similarly, with a longer ultrasonication time, the rupture of the sludge floc and cell membranes was more effective. In section 3.2.1 turbidity was reported as a measurement of sludge disintegration. In this section, results are focused on reporting the quality of the supernatant (final effluent from WWTP) in terms of SCOD and of its main components (SMP).

According to the SCOD results (Fig.6.a), it was observed an increment in SCOD value with the increase of the power input. In addition, an increment in SCOD with 10 minutes of ultrasonication, compared to 5min, was also observed for all experiments. The increase in SCOD value is related to the organic matter released into water due to the microorganism's cell lysis. As the microorganism's cells are mostly composed by protein, their lysis result in more soluble microbial proteins (SMPp) released in the water comparing with soluble microbial carbohydrates (SMPc) [42,43]. The increase in SMPp concentration was observed after all the US experiments (Fig.6.b), suggesting that the hydro-mechanical shear forces, during the US, were sufficient to promote the rupture of the sludge floc.
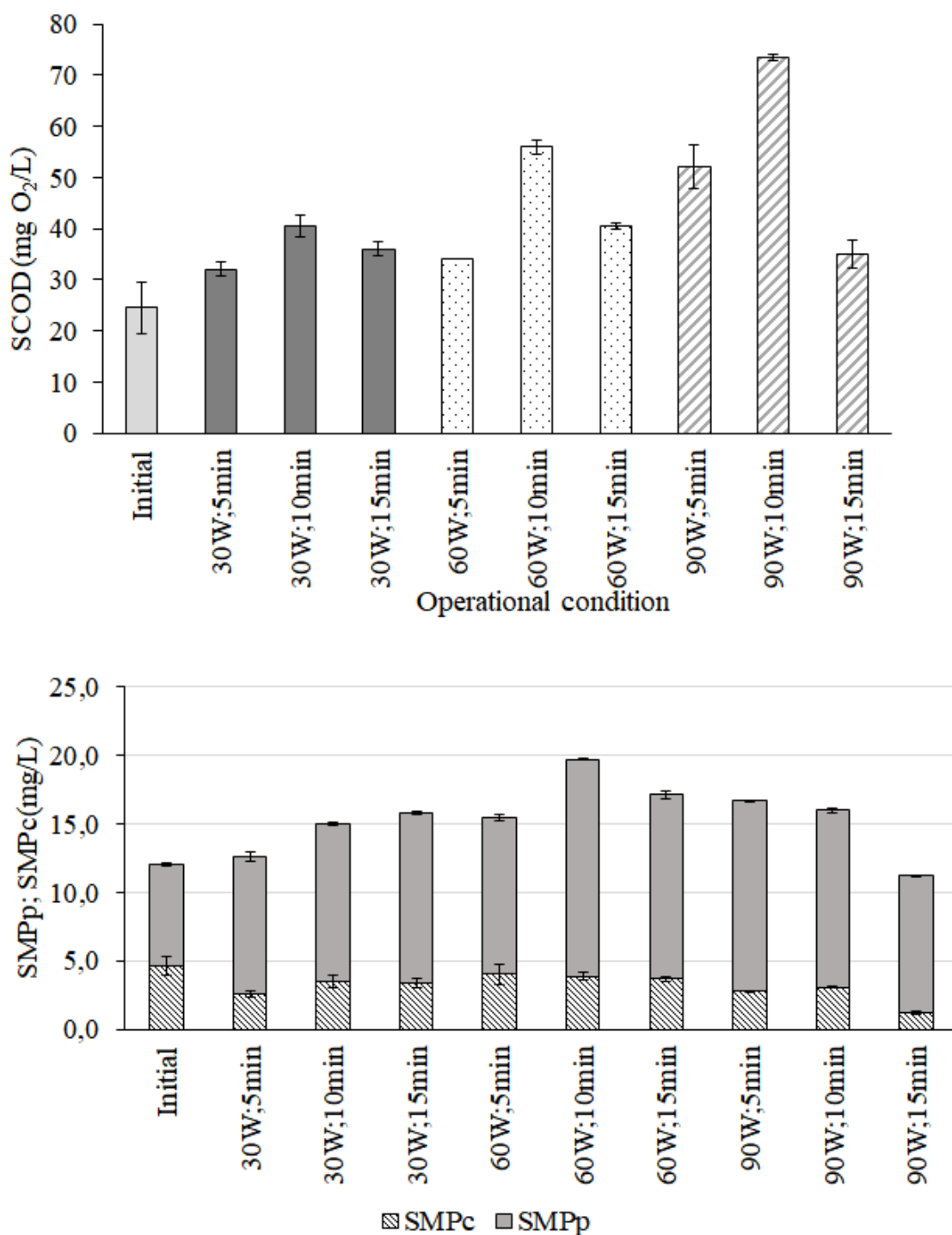
Fig.6. Mixed liquor disintegration in terms of SCOD released concentration (a) and carbohydrates and proteins concentrations (b)

However, for all powers tested it was observed that, when the time was increased from 10 minutes up to 15 minutes, a decrease of SCOD occurred. This reduction can be

justified by the sonochemical effects. In this process, due to the acoustic cavitation, microbubbles are formed and violently collapsed, resulting in high temperature and pressure points. The result of this mechanism is the sonochemical effect, which results in the generation of hydroxyl radicals [44]. Hydroxyl radicals have a high oxidant capacity and react with organic species fast and in a nonspecific way [45]. The reaction of •OH with the extracellular polymeric substances (EPS) - present in the sludge floc - can also promote its rupture and the release of soluble organics as SMP, or even its mineralization into CO_2 and H_2O [42]. When the mixed liquor was submitted to 15 minutes of US at any tested power, the oxidation of soluble organic matter due to the sonochemical effects, was more evident, resulting in a final value of SCOD lower than 10min. Analyzing the SMPc results after the ultrasonication, a reduction in its concentration was observed compared to the initial value. This can be another evidence of the oxidation of soluble organic matter by the generated hydroxyl radicals. Based on these results, it was established a hypothesis that a maximum cell lysis was achieved at 10min. After that, cell lysis still occurred, but the mineralization of SCOD seemed to be more intense. Wang et al. [45] reported that the sludge disintegration during the ultrasonication is performed by both hydro-mechanical shear forces and oxidizing effect. To mask the oxidizing effect of radical •OH, the authors proposed the addition of $NaHCO_3$ in the sludge before the US. Al-Juboori et al. [46] investigated the removal of dissolved organic carbon (DOC) present in Narda lagoon (Autralia) applying a 20khz frequency, power input of 93W and 143W and treatment time of 5 and 15min. The authors used real water, which contains a high concentration of microbes, to assess the influence of the microbe's presence in the ultrasonication process. They concluded that the release of microbial cell lysis products and the reduction of DOC occurred simultaneously when applying US to water with a high microbial load.

With the application of 30W there was a decrease of SCOD in 12% between 10 and 15min. For 60W and 90W a reduction of 28% and 58% were obtained, respectively (Fig.7.a). The explanation of the relation between the reduction of SCOD and the power input is that the power intensity is linked to the size of the cavitation bubbles, the temperature, and the internal pressure of the bubbles during their collapse. Increasing the power intensity results in a greater number of collapsed bubbles, higher temperature and greater sonochemical effects [47]. This may be the reason why the increase in the

power produced a proportional SCOD reduction between 10 and 15min test. The results of the sonochemical effects measured at 5, 10, 15 and 20 min, were plotted in Fig.7(b) in order to assess the hydroxyl radical production in the system. As expected, the sonochemical effects, increased with both exposition time and power applied.
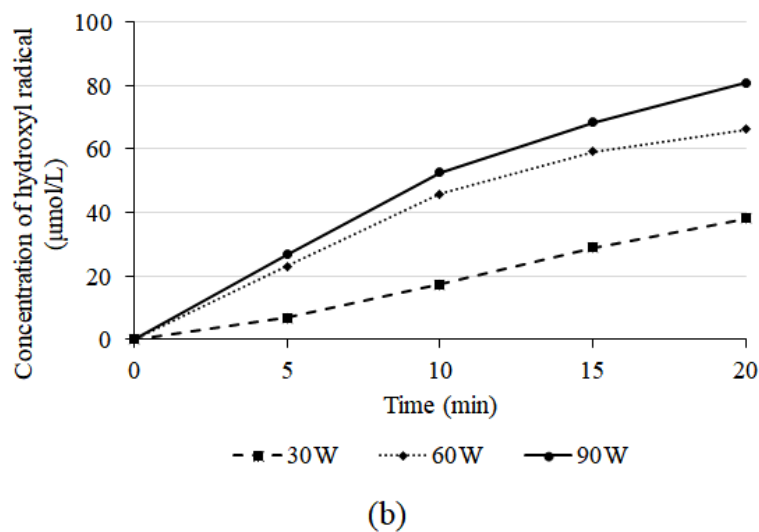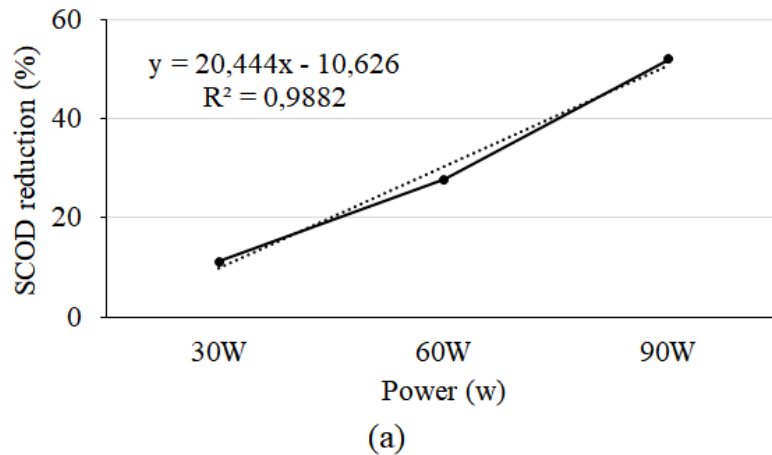

(a)

(b)

Fig.7. SCOD reduction with power at 10min and 15min of US (a); Production of free radicals in function of time and power (b)

3.2.3. Optimized extraction model

From the results obtained in experimental design (section 3.2.1), the optimum point of maximum recovery of MPs and minimum turbidity of the supernatant was predicted. According to the response surface established by the software (Fig.8), a test time of 8 min 45 sec at a power of 54 W was ideal. These operational conditions would

theoretically result in a recovery percentage of 50 MPs and a turbidity of 134 NTU (Table 4). Due to the power adjustment possibilities in the US equipment, it was not possible to apply 54 W. Therefore, 50 W was established as the power for the optimal point test. According to the established model, the expected response for the percentage of MPs recovery was 50% and for the turbidity 136 NTU. When carrying out the extraction process, $48 \pm 4\%$ of MPs were recovered and a turbidity of 179 ± 6 NTU was produced. Moreover, an increase in SCOD was observed (45 ± 3 mgO$_2$/L after US). The results obtained under the optimal condition showed a high correlation between the expected values and the real ones, and therefore, it showed a relevant step in the possible application of US to remove MPs from activated sludge.
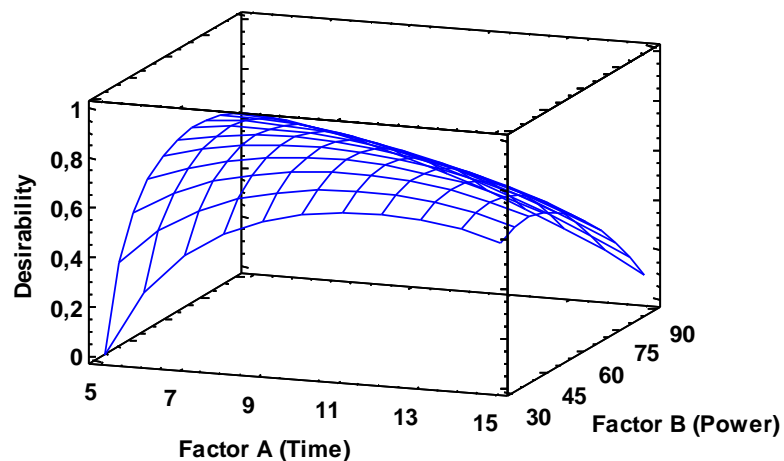

Fig.8. Response surface plot for optimization

Table 4 - Ultrasound optimized operational conditions for mixed liquor.

Factor	Value	Parameter	
Factor_A (min)	8 min 45 sec	%Recovery (MPs)	Maximize
Factor_B (W)	54	Turbidity (NTU)	Minimize
	Predicted response	**Real response**	
%Recovery (MPs)	50	48±4	
Turbidity (NTU)	136	179±6	

3.2.4. Concentrated mixed liquor

The extraction of MPs from WWTP sludge may be influenced by the concentration of total solids (TS) in the sludge. A high concentration of TS can absorb acoustic energy and reduce the efficiency of the sludge floc disintegration [48], and consequently decrease the release of MPs. Applying the optimum point established in section 3.2.3. for the mixed liquor, 48 ± 4% of PE microspheres were recovered by the supernatant. However, when the same experimental conditions were applied to the concentrated mixed liquor, only 12 ± 4% recovery was achieved. The high concentration of TS, compromised the cavitation process, making difficult to detach the MPs retained in the sludge. However, submitting the sludge to a higher power and time (90 W; 30 min) the recovery of MPs reached 38 ± 4% (Table 5).

Table 5 – MPs recovery (%) and SCOD concentrations achieved at different ultrasound operational conditions for concentrated mixed liquor.

Operational condition	%Recovery (MPs)	SCOD (mg/L)
Initial	2 ± 1	39 ± 1
80 kHz; 50 W; 8 min 45 sec	12 ± 4	61 ± 4
80 kHz; 90 W; 30 min	38 ± 4	188 ± 6
80 kHz; 90 W; 60 min	29 ± 1	310 ± 4

On the contrary, upon being subjected to US, the SCOD value increased instead of decreasing due to the sludge fragmentation, that caused a release of more organic products. In addition, a longer US time (comparing the same power of 90 W) resulted in a greater disintegration of the sludge. Although the US process produces free radicals (extremely oxidizing), results suggested that the sludge disintegration process by hydro-mechanical was higher than the sonochemical effects. Tian et al. [49] studied the ultrasonication effects in the activated sludge (16.2–17.2 g/L of TS), under 20 kHz frequency, and they also observed a low influence of free radicals in the ultrasonication process and the predominance of hydro-mechanical shear forces in the sludge disintegration. The results of SMPp and SMPc for concentrated mixed liquor (Fig.9) corroborate that. At the optimum conditions for the mixed liquor (50 W; 8 min 45 sec), the concentrated mixed liquor suffered a low disintegration. However, when 90W;60min was applied, an increment of more than 450% and 390% of SMPp and SMPc were achieved, respectively. According to these results and the ones regarding the recovery of MPs, the degree of MPs recovery seems to be related to the extent of floc disintegration, as it was also concluded in the section 3.2.1 for the mixed liquor. Nevertheless, an intense sludge fragmentation can be negative for the recovery of MPs. After 90 W; 60 min of ultrasonication a reduction in the recovery of MPs compared to 30 min was observed. Our hypothesis is that, under extreme sludge disintegration conditions, the sample are not able to decant, and the consequence is that the sample is more homogeneous with a more intense mixture of sludge and MPs. Therefore, without a separation of water from the sludge, the buoyancy of MPs is seriously compromised.
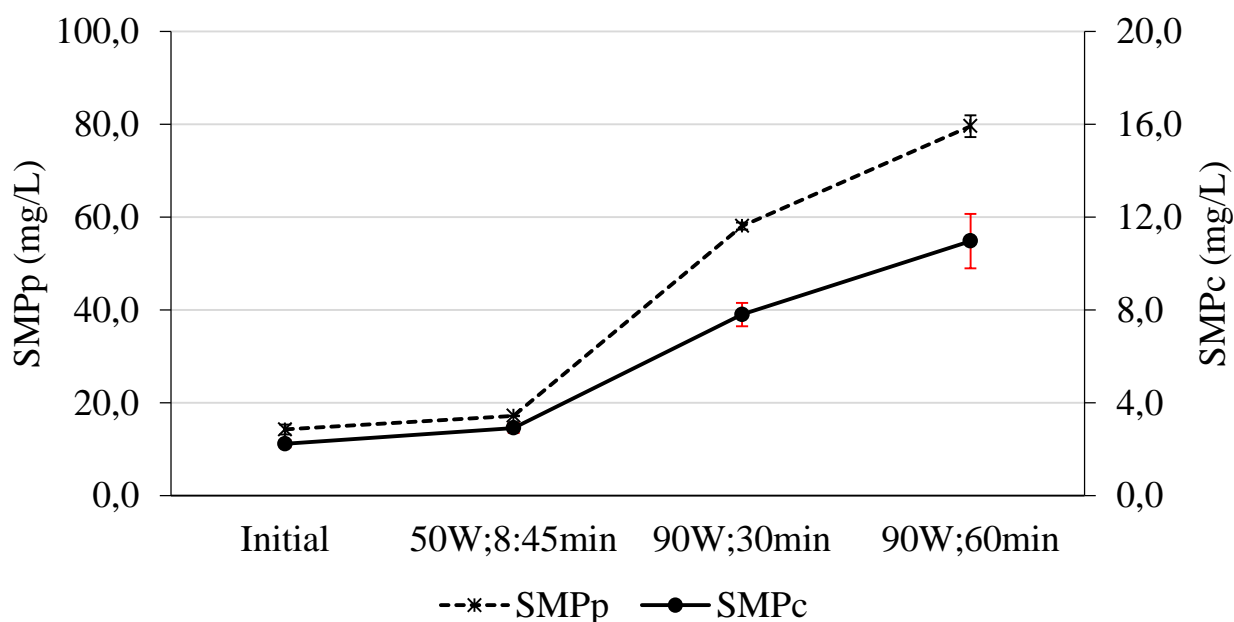
Fig.9. Released SMPp and SMPc after ultrasonication of concentrated mixed liquor

This is the first investigation into how the US can accomplish the extraction of MPs from activated sludge, and therefore the results obtained in this study cannot be compared with previous ones. However, this research provides important information about the viability of treating the activated sludge for the removal PE microspheres. Several microplastics can be found in activated sludge (microfibers, fragments and spheres), of different sizes and compositions, thus further research must be carried out to evaluate the potential for removing these MPs through US.

3.3. Particle size distribution of PE microspheres after ultrasonication

According to the results of the particle size distribution in the supernatant for the optimum point (80 kHz; 50 W; 8 min 45 sec) applied to the mixed liquor, and for the best conditions applied to the concentrated sludge (80 kHz; 90 W; 30 min), the fragmentation of the PE microspheres into smaller fragments was not observed. This result would demonstrate that PE microspheres fragmentation should be caused by factors like biodegradation, UV irradiation, and not just mechanical processes such as US [41].

4. Conclusions

The main conclusion of this work is that ultrasonication can become an excellent tool to assist in the separation of PE microspheres, a type of MPs, from the activated sludge. It was possible to remove $48 \pm 4\%$ of MPs that were retained in the mixed liquor by applying of ultrasonication optimal conditions at a frequency of 80 kHz (power input 50 W and time 8 min 45 sec). After the US, the PE microspheres were transferred to the water column phase due to the disruption of the sludge floc by cavitation process, which allowed its extraction and therefore, the reduction of the concentration of MPs in the sludge. In addition, the quality of the final effluent was not compromised as showed the SCOD and SMP measured values.

Regarding the concentrated mixed liquor, when these optimal operational conditions were applied, only $12 \pm 4\%$ of MPs could be recovered from the activated sludge. The higher total solid concentration compromised the cavitation process, leading to a poor sludge disintegration compared to that of the mixed liquor. Since the sludge was insufficiently disintegrated, only a small amount of MPs were recovered. However, when the concentrated mixed liquor was exposed to a higher power and a longer US time, (80 kHz; 90 W; 30 min) $38 \pm 4\%$ of MPs were detached from the sludge floc and moved to the sample surface. Moreover, the fragmentation of the MPs into NPs after the US for both mixed liquor and concentrated mixed liquor was not observed. Based on these results, the US process has an interesting potential for the extraction of MPs from a biomass matrix. Further studies should be developed to better understand the process of extracting MPs via US and to study the feasibility of its application on an industrial scale. The reduction of the concentration of MPs in the activated sludge deserves attention, since mostly MPs that enter the secondary treatment are retained in the sludge, and this material is commonly applied as fertilizers in agricultural lands leading to their contamination by these synthetic polymers.

REFERENCES

[1]   M.A. Abu-hassan, D. Mantzavinos, I.S. Metcalfe, Wet air oxidation and ultrasound for the removal of linear alkylbenzene sulfonates from wastewater: the beneficial role of catalysis, 33 (2005). https://doi.org/10.1007/s11244-005-2520-6.

[2]   N. Ghasemi, G. Gbeddy, P.Egodawatta, F. Zare, A. Goonetilleke, Removal of polycyclic aromatic hydrocarbons from wastewater using dual-mode ultrasound system, Water and Environment Journal (2017) 1–10. https://doi.org/10.1111/wej.12540.

[3]   P. Chowdhury, T. Viraraghavan, Sonochemical degradation of chlorinated organic compounds, phenolic compounds and organic dyes – A review, Sci. Total Environ. 407 (2009) 2474–2492. https://doi.org/10.1016/j.scitotenv.2008.12.031.

[4]   M. Zhang, Z. Zhang, S. Liu, Y. Peng, J. Chen, S. Yoo Ki, Ultrasound-assisted electrochemical treatment for phenolic wastewater, Ultrason. Sonochem. 65 (2020) 105058. https://doi.org/10.1016/j.ultsonch.2020.105058.

[5]   S. Chandak, P.K. Ghosh, P.R. Gogate, Treatment of real pharmaceutical wastewater using different processes based on ultrasound in combination with oxidants, Process Saf. Environ. Prot. 137 (2020) 149–157. https://doi.org/10.1016/j.psep.2020.02.025.

[6]   M. Vázquez-López, L.E. Amabilis-Sosa, G.E. Moeller-Chávez, A. Roé-Sosa, P. Neumann, G. Vidal, Evaluation of the ultrasound effect on treated municipal wastewater, Environ. Technol. 40 (2019) 3568–3577. https://doi.org/10.1080/09593330.2018.1481889.

[7]   Blume, T., Leis, U. Improving chlorine disinfection of wastewater by ultrasound application. Water science and technology 52 (10-11) (2005), 139-144.

[8]   X. Zhou, J. Zhao, Z. Li, J. Song, X. Li, X. Yang, D. Wang, Enhancement effects of ultrasound on secondary wastewater effluent disinfection by sodium hypochlorite and disinfection by-products analysis, Ultrason. Sonochem. 29 (2016) 60–66. https://doi.org/10.1016/j.ultsonch.2015.09.001.

[9]   J. Lin, Y. Hu, G. Wang, W. Lan, Sludge reduction in an activated sludge sewage treatment process by lysis-cryptic growth using ClO2-ultrasonication disruption, Biochem. Eng. J. 68 (2012) 54–60. https://doi.org/10.1016/j.bej.2012.07

[10]   P.M. Romero-Pareja, C.A. Aragon, J.M. Quiroga, M.D. Coello, Evaluation of a biological wastewater treatment system combining an OSA process with ultrasound for sludge reduction, Ultrason. Sonochem. 36 (2017) 336–342. https://doi.org/10.1016/j.ultsonch.2016.12.006.

[11]   J. Quarmby, J.R. Scott, A.K. Mason, G. Davies, S.A. Parsons, The Application of Ultrasound as a Pre-Treatment for Anaerobic Digestion, Environ. Technol. 20 (1999) 1155–1161. https://doi.org/10.1080/09593332008616913.

