Biobed biopurification systems with agroindustrial wastes to remove organic contaminants from water: microbiological and molecular aspects

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Biobed biopurification systems with agroindustrial wastes to remove organic contaminants from water: microbiological and molecular aspects

Memoria presentada por la Licenciada en Biología y Bioquímica

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Para aspirar al Título de Doctora por la Universidad de Granada

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ESTACIÓN EXPERIMENTAL DEL ZAIDÍN, CSIC. UNIVERSIDAD DE GRANADA

Esta Tesis Doctoral ha sido realizada en el Departamento de Protección Ambiental, dentro de los grupos de investigación “Relaciones Planta-Suelo” y “Microbiología Ambiental y Biodegradación”, de la Estación Experimental del Zaidín (EEZ-CSIC), perteneciente al Consejo Superior de Investigaciones Científicas, en Granada, gracias a la ayuda para contratos predoctorales para la formación de doctores 2014 (FPI) (BES-2014-070043). El estudio se encuadró dentro de los objetivos científicos-técnicos propuestos en los proyectos de investigación subvencionados por la Comisión Interministerial de Ciencia y Tecnología dentro del Programa de I+D+i orientada a los Retos de la Sociedad y cofinanciado con fondos FEDER, CTM2013-44271-R “Sistemas de biorremediación biobeds con residuos agroindustriales para eliminar contaminantes orgánicos de aguas: biodisponibilidad y aspectos microbiológicos y moleculares” y CTM2017-86504-R “Innovación de sistemas de biorremediación biobed mediante bioaumentación y nuevos materiales adsorbentes para eliminar contaminantes emergentes y plaguicidas de las aguas”. Algunas de las actividades científicas incluidas en este trabajo fueron realizadas en la sección de Microbiología del Departamento de Biología de la Universidad de Copenhague durante la estancia breve realizada en el extranjero por la Lcda. Ines Mª Aguilar Romero durante el periodo de disfrute de la Beca FPI concedida.

“Nuestras vidas son tejidas
por el mismo hilo que nuestros sueños”
William Shakespeare

“No dejes apagar el entusiasmo,
virtud tan valiosa como necesaria;
trabaja, aspira, tiende siempre hacia la altura”
Rubén Darío

“El éxito no es la clave de la felicidad,
la felicidad es la clave del éxito.
Si amas lo que haces, tendrás éxito”
Albert Schweitzer

“Un día sin sonreír, es un día perdido”
Charles Chaplin

A mis padres

A mis hermanos

A Santi

A la memoria de mis abuelos

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RESUMEN

El deterioro de la calidad del agua debido a la presencia de contaminantes orgánicos generados por las actividades industriales, agrícolas y urbanas conlleva una problemática ambiental ya que afecta a la calidad de los ecosistemas y a la salud humana. Junto a los plaguicidas, comienzan a ser un problema la acumulación de otros contaminantes emergentes como los productos farmacéuticos y de cuidado personal (PPCP-Pharmaceutical and personal care products). En general, la degradación de estos contaminantes durante el tratamiento convencional en plantas depuradoras de aguas residuales basado en lodos activados suele ser incompleta y permanecen en los efluentes que son descargados al medio ambiente (Fekadu et al., 2019; Petrović et al., 2003; Rosal et al., 2010). Estas plantas depuradoras carecen de medios para detectar y eliminar completamente estos contaminantes ya que en principio no se diseñaron para este fin. En consecuencia, la entrada incontrolada de estas sustancias en los ecosistemas contribuye a su detección en aguas superficiales y subterráneas, suelo, sedimentos, sólidos en suspensión e incluso en el agua de consumo humano (Houtman, 2010; Jurado et al., 2019; Wilkinson et al., 2017). Estos contaminantes son nocivos para numerosos organismos terrestres y acuáticos, actuando generalmente como disruptores endocrinos (Farré et al., 2008; Houtman, 2010; Nilsen et al., 2019). Por estos motivos, y teniendo en cuenta la creciente demanda de este recurso natural indispensable para el desarrollo de la humanidad y del medioambiente, así como las exigencias de la Directiva Marco del agua (2000/60/CE) referente a la recuperación de la calidad química y ecológica de las aguas, es una prioridad científico-técnica de interés mundial desarrollar sistemas que permitan eliminar de forma eficiente y sostenible los contaminantes orgánicos de las aguas y evitar así su impacto en otros compartimentos ambientales (Grassi et al., 2013). Entre las técnicas de biorremediación destacan los sistemas de biopurificación (BPSs-Biopurification systems), también conocidos como biobeds o “camas biológicas”, los cuales son de fácil implantación y han resultado ser muy eficientes para evitar contaminaciones puntuales que puedan ocurrir durante la carga y limpieza de la maquinaria de dispersión de pesticidas (Grassi et al., 2013). Estos sistemas consisten en excavaciones o contenedores rellenos de biomezclas activas que retienen y degradan los contaminantes, y han llamado la atención en numerosos países donde se está trabajando para adaptarlo a las condiciones y aplicaciones

locales (De Wilde et al., 2007; Dias et al., 2020). La biomezcla original consiste en suelo, turba (materia orgánica estable) y paja (agente estructurante) en proporciones 25:25:50 % en volumen. No obstante, la escasez de estos materiales en algunas regiones ha llevado a la búsqueda de otros materiales alternativos más disponibles y económicos en la zona.

En España, nuestro grupo de investigación ha desarrollado por primera vez biomezclas a partir de residuos orgánicos de bajo coste muy abundantes en nuestra región procedentes de la agroindustria del aceite de oliva (alperujo y la poda de olivar), residuos de invernadero y de la producción del vino o alcohol (lodos de vinaza, orujillo y sarmientos). Estudios previos han descrito la eficacia de estas biomezclas para eliminar diversos tipos de pesticidas y se ha seleccionado la biomezcla más óptima para extender la aplicación de estos sistemas de biopurification para eliminar los PPCPs presentes en aguas residuales (Delgado-Moreno et al., 2019, 2017a).

En esta memoria de tesis doctoral se inicia una investigación de carácter multidisciplinar que aborda diversos aspectos bioquímicos, microbiológicos y genéticos de un sistema de biopurificación, a fin de demostrar su utilidad para eliminar de forma eficiente tres PPCPs, ibuprofeno, diclofenaco y triclosan, que frecuentemente se detectan en los recursos hídricos. Asimismo, se ha estudiado la ecotoxicidad de estos compuestos y su impacto sobre la biodiversidad de las poblaciones microbianas empleando técnicas microscópicas y de secuenciación masiva. En esta tesis doctoral también se desarrollan técnicas de bioaumentación a fin de promover una eliminación más rápida de los PPCPs, así como de dos herbicidas fenilurea, diuron y linuron, y de sus metabolitos más recalcitrantes en los BPSs. Para ello, se ha realizado la búsqueda de microorganismos autóctonos o exógenos resistentes o potencialmente degradadores de estos compuestos.

Considerando que los métodos convencionales de bioaumentación usan tecnologías complejas para su aplicación en campo, se ha diseñado una técnica novedosa, sencilla y de bajo coste para incrementar la eficacia de los BPSs mediante la bioaumentación con microorganismos autóctonos, con la ventaja adicional de que se puede realizar a escala de campo. Esta nueva alternativa de bioaumentación se basa en la aplicación de extractos líquidos aireados ricos en microorganismos procedentes de medios previamente expuestos a los

contaminantes. Para la obtención de estos extractos serán muy útiles las propias biomezclas que conforman los BPSs que tras un periodo de funcionamiento están envejecidas o agotadas. En cuanto a la bioaumentación con bacterias exógenas, se ha aislado y caracterizado genéticamente una cepa bacteriana capaz de mineralizar ibuprofeno, con el fin de determinar los genes implicados en la ruta de degradación de este compuesto. Además, se ha estudiado la supervivencia de esta cepa, así como su desarrollo en diferentes ambientes con el fin de proponer posibles aplicaciones para promover la eliminación de ibuprofeno y/o sus metabolitos de las aguas residuales de forma rápida y eficaz.

Esta actividad investigadora se ha reunido en tres capítulos que se comentan a continuación.

En el primer capítulo se determinó el potencial de un sistema de biopurificación modelo constituido por una biomezcla compuesta por suelo, vermicompost de alperujo y poda de olivar para degradar ibuprofeno, diclofenaco y triclosan. En estos BPSs construidos a escala microcosmo se aplicaron los contaminantes tanto individualmente como en mezcla a altas concentraciones. De este modo se han podido conocer las interacciones existentes entre los contaminantes y su repercusión en las cinéticas de disipación de cada uno de ellos, así como el posible efecto tóxico de los PPCPs sobre las comunidades bacterianas indígenas de la biomezcla. Asimismo, se estudiaron e identificaron las comunidades microbianas resistentes a los contaminantes y los principales grupos bacterianos potencialmente involucrados en la degradación de estos PPCPs. Los resultados revelaron que esta biomezcla elimina eficientemente el ibuprofeno seguido del diclofenaco, mientras que el triclosan requiere un periodo de incubación más largo. Además, la disipación del diclofenaco se ve reducida en presencia de los otros PPCPs. El análisis microscópico de células bacterianas vivas mediante la técnica LIVE/DEAD® puso de manifiesto que el triclosan causó un impacto negativo inicial sobre la viabilidad bacteriana. No obstante, este efecto fue mitigado cuando los PPCPs se aplican simultáneamente. Los análisis estadísticos realizados revelaron que las comunidades bacterianas de los microcosmos se ven afectadas principalmente por el tiempo de incubación más que por los PPCPs aplicados. Sin embargo, estos contaminantes afectaron de manera diferente la composición y abundancia relativa de los taxones

bacterianos. Tanto el ibuprofeno como el diclofenaco inicialmente aumentaron la diversidad y riqueza bacteriana, mientras que la exposición a triclosan generalmente provocó un efecto opuesto, sin una recuperación completa al final del período de incubación. El triclosan fue el compuesto que causó un mayor impacto en las comunidades bacterianas, afectando negativamente la abundancia relativa de *Acidobacteria*, *Methylophilales* y *Legionellales*. Por último, se buscaron biomarcadores consistentes en unidades taxonómicas operacionales (OTU-Operational Taxonomic Unit) predominantes a altas concentraciones de PPCPs y que, por lo tanto, probablemente alberguen mecanismos de degradación y / o detoxificación.

En el segundo capítulo se ensaya por primera una técnica de bioaumentación sencilla y de bajo coste para acelerar la degradación de contaminantes orgánicos y facilitar su aplicación a escala de campo. Esta técnica consiste en la aplicación de extractos líquidos aireados ricos en microorganismos, generados a partir de distintos tipos de biomezclas previamente expuestas a los contaminantes. El potencial de degradación de estos extractos se ensaya tanto en sistemas acuosos como en los BPSs.

El primer estudio de bioaumentación basado en estos extractos se orienta a acelerar la eliminación de pesticidas. Para ello, se emplean biomezclas constituidas por residuos agroindustriales de la producción de aceite de oliva procedentes de estudios anteriores realizados a escala piloto y, por tanto, ya aclimatadas a estos contaminantes. Los resultados obtenidos demuestran que los extractos están enriquecidos en poblaciones microbianas que mejoran la eliminación de los herbicidas fenilurea, diuron y linuron, usados como compuestos modelo, así como de su metabolito más recalcitrante y tóxico, la 3,4-dicloroanilina. De los extractos ensayados, el más efectivo proviene de una biomezcla compuesta por vermicompost de alperujo. El análisis de las comunidades bacterianas enriquecidas en esta biomezcla tras cinco meses de incubación con los contaminantes y de su correspondiente extracto, permitió discriminar grupos de microorganismos potencialmente implicados en la degradación de estos herbicidas. En la biomezcla, se observó un incremento en la abundancia relativa de los taxones *Chloroflexi*, *Acidobacteria*, *Gemmatimonadetes*, *Firmicutes*, *Deinococcus-Thermus* y *Proteobacteria* tras la

aplicación de pesticidas. En el extracto correspondiente de esta biomezcla se observa un enriquecimiento de los géneros *Dokdonella* y *Pseudomonas*.

El segundo estudio de bioaumentación con extractos líquidos aireados se enfoca a la eliminación de PPCPs. Con este fin, se llevó a cabo una aclimatación de la biomezcla de vermicompost de alperujo con los PPCPs para enriquecerla en microorganismos degradadores autóctonos. Seguidamente se comprobó la eficacia del extracto líquido aireado procedente de esta biomezcla para acelerar la eliminación de estos contaminantes y de sus principales productos de degradación. Los resultados mostraron que este extracto mejora la degradación del diclofenaco y del triclosan en los BPSs que son relativamente más persistentes en la biomezcla, así como del ibuprofeno en sistemas acuosos contaminados, y además evita la acumulación de metabolitos secundarios como el metil-triclosan y metabolitos hidroxilados del ibuprofeno. El análisis de las poblaciones microbianas permitió conocer los cambios poblacionales ocurridos en la biomezcla tras la aclimatación con los PPCPs y en el extracto líquido a fin de identificar los grupos resistentes y potencialmente involucrados en la mejora de la degradación de estos contaminantes. Los OTUs más dominantes detectados en el extracto pertenecieron a los grupos taxonómicos *Flavobacterium*, *Thermomicrobia*, *Nonomuraea* y *Fluviicola*.

El tercer capítulo está enfocado a la búsqueda de microorganismos exógenos con capacidad de degradar o resistir altas concentraciones de PPCPs a fin de utilizarlos para incrementar la eliminación de estos contaminantes emergentes tanto en los BPSs como en efluentes secundarios de plantas de tratamiento de aguas residuales. Para ello se llevaron a cabo enriquecimientos a partir de sedimentos del río Elba (Hamburgo, Alemania) expuestos a vertidos de contaminantes procedentes de la industria farmacéutica. En un primer estudio se aislaron y secuenciaron 5 cepas tolerantes a altas concentraciones de diclofenaco. Una de estas cepas pertenece al género *Achromobacter* y las otras cuatro al género *Pseudomonas*. Seguidamente, en un segundo estudio, se aisló una cepa capaz de utilizar ibuprofeno como única fuente de carbono y energía que se identificó como *Sphingopyxis granuli* RW412. El análisis genómico de esta cepa reveló que alberga genes con una similitud muy alta con los genes *ipf*, implicados en la ruta degradación de ibuprofeno propuesta por Murdoch and Hay (2013).

Para confirmar que RW412 degrada ibuprofeno mediante dicha ruta e identificar los metabolitos intermediarios producidos, se purificó la proteína IpfF que inicia la unión específica de coenzima A (CoA) al ibuprofeno generándose ibuprofenil-CoA. Este complejo se identificó por primera vez en este estudio mediante la técnica LC-MS. Este hallazgo, permite demostrar el primer paso en la ruta de degradación del ibuprofeno que se predice en la bibliografía existente. Asimismo, se determinó e identifico otro metabolito intermediario del proceso de degradación mediante las técnicas LC-MS y RMN, el 4-isobutilcatecol confirmando, por primera vez, que su estructura presenta grupos hidroxilo en las posiciones 1 y 2, lo que facilita la acción de extradiol dioxigenasas para su completa mineralización.

Por último, en este capítulo se han desarrollado distintos ensayos de bioaumentación con esta cepa exógena con el propósito de mostrar su potencial para acelerar la descontaminación del ibuprofeno tanto en sistemas de biopurificación como en efluentes secundarios. Los resultados obtenidos mostraron que la inoculación de la cepa RW412 aceleró la disipación de este contaminante en los BPSs evitando la acumulación de metabolitos hidroxilados y carboxilados del ibuprofeno. Asimismo, esta cepa fue capaz de eliminar el ibuprofeno mucho más rápido que la propia comunidad bacteriana autóctona que habita los efluentes secundarios procedentes de la planta de tratamiento (EDAR, Granada), eliminando el 100% de este contaminante en un sólo día, lo que indica que esta cepa posee un alto potencial de degradación para el ibuprofeno en estos efluentes.

I. GENERAL INTRODUCTION

1. ORGANIC CONTAMINANTS: Water pollution and environmental concern.

Water is an indispensable natural resource for humans and ecosystems. The deterioration of water quality and the scarcity of this resource in some regions as a result of increased water consumption, climate change and anthropogenic activities are of global concern (Sousa et al., 2018). The production and use of numerous chemical substances in medicine, industry and agriculture generate different types of pollutants such as metals, polycyclic aromatic hydrocarbons, polychlorinated biphenyl compounds, dioxins, dibenzofurans and chlorinated pesticides (Poynton and Robinson, 2018; Sousa et al., 2018). The main routes of entry for organic pollutants into environmental compartments are shown in Figure 1. Most of these compounds are apolar, toxic, persistent, and bioaccumulative, whose presence in the environment requires regulation by the implementation of different laws (Cuerda-Correa et al., 2020). In the past, attention focused on the direct effects of individual pollutants and their negative impact on ecosystems in the short term. However, a new environmental concern has emerged when evaluating the chronic effects caused by pollutants after a long period of exposure, even at low concentrations (Oller et al., 2011). In this context, the continuous and uncontrolled release of these chemical compounds into the environment contributes to their possible accumulation in aquatic environmental compartments (water, sediment and biota), which can pose harmful effects on both aquatic ecosystems and human health (Ribeiro et al., 2016). For this reason, efforts by the scientific community have focused on studying the impact of persistent organic compounds on the environment and different means to eliminate or prevent their entry into ecosystems.

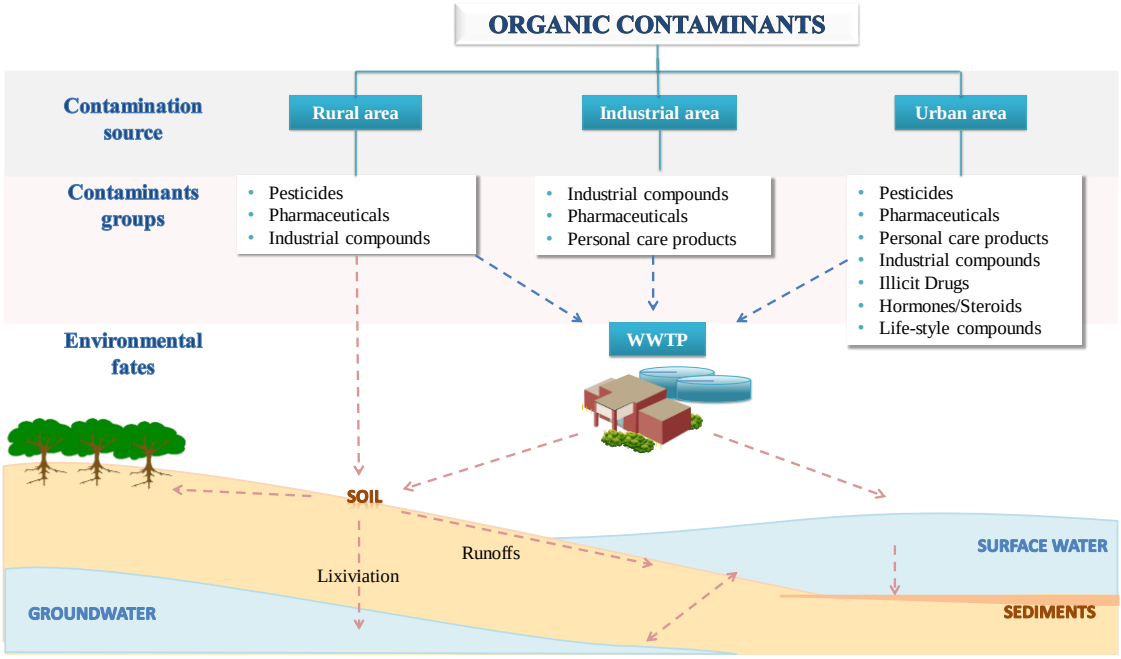


Figure 1. Main sources and environmental fates of different groups of organic contaminants.

In order to regulate the presence of some pollutants and to maintain suitable ecological and chemical quality of water resources, the European Union Water Framework Directive (EU WFD) was launched to monitor certain substances, giving rise to a great improvement in water protection policy (Sousa et al., 2018). The complete list of substances, the matrices that should be monitored as well as possible analysis techniques were published in Decision 2015/495/EU (2015) (Sousa et al., 2019). The European Union has developed even more restrictive directives to preserve the quality of water resources by reducing pollution levels and promoting their sustainable management. To achieve the goal to recycle and safely reuse wastewater which was set by European countries to be achieved by 2030 (UN Sustainable Development Goal on Water, SDG 6), effective strategies are required to purify wastewater from agricultural and industrial activities. This regulation is not exclusive to Europe. The United States Environmental Protection Agency (USEPA) has also developed a list of priority pollutants in which they identify an extensive variety of chemical compounds which have been detected in wastewater and storm water runoff that may pose a risk to ecosystems (Ebele et al., 2017).

1.1. EMERGING CONTAMINANTS: Environmental fate, ecotoxicological risk assessment and biotransformation.

In recent years, the development of more advanced and sensitive analytical techniques has allowed the detection of compounds of different chemical natures in waters and soil at very low concentrations (trace levels, ≤ ng L^{-1}). These compounds are known as "emerging contaminants". This term includes recently detected pollutants in the environment, and those compounds that potentially cause harmful effects on ecosystems or humans but the extent of which still has not been well established and consequently remains unregulated. (Houtman, 2010; Rivera-Utrilla et al., 2013).

In general, emerging contaminants are also constantly being introduced into the environment through different anthropogenic sources, such as industrial, rural and urban activities (Figure 1). These compounds are highly resistant to biodegradation, and so their removal during conventional activated sludge-based wastewater treatment is often incomplete. As a result, they remain in the effluents and enter surface and groundwater (Jackson and Sutton, 2008; Kostich et al., 2014). Thus, municipal sewage, wastewater treatment plants (WWTPs), accidental leaks, and landfills constitute important dispersion sources for these pollutants in the environment (Gomes et al., 2017). Moreover, the reuse of wastewater for irrigation purposes in areas with water shortages and the application of biosolids (treated sludge) and manures as fertilizers for agriculture are also a relevant entrance pathway for emerging contaminants into soil (Christou et al., 2019; Grassi et al., 2013; Verlicchi and Zambello, 2015). Consequently, these compounds may enter into plant tissues through root absorption (Li et al., 2020; Madikizela et al., 2018). Moreover, by runoff and lixiviation, they may also reach surface and ground waters to pose a threat to drinking water (Ebele et al., 2017; Schriks et al., 2010).

Table 1 summarizes the different types of emerging pollutants, including the main sub-groups and also examples of the most frequently detected compounds in environmental compartments.

Table 1. Types of emerging contaminants and examples of each sub-group.

Groups	Main subgroups	Examples
Pharmaceuticals	Analgesics	Paracetamol, Codeine
	Anti-inflamatorics	Ibuprofen, diclofenac
	Antibiotics	Amoxicillin, erythromycin
	Lipid regulator	Bezafibrate, gemfibrozil
	Antidepressant	Amitriptyline, fluoxetine
	Others	Atenolol, hydrochlorothiazide
Personal care products	Disinfectants	Triclosan, triclorocarban
	Fragrances	Musk xylol, isobornyl acetate
	Ultraviolet filters	Benzophenone-4, homosalate
Industrial compounds	Anti-oxidants	Butylated hydroxyanisole
	Phenols	Bisphenol-A; bisphenol F
	Phthalates	Diethy phthalate, dibutyl phthalate
	Flame retardants	Polybrominated diphenylethers
	Triazoles	Benzotriazole and tolyltriazole
Life-style compounds		Caffeine, nicotine
Synthetic hormones		Estrone, 17β-estradiol
Illicit drugs		Cocaine, methadone

Although emerging contaminants are often found at trace levels, their continuous release into ecosystems poses a risk to non-target organisms and public health due to bioaccumulation processes and to adsorption to sediments and suspended particles in aquatic ecosystems (Gomes et al., 2017; Verlicchi et al., 2012). Long-term exposure of these pollutants can cause chronic toxicity, endocrine disruption, or the development of bacterial resistance (Gavrilescu et al., 2015). Among the emerging contaminants most frequently detected in aquatic ecosystems are pharmaceuticals and personal care products (PPCPs) which are considered as priority compounds due to their harmful effects.

Pharmaceuticals and personal care products

Pharmaceutical products are therapeutic drugs used for the treatment or prevention of human and animal diseases, whilst personal care products are substances used mainly to improve hygiene and the quality of daily life (Boxall et

al., 2012). However, PPCPs are considered a unique group of emerging contaminants for their ability to induce physiological effects in non-target organisms, even at very low concentrations (Ebele et al., 2017).

As observed for emerging contaminants, PPCPs are commonly detected in surface and groundwaters at different concentrations (ng L^{-1} or μg L^{-1}) due to their continuous introduction into aquatic ecosystems mainly through sewage treatment effluents (Grassi et al., 2013). Many of these compounds have aromatic structures with different functional groups which are responsible for their stability and low water solubility based on their octanol/water partition coefficients (Log K_{ow}). Thus, their bioavailability and biodegradability are low. In this study, three PPCPs with different properties which are frequently detected in waters were selected (Table 2).