[12]   X. Li, S. Guo, Y. Peng, Y. He, S. Wang, L. Li, M. Zhao, Anaerobic digestion using ultrasound as pretreatment approach: Changes in waste activated sludge, anaerobic digestion performances and digestive microbial populations, Biochem. Eng. J. 139 (2018) 139–145. https://doi.org/10.1016/j.bej.2017.11.009.

[13]   H.S. Auta, C.U. Emenike, S.H. Fauziah, Distribution and importance of microplastics in the marine environment A review of the sources, fate, effects, and potential solutions, Environ. Int. 102 (2017) 165–176. https://doi.org/10.1016/j.envint.2017.02.013.

[14]   S.A. Carr, J. Liu, A.G. Tesoro, Transport and fate of microplastic particles in wastewater treatment plants., Water Res. 91 (2016) 174–82. https://doi.org/10.1016/j.watres.2016.01.002.

[15]   M. Cole, H. Webb, P.K. Lindeque, E.S. Fileman, C. Halsband, T.S. Galloway, Isolation of microplastics in biota-rich seawater samples and marine organisms, Sci. Rep. 4 (2014) 1–8. https://doi.org/10.1038/srep04528.

[16]   H. Bouwmeester, P.C.H. Hollman, R.J.B. Peters, Potential Health Impact of Environmentally Released Micro- and Nanoplastics in the Human Food Production Chain: Experiences from Nanotoxicology, Environ. Sci. Technol. 49 (2015) 8932–8947. https://doi.org/10.1021/acs.est.5b01090.

[17]   B. Hu, Y. Li, L. Jiang, X. Chen, L. Wang, S. An, F. Zhang, Influence of microplastics occurrence on the adsorption of 17 β -estradiol in soil, J. Hazard. Mater. 400 (2020) 123325. https://doi.org/10.1016/j.jhazmat.2020.123325.

[18]   M.D. Sharma, A.I. Elanjickal, J.S. Mankar, R.J. Krupadam, Assessment of cancer risk of microplastics enriched with polycyclic aromatic hydrocarbons, J. Hazard. Mater. 398 (2020) 122994. https://doi.org/10.1016/j.jhazmat.2020.122994.

[19]   G. Kalčíková, B. Alič, T. Skalar, M. Bundschuh, A.Ž. Gotvajn, Wastewater treatment plant effluents as source of cosmetic polyethylene microbeads to freshwater, Chemosphere. 188 (2017) 25–31. https://doi.org/10.1016/j.chemosphere.2017.08.131.

[20]   C. Edo, M. González-Pleiter, F. Leganés, F. Fernández-Piñas, R. Rosal, Fate of microplastics in wastewater treatment plants and their environmental dispersion with effluent and sludge, Environ. Pollut. 259 (2020). https://doi.org/10.1016/j.envpol.2019.113837.

[21]   J. Talvitie, A. Mikola, O. Setälä, M. Heinonen, A. Koistinen, How well is microlitter purified from wastewater? – A detailed study on the stepwise removal of microlitter in a tertiary level wastewater treatment plant, Water Res. 109 (2017) 164–172. https://doi.org/10.1016/j.watres.2016.11.046.

 [22]   D. Lobelle, M. Cunliffe, Early microbial biofilm formation on marine plastic debris, Mar. Pollut. Bull. 62 (2011) 197–200. https://doi.org/10.1016/j.marpolbul.2010.10.013.

[23]   E.R. Zettler, T.J. Mincer, L.A. Amaral-Zettler, Life in the "plastisphere": Microbial communities on plastic marine debris, Environ. Sci. Technol. 47 (2013) 7137–7146. https://doi.org/10.1021/es401288x.

[24]   C. Bretas Alvim, S. Castelluccio, E. Ferrer-Polonio, M.A. Bes-Piá, J.A. Mendoza-Roca, J. Fernández-Navarro, J.L. Alonso, I. Amorós, Effect of polyethylene microplastics on activated sludge process - Accumulation in the sludge and influence on the process and on biomass characteristics, Process Saf. Environ. Prot. 148 (2021) 536–547. https://doi.org/10.1016/j.psep.2020.10.014.

[25]   M. Wu, W. Tang, S. Wu, H. Liu, C. Yang, Fate and effects of microplastics in wastewater treatment processes, Sci. Total Environ. 757 (2021) 143902. https://doi.org/10.1016/j.scitotenv.2020.143902.

[26]   F. Corradini, P. Meza, R. Eguiluz, F. Casado, E. Huerta-Lwanga, V. Geissen, Evidence of microplastic accumulation in agricultural soils from sewage sludge

disposal, Sci. Total Environ. 671 (2019) 411–420. https://doi.org/10.1016/j.scitotenv.2019.03.368.

[27]   P. van den Berg, E. Huerta-Lwanga, F. Corradini, V. Geissen, Sewage sludge application as a vehicle for microplastics in eastern Spanish agricultural soils, Environ. Pollut. 261 (2020) 114198. https://doi.org/10.1016/j.envpol.2020.114198.

[28]   L. Nizzetto, M. Futter, S. Langaas, Are Agricultural Soils Dumps for Microplastics of Urban Origin?, Environ. Sci. Technol. 50 (2016) 10777–10779. https://doi.org/10.1021/acs.est.6b04140.

[29]   E.L. Ng, E. Huerta Lwanga, S.M. Eldridge, P. Johnston, H.W. Hu, V. Geissen, D. Chen, An overview of microplastic and nanoplastic pollution in agroecosystems, Sci. Total Environ. 627 (2018) 1377–1388. https://doi.org/10.1016/j.scitotenv.2018.01.341.

[30]   T. Bosker, L.J. Bouwman, N.R. Brun, P. Behrens, M.G. Vijver, Microplastics accumulate on pores in seed capsule and delay germination and root growth of the terrestrial vascular plant Lepidium sativum, Chemosphere. 226 (2019) 774–781. https://doi.org/10.1016/j.chemosphere.2019.03.163.

[31]   K. Dietz, S. Herth, Plant nanotoxicology, Trends Plant Sci. 16 (2011) 582–589. https://doi.org/10.1016/j.tplants.2011.08.003.

[32]   G. Oliveri Conti, M. Ferrante, M. Banni, C. Favara, I. Nicolosi, A. Cristaldi, M. Fiore, P. Zuccarello, Micro- and nano-plastics in edible fruit and vegetables. The first diet risks assessment for the general population, Environ. Res. 187 (2020) 109677. https://doi.org/10.1016/j.envres.2020.109677.

org/10.1016/j.chemosphere.2019.03.163.

[33]   W. Wei, Q.S. Huang, J. Sun, J.Y. Wang, S.L. Wu, B.J. Ni, Polyvinyl Chloride Microplastics Affect Methane Production from the Anaerobic Digestion of Waste Activated Sludge through Leaching Toxic Bisphenol-A, Environ. Sci. Technol. 53 (2019) 2509–2517. https://doi.org/10.1021/acs.est.8b07069.

[34]   E. Ferrer-Polonio, J. Fernández-Navarro, J.L. Alonso-Molina, J.A. Mendoza-Roca, A. Bes-Piá, I. Amorós, Towards a cleaner wastewater treatment: Influence of

folic acid addition on sludge reduction and biomass characteristics, J. Clean. Prod. 232 (2019) 858–866. https://doi.org/10.1016/j.jclepro.2019.06.021.

[35]   C. Bretas Alvim, M.A. Bes-Piá, J.A. Mendoza-Roca, Separation and identification of microplastics from primary and secondary effluents and activated sludge from wastewater treatment plants, Chem. Eng. J. 402 (2020) 126293. https://doi.org/10.1016/j.cej.2020.126293.

[36]   Z. Meng, Z. Zhou, D. Zheng, L. Liu, J. Dong, Y. Yang, X. Li, T. Zhang, Optimizing dewaterability of drinking water treatment sludge by ultrasound treatment: Correlations to sludge physicochemical properties, Ultrason. Sonochem. 45 (2018) 95–105. https://doi.org/10.1016/j.ultsonch.2018.02.027.

[37]   X. Feng, H. Lei, J. Deng, Q. Yu, H. Li, Physical and chemical characteristics of waste activated sludge treated ultrasonically, Chem. Eng. Process. Process Intensif. 48 (2009) 187–194. https://doi.org/10.1016/j.cep.2008.03.012.

[38]   E. Zuriaga-Agustí, A. Bes-Piá, J.A. Mendoza-Roca, J.L. Alonso-Molina, Influence of extraction methods on proteins and carbohydrates analysis from MBR activated sludge flocs in view of improving EPS determination, Sep. Purif. Technol. 112 (2013) 1–10. https://doi.org/10.1016/j.seppur.2013.03.048.

[39]   B. Frølund, R. Palmgren, K. Keiding, P.H. Nielsen, Extraction of extracellular polymers from activated sludge using a cation exchange resin, Water Res. 30 (1996) 1749–1758. doi:10.1016/0043-1354(95)00323-1.

 [40]   P. Wang, C. Cheng, Y. Ma, M. Jia, Degradation behavior of polyphenols in model aqueous extraction system based on mechanical and sonochemical effects induced by ultrasound, Sep. Purif. Technol. 247 (2020) 116967. https://doi.org/10.1016/j.seppur.2020.116967.

[41]   M. Enfrin, J. Lee, Y. Gibert, F. Basheer, L. Kong, L.F. Dumée, Release of hazardous nanoplastic contaminants due to microplastics fragmentation under shear stress forces, J. Hazard. Mater. 384 (2020) 121393. https://doi.org/10.1016/j.jhazmat.2019.121393.

[42]   R. Guan, X. Yuan, Z. Wu, L. Jiang, Y. Li, Principle and application of hydrogen peroxide based advanced oxidation processes in activated sludge treatment: A review, Chem. Eng. J. 339 (2018) 519–530. https://doi.org/10.1016/j.cej.2018.01.153.

[43]   G.J. Xie, B.F. Liu, Q. Wang, J. Ding, N.Q. Ren, Ultrasonic waste activated sludge disintegration for recovering multiple nutrients for biofuel production, Water Res. 93 (2016) 56–64. https://doi.org/10.1016/j.watres.2016.02.012.

[44]   K.R. Morison, C.A. Hutchinson, Limitations of the Weissler reaction as a model reaction for measuring the efficiency of hydrodynamic cavitation, Ultrason. Sonochem. 16 (2009) 176–183. https://doi.org/10.1016/j.ultsonch.2008.07.001.

[45]   N.N. Mahamuni, Y.G. Adewuyi, Ultrasonics Sonochemistry Advanced oxidation processes (AOPs) involving ultrasound for waste water treatment : A review with emphasis on cost estimation, Ultrason. - Sonochemistry. 17 (2010) 990–1003. https://doi.org/10.1016/j.ultsonch.2009.09.005.

[45]   F. Wang, Y. Wang, M. Ji, Mechanisms and kinetics models for ultrasonic waste activated sludge disintegration, J. Hazard. Mater. 123 (2005) 145–150. https://doi.org/10.1016/j.jhazmat.2005.03.033.

[46]   R.A. Al-Juboori, T. Yusaf, V. Aravinthan, L. Bowtell, Investigating natural organic carbon removal and structural alteration induced by pulsed ultrasound, Sci. Total Environ. 541 (2016) 1019–1030. https://doi.org/10.1016/j.scitotenv.2015.09.143.

[47]   P. Kanthale, M. Ashokkumar, F. Grieser, Sonoluminescence, sonochemistry (H2O2 yield) and bubble dynamics: Frequency and power effects, Ultrason. Sonochem. 15 (2008) 143–150. https://doi.org/10.1016/j.ultsonch.2007.03.003.

[48]   V.K. Tyagi, S.L. Lo, L. Appels, R. Dewil, Ultrasonic treatment of waste sludge: A review on mechanisms and applications, Crit. Rev. Environ. Sci. Technol. 44 (2014) 1220–1288. https://doi.org/10.1080/10643389.2013.763587.

[49]   X. Tian, W.J. Ng, A.P. Trzcinski, Optimizing the synergistic effect of sodium hydroxide/ultrasound pre-treatment of sludge, Ultrason. Sonochem. 48 (2018) 432–440. https://doi.org/10.1016/j.ultsonch.2018.07.005.

Capítulo VII

Methodology for removing microplastics and other anthropogenic microparticles from sludge dehydrating system

Autores – C.Bretas Alvim, S. Navajas Valiente, M.A. Bes-Piá, J.A. Mendoza-Roca

Abstract

Anthropogenic microparticles (e.g., microplastics) are present in sewage plants, especially in sludge streams. However, the lack of standardized protocols to scrutinize the presence of anthropogenic microparticles in sludge makes the comparison between studies unfeasible. To tackle the knowledge gap regarding the efficiency of methodologies on the extraction of anthropogenic microparticles from the complex organic matrix, dewatered sludge, and digested sludge was treated with peroxidation and density separation, and the recovery of microparticles from these samples was investigated. The results showed that with the use of a higher density solution (NaI, 1.5 g/cm^3) a much better recovery of anthropogenic microparticles from sludge samples (approximately 1000 microparticles/g-dw and 2000 microparticles/g-dw, from dewatered and digested sludge, respectively) was achieved in comparison with the use of a lower density solution (NaCl, 1.2 g/cm^3) (200 microparticles/g-dw and 600 microparticles/g-dw from dewatered and digested sludge, respectively). Moreover, although the use of peroxidation is an essential step to break down the sludge structure and to release microparticles to the liquid phase, the use of peroxidation after or before density separation did not affect the overall recovery of microparticles. Polyethylene, polypropylene, and copolymer ethylene-ethyl-acrylate were the main microplastic fragments identified in digested sludge and dewatered sludge. However, no relation was observed between the method applied and the polymer recovered. Regarding the presence of anthropogenic microparticle in centrifuge effluent, 450 ± 212 microparticles/L were counted, and although little is known about this stream, in can be a relevant source of anthropogenic microparticles.

1. Introduction

Microplastics (MPs) correspond to a class of anthropogenic microparticles that have been constantly identified in oceans (Lusher et al., 2014; Mu et al., 2019), rivers (Barrows et al., 2018), groundwater (Panno et al., 2019; Samandra et al., 2022) and terrestrial ecosystems (Nizzetto et al., 2016; Ng et al., 2018). The potentially harmful effects of MPs on aquatic and terrestrial environments have been investigated by the scientific community. Recent studies indicate that besides the leaching of chemical additives - added to the formulation of plastics (such as colorants, stabilizers, plasticizers) - these microparticles are capable of adsorbing persistent organic substances, metals and pathogens, acting as vectors for their transport to other environments (Li et al., 2018; Ren et al., 2021). They can even enhance the spread of antibiotic-resistant bacteria (Arias-Andres et al., 2018; Pham et al, 2021). However, concerns go beyond the presence of synthetic materials, such as MPs, on ecosystems. Since natural textile fibers (for instance, made of cellulose) are more biodegradable than MPs, chemical additives may be released faster and it could be an aggravating factor (Kim et al., 2021; Ladewig et al., 2015; Remy et al.,2015).

Several studies have shown that wastewater treatment plants can significantly remove anthropogenic microparticles from the water stream (Carr et al., 2016; Murphy et al., 2016; Simon et al., 2018). However, the removed microparticles are largely retained in the primary and secondary sludge (Gies et al., 2018; Edo et al., 2020) which is usually forwarded to stabilization for use in agricultural fields. Anaerobic digestion is usually employed in wastewater treatment plants (WWTPs) for sludge stabilization and energy production. In this process, anaerobic microorganisms use the primary and secondary sludge as organic matter for their metabolism and in return, biogas is produced (Tayibi et al., 2021). After digestion, the sludge passes through centrifugation, a mechanical way to produce dewatered sludge, which finally can be applied as fertilizer on agricultural lands. The application of biosolids on lands drives to a significant input of MPs in soils. Talvitie et al. (2017) performed a study on the abundance of anthropogenic microparticles in different stages of a WWTP in Finland, including centrifugation system, and reported that approximately 80% of microlitter removed from the process were retained in dewatered sludge (1.51×10^{11} Microlitter/d). Van den Berg et al. (2020) studied the presence of MPs in agricultural fields in the east

of Spain. Results showed that an amount of 930 ± 740 MPs/kg (with density < 1 g/cm^3) and 1100 ± 570 MPs/kg (with density > 1 g/cm^3) were found in soil without application of sewage sludge. However, soils which received sewage sludge contained around 2130 ± 950 MPs/kg (with density < 1 g/cm^3) and 3060 ± 1680 MPs/kg (with density > 1 g/cm^3). The authors also detected an average of 18,000 ± 15,940 MPs/kg (with density < 1 g/cm^3) and 32,070 ± 19,080 MPs/kg (with density > 1 g/cm^3) in sewage sludge, which leads to a direct input of MPs in agricultural fields through biosolids application.

Despite the evidence of the accumulation of anthropogenic microparticles (including MPs) in sewage sludge, there are no standard methodologies that allow the extraction of these particles from complex organic matrices. Due to the high organic load content in the sewage sludge samples, the extraction of anthropogenic microparticles for their quantification and subsequent identification can be challenging. Authors have reported several methods to extract anthropogenic microparticles from sludge samples including a purification step and density separation with salt solutions (NaCl, NaI, ZnCl$_2$). Magni et al. (2019) used a hypersaline NaCl solution (1.2 g/cm^3), which had the disadvantage of no separating high density microplastics as polyvinylchloride (1.3 – 1.7 g/cm^3). The use of ZnCl2 was proposed by Mintenig et al. (2017). In this case the objective was the separation of the inorganic materials from the microplastics using a 1.6 g/cm^3 solution. Hurley et al. (2018) utilized a 1.8 g/cm^3 NaI solution for the separation. These authors tested the separation procedures with three materials to evaluate the recovery efficiency. In spite of the high concentration, the recovery of PET fibers did not reach 80% after two extractions, which showed that the form of the microparticles influences their separation.

Few researchers have evaluated the effectiveness of protocols applied (Sakali et al., 2021; Hurley et al., 2018). According to Li et al (2020) the encapsulation of MPs by extracellular polymeric substances, secreted by sludge, may leads to the overestimation of the number of MPs when only density separation is used. It is due to the great bound between MPs and the sludge floc, and to improve the extraction processes some purification step is required (Lares et al., 2019; Li et al., 2020). Oxidative treatments (e.g. Peroxidation and Fenton's reaction) are the main purification reported to treat WWTP samples, due to their efficiency in removing organic matter and since they do not compromise the polymeric identification of the microparticles (Bretas Alvim et al.,

2020; Li et al., 2020; Tagg et al., 2015). Enzymatic processes and alkaline and acid treatments have also been investigated as purification step in studies of MPs, nevertheless, the first one can be very complex and expensive (Mintenig et al., 2017) and the last ones can be very aggressive to MPs (Catarino et al., 2017; Dehaut et al. 2016; Enders et al., 2017; Li et al., 2020).

This study aimed to explore different routes, using peroxidation as purification step and density separation, to extract anthropogenic microparticles from both digested, and dewatered sludge and the water separated from the sludge in the dehydration process. This drives the development of protocols for measuring anthropogenic microparticles (as MPs) in three streams, with very different characteristics, from the sludge treatment process of a WWTP. Moreover, the effectiveness of the protocols proposed was investigated. Unlike other studies, a comparison of the protocols applied to three samples from the sludge treatment line with very different characteristics in terms of total solids has been performed in this work.

2. Materials and methods

2.1. Sample collection

This study was performed with samples collected from a WWTPs located in *Comunitat Valenciana* (Spain), which treats municipal wastewater. Samples of digested sludge (labeled as stream 1), dewatered sludge (labeled as stream 2), and centrifuge effluent (named *sludge liquor* and also *centrate* if the equipment is a centrifuge) (labeled as stream 3) (Fig. 1) were collected on the same day and kept in the fridge at 4 ℃ until analysis of anthropogenic microparticle content. The samples were characterized in terms of water content for dewatered sludge, total solids, and total suspended solids for digested sludge and effluent from the centrifuge. The dewatered sludge water content was calculated after oven-drying 5 g of the wet sample at 105 ℃ for 2 h. The total solids were calculated after oven-drying 5 mL of digested sludge and 50 mL of centrifuge effluent at 105 ℃ until samples were dried (after 24 h). Total suspended solids were calculated after filtering 5 mL of digested sludge and 50 mL of centrifuge effluent on a glass fiber membrane (VWR, 1 μm, 90 mm diameter) and oven-drying at 105 ℃ for 2 h.
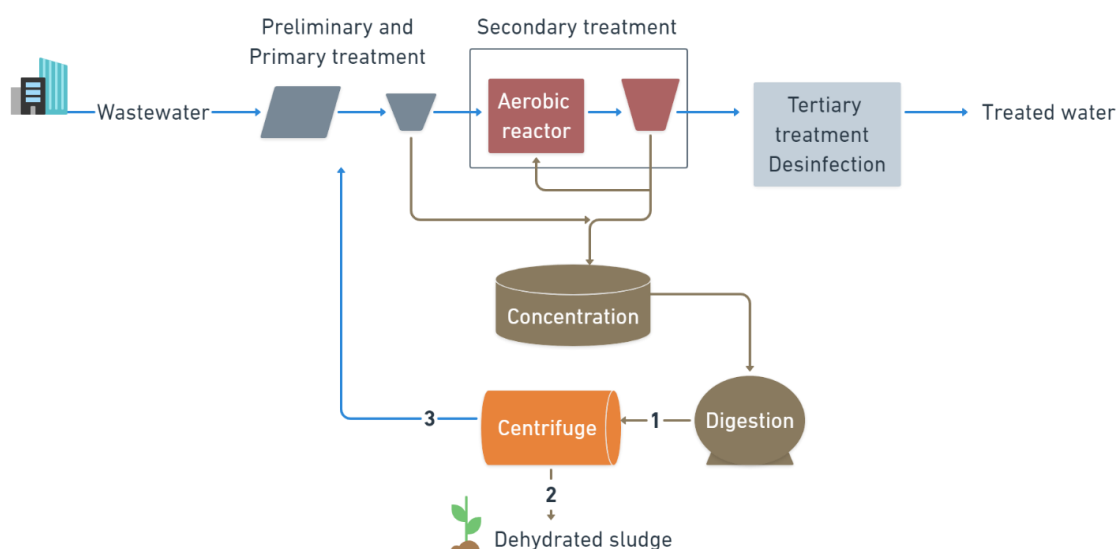
Fig. 1. Flow chart illustrating the sampling points (1 - digested sludge, 2 - dewatered sludge, and 3 - centrifuge effluent)

2.2. Quality assurance and Quality control (QA/Qc)

To ensure the quality of the tests carried out, all the materials used (tweezers, Petri dishes, pipettes, beakers) were carefully cleaned with soap, deionized water, and ethanol. Glass fiber membranes (VWR, 1 μm, 90 mm diameter) were collected directly from the box and inspected with a stereomicroscope (LEICA MZ APO) to assess the presence of contaminants. To evaluate the possible air contamination, two glass fiber membranes were inspected with the stereomicroscope and placed on an open Petri dish and left on the workbench for 24 hours. The filters were analyzed again with the stereomicroscope to recount microparticles. Furthermore, the presence of microparticles in the deionized water - used for cleaning materials - was also checked. For that, 1L of deionized water was filtered through a glass fiber membrane (which was previously inspected to eliminate possible initial contaminants). The filter was dried in an oven at 50 ºC for 2 hours and analyzed with a magnifying glass.