Table 2. Main physicochemical properties of selected PPCPs; ibuprofen, diclofenac and triclosan.

PPCP	Molecular Weight (g mol^{-1})	Water Solubility (mg L^{-1})	pK_a	log K_{ow}
Ibuprofen	206.3	21	4.91	3.97
Ibuprofen sodium salt	228.3	1 x 10^5	4.85	0.92
Diclofenac	296.1	2.37	4.15	4.51
Diclofenac sodium salt	318.1	50 x 10^3	4.0	0.7
Triclosan	289.5	10	7.90	4.76

The removal capacities by sewage treatment plants reported for these PPCPs with respect to the amounts determined in the influents are: 74.2 % for IBP, 34.6 % for DCF and 76.8 for TCS (Deblonde et al., 2019). Due to their high worldwide consumption, biological activity, continuous release and persistence in ecosystems, these PPCPs are considered target compounds to be removed from the environment.

1.1.1. Ibuprofen

Ibuprofen (2-(4-isobutylphenyl)-propionic acid) belongs to the family of pharmaceuticals classified as non-steroidal anti-inflammatory drugs (NSAID),

and is one of the most widely used drugs worldwide for its analgesic, antipyretic and anti-inflammatory properties (Figure 2). This drug is used for the treatment of arthritis and moderate pain including headache and dental pain (Rainsford, 2009).

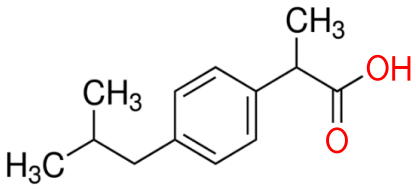


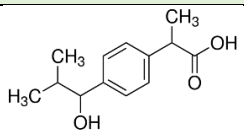
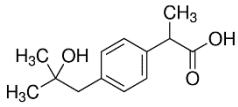
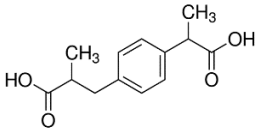
Figure 2. Chemical structure of ibuprofen (CAS 15687-27-1).

1.1.1.1. Environmental fate of ibuprofen

The annual production of ibuprofen ranges in millions of tons, and the yearly average consumption of this NSAID is between 58 and 300 tons in countries such as Poland, England and Germany (Marchlewicz et al., 2015). This large consumption has led to the presence of this drug in many aquatic ecosystems due to its continuous discharge in municipal, hospital and industrial effluents (Gutiérrez-Noya et al., 2020). However, others countries such as Norway or Denmark show relatively low ibuprofen consumption (Gutiérrez-Noya et al., 2020). Approximately 15% of the ibuprofen consumed by humans is excreted in an unchanged form, and the rest is excreted as glucuronide conjugates or as metabolites (Żur et al., 2018). The main metabolites in humans are carboxyibuprofen and 2-hydroxyibuprofen which represent 43% and 26% of the total excreted ibuprofen, respectively. Moreover, others metabolites such as 1-hydroxyibuprofen and glucuronic acid conjugates can be present in urine at lower concentrations (Farré et al., 2008; Ferrando-Climent et al., 2012). Both carboxyibuprofen and ibuprofen show the highest removal rates in WWTPs followed by 2-hydroxyibuprofen and 1-hydroxyibuprofen, which are considered the most recalcitrant of these metabolites (Ferrando-Climent et al., 2012). The detection of these compounds in WWTP effluents confirmed the inadequacy of this system to completely eliminate ibuprofen and its metabolites from wastewater before its discharge into the environment. Consequently, ibuprofen has been found at different concentrations in various environmental

compartments such as surface and groundwater, sediments and even in drinking water. Moreover, ibuprofen has been detected in soils irrigated with wastewater containing pharmaceuticals residues (Żur et al., 2018).

Table 3. Main physicochemical properties of ibuprofen metabolites.

Compound	Structure	Molecular Weight (g/mol)	pKa	Log Kow
1-Hydroxyibuprofen		222.28	4.55	2.69
2-Hydroxyibuprofen		222.28	4.63	2.37
Carboxyibuprofen		236.26	3.97	2.78

1.1.1.2. Ecotoxicology of ibuprofen

Several authors have observed a negative impact on aquatic organisms including fish, bivalves and crustaceans after prolonged exposure to ibuprofen. Yang et al. (2019) investigated the effects of ibuprofen and other two pharmaceuticals on the enzymatic activities in the brain and liver of the freshwater crucian carp, *Carassius auratus*. The results obtained demonstrated that exposure to ibuprofen and a PPCP mixture at environmentally relevant concentrations can be harmful to fish. In addition, Parolini et al. (2011) demonstrated that continued exposure to ibuprofen at environmental concentrations alters the activity of enzymes involved in oxidative stress such as catalase or superoxide dismutase causing moderate cellular and genetic damage to zebra mussel (*Dreissena polymorpha*). Furthermore, ibuprofen can act as an endocrine disruptor by increasing the production of 17β-estradiol and aromatase activity and reducing testosterone production in the freshwater species *Oryzias latipes, Daphnia magna* and *Moina macrocopa* (Han et al., 2010). Wang et al. (2016) also investigated the effects of ibuprofen exposure on the growth and reproduction of *Daphnia magna*. Their results revealed a significant decrease in

the total amount of eggs produced per female, the total number of brood per female, and body length.

1.1.1.3. Biodegradation of ibuprofen

Different biotransformation or biodegradation pathways of ibuprofen have been suggested (Figure 3). Chen and Rosazza (1994) described a bacterial strain, *Nocardia* sp. NRRL 5646, capable of biotransforming ibuprofen by a carboxylic acid reductase enzyme system with R(-)-enantioselective properties. The two major metabolites produced from racemic ibuprofen were ibuprofenol and ibuprofen acetate. Furthermore, Quintana et al. (2005) identified two hydroxyibuprofen isomers as intermediates during ibuprofen removal by microorganisms from activated sludge.

It is known that biological transformation is considered an important removal mechanism in wastewater treatment. In fact, several bacterial strains isolated from activated sewage sludge have the ability to use ibuprofen as a sole carbon and energy source. Murdoch and Hay (2013, 2005) proposed that an isolate, *Sphingomonas* sp. strain Ibu-2, degrades ibuprofen by CoA ligation followed by ring dioxygenation at the 1,2 position, giving rise to isobutylcatechol which is subsequently metabolized via *meta*-cleavage. Instead, *Variovorax* sp. strain Ibu-1, appears to transform ibuprofen to trihydroxyibuprofen and then, a *meta* ring-fission pathway could occur (Murdoch and Hay, 2015). Additionally, Salgado et al. (2020) isolated *Patulibacter medicamentivorans* from sludge, another strain with the ability to degrade ibuprofen. They analysed the toxicity and the biodegradability potential of the detected metabolites and proposed two possible biodegradation pathways for this strain, the first through the formation of isobutylbenzene or 2-phenylpropanoic acid with posterior ring cleavage. The other mechanism used by this strain could be by hydroxylation and carboxylation.

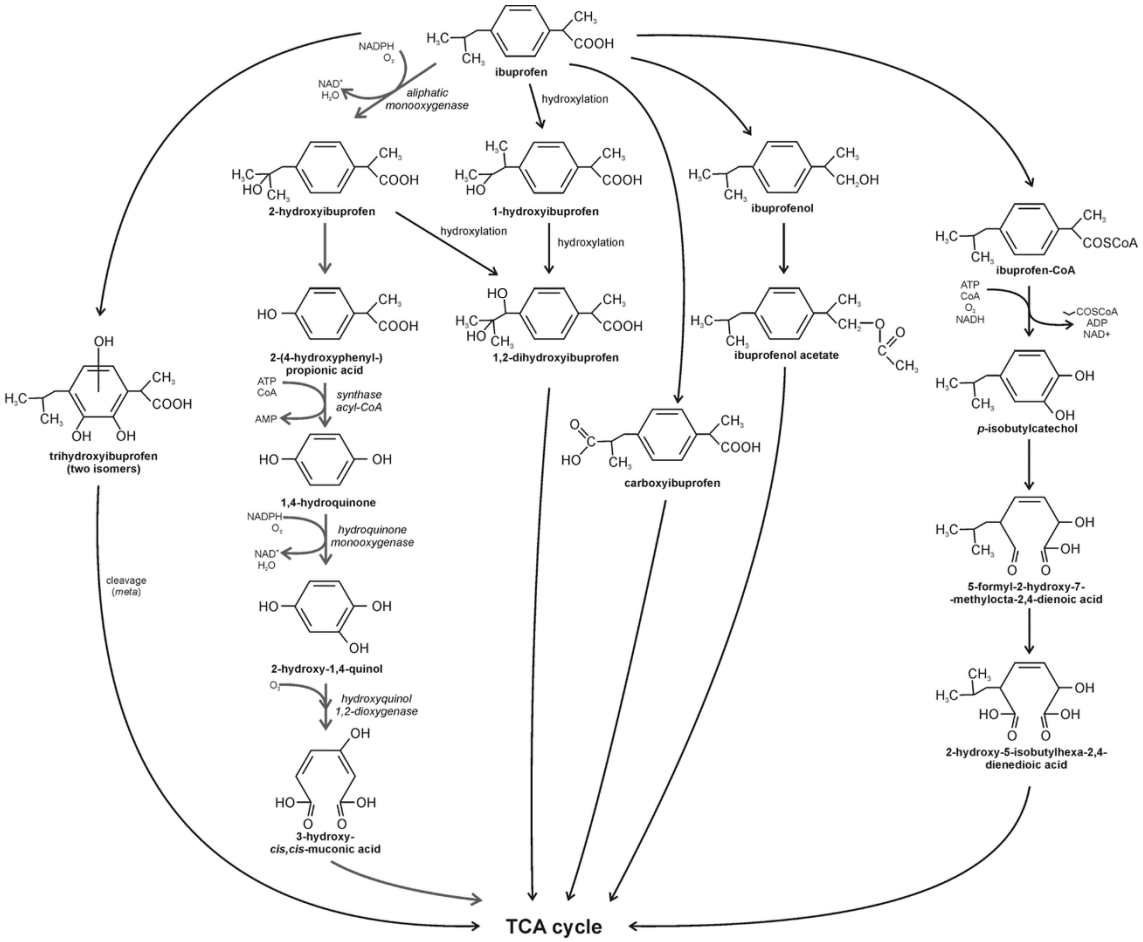


Figure 3. Suggested biodegradation pathways of ibuprofen (Żur et al., 2018).

On the other hand, microorganisms isolated from surface water bodies and soils which are capable of eliminating ibuprofen have also been obtained. In fact, a community composed of *Comamonas aquatic* and *Bacillus* sp. from river surface water efficiently removes this contaminant (Fortunato et al., 2016). Moreover, *Bacillus thuringiensis* B1, isolated from soil from a chemical factory, eliminates ibuprofen even in the presence of other aromatic compounds or heavy metals (Marchlewicz et al., 2017). In accordance with the specific activity of enzymes after induction by ibuprofen and the intermediates detected by GC-MS, a degradation pathway was proposed. In these pathways, it is proposed that the aliphatic chain and the aromatic ring of ibuprofen are hydroxylated by aliphatic monooxygenase and hydroquinone monooxygenase enzymes, respectively, giving rise to 2-hydroxy-1,4-quinol. Then, this intermediate could be subject to *ortho* cleavage of the aromatic ring by the activity of hydroxyquinoline 1,2-dioxygenase (Figure 3).

Degradation of ibuprofen can also be mediated by white-rot fungi. These degrading fungi produce the metabolite 1,2 –hydroxyibuprofen, which can be even more toxic than the parent compound (Marco-Urrea et al., 2009). However, this possible degradation pathway is still unconfirmed.

1.1.2. Diclofenac

Diclofenac (2-[2-(2,6-dichloroanilino)phenyl]acetic acid) is a NSAID commonly used since the 1970s to relieve pain, reduce swelling and ease inflammation in conditions affecting joints, muscles and tendons (Figure 4). This drug is consumed as oral tablets or as a topical gel for the treatment of arthritis or acute injuries (Lonappan et al., 2016). Furthermore, the use of pharmaceuticals containing diclofenac is approved in some countries for veterinary applications in domestic livestock such as horses, pigs and cattle (Green et al., 2016).

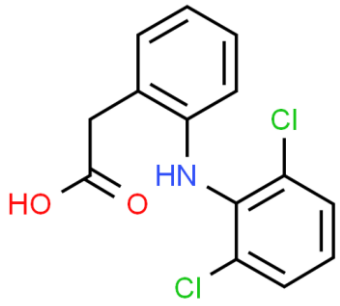


Figure 4. Chemical structure of diclofenac (CAS 15307-86-5).

1.1.2.1. Environmental fate of diclofenac

Diclofenac is sold under numerous trade names for human and veterinary use and is an over-the-counter medicine, making it difficult to calculate its global consumption. According to International Marketing Services health data analyses from 86 countries, the global annual consumption of diclofenac is approximately 1.450 tons, with 39.5% consumed in Asia and 28.7% in Europe (Acuña et al., 2015; Lonappan et al., 2016). Nevertheless, this estimation does not cover the consumption of diclofenac for veterinary uses.

Most orally administered diclofenac is metabolized by the human body and excreted in a conjugated form or as hydroxyl derivatives, and only < 1% is

eliminated as unmodified diclofenac. On the other hand, after topical application of diclofenac gel formulations, between 6-7% of this compound is absorbed by the skin while the remaining part is probably washed off and ends up in the wastewater (Davies and Andersen, 1997; Vieno and Sillanpää, 2014). In fact, this drug is frequently detected in the influents of WWTPs due to its continuous introduction from pharmaceutical industry, hospital and household drainage (Verlicchi et al., 2012).

The removal rate of diclofenac by the conventional process in WWTPs ranges to between 30-70%, which has led to the detection of this contaminant and its metabolites in many freshwater ecosystems worldwide (Acuña et al., 2015). In surface water, photodegradation is the main removal process for this pharmaceutical (Buser et al., 1998). Although further studies are required to investigate the toxicity of photodegradation products, some authors suspect that these metabolites can be more harmful than diclofenac. Moreover, diclofenac can interact with organic, inorganic compounds or even metabolites present in WWTP sludge to generate complexes of generally unknown toxicity that can also be released into the environment (Lonappan et al., 2016).

Many authors have investigated the occurrence of diclofenac in aquatic environments (surface water, groundwater, drinking water, wastewater), soil compartments (sediments, suspended solid and sewage sludge) and biota (aquatic and terrestrial organisms) (Sathishkumar et al., 2020). In general, the recorded concentrations of diclofenac in aquatic environments range from a few nanograms per litre to several micrograms per litre.

1.1.2.2. Ecotoxicology of diclofenac

The observed concentrations of diclofenac in ecosystems are of environmental concern as they can give rise to adverse effects on non-target organisms. For this reason, diclofenac was included in the watch list of substances in the EU that require environmental monitoring in Directive 2013/39/EU (Sathishkumar et al., 2020; Vieno and Sillanpää, 2014).

Several studies have shown that diclofenac is bioaccumulated and causes harmful effects on fish organs such as liver, kidneys and gills (Schwaiger et al.,

2004; Triebskorn et al., 2007) and delays the hatching time of eggs (Lee et al., 2011). In addition, diclofenac can affect other aquatic organisms such as mussels (Gonzalez-Rey and Bebianno, 2014; Quinn et al., 2011) and microcrustaceans (Ferrari et al., 2003). Haap et al. (2008) determined the harmful effect of diclofenac using acute toxicity tests for *Daphnia magna* and summarized studies which evaluate the ecotoxicology of this contaminant on bacteria, microalgae, crustaceans and fish.

On the other hand, it is important to highlight the negative impact of diclofenac on vulture populations in Asian countries (India, Nepal, Pakistan and Bangladesh). This drug caused a rapid decrease in the population of 'Gyps vultures' due to the accumulation of uric acid crystals in visceral organs after consumption of diclofenac-medicated domestic animals (Naidoo and Swan, 2009; Oaks et al., 2004; Sathishkumar et al., 2020).

Finally, the occurrence of diclofenac in soil through the use of sludge from WWTPs as a source of nutrients could generate phytotoxicity (Feito et al., 2012; Kummerová et al., 2016).

1.1.2.3. Biodegradation of diclofenac

The amount of diclofenac that reaches the ecosystems through sludge or effluents from the WWTPs is mitigated by natural processes such as biodegradation, soil retention or phototransformation (Lonappan et al., 2016). However, as indicated above, the concentrations detected in the environment can pose an ecological and human health risk. In order to avoid the presence of this drug and its metabolites in the environment, numerous physical, chemical and biological techniques have been investigated. Focusing on bioremediation techniques, the biotransformation of diclofenac mediated by microbial enzymes is of increasing interest because it is a fast, simple and eco-friendly strategy (Sathishkumar et al., 2020). Different white-rot fungal strains produce ligninolytic enzymes that catalyse the degradation of contaminants including PPCPs (Naghdi et al., 2018). Crude or purified laccases and peroxidases with high redox potential such as lignin peroxidase and versatile peroxidases from *Trametes versicolor*, *Myceliophthora thermophila*, *Phanerochaete*

chrysosporium and *Bjerkandera adusta,* or whole fungal cultures efficiently remove diclofenac (Eibes et al., 2011; Lloret et al., 2010; Marco-Urrea et al., 2010; Rodriguez-Rodriguez et al., 2011; Zhang and Geißen, 2010).

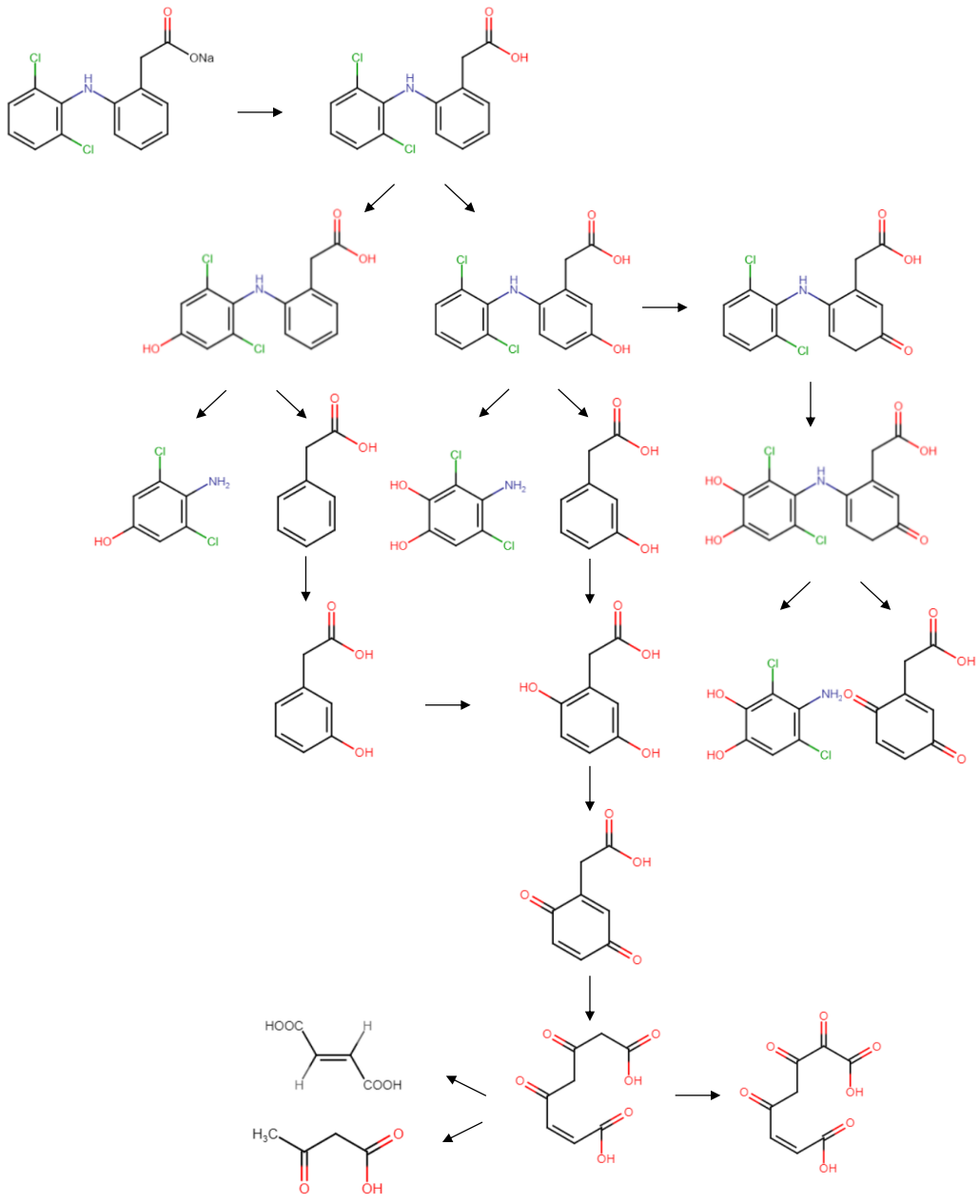


Figure 5. Proposed pathways for diclofenac biodegradation by *R. ruber* IEGM 346 (Ivshina et al., 2019).

Several microorganisms have shown the ability to biotransform this micro-pollutant. The bacterial strain, *Brevibacterium* sp. D4, isolated from activated sludge of a WWTP showed high biodegradation efficiency by dissipating up to

90% of the initial diclofenac via co-metabolism with acetate (Bessa et al., 2017). Similarly, the capacity of diclofenac biodegradation by another environmental isolate, *Labrys portucalensis* F11 strain, was improved by supplying acetate, achieving complete disappearance of the contaminant within 6 days (I. S. Moreira et al., 2018). Moreover, Murshid and Dhakshinamoorthy (2019) reported on a microbial consortium composed of *Alcaligenes faecalis*, *Staphylococcus aureus*, *Staphylococcus haemolyticus* and *Proteus mirabilis* with the ability to biotransform up to 134 mg L^{-1} of diclofenac sodium salt in 120 h. Recently, Ivshina et al. (2019) reported that the *Rhodococcus ruber* strain IEGM 346 is capable of completely biodegrading diclofenac and proposed a catabolic pathway base on the degradation products detected in the culture medium (Figure 5). Finally, several studies revealed that algae also show high diclofenac removal efficiencies (Ouada et al., 2019; Rabello et al., 2019).

1.1.3. Triclosan

Triclosan (5-chloro-2-(2,4-dichlorophenoxy)-phenol) (Figure 6) is a broad-spectrum antimicrobial agent that targets a specific bacterial fatty acid biosynthetic enzyme, enoyl-[acyl-carrier protein] reductase (Schweizer, 2001). This agent mainly exhibits antibacterial, but also some antifungal and antiviral activity, and is used in a wide range of personal care products, household items and medical devices including hand soaps, surgical scrubs, shower gels, deodorant soaps, toothpastes and mouthwashes (Fang et al., 2010; Halden et al., 2017; Schweizer, 2001).

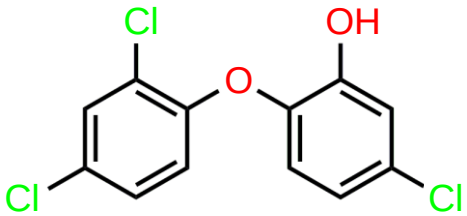


Figure 6. Chemical structure of triclosan (CAS 3380-34-5).

1.1.3.1. Environmental fate of triclosan

Despite certain restrictions established by the European Food Safety Authority and by the Food and Drug Administration in United States, products containing triclosan are widely used worldwide (Lee et al., 2019; Peng et al., 2019). Most of the triclosan present in these products is washed off during normal use, reaching WWTPs at concentrations in the order of 1 to 10 μg L^{-1} through hospital, industrial and domestic drainage (Anger et al., 2013). Unfortunately, like most emerging contaminants, triclosan is not efficiently removed from wastewater by the conventional processes carried out in WWTPs (Zheng et al., 2020). Hence, this product is commonly detected in surface waters receiving the effluents from WWTPs as well as in groundwater, and soil amended with WWTP sludge or irrigated with wastewater effluent (López-Pacheco et al., 2019; Wang et al., 2014). Furthermore, triclosan could eventually reach humans by drinking contaminated water or by consuming animals or vegetables exposed to triclosan. This agent is potentially bioaccumulative in both animal and plant tissues due to its lipophilic nature (log K_{ow} = 4.76) (Maulvault et al., 2019; Pannu et al., 2012). Moreover, during sewage treatment, triclosan can be transformed into methyl triclosan by biomethylation (Quan et al., 2019). This metabolite is even more harmful than triclosan because it is more persistent in the environment and more bioaccumulative in aquatic biota (Fu et al., 2020).