2.3. Considerations about the efficiency of the anthropogenic microparticles separation with the used procedures

Due to the high concentration of organic solids both in digested sludge and especially in dewatered sludge samples, scrutinizing the total number of anthropogenic microparticles in these samples is a very hard task. Thus, to estimate the total amount of

anthropogenic microparticles (with a size higher than 150 μm) in these samples, a chemical procedure to reduce the organic matter content was applied. For that, 5 g of dewatered sludge were mixed with 30 mL H_2O_2 (35%) and 25 mL of digested sludge were mixed with 25 mL H_2O_2 (35%). Both samples were heated up for 4 h at 60 ℃. After digestion, the samples were passed through a stainless sieve of aperture 150 μm to retain particle size of interest. The sieve was rinsed with deionized water and the resulting sample was filtered through a glass fiber membrane. The reason why the sludge samples were not filtered directly on a glass fiber membrane was due to the possibility of complete blockage of the filter and the infeasibility of counting retained particles due to the high concentration of organic matter and solids. The use of sieves is a common technique to separate specific sizes of anthropogenic microparticles and also to reduce the amount of solids to filtrate, avoiding the filter clogging (Hidalgo-Ruz et al., 2012; Wang, W. and Wang, J., 2018).

In sewage sludge, fibers have been reported as the main anthropogenic microparticle found and their presence in WWTPs is principally related to their release during laundry (Li et al., 2019; Alavian Petroody et al., 2021). Regarding the sampling of fibers, if these particles collide horizontally on the sieve, and their length is higher than the sieve aperture, it is expected that they would be retained by the system. However, due to the small thickness of fibers (around 10 – 20 μm), they could pass longitudinally through a sieve of aperture 150 μm and their number could be underestimated. Thus, to predict the possible loss of fibers during filtration processes, 10 L of tap water were passed through the stainless sieve (150 μm) and the retained material was rinsed with deionized water. Finally, the sample was filtered through a glass fiber membrane to count microparticles (Sample A). Assuming that fiber could not pass through the glass fiber membrane aperture (1 μm), 10 L of tap water was also filtered through the membrane, and the retained particles were counted under the stereomicroscope (Sample B). The retention of fibers on the sieve system was estimated by Equation 1.

$$\frac{Sample\ A\ (number\ of\ fibers)}{Sample\ B\ (number\ of\ fibers)} \times 100\% = \%\ Retention\ of\ fibers \qquad (1)$$

Based on the percentage of retention of fibers determined by Equation 1, the efficiency of the protocols for the recovery of microparticles from dewatered sludge and digested sludge was estimated.

2.4. Microparticles separation protocols

Initially, to find the most suitable protocol for the separation of anthropogenic microparticles from complex organic matter dewatered sludge samples were used. Two routes were tested using density separation together with peroxidation. The protocols are thoroughly described in the following sub-sections. The most suitable protocol for dewatered sludge was the one applied for digested sludge samples.

2.4.1. Protocol 1 – Density separation followed by peroxidation

Protocol 1 was based on the application of density separation to initially extract microparticles from dewatered sludge to liquid phase, followed by peroxidation of the remained solid (Fig. S1).

Solutions of NaCl and NaI were prepared with densities of 1.2 g/mL and 1.5 g/mL, respectively. To achieve the established densities Equation 2 proposed by Quinn et al. (2017) was applied. Briefly, the final weight of the flask containing the salt dissolved in deionized water was subtracted from the weight of the flask container and divided per the volume of the flask. To adjust these densities, 36 g of NaCl and 130 g of NaI were mixed with deionized water up to reach 100 mL of total volume.

$$Density = \frac{weight\ of\ flask\ containing\ salt\ solution\ (g) - weight\ of\ flask\ (g)}{total\ soluton\ volume\ (cm^3)}$$

(2)

Samples of 5 g (wet weight; w.w) of dewatered sludge were mixed with 50 mL of brine solution (NaCl or NaI). When the sludge was solubilized, the sample was allowed to settle for 24 h and 48 h when NaCl and NaI were used, respectively. The supernatant was collected and filtered through a glass fiber membrane (1µm) and dried in an oven at 50 ºC for 2 h. This first collected sample was named "*Extraction 1*". After the removal of the supernatant, 25 mL of NaCl solution was added to the rest of the sample. After mixing, it was settled for 24 h. Once again, the supernatant was collected

and filtered through a glass fiber membrane, and this fraction was named "*Extraction 2*". The settled solid fraction was submitted to a peroxidation with 30 mL H_2O_2 (35%) for 4 h at 60 ºC. The oxidized sample was settled for 2 h. The final supernatant was filtered, and this sample was named "*Supernatant H_2O_2*". The sedimentation time was initially set up and adjusted, when necessary, as explained in the results section.

The number of particles bigger than 150 μm retained on glass fiber membrane from *Extraction 1*, *Extraction 2,* and *Supernatant H_2O_2* was counted under the stereomicroscope with magnification adjusted between 8 X and 80 X. The number of microparticles was expressed per gram of dried weight (d.w) and the shape of microparticles was cataloged as fiber, fragment, or sphere.

The same protocol was performed using a NaI solution with higher density to assess the influence of density separation on microparticles extraction. As in the procedure with NaCl, the sedimentation time was initially set up and adjusted, when necessary, as detailed in the results section.

2.4.2. Protocol 2 - Peroxidation followed by density separation

Protocol 2 started with a peroxidation to reduce the organic matter before density separation (Fig. S2). Samples of 5 g (w.w) of dewatered sludge were mixed with 30 mL H_2O_2 (35%) for 4 h at 60 ºC. When this stage was finished the oxidized sample was settled for 2 h and the supernatant was filtered through a glass fiber membrane. This sample, which originated from peroxidation was named "*Supernatant H_2O_2*", similarly to Protocol 1. The settled phase was mixed with 50 mL of NaCl or NaI for the first density separation and then settled for 24 h before collecting supernatants (*Extraction 1*). After that, this extraction process was repeated by adding 25 mL of NaCl or NaI to the settled phase obtaining the *Extraction 2* supernatant after 24 h. The number of microparticles bigger than 150 μm retained on glass fiber membrane from *Extraction 1, Extraction 2,* and *Supernatant H_2O_2* was counted under the stereomicroscope as performed in Protocol 1 and the number of microparticles was expressed per gram of dried weight (d.w).

2.5. Centrifuge effluent

The centrifuge effluent was treated with peroxidation as suggested by Bretas Alvim et al. (2020) for purification of effluents. A volumetric relation of 1:100 (H_2O_2: Sample)

was applied and the sample was heated up for 2 h at 60 ºC. After peroxidation, the sample was directly filtered through a glass fiber membrane and dried at 50 °C in an oven for 2 h.

2.6. Visual sorting and polymer identification

The visual sorting was performed as reported by Bretas Alvim et al. (2020) and a stereomicroscope (LEICA MZ APO) with magnification between 8 X and 80 X was used. Microparticles ≥ 150 μm were considered, and the number of particles in sludge samples were reported per gram of dry weight (as above mentioned) and per liter in centrifuge effluent. After visual sorting, microparticles with homogenous color, rigid structure (they did not break down when were pressed by forceps), and in the case of fiber, regular thickness over the entire length, were classified as anthropogenic microparticles. Thereafter, anthropogenic microparticles were characterized with a microscope Fourier Transform-Infrared spectroscopy (FTIR, Bruker) under attenuated total reflection (ATR) mode (μ-ATR-FTIR) for polymeric identification. The ATR crystal used was a germanium one. The μ-ATR-FTIR was operated under the spectral resolution of 6 cm^{-1}, sample scan 128, and spectrum with wavelengths between 600 and 4000 cm^{-1}. All spectra were analyzed with the software Bio-Rad KnowItAll® Informatics System 2020, applying baseline correction and no-ATR correction. Moreover, the CO_2 peaks (2390 – 2285 cm^{-1}) were removed from spectra before identification. Since this method for identifying anthropogenic microparticles is very time-consuming, a subset of fragments was analyzed with μ-ATR-FTIR. Herein, only fragments were cataloged according to their polymer type.

3. Results and discussion

3.1. Samples characterization

Dewatered sludge was characterized by measuring moisture content and total solids (TS), total suspended solids (TSS), Total Volatile Solids (TVS), Dissolved Total Nitrogen (dTN), and alkalinity were assessed for digested sludge and effluent from centrifuge (Table 1).

Table 1 - Physicochemical characteristics of sludge samples and centrifuge effluent

Sample	Parameter				
	Moisture content (%)				
Dewatered sludge	73%				
	TS (g/L)	TSS (g/L)	TVS (g/L)	dTN (mg/L)	Alkalinity (mgCaCO$_3$/L)
Digested sludge	14.12 ± 0.57	11.55 ± 0.07	6.49 ± 0.5	910 ± 10	3500 ± 50
Centrifuge effluent	3.39 ± 0.12	0.07 ± 0.01			

3.2. QA/QC

Two glass fiber membranes were collected directly from the box and inspected under the stereomicroscope. No microparticles were identified in initial filters, and therefore the possibility of samples contamination by the filter was discharged. To assess airborne contamination, two glass fiber membranes were placed in a workbench for 24 h and after that, 1 ± 0 colored fiber was identified. Fragments and spheres were not found in the filters. Previous studies have reported the presence of anthropogenic microparticles, especially fibers in remote areas (Adams et al., 2021; Ambrosini et al., 2019) which could be related to their transport through the atmosphere. Thereby, airborne contamination can be a constant source of anthropogenic microparticles and difficult to control due to the high mobility of these materials (Bullard et al., 2021; Evangeliou et al., 2020; Mishra et al., 2020; Pedrotti et al., 2021).

The presence of anthropogenic microparticles in deionized water was also investigated in this work and an amount of 10 microparticles/L was identified. All microparticles were cataloged as colored fibers. These results suggest that cross-contamination could occur to some extent during the cleaning of materials when deionized water was used. Besides, as above mentioned, airborne contamination could input anthropogenic microparticles into the system and could be the reason for the presence of fibers in deionized water. To minimize cross-contamination, the water container was rapidly closed after the collection of deionized water to avoid microparticles deposition. In addition, all beakers were covered with aluminum foil and Petri dishes were kept closed until their use.

3.3. Effectiveness of Protocol 1 and 2 in removing anthropogenic microparticles from dewatered sludge

Regarding the number of microparticles recovered from dewatered sludge in both protocols (Table 2), NaCl solution extracted around 6-fold lower compared to NaI, which could be attributed to the lower density of NaCl and the worst flotation of particles.

Table 2 -Total microparticles recovered from dewatered sludge in Protocol 1 and Protocol 2 per gram (d.w)

Method	NaCl	NaI
Protocol 1	236 ± 21	1567 ± 199
Protocol 2	250 ± 91	1250 ± 114

The use of NaI solution promoted an extensive recovery of fibers, increasing more than 9-fold and 5-fold compared to NaCl in Protocol 1 and Protocol 2, respectively. An amount of 1428 ± 177 fiber/g-d.w and 908 ± 152 fiber/g-d.w were collected from sludge when NaI was used in Protocol 1 and Protocol 2, respectively. Higher recovery of fragments was also achieved when NaI was used for density separation, especially in Protocol 2 (Fig. 2). Concerning the number of fragments, 139 ± 21 fragments/g-d.w and 343 ± 39 fragments/g-d.w were counted in Protocol 1 and Protocol 2, respectively, using NaI solution. The density separation with NaCl resulted quite less effective since it was able to recover just 152 ± 23 fiber/g-d.w and 156 ± 103 fiber/g-d.w in Protocol 1 and Protocol 2, respectively, and 84 ± 2 fragments/g-d.w in Protocol 1 and 95 ± 12 fragments/g-d.w in Protocol 2. Some figures from Protocol 1 and Protocol 2 performed with NaCl were provide in supplementary material (S3).
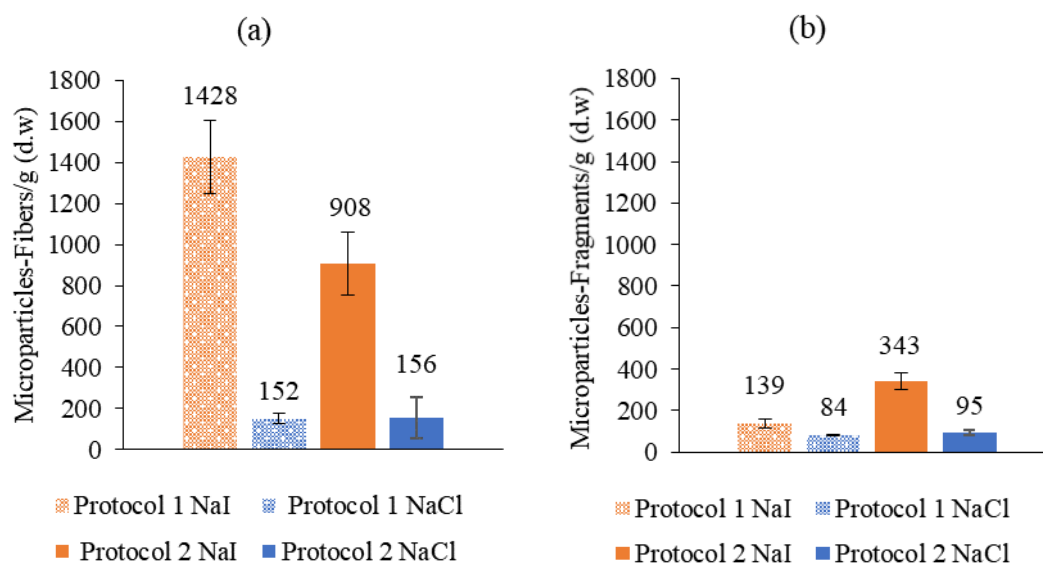
Fig. 2. Fibers (a) and fragments (b) recovered from dewatered sludge with Protocol 1 and Protocol 2

Analyzing each stage of Protocol 1 with NaCl, the amount of recovered fibers (20 ± 7 fibers/g-d.w and 21 ± 1 fibers/g-d.w) and fragments (20 ± 5 fragments/g-d.w and 13 ± 2 fragments/g-d.w) from sludge was quite similar in *Extraction 1* and *Extraction 2* (Fig. 3a). However, a great improvement in the global recovery was achieved when the sample was next submitted to peroxidation which resulted in a higher number of both fibers (112 ± 15 fibers/g-d.w) and fragments (50 ± 5 fragments/g-d.w). The use of NaI solution in Protocol 1 improved the recovery of fibers and fragments, especially of fibers. However, with this solution, it was not observed a higher recovery after peroxidation than in the extractions (Fig. 3b).

In Protocol 2, when NaCl was used, 55 ± 2 fiber/g-d.w, 56 ± 43 fiber/g-d.w, and 57 ± 44 fiber/g-d.w were collected from *Supernatant H_2O_2*, *Extraction 1* and *Extraction 2*, respectively. About the number of fragments, 48 ± 29 fragments/g-d.w, 47 ± 2 fragments/g-d.w and 16 ± 6 fragments/g-d.w, were extracted from Supernatant H_2O_2, *Extraction 1* and *Extraction 2*, respectively (Fig. 3c). Regarding the recovery of particles with NaI, after peroxidation 41 ± 12 fiber/g-d.w and 72 ± 1 fragments/g-d.w were counted in *Supernatant H_2O_2* sample, similarly to samples treated with NaCl (Fig. 3d). In this stage, samples showed a similar behavior as expected since the initial peroxidation procedure was the same for sludge samples. The explanation for the

recovery of microparticles after peroxidation could rely on the disruption of sludge structure by the peroxidation and the release of anthropogenic particles to the supernatant.

When the settled solid phase was submitted to density separation with NaI, 467 ± 95 fiber/g-d.w and 90 ± 17 fragments/g-d.w in *Extraction 1* and, 400 ± 70 fiber/g-d.w and 182 ± 57 fragments/g-d.w in *Extraction 2* were obtained. These results were much higher than the values obtained with NaCl solution. The higher density of NaI solution seemed to be enough to promote the better flotation of anthropogenic microparticles, whereas the density separation with NaCl solution was not sufficient to extract properly anthropogenic particles from sludge to liquid phase.
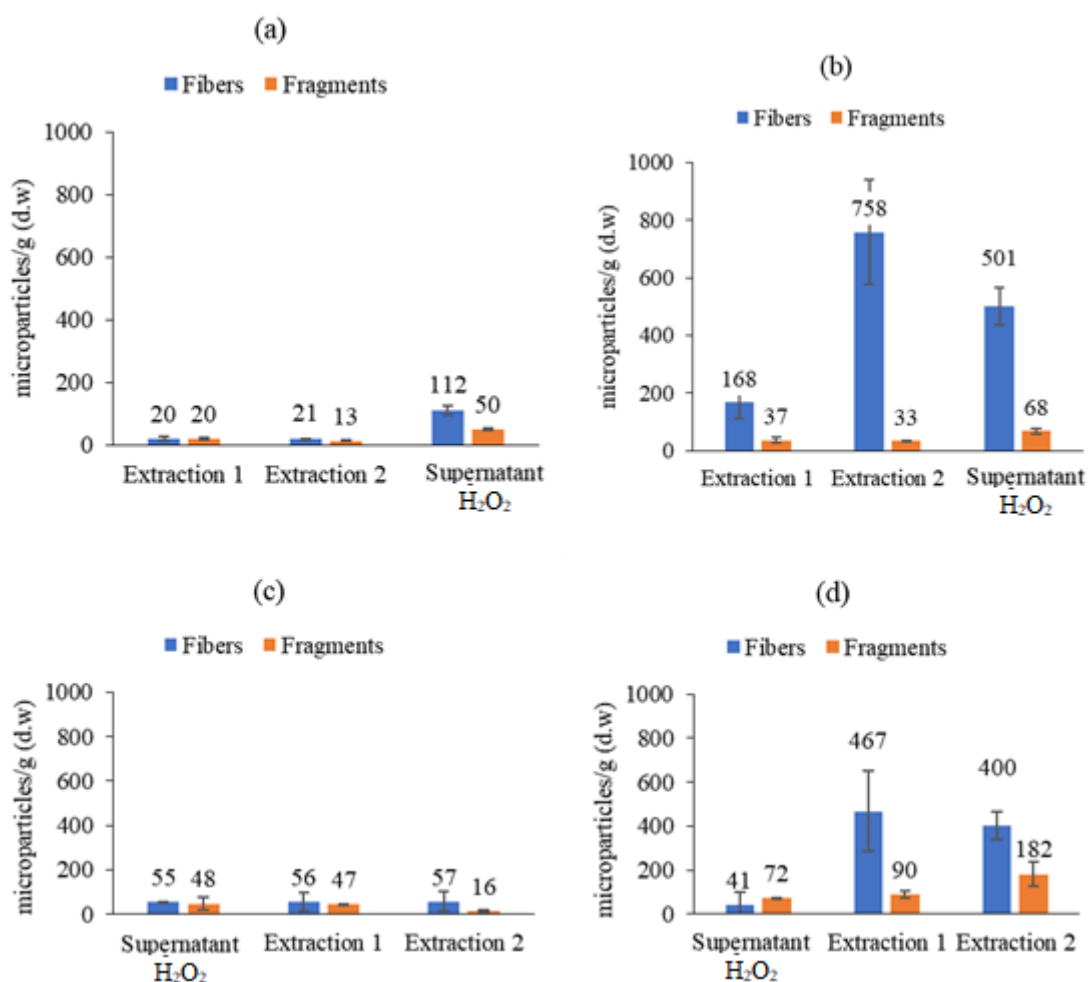

Fig. 3. Protocol 1 for dewatered sludge with NaCl (a) and NaI (b); Protocol 2 for dewatered sludge with NaCl (c) and NaI (d)

Although more microparticles were recovered in Protocol 1, the reaction between NaI solution (it remained on sludge after density separation) and H_2O_2 in Protocol 1 was extremely violent and very difficult to control since concentrated H_2O_2 was directly added to NaI. In Protocol 2 the NaI and H_2O_2 produced a reaction easier to handle, which could be explained by the lower H_2O_2 concentration in sludge when NaI was added since the peroxide was largely consumed during sample oxidizing previously to NaI addition. Thus, Protocol 2 was adopted as the safest and best method for recovering anthropogenic particles from a complex organic matrix.

The peroxidation of samples before density separation was also investigated by Li et al. (2019) for the separation of MPs from soil and sewage samples and the authors observed that the peroxidation before density separation contributed to greatly improving the extraction of the MPs from the samples. Therefore, it can be highlighted that the density of the salt solution and the use of some digestion processes (such as peroxidation, Fenton) contribute in different ways for the success of the extraction of MPs from samples with high organic matter content, nevertheless, both processes are relevant.

The use of different protocols to analyze anthropogenic microparticles in dewatered sludge has been reported in other studies (Table 3). In this research, a higher concentration of anthropogenic microparticles was found in the dewatered sludge compared to the values observed by Talvitie et al. (2017). This significant difference could be attributed to the treatment applied before microparticles quantification. Talvitie et al. (2017) did not use chemical treatments such as peroxidation. Only a filtration system for physical separation was adopted. As noted in this study (it was also reported by Li et al. (2019), the use of peroxidation for treating sludge samples is a fundamental step to promote the disruption of sludge flocs and to release anthropogenic microparticles to the liquid phase. Therefore, the absence of chemical treatment (e.g., oxidative process) could be the reason for the high discrepancy between the results stated herein and those observed by Talvitie et al. (2017). On the other hand, Salmi et al. (2021) found a concentration of 9379 ± 4474 MPs/g-dw in dewatered sludge after treating the sludge samples with enzymatic and oxidative processes, conjugated with density separation. Other authors observed lower concentrations of microparticles even when purification steps and density separation were employed (Jiang et al., 2020; Liu et al., 2019; Alavian Petroody et al., 2021).

Since the application of different protocols to extract microparticles from the complex organic matrix, as dewatered sludge, could result in divergences in concentration results, the comparison of microparticles amount between studies can be a hard task. Not just the treatments employed can interfere with the results, but also the treatments applied in WWTPs, the wastewater flow, and the particle size detection limit. Therefore, the determination of standard protocols is crucial to understanding the presence and fate of anthropogenic microparticles in sewage systems.

Table 3

Concentration of anthropogenic microparticles in dewatered sludge

Detection limit	Treatment	Concentration	Reference
150 µm	Purification (H_2O_2) + Density Separation (NaI)	1567 ± 199 microparticles/g-dw	This study
20 µm	-	186.7 ± 26.0 microparticles/g-dw	Talvitie et al. (2017)
20 µm	Enzymatic treatment + H_2O_2 + Density separation ($ZnCl_2$)	9379 ± 4474 MPs/g-dw	Salmi et al. (2021)
20 µm	Purification (H_2O_2) + Density Separation (NaI and NaCl)	240.3 ± 31.4 MPs/g-dw	Liu et al. (2019)
37 µm	Purification (H_2O_2) + Density Separation (NaI) + Rose Bengal	120 ± 16 MPs/g-dw	Alavian Petroody et al. (2021)

3.4. Sludge from anaerobic digestion - Protocol 2

According to the results of section 3.3, Protocol 2 was considered the most suitable for removing anthropogenic microparticles from dewatered sludge. As a result, it was also applied to sludge samples from anaerobic digestion with slight modifications. Chemical digestion of 25 mL of sludge with 35 % wt. H_2O_2 was applied using a volumetric relation of [1:1; Sludge: H_2O_2]. The procedure described in section 2.4.2 was

carried out with a sedimentation time of 24h, for *Extraction 1* and *Extraction* 2, regardless of the salt solution used.

As observed for dewatered sludge, NaI had a better performance in the recovery of anthropogenic microparticles from sludge compared to NaCl. 333 ± 2 fiber/g-d.w and 1086 ± 623 fiber/g-d.w were recovered from digested sludge when NaCl and NaI solutions were used, respectively. Concerning the number of fragments, 268 ± 94 fragments/g-d.w and 946 ± 20 fragments/g-d.w were counted when NaCl and NaI were used, respectively. These values represent the total number of fibers and fragments measured for each solution.

For each stage of the protocol (Fig. 4), 176 ± 80 fiber/g-d.w, 51 ± 12 fiber/g-d.w, and 106 ± 66 fiber/g-d.w were counted in *Supernatant H_2O_2*, *Extraction 1* and *Extraction 2*, respectively, with NaCl. Regarding the number of fragments, 106 ± 42 fragments/g-d.w, 75 ± 30 fragments/g-d.w and 86 ± 22 fragments/g-d.w were counted in *Supernatant H_2O_2*, *Extraction 1* and *Extraction 2*, respectively. Similar to dewatered sludge, when NaI was used as a solution an improvement in microparticles recovery was achieved. After peroxidation 48 ± 12 fiber/g-d.w and $123 \pm$, 26 fragments/g-d.w were numbered in sample "*Supernatant H_2O_2*". When the settled phase was then submitted to density separation with NaI, 650 ± 407 fiber/g-d.w and 388 ± 228 fiber/g-d.w, and 392 ± 54 fragments/g-d.w and 431 ± 48 fragments/g-d.w were obtained from *Extraction 1* and *Extraction 2*, respectively. These results reinforce the conclusion that peroxidation disrupts the sludge structure, and the use of a high-density salt solution improves particles separation from sludge to liquid phase, as also described in section 3.3 for dewatered sludge.
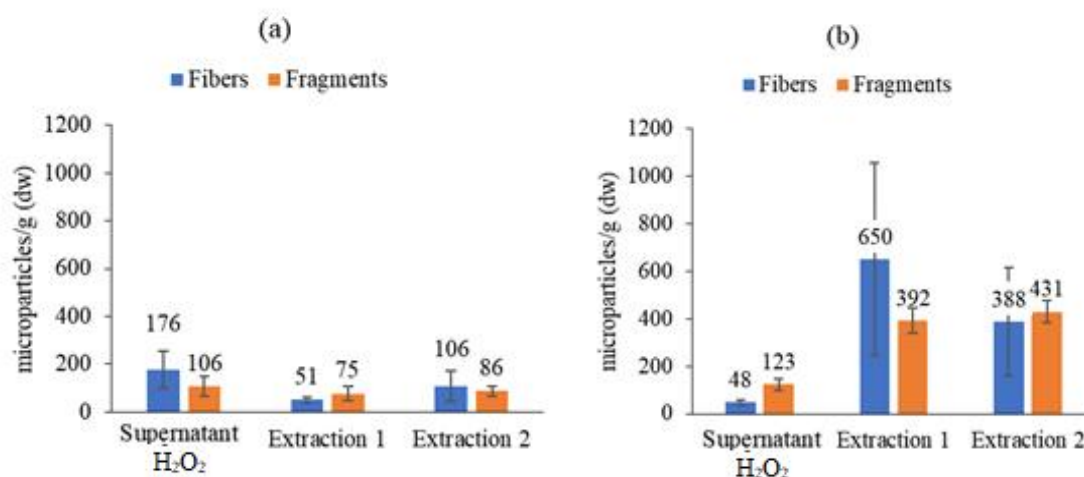
Fig. 4. Protocol 2 for digested sludge with NaCl (a) and NaI (b)

In Table 4 the concentrations of anthropogenic microparticles in digested sludge reported in previous works are described. As mentioned in the discussion of dewatered sludge results, the lack of standard protocols makes the comparison between studies very difficult. Besides, the representation of results in terms of the number of microparticles per mass or volume can also be an aggravating factor in drawing a parallel between the studies. Once again, the necessity of standard protocols for studying anthropogenic microparticles as well as MPs is highlighted.

Table 4 – Concentration of anthropogenic microparticles in digested sludge

Detection limit	Concentration	Reference
150 µm	2033 ± 603 microparticles/g-dw	This study
10 µm	6360 ± 0.8 MPs/g-dw	Chand et al. (2021)
37 µm	238 ± 31 MPs/g-dw	Alavian Petroody et al. (2021)
20 µm	102 ± 20 MPs/L	Salmi et al. (2021)
≥ 20 µm	4800 MPs/L	Nakao et al. (2021)

3.5. Centrifuge effluent

When digested sludge is subjected to a centrifugation process to finally produce dewatered sludge, a stream of water (centrifuge effluent) is generated and recirculated to the WWTPs. Herein, the number of particles in this stream was also investigated. An amount of 450 ± 212 microparticles/L were counted, and a portion of 98 ± 2% fibers and 2 ± 2% fragments were cataloged. In another research, Alavian Petroody et al. (2021) observed a reduction in MPs concentration in sludge after dewatering. The authors related the decrease to the return of MPs to the system in the rejected water stream produced from dewatering. In this context, Talvitie et al. (2017) found that after centrifugation around 20% of microparticles return to the system in centrifuge effluent and an amount of 12866.7 ± 275.4 microparticles/L was reported for this sample type. The expressive difference between microparticle concentrations found herein and the one reported by Talvitie et al. (2017) could be related to the discrepancy of methodologies used in the studies. Talvitie et al. (2017) used a smaller detection limit,

and this could have contributed to a higher concentration of microparticles identified. Besides, the configuration of the WWTP and the treatments employed in facilities would also contribute to different results.

So far, little is known about the presence of anthropogenic microparticles, as well MPs, in centrifuge effluent and how the continuous recirculation of these particles to the system could affect its performance. Thereby, based on the results reported herein and on previous studies (Table 5), centrifuge effluent could be a relevant, and constant, input of anthropogenic microparticles back to wastewater treatment processes and should also be better investigated.

Table 5 - Concentration of anthropogenic microparticles in centrifuge effluent

Detection limit	Concentration	Reference
150 μm	450 ± 212 microparticles/L	This study
≥ 20 μm	650 MPs/L	Nakao et al. (2021)
20 μm	10400 ± 3464 MPs/L	Salmi et al. (2021)
≥ 20 μm	12866.7 ± 275.4 microparticles/L	Talvitie et al. (2017)

3.6. Estimation of total particles in samples and efficiency of separation protocols

To assess the total particles in dewatered sludge and the efficiency of Protocols 1 and 2, this sample was prepared as described in section 2.3. Firstly, the possible loss of fibers through the sieve (used just in this experimentation step) was evaluated for calculations. It was observed that only 20% of total fibers in tap water were retained on the sieve. The remaining fraction passed longitudinally through the sieve aperture (150 μm), which is much larger than the thickness of fibers (around 10 – 20 μm). Therefore, for calculations, it was considered that the number of fibers from dewatered sludge retained on the sieve corresponded to 20% of the total present in the sample. The fragments (≥150 μm) were completely retained by the sieve. After applying these considerations, a total of 2966 microparticles/g-dw (83% fibers and 17% fragments) in dewatered sludge were estimated.

Based on the number of microparticles estimated in dewatered sludge, it was observed that Protocol 1 and 2 with NaCl were able to extract just 8% of the total

microparticles (Fig. 5a). However, the use of NaI increased to 53% and 42% of the total of microparticles extracted from dewatered sludge, in Protocol 1 and Protocol 2, respectively. These results suggest that the order of application of peroxidation and density separation seems not to alter the overall recovery of anthropogenic microparticles and corroborate the observations reported by Hurley et al. (2018). The authors also stated that when density separation and peroxidation are applied for extracting MPs from sludge samples, the order of the processes does not significantly impact the efficiency. However, apparently, the density of the salt solution has a much higher influence on the separation process. Regarding the digested sludge, the number of microparticles estimated in this sample was 3088 microparticles/g-dw and it was also observed that the use of NaI promoted a greater extraction of microparticles from the sample which reached 66% of the total (Fig.5b). Comparing the recoveries of microparticles from dewatered sludge with those from digested sludge when Protocol 2 was applied, it was observed that dewatered sludge achieved lower recovery percentages. This worse performance could be related to the fact that the dewatered sludge must be solubilized during peroxidation or density separation to release the microparticles to the liquid phase. If some flocs were not well dissolved, the particles would remain trapped in the sludge making their extraction unfeasible.
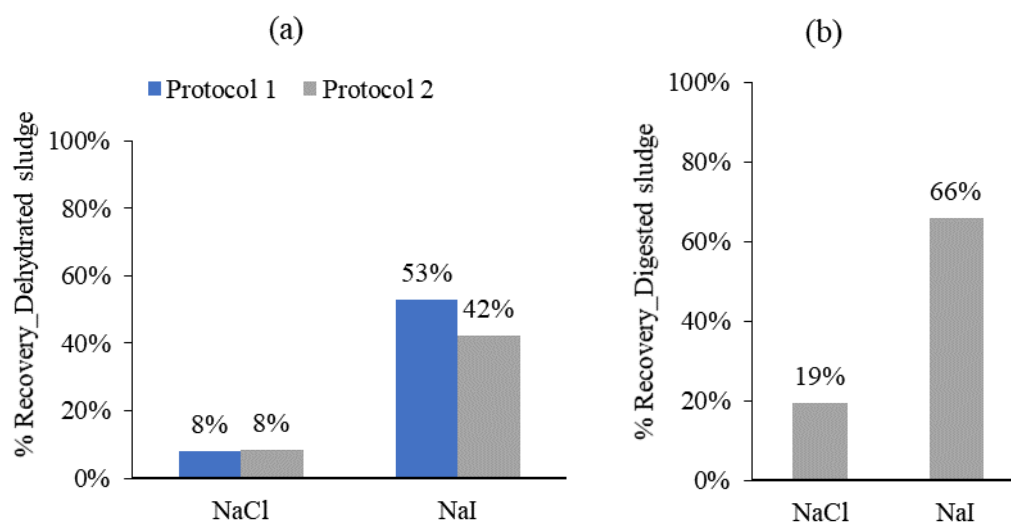

Fig. 5. The overall recovery of anthropogenic microparticles from dewatered sludge (a) and digested sludge (b)

As shown herein, Li et al. (2019) observed that the use of NaI resulted in a higher recovery of MPs from sludge than NaCl and stated that the higher the density of the salt solution the better the extraction of MPs from sludge, and Quinn et al. (2017) also stated that the recovery of MPs from sediments increased with the increment of solution density. In a recent study on considerations for density separation in the extraction of MPs from sediments (Cutroneo et al. 2021), was stated that NaCl is the most salt used for extracting MPs from sediments (45.6% of the publications analyzed by the authors). The use of NaI was reported in more than 17% of the publications analyzed. The predominance of the use of NaCl can be related to the inexpensive and environmentally friendly features of this salt. However, the low final density of NaCl solution may compromise the extraction of denser particles, and then, NaI can be an option to recover dense materials. Economic and environmental issues also should be considered for the assessment of the most suitable protocols for recovering MPs. In this context, the cost of NaI can be 70 times more expensive than the cost of NaCl (Claessen et al., 2013). And nevertheless, the hazard to the aquatic environment of NaI makes this compound a residue that requires precaution (Cutroneo et al. 2021). This discussion suggests that better results can be achieved when a high-density solution is used to recover MPs from complex organic samples, but at the same time, economic and environmental aspects must be considered for determining properly the management of residues and safety protocols.

3.7. Polymeric identification of the fragments in sludge samples

In Fig. 6 fragments collected from dewatered sludge which were classified as anthropogenic microparticles due to their structure, color, and shape are shown.
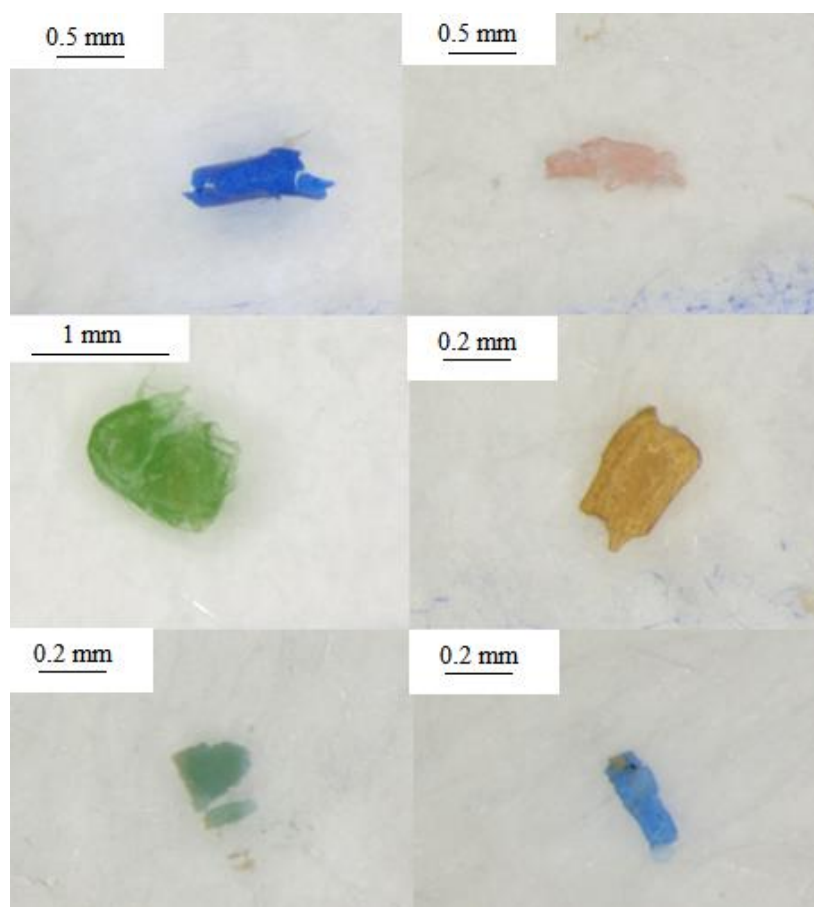
Fig. 6. Anthropogenic fragments extracted from dewatered sludge

Regarding the identification of the polymer, no tendency was observed in terms of recovered polymer and the protocol applied to sludge samples, nevertheless more than 90% of the fragments separated from digested and dewatered sludge samples were identified as microplastics and from centrifuge effluent 100%. This lack of pattern could be related to the fact that both peroxidation and density separation would promote the resuspension of organic and inorganic matter from sludge, which would affect the final density of the supernatant. The support for this hypothesis was the identification of silicon dioxide microparticles - which have a much higher density (2.65 g/cm^3) compared to the salt solutions used – among the microparticles extracted from digested and dewatered sludge. Due to the high density of silicon dioxide microparticles, the extraction of this material by flotation was initially unexpected. Their recovery could be explained by the increase of the final density of the supernatant due to the resuspension of organic and inorganic matter from sludge as commented above.

Concerning the composition of digested sludge and dewatered sludge, an increase in the percentage related to the presence of polyethylene (PE) MPs and polystyrene (PS) MPs in the dewatered sludge compared to the digested sludge was found. Furthermore, a reduction of polyethylene terephthalate (PET) MPs, polyurethane (PU) MPs, and polypropylene (PP) MPs in dewatered sludge compared to digested sludge was also appreciated (Fig.S4). The copolymer ethylene-ethyl-acrylate was quantified in a similar amount in both samples digested and dewatered sludge. Due to its toughness, flexibility, and elasticity - what makes the copolymer ethylene-ethyl-acrylate an impact-resistant material - it has been frequently used in automobile, packing, and adhesive industries (Li et al., 2013; Zhong and Sun, 2002). Alavian Petroody et al. (2021) and Nakao et al. (2021) also reported an increase in the number of PE MPs and a reduction in PET MPs compared to digested sludge. However, unlike the results presented here, Alavian Petroody et al. (2021) observed an increase in PP MPs and a reduction in PS MPs in the dewatered sludge, compared to digested sludge. The relation between the increased or reduction of certain polymer types in dewatered sludge samples compared to digested sludge ones and their densities is not clear since no pattern can be established. The reasons why the presence of some polymers increases or decreases in dewatered sludge after dewatering seem to go beyond the density of the polymers (Table S1) and could also be linked to the size and shape of the microparticles.

It has been demonstrated that anthropogenic microparticles, such as MPs, are greatly retained in the sludge stream in WWTPs and accumulated in biosolids, which can be applied as fertilizer in agricultural lands. The concerns on disposal of MPs in soils are based on the capacity of these particles to retain and transport pathogens, heavy metals, and persistent organic compounds. However, by now there is a lack of standard methodologies to assess the presence of MPs in the organic matrix for WWTPs. Some studies have been proposing the use of density separation for extracting these particles, but insufficient information about the effectiveness of protocols is available. Furthermore, unfortunately by now no environmental law has regulated the disposal of MPs in soil through biosolid application, which makes difficult to establish suitable methods for treating sludge samples and optimizing them for quantifying and identifying MPs according to size, shape, concentration, and polymer considered as a hazard for environment.

4. Conclusions

The use of NaI solution to extract anthropogenic microparticles from sludge samples resulted in more efficient protocols compared to the use of NaCl, which can be related to the higher density of NaI. More than 1000 microparticles/g-dw and more than 200 microparticles/g-dw were recovered from dehydrated sludge when NaI and NaCl were applied, respectively. From digested sludge more than 2000 microparticles/g-dw and more than 600 microparticles/g-dw were recovered when NaI and NaCl were applied, respectively. Furthermore, the use of peroxidation is a fundamental step to disrupt the sludge structure and to release the microparticle to the liquid phase. When peroxidation and density separation were used to recover microparticles from sludge samples the order of the processes did not affect the overall recovery, however when NaI was applied the peroxidation prior to density separation resulted in a safety protocol and the reaction was easier to handle. Herein, the most suitable protocol for extracting anthropogenic microparticles from sludge samples was peroxidation followed by the use of NaI for density separation. Regarding the centrate, as this stream can be an important input of anthropogenic microparticles back to the WWTP, it also deserves attention and should be further investigated in future studies.

Summarizing, according to our results, 1 kg of dried sludge from a WWTP could contain more than 10^6 MPs, which will return to the environment. If it is considered that the main way of management of the sludge in many countries is the agricultural use, MPs will be accumulated on the agricultural soils. The environmental consequences can be not only their accumulation in the environment but also the transport of toxic pollutants, since they can act as vector of these compounds.

Future works have to be focused on elaborating standardized protocols for quantification and characterization of microparticles, such as MPs, in order to compare results from different works and to draw conclusions properly. These protocols will have to be different according to the solids content of the sludge samples. Furthermore, tests to check the efficiency of microparticles separation from the samples is an important challenge in the next future. After the correct and standardized characterization of the samples, research should be focused on MPs removal from the sludge to avoid their return to the environment.

Acknowledgments

REFERENCES

Adams, J.K., Dean, B.Y., Athey, S.N., Jantunen, L.M., Bernstein, S., Stern, G., Diamond, M.L., Finkelstein, S.A., 2021. Anthropogenic particles (including microfibers and microplastics) in marine sediments of the Canadian Arctic. Sci. Total Environ. 784, 147155. https://doi.org/10.1016/j.scitotenv.2021.147155

Alavian Petroody, S.S., Hashemi, S.H., van Gestel, C.A.M., 2021. Transport and accumulation of microplastics through wastewater treatment sludge processes. Chemosphere 278, 130471. https://doi.org/10.1016/j.chemosphere.2021.130471

Ambrosini, R., Azzoni, R.S., Pittino, F., Diolaiuti, G., Franzetti, A., Parolini, M., 2019. First evidence of microplastic contamination in the supraglacial debris of an alpine glacier. Environ. Pollut. 253, 297–301. https://doi.org/10.1016/j.envpol.2019.07.005

Arias-Andres, M., Klümper, U., Rojas-Jimenez, K., Grossart, H.-P., 2018. Microplastic pollution increases gene exchange in aquatic ecosystems. Environ. Pollut. 237, 253–261. https://doi.org/10.1016/j.envpol.2018.02.058

Barrows, A.P.W., Christiansen, K.S., Bode, E.T., Hoellein, T.J., 2018. A watershed-scale, citizen science approach to quantifying microplastic concentration in a mixed land-use river. Water Res. 147, 382–392. https://doi.org/10.1016/j.watres.2018.10.013

Bretas Alvim, C., Bes-Piá, M.A., Mendoza-Roca, J.A., 2020. Separation and identification of microplastics from primary and secondary effluents and activated sludge from wastewater treatment plants. Chem. Eng. J. 402, 126293. https://doi.org/10.1016/j.cej.2020.126293

Bullard, J.E., Ockelford, A., O'Brien, P., McKenna Neuman, C., 2021. Preferential transport of microplastics by wind. Atmos. Environ. 245, 118038. https://doi.org/10.1016/j.atmosenv.2020.118038

Carr, S.A., Liu, J., Tesoro, A.G., 2016. Transport and fate of microplastic particles in wastewater treatment plants. Water Res. 91, 174–82. https://doi.org/10.1016/j.watres.2016.01.002

Catarino, A.I., Thompson, R., Sanderson, W., Henry, T.B., 2017. Development and optimization of a standard method for extraction of microplastics in mussels by enzyme digestion of soft tissues. Environ. Toxicol. Chem. 36, 947–951. https://doi.org/10.1002/etc.3608.

Chand, R., Rasmussen, L.A., Tumlin, S., Vollertsen, J., 2021. The occurrence and fate of microplastics in a mesophilic anaerobic digester receiving sewage sludge, grease, and fatty slurries. Sci. Total Environ. 798, 149287. https://doi.org/10.1016/j.scitotenv.2021.149287

Claessens, M., Van Cauwenberghe, L., Vandegehuchte, M.B., Janssen, C.R., 2013. New techniques for the detection of microplastics in sediments and field collected organisms. Mar. Pollut. Bull. 70, 227–233. https://doi.org/10.1016/j.marpolbul.2013.03.009

Cutroneo, L., Reboa, A., Geneselli, I., Capello, M., 2021. Considerations on salts used for density separation in the extraction of microplastics from sediments. Mar. Pollut. Bull. 166, 112216. https://doi.org/10.1016/j.marpolbul.2021.112216

Dehaut, A., Cassone, A.L., Frère, L., Hermabessiere, L., Himber, C., Rinnert, E., Rivière, G., Lambert, C., Soudant, P., Huvet, A., Duflos, G., Paul-Pont, I., 2016. Microplastics in seafood: Benchmark protocol for their extraction and characterization. Environ. Pollut. 215, 223–233. https://doi.org/10.1016/j.envpol.2016.05.018

Edo, C., González-Pleiter, M., Leganés, F., Fernández-Piñas, F., Rosal, R., 2020. Fate of microplastics in wastewater treatment plants and their environmental dispersion with effluent and sludge. Environ. Pollut. 259, 113837. https://doi.org/10.1016/j.envpol.2019.113837

Enders, K., Lenz, R., Beer, S., Stedmon, C.A., 2017. Extraction of microplastic from biota: Recommended acidic digestion destroys common plastic polymers. ICES J. Mar. Sci. 74, 326–331. https://doi.org/10.1093/icesjms/fsw173

Evangeliou, N., Grythe, H., Klimont, Z., Heyes, C., Eckhardt, S., Lopez-Aparicio, S., Stohl, A., 2020. Atmospheric transport is a major pathway of microplastics to remote regions. Nat. Commun. 11. https://doi.org/10.1038/s41467-020-17201-9

Gies, E.A., LeNoble, J.L., Noël, M., Etemadifar, A., Bishay, F., Hall, E.R., Ross, P.S., 2018. Retention of microplastics in a major secondary wastewater treatment plant in Vancouver, Canada. Mar. Pollut. Bull. 133, 553–561. https://doi.org/10.1016/j.marpolbul.2018.06.006

Hidalgo-Ruz, V., Gutow, L., Thompson, R.C., Thiel, M., 2012. Microplastics in the marine environment: a review of the methods used for identification and quantification. Environ. Sci. Technol. 46, 3060–3075. https://doi.org/10.1021/es2031505.