1.1.3.2. Ecotoxicology of triclosan

Numerous studies suggest that triclosan is an environmental stressor and causes toxic effects on bacteria, algae, crustaceans, oligochaetes, fish, insects, molluscs and amphibians (Orvos et al., 2002; Zheng et al., 2019). As an antimicrobial agent, triclosan alters the taxonomic composition of microbial communities and reduces significantly species richness and diversity (Clarke et al., 2019; Oh et al., 2019; Peng et al., 2019). Moreover, triclosan may promote broad resistance phenotypes in microorganisms and contribute to the spread of antibiotic resistance in different ecosystems (Carey and McNamara, 2014).

On the other hand, triclosan is considered an endocrine disruptor due to its estrogenic, antiestrogenic, androgenic and antithyroid activity in both aquatic

animals and mammals causing diverse physiological alterations such as adverse reproductive effects at concentrations detected in the environment (Crofton et al., 2007; Jung et al., 2012). According to Stenzel et al. (2019), this agent is harmful to adult zebrafish and their offspring, delays metamorphosis and impairs fecundity and fertility after larval exposure to environmentally relevant concentrations of triclosan. Therefore, the use of products containing triclosan can pose a risk to aquatic ecosystems and human health (Crofton et al., 2007; Fu et al., 2019; Olaniyan et al., 2016).

1.1.3.3. Biodegradation of triclosan

Biodegradation is considered the most economical and ecological process to remove contaminants. Although the complex chemical structure of some organic compound hinders degradation by microorganisms, many reports claim that triclosan can be transformed by bacteria, fungi and algae (Hundt et al., 2000; Kim et al., 2011; Wang et al., 2013). Interestingly, two fungal strains, *Rhodotorula mucilaginosa* and *Penicillium* sp. showed the ability to transform triclosan to 2,4-dichlorophenol (Tastan et al., 2016). Moreover, Lee et al. (2012) demonstrated that a bacterium isolated from activated sludge, *Sphingopyxis* strain KCY1, was capable of dechlorinating triclosan with a stoichiometric release of chloride, but did not completely mineralize it. They detected five metabolites and suggested that triclosan biotransformation occurs via a 2,3-dioxigenase pathway. In contrast, *Sphingomonas* strain YL-JM2C completely mineralized triclosan at an initial concentration of 5 mg L^{-1} in 72 h, in which the enzyme chlorohydroquinone dehalogenase was suggested to play an important role (Mulla et al., 2016). Based on the detection of three main metabolites during the triclosan biodegradation process, 2,4-dichlorophenol, 2-chlorohydroquinone and hydroquinone, a possible degradation pathway by this strain was proposed. Similarly, Wang et al. (2018) found a strain capable of using triclosan as a sole carbon source, *Dyella* sp. WW1, and determined the optimal conditions to degrade this contaminant. In addition, six intermediate products were detected in the culture medium, leading to two hypothetical biodegradation pathways in which the introduction of hydroxyl radicals occurs first followed by a meta-cleavage pathway and dechlorination (Figure 7).

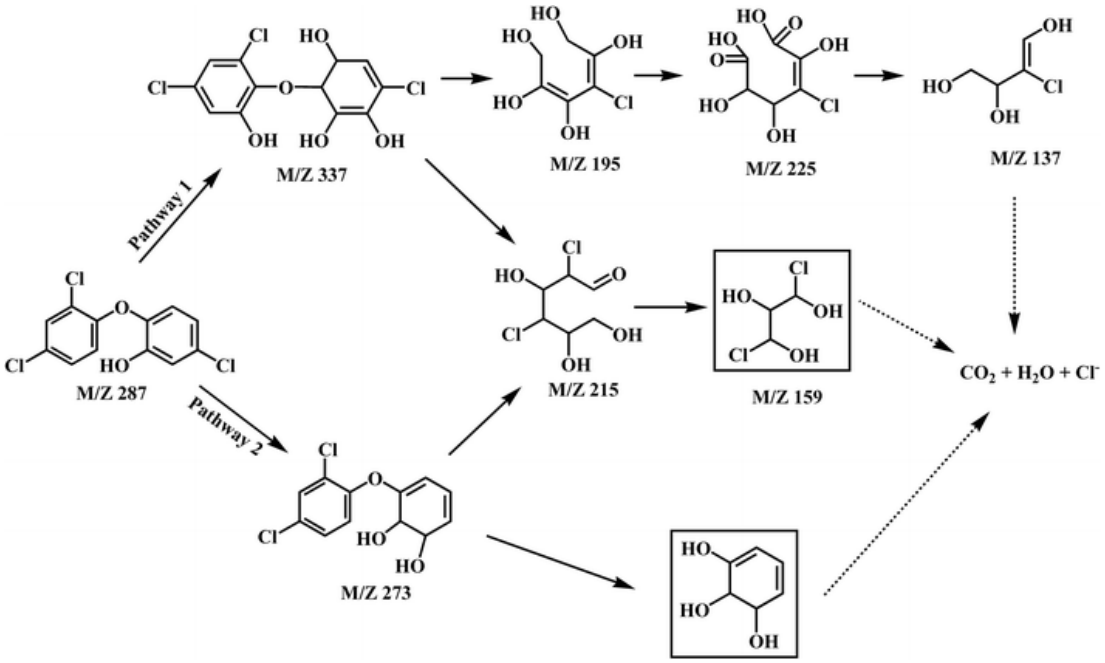


Figure 7. The suggested biodegradation pathways for triclosan by *Dyella* sp. WW1 (Wang et al., 2018).

1.2. PESTICIDES AND PLANT PROTECTION PRODUCTS: Environmental fate, ecotoxicological risk assessment and biotransformation.

Pesticides and plant protection products (or phytosanitary products) are widely used chemicals, mainly in agriculture, to protect crops from pests, diseases and weeds which must be prevented or controlled in order to reduce or eliminate yield losses and maintain high product quality (Damalas and Eleftherohorinos, 2011). However, the excessive and continuous application of agrochemical products can give rise to residues in soil, groundwater and surface water. The inadequate management of pesticides has been a worldwide concern for decades owing to their potential harmful risks to public health and their negative impact on natural ecosystems (De Wilde et al., 2007).

Different directives have been developed to regulate the use of these substances. The pesticide legislation in the European Union (EU) is considered

the most complete and stringent worldwide, which was reinforced in 2010 with the launch of the "Thematic Strategy on the Sustainable Use of Pesticides" (Kudsk and Mathiassen, 2020). In Spain, Directive 2009/128/EC was adopted in order to achieve a sustainable use of phytosanitary products and reduce environment and human health risks. Unfortunately, pesticides banned in the EU continue to be used in other regions of the world due to the enormous economic interest in maintaining high agricultural production and export values. In fact, approximately 27% of all agricultural pesticides used in the United States are banned in the EU (Donley, 2019).

The removal of the systemic fungicides tebuconazole and metalaxyl, the insecticides dimethoate and imidacloprid, and the herbicides oxyfluorfen and diuron, which are frequently used in agriculture to protect crops (Table 4), has been studied in unamended and amended soils with agroindustrial wastes after vermicomposting process (Castillo-Diaz et al., 2017), as well as in bioremediation systems (Castillo et al., 2016; Delgado-Moreno et al., 2017a; Romero et al., 2019). In this doctoral thesis some of these pesticides were also assayed taking into account their extensive use and possible harmful effects on ecosystems. Accordingly, the application of some pesticides, such as diuron, is restricted in vulnerable areas.

Table 4. Main physicochemical properties of selected pesticides.

Pesticide	Molecular Weight ($g\ mol^{-1}$)	Water Solubility ($mg\ L^{-1}$)	Log K_{ow}	K_{oc}
Tebuconazole	307.82	36	3.7	470-6000
Metalaxyl	279.33	8400	1.75	162
Dimethoate	229.26	25900	0.75	5.2-50
Imidacloprid	255.66	610	0.57	156-800
Oxyfluorfen	361.7	0.116	4.86	8900
Diuron	233.09	35.6	2.87	680
Linuron	249.09	63.8	3.00	843

Data from Pesticide Properties Database (PPDB) and PubChem database.
Kow: octanol-water partition coefficient.
Koc: organic-carbon partition coefficient

Phenylurea herbicides

The phenylurea herbicide group is one of the main classes of pesticides used extensively worldwide. These herbicides inhibit the Hill reaction in photosynthesis by blocking the transport of electrons at the reaction centre of photosystem II, limiting CO_2 fixation and the production of ATP (Allen et al., 1983; Snel et al., 1998). In soil, the biodegradation of phenylurea pesticides such as diuron and linuron, often results in the formation of the metabolite 3,4 dichloroaniline (3,4-DCA). This metabolite is strongly adsorbed to soil particles, being more recalcitrant and toxic than the parent compounds (Fernández-Bayo et al., 2009). In general, dichloroanilines also result from the biodegradation of other chemicals since these molecules are widely used as intermediates in the synthesis of azo dyes, paints, herbicides, cosmetics and other industrial products. Their stability and toxicity also render them hazardous when released into the environment (Yao et al., 2011). The recalcitrance of the parent compounds and of the metabolites were the main reasons for selecting these phenylurea herbicides as target pesticides for this study.

1.2.1. Diuron

Diuron (3-(3,4-Dichlorophenyl)-1,1-dimethylurea) (Figure 8) is a systemic broad-spectrum phenylurea herbicide. It is easily absorbed by the root system of plants and rapidly translocated by transpiration to stems and leaves mainly through the xylem. Diuron has been used to control broadleaf and grassy weeds as well as mosses on agricultural land and non-crop areas.

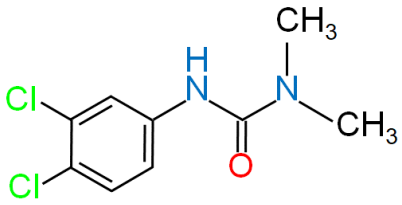


Figure 8. Chemical structure of diuron (CAS Number 330-54-1).

1.2.1.1. Environmental fate of diuron

Diuron have been extensively used on agricultural crops such as cotton, sugar cane, fruits and wheat, and on non-crops areas such as railway lines and roads at application rates of up to 3 Kg $ha^{-1}year^{-1}$ (Giacomazzi and Cochet, 2004). Moreover, this pesticide is also used as a biocide in paints (antifouling paints) and as an algaecide in fountains, ornamental ponds and aquaria (Singh and Singh, 2016). Consequently, diuron has been found in different environmental compartments. In soil, the persistence of diuron is governed by its physicochemical properties, as well as by the structure and composition of the soil and the organic amendments it contains (Landry et al., 2004; Thevenot and Dousset, 2015). Although this herbicide is characterized by its low mobility in soil due to its hydrophobicity and relatively low solubility in water, runoff and leaching may result in the migration of diuron to surface and groundwater after intensive rainfall (Arias-Estévez et al., 2008; Guimarães et al., 2019). Several authors claim that organic amendments reduce the mobility of diuron and their metabolites (Cabrera et al., 2010; Fernández-Bayo et al., 2015; Romero et al., 2010). However, other authors confirm that the addition of organic amendments promotes leaching of this contaminant by the formation of a mobile complex between diuron and dissolved organic matter (Cox et al., 2007; Thevenot et al., 2009). As a result, diuron has been detected in both surface and groundwater at concentrations that exceed the EU threshold limit value (0.1 $\mu g\ L^{-1}$) (Lapworth and Gooddy, 2006; Struger et al., 2011).

1.2.1.2. Ecotoxicology of diuron

The toxic effect of this herbicide has been studied in many organisms such as mammals, birds, fish, amphibians, and aquatic and terrestrial invertebrates (El-Nahhal, 2018; Giacomazzi and Cochet, 2004). Although diuron is slightly toxic to birds and aquatic invertebrates, the toxicity to fish is high even at environmental concentrations. In fact, exposure of goldfish to 5 $\mu g\ L^{-1}$ of diuron can induce variations in fish behaviour by altering the chemical perception of natural substances of ecological importance (Saglio and Trijasse, 1998). Moreover, several studies have shown that diuron is harmful to photosynthetic organisms

even at low concentrations. Recently, Moon et al. (2019) evaluated the toxic effects of three alternative antifouling biocides, including diuron, on non-target marine fish. Results showed that diuron can cause nervous system and muscle cell developmental defects as well as immune system disruption in non-target marine organisms. In addition, diuron could induce direct genotoxic activity in mammalian cells and exert systemic toxicity (Domingues et al., 2011; Federico et al., 2011).

On the other hand, when diuron is degraded by microorganisms in the environment, the main metabolite of phenylurea herbicides, 3,4-dichloroaniline (3,4-DCA) (Crossland, 1990), is released into soil or water (EC, 2006). According to the European Commission, the registered range of 3,4-DCA is between 0.05 and 1.5 μg L^{-1} in surface waters and the value of the predicted concentration without effect (0.2 μg L^{-1}) is frequently exceeded (IHCP, 2006). This compound is considered even more hazardous than the parent herbicides due to its high persistence in the environment (Yuan et al., 2017). Many authors confirmed the toxic effects of this compound on various organisms such as mammals, fish, protozoa, microalgae, crustaceans, insects, and bacteria (Giacomazzi and Cochet, 2004; Philippe et al., 2019). Also, 3,4-DCA poses a risk to human health (IHCP, 2006). Consequently, diuron was considered as a "Priority Hazardous Substance" that presents a significant risk to aquatic ecosystems, and is included in the EU Water Framework Directive's list of priority substances (Directive 2008/105/CE). However, despite restrictions, diuron is still detected in both surface and groundwater indicating the continued use of this herbicide (Rico et al., 2019; Teijon et al., 2010).

1.2.1.3. Biodegradation of diuron

In the environment, diuron can be degraded by abiotic processes through hydrolysis (Salvestrini, 2013) or photolysis (López-Ramón et al., 2019), but microbial degradation is considered the main process for removal of this herbicide from ecosystems (Sharma et al., 2010). Under aerobic conditions, microorganisms perform an *N*-demethylation of the urea group and subsequent hydrolysis, biotransforming diuron into metabolites such as DCPMU (1-(3,4-dichlrophenyl)-3-methylurea), DCPU (1-3,4-dichlorophenylurea) and 3,4-DCA

(Figure 9) (Gooddy et al., 2002; Sørensen et al., 2003). Turnbull et al. (2001) reported that a soil isolate, *Arthrobacter globiformis* strain D47, was able to degrade a wide range of urea-based herbicides and to transform diuron directly into 3,4-DCA through the action of the *puh*A gene that encodes a phenylurea hydrolase which cleaves the carbonyl bond in this group of herbicides. Interestingly, another gene with diuron hydrolase activity (*puh*B) implicated in diuron degradation was identified in *Mycobacterium brisbanense* JK1 (Khurana et al., 2009). Although these genes were not detected in all samples showing diuron biodegradation potential, the abundance of *puh*B was correlated with the presence of diuron-degrading microorganisms (Hussain et al., 2015). In a previous study, the addition of vermicompost was shown to favour the proliferation of diuron degraders, increasing the soil diuron-depuration capability (Castillo Diaz et al., 2016). It was observed that the soil history of diuron application counterbalanced the vermicompost-amendment effect. Moreover, the addition of vermicompost from olive mill wastes increased bacterial and *puh*B abundance, corroborating its effectiveness as a tool to enhance diuron biodegradation in soil.

The 3,4-DCA metabolite can be further degraded through different pathways, including dehalogenation and hydroxylation of the aromatic ring (Hussain et al., 2015). For instance, Hongsawat and Vangnai (2011) reported that a soil isolate, *Acinetobacter baylyi* strain GFJ2, completely degraded 4-chloroaniline and 3,4-dichloroaniline. They proposed a biodegradation pathway where a dechlorination reaction may be involved in the biodegradation of 3,4-DCA (Figure 9, pathways marked in green). Sharma et al. (2010) isolated and identified the bacterial strain *Micrococcus* sp. PS-1 which was able to grow with diuron as a sole carbon source via the production of 3,4-DCA. They identified six metabolites and proposed a possible 3,4-DCA degradation pathway (Figure 9, pathway marked in red).

On the other hand, alternative pathways for diuron degradation may exist which do not imply the production of the 3,4-DCA intermediate. In a recent study, Moretto et al. (2019) obtained a bacteria isolated from a sugarcane cultivation area, identified as *Escherichia fergusonii*, and suggested that this isolate may degrade diuron by two new pathways by replacing a chlorine atom (Figure 9, pathways marked in blue).

Fungal species such as *Fusarium* sp., *Aspergillus niger* or *Mortierella* sp., have also been reported to degrade phenylurea herbicides and its main metabolite 3,4-DCA (Castillo et al., 2014; de Lima et al., 2018; Ellegaard-Jensen et al., 2014; Hussain et al., 2015).

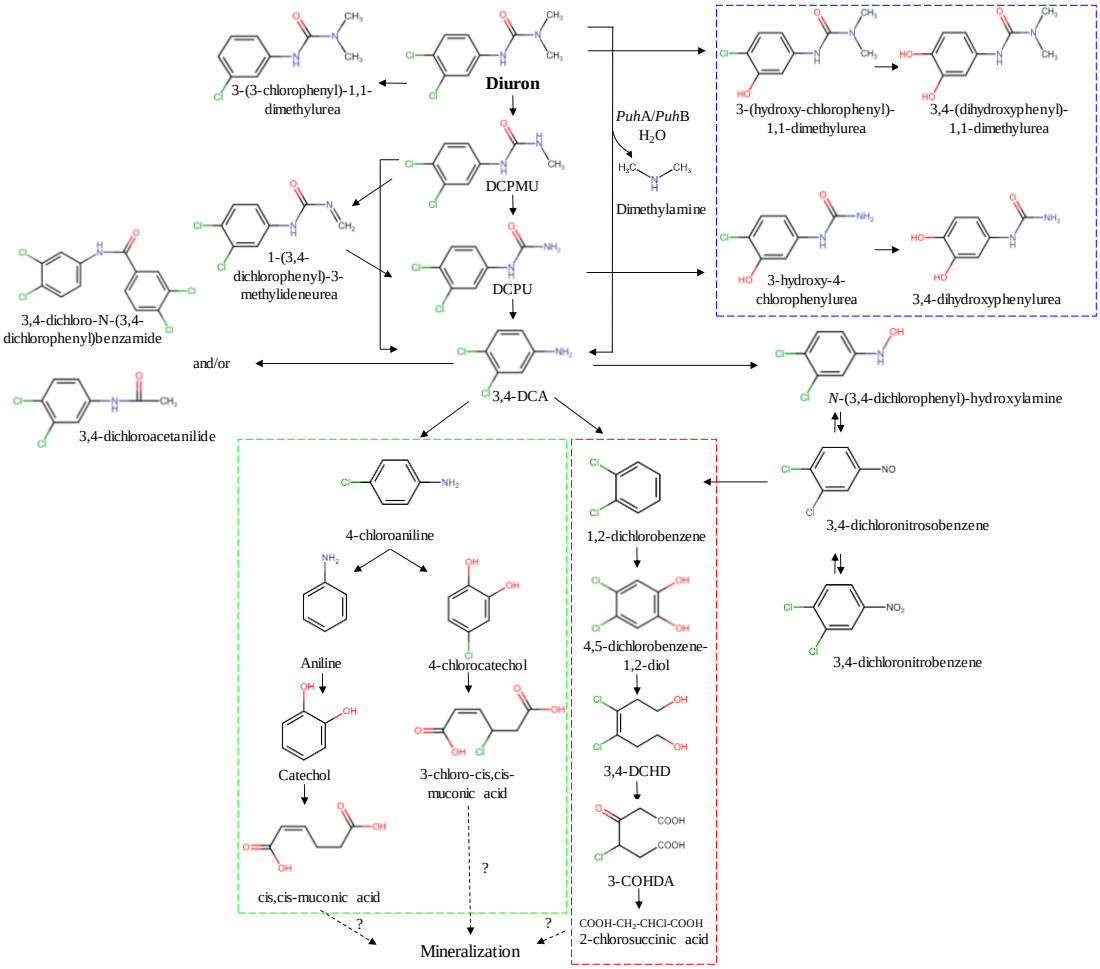


Figure 9. Biodegradation pathways of diuron and 3,4-DCA mediated by microorganisms. DCPMU: (1-(3,4-dichlrophenyl)-3-methylurea); DCPU: (1-3,4-dichlorophenylurea); 3,4-DCA: (3,4-dichloroaniline); 3,4-DCHD: (Z)-3,4-dichlorohex-3-ene-1,6-diol; 3-COHDA: (3-chloro-4-oxohexanedioic acid).

1.2.2. Linuron

Linuron (3-(3,4-dichlorophenyl)-1-methoxy-1-methylurea) (Figure 10) is an important phenylurea herbicide, which has been widely used in orchards, tree

nurseries and crop areas as nonselective pre-emergent herbicides to control broad-leafed weeds (Satsuma, 2010).

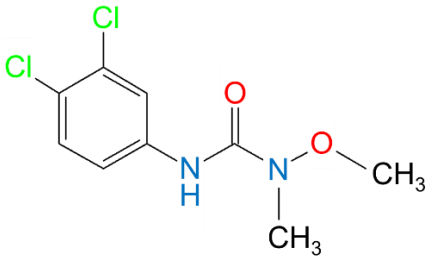


Figure 10. Chemical structure of linuron (CAS Number 330-55-2)

1.2.2.1. Environmental fate of linuron

Linuron has been used in crops such as corn, soybeans, carrots, potatoes or fruit trees at application rates between 0.28 and 4.3 kg $ha^{-1}year^{-1}$, depending on the type of crop and soil. Due to long-term and widespread application and its relatively high water solubility, linuron has been detected in different ecosystems, including soil, surface and groundwater (Caux et al., 1998).

1.2.2.2. Ecotoxicology of linuron

Unfortunately, the presence of linuron residues in the environment can cause toxic and carcinogenic effects in various types of non-target organisms. (Gatidou et al., 2015; Van Geest et al., 1999; Zhang et al., 2020). Linuron can negatively affect normal embryonic development in amphibians and cause damage in the brain tissues of fish (Quintaneiro et al., 2018; Topal et al., 2017). Furthermore, linuron is considered an endocrine disruptor which competitively binds to the androgen receptor in fish, mammals, and amphibians and can cause reproductive malformations in male rats (Lambright et al., 2000; Orton et al., 2009; Uren Webster et al., 2015; Wolf et al., 1999). To address this issue, Europe has been evaluating the risks associated with the use of this active substance for the environment (EFSA).

1.2.2.3. Biodegradation of linuron

Biodegradation is considered the most significant mechanism for the dissipation of linuron from the environment (Sheets, 1964). Numerous bacterial strains belonging to the genera *Bacillus*, *Streptomyces*, *Arthrobacter*, *Micrococcus, Mycobacterium, Diaphorobacter* and *Variovorax*, are involved in the biotransformation of linuron (Breugelmans et al., 2007; Cullington and Walker, 1999; Shelton et al., 1996; Wallnöfer, 1969; Zhang et al., 2020). Interestingly, several *Variovorax* strains isolated from different parts of the world showed the ability to mineralize this herbicide (Breugelmans et al., 2007; Satsuma, 2010; Sørensen et al., 2008). *Variovorax* sp. WDL1 was able to grow with linuron as a sole carbon and energy source. In addition, some fungal species such as *Phanerochaete chrysosporium* and *Trichoderma asperellum* can also be implicated in the biodegradation of linuron leading to a decrease in the effect of this herbicide on plants (Danilovic et al., 2015).

In bacteria, cleavage of the amide bond and simultaneous formation of 3,4-dichloroaniline (3,4-DCA) and N,O-dimethylhydroxylamine (N,O-DMHA) occur in the first steps in the biodegradation of linuron (Figure 11). Currently, six hydrolases called PuhA, PuhB, HylA, LibA, Phh, and TccA2 which were identified from *Arthrobacter globiformis* D47, *Mycobacterium brisbanense* JK1, *Variovorax sp. WDL1*, *Variovorax sp*. SRS16 and *Diaphorobacter sp*. LR2014-1, respectively, are associated with this first stage of linuron biodegradation (Bers et al., 2013, 2011; Khurana et al., 2009; Gillian A. Turnbull et al., 2001; Zhang et al., 2018). Recently, Zhang et al. (2020) obtained a linuron-degrading bacterial strain, *Sphingobium sp*. SMB, and identified another amidohydrolase (LahB) responsible for the biotransformation of linuron into 3,4-DCA and N,O-DMHA. On the other hand, Werner et al. (2020) investigated the new PromA g plasmids which carry genes involved in linuron degradation. Their results also demonstrated that these plasmids could be transferred between *Hydrogenophaga* and *Variovorax* genera when they coexist in the same environment.