Hurley, R.R., Lusher, A.L., Olsen, M., Nizzetto, L., 2018. Validation of a Method for Extracting Microplastics from Complex, Organic-Rich, Environmental Matrices. Environ. Sci. Technol. 52, 7409–7417. https://doi.org/10.1021/acs.est.8b01517

Jiang, J., Wang, X., Ren, H., Cao, G., Xie, G., Xing, D., Liu, B., 2020. Investigation and fate of microplastics in wastewater and sludge filter cake from a wastewater treatment plant in China. Sci. Total Environ. 746, 141378. https://doi.org/10.1016/j.scitotenv.2020.141378

Kim, L., Kim, S.A., Kim, T.H., Kim, J., An, Y.-J., 2021. Synthetic and natural microfibers induce gut damage in the brine shrimp Artemia franciscana. Aquat. Toxicol. 232, 105748. https://doi.org/10.1016/j.aquatox.2021.105748

Ladewig, S.M., Bao, S., Chow, A.T., 2015. Natural Fibers: A Missing Link to Chemical Pollution Dispersion in Aquatic Environments. Environ. Sci. Technol. 49, 12609–12610. https://doi.org/10.1021/acs.est.5b04754

Lares, M., Ncibi, M.C., Sillanpää, Markus, Sillanpää, Mika, 2019. Intercomparison study on commonly used methods to determine microplastics in wastewater and sludge samples. Environ. Sci. Pollut. Res. 26, 12109–12122. https://doi.org/10.1007/s11356-019-04584-6

Li, J., Zhang, K., Zhang, H., 2018. Adsorption of antibiotics on microplastics. Environ. Pollut. 237, 460–467. https://doi.org/10.1016/j.envpol.2018.02.050

Li, Q., Wu, J., Zhao, X., Gu, X., Ji, R., 2019. Separation and identification of microplastics from soil and sewage sludge. Environ. Pollut. 254, 113076. https://doi.org/10.1016/j.envpol.2019.113076

Li, Z.L., Li, L., Deng, X.X., Lv, A., Wang, C.H., Du, F.S., Li, Z.C., 2013. Ethylene-ethyl acrylate copolymers via ADMET polymerization: Effect of sequence distribution on thermal properties. J. Polym. Sci. Part A Polym. Chem. 51, 2900–2909. https://doi.org/10.1002/pola.26682

Li, X., Chen, L., Ji, Y., Li, M., Dong, B., Qian, G., Zhou, J., Dai, X., 2020. Effects of chemical pretreatments on microplastic extraction in sewage sludge and their physicochemical characteristics. Water Res. 171, 115379. https://doi.org/10.1016/j.watres.2019.115379

Liu, X., Yuan, W., Di, M., Li, Z., Wang, J., 2019. Transfer and fate of microplastics during the conventional activated sludge process in one wastewater treatment plant of China. Chem. Eng. J. 362, 176–182. https://doi.org/10.1016/j.cej.2019.01.033

Lusher, A.L., Burke, A., O'Connor, I., Officer, R., 2014. Microplastic pollution in the Northeast Atlantic Ocean: Validated and opportunistic sampling. Mar. Pollut. Bull. 88, 325–333. https://doi.org/10.1016/j.marpolbul.2014.08.023

Magni, S., Binelli, A., Pittura, L., Avio, C.G., Della Torre, C., Parenti, C.C., Gorbi, S., Regoli, F., 2019. The fate of microplastics in an Italian Wastewater Treatment Plant. Sci. Total Environ. 652, 602–610. https://doi.org/10.1016/j.scitotenv.2018.10.269

Mintenig, S.M., Int-Veen, I., Löder, M.G.J., Primpke, S., Gerdts, G., 2017. Identification of microplastic in effluents of waste water treatment plants using focal plane array-based micro-Fourier-transform infrared imaging. Water Res. 108, 365–372. https://doi.org/10.1016/j.watres.2016.11.015

Mishra, S., Singh, R.P., Rath, C.C., Das, A.P., 2020. Synthetic microfibers: Source, transport and their remediation. J. Water Process Eng. 38, 101612. https://doi.org/10.1016/j.jwpe.2020.101612

Mu, J., Zhang, S., Qu, L., Jin, F., Fang, C., Ma, X., Zhang, W., Wang, J., 2019. Microplastics abundance and characteristics in surface waters from the Northwest Pacific, the Bering Sea, and the Chukchi Sea. Mar. Pollut. Bull. 143, 58–65. https://doi.org/10.1016/j.marpolbul.2019.04.023

Murphy, F., Ewins, C., Carbonnier, F., Quinn, B., 2016. Wastewater Treatment Works (WwTW) as a Source of Microplastics in the Aquatic Environment. Environ. Sci. Technol. 50, 5800–5808. https://doi.org/10.1021/acs.est.5b05416

Nakao, S., Akita, K., Ozaki, A., Masumoto, K., Okuda, T., 2021. Circulation of fibrous microplastic (microfiber) in sewage and sewage sludge treatment processes. Sci. Total Environ. 795, 148873. https://doi.org/10.1016/j.scitotenv.2021.148873

Ng, E.-L., Huerta Lwanga, E., Eldridge, S.M., Johnston, P., Hu, H.-W., Geissen, V., Chen, D., 2018. An overview of microplastic and nanoplastic pollution in agroecosystems. Sci. Total Environ. 627, 1377–1388. https://doi.org/10.1016/j.scitotenv.2018.01.341

Nizzetto, L., Futter, M., Langaas, S., 2016. Are Agricultural Soils Dumps for Microplastics of Urban Origin? Environ. Sci. Technol. 50, 10777–10779. https://doi.org/10.1021/acs.est.6b04140

Panno, S. V., Kelly, W.R., Scott, J., Zheng, W., McNeish, R.E., Holm, N., Hoellein, T.J., Baranski, E.L., 2019. Microplastic Contamination in Karst Groundwater Systems. Groundwater 57, 189–196. https://doi.org/10.1111/gwat.12862

Pedrotti, M.L., Petit, S., Eyheraguibel, B., Kerros, M.E., Elineau, A., Ghiglione, J.F., Loret, J.F., Rostan, A., Gorsky, G., 2021. Pollution by anthropogenic microfibers in North-West Mediterranean Sea and efficiency of microfiber removal by a wastewater treatment plant. Sci. Total Environ. 758, 144195. https://doi.org/10.1016/j.scitotenv.2020.144195

Pham, D.N., Clark, L., Li, M., 2021. Microplastics as hubs enriching antibiotic-resistant bacteria and pathogens in municipal activated sludge. J. Hazard. Mater. Lett. 2, 100014. https://doi.org/10.1016/j.hazl.2021.100014

Quinn, B., Murphy, F., Ewins, C., 2017. Validation of density separation for the rapid recovery of microplastics from sediment. Anal. Methods 9, 1491–1498. https://doi.org/10.1039/c6ay02542k

Remy, F., Collard, F., Gilbert, B., Compère, P., Eppe, G., Lepoint, G., 2015. When Microplastic Is Not Plastic: The Ingestion of Artificial Cellulose Fibers by Macrofauna Living in Seagrass Macrophytodetritus. Environ. Sci. Technol. 49, 11158–11166. https://doi.org/10.1021/acs.est.5b02005

Ren, Z., Gui, X., Xu, X., Zhao, L., Qiu, H., Cao, X., 2021. Microplastics in the soil-groundwater environment: Aging, migration, and co-transport of contaminants – A critical review. J. Hazard. Mater. 419, 126455. https://doi.org/10.1016/j.jhazmat.2021.126455

Sakali, A., Coello, D., Haïlaf, A., Egea-Corbacho, A., Albendín, G., Arellano, J., Brigui, J., Quiroga, J.M., Rodríguez-Barroso, R., 2021. A new protocol to assess the microplastics in sewage sludge. J. Water Process Eng. 44, 102344. https://doi.org/10.1016/j.jwpe.2021.102344

Salmi, P., Ryymin, K., Karjalainen, A.K., Mikola, A., Uurasjärvi, E., Talvitie, J., 2021. Particle balance and return loops for microplastics in a tertiary-level wastewater treatment plant. Water Sci. Technol. 84, 89–100. https://doi.org/10.2166/wst.2021.209

Samandra, S., Johnston, J.M., Jaeger, J.E., Symons, B., Xie, S., Currell, M., Ellis, A. V., Clarke, B.O., 2022. Microplastic contamination of an unconfined groundwater aquifer in Victoria, Australia. Sci. Total Environ. 802, 149727. https://doi.org/10.1016/j.scitotenv.2021.149727

Simon, M., van Alst, N., Vollertsen, J., 2018. Quantification of microplastic mass and removal rates at wastewater treatment plants applying Focal Plane Array (FPA)-based Fourier Transform Infrared (FT-IR) imaging. Water Res. 142, 1–9. https://doi.org/10.1016/j.watres.2018.05.019

Tagg, A.S., Sapp, M., Harrison, J.P., Ojeda, J.J., 2015. Identification and Quantification of Microplastics in Wastewater Using Focal Plane Array-Based Reflectance Micro-FT-IR Imaging. Anal. Chem. 87, 6032–6040. https://doi.org/10.1021/acs.analchem.5b00495

Talvitie, J., Mikola, A., Setälä, O., Heinonen, M., Koistinen, A., 2017. How well is microlitter purified from wastewater? – A detailed study on the stepwise removal of microlitter in a tertiary level wastewater treatment plant. Water Res. 109, 164–172. https://doi.org/10.1016/j.watres.2016.11.046

Tayibi, S., Monlau, F., Bargaz, A., Jimenez, R., Barakat, A., 2021. Synergy of anaerobic digestion and pyrolysis processes for sustainable waste management: A critical review and future perspectives. Renew. Sustain. Energy Rev. 152, 111603. https://doi.org/10.1016/j.rser.2021.111603

van den Berg, P., Huerta-Lwanga, E., Corradini, F., Geissen, V., 2020. Sewage sludge application as a vehicle for microplastics in eastern Spanish agricultural soils. Environ. Pollut. 261, 114198. https://doi.org/10.1016/j.envpol.2020.114198

Wang, W., Wang, J., 2018. Investigation of microplastics in aquatic environments: an overview of the methods used, from field sampling to laboratory analysis. TrAC Trends Anal. Chem. (Reference Ed.) 108, 195–202. https://doi.org/10.1016/j.trac.2018.08.026.

Zhong, Z., Sun, S.X., 2003. Properties of soy protein isolate/poly(ethylene-co-ethyl acrylate-co-maleic anhydride) blends. J. Appl. Polym. Sci. 88, 407–413. https://doi.org/10.1002/app.11714

Supplementary Material

Methodology for removing microplastics and other anthropogenic microparticles from sludge dewatering system

Authors – C.Bretas Alvim, S. Navajas Valiente, M.A. Bes-Piá, J.A. Mendoza-Roca

* Corresponding author: *claal@posgrado.upv.es*

Supplementary tables and figures

Supplementary tables :

Table S1. Density of microplastics according to polymer type
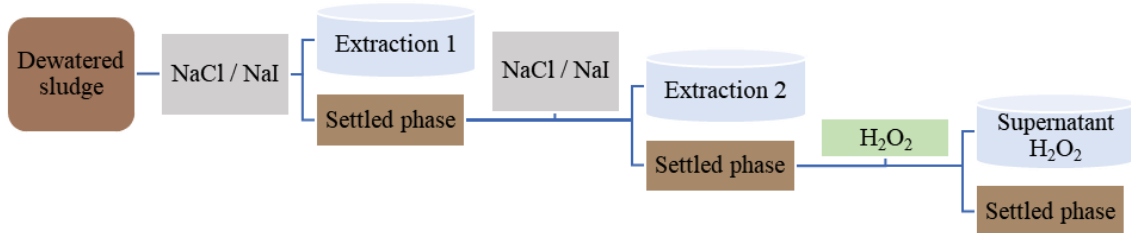
Supplementary figures :

Fig. S1 Protocol 1 – Density separation procedure followed by peroxidation

Fig. S2. Protocol 2 – Peroxidation followed by density separation

Fig. S3. Types of microplastics identified in sludge samples. Protocol 1- Sample of dewatered sludge with NaCl (a); settled phase with H_2O_2 (b, c); supernatant separation after peroxidation (d). Protocol 2- Sample of dewatered sludge with H_2O_2 (a); peroxidation after 30 min of reaction (b); supernatant separation after density separation with NaCl (c).

Fig. S4. Types of microplastics identified in sludge samples

Table S1. Density of microplastics according to polymer type

Polymer	Density (g/cm^3)
PET	1.37-1.45
PC	1.20
PS	1.04-1.1
PA	1.02-1.05
PE	0.91-0.97
ETHYLENE-ETHYL-ACRYLATE	0.93
PP	0.9-0.91

Fig. S1 Protocol 1 – Density separation procedure followed by peroxidation
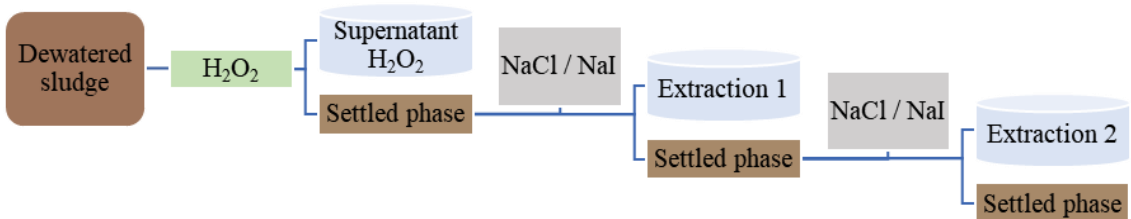

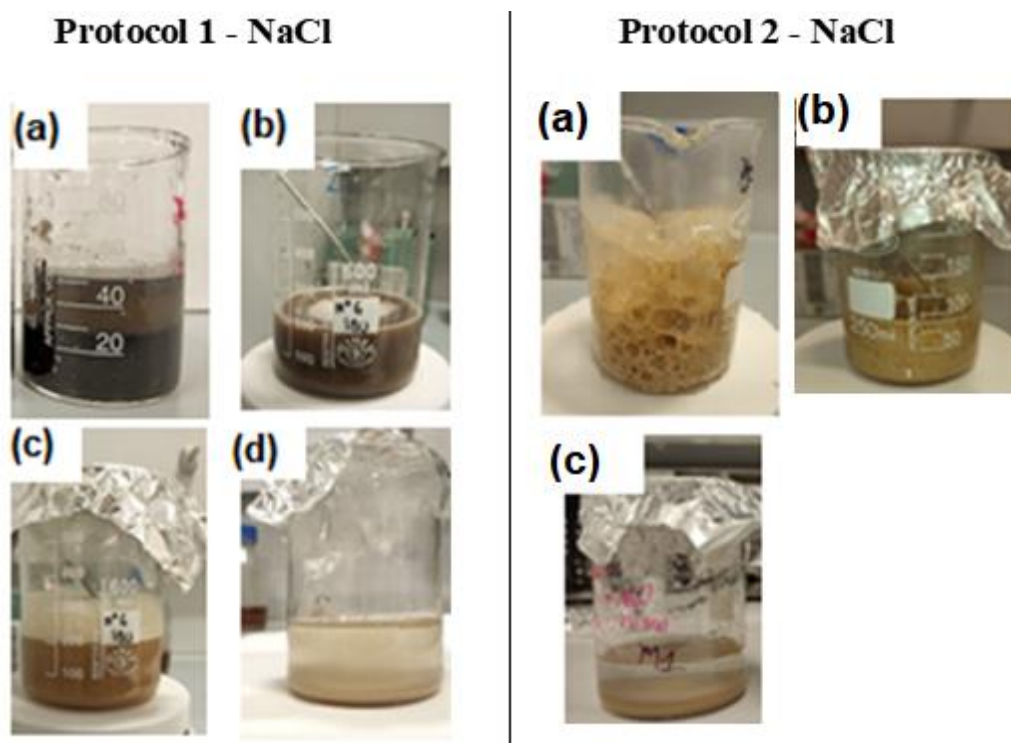**Fig. S2.** Protocol 2 – Peroxidation followed by density separation

Fig. S3. Types of microplastics identified in sludge samples. Protocol 1- Sample of dewatered sludge with NaCl (a); settled phase with H_2O_2 (b, c); supernatant separation after peroxidation (d). Protocol 2- Sample of dewatered sludge with H_2O_2 (a); peroxidation after 30 min of reaction (b); supernatant separation after density separation with NaCl (c).
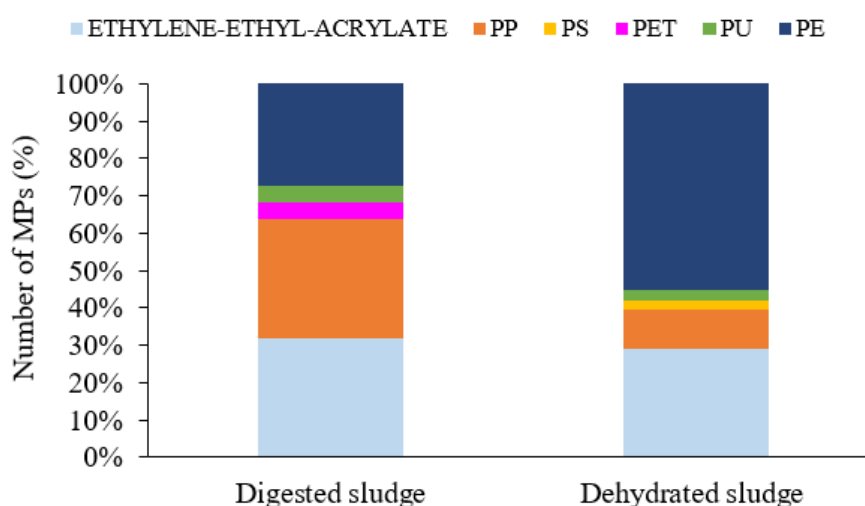

Fig. S4. Types of microplastics identified in sludge samples

Capítulo VIII

# Identification of microfibers in drinking water with Nile Red. Limitations and strengths

Autores– C.Bretas Alvim[1], M.A Bes-Piá[1], J.A. Mendoza-Roca[1], J.L. Alonso[2],

[1] Instituto de Seguridad Industrial, Radiofísica y Medioambiental, Universitat Politècnica de València, Camino de Vera, s/n, Valencia 46022, Spain

[2] Instituto de Ingeniería del Agua y Medio Ambiente, Universitat Politècnica de Valencia, Valencia 46022, Spain

Graphical Abstract
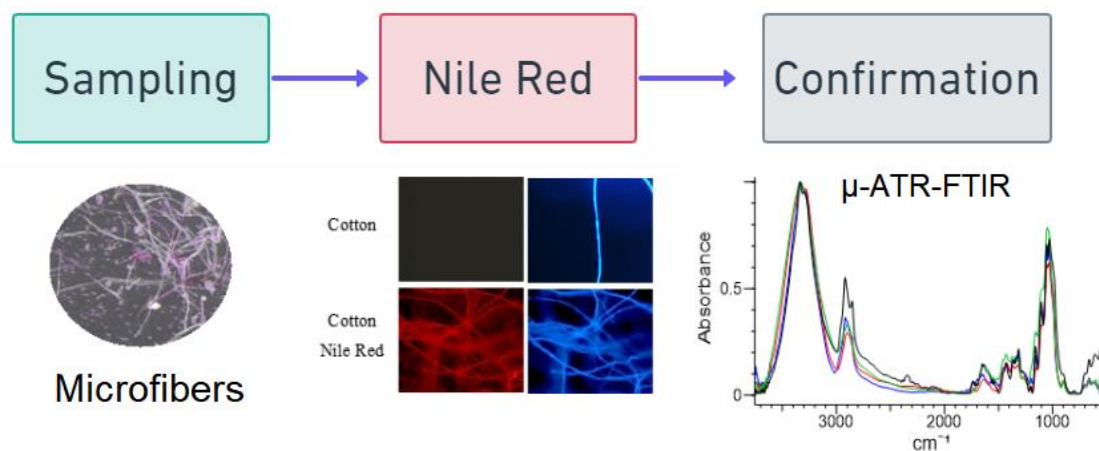
Abstract

Traditional drinking water treatment plants (DWTP) are not specifically designed for removing microplastics (MPs) from drinking water. Therefore, this emergent contaminant has been identified in water sources and final drinking water. Microfibers from plastic-based materials are a relevant category of MPs that has attracted attention in recent studies due to their significant presence in environmental samples. This study investigated the presence of microfibers (> 10 μm) in the raw water and the final drinking water of a DWTP located in Spain. It was observed that the DWTP studied was capable of removing 86% of microfibers from raw water. Even with this high removal rate, an amount of 613 microfibers/L (including natural, artificial, and synthetic materials) was counted in drinking water after disinfection. The reactive Nile Red was tested to investigate its use for staining microfibers from the mentioned DWTP and also from the tap and bottled water samples facilitating their identification. It was observed that microfibers from environmental samples can have different manifestations of fluorescence compared to virgin polymers, and it could be related to degrees of degradation and/or adsorption of organic and inorganic material on the microfibers' surfaces. For this reason, the use of Nile Red could help understand the levels of degradation of these materials in the environment, and thus its use could go beyond the identification of MPs, which was initially proposed in previous studies.

Abbreviations

CA – cellulose acetate

DWTP – drinking water treatment plant

EPS - expanded polystyrene

HDPE - high-density polyethylene

LDPE - low-density polyethylene

MPs - microplastics

PAN – Polyacrylonitrile

PC – Polycarbonate

PE – Polyethylene

PET – polyethylene terephthalate

PP – Polypropylene

PS – Polystyrene

PUR – polyurethane

PVC – polyvinyl chloride

WWTP – wastewater treatment plant

1. Introduction

Textile microfibers are fibers less than 5 mm in length and approximately 10-20 μm thick. Their origin can be related to the textile production processes and they can also be formed from the detachment of clothing items (Napper and Thompson, 2016; Henry et al., 2019). According to Browne et al. (2011), more than 1,900 polyester fibers (PET) can be released into the water after washing synthetic clothes. In other studies, Almroth et al. (2018) described that a wool garment could shed approximately 110,000 fibers when washed and Sillanpää and Sainio (2017) concluded that 2.5×10^5 and 1.3×10^7 PET and cotton microfibers, respectively, could be detached in the first washes. In another research, Athey et al. (2020) studied the washing of denim garments (jeans) and found that $56,000 \pm 4,100$ fibers can be shed per wash cycle. The number of fibers and microfibers that are shed from each garment will depend on various factors such as the properties of the fabric, temperature, time, and speed of washing, as well as the products used as detergents and softeners (Hernández et al., 2017; De Falco et al., 2018). After being released from the fabrics, the microfibers will be present in the wastewater produced during laundry, which is discharged into wastewater treatment plants (WWTPs). Although WWTPs have been shown to have a high retention capacity (over 98%) of microparticles, including microfibers, due to the large volumes of effluent treated daily, the concentration of microfibers in the final treated water is not negligible (Lares et al., 2018; Kazour et al., 2019). For example, Pedrotti et al. (2021) reported a concentration of 3.6×10^4 synthetic microfibers/m^3 in the final effluent of a WWTP in France. In this way, the final effluents from WWTPs, when are discharged into rivers, oceans, and seas, contribute significantly to the entry of microfibers into water resources. Finally, if drinking water treatment plants (DWTPs) collect natural waters contaminated with microfibers, these particles may end up in the final drinking water. In other words, microfibers are present in all water cycle.

The presence of microfibers in drinking water has been described in recent studies (Kosuth et al., 2018; Mason et al., 2018; Kankanige and. Babel, 2020). However, the differences between methodologies make the direct comparison between the reported results difficult. In addition to the methodology used in each study, it is important to consider that DWTPs can catch water from different sources, such as rivers, reservoirs, and groundwater. These different water intake points will influence

the greater or lesser presence of microfibers in drinking water (Pivokonsky et al., 2018; Wang et al.; 2020). When drinking water is collected for studying the presence of MPs (e.g., microfibers), spectrometric techniques such as Raman and FTIR are commonly used. These techniques allow the identification of the polymer chain and the classification between synthetic, natural, and artificial materials. However, the use of spectrometry is an expensive and time-consuming procedure, since each material must be identified individually, increasing the analysis time (Prata et al., 2021; Shruti et al., 2022). In addition, finding the best-operating conditions for the equipment for the correct generation of spectra (with high signal and low noise) is a task that requires a high knowledge of the equipment and the management of the samples, which adds more complexity to the process.

Recent studies have suggested the use of staining techniques with Nile Red to aid the visual identification of MPs and natural materials (Maes et al., 2017; Erni-Cassola et al., 2017; Konde et al., 2020). With staining methods, unlike spectrometry, it is not possible to identify the polymer chain, and therefore a precise classification of materials cannot be made. However, the advantage of using staining techniques could be the fastest visual sorting of materials that may or may not be MPs.

Nile Red corresponds to a neutral aromatic molecular structure composed of two heteroatoms (nitrogen and oxygen) and with intense fluorescence properties in the apolar medium. Therefore, Nile Red is a reagent that is characterized by the effect of solvatochromism, which means that its fluorescence is linked to the polarity of the solvent used for its dissolution (Martínez et al. 2016; Maes et al, 2017). Since this dye has low solubility in water and does not show fluorescence in this solvent (Martínez et al. 2016), different solvents, such as methanol, ethanol, and acetone, have been used for the preparation of Nile Red solutions. The choice of solvent must be based on its capacity of not damaging the microparticles. In this context, Konde et al. (2020) suggested that acetone can result in the surface degradation of some plastics and therefore other solvents such as methanol or ethanol could be more recommended. In addition to the choice of solvent, the concentration of the solution, the temperature, and the reaction time are parameters that may govern the effectiveness of the staining. The adoption of higher reaction temperatures (60 ºC – 70 ºC) has shown more satisfactory staining results than staining at room temperature (Konde et al., 2020; Wang et al.,

2020). When the polymer chains are subjected to higher temperatures, the polymer chain is loose, and Nile Red can interact better with the polymer structure. Finally, when the polymer reaches room temperature its structure becomes dense, and Nile Red stays trapped in the polymer (Shruti et al., 2022).

This study aims to evaluate the efficiency of Nile Red to stain natural, artificial, and synthetic textile microfibers, which may improve the visual identification of these particles in drinking water samples. Furthermore, limitations of this technique have been brought out. For that, virgin microfibers of cotton, wool, rayon, polyester (PET), and polypropylene (PP) were used to scrutinize the experimental conditions for staining and to compare their identification with the eventual identification of microfibers of the same materials in a water sample. The differences of fluorescence of microfibers of the same materials (comparing pristine and found in water samples microfibers) led to relate fluorescence with polymer degradation, which is hardly described in the literature until now.

2. Materials and methods

2.1. QA/QC

To ensure the quality of the tests carried out, all the materials used (tweezers, Petri dishes, pipettes) were carefully cleaned with soap, deionized water, and ethanol. Polycarbonate filters (PCTE, 47 mm diameter, 10 μm pore size, Whatman) were collected directly from the box and inspected with a stereomicroscope (LEICA MZ APO) to check the eventual presence of cross-contamination. To evaluate possible airborne contamination, two PCTE filters were inspected with the stereomicroscope and then were placed in an open Petri dish and left on the workbench for 24 hours. After that, the filters were analyzed with the stereomicroscope for counting the particles deposited on them.

2.2. Microfibers used to assess the efficiency of Nile Red

Virgin polymers (natural, artificial, and synthetics) - with microfiber shape - were used as patterns in this study. The fibers were cut from sewing threads with lengths less than 5 mm and thickness around 10 – 20 μm. In this work,

white/transparent and light-colored microfibers were used for two main reasons: firstly, the use of staining techniques for the identification of microfibers is more efficient for the recognition of white and transparent materials, since the presence of additives and dyes can worsen the staining process (Nel et al., 2011). Secondly, the number of white/transparent microfibers can be underestimated due to the greater difficulty of their visual perception (Stolte et al., 2015; Briain et al., 2020; Shruti et al., 2022) and the use of staining techniques could improve their identification. Natural (cotton and wool), artificial (rayon), and synthetic (PET - polyester, PP - polypropylene) microfibers were used in this study. The confirmation of the polymer type of virgin microfibers was performed with a Fourier-Transformed-Infrared in mode Attenuated Total Reflectance (ATR-FTIR) with a germanium crystal (Bruker). The equipment was operated with a spectral resolution of 6 cm^{-1}, 32 scans, and a range of wavelengths between 600 and 4000 cm^{-1}. In addition, a microscope was coupled with the equipment to allow the perfect alignment between the microfiber and the ATR-FTIR.

2.3. Drinking water samples

To scrutinize the efficiency of a Drinking Water Treatment Plant (DWTP) located in Spain to remove microfibers, water samples were collected in four stages of treatment. The samples analyzed were the raw water, collected from a reservoir, (labeled as stream 1), the effluent of sand filtration (labeled as stream 2), the effluent of disinfection stage (labeled as stream 3), and the water stored for supply (labeled as stream 4). Samples were taken in glass containers on the same day from this DWTP which was named DWTP-A. 100 mL of raw water (stream 1) were treated with a chemical digestion procedure to reduce organic matter. The chemical digestion was carried out with hydrogen peroxide (H_2O_2, 35%) at 60 ± 2 ºC for 2 hours, using a volumetric ratio of 1:10 [H_2O_2:sample]. After chemical digestion, the sample was filtered on a PCTE filter and stored in a Petri dish. For the other samples (2, 3, and 4), 1 L of water was vacuum filtered through a PCTE filter without chemical digestion. All PCTE filters were dried in an oven at 60 ºC for 1 hour for completely removing the water. Finally, the filters were left in a desiccator (approximately 1 hour) until reaching room temperature. The material retained on the filters was visually analyzed with the above-referred stereomicroscope with a magnification adjusted between 8 X and 80 X

for counting microfibers >10 µm. A black background was used for counting white/transparent microfibers and a white background for counting the light-colored ones. Microfibers were quantified in all samples, and stream 3 was used for testing the viability of the staining method. Besides the samples collected from stream 3 (DWTP-A) and microfibers from tap water (distributed by a DWTP-B) were used to investigate the protocol with Nile Red. After being stained with Nile Red, the microfibers were randomly selected from the samples and positioned on the PCTE filter as described in the next section.

2.4. Microfibers stain procedure with Nile Red

The staining method used in this study was based on the methodology reported by Erni-Cassola et al. (2017). A stock solution of 10 mg/L Nile Red (technical grade, N3013, Sigma-Aldrich) was prepared in methanol. The stock solution was covered with aluminum foil and kept in a fridge. Two more solutions of Nile Red with concentrations of 5 mg/L and 1 mg/L were prepared from the stock solution, covered with aluminum foil, and kept in a fridge until their use. The three concentrations of Nile Red (1 mg/L, 5 mg/L, and 10 mg/L) were tested to determine the best concentration to stain microfibers considering the fluorescence of microfibers and the lowest background signal. To set up the best concentration for staining microfibers, virgin polyester microfibers (PET) were used since polyester MPs have been commonly found in drinking water, and the Nile Red has been reported for dying MPs (Erni-Cassola et al. ,201719; Prata et al., 2019). The PET microfibers were carefully positioned on the PCTE filter, and the filters were coated with Nile Red solution. The filters were kept in an oven for 30 min at 60 ºC. Finally, the filters were left in a desiccator (approximately 1 hour) until reaching room temperature.

The fluorescence of the microfibers before and after staining was evaluated with an epifluorescence microscope (Olympus BX50, Paris) equipped with a 100 W mercury high-pressure lamp and a set of filters. The filters were the following: U-MWB (filter cube for blue excitation (456-490 nm) and green emission consisting of a set of mirrors DM500, BP450-480, and BA515), U-MWIB (filter cube for broadband green excitation (534-558 nm) and red emission consisting of a set of mirrors DM570, BP510-550, and

BA590) and U-MWIG (filter cube for broadband ultraviolet excitation (365 nm) and blue emission consisting of a set of mirrors DM400, BP330-385, and BA420). The microscope was also equipped with an AxioCan ICc5 camera (Zeiss) and the software Zen 3.1 (Zeiss) was used to process the data. When the best concentration of Nile Red was selected, it was applied to stain the microfibers of the water samples of the DWTPs. Finally, a relation between the fluorescence emitted by the fibers and their polymer type was established.

The fluorescence emitted by particles stained with Nile Red depends on their surface morphology and properties, color, and whether the particles are degraded. Therefore, it is very important to scrutinize the fluorescence of particles collected from environmental samples and to relate it to their polymeric structure. As a result, drinking water samples prepared as described in section 2.3 were covered with the optimal concentration of Nile Red and kept in an oven for 30 min at 60 ºC. With the stereomicroscope, the microfibers retained on the PCTE filter were randomly positioned in the numbered positions written on a microscope slide placed under the PCTE filter (Fig. 1). The fluorescence of the positioned microfibers was obtained one by one with the epifluorescence microscope and finally, their polymeric composition was identified with the μ-ATR-FTIR equipment.
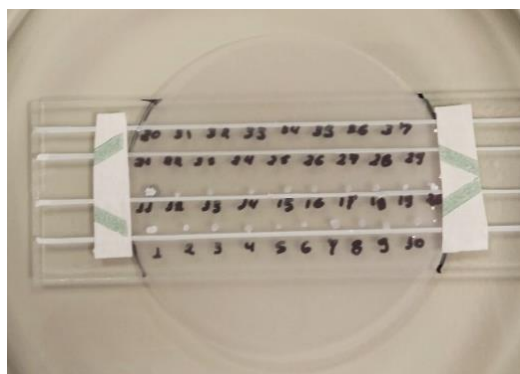

Fig. 1. PCTE filter positioned on a microscope slide to array microfibers stained with Nile Red

3. Results

3.1. QA/QC

Two PCTE filters were randomly collected from the box and analyzed with a stereomicroscope and no microfiber was identified, excluding the possibility of initial contamination by the filters. Regarding airborne contamination, after 24 hours of exposure, the presence of 33 ± 12 microfibers per filter was observed. $97 \pm 1\%$ of the microfibers identified in the filters were white. According to these results, it is estimated that about 1.4 ± 0.5 microfibers/h could be deposited on the surfaces where the tests were carried out. This value indicates that it is very difficult for the sampling system to be free from the influence of air pollution. To minimize the effects of airborne contamination on the samples, not only the materials were continuously washed with water, soap, and then alcohol, but also the Petri dishes. They were inspected with a magnifying glass for removing contaminants and all materials were covered with aluminum foil before being used.

3.2. Quantification and identification of microfibers in drinking water

Concerning the shape and color of microparticles in DWTP-A, microfibers corresponded to more than 75 % of microparticles in all samples and white/transparent and light-colored microfibers were the main colors observed (more than 95 %). Table 1 shows the number of microfibers counted in the four samples analyzed and their turbidity. It can be observed that the DWTP-A showed an efficiency of 86 % for removing microfibers from raw water after disinfection (Table 1). However, the lower concentration of microfibers in the storage tank in comparison with those measured in the water after disinfection was unexpected since there is no treatment step between both sampling points. A recent study by Chu et al. (2022) showed an 86% decrease in the concentration of microparticles in the tap water provided by a DWTP compared to the water at the exit of the DWTP. According to the authors, this result was caused by the adsorption of microparticles in the drinking water supply system. Therefore, it is hypothesized that the decrease in the concentration of microfibers between disinfection and the storage tank observed herein could be linked to the adsorption of microparticles in the pumping and distribution system to the storage tank.

Since microparticles – including microfibers - have a size between 5 mm and 1 μm, and therefore are particles in suspension, it can be inferred that the reduction of the turbidity of the water could corroborate the reduction of the number of microfibers/L. In this way, the physicochemical characterization of the samples revealed a reduction in the turbidity value of the water in the storage tank (0.297 NTU) in comparison with water after disinfection (0.617 NTU).

Table 1- Microfibers/L and turbidity values at the analyzed collection points

Sample	Turbidity (NTU)	Microfibers/L
1	13	4450
2	0.515	833
3	0.617	613
4	0.297	549

It was also observed that most particles in tap water (distributed by DWTP-B) corresponded to microfibers ($76 \pm 7\%$ of the total microparticles). The predominance of microfibers in drinking water has also been reported in previous studies (Kankanige and. Babel, 2020; Wang et al., 2020). The presence of microfibers in environmental samples has been related to their release during laundry. In our research, interesting evidence of the presence of textile microfibers in drinking water was the finding of blue fibers in all samples (Fig.2a), which after their inspection showed the presence of the additive indigo blue, usually applied for dying jeans. The characteristic peaks in the cotton spectra were also seen in the blue fibers, but the spectra of these fibers also revealed peaks at 1623, 1609, 1584, 1481, and 1459 cm^{-1}, which could be related to the aromatic ring of the dye (Baran et al., 2010; Lee et al., 2013). To validate this hypothesis, blue fibers from a jeans garment were also studied and the spectra matched with blue fibers collected from the samples (Fig.2b). Another relevant observation was the appearance of degradation of the blue fibers in Fig.2a since part of its color seemed to be leached from the fiber. The fact that the microfibers of environmental samples can undergo degradation processes is an important observation to understand the results of Nile Red. 67 % and 80 % of blue fibers analyzed from bottled and tap water, respectively, were identified as cotton fiber with indigo blue dye. These microfibers

would be released from denim clothes in washing machines and ended in raw water taken by DWTPs, which were not able to retain 100% of these small fibers. Microfibers from other materials can derive from contamination during transportation, storage, and water pipes. Even the indiscriminate disposal of face masks used in the current pandemic scenario (COVID-19) could be also a relevant source of fibers (such as cellulose, rayon, polyester, polypropylene, and others) to water pollution (Fadare et al., 2020).
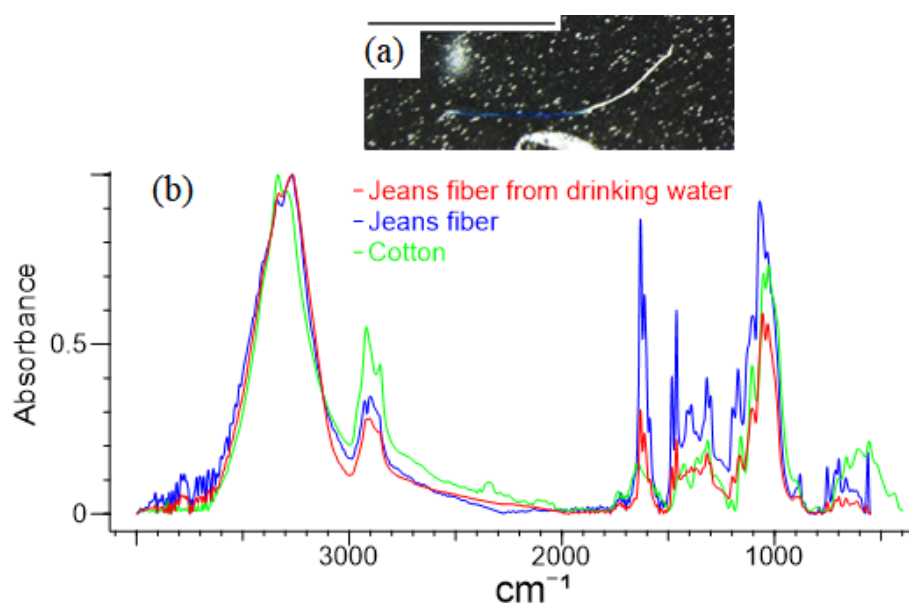

Fig.2. Blue fiber found in drinking water (a); μ-ATR-FTIR spectra of blue fiber, jeans fiber, and cotton fiber (b)

Regarding the polymer types in samples from DWTP-A after disinfection (sampling point 3), polyester and nylon, and also cotton and rayon microfibers were found. In tap water distributed by DWTP-B were also found polyester, nylon, cotton, and rayon. Although the presence of MPs in drinking water is evident, the harmfulness of this contaminant to human health is still not very clear due to the scarcity of research on this field. The translocation process and the extent of the toxicity of microfibers, and MPs in general, can depend on the size of the particles, their shape (fiber, sphere, or fragment), the type of polymer, and the exposure dose (Lithner et al., 2011; Prüst et al.,

2020; Banerjee and Shelver, 2021). Therefore, accurately inferring the hazard related to the ingestion of MPs is an extremely complicated task. Studies indicate that MPs smaller than 150 μm can be transferred from the gastrointestinal tract to the lymphatic and circulatory systems. However, it is estimated that the human body absorbs ≤ 0.3% of MPs and that only the smallest particles (≤ 20 μm) can enter the organs. Access to all organs and crossing cell barriers would be feasible for particles ≤ 0.1 μm (Barboza et al., 2018). The translocation capacity of MPs in the human organism can be so extensive that it can even reach the placenta in pregnant women. These particles could be transported through the respiratory or gastrointestinal system to the blood system, and finally, reach the placenta. Once in the placenta, MPs can alter the mechanisms of immunity and communication between the embryo and the uterus during pregnancy, which can also result in complications, such as pre-eclampsia and limitations of fetal growth (Ragusa et al., 2021).

Besides the presence of MPs in drinking water, natural and artificial materials also deserve attention. Although cellulose-based microfibers are more rapidly biodegradable, the risk they pose to the environment cannot be ignored. As synthetic microfibers, natural and artificial ones contain chemical additives that can be leached into the environment and can result in harmful effects to vertebrates and invertebrates through the ingestion of these microparticles. Additionally, the greater biodegradability of cellulosic-based microfibers (natural and artificial) could be an aggravating factor by accelerating the release of additives in the environment or in the organism of living beings which have ingested them (Ladewig et al., 2015; Remy et al., 2015). In a recent study by Kim et al. (2021), it was reported that the ingestion of artificial lyocell microfibers led to intestinal damage in the *Artemia franciscana* shrimp species, as in the case of synthetic microfibers (PP and PET). It demonstrated that the possible toxic effects of the intake of natural and artificial microfibers should also be considered, in addition to those of synthetic microfibers. In another study, a mean of 1.38 ± 0.79 fibers (0.1 mm to 6 mm) per organism was found in the intestine of invertebrates belonging to the phylum Arthropoda, collected in the Bay of Calvi (Corsica). When carrying out the analysis of blue and red fibers, it was observed that, although the polymeric base of the fibers was of cellulosic origin, the dyes "Direct Blue 22" and "Direct Red 28" were also identified via Raman. According to the authors, isolated cellulose would not be an

environmental problem. However, when it is treated with additives such as dyes, its toxic potential can be high. For example, the "Direct Red 28" dye identified in fibers ingested by invertebrates is classified as carcinogenic, mutagenic, or toxic. In the human intestine, there are bacteria capable of reducing chemical bonds of this additive, which gives rise to benzidine molecules, which are related to bladder and pancreatic cancer in humans (Remy et al., 2015).

3.3. Determination of the optimal concentration of Nile Red and the fluorescence of virgin polymers

To determine the optimal concentration of Nile Red for staining microfibers, initially, the inherent fluorescence of the PET microfibers was analyzed. It was observed that unstained PET microfibers did not show any fluorescence (Fig. 3). Three different concentrations of Nile Red (1 mg/L, 5 mg/L, and 10 mg/L) were tested to determine the best concentration for staining the microfibers. Results are shown in Fig. 3. After staining, with 1 mg/L Nile Red, it was observed that the PET microfibers emitted a red fluorescence, which was not previously appreciated in the unstained microfiber. The increase in the concentration of Nile Red to 5 mg/ L and 10 mg/L caused, in both cases, the increase of the background fluorescence and therefore, it was decided to use a concentration of 1 mg/L of Nile Red. These results are coincident with the ones reported by Erni-Cassola et al. (2017). This concentration was finally used to evaluate the staining of the other microfibers (natural, artificial, and synthetic) used in this study.
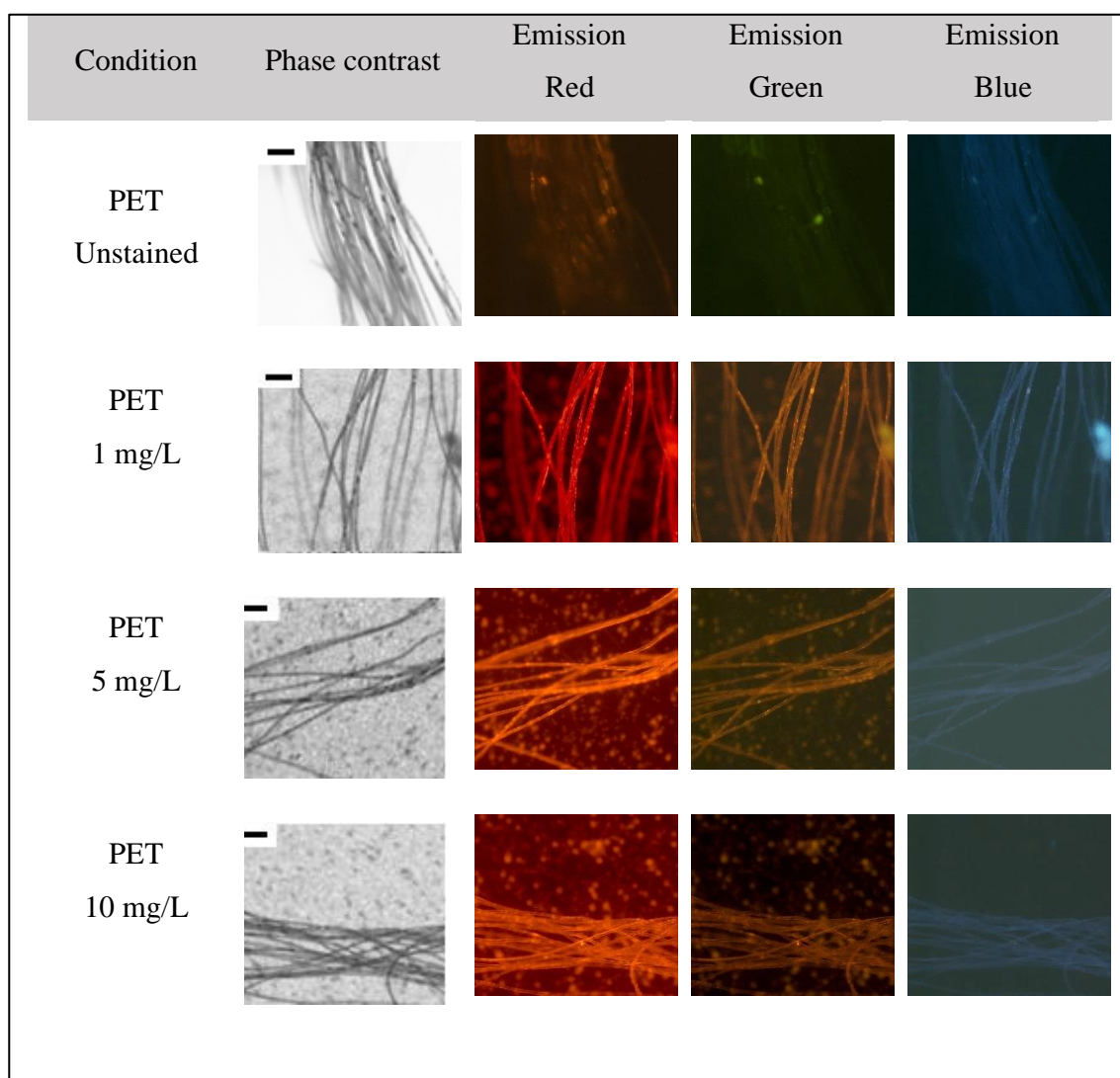
Fig. 3. PET fluorescence after staining with different concentrations of Nile Red solution (Scale bar 100 µm; magnification 100 X)

As performed with PET microfibers, the microfibers of PP, rayon, cotton, and wool were analyzed in the epifluorescence microscope before the use of Nile Red and the inherent fluorescence of the microfibers was determined (Fig. 4). From the results of the inherent fluorescence of the microfibers, it was stated that cotton microfibers exhibited an intense blue fluorescence when excited at 365 nm. No fluorescence was observed when this natural polymer was excited at 456 – 490 nm and 534 – 558 nm. Wool showed slight fluorescence when excited. The rest of the microfibers (PET, PP, and Rayon) did not show any fluorescence before staining.