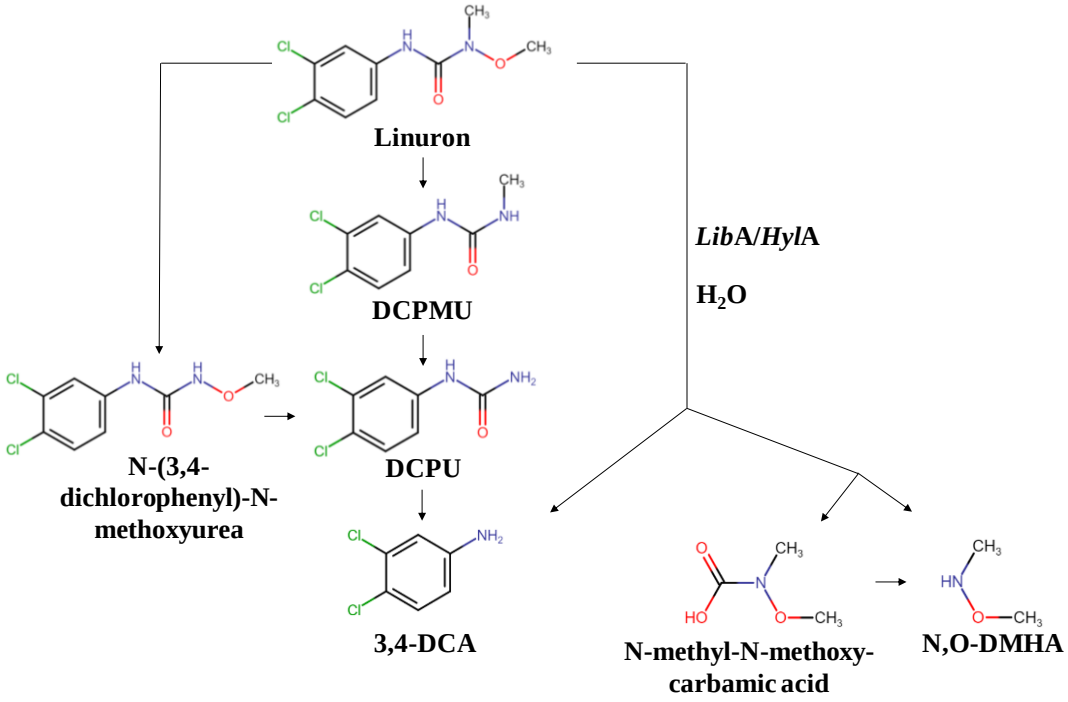


Figure 11. Biodegradation pathways of linuron (Hussain et al., 2015). DCPMU: (1-(3,4-dichlrophenyl)-3-methylurea); DCPU: (1-3,4-dichlorophenylurea); 3,4-DCA: (3,4-dichloroaniline); N,O-DMHA: (N,O-dimethylhydroxylamine).

2. ENVIRONMENTAL REMEDIATION: Bioremediation strategies and technologies.

Processes to remove organic contaminants from the environment comprise a wide range of technologies, mainly based on physical, chemical, and biological processes or combinations of them (Megharaj et al., 2011; Tolboom et al., 2019). Emerging contaminants and pesticides in wastewaters can be removed by adsorption mechanisms using different adsorbent materials such as active carbon, biochars or hydrochars (Delgado-Moreno et al., 2020), by redox mechanisms such as advanced oxidation, enzyme oxidation, and chemical reduction; or by bioremediation strategies including bioadsorption, biological uptake or biotransformation (Quan et al., 2019). These strategies show several disadvantages such as the need to treat the waste generated after the adsorption processes, the formation of halogenated species during the redox processes, or

the necessity to implement pre-treatments before biodegradation (Luo et al., 2019). However, biodegradation is considered the most economical and ecological process to remove contaminants.

2.1. BIOREMEDIATION STRATEGIES

Bioremediation approaches are considered the most promising, economical and eco-friendly technologies (Gaur et al., 2018; Tyagi et al., 2011). Natural transformation processes (bioattenuation), the application of exogenous microbial cultures (bioaugmentation) as well as the addition of stimulating material (biostimulation) to the indigenous microbial population to improve the degradation, metabolism or immobilization of contaminants are the most commonly used strategies for *in situ* bioremediation of chronically contaminated sites or accidental spills (Ossai et al., 2020). Some bioremediation techniques, such as phytoremediation, phycoremediation, and mycoremediation, are specifically designed to use plants, algae, and fungi, respectively, in order to degrade or extract organic pollutants from soil, sediment, surface water, and groundwater (Ossai et al., 2020).

2.1.1. Bioattenuation

Bioattenuation is based on the degradation, transformation or immobilization of contaminants by naturally occurring physical, chemical and biological processes such as dissolution, volatilization, stabilization or biodegradation by indigenous microbial populations (Vásquez-Murieta et al., 2016). Generally, this bioremediation strategy is used in sites with low contamination levels or in areas where other remediation techniques cannot be adopted to dissipate the contaminant (Ossai et al., 2020). However, this strategy requires a long period of time.

2.1.2. Biostimulation

Biostimulation is the addition of nutrients, metabolic precursors, cofactors (e.g. metals) or any stimulant material (e.g. organics amendments, bio-surfactants or biopolymers) to a contaminated site to promote the growth and

activity of native microbial communities in order to enhance remediation. In general, parameters that tend to limit microbial performance, such as nitrogen, phosphorus, potassium, carbon or micronutrients, are added or their use optimized (Megharaj et al., 2011; Nikolopoulou and Kalogerakis, 2009).

Biostimulation by organic amendments is a widely used bioremediation strategy. The application of organic materials, such as animal manures, sugarcane bagasse, straw, compost or vermicompost among others, improves the aeration, porosity and water-holding capacity of soils, and increases the nutrient content, promoting microbial activity and soil biodiversity (Castillo Diaz et al., 2017; Ren et al., 2018; Romero et al., 2010). However, it is important to highlight the fact that some organic amendments, such as sewage biosolids, can contain components which pose a risk to human, animal and/or plant health (Goss et al., 2013).

2.1.3. Bioaugmentation

Bioaugmentation involves the addition of cultures containing pre-adapted indigenous or exogenous microorganisms, or even genetically modified microbes with proven capabilities to degrade the target recalcitrant toxic compounds in a contaminated site. Furthermore, bioaugmentation approaches include the introduction of biodegradation-relevant genes that encode enzymes with specific catabolic activities into indigenous microorganisms (El Fantroussi and Agathos, 2005).

Bioaugmentation is suitable for areas with low microbial populations or with native populations which lack the metabolic capacity necessary to attack/remove/degrade the contaminant (Tyagi et al., 2011). However, some problems may arise after bioaugmentation such as poor adaptation of the inoculated microorganisms to the environment, poor competition with the indigenous population, the preferred use of other substrates than the pollutant or even predation by protozoa (Goldstein et al., 1985; Megharaj et al., 2011). Numerous studies have reported bacterial and fungal strains, belonging to the genera *Alcaligenes, Arthrobacter, Bacillus, Brucella, Burkholderia, Catellibacterium, Pichia, Pseudomonas, Rhodococcus, Serratia, Sphingomonas, Stenotrophomonas, Streptomyces* and *Verticillum,* which have potential

applications in bioaugmentation technologies to bioremediate soil contaminated with pesticides (Cycoń et al., 2017).

The addition of a microbial consortium is one of the most widely used bioaugmentation strategies because it represents more closely real conditions and often provides adequate metabolic capacity and robustness for environmental applications (Tyagi et al., 2011). Consortia usually consist of multi-component cultures based on several microorganisms involved in the degradation of the target compound. However, since the vast majority of viable microorganisms are not cultivable, some authors researched the use of materials previously exposed to contaminants, which harbour adapted microorganisms, as a bioaugmentation method (Sniegowski et al., 2011; Sniegowski and Springael, 2015).

In addition, aqueous extracts from organic materials rich in nutrients and beneficial microorganisms, such as compost and vermicompost, have been used as low-cost methods to effectively control pests (Salter and Edwards, 2010; Scheuerell and Mahaffee, 2002; Stewart-Wade, 2020). The application of these extracts known as compost teas and vermicompost teas, is considered a novel approach for bioremediation (Haller et al., 2016). However, the microorganisms responsible for their agronomic or environmental effectiveness and their application to enhance the biodegradation of persistent organic contaminants, such as emerging contaminants and pesticides, have hardly been investigated.

2.2. BIOREMEDIATION TECHNOLOGY

Over the past decades, unconventional remediation technologies have improved thanks to the development of new techniques that provide more advanced removal of contaminants from the environment. The main remediation technologies are based on biological degradation, physical adsorption, advanced chemical oxidation or combinations of these technologies (Rasheed et al., 2019; Tolboom et al., 2019). Figure 12 shows a summary of some of the most common techniques used for the remediation of emerging contaminants and pesticides.

The most frequently used methods are based on physical-chemical techniques but, these tend to be expensive and their by-products may be dangerous for the environment. On the other hand, bioremediation techniques based on the use of organisms or their products are more suitable natural treatments to reduce the adverse effects of pollutants on the ecosystems (Quintella et al., 2019). Within the biological degradation techniques, biopurification systems which are simple and effective bioremediation methods designed to minimize environmental contamination, have attracted increasing attention in numerous countries during recent decades.

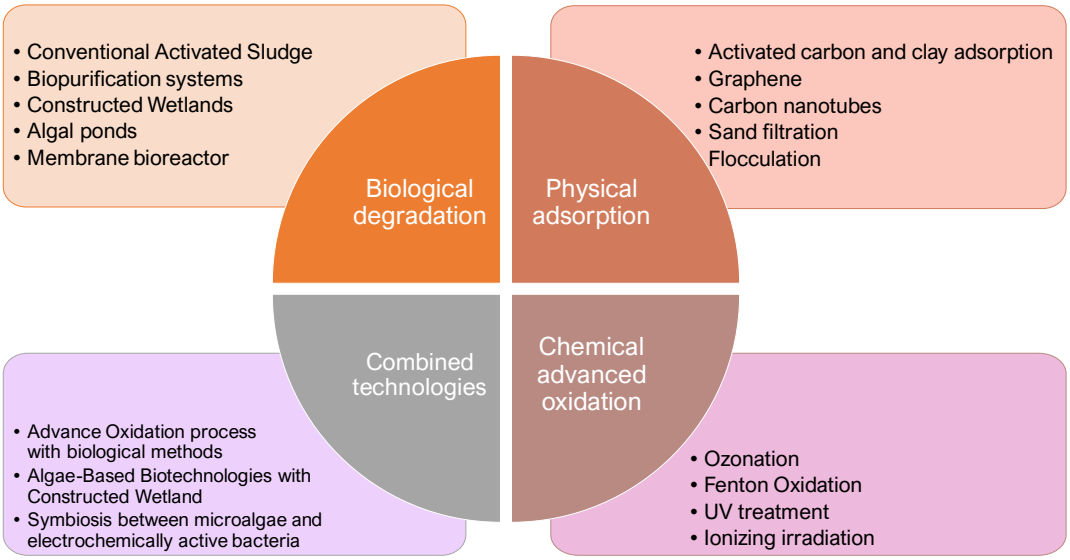


Figure 12. Remediation processes and techniques for the removal of emerging contaminants and pesticides (modified from Tolboom et al., 2019).

2.2.1. Biopurification systems

Biopurification systems (BPSs), or biobeds, are sustainable bioremediation techniques originating from Sweden in the 1990s, which were developed to collect and mitigate pesticide spills on farms. In its simple form, a BPS is a cheap construction consisting of an excavation in the ground lined with a layer of clay, filled with an active biomixture composed of soil, peat and straw in volumetric proportions of 1:1:2 and with a grass layer on the surface (De Wilde et al., 2007). The active biomixture not only retains organic contaminants through adsorption and absorption processes, but also allows the establishment and development of a microbial community with the catabolic capacity to degrade chemical

compounds. Moreover, BPSs are being investigated in different countries around the world due to their numerous advantages such as low cost, easy implantation, versatility and efficiency.

2.2.1.1. Components of biopurification systems

The efficiency of the retention and dissipation of contaminants depends on the physical, chemical and biological characteristics of each of the components of these systems. Furthermore, abiotic factors such as humidity and temperature can influence the correct operation of these systems. Figure 13 shows the different parts of a basic BPS.

- Clay layer

The clay layer is placed at the bottom of the BPS and works to decrease the leaching of polluted water and increasing the retention time of the pesticides in the BPS due to its low permeability and high sorption capacity (Castillo et al., 2008).

- Soil

The soil is an important source of pesticide-degrading microorganisms. Furthermore, it plays an important role as a component in the BPSs because it provides sorption capacity to these systems. Due to its nature and complex characteristics (texture and composition), the soil contributes to the retention of contaminants in the BPSs. Although there is evidence that soil texture influences the degradation rate of pesticides, this factor has a negligible effect on the degradation potential of the biomixture. However, the use of a soil previously exposed to the target pesticides improves the efficiency of pesticide dissipation compared to unexposed soil (Sniegowski et al., 2012).

- Humidified organic substrates

Humidified organic substrates help to maintain the moisture of the biomixture, and contribute to the sorption capacity and abiotic degradation of pesticides. In early Swedish BPSs, peat was used as humidifying material. However, peat is a scarce and expensive natural resource in some regions, making its use inappropriate and inadvisable. Consequently, the implementation of BPSs in countries with low availability to peat led to the replacement of this component

by other more accessible humidified organic substrates found in those regions, such as urban or garden compost (Chin-Pampillo et al., 2015).

- Lignocellulosic wastes

Straw is the lignocellulosic waste typically used in BPS. It is an additional source of nutrients that supports the growth of lignin-degrading microorganisms, such as white rot fungi, which produce catalytic enzymes that degrade a wide range of chemicals. These enzymes are suitable for the degradation of pesticide mixtures due to their broad specificity (Castillo et al., 2008). However, active biomixtures quickly mineralize straw with annual losses of around 10%, leading to the need to incorporate fresh straw every year. In addition, due to the unavailability of straw on a large scale in some regions or due to its demand as animal feed, other alternative lignocellulosic materials are being tested as replacements (Dias et al., 2020; Diez et al., 2013).

- Grass layer

The grass layer contributes to control moisture in the BPS by creating an upward transport of water by evapotranspiration. Moreover, this layer maintains an optimal temperature for microbial activity, avoids preferential flow and can indicate pesticides spills (De Wilde et al., 2007).

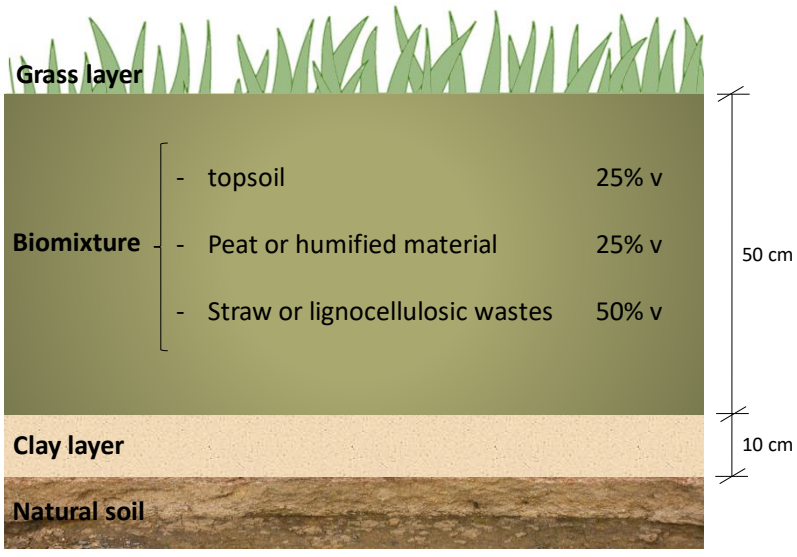


Figure 13. Schematic representation of a Swedish BPS.

2.2.1.2. Applications of biopurification systems

Biopurification systems are commonly used to control point-source contamination caused by spills of pesticide contaminated water from agricultural activities (Castillo et al., 2008; Dias et al., 2020). For instance, accidental spilling of pesticide concentrates during the filling or preparation of spraying equipment can lead to point-source contamination with a high environmental risk (Castillo et al., 2008). Similarly, the elimination of remaining agrochemicals in containers after use or those washed off during the cleaning of equipment releases agrochemical active ingredients at concentrations higher than the recommended application dose for agricultural crops. Therefore, the correct disposal of these polluted waters is important to prevent pesticides from reaching surface waters and aquifers. BPSs are suitable methods of remediating concentrated pesticide spills and to safely dispose of waters containing agrochemical residues, since these systems efficiently mitigate pesticide leaching. For this reason, the FAO recommended the use of these systems to reduce contamination of soil and water resources during pesticide management (http://www.fao.org/3/a-i5888s.pdf).

2.2.1.3. Types of biopurification systems

Biopurification systems have attracted attention in some countries, where they have been modified and adapted to local conditions, and renamed to biomassbed, biofilter, Phytobac and biobac (Castillo et al., 2008).

- Biomassbed

The biomassbed is an Italian bioremediation system developed for the treatment and decontamination of large volumes of water contaminated with pesticides from the filling and washing of spraying equipment. In these systems, biomixtures are used as a filter through which the contaminated water circulates. Due to the high cost of peat in Italy, this material was replaced with other, more accessible, organic materials such as urban and garden compost or citrus peel (Vischetti et al., 2004).

- Biofilter

Biofilters originated in Belgium and consist of a system of two or three units of 1 m^3 plastic containers filled with a biomixture and stacked vertically with

connecting plastic valves and pipes. These biofilters were designed to treat large volumes of effluents with low concentrations of contaminants that are recycled with a pump, or to treat small volumes of highly contaminated water. The number of containers depends on the sprayer and the hydraulic load (De Wilde et al., 2007).

- Phytobac®

The Phytobac®, developed by Bayer Crop-Science in France, is a 60 cm deep excavation covered by watertight materials to completely retain effluents and prevent leaching of contaminants. The biomixture of this system is composed only of farm soil (70%) and straw (30%). The Phytobac lacks the grass cover layer and is protected from rainfall by a waterproof cover. In this system, moisture control is important in order to avoid drying of the materials or saturation of the system because the water content is only regulated by evaporation. In addition, the Phytobac can be connected to a buffer tank to collect the effluents and recirculate them back onto the system (Castillo et al., 2008).

- Biobac

The biobac consists of a tank filled with a mixture of farm soil and straw similar to the biomixture used in Phytobacs. However, in this system, the farm soil used has previously been exposed to contaminants and therefore contains adapted microorganisms that improve the degradation capacity of the system. These microbial populations are maintained and stimulated through the input of a supplementary source of carbon and energy (Castillo et al., 2008).

2.2.1.4. Improvements in biopurification systems

Research on BPS generally focuses on the development of effective biomixtures to degrade a specific type of agrochemical such as carbofuran (Chin-Pampillo et al., 2015; Ruiz-Hidalgo et al., 2016), or complex mixtures of pesticides present in wastewaters produced by on-farm activities (Delgado-Moreno et al., 2017a; Huete-Soto et al., 2017; Masís-Mora et al., 2019). Our research group has developed biomixtures composed of vermicomposts obtained from different local agricultural wastes derived from the wine and olive oil agroindustries (Delgado-Moreno et al., 2017b; Romero et al., 2019). Unlike peat, vermicomposts harbour

a wide variety and quantity of microorganisms and have a high microbial functional diversity (Fernández-Gómez et al., 2011).

On the other hand, after evaluating the structure and composition of BPS, different strategies can be developed to increase the degradation rate of pesticides in these systems. Bioaugmentation is considered a suitable method to increase the biodegradation process, through the inoculation of microorganisms with known capacities to degrade pesticides (Castillo Diaz et al., 2016; Saez et al., 2018). Furthermore, this strategy is recommended to improve the removal rate of highly recalcitrant pesticides, such as triazoles and neonicotinoids (Dias et al., 2020; Rodríguez-Castillo et al., 2019). Another approach to improve biodegradation, even of pesticide mixtures, is by adding pesticide-primed material to the BPS which harbour a pesticide-degrading microbial community (Sniegowski and Springael, 2015). Pesticide-primed material refers to soils with long-term treatments with pesticides or to biomixtures from BPSs which have been in operation. This can be considered a method of revaluing used biomixtures which should be replaced every five or eight years to maintain the effectiveness of BPS. However, exhausted biomixtures may also contain remnants of pesticides and must be disposed of properly. Land dispersion, landfill disposal or incineration are the main disposal techniques used to remove exhausted biomixtures. Nevertheless, composting of these residual biomixtures is considered a low cost option (De Wilde et al., 2007). A vermicomposting system has recently been proposed as a low cost bioremediation strategy to eliminate pesticides from these biomixtures (Delgado-Moreno et al., 2020). Moreover, Lescano et al. (2020) reported that the addition of earthworms within BPS also improves the degradation of glyphosate and its main metabolite aminomethylphosphonic acid.

2.2.1.5. Global use of biopurification systems

Biopurification systems are being implemented in different countries around the world as bioremediation techniques to avoid punctual environmental contamination caused by the use and management of pesticides in agriculture. The types of BPSs implanted in different countries depend on regional, cultural, socio-economic and scientific-technological aspects. In some countries, BPSs are

considered systems for good agricultural practice. However, in Spain they have not been implemented yet. Figure 14 shows an updated map indicating the countries where BPSs are currently registered, used or tested.

Since BPSs were originated in Sweden in the 1990s, this technique has been adopted in many countries in Europe. In 2013, around 5000 BPSs had already been put into use in the European continent. In recent years, the Swedish Institute of Agricultural and Environmental Engineering in cooperation with the Food and Agriculture Organization of the United Nations (FAO) initiated a campaign to spread the use of these systems in other regions of the world in order to minimize contamination during pesticide management and promote good agricultural practices. As a result, the use of these systems has spread to China and Latin America. Currently, it is estimated that around 2000 BPSs have been implanted in Latin America, mainly in Guatemala and Chile (http://biobeds.net/en/home-eng/).

Figure 14. Global distribution of the use and development of biobeds biopurification systems.

2.2.1.6. Innovative approaches for biopurification systems

The effectiveness of BPSs for the treatment of wastewater effluents from industries, hospitals or WWTPs has recently been investigated (Delgado-Moreno et al., 2019). Some agro-industrial activities such as fruit-packaging, seed-coating and bulb disinfestation, produce effluents contaminated with high pesticide loads which constitute an important source of contamination of natural water resources. This is the case of post-harvest fruit washing that generates large volumes of wastewater containing high concentrations of fungicides and antioxidants used to prevent the deterioration of fruit (Karanasios et al., 2012). The European Commission acknowledged the environmental risk caused by the uncontrolled release of these wastewater effluents from these agro-industries, and imposed the implementation of efficient bioremediation techniques for its treatment (Papazlatani et al., 2019). Within these recommended techniques, BPSs have been proposed as a possible solution for the remediation of post-harvest wastewaters (Karanasios et al., 2012; Papazlatani et al., 2019).

In addition, BPSs have proven to be effective in removing other types of contaminants such as antibiotics or PPCPs (Jiménez-Gamboa et al., 2018). Recently, Delgado-Moreno et al. (2019) claimed that BPSs can be a viable strategy to eliminate PPCPs such as ibuprofen, diclofenac and triclosan, present in the effluents of WWTPs and prevent their entry into the environment. However, the large volumes of effluents produced by WWTPs or by agro-industrial activities require adaptations of BPS water management protocols and biomixture content to effectively remove the different types of contaminants at a high scale.

II. OBJECTIVES

The continuous and uncontrolled release of organic pollutants such as pesticides and pharmaceutical and personal care products (PPCPs) originating from agricultural, industrial and urban activities affects water quality and can have a negative impact on ecosystems and human health. Currently, treatment of these wastewaters is expensive and the elimination of pollutants by conventional activated sludge treatment is insufficient. For this reason, it is necessary to develop affordable systems to prevent the transfer of these pollutants to the environment. Biopurification systems (BPSs) have proven to be a highly efficient technology to retain and degrade pesticides with diverse physicochemical characteristics. The effectiveness of BPSs is closely related to the composition of the biomixtures of which they are composed. Our research group has developed biomixtures based on low-cost agro-industrial wastes abundant in Spain, which are very efficient in the removal of pesticides and PPCPs even at high concentrations. However, despite the high decontamination potential of these systems little is known of the functioning of these BPSs at the microbiological and molecular level and how they could be improved to reduce the degradation time and elimination of even the most recalcitrant pollutants. Thus, the main objective of this thesis is to deepen our understanding of these aspects of BPSs and to improve their efficiency. For this purpose, the thesis has been oriented towards the following specific objectives:

Objective 1: To determine the effects of PPCPs on bacterial viability and the microbial community structure of BPSs as well as to identify the main bacterial groups potentially involved in the degradation of these compounds.

Objective 2: To develop a novel low-cost bioaugmentation strategy based on aqueous extracts rich in microorganisms to improve the elimination of pesticides and PPCPs as well as their main degradation products from BPSs and contaminated aqueous systems.