Fig.4 also shows the images of the natural, synthetic, and artificial fibers after being stained with 1 mg/L of Nile Red. The fluorescence emitted by wool does not seem to be affected by the presence of Nile Red. The PP microfibers showed a slight red and strong green fluorescence. Cotton exhibited red fluorescence apart from the blue fluorescence naturally manifested without Nile Red. Finally, Rayon showed a slight red fluorescence. Based on these results, it can be stated that the identification of synthetic, natural, and artificial microfibers by fluorescence implies the evaluation of different excitation wavelengths. For instance, if just the red fluorescence was used to identify the presence of microfibers, PET and Rayon polymers would be bright, which could lead to a misinterpretation of results.
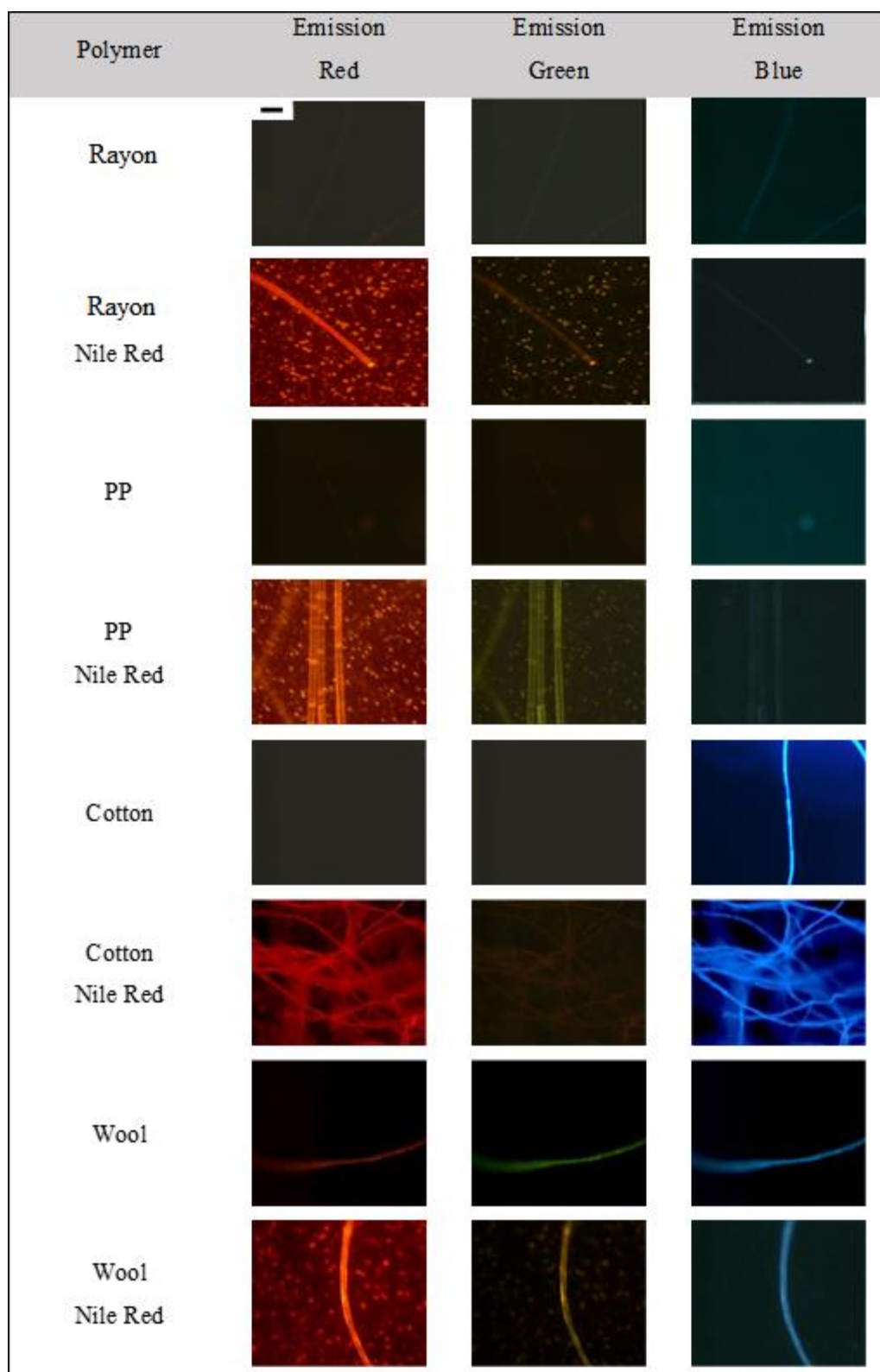
Fig.4. Inherent fluorescence of textile microfibers and fluorescence after staining with 1mg/L Nile Red (Scale bar 100 µm; magnification 100 X)

Although the material identification of MPs with spectrometric techniques is very precise, the use of these technologies can be expensive and very time-consuming. For this reason, Nile Red can be applied for staining MPs not only for distinguishing MPs from natural microfibers but also as an alternative technique for material identification. Table 2 shows the materials and staining conditions used in this work with those of other authors (Erni-Cassola et al., 2017; Maes et al., 2017; Prata et al., 2019). Some differences in results can be observed even when similar protocols were applied. For instance, Erni-Cassola et al. (2017) found that all MPs fragments tested by them (except the black tire rubber) emitted in green after being stained with Nile Red. The authors stated that the use of green fluorescence was more suitable for the identification of MPs (compared to red fluorescence) because synthetic polymers fluoresced better under this condition. When red fluorescence was tested, weak or null fluorescence was emitted by MPs (PP, PET, and PS). In addition, Erni-Cassola et al. (2017) stated that chitin (a natural polymer) also showed green fluorescence after staining with Nile Red, which could lead to a misinterpretation of results and overestimation of MPs. However, in this work, a very similar staining protocol has led to null green fluorescence of PET and a slight green fluorescence of PP microfibers. These differences in results may be related to microscope conditions (e.g., filter setup, wavelengths, light), or even could be related to the particles' shape. In another study, Prata et al. (2019) tested several wavelengths to find the best conditions to identify MPs, observing that different materials had better fluorescence under different wavelengths. For instance, at 470 nm only virgin HDPE and PVC and weathered PE fibers did not show any fluorescence with Nile Red. However, under this wavelength, natural organic matter also fluoresced. At 530 nm almost all textile material fluoresced, except viscose and wool, CA and some fragments of natural organic matter also fluoresced at this wavelength. Unlike the results observed by Prata et al. (2019), in this study wool microfibers showed a red fluorescence under 534 – 558 nm. Consequently, it is paramount to validate the applied protocol based on the experimental conditions. e.g., the microscopy used, solvent, and concentration of Nile Red among others. Once these considerations had been taken into account, a procedure consisting in varying the excitation wavelength could help to identify the materials of the microfibers saving time and costs.

Table 2 – Studies related to the use of Nile Red for identifying MPs and experimental conditions applied

Concentration	Solvent	Conditions	Reference
1 mg/L	Methanol	60 min at 60 ℃	This study
1 mg/L	Methanol	10 min at 60 ℃	Erni-Cassola et al. (2017)
10 mg/L	Acetone	30 min	Maes et al. (2017)
10 mg/L	Ethanol	30 min	Prata et al. (2019)
Polymers and shape			**Reference**
Virgin microfibers (PP, PET, Cotton, wool, and rayon). Weathered microfibers from drinking water.			This study
Virgin fragments (PE, PET, PVC, PS, PC, PUR, black tire rubber) and nylon-6 fibers. Weathered polymers collected from a beach in the UK.			Erni-Cassola et al. (2017)
Virgin fragments (LDPE, HDPE, PP, PS, PET, EPS, CA, PVC, and virgin microfiber (Cotton, Linen, Polyester, cotton and polyester, nylon, and rayon). Weathered polymers collected from a beach in Portugal. Organic materials (algae, driftwood, feathers, bivalve shell, charcoal, fish muscle, shrimp chitin shell, shrimp muscle, palm fat, paraffin)			Prata et al. (2019)

3.4. Nile Red fluorescence of microfibers isolated from drinking water (DWTP-A) and determination of polymer type

Regarding the fluorescence of microfibers isolated from drinking water samples, it was observed that some microfibers showed an evident blue fluorescence pattern, whereas no green and red fluorescence were observed. The spectra obtained for all microfibers with this fluorescence pattern were identified as PET (Fig. 5). However, based on the fluorescence results for virgin polymers after staining (Fig. 4), the PET microfibers showed only red fluorescence. The discrepancy could be related to the fact

that microfibers found in the environment can undergo numerous degradation processes or adsorb organic or inorganic materials, which can alter their surface chemical structure. Since the manifestation of fluorescence by Nile Red is linked to the polarity of the surface of the polymers (Maes et al., 2017), the modifications on the polymeric chain of microfibers due to degradation processes or adsorption of foreign compounds could influence the fluorescence generated by Nile Red. Similar results were also observed by Prata et al. (2019). The authors stated that PE virgin fluoresced after being stained with Nile Red, however, weathered PE did not. In addition to the degradation processes, the presence of additives in the fiber's structure could also result in different fluorescence. This initial observation indicates that the Nile Red staining process could be affected by specific external parameters such as the surface of the microfibers. Therefore, microfibers could present different manifestations of fluorescence for the same polymeric matrix.
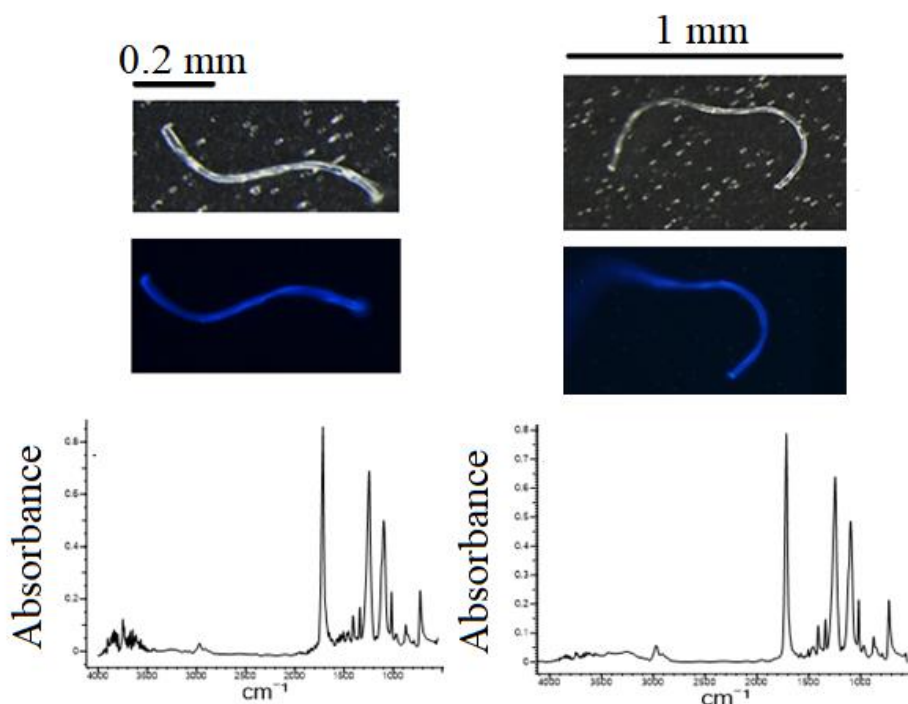

Fig.5. Microfibers collected in drinking water with blue fluorescence identified as PET

In addition to PET microfibers, natural cotton microfibers were also identified in drinking water. Unlike the PET microfibers, the cellulose microfibers did not show a single fluorescence pattern. According to the fluorescence results, cotton microfibers

could have three different manifestations of fluorescence, which were called Pattern 1, Pattern 2, and Pattern 3. The fluorescence emission patterns (Fig.6). correspond to blue and red fluorescence, non-existent or slight red fluorescence, and green and red fluorescence, respectively.
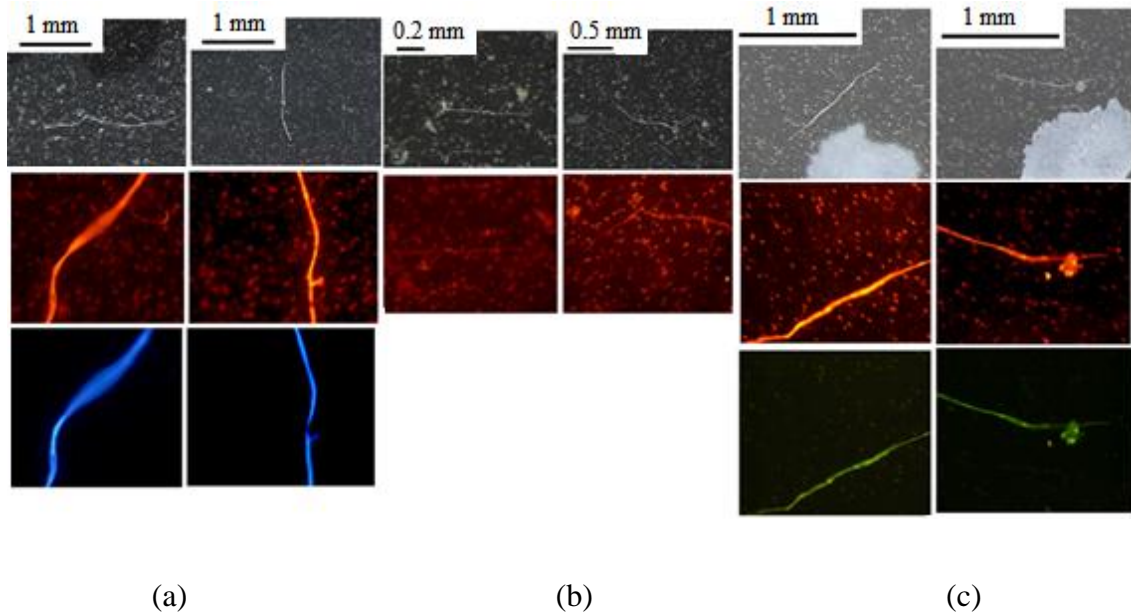

(a)   (b)   (c)

Fig.6. Fluorescence Pattern 1 (a), Pattern 2 (b) and Pattern 3 (c) for cotton microfibers

Pattern 1 was expected in agreement with the results presented in Fig.4, in which the virgin cotton fiber emitted blue and red fluorescence after staining with Nile Red. However, Patterns 2 and 3 were novel. Fig.7 shows the spectra of the microfiber material obtained by µ-ATR-FTIR.
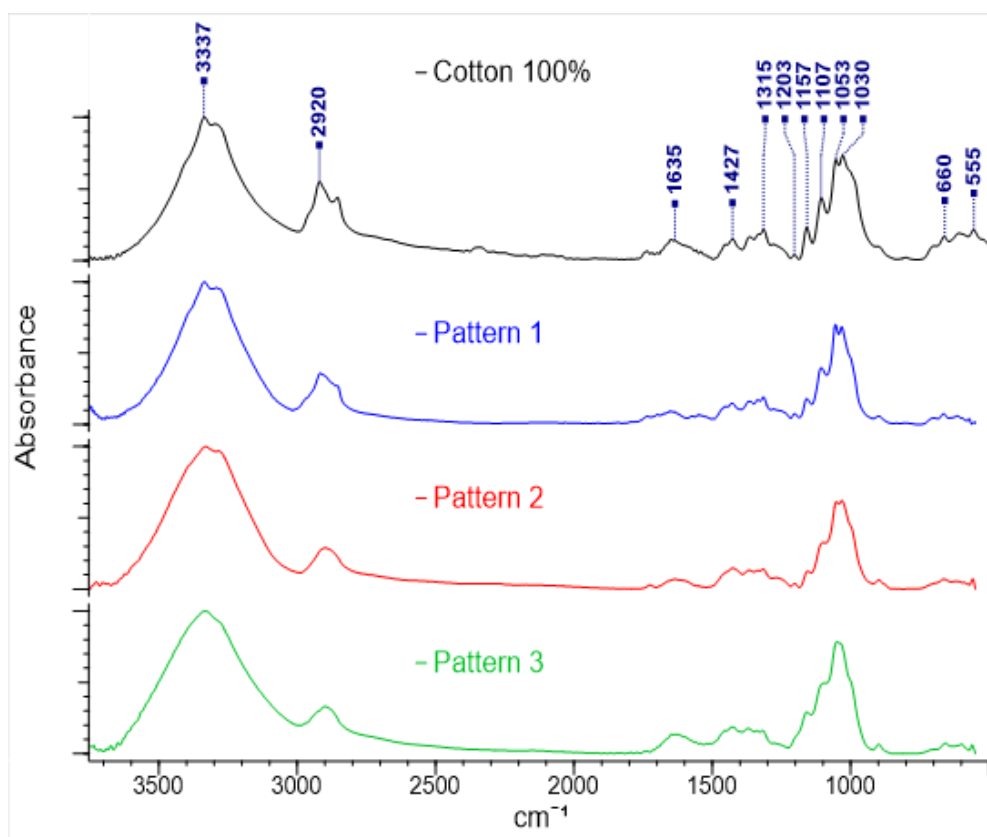
Fig.7. μ-ATR-FTIR spectra corresponding to cotton fibers fluorescence patterns

According to the μ-ATR-FTIR spectra, the manifestation of different fluorescence patterns could be related to the degree of degradation of the fibers. In Pattern 1 it was observed that the characteristic peaks of virgin cotton fibers were maintained. It could be assumed that cotton microfibers with this fluorescence pattern are approximate to virgin cotton fibers recently disposed of in the environment, with little degradation. However, in Pattern 2 it was observed that the peaks located at 1030 $\pm$ 6 cm^{-1}, 1107 $\pm$ 6, and 1157 $\pm$ 6 cm^{-1} lost intensity compared to the virgin cotton structure and showed less pronounced picks. Finally, in Pattern 3 the peaks 1030 $\pm$ 6 cm^{-1}, and 1203 $\pm$ 6 cm^{-1} were almost imperceptible, and the peaks 1107 $\pm$ 6 cm^{-1}, 1157 $\pm$ 6 cm^{-1} were further reduced compared to Pattern 2. The progressive smoothing of the peaks in the cotton structure could be related to breaks in the cellulose chain due to degradation processes. Li et al. (2010) reported similar results in their study on the biodegradability of cotton fibers. The authors also observed that in the spectra of cotton fibers after going through a degradation process via composting, the peaks at 1032 cm^{-1}

and 1061 cm^{-1} suffered an expressive reduction. Furthermore, the decrease in the intensity of the peaks at 1169 cm^{-1} and 1111 cm^{-1} was also detected. The reduction of the peaks was related to the breaking of the main chains of the cellulose. Coletti et al. (2021) investigated whether the degradation processes could influence the identification of fibers by ATR-FTIR and observed that, indeed, the degradation of the natural fibers (flax and hemp) studied affected their FTIR spectra in such a way that the two materials can become indistinguishable.

Related to rayon, this artificial microfiber collected from drinking water also presented two fluorescence patterns. Fluorescence pattern 1 corresponds to the manifestation of blue and red fluorescence and pattern 2 corresponds to absence or slight red fluorescence (Fig.8). However, in this case, it was not possible to link their relationship with the degradation processes. It would be necessary to analyze more microfibers of this material to improve the understanding of the phenomenon.
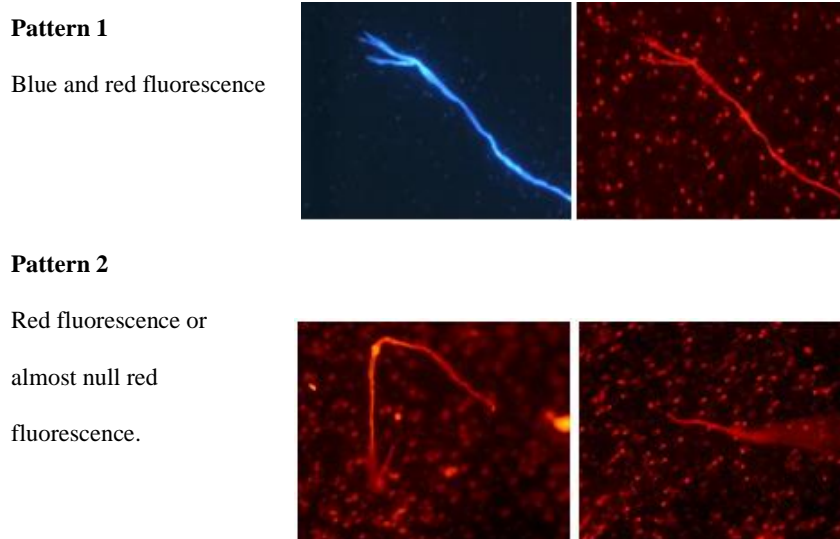
Pattern 1

Blue and red fluorescence

Pattern 2

Red fluorescence or

almost null red

fluorescence.

Fig.8. Fluorescence patterns for rayon microfibers from drinking water

Table 3 summarizes the fluorescence patterns observed herein for each microfiber identified in drinking water. Due to the coincidence of fluorescence patterns 1 and 2 between cotton and rayon microfibers, these materials could not be differentiated after staining with Nile Red. However, the global presence of natural and artificial microfibers (cotton and rayon) and synthetic microfibers (polyester) could be estimated. In this study, just the fluorescence of cotton, rayon, and polyester from

drinking water samples was determined since they were the predominant microfibers in the samples. Nevertheless, other natural, artificial, and synthetic polymers should be investigated for understanding their interactions with the Nile Red reagent.