Objective 3: To increase the dissipation rate of PPCPs and avoid the accumulation of their main metabolites in BPSs and in aqueous systems by bioaugmentation with exogenous degrading bacterial strains isolated from polluted environments.

III. GENERAL MATERIALS AND METHODS

1. BIOMIXTURES

Three biomixtures were used to construct the biopurification systems at pilot and microcosm scale (Table 1). The first biomixture containing soil, peat and straw (1:1:2) comprises the components traditionally used in these systems worldwide. The other two novel biomixtures are composed of soil, organic wastes from the olive oil agroindustry and olive tree prunings as a texturizing agent at the same ratio.

Table 1. Composition of different biomixtures.

Biomixtures	% Content by volume in the biomixtures					
	Soil	Peat	Straw	Wet olive mill Cake	Vermicompost of wet olive mill cake	Olive tree Pruning
SPS	25	25	50	-	-	-
SCP	25	-	-	25	-	50
SVP	25	-	-	-	25	50

The silty clay loam soil was collected from an olive orchard (0-25 cm depth) placed in the south of Spain (37° 22′19.41″N, 3° 36′5.54″W). The peat was supplied by Agia S.L. (Padul, Granada, Spain) while the agroindustrial wastes were obtained from Romeroliva, S.L. (Deifontes, Spain). Wet olive mill cake is the main waste product generated during the two-phase olive oil extraction process. This waste was vermicomposted with manure at a 4:1 ratio using the earthworm specie *Eisenia fetida* as described by Castillo et al. (2013). All components were air dried, ground and passed through a 4 mm sieve prior to use. The description of each component of the biomixtures were reported by Delgado-Moreno et al. (2017). Table 2 summarizes the main physicochemical properties of the biomixtures.

Table 2. Physicochemical properties of biomixtures.

Biomixture	WHC (%)	pH	EC mS cm^{-1}	OC g Kg^{-1}	C/N ratio	WSC g Kg^{-1}	HA g Kg^{-1}	Lig./Cel./Hemic. (%)
SPS	79.3	7.1	3.4	161	29.1	1.5	39.7	2.8/2.8/3.4
SCP	86.3	8.1	1.9	213	24.4	13.7	19.7	7.5/8.2/7.8
SVP	64.7	7.9	1.1	216	20.1	7.3	17.4	8.6/6.3/8.2

Water holding capacity (WHC), Electrical conductivity (EC), Organic carbon (OC), Water soluble carbon (WSC), Humic acid (HA) and percentages of Lignin/Cellulose/Hemicellulose (Lig./Cel./Hemic).

2. PREPARATION OF AQUEOUS EXTRACTS WITH AUTOCHTHONOUS MICROBIAL CONSORTIA FROM BIOMIXTURES IN A LAB-SCALE BIOREACTOR

The aqueous extracts were obtained from initial uncontaminated biomixtures, from the residual biomixtures of biopurification system exposed to different pesticides at large scale or from biomixtures acclimatized to different PPCPs according to the procedure described by Salter and Edwards (2010). For this purpose, a sample of the biomixture was placed in a nylon bag (50 µm mesh) and then extracted in distilled water in a dry weight:volume ratio of 1:10 in a glass microreactor aerated by a Tetra APS 400 air pump (Tetra GmbH, Germany) for 48 hours at 30 ºC in darkness. Finally, the extracts were left to settle for 1 hour and the sediment was discarded.

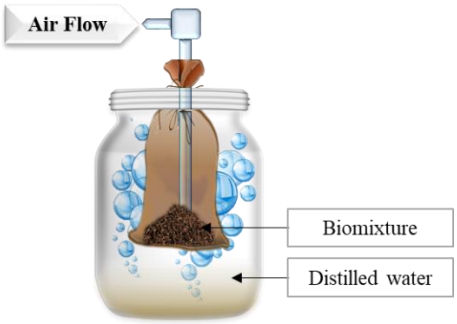


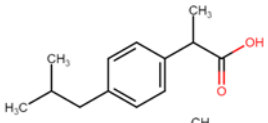
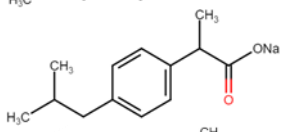
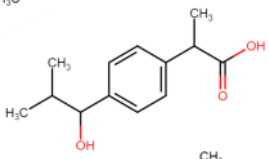
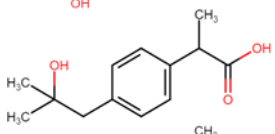
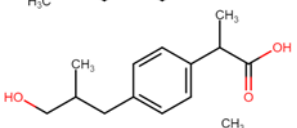
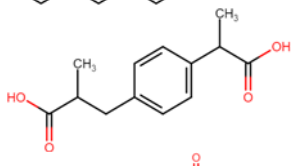
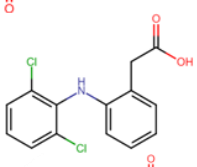
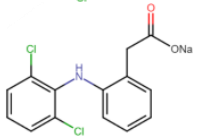
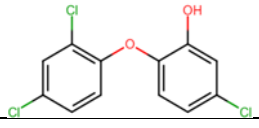
Figure 1. Scheme of lab-scale bioreactor used to obtain aqueous extracts.

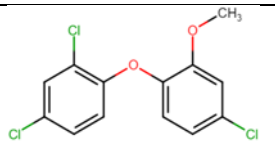
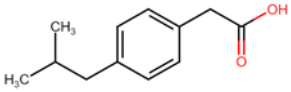
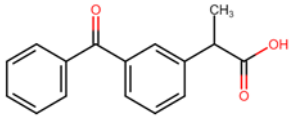
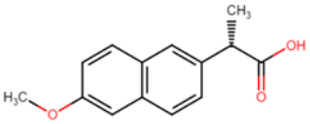
3. CHEMICAL COMPOUNDS

All chemicals compounds used were of the highest purity commercially available. Standard chemical solutions were prepared in HPLC-grade organic solvents, while experimental solutions were prepared in MilliQ water Type 1 Ultrapure Water Systems (Merck Millipore, Darmstadt, Germany).

3.1. Pharmaceutical and personal care products (PPCPs):

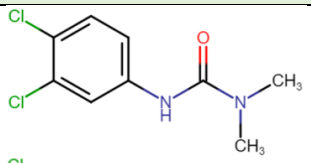
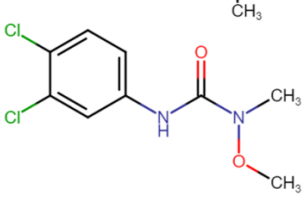
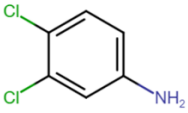
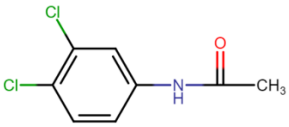
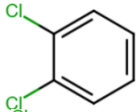
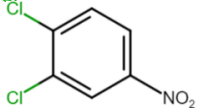
Table 3. Chemical structure and acronyms of the PPCPs and their main degradation products analysed in this study.

Compound	Acronym	Chemical structure
Ibuprofen	IBP	
Ibuprofen sodium salt	IBP	
1-Hydroxyibuprofen	1-OH IBP	
2-Hydroxyibuprofen	2-OH IBP	
3-Hydroxyibuprofen	3-OH IBP	
Carboxyibuprofen	CBX IBP	
Diclofenac	DCF	
Diclofenac sodium salt	DCF	
Triclosan	TCS	

Methyl triclosan	M-TCS	
Ibufenac	-	
Ketoprofen	-	
Naproxen	-	

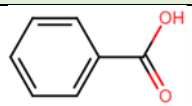
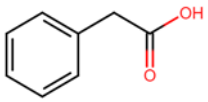
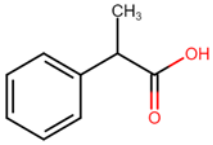
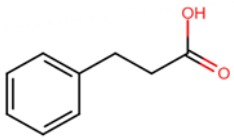
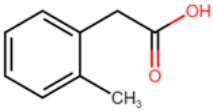
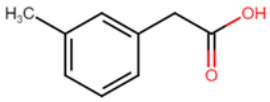
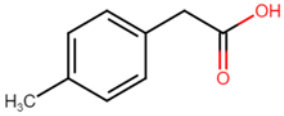
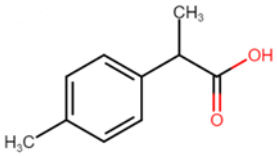
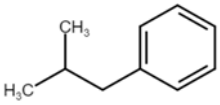
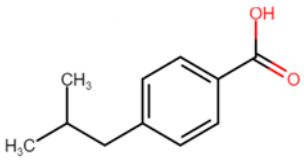
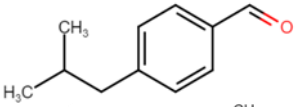
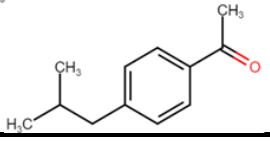
3.2. Phenylurea herbicides:

Table 4. Chemical structure and acronyms of phenylurea herbicides and their main degradation products analysed in Chapter 2.

Compound	Acronym	Chemical structure
Diuron	-	
Linuron	-	
3,4-Dichloroaniline	3,4-DCA	
3,4-Dichloroacetanilide	3,4-DCAN	
3,4-Dichlorobenzene	3,4-DCB	
3,4-Dichloronitrobenzene	3,4-DCNB	

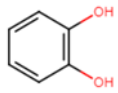
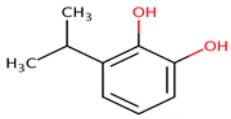
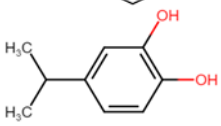
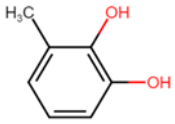
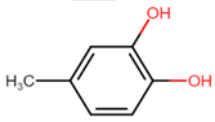
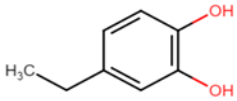
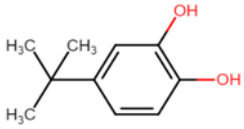
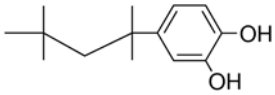
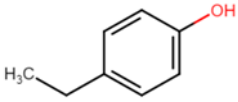
3.3. Structural analogues of ibuprofen:

Table 5. Chemical structure of ibuprofen structural analogues tested as possible substrates for the RW412 strain in Chapter 3.

Compound	Chemical structure
Benzoic acid	
Phenylacetic acid	
2-Phenylpropanoic acid	
3-Phenylpropanoic acid	
2-Methylphenylacetic acid	
3-Methylphenylacetic acid	
4-Methylphenylacetic acid	
2-(4-Methylphenyl)propionic acid	
Isobutylbenzene	
4-Isobutylbenzoic acid	
4-Isobutylbenzaldehyde	
4-Isobutylacetophenone	

3.4. Possible catabolites of ibuprofen:

Table 6. Chemical structure of possible intermediates of ibuprofen degradation analysed in Chapter 3.

Compound	Chemical structure
Catechol	
3-Isopropylcatechol	
4-Isopropylcatechol	
3-Methylcatechol	
4-Methylcatechol	
4-Ethylcatechol	
4-*tert*-Butylcatechol	
4-*tert*-Octylcatechol	
4-Ethylphenol	

4. ANALYTICAL METHODS

4.1. High Performance Liquid Chromatography analysis

The concentration of PPCPs (ibuprofen, diclofenac and triclosan) and phenylurea herbicides (diuron and linuron) as well as its main metabolite 3,4-dichloroaniline (3,4-DCA) were determined by high-performance liquid

chromatography (HPLC). The chromatograph (HPLC series 1100 system, Agilent Technologies) had a Zorbax RX-C8 column (5 μm, 2.1 x 150 mm) and an Eclipse XDB-C8 precolumn (5 μm, 2.1 x 12.5 mm). The mobile phase used was acetonitrile and acidified water (pH 3) with H_2SO_4 in a 50:50 ratio for PPCPs analysis and in a 40:60 ratio for the phenylurea herbicides and 3,4-DCA analysis. The flow was 0.2 mL min^{-1}, the oven temperature was 40 ºC and the injection volume was 10 μL. The wavelengths were 275 nm for DCF, 220 nm for IBP and TCS, and 254 nm for phenylurea herbicides. The limit of detection was 0.2 and 0.05 mg L^{-1} for PPCPs and herbicides, respectively.

4.2. Gas Chromatography/Mass Spectrometer analysis

The metabolites derived from the transformation of ibuprofen, triclosan and 3,4-DCA were analysed by gas chromatography/mass spectrometry (GC/MS) using a gas chromatograph (Varian 450) with a 240-MS detector (Agilent Technologies, CA, USA) and a chromatographic J&W Scientific DB 5-MS stationary phase column (Agilent Technologies, CA, USA).

Ibuprofen metabolites (1-hydroxyibuprofen, 2-hydroxyibuprofen, 3-hydroxyibuprofen and carboxyibuprofen) were analysed in aqueous solutions inoculated with different extracts as well as in BPS microcosms. Samples were derivatized at 70 ºC for 1 hour with the silylating agent N,O-bis(trimethylsilyl) trifluoroacetamide and trimethylchlorosilane (BSTFA/TMCS, 99:1; Sigma-Aldrich) and then analysed by GC/MS. The internal standard was 2-naftoic acid (Sigma-Aldrich). High purity helium was used as carrier gas with a flow rate of 1 mL min^{-1}. The injected sample volume was of 10 μL and the injector temperature was 280 °C. Temperature gradient was as follows: 90 °C for 5 min, then increased to 280 °C at a rate of 20 °C min^{-1} and held at 300 °C for 1 min. The total time of the analysis was 17 min. The ionization performed was by electron impact at 70 eV. Acquisition was carried out in the scan mode (mass scanning range of 50-600 m/z).

On the other hand, methyl triclosan (M-TCS) was analysed in BPS microcosms. In this case, derivatization was not necessary and n-eicosane provided by Merck KGaA (Darmstadt, Germany) was used as an internal standard. The injected

sample volume was 1 µL. The column temperature was initially set at 70 °C for 2 min, then increased to 205 °C at a rate of 10 °C min^{-1} and held for 5 min, increased again to 280 °C at a rate of 20 °C min^{-1} and held for 5 min, and finally increased to 300 °C at a rate of 50 °C min^{-1} and held for 10 min. The total time of the analysis was 29 min. All other conditions are similar to those described above. Identifications were confirmed by coincidence in retention times and mass spectra of pure standards.

Finally, the metabolites derived from 3,4-DCA transformation were analysed by solid-phase microextraction with 2,4,5-trichloroaniline as an internal standard according to the method described by Castillo et al. (2014).

5. MOLECULAR BIOLOGY TECHNIQUES

5.1. Identification of bacterial isolates, DNA extraction, PCR amplification

To characterize each isolate, pure cultures of each strain were grown overnight in Luria-Bertani (LB) medium (Sambrook et al., 1989) at 30 °C on a shaker at 200 rpm. Total genomic DNA was extracted using the Wizard genomic DNA purification kit (Promega) following the manufacturer's recommendations. The quality and quantity of the DNA were assessed with the Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific) and Qubit (Thermo Fisher Scientific). The strains were identified by sequencing the amplification products of the 16S rRNA gene and other housekeeping genes *rpoB*, *rpoD* and *gyrB* and comparing in public databases by BLAST (Altschul et al., 1990). To amplify the 16S rRNA gene, PCR was performed with 10 ng of genomic DNA in a final volume of 20 µL with 10 µL of KAPA2G Fast HotStart DNA polymerase mix (Kapa Biosystems, Massachusetts, United States) and 500 nM of primers GM3F and GM4R (Table 7). The cycling conditions applied were 95 °C for 5 min, followed by 30 cycles of denaturation at 95 °C for 25 s, annealing at 60 °C for 25 s and extension at 72 °C for 35 s with a final extension step at 72 °C of 5 min. The housekeeping genes were amplified as described by Pascual et al. (2010) using the primers described in Table 7.

Table 7. Sequence of primers used to identify bacterial isolates.

Locus	Primer	Sequence (5´→ 3´)
rpoB	LAPS	TGGCCGAGAACCAGTTCCGCGT
	LASP27	CGGCTTCGTCCAGCTTGTTCAG
rpoD	70F	ACGACTGACCCGGTACGCATGTAYATGMGNGARATGGGNACNGT
	70R	ATAGAAATAACCAGACGTAAGTTNGCYTCNACCATYTCYTTYT
gyrB	up1E	GAAGTCATCATGACCGTTCTGCAYGCNGGNGGNAARTTYRA
	up2AR	AGCAGGGTACGGATGTGCGAGCCRTCNACRTCNGCRTCNGYCAT
16S rRNA	GM3F	AGAGTTTGATCMTGGC
	GM4R	TACCTTGTTACGACTT

6. DATA ANALYSIS

6.1. Data modeling

The single first-order model ($C_t = C_0 \times e^{-kt}$) was tested to describe the dissipation kinetics of both PPCPs in BPS microcosms and phenylurea herbicides in aqueous solutions using the ModelMaker 4.0 modelling program (Cherwell Scientific Ldt., Oxford, UK). C_0 refers to the initial concentration (μg g^{-1}) of organic contaminant and C_t is the concentration at time *t* (days), while *k* is the degradation rate constant ($days^{-1}$). The chi-square (χ^2), determination coefficient (R^2) and scaled error (err_{scaled}) values were used as criteria to ensure that the theoretical kinetics fit the experimental data. All single first-order kinetic parameters, DT_{50} and DT_{90} values which represent the time required for the 50% and 90% dissipation of the initial concentration, respectively, were also determined using the ModelMaker 4.0 modelling program.

6.2. Bioinformatic data analysis

The 16S rRNA gene amplicon sequences obtained by Illumina were analysed using QIIME version 1.9.0 (Caporaso et al., 2010b). Forward and reverse sequences were joined, fastq files were filtered for quality (Phred 20) and potential chimeras were detected and removed from the dataset using usearch61

(Edgar et al., 2011). The remaining sequences were clustered into operational taxonomic units (OTUs) at a similarity threshold of 97% following an open reference OTU picking strategy using the UCLUST program (Edgar et al., 2011). Representative sequences of each OTU were aligned against the SILVA database v.132 (Quast et al., 2013) using the PyNAST program (Caporaso et al., 2010a).

6.3. Ecological and statistical analysis

Alpha diversity indices (Chao1, Shannon diversity index and Simpson's Evenness), and beta diversity of bacterial communities were analysed using QIIME v1.9.1 (Caporaso et al., 2010b). The rarefaction curves of observed OTUs based on data rarefied to the number of reads found in the least abundant sample, and the principal component analysis (PCoA) based on Bray-Curtis distances, as well as PERMANOVA analysis were performed using QIIME. Hierarchical cluster analysis of bacterial community composition at the phylum level based on the Bray-Curtis similarity index and the percentage of similarity between means of groups of samples (n=3) using the Unweighted Pair Group Method with Arithmetic mean (UPGMA) were determined using PAST software version 3.21 (https://folk.uio.no/ohammer/past/). Similarly, Non-metric Multidimensional Scaling (NMDS) ordination, heat map and radar chart plot were performed with the aid of PAST software. SPSS Statistical Software Package version 25 (IBM Corporation, New York, USA) was used for one-way ANOVA at a significance level of 0.05.

IV. RESULTS AND DICUSSION

CHAPTER 1

Bacterial ecotoxicity and shifts in bacterial communities associated with the removal of ibuprofen, diclofenac and triclosan in biopurification systems

The results of Chapter 1 have been published in the journal *Science of The Total Environment,*

Aguilar-Romero, I., Romero, E., Wittich R. M., van Dillewijn P. 2020. Bacterial ecotoxicity and shifts in bacterial communities associated with the removal of ibuprofen, diclofenac and triclosan in biopurification systems. *Science of The Total Environment*, 741, 140461.
https://doi.org/10.1016/j.scitotenv.2020.140461

Resumen

La proliferación y los posibles efectos adversos de contaminantes emergentes, como los productos farmacéuticos y de cuidado personal (PPCPs) en las aguas y el medio ambiente, es un motivo de creciente preocupación. En este capítulo se investiga la disipación de tres PPCPs: ibuprofeno (IBP), diclofenaco (DCF) y triclosán (TCS), por separado y en mezcla, en un sistema de biopurificación (BPS) a escala microcosmos, prestando especial atención a sus efectos sobre la ecotoxicidad bacteriana, así como en la estructura y composición de la comunidad bacteriana. Los resultados revelan que los BPSs disipan eficientemente IBP y DCF, eliminando un 90% de estos contaminantes tras 45 y 84 días de incubación, respectivamente. Sin embargo, la eliminación de TCS requirió un período de incubación más largo de 127 días para alcanzar una eliminación del 90%. La disipación de los PPCPs fue más lenta cuando se aplicaron en mezcla en los microcosmos. TCS tuvo un efecto negativo inicial sobre la viabilidad bacteriana causando una disminución entre un 34-43% según los recuentos de células bacterianas vivas realizados mediante la técnica de microscopía LIVE / DEAD®; sin embargo, este efecto se mitigó cuando los tres PPCPs se aplicaron simultáneamente en los BPSs. Las comunidades bacterianas en los microcosmos se vieron más afectadas por el tiempo de incubación que por los PPCPs utilizados. No obstante, los PPCPs afectaron de manera diferencial la composición y abundancia relativa de taxones bacterianos. IBP y DCF inicialmente aumentaron la diversidad y riqueza bacteriana, mientras que la exposición a TCS generalmente provocó un efecto opuesto sin una recuperación completa al final del período de incubación. TCS afectó negativamente la abundancia relativa de *Acidobacteria*, *Methylophilales* y *Legionellales*, y causó un mayor impacto en los grupos bacterianos. Por último, se buscaron biomarcadores consistentes en unidades taxonómicas operacionales (OTU-Operational Taxonomic Unit) predominantes a altas concentraciones de PPCPs y que, por lo tanto, probablemente alberguen mecanismos de degradación y / o desintoxicación. Este estudio revela por primera vez el efecto de los PPCPs sobre la ecotoxicidad bacteriana y sobre la diversidad en los microcosmos del sistema de biopurificación y también facilita el diseño de nuevas aplicaciones de las biomezclas para eliminar los PPCPs.

Abstract

The proliferation and possible adverse effects of emerging contaminants such as pharmaceutical and personal care products (PPCPs) in waters and the environment is a cause for increasing concern. We investigated the dissipation of three PPCPs: ibuprofen (IBP), diclofenac (DCF) and triclosan (TCS), separately and in mixtures, in the ppm range in biopurification system (BPS) microcosms, paying special attention to their effect on bacterial ecotoxicity, as well as bacterial community structure and composition. The results reveal that BPS microcosms efficiently dissipate IBP and DCF with 90% removed after 45 and 84 days of incubation, respectively. However, removal of TCS required a longer incubation period of 127 days for 90% removal. Furthermore, dissipation of the PPCPs was slower when a mixture of all three was applied to BPS microcosms. TCS had an initial negative effect on bacterial viability by a decrease of 34–43% as measured by live bacterial cell counts using LIVE/DEAD® microscopy; however, this effect was mitigated when the three PPCPs were present simultaneously. The bacterial communities in BPS microcosms were more affected by incubation time than by the PPCPs used. Nonetheless, the PPCPs differentially affected the composition and relative abundance of bacterial taxa. IBP and DCF initially increased bacterial diversity and richness, while exposure to TCS generally provoked an opposite effect without full recovery at the end of the incubation period. TCS, which negatively affected the relative abundance of *Acidobacteria*, *Methylophilales*, and *Legionellales*, had the largest impact on bacterial groups. Biomarker OTUs were identified in the BPS microcosms which were constrained to higher concentrations of the PPCPs and thus are likely to harbour degradation and/or detoxification mechanisms. This study reveals for the first time the effect of PPCPs on bacterial ecotoxicity and diversity in biopurification system microcosms and also facilitates the design of further applications of biomixtures to eliminate PPCPs.

1. INTRODUCTION

Pharmaceuticals and personal care products (PPCPs), which are used as medicinal drugs or to improve the quality of daily life, are considered to be emerging contaminants of public concern. PPCPs are increasingly being detected in aquatic environments, such as water, sediment and biota (Ebele et al., 2017; Peng et al., 2019). The presence of PPCPs in surface water may have negative ecological effects even at low concentrations due to their continuous introduction into environments from different anthropogenic sources (Ebele et al., 2017; Houtman, 2010). In rural areas with low population densities, untreated sewage is directly discharged into the sea and rivers (Daughton and Ternes, 1999). However, effluents from wastewater treatment plants (WWTPs) are the main route of entry of PPCPs into aquatic environments due to their incomplete elimination (Couto et al., 2019; Fekadu et al., 2019; Petrie et al., 2015; Petrović et al., 2003; Rosal et al., 2010). PPCPs include the non-steroidal anti-inflammatory drugs ibuprofen (IBP) and diclofenac (DCF), as well as the antimicrobial agent triclosan (TCS), which are commonly detected in surface water at concentrations of ng per litre (ppb) (Wilkinson et al., 2017). Moreover, TCS has been detected in drinking water and in plants cultivated in soils amended with biosolids from WWTPs or irrigated with sewage (Olaniyan et al., 2016). These contaminants have been shown to have harmful effects on aquatic organisms such as fish, algae and invertebrates (Lonappan et al., 2016; Olaniyan et al., 2016). TCS, which can cause endocrine disruptions and affect different tissues, also poses a threat to human health (Gee et al., 2008; Geens et al., 2012; Jung et al., 2012). Thus, an appropriate technology needs to be developed to efficiently remove PPCPs before effluent is discharged (Grassi et al., 2013).

Bioremediation strategies include biopurification systems (BPSs), also known as biobeds, which have been used successfully on farms to remove organic pollutants from wastewater and to control point-source contamination (Castillo et al., 2008; De Wilde et al., 2007; Dias et al., 2020; Karas et al., 2016). BPSs are large containers or lined excavations filled with a biomixture consisting of topsoil and organic materials which harbour active microorganisms which can facilitate the co-metabolic degradation of pollutants or become adapted to eliminate these

pollutants (Aguilar-Romero et al., 2019; Castillo Diaz et al., 2016; Karanasios et al., 2012; Sniegowski et al., 2011). The components of the biomixture determine the degree of adsorption of the contaminants and contribute to their bioavailability and toxicity for bacterial and fungal degraders (Castillo et al., 2008; Vischetti et al., 2004). Recently, Delgado-Moreno et al. (2019) suggested that BPSs based on biomixtures composed of available and sustainable local organic materials such as agro-industrial olive oil waste, could be a workable strategy to remove PPCPs from wastewaters generated by the pharmaceutical industry and hospitals, or effluents from wastewater treatment plants.

The efficiency of BPSs in removing contaminants such as pesticides has been ascribed to the functioning and resilience of the microbial communities of the biomixture used (Castro-Gutiérrez et al., 2019, 2018, 2017; Dealtry et al., 2016; Diez et al., 2018, 2017; El Azhari et al., 2018; Góngora-Echeverría et al., 2018; Holmsgaard et al., 2017; Marinozzi et al., 2013; Tortella et al., 2013). However, little is known regarding the impact of PPCPs on microbial populations in this system. The current study investigates the potential removal of three PPCPs, separately and in mixtures, in BPS microcosms, how interactions between contaminants can affect their dissipation kinetics, their possible toxic effects on indigenous microbial communities in BPS microcosms, the resilience of these communities as well as the main bacterial groups potentially involved in PPCP bioremoval. To the best of our knowledge, this study represents the first investigation of the effect of PPCPs on bacterial ecotoxicity and bacterial community structure in biopurification system microcosms or any other soil-based bioremediation system.

2. MATERIALS AND METHODS

2.1. Chemicals

Ibuprofen (IBP) (≥98% purity), diclofenac sodium salt (DCF) (≥98.5% purity) and triclosan (TCS) (>97% purity) were purchased from Sigma-Aldrich (Steinheim, Germany). HPLC-grade solvents acquired from Scharlau (Barcelona, Spain) and MilliQ water were also used.

2.2. Degradation study in BPS microcosms

Biopurification system (BPS) microcosms were constructed using a biomixture containing agricultural silty clay loam soil, vermicompost from wet olive cake and olive tree pruning (1,1,2, v,v,v). Physicochemical properties of this biomixture are described in the General Materials and Methods section. Each BPS microcosm was prepared in triplicate using the biomixture contaminated with either 100 μg g^{-1} of IBP, 20 μg g^{-1} of DCF or 20 μg g^{-1} of TCS, or all together in a mixture. The quantity of IBP used was higher than that for the other compounds due to the higher concentrations found in influents (Couto et al., 2019; Petrie et al., 2015; Wilkinson et al., 2017) and surface waters (Fekadu et al., 2019). To apply IBP, DCF and TCS, either separately or in a mixture, 1 g of silica sand, placed in a 200 mL glass container used to house the microcosms, was spiked with an acetone solution containing each compound. After solvent evaporation, 60 g of the biomixture was added and mixed in an end-over-end rotary shaker for 15 min at room temperature. The biomixtures were moistened to 75% of their field capacity and the microcosms were incubated in darkness in a thermostatic chamber at 20 °C. Moisture content was maintained by adding sterile distilled water weekly. For sterilized controls, biomixtures were autoclaved at 121 °C for 20 min as previously described by Aguilar-Romero et al. (2019). These autoclaved biomixtures were prepared in parallel as abiotic controls while BPS microcosms with non-contaminated biomixtures were run in parallel to determine the effect of experimental conditions on microbial populations.

2.3. PPCP analysis

The PPCPs were extracted on day 0, 7, 14, 21, 28, 50 and 79 from 3 g (dry-weight) of biomixture in each BPS microcosm by adding 6 mL of acetonitrile acidified with 1% acetic acid followed by vortexing for 1 min. Then, 1 g of a QuEChERS extraction salts mixture (Agilent Technologies, Santa Clara, CA, USA) was added and vortexed again for 1 min. Samples were centrifuged for 5 min at 3500 rpm, and 1 mL supernatant was diluted (1:1) with MilliQ water, filtered using a 0.45 μm PTFE filter and analysed by high-performance liquid chromatography, under the conditions described in the General Materials and Methods section. The extraction method recovery rates for IBP, DCF and TCS

were 87%–92%, 82%–90% and 82%–106%, respectively (Delgado-Moreno et al., 2019).

2.4. Total DNA isolation and amplicon sequencing analysis

To determine the effect of PPCPs on the indigenous bacterial community, total DNA was extracted from 0.5 g (wet weight) of the biomixture from each BPS microcosm at different times using the FastDNA® Spin Kit for Soil (MP Biomedicals, Solon, OH, USA). The DNA was quantified using the Qubit dsDNA BR Assay kit (Live Technologies, Invitrogen, USA). DNA samples were then submitted for high-throughput 16S rRNA gene amplicon sequencing to Integrated Microbiome Resource (www.cgeb-imr.ca, Halifax, Nova Scotia, Canada). Primers for the V4-V5 variable regions of the 16S rRNA gene (Walters et al., 2016) were used and the amplified products were sequenced with the aid of Illumina MiSeq using 2 × 300 bp PE v3 chemistry (Comeau et al., 2017). All sequence files were submitted to the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA - https://www.ncbi.nlm.nih.gov/sra) and can be accessed in BioProject PRJNA603893.

2.5. Bioinformatics data analysis

Amplicon sequences of the V4-V5 region of bacterial 16S rRNA gene were processed using QIIME as described in the General Materials and Methods section.

2.6. Quantitative PCR (qPCR)

Total bacterial community and the abundance of important taxonomic groups, *Acidobacteria* and *Alpha-*, *Beta-* and *Gammaproteobacteria*, were quantified by real-time PCR assays using taxon-specific 16S rRNA primers under thermal conditions described by Philippot et al. (2011). The real-time PCR reactions were carried out in a volume of 12.5 µl using 6.25 µl of iQ™ SYBR® Green Supermix (Bio-Rad Laboratories, Hercules, CA, USA), 400 nM of each primer and 1 ng of DNA. All samples were run in triplicate on the MyiQ2 system (Bio-Rad) real-time PCR instrument. Serial dilutions of linearized pCR2.1-TOPO® containing cloned

sequences of the 16S rRNA gene specific for each taxon were used for standard curves. The PCR efficiency values ranged from 81 to 103%. To check for the presence of inhibitors in the DNA used for qPCR assays, DNA samples from the BPS microcosms were mixed with a known amount of standard DNA prior to qPCR. No inhibitors were detected in the assays.

2.7. Toxicological analysis

To determine the toxic effects of PPCPs on microbial populations, the number of live microorganisms was determined in non-sterile samples at different times by microscopic analyses using the LIVE/DEAD® BacLight™ Bacterial Viability Kit (Molecular Probes®, Life Technologies, USA). For this purpose, the microorganisms from 0.5 g of the PPCP-treated and non-treated biomixture were extracted in 50 mL of phosphate-buffered saline (PBS) for 1 h by shaking at 30 °C in triplicate. A double-concentrated working solution of the LIVE/DEAD BacLight staining reagent mixture was then prepared following the manufacturer's instructions and mixed with an equal volume of the bacterial suspension. This mixture was incubated at room temperature in the dark for 15 min. Cell counts were performed from 10 µL with the aid of a Neubauer chamber.

2.8. Data modeling and statistical analysis

Data modeling and ecological and statistical analysis were performed as described in the General Materials and Methods section. For these analyses, the outlier sample of BPS microcosm 2 contaminated with triclosan at day 7, T27, was not taken into account. In order to detect biomarker OTUs which showed differences in relative abundances between control and PPCP treatments at each time point, the linear discriminant analysis (LDA) effect size (LEfSe) algorithm (Segata et al., 2011) was used in the MicrobiomeAnalyst pipeline (Chong et al., 2020). The SPSS Statistical Software Package version 25 (IBM Corporation, New York, USA) was used for one-way ANOVA at a significance level of 0.05.

3. RESULTS AND DISCUSSION

3.1. PPCP removal in BPS microcosms

To determine how PPCPs affect indigenous microbial populations in the BPS biomixture, the disappearance of each compound was measured over time in BPS microcosms treated with IBP, DCF, and TCS, both individually and simultaneously. The dissipation curves of the PPCPs are shown in Figure 1. Except for the sterile controls, the single first-order model accurately fitted all the experimental data and showed low chi-square and scaled error values ($\chi_{5,0.05}^2$ = 11.070, err_{scaled} < 6.24). The R^2 values were over 0.92 except for TCS (Table 1).

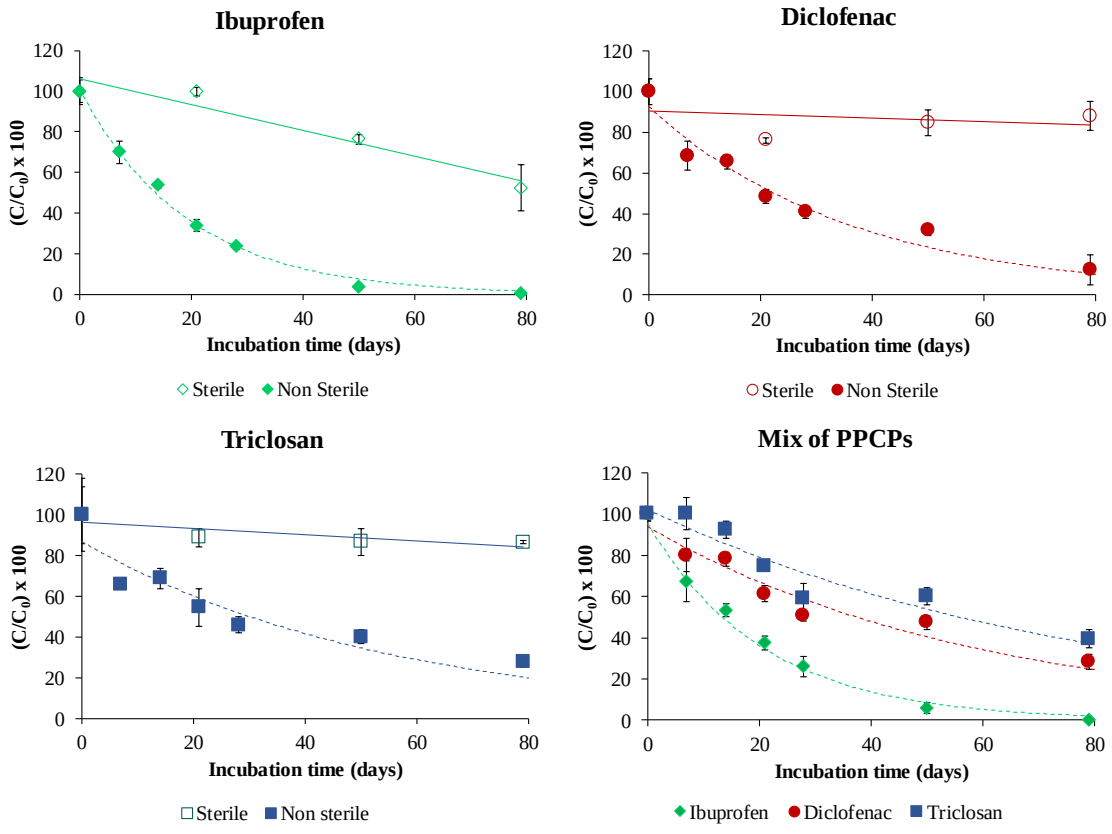


Figure 1. Dissipation of ibuprofen, diclofenac and triclosan applied separately or in mixture in non-sterilized (black symbols) and sterilized (white symbols) BPS microcosms. Lines represent the model fit to experimental data. Error bars indicate standard deviation (n=3).

Despite an initial 5-fold higher concentration in BPS microcosms than that of the other compounds, IBP showed the highest dissipation rate constant k, with a DT90 of 45 days and was no longer detectable (less than 0.9%) after 79 days of incubation. DCF and TCS dissipated more slowly than IBP and required 84 and 127 days, respectively, for 90% removal (DT_{90}) when applied individually. Dissipation of IBP, DCF and TCS was even slower (DT_{90} of 47, 137 and 180 days, respectively), when applied simultaneously. In all cases, *k* values were higher when the PPCPs were applied separately rather than simultaneously (Table 1). However, the difference in this increase was only statistically significant in samples contaminated with DCF (ANOVA, $p = 0.012$). This indicates that the removal of DCF in BPS microcosms was affected by the presence of either TCS or IBP or both. This could be attributed to the possible negative effect of these compounds on groups of microorganisms involved in DCF dissipation. According to the results obtained by Delgado-Moreno et al. (2019), when a mixture of the three PPCPs was applied, the dissipation rates for DCF and TCS were 3- and 2-fold higher, respectively, than the values obtained in our study. Nevertheless, the 5-fold higher amount of IBP applied in this study could negatively impact microbial populations harboured in the BPS microcosms which are responsible for DCF and TCS dissipation. This could also be explained by the existence of microorganisms with substrate preferences. For instance, (Z. Lu et al., 2019) have reported that the bacterial strain *Pseudoxanthomonas* sp. DIN-3, which has the ability to remove DCF, IBP, and Naproxen (each at 50 µg/L), eliminated IBP more effectively when confronted with this PPCP only than in a mixture with the other compounds.

On the other hand, results from sterilized control treatments revealed that, after 79 days of incubation, the amounts dissipated of IBP, DCF and TCS were 47.6, 7.3 and 13.43%, respectively (Figure 1). These percentages indicate that some abiotic dissipation occurs, especially in microcosms treated with IBP. Under these conditions, the removal of PPCPs could be influenced by the presence of clay particles and solar radiation via photochemical reactions generating metabolites (Aranami and Readman, 2007; Lonappan et al., 2016; Maldonado-Torres et al., 2018). In fact, two likely abiotically formed metabolites of IBP and DCF, 1-(4-isobutylphenyl)ethanone and 2-(9H-carbazol-1-yl) acetic

acid, respectively, were detected in the same type of BPS in a previous study (Delgado-Moreno et al., 2019).

Table 1. Single first-order kinetic parameters and percentages removed (D) of ibuprofen, diclofenac and triclosan applied separately and in mixture in BPS microcosms.

	PPCPs applied individually			PPCPs applied in mixture		
	Ibuprofen	Diclofenac	Triclosan	Ibuprofen	Diclofenac	Triclosan
C_0 ± sd (%)	100.8 ± 3.9	92.8 ± 2.9	87.7 ± 10.1	99.1 ± 1.0	93.9 ± 3.9	102.3 ± 2.3
k x 10^2 ± sd (d^{-1})	5.2 ± 0.5	2.8 ± 0.4	1.9 ± 0.5	4.9 ± 0.7	1.7 ± 0.0	1.3 ± 0.1
R^2	0.99	0.94	0.79	0.98	0.92	0.88
Err_{scaled}	2.02	4.41	6.24	1.89	4.36	5.3
DT_{50} ± sd (d)	14 ± 0	25 ± 4	38 ± 11	14 ± 2	41 ± 1	54 ± 2
DT_{90} ± sd (d)	45 ± 2	84 ± 12	127 ± 37	47 ± 6	137 ± 4	180 ± 8
D ± sd (%)	99.9 ± 0.1	87.6 ± 7.3	72.2 ± 2.8	100.0 ± 0.0	71.8 ± 3.7	60.7 ± 4.5

3.2. Bacterial community structure, dynamics and composition in BPS microcosms

To determine the response of bacterial communities to PPCPs in BPS microcosms, amplicon products of V4-V5 variable regions of the 16S rRNA gene were analysed at different times. A total of 1,024,229 sequences were obtained after quality filtering and the removal of possible chimera sequences. The sequencing depth of these samples was sufficient to cover the full range of diversity as indicated by rarefaction curves (Supplementary Figure S1A).

3.2.1. Bacterial richness and diversity in BPS microcosms

Bacterial community alpha diversity in the BPS microcosms was determined over a period of 50 days (Table 2). In control BPS microcosms, bacterial richness (chao1), number of observed OTUs, as well as the Shannon diversity and Simpson's evenness indices, generally increased over time, dipped between day 7 and 14, before increasing up to the end of the incubation period at day 50. Similar tendencies were observed in BPS microcosms exposed to PPCPs, although punctually significant differences were observed with respect to the control.

Overall, BPS microcosms exposed to IBP and DCF showed significantly higher alpha diversity, as indicated by Shannon and Simpson indices (ANOVA, $p < 0.05$), than controls at day 7, and all the indices for IBP showed increased alpha diversity (ANOVA, $p < 0.05$) after 14 days (Table 2). After 50 days, the number of observed OTUs and the Shannon diversity for DCF were significantly higher (ANOVA, $p < 0.05$). Jiang et al. (2017) have also observed an increase in Shannon diversity in sequence batch reactors exposed to IBP and/or DCF after 130 days, thus indicating the resilience potential of the bacterial community in response to low concentrations of these two non-steroidal anti-inflammatory drugs.

The alpha diversity parameters of TCS-exposed BPS microcosms were significantly lower than those of the control after 50 days ($p < 0.05$; Table 2). This clearly indicates that TCS negatively affects the richness and diversity of BPS microcosm bacterial populations. Recent studies have demonstrated that TCS is an environmental stressor, alters the community structure and reduces species diversity and richness (Clarke et al., 2019; Oh et al., 2019; Peng et al., 2019). Following simultaneous treatment with the three PPCPs, alpha diversity values were generally lower than those for IBP and DCF and higher than those for TCS alone, but did not significantly differ from those of the controls, except for a higher evenness value after 7 days. Therefore, the negative impact generated by TCS on its own may be compensated by the increase in richness and diversity caused by DCF and especially by IBP. With regard to other studies of BPS, the increased application of pesticide has been shown to decrease bacterial diversity in some BPSs (Holmsgaard et al., 2017), while in others, the pesticides introduced had a limited or only transient effect (Castro-Gutiérrez et al., 2017; Diez et al., 2018, 2017; El Azhari et al., 2018; Góngora-Echeverría et al., 2018; Marinozzi et al., 2013; Tortella et al., 2013). Although these studies indicate that certain pesticides had concentration-dependent effects, they mostly show that BPS bacterial communities were resilient to these types of contaminants. Similarly, in this study, even under treatment with TCS, the bacterial community in BPS microcosms was resilient, with diversity improving once the levels of the contaminant decreased or were mitigated by the effect of IBP and DCF.

Table 2. Alpha diversity indices (Chao1, Shannon and Simpson evenness) and Observed OTUs of the non-contaminated (control) and contaminated BPS microcosms with ibuprofen, diclofenac and triclosan applied separately or in mixture. Each value is the mean of three replicates ± standard deviation.

Treatments	Days	Chao1	Observed OTUs	Shannon	Simpson
	0	1503 ± 41	687 ± 24	7.25 ± 0.06	0.973 ± 0.002
Control	7	1547 ± 34	701 ± 27	6.93 ± 0.05	0.939 ± 0.005
	14	1351 ± 69	674 ± 34	7.00 ± 0.33	0.949 ± 0.017
	21	1560 ± 151	811 ± 42	8.13 ± 0.16	0.989 ± 0.002
	28	1696 ± 100	860 ± 41	8.38 ± 0.13	0.992 ± 0.001
	50	1675 ± 42	842 ± 40	8.34 ± 0.19	0.991 ± 0.003
Ibuprofen	7	1678 ± 89	751 ± 24	7.56 ± 0.05*	0.975 ± 0.002*
	14	1726 ± 67*	826 ± 21*	8.01 ± 0.15*	0.984 ± 0.004*
	21	1553 ± 124	812 ± 89	8.22 ± 0.38	0.991 ± 0.003
	28	1902 ± 65*	907 ± 24	8.50 ± 0.05	0.993 ± 0.000
	50	1802 ± 165	903 ± 41	8.63 ± 0.13	0.994 ± 0.001
Diclofenac	7	1709 ± 125	765 ± 53	7.46 ± 0.27*	0.965 ± 0.008*
	14	1462 ± 198	735 ± 65	7.68 ± 0.32	0.982 ± 0.004*
	21	1565 ± 154	837 ± 57	8.33 ± 0.16	0.992 ± 0.001
	28	1718 ± 134	885 ± 40	8.44 ± 0.04	0.993 ± 0.000
	50	1784 ± 104	934 ± 32*	8.71 ± 0.1*	0.994 ± 0.001
Triclosan	7	1636 ± 178	729 ± 64	7.35 ± 0.53	0.962 ± 0.021
	14	1274 ± 110	633 ± 50	6.90 ± 0.34	0.951 ± 0.012
	21	1390 ± 76	745 ± 51	7.92 ± 0.25	0.987 ± 0.003
	28	1671 ± 141	837 ± 59	8.27 ± 0.13	0.992 ± 0.001
	50	1471 ± 84*	751 ± 37*	7.87 ± 0.17*	0.983 ± 0.007
Mix of PPCPs	7	1296 ± 301	597 ± 95	6.76 ± 0.55	0.958 ± 0.011*
	14	1440 ± 122	697 ± 60	7.50 ± 0.35	0.976 ± 0.008
	21	1413 ± 158	764 ± 93	7.99 ± 0.52	0.988 ± 0.006
	28	1583 ± 145	820 ± 43	8.34 ± 0.15	0.993 ± 0.001
	50	1666 ± 88	814 ± 32	8.29 ± 0.09	0.992 ± 0.001

∗ Significant differences between non-contaminated (control) and contaminated BPS microcosms (ANOVA, p b 0.05).

3.2.2. Bacterial community structure and composition in BPS microcosms

The bacterial community in all of the BPS microcosms was composed of 38 different phyla, 10 of which accounted for 98–99% of the total OTUs detected (Figure 2A). *Proteobacteria* were the most abundant phylum (38.6–57.5%), followed by *Bacteroidetes* (13.5–48.4%), *Actinobacteria* (3.6–11.2%), *Planctomycetes* (3.1–9.4%), *Acidobacteria* (0.7–6.4%) and *Verrucomicrobia* (0.3–5.6%).