Table 3 - Microfiber fluorescence patterns after staining with Nile red identified in drinking water (n = number of samples)

Polymer		Fluorescence pattern			n
Cotton	1	Blue	Red		9
Rayon	1	Blue	Red		1
Cotton	2		Red/Null		6
Rayon	2		Red/Null		2
Cotton	3		Red	Green	3
PET (polyester)		Blue			5
Total					**26**

3.5. Application of the Nile Red staining method to estimate microfibers in tap water (DWTP-B)

To determine the feasibility of using the Nile Red staining method in terms of the percentage estimation of synthetic, natural, and artificial microfibers in drinking water, a sampling of 29 randomly selected microfibers from the tap water sample was taken. The fluorescence of every microfiber was measured based on fluorescence patterns described in table 3 (Fig. 9b). Results showed that 93% of microfibers followed a fluorescence pattern that would correspond to natural/artificial microfibers whereas only 7% of PET fibers. To check these results, the material identification of microfibers with μ-ATR-FTIR was carried out (Fig. 9a). Comparing the fluorescence after the staining, it can be stated that Nile Red could help in the estimation of MPs (synthetic microfibers) and other microfibers (natural/artificial) present in drinking water. Further analysis should be carried out in order to confirm the feasibility of the method.
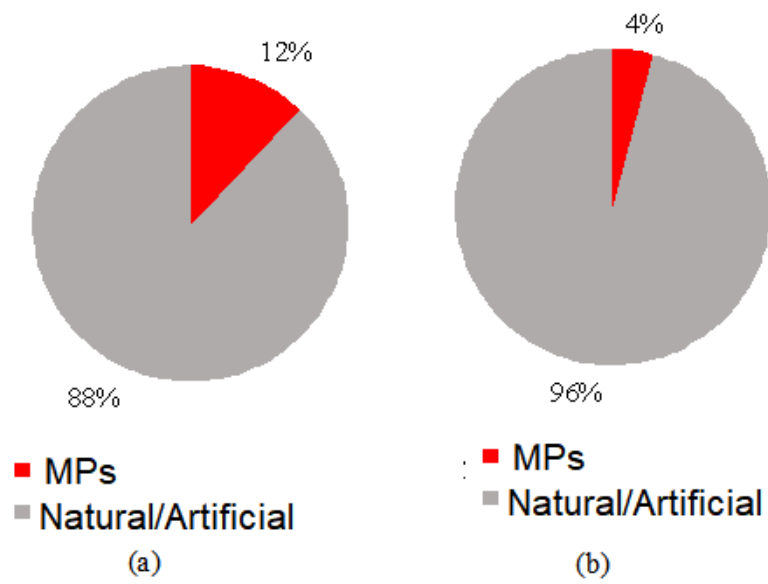
Fig.9. Microfibers material identification with (a) µ-ATR-FTIR s and (b) fluorescence emission

Conclusions

This study investigated the occurrence of microfibers in the water of two DWTPs. In DWTP-A, microfibers were eliminated in a percentage of 86% (comparing raw water and water after disinfection). Microfibers were identified in all the samples as most of the microparticles (75 % of microparticles identified).

FTIR results showed that synthetic, natural, and artificial polymers were present in all the samples. Concerning the use of Nile Red to identify microfibers material in drinking water, it was observed that its use for the visual sorting of microfibers - between natural, artificial, and synthetic -is not an obvious procedure since the manifestation of fluorescence can be influenced by the level of degradation of the material. Moreover, since the fluorescence of the materials could be affected by the applied experimental conditions such as the microscopy (lamp and a set of filters), solvent, and concentration of Nile Red, it is important to validate the method proposed by staining virgin polymers and compare its efficiency when the method is applied to microfibers from drinking water samples. Taking into account these considerations, the use of Nile Red could help to identify the material of the microfibers. For it, the fluorescence of the microfibers after staining with Nile Red using different excitation wavelengths is proposed from the results of this work. In the analyzed samples of tap water (DWTP-B) with this methodology, it was identified 4% of synthetic microfibers. In addition, the staining with Nile Red could also be an interesting tool for helping in understanding the levels of degradation of these materials in the environment, and even in estimating the time they have been in nature.

Acknowledgments

REFERENCES

Almroth, C.B.M., Åström, L., Roslund, S., Petersson, H., Johansson, M., Persson, N.K., 2018. Quantifying shedding of synthetic fibers from textiles; a source of microplastics released into the environment. Environ. Sci. Pollut. Res. 25, 1191–1199. https://doi.org/10.1007/s11356-017-0528-7.

Athey, S.N., Adams, J.K., Erdle, L.M., Jantunen, L.M., Helm, P.A., Finkelstein, S.A., Diamond, M.L., 2020. The Widespread Environmental Footprint of Indigo Denim Microfibers from Blue Jeans. Environ. Sci. Technol. Lett. 7, 840–847. https://doi.org/10.1021/acs.estlett.0c00498

Banerjee, A., Shelver, W.L., 2021. Micro- and nanoplastic induced cellular toxicity in mammals: A review, Sci. Total Environ. 755 (2021) 142518. https://doi.org/10.1016/j.scitotenv.2020.142518.

Baran, A., Fiedler, A., Schulz, H., Baranska, M., 2010. In situ Raman and IR spectroscopic analysis of indigo dye. Anal. Methods 2, 1372–1376. https://doi.org/10.1039/c0ay00311e

Barboza, L.G.A., Dick Vethaak, A., Lavorante, B.R.B.O., Lundebye, A.K., Guilhermino, L., 2018. Marine microplastic debris: An emerging issue for food security, food safety, and human health. Mar. Pollut. Bull. 133, 336–348. https://doi.org/10.1016/j.marpolbul.2018.05.047

Briain, O. Ó., Marques Mendes, A.R., McCarron, S., Healy, M.G., Morrison, L., 2020. The role of wet wipes and sanitary towels as a source of white microplastic fibres in the marine environment. Water Res. 182, 116021. https://doi.org/10.1016/j.watres.2020.116021.

Browne, M.A., Crump, P., Niven, S.J., Teuten, E., Tonkin, A., Galloway, T., Thompson, R., 2011. Accumulation of microplastic on shorelines woldwide: Sources and sinks. Environ. Sci. Technol. 45, 9175–9179. https://doi.org/10.1021/es201811s.

Chu, X., Zheng, B., Li, Z., Cai, C., Peng, Z., Zhao, P., Tian, Y., 2022. Occurrence and distribution of microplastics in water supply systems: In water and pipe scales. Sci. Total Environ. 803, 150004. https://doi.org/10.1016/j.scitotenv.2021.150004.

Coletti, F., Romani, M., Ceres, G., Zammit, U., Guidi, M.C., 2021. Evaluation of microscopy techniques and ATR-FTIR spectroscopy on textile fibers from the Vesuvian area: A pilot study on degradation processes that prevent the characterization of bast fibers. J. Archaeol. Sci. Reports 36, 102794. https://doi.org/10.1016/j.jasrep.2021.102794

De Falco, F. De, Pia, M., Gentile, G., Di, E., Escudero, R., Villalba, R., Mossotti, R., Montarsolo, A., Gavignano, S., Tonin, C., Avella, M., 2018. Evaluation of microplastic release caused by textile washing processes of synthetic fabrics. Environ. Pollut. 236, 916–925. https://doi.org/10.1016/j.envpol.2017.10.057.

Erni-Cassola, G., Gibson, M.I., Thompson, R.C., Christie-Oleza, J.A., 2017. Lost, but found with nile red: a novel method for detecting and quantifying small microplastics (1 mm to 20 µm) in environmental samples. Environ. Sci. Technol. 51, 13641–13648. https://doi.org/10.1021/acs.est.7b04512.

Fadare, O.O., Okoffo, E.D., 2020. Covid-19 face masks: A potential source of microplastic fibers in the environment. Sci. Total Environ. 737, 140279. https://doi.org/10.1016/j.scitotenv.2020.140279.

Henry, B., Laitala, K., Klepp, I.G., 2019. Microfibres from apparel and home textiles: Prospects for including microplastics in environmental sustainability assessment. Sci. Total Environ. 652, 483–494. https://doi.org/10.1016/j.scitotenv.2018.10.166.

Hernandez, E., Nowack, B., Mitrano, D.M., 2017. Polyester Textiles as a Source of Microplastics from Households: A Mechanistic Study to Understand Microfiber Release during Washing. Environ. Sci. Technol. 51, 7036–7046. https://doi.org/10.1021/acs.est.7b01750.

Kankanige, D., Babel, S., 2020. Smaller-sized micro-plastics (MPs) contamination in single-use PET-bottled water in Thailand. Sci. Total Environ. 717, 137232. https://doi.org/10.1016/j.scitotenv.2020.137232.

Kazour, M., Terki, S., Rabhi, K., Jemaa, S., Khalaf, G., Amara, R., 2019. Sources of microplastics pollution in the marine environment: Importance of wastewater treatment plant and coastal landfill. Mar. Pollut. Bull. 146, 608–618. https://doi.org/10.1016/j.marpolbul.2019.06.066.

Kim, L., Kim, S.A., Kim, T.H., Kim, J., An, Y.-J., 2021. Synthetic and natural microfibers induce gut damage in the brine shrimp Artemia franciscana. Aquat. Toxicol. 232, 105748. https://doi.org/10.1016/j.aquatox.2021.105748.

Konde, S., Ornik, J., Prume, J.A., Taiber, J., Koch, M., 2020. Exploring the potential of photoluminescence spectroscopy in combination with Nile Red staining for microplastic detection. Mar. Pollut. Bull. 159, 111475. https://doi.org/10.1016/j.marpolbul.2020.111475.

Kosuth, M., Mason, S.A., Wattenberg, E. V., 2018. Anthropogenic contamination of tap water, beer, and sea salt. PLoS One 13, 1–18. https://doi.org/10.1371/journal.pone.0194970.

Ladewig, S.M., Bao, S., Chow, A.T., 2015. Natural Fibers: A Missing Link to Chemical Pollution Dispersion in Aquatic Environments. Environ. Sci. Technol. 49, 12609–12610. https://doi.org/10.1021/acs.est.5b04754.

Lares, M., Ncibi, M.C., Sillanpää, Markus, Sillanpää, Mika, 2018. Occurrence, identification and removal of microplastic particles and fibers in conventional activated sludge process and advanced MBR technology. Water Res. 133, 236–246. https://doi.org/10.1016/j.watres.2018.01.049.

Lee, J., Kang, M.H., Lee, K.B., Lee, Y., 2013. Characterization of natural dyes and traditional korean silk fabric by surface analytical techniques. Materials (Basel). 6, 2007–2025. https://doi.org/10.3390/ma6052007.

Li, L., Frey, M., Browning, K.J., 2010. Biodegradability study on cotton and polyester fabrics. J. Eng. Fiber. Fabr. 5, 42–53. https://doi.org/10.1177/155892501000500406.

Lithner, D., Larsson, Å., Dave, G., 2011. Environmental and health hazard ranking and assessment of plastic polymers based on chemical composition. Sci. Total Environ. 409, 3309–3324. https://doi.org/10.1016/j.scitotenv.2011.04.038.

Maes, T., Jessop, R., Wellner, N., Haupt, K., Mayes, A.G., 2017. A rapid-screening approach to detect and quantify microplastics based on fluorescent tagging with Nile Red. Nat. Publ. Gr. 1–10. https://doi.org/10.1038/srep44501.

Martinez, V., Henary, M., 2016. Nile Red and Nile Blue: Applications and Syntheses of Structural Analogues. Chem. Eur. J., 22(39), 13764–13782. https://doi.org/10.1002/chem.201601570.

Mason, S.A., Welch, V.G., Neratko, J., 2018. Synthetic polymer contamination in bottled water. Front. Chem. 6, 1–17. https://doi.org/10.3389/fchem.2018.00407.

Napper, I.E., Thompson, R.C., 2016. Release of synthetic microplastic plastic fibres from domestic washing machines: Effects of fabric type and washing conditions. Mar. Pollut. Bull. 112, 39–45. https://doi.org/10.1016/j.marpolbul.2016.09.025.

Nel, H.A., Chetwynd, A.J., Kelleher, L., Lynch, I., Mansfield, I., Margenat, H., Onoja, S., Goldberg Oppenheimer, P., Sambrook Smith, G.H., Krause, S., 2021. Detection limits are central to improve reporting standards when using Nile red for microplastic quantification. Chemosphere 263. https://doi.org/10.1016/j.chemosphere.2020.127953.

Pedrotti, M.L., Petit, S., Eyheraguibel, B., Kerros, M.E., Elineau, A., Ghiglione, J.F., Loret, J.F., Rostan, A., Gorsky, G., 2021. Pollution by anthropogenic microfibers in North-West Mediterranean Sea and efficiency of microfiber removal by a wastewater treatment plant. Sci. Total Environ. 758, 144195. https://doi.org/10.1016/j.scitotenv.2020.144195.

Pivokonský, M., Cermakova, L., Novotna, K., Peer, P., Cajthaml, T., Janda, V., 2018. Occurrence of microplastics in raw and treated drinking water. Sci. Total Environ. 643, 1644–1651. https://doi.org/10.1016/j.scitotenv.2018.08.102.

Prata, J.C., Reis, V., Matos, J.T.V., da Costa, J.P., Duarte, A.C., Rocha-Santos, T., 2019. A new approach for routine quantification of microplastics using Nile Red and automated software (MP-VAT). Sci. Total Environ. 690, 1277–1283. https://doi.org/10.1016/j.scitotenv.2019.07.060.

Prata, J.C., da Costa, J.P., Fernandes, A.J.S., da Costa, F.M., Duarte, A.C., Rocha-Santos, T., 2021. Selection of microplastics by Nile Red staining increases

environmental sample throughput by micro-Raman spectroscopy. Sci. Total Environ. 783, 146979. https://doi.org/10.1016/j.scitotenv.2021.146979.

Prüst, M., Meijer, J., Westerink, R.H.S., 2020. The plastic brain: neurotoxicity of micro- and nanoplastics, Part. Fibre Toxicol. 17, 24. https://doi.org/10.1186/s12989-020-00358-y.

Ragusa, A., Svelato, A., Santacroce, C., Catalano, P., Notarstefano, V., Carnevali, O., Papa, F., Rongioletti, M.C.A., Baiocco, F., Draghi, S., D'Amore, E., Rinaldo, D., Matta, M., Giorgini, E., 2021. Plasticenta: First evidence of microplastics in human placenta. Environ. Int. 146, 106274. https://doi.org/10.1016/j.envint.2020.106274.

Remy, F., Collard, F., Gilbert, B., Compère, P., Eppe, G., Lepoint, G., 2015. When Microplastic Is Not Plastic: The Ingestion of Artificial Cellulose Fibers by Macrofauna Living in Seagrass Macrophytodetritus. Environ. Sci. Technol. 49, 11158–11166. https://doi.org/10.1021/acs.est.5b02005.

Shruti, V.C., Pérez-Guevara, F., Roy, P.D., Kutralam-Muniasamy, G., 2022. Analyzing microplastics with Nile Red: Emerging trends, challenges, and prospects. J. Hazard. Mater. 423, 127171. https://doi.org/10.1016/j.jhazmat.2021.127171.

Sillanpää, M., Sainio, P., 2017. Release of polyester and cotton fibers from textiles in machine washings. Environ. Sci. Pollut. Res. 24, 19313–19321. https://doi.org/10.1007/s11356-017-9621-1.

Stolte, A., Forster, S., Gerdts, G., Schubert, H., 2015. Microplastic concentrations in beach sediments along the German Baltic coast. Mar. Pollut. Bull. 99, 216–229. https://doi.org/10.1016/j.marpolbul.2015.07.022.

Wang, Z., Lin, T., Chen, W., 2020. Occurrence and removal of microplastics in an advanced drinking water treatment plant (ADWTP). Sci. Total Environ. 700, 134520. https://doi.org/10.1016/j.scitotenv.2019.134520.

Capítulo IX

Conclusiones

La presencia de MPs y NPs ha sido investigada en los últimos años de modo frecuente en el ciclo del agua. Sin embargo, la falta de metodologías estandarizadas para su cuantificación e identificación hace que los análisis de estas partículas generen discrepancias en la discusión de resultados en la comunidad científica. Con la intención de mejorar la discusión de los resultados entre investigadores, en esta tesis se proponen metodológicas para la separación, cuantificación e identificación de MPs en muestras de diferentes corrientes, tanto de la línea de aguas como de la línea de fangos de una Estación de Depuración de Aguas Residuales, así como el estudio de la presencia de microfibras naturales, artificiales y sintéticas en el agua natural y potable.

Con la relación al uso de digestiones químicas para la preparación de muestras, se demostró que la oxidación química con peróxido de hidrógeno es un proceso efectivo para reducir considerablemente la concentración de materia orgánica y sólidos en suspensión de muestras de efluentes de los tratamientos primarios y secundarios, así como de fango activo, permitiendo la separación efectiva y la clasificación visual de posibles microplásticos. Las condiciones en las que la peroxidación resultó ser más efectiva para la disminución de los sólidos en suspensión fueron: tiempo de oxidación de 2 horas y temperatura de 60ºC. La proporción en volumen H_2O_2 (35%)/muestra fue de 1/100 para el efluente primario y 2/1 para el fango activo. No fue necesaria la peroxidación mara muestras de efluentes secundarios por su bajo contenido en materia orgánica. En cuanto a los tamaños de los MPs identificados en muestras de la EDAR, se ha optado por un protocolo de filtración que permite la determinación de MPs de un tamaño de entre 150 μm y 5 mm. De las muestras analizadas, se concluye que la mayor cantidad de MPs se concentra en los fangos, habiendo determinado una concentración media de 280 micropartículas/L (o 112 micropartículas/gMS) en el fango activo. En efluente secundario se determinaron menos de 3 micropartículas/L (0,44 MPs/L), que, si bien parece una concentración pequeña, significa que cada día se vierten millones de

MPs a las aguas naturales, ya que las depuradoras de aguas residuales no están diseñadas para la separación de MPs.

Se observó que los espectros obtenidos vía FTIR tras la peroxidación no mostraron interferencia de materiales orgánicos, lo que corrobora la eficiencia del método de digestión basado en la peroxidación. Además, la peroxidación no comprometió la identificación de polímeros vía ATR-FTIR, puesto que los picos más característicos de los espectros de los polímeros no se modificaron. Respecto a la forma de las micropartículas encontradas en las muestras analizadas, las microfibras son la fracción más abundante de las micropartículas identificadas en las muestras de efluente primario, secundario y fango activo, constituyendo más del 90%. Las microfibras pueden ser naturales, artificiales y sintéticas, siendo estas últimas las que se consideran microplásticos. Con relación a la identificación de micropartículas presentes en el efluente secundario, se observó que las microfibras naturales de algodón fueron las más abundantes. Sin embargo, este polímero con una base natural, también pueden ser considerado un problema ambiental similar a los microplásticos, ya que lleva en su composición diferentes aditivos (como colorantes y estabilizantes). Con relación a los fragmentos caracterizados, los polímeros PE y PP fueron los más abundantes.

La elevada presencia de micropartículas antropogénicas de origen natural y artificial observada en las muestras estudiadas jutifica la relevancia de estudiar no solo la presencia de MPs sino también la nocividad que pueden representar estos materiales para el medio ambiente. Por lo tanto, en muestras complejas como fango digerido y fango deshidratado se investigó la efectividad de diferentes protocolos para la extracción de micropartículas antropogénicas (entre 150 μm y 5 mm). La metodología seleccionada consistió en la combinación de la peroxidación (tiempo igual a 4 horas, temperatura igual a 60ºC) y la separación por densidad, siendo más efectivo el uso de una disolución de NaI (densidad = 1,5 g/mL) debido a su mayor densidad con relación a la disolución de NaCl. Cabe resaltar que el orden de las etapas (peroxidación y separación por densidad) en la extracción de micropartículas en muestras complejas no afectó al resultado final. Sin embargo, el uso de la peroxidación demostró ser una etapa fundamental para romper la estructura del lodo y liberar las micropartículas a la fase líquida. La caracterización del licor del fango procedente de la etapa de centrifugación indica que esta corriente es una fuente importante de microplásticos en la EDAR, y

también merece atención su caracterización. Las muestras de fango deshidratado son las que más micropartículas contenían. En concreto, se estimó que las muestras analizadas podrían contener aproximadamente 2966 micropartículas /gMS lo que confirma los resultados discutidos anteriormente en que se observó una acumulación significativa acumulo de micropartículas en el fango.

Durante la operación del reactor biológico secuencial (SBR) de laboratorio al que se añadieron en el agua residual simulada microesferas de PE (PE-MPs), se observó que el 98 ± 2 % de los PE-MPs introducidos al sistema se concentraban en el licor de mezcla, y la fracción restante salía por el efluente. Este resultado corrobora las observaciones anteriores, es decir, la mayor parte de los MPs que entran al sistema secundario de tratamiento de aguas residuales pasan al fango y quedan retenidos en su matriz orgánica. Aunque que se haya observado una elevada retención de microesferas PE-MP en el fango, éstas no interfirieron en la capacidad de depuración del reactor SBR en términos de eliminación de la materia orgánica y proceso de nitrificación. Sin embargo, la diversidad de la comunidad microbiana del fango activo se vio reducida. Las medidas realizadas respecto a las actividades enzimáticas (MHEA) en el SBR con PE-MP revelaron un aumento de la actividad de la proteasa lo que podría estar relacionado con la degradación parcial de las microesferas plásticas (PE-MP). Con relación a los nanoplásticos de poliestireno de 100 nm de diámetro (PS-NP, 2 μg/L), estos tampoco interfirieron negativamente en el proceso de depuración por fangos activos, no viéndose afectadas la eliminación de la materia orgánica ni el proceso de nitrificación durante el periodo del ensayo. La identificación de una menor abundancia relativa de bacterias *Nitrotoga* en el SBR con NPs respecto al reactor SBR de control, sugiere que los NPs ensayados podrían inhibir el desarrollo de este género bacterias y, a largo plazo, el proceso de nitrificación podría verse comprometido. Además, la abundancia ligeramente superior del filo *Patescibacteri*a en el SBR con nanopartículas respecto al SBR-Control, sugiere que este filo puede tener cierta sensibilidad a la presencia de nanopartículas de poliestireno (de 100 nm), lo cual podría considerarse como un bioindicador de la contaminación por MPs/NPs.

La técnica de ultrasonidos (US) puede convertirse en una excelente herramienta para reducir la concentración de microplásticos en el fango, ya que genera la disrupción de los flóculos contenidos en el fango mediante un proceso de cavitación. La técnica ha

permitido separar el $48 \pm 4\%$ de microplásticos de polietileno contenidos en muestras de fango activo en las condiciones óptimas de operación (frecuencia de 80 kHz, potencia de entrada 50 W y tiempo 8 min 45 seg). La efectividad de la técnica de US depende de la concentración de sólidos en las muestras, siendo necesario adaptar las condiciones óptimas de operación para conseguir rendimientos de separación aceptables de microplásticos. Así, en el caso del fango secundario concentrado, se consiguió separar un 38 ± 4 % de los microplásticos en las condiciones óptimas de operación (80 kHz; 90 W; 30 min).

Finalizando el estudio de la presencia de MPs en el ciclo del agua se comprobó que estas micropartículas también se encuentran presentes en las aguas naturales, siendo mayoritariamente microfibras, que igualmente pueden ser clasificadas como naturales, artificiales o sintéticas. Incluso tras su paso por una estación de tratamiento de agua potable se detectaron más de 500 microfibras/L. Los resultados mostraron que las microfibras pueden emitir fluorescencia en función del polímero, ya sea como fluorescencia natural o tras tinción y excitación a diferentes longitudes de onda. En este sentido, con relación al uso de la técnica de tinción con Rojo Nilo para mejorar la identificación visual de las microfibras en muestras de aguas naturales y potables, se observó que diferentes polímeros vírgenes (sintéticos, naturales y artificiales) manifiestan diferentes fluorescencias. Sin embargo, tras analizar estos mismos polímeros, pero siendo extraídos de muestras reales, se apreciaron diferentes fluorescencias. Esta discrepancia entre resultados de materiales vírgenes y muestras reales podría estar relacionada con diferentes grados de degradación de los materiales, adsorción de materia orgánica o presencia de diferentes aditivos. Sin embargo, la investigación sobre el uso de las técnicas de tinción aplicada a la identificación rápida y eficaz de microplásticos y microfibras se postula como una opción muy interesante a considerar en este campo, dado el coste en tiempo y dinero que implica el uso de las técnicas espectroscópicas.