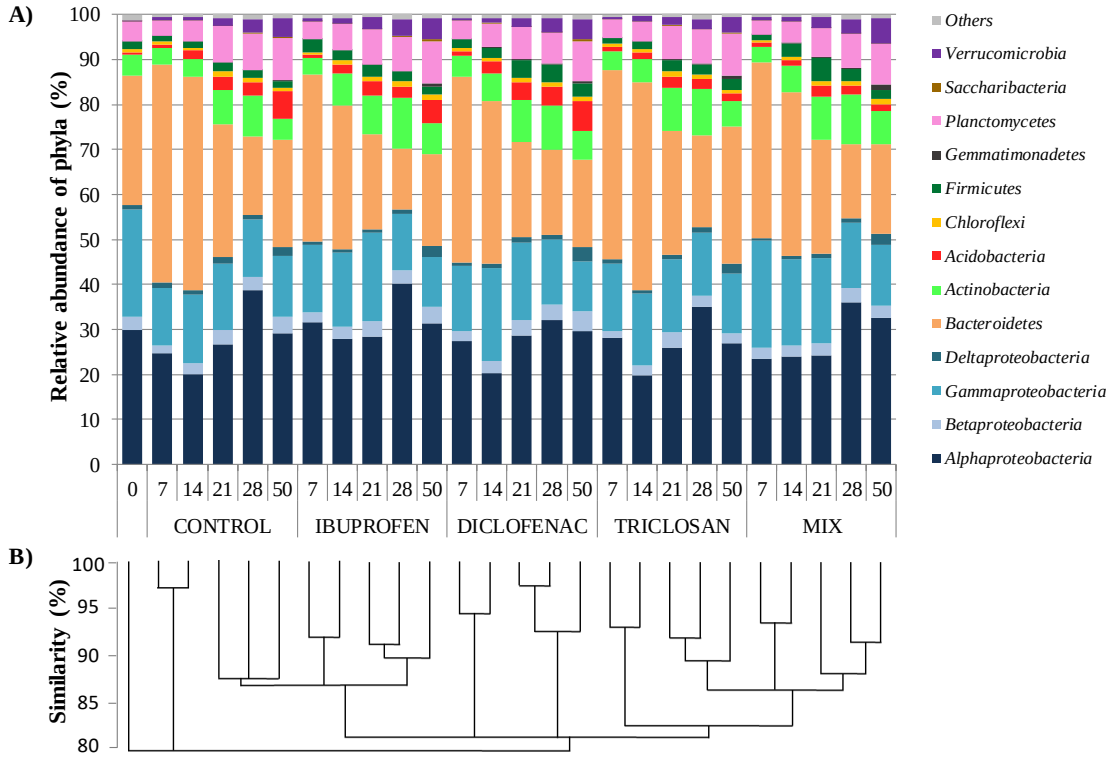


Figure 2. Relative abundance of the most dominant phyla (>1% in any sample) and classes of *Proteobacteria* at different incubation times (days) in non-contaminated (control) and contaminated BPS microcosms with ibuprofen, diclofenac and triclosan, applied separately or in mixture (A). Hierarchical cluster analysis of the bacterial community composition based on Bray-Curtis distances (B).

Hierarchal clustering analysis of bacterial community composition over time grouped bacterial communities mainly according to the contaminant applied

(Figure 2B). The main cluster showed 82% similarity between microcosms treated with DCF and the other treatments. Within this cluster, BPS microcosms treated with IBP presented a cluster with 87% similarity to control microcosms at day 21, 28 and 50, while those contaminated with TCS clustered more closely with those treated simultaneously with the three PPCPs, especially at day 21, 28 and 50. Within each subcluster, the bacterial communities at initial times (day 7 and 14) grouped together in all cases.

Principal component analysis show that 31.63% of the diversity variations (PC1) in bacterial community structures were affected mainly by incubation time, with higher similarity between communities observed in the initial time period (day 0, 7 and 14) than those found later at day 21, 28 and 50 (Figure 3).

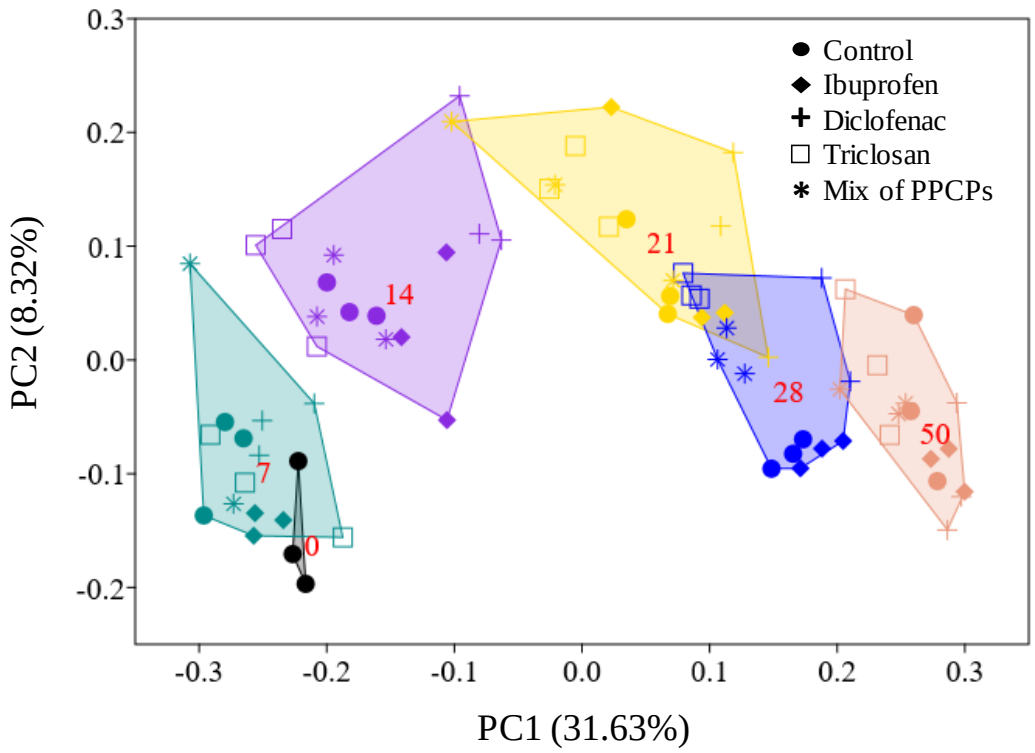


Figure 3. Principal coordinates analysis of bacterial communities in the BPS microcosms based on Bray-Curtis distances. The numbers in red indicate the incubation time (days).

The permutational multivariate analysis of variance (PERMANOVA) of the data also indicates that incubation time had the strongest effect on the bacterial community composition of BPS microcosms (PERMANOVA, $p = 0.001$), followed

by the effect of contamination with the different PPCPs (PERMANOVA, $p < 0.005$). Similarly, nonmetric multidimensional scaling (NMDS) analysis of the bacterial communities also grouped the samples according to incubation time (Supplementary Figure S1B). Thus, the main determining factor which affected the bacterial community composition of BPS microcosms was incubation time and, to a lesser extent, treatment with PPCPs. Similarly, aging of the BPS and incubation time have also been observed to have a stronger impact in other BPSs, in which the effects of pesticides were studied (Castro-Gutiérrez et al., 2017; Diez et al., 2017; El Azhari et al., 2018; Marinozzi et al., 2013), even when co-applied with an antibiotic (Castro-Gutiérrez et al., 2019, 2017).

3.2.2.1. Impact of PPCPs on the composition and specific taxa of bacterial communities

In order to analyse the response of bacterial communities in BPS microcosms to exposure to PPCPs, differences in the relative abundances of bacterial groups between the treated BPS microcosms and controls were compared. We also used linear discriminant analysis (LDA) effect size (LEfSe) to find biomarkers consisting of differentially abundant OTUs in BPS microcosms (Figure 4, Table S1). These analyses indicate that all PPCP treatments had a negative effect on *Bacteroidetes* (Figure 2A) particularly on 9 OTUs belonging to the *Cytophagaceae* and *Chitinophagaceae* families which proliferated mainly in the control BPS microcosms (Figure 4). Other taxa strongly affected by PPCPs included an OTU belonging to the *Planctomycetaceae* family and two OTUs of the alphaproteobacterial family *Hyphomonadaceae* (Supplementary Figure S4, Control). OTUs of the betaproteobacterial family *Methylophilaceae* were also disfavoured in treated BPS microcosms (Figure 4, Control), suggesting that these biomarkers are especially sensitive to PPCPs.

- Impact of ibuprofen on BPS microcosm bacterial communities

Treatment with ibuprofen had a marked effect on proteobacterial populations in BPS microcosms, especially on those of *Alphaproteobacteria* which increased

significantly in the first 14 days (ANOVA, $p < 0.05$) (Figure 2A). During this period, the relative abundance (RA) of the family *Sphingomonadaceae* increased (Supplementary Figure S2). Several of the biomarker OTUs detected in IBP-treated BPS microcosms belong to this family and were identified as *Novosphingobium* OTUs (Figure 4). Recently, Navrozidou et al. (2019) also reported the enrichment of *Novosphingobium* in activated sludge exposed to high doses of IBP. IBP-exposed BPS microcosms were also enriched in a biomarker associated with *Sphingomonas* (Figure 4). Previous studies have demonstrated that specialized bacteria belonging to these genera are capable of degrading PPCPs (Zhou et al., 2013). Moreover, Murdoch and Hay (2005) isolated a *Sphingomonas* strain from activated sludge which can grow with IBP as a sole carbon and energy source through its transformation into a metabolizable alkylcatechol intermediate (Murdoch and Hay, 2013). In the current study, although the *Novosphingobium* and *Sphingomonas* biomarkers were mostly constrained to mid-to-low concentrations of IBP, one biomarker, belonging to the same taxonomic order and specifically to the family *Erythrobacteraceae*, was constrained to higher concentrations (Supplementary Figure S3A), thus indicating its possible involvement in IBP degradation. In BPS microcosms exposed to IBP, the relative abundance of *Gammaproteobacteria* increased at punctual moments (Figure 2A), as reflected by increases in the dominant families *Sinobacteraceae* and *Pseudomonadaceae* (Supplementary Figure S2). Among the biomarker OTUs detected (Figure 4), one OTU was identified from each of these families, including the species *Pseudomonas stutzeri* which was constrained to higher concentrations of IBP in the BPS microcosms (Supplementary Figure S3A). As this species is associated with polycyclic aromatic hydrocarbon degradation (Wang et al., 2019), the identified biomarker could be an interesting candidate for studying IBP degradation. While IBP had a positive effect on some bacterial taxa in the BPS microcosms, exposure to this PPCP significantly decreased the RA of *Bacteroidetes* (ANOVA, $p < 0.05$) by 3.8–15.6%, which mainly affected the *Cytophagaceae* and *Chitinophagaceae* families (Supplementary Figure S2). Similarly, a decrease in *Bacteroidetes* and an increase in *Alphaproteobacteria* communities have been observed in activated sludge treated with high concentrations of IBP (Davids et al., 2017).

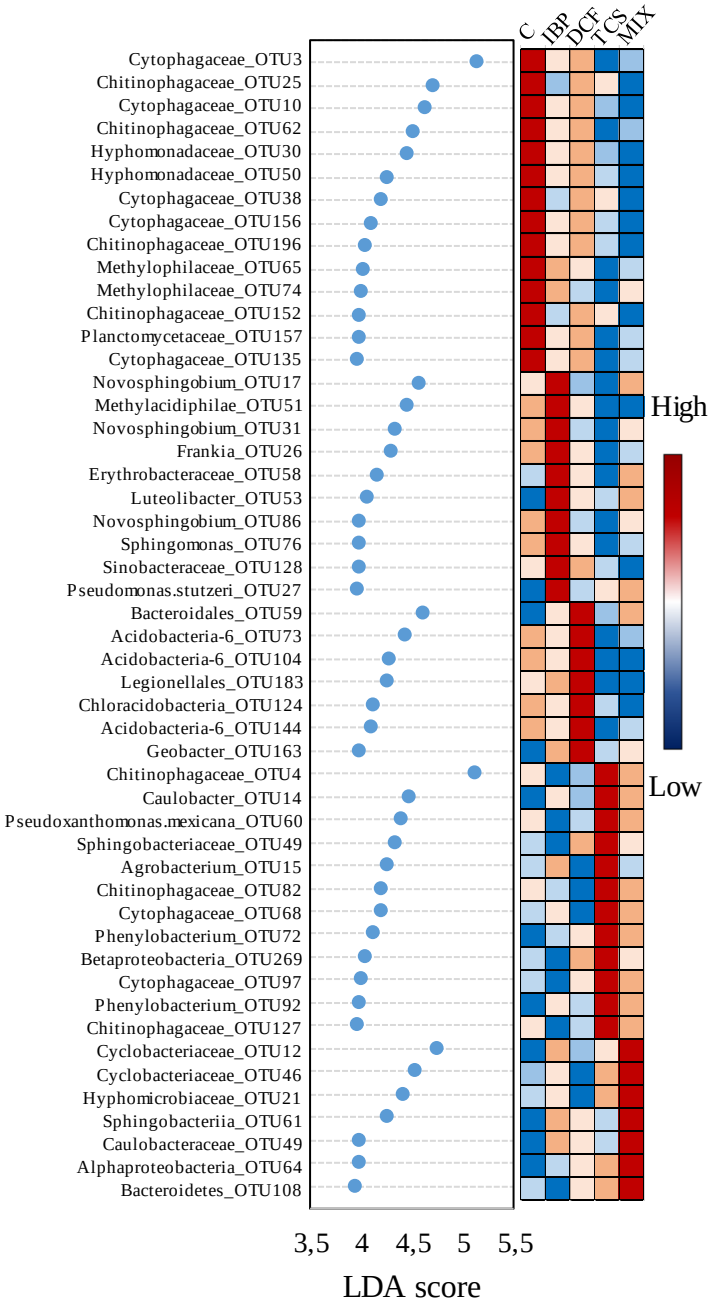


Figure 4. Biomarker OTUs identified by LEfSe (Segata et al., 2011) with the highest linear discriminant analysis (LDA) scores and organized by their respective abundance in each treated BPS microcosm. The heatmap shows the comparative abundance of biomarker OTUs in each BPS microcosm. C indicates control, IBP indicates ibuprofen-exposed BPS microcosms, DCF indicates diclofenac-exposed BPS microcosms, TCS indicates triclosan exposed BPS microcosms, and MIX indicates BPS microcosms treated with PPCP mixture.

In the IBP-exposed BPS microcosms, the RA of the *Verrucomicrobia* phylum showed enrichment during the incubation period (Figure 2A). Two of the biomarkers identified belong to the family *Verrucomicrobiaceae* and another to the verrucomicrobial class *Methylacidiphilae* (Figure 4). The former, identified as *Luteolibacter,* responded to the highest concentrations of ibuprofen (Supplementary Figure S3A), suggesting that it directly interacts with IBP. The RA of the phylum *Actinobacteria* showed an increase in the IBP-treated BPS microcosms after 14 days as compared to the control (Figure 2A), while an OTU identified as Frankia was found to be a biomarker belonging to this phylum (Figure 4). Although species belonging to this genus have been reported to degrade atrazine (Rehan et al., 2017) or naphthalene (Baker et al., 2015), this biomarker was constrained to lower concentrations of IBP (Supplementary Figure S3A).

- Impact of diclofenac on BPS microcosm bacterial communities

Generally, DCF caused less evident changes in the relative abundance of *Proteobacteria* in BPS microcosms than the other PPCPs. However, an increase in the relative abundance of *Gammaproteobacteria* was observed at day 14 (Figure 2A). Within this class, significant increases in the RA of *Legionellales* were detected at certain time points (ANOVA, $p < 0.05$; Supplementary Figure S4C). One of the biomarker OTUs identified in DCF-treated BPS microcosms which belongs to the order *Legionellales* (Figure 4), was constrained to higher concentrations of DCF than the other biomarkers (Supplementary Figure S3B), indicating that it somehow interacts with this PPCP. Curiously, although the relative abundance of *Deltaproteobacteria* was low in all BPS microcosms, one of the biomarker OTUs in the DCF-treated BPS microcosms was identified as *Geobacter*. Although constrained to lower concentrations of DCF, *Geobacter* has been shown to be involved in diclofenac degradation in an anaerobic anodic chamber (Qiu et al., 2020). As observed for the IBP-treated BPS microcosms, the RA of *Bacteroidetes* was negatively affected by DCF (Figure 2A). Nonetheless, although one of the biomarker OTUs belongs to the bacteroidetal class *Bacteroidales* (Figure 4), it appeared to be constrained to the lowest

concentrations of DCF (Supplementary Figure S3B). The RA of *Acidobacteria* was observed to have increased significantly at day 21 and 28 in DCF-treated BPS microcosms (ANOVA, $p < 0.05$; Figure 2A). While a number of the high-scoring biomarkers found in BPS microcosms exposed to DCF belong to the actinobacterial classes *Acidobacteria*-group 6 and *Chloracidobacteria*, most of these OTUs were constrained to the lowest DCF concentrations (Supplementary Figure S3B).

- Impact of triclosan on BPS microcosm bacterial communities

Triclosan did not greatly affect *Alphaproteobacteria* as compared to control conditions. Nonetheless, an initial increase in the relative abundance of *Rhizobiales* was observed (Supplementary Figure S4), which coincides with one of the biomarker OTUs identified in TCS-exposed BPS microcosms as belonging to Agrobacterium (Figure 4). This genus includes strains which have been shown, with the aid of a multidrug efflux pump, to be resistant to triclosan (Nuonming et al., 2018). When correlated to TCS concentrations using NMDS analysis (Supplementary Figure S3C), the Agrobacterium OTU was found to have a particularly strong impact at higher TCS concentrations. The RA of the alphaproteobacterial family *Caulobacteraceae* (Supplementary Figure S2) increased by 1–2% between day 14 and 28 with TCS. Several of the biomarkers identified belong to this family and are related to the genera *Caulobacter* and *Phenylobacterium*, which are associated with the degradation of other organic pollutants such as polyaromatic hydrocarbons and oil hydrocarbons, respectively (Lopez-Echartea et al., 2020; C. Lu et al., 2019). Moreover, given its particular importance at higher TCS concentrations (Supplementary Figure S3C), the *Caulobacter* OTU could be a good candidate for identifying TCS resistance and degradation mechanisms. Despite the increase in the relative abundance of *Betaproteobacteria* after 14 days and the identification of a betaproteobacterial biomarker, the RA of the order *Methylophilales* decreased significantly in BPS microcosms exposed to TCS (ANOVA, $p < 0.05$; Supplementary Figure S4B). Although the RA of *Gammaproteobacteria* was similar in both TCS-exposed BPS microcosms and the control, that of the class *Legionellales* decreased (ANOVA, $p < 0.05$; Supplementary Figure S4C). On the other hand, the RA of the family

Xanthomonadaceae increased at intermediate time points (Supplementary Figure S2), and one of the biomarkers in the BPS microcosms exposed to TCS, specifically *Pseudoxanthomonas mexicana*, was identified to belong to this family. (Oh et al., 2019) have also reported an increase in *Xanthomonadaceae*, specifically the genus *Pseudoxanthomonas*, in TCS-amended activated sludge reactors. Moreover, Zhang et al. (2019) found that *P. mexicana* is resistant to triclosan and that the abundance of this species increases in TCS-exposed river biofilms. On the other hand, the increase in the *Verrucomicrobia* phylum was 2-fold less pronounced during the incubation period in BPS microcosms exposed to TCS, and the RA of the *Acidobacteria* phylum was found to be drastically reduced after 28 and 50 days (Figure 2A), which mainly affected the classes Acidobacteria-6 and *Chloracidobacterias* (Supplementary Figure S5B). Compared to BPS microcosms treated with other PPCPs, the RA of the *Bacteroidetes* phylum did not decrease markedly when exposed to TCS. In fact, 6 of the biomarker OTUs detected in the TCS-treated BPS microcosms belong to the bacteroidetal families *Chitinophagaceae*, *Cytophagaceae* and *Sphingobacteriaceae*. Given their particular importance at higher TCS concentrations (Supplementary Figure S3C), two of these *Chitinophagaceae* biomarkers and the *Sphingobacteriaceae* biomarker could be good candidates for identifying TCS resistance and degradation mechanisms.

- Impact of PPCP mixture on BPS microcosm bacterial communities

In general, comparisons of the RA of bacterial taxa in BPS microcosms show that those treated with the PPCP mixture shared most similarity with those treated with TCS. For instance, as with those amended with TCS, the RA of the alphaproteobacterial family *Caulobacteraceae* (Supplementary Figure S2) increased in BPS microcosms treated with the PPCP mixture, in which one of the biomarkers was found to belong to this family (Figure 4). On the other hand, the RA of the alphaproteobacterial family *Hyphomicrobiaceae* (Supplementary Figure S2) in the BPS microcosms treated with the PPCP mixture differed from the other treatments, as highlighted by the identification of a hyphomicobiaceal biomarker (Figure 4). Once again, the drastic reduction observed for

Acidobacteria (Figure 2A) and its classes *Acidobacteria*-group 6 and *Chloracidobacteria* (Supplementary Figure S5B) was similar to that observed in the TCS-treated BPS microcosms (Figure 2A). As acidobacterial biomarkers were identified in DCF-treated BPS microcosms, the reduction in *Acidobacteria* could help to explain why the dissipation of DCF was reduced in BPS microcosms exposed to the PPCP mixture. Although the RA of *Bacteroidetes* is lower in BPS microcosms treated with the PPCP mixture, the RA of the bacteroidetal class *Sphingobacteriia* increased in a manner similar to that observed in TCS-exposed BPS microcosms. Moreover, one of the biomarkers identified in the BPS microcosms treated with the PPCP mixture was found to belong to this class (Figure 4). Other biomarkers identified in BPS microcosms treated with the PPCP mixture belong to the bacteroidetal family *Cyclobacteriaceae*. The RA of this family increased markedly in these BPS microcosms (Supplementary Figure S2), and one of the cyclobacteriaceal biomarkers was the only one to be constrained to higher PPCP concentrations (Supplementary Figure S3D), thus suggesting that this OTU may interact with the PPCPs studied.

3.2.3. Quantification by qPCR of abundance of bacterial taxa

In order to verify the results of the bacterial community analysis based on 16S rRNA amplicon sequences, the abundance of *Alpha-*, *Beta-*, *Gammaproteobacteria* and *Acidobacteria*, the taxa most affected by contamination with PPCPs, was quantified using real-time PCR assays (Supplementary Table S2). As with 16S rRNA bacterial community data, no significant difference in the abundance of *Alpha-*, or *Gammaproteobacteria* at the class level was observed in any microcosms between initial (day 7) and final (day 50) time points. Nevertheless, the bacteria belonging to the *Betaproteobacteria* class were enriched in BPS microcosms after contamination with IBP or DCF but not those exposed to TCS or the PPCP mixture. Moreover, in accordance with the 16S rRNA community study results, the abundance of the phylum *Acidobacteria* increased in control samples and in BPS microcosms treated with IBP and DCF, but no significant differences were observed in BPS microcosms exposed to TCS and the PPCP mixture. The results obtained by qPCR analysis were thus consistent with 16S rRNA amplicon sequencing data.

3.3. Toxicological analysis

In order to discern whether the PPCPs at the concentrations used in this study have a lethal effect on total bacterial populations in the BPS microcosms, the number of live bacterial cells was determined using LIVE/DEAD® microscopy (Figure 5).

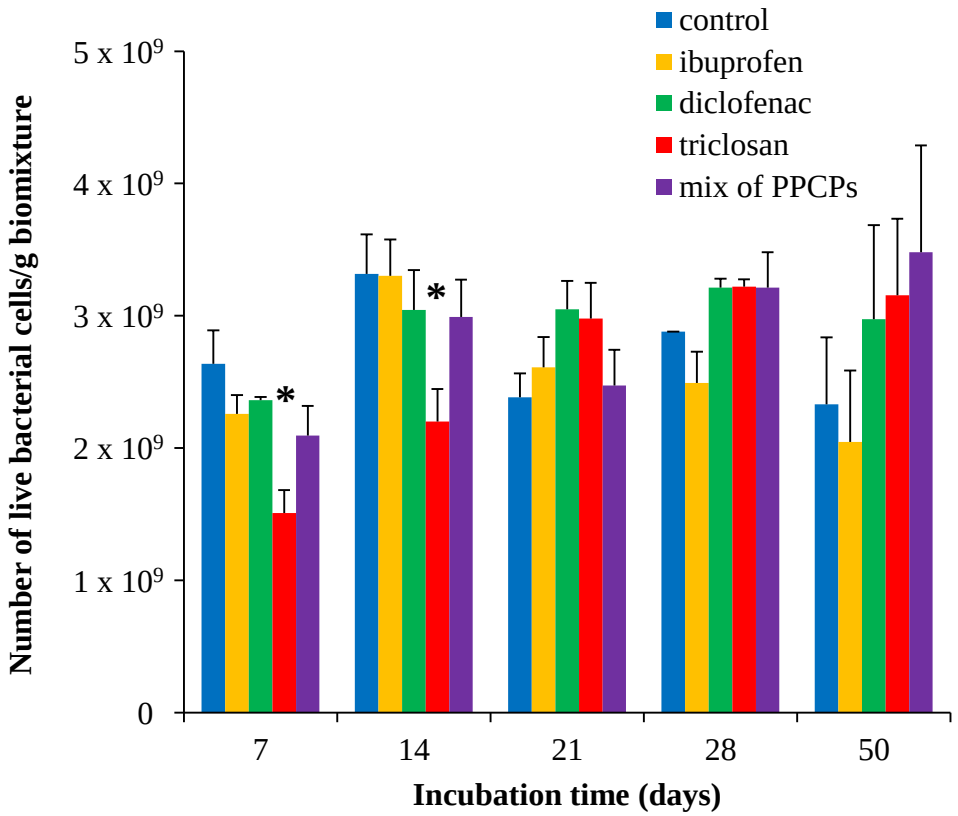


Figure 5. Number of live bacterial cells determined using the LIVE/DEAD® BacLight™ Bacterial Viability Kit per gram of non-contaminated (control) and BPS microcosms contaminated with ibuprofen, diclofenac and triclosan, applied separately or in mixture at different incubation times. The number of live bacterial cells in the BPS microcosms was initially $1.03 \times 10^9 \pm 5.48 \times 10^7$ cells/g. *Significant differences among non-contaminated and contaminated BPS microcosm samples (ANOVA homogeneity test; $p < 0.01$). Error bars represent the standard error for each sample ($n > 2$).

Prior to contamination, the number of live bacterial cells in BPS microcosms was $1.03 \times 10^9 \pm 5.48 \times 10^7$ cells/g. Throughout the incubation period, the number of cells in BPS microcosms treated with IBP or DCF did not significantly differ from that in non-contaminated control BPS microcosms. However, after 7 and 14 days, BPS microcosms exposed to TCS contained 43% and 34% fewer live bacterial cells, respectively, than control BPS microcosms (ANOVA, $p > 0.01$) before recovering in later periods. This may be due to the fact that the TCS-treated BPS microcosms still contained more than 65% of the initially applied biocide at these time points. Nevertheless, when a mixture of the three PPCPs was applied and TCS dissipation was much slower, the decrease observed in viability during the same period was not found to be significant. These results suggest that, in the absence of other PPCPs, the bactericidal effect of TCS is related to its concentration in the BPS microcosms, although the bacterial community eventually recovered. On the other hand, the lethal effect of TCS was mitigated in BPS microcosms contaminated with the three PPCPs, possibly due to an increase in resistant populations induced by IBP and/or DCF. Further studies with different PPCP concentrations will be needed to determine the toxicity limits of the PPCPs in BPS microcosms.

Supplementary material

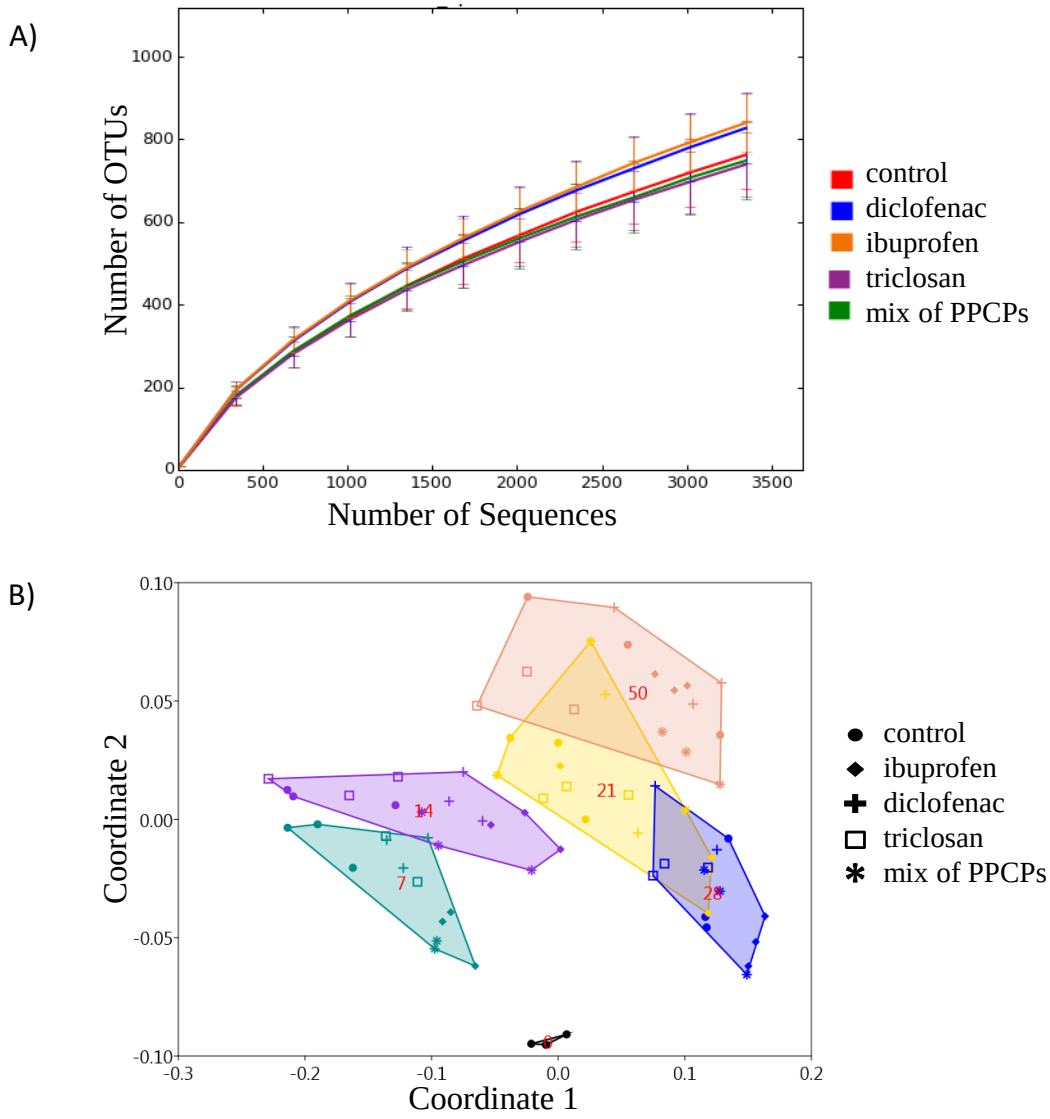


Figure S1. Rarefaction curves for the Operational Taxonomic Units (OTUs) of the non-contaminated (control) and contaminated BPS microcosms with ibuprofen, diclofenac and triclosan, applied separately and in mixture (A). Nonmetric Multidimensional Scaling (NMDS) ordination of the bacterial community composition based on Bray-Curtis distances. The numbers in red indicate the incubation time (days) (B).

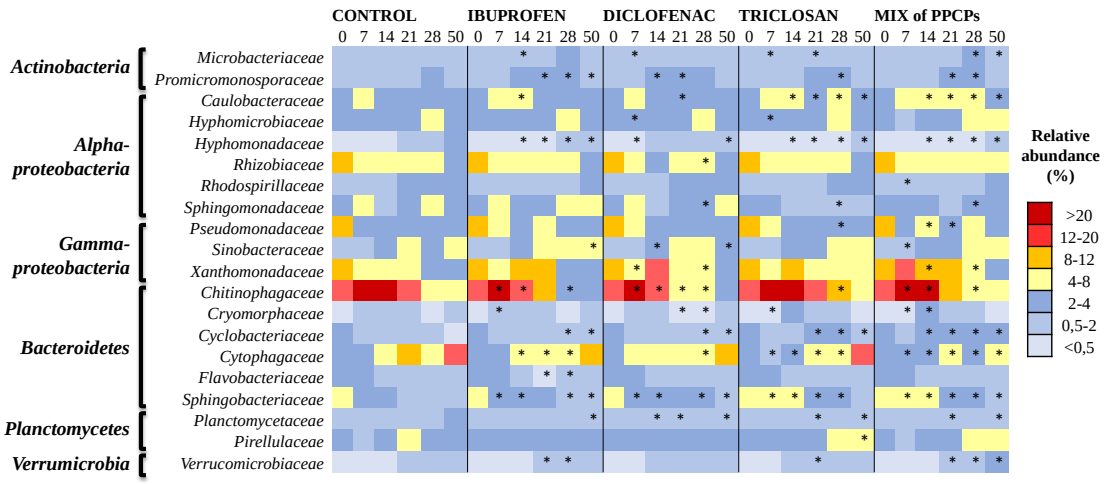


Figure S2. Heatmap of the most dominant families (>2% in any sample) in non-contaminated (control) and BPS microcosms contaminated with ibuprofen, diclofenac and triclosan, applied separately or in mixture at different incubation times (days). *Significant differences among non-contaminated and contaminated BPS microcosms samples (ANOVA homogeneity test; $p < 0.05$).

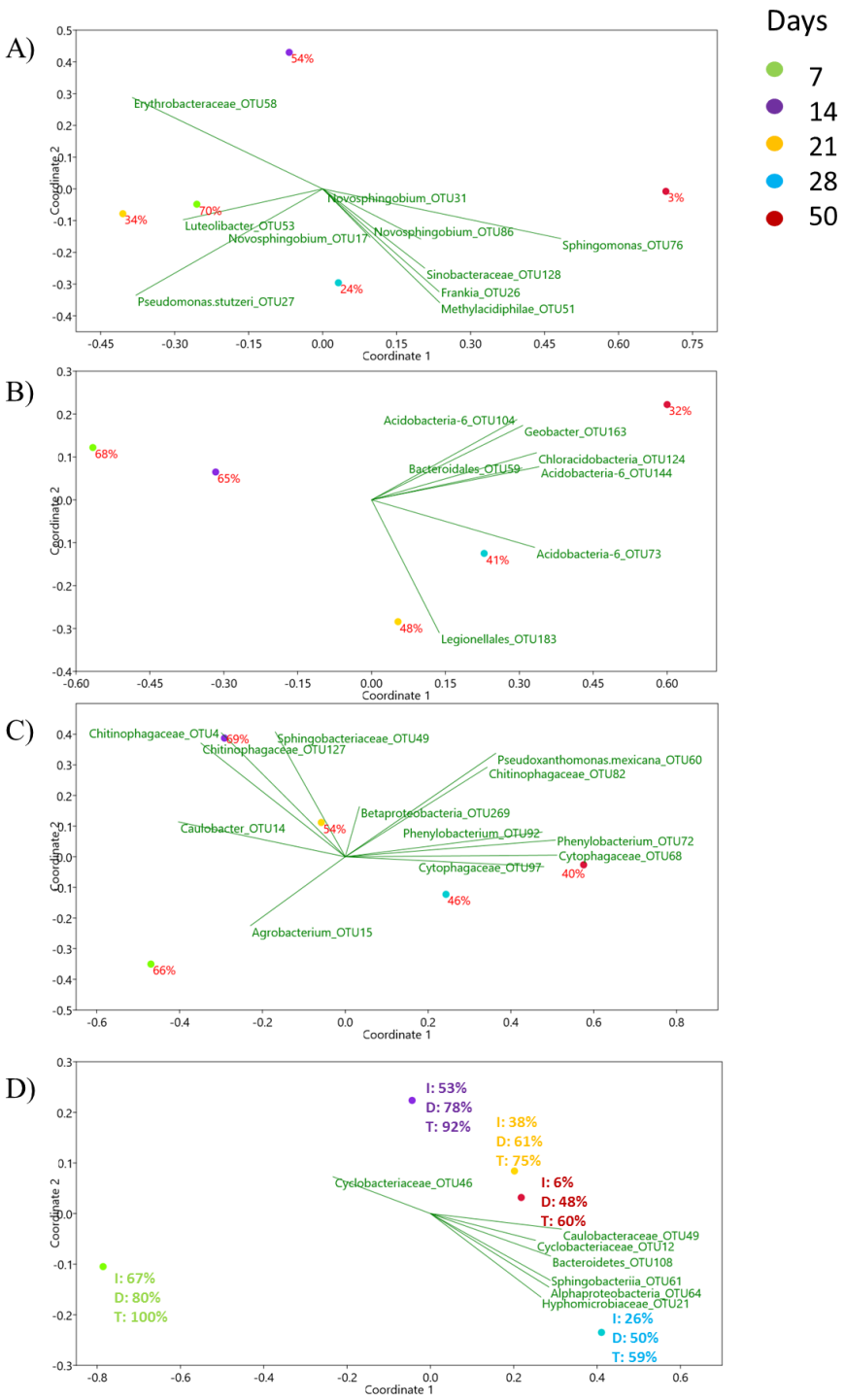


Figure S3. Nonmetric Multidimensional Scaling (NMDS) ordination based on Bray-Curtis distances of the concentrations of PPCPs constrained by biomarker OTUS in IBP-treated BPS microcosms (A), DCF-treated BPS microcosms (B), TCS-treated BPS microcosms (C) and BPS microcosms treated with the mixture of PPCPS (D). Numbers represent % C/C_0 remaining of each PPCP in the BPS microcosms and I indicates the concentration of IBP, D the concentration of DCF, and T the concentration of TCS in the PPCP mixture (D).

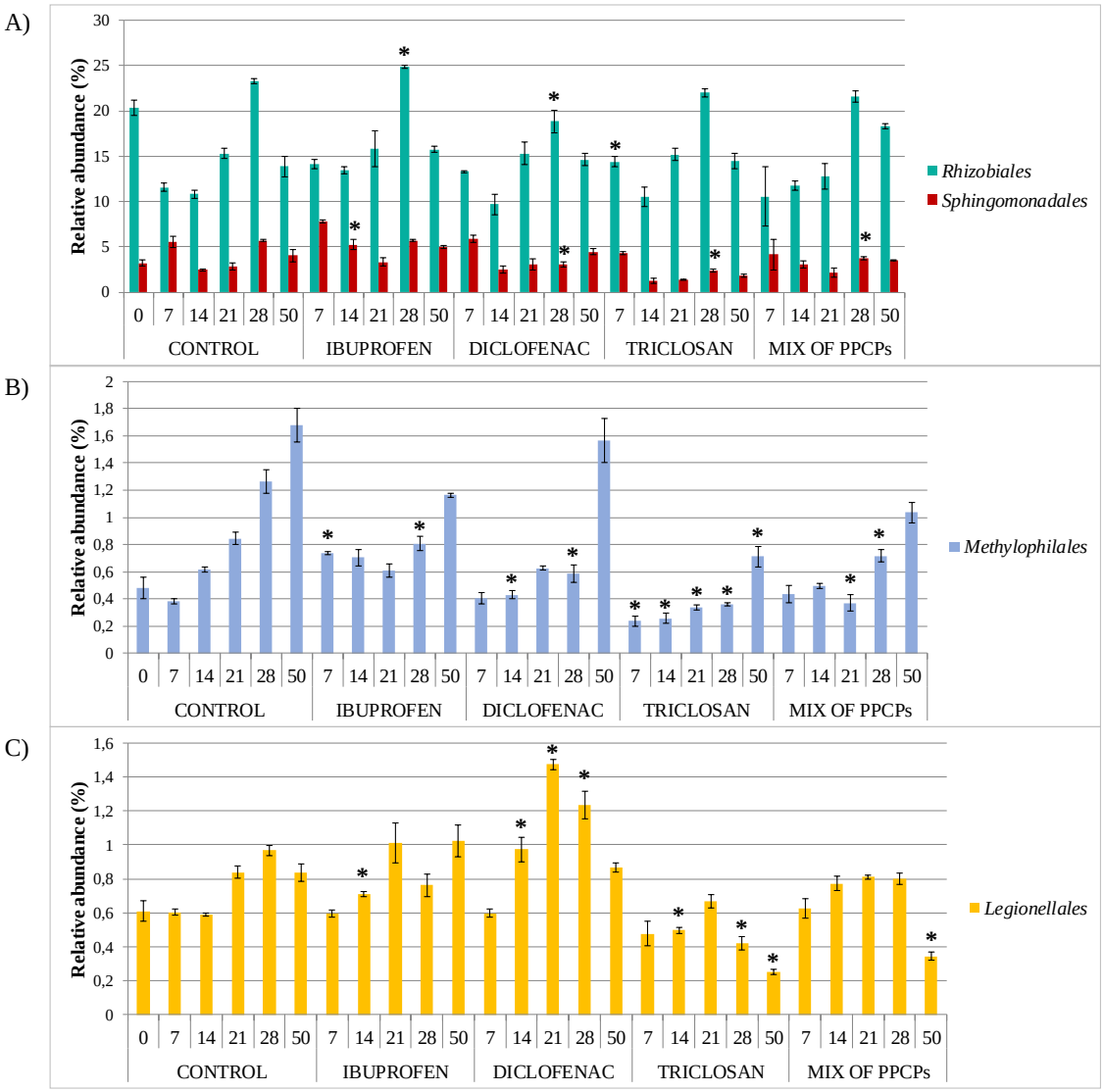


Figure S4. Relative abundance of affected orders within the classes *Alphaproteobacteria* (A), *Betaproteobacteria* (B) and *Gammaproteobacteria* (C). *Significant differences among non-contaminated (control) and contaminated BPS microcosms with ibuprofen, diclofenac and triclosan, applied separately and in mixture (ANOVA homogeneity test; $p < 0.05$). Error bars represent the standard error for each sample (n=3).

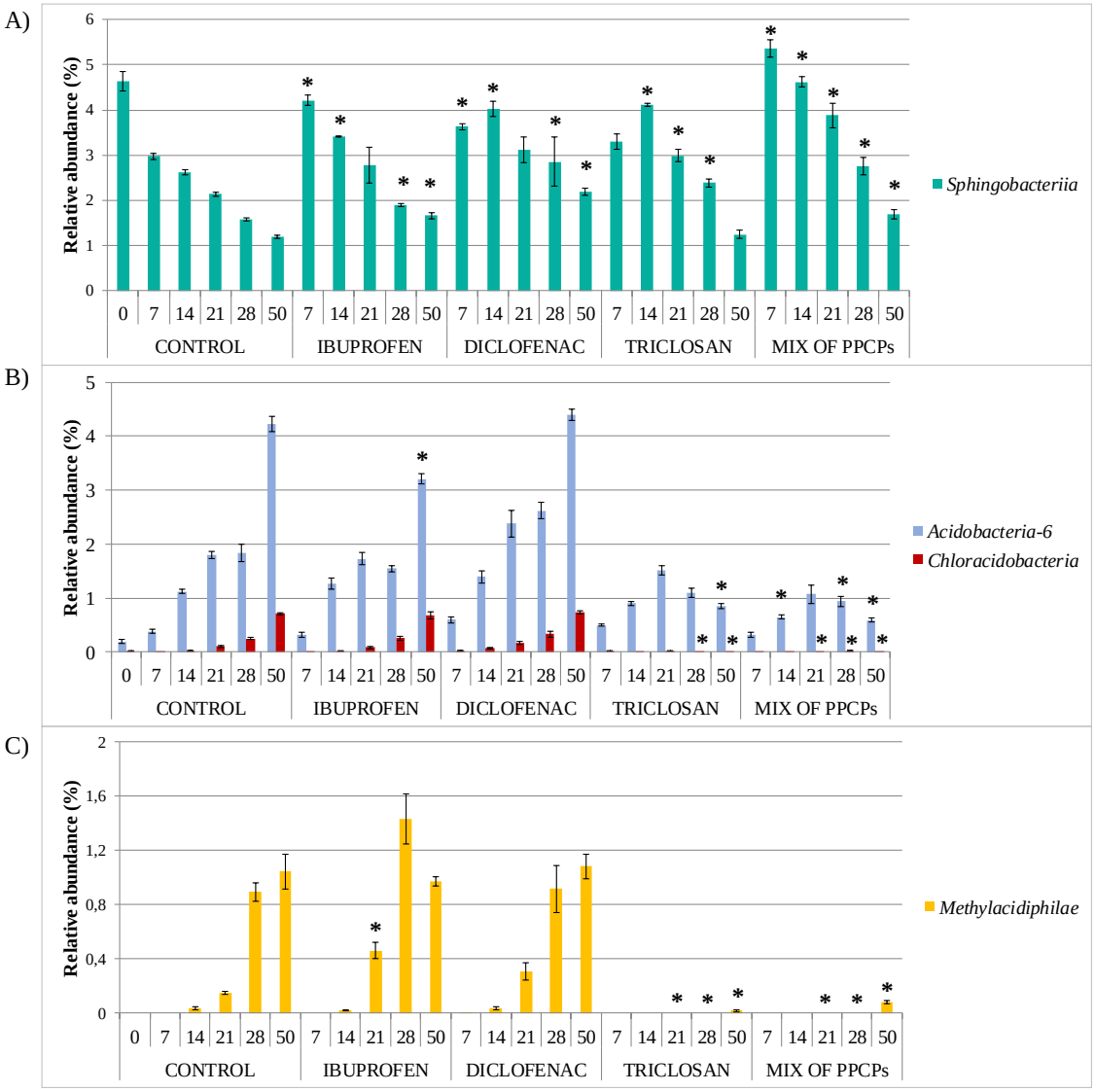


Figure S5. Relative abundance of affected classes within the phylum *Bacteroidetes* (A), *Acidobacteria* (B) and *Verrucomicrobia* (C). *Significant differences among non-contaminated (control) and contaminated BPS microcosms with ibuprofen, diclofenac and triclosan, applied separately and in mixture (ANOVA homogeneity test; $p < 0.05$). Error bars represent the standard error for each sample (n=3).

Table S1. Identification of the 50 biomarker OTUs detected in BPS microcosms with the highest L

Biomarker	OTU name from QIIME analyses	LDA Score	Phylum	Class	Order	Family
				CONTROL		
Cytophagaceae_OTU3	JQ978597.1.1486	5.12	Bacteroidetes	Cytophagia	Cytophagales	Cytophagaceae
Cytophagaceae_OTU10	FPLS01024837.16.1519	4.62	Bacteroidetes	Cytophagia	Cytophagales	Cytophagaceae
Cytophagaceae_OTU38	AUOS02000042.10667.12167	4.19	Bacteroidetes	Cytophagia	Cytophagales	Cytophagaceae
Cytophagaceae_OTU156	New.ReferenceOTU33	4.09	Bacteroidetes	Cytophagia	Cytophagales	Cytophagaceae
Cytophagaceae_OTU135	EU676411.1.1476	3.94	Bacteroidetes	Cytophagia	Cytophagales	Cytophagaceae
Chitinophagaceae_OTU25	KJ081628.1.1526	4.69	Bacteroidetes	Saprospirae	Saprospirales	Chitinophagacea
Chitinophagaceae_OTU62	KC255272.1.1496	4.5	Bacteroidetes	Saprospirae	Saprospirales	Chitinophagacea
Chitinophagaceae_OTU196	New.ReferenceOTU66	4.03	Bacteroidetes	Saprospirae	Saprospirales	Chitinophagacea
Chitinophagaceae_OTU152	HQ120071.1.1501	3.97	Bacteroidetes	Saprospirae	Saprospirales	Chitinophagacea
Hyphomonadaceae_OTU30	FPLS01011561.10.1468	4.43	Proteobacteria	Alpha-proteobacteria	Rhodo-bacterales	Hyphomonadace
Hyphomonadaceae_OTU50	KF945044.1.1228	4.25	Proteobacteria	Alpha-proteobacteria	Rhodo-bacterales	Hyphomonadace
Methylophilaceae_OTU65	LXTQ01000003.3712.5257	4.01	Proteobacteria	Beta-proteobacteria	Methylo-philales	Methylophilacea
Methylophilaceae_OTU74	KP770039.1.1543	3.99	Proteobacteria	Beta-proteobacteria	Methylo-philales	Methylophilacea
Planctomycetaceae_OTU157	New.ReferenceOTU18	3.96	Planctomycetes	Plancto-mycetia	Plancto-mycetales	Planctomycetace
				IBUPROFEN		
Frankia_OTU26	New.ReferenceOTU9	4.29	Actinobacteria	Actinobacteria	Actino-mycetales	Frankiaceae
Novosphingobium_OTU17	KP284176.1.1502	4.55	Proteobacteria	Alpha-proteobacteria	Sphingo-monadales	Sphingomonadac
Novosphingobium_OTU31	KX427081.1.1506	4.32	Proteobacteria	Alpha-proteobacteria	Sphingo-monadales	Sphingomonadac
Novosphingobium_OTU86	AB088760.1.1473	3.97	Proteobacteria	Alpha-proteobacteria	Sphingo-monadales	Sphingomonadac
Sphingomonas_OTU76	CP006644.5474886.5476376	3.97	Proteobacteria	Alpha-proteobacteria	Sphingo-monadales	Sphingomonadac
Erythrobacteraceae_OTU58	KP122961.1.1482	4.15	Proteobacteria	Alpha-proteobacteria	Sphingo-monadales	Erythrobacterace
Sinobacteraceae_OTU128	FPLL01000547.13.1528	3.97	Proteobacteria	Gamma-proteobacteria	Xantho-monadales	Sinobacteraceae
Pseudomonas.stutzeri_OTU27	New.ReferenceOTU110	3.95	Proteobacteria	Gamma-proteobacteria	Pseudo-monadales	Pseudomonadace
Methylacidiphilae_OTU51	FPLS01010523.11.1518	4.44	Verruco microbia	Methyl-acidiphilae	Methyl-acidiphilales	LD19
Luteolibacter_OTU53	GU444072.1.1508	4.04	Verruco microbia	Verruco-microbiae	Verruco-microbiales	Verrucomicrobia

				DICLOFENAC		
Bacteroidales_OTU59	FQ660068.1.1348	4.6	Bacteroidetes	Bacteroidia	Bacteroidales	
Acidobacteria-6_OTU73	CP015136.651073.652614	4.42	Acidobacteria	Acidobacteria-6	iii1-15	
Acidobacteria-6_OTU104	MEDJ01000003.85717.87273	4.27	Acidobacteria	Acidobacteria-6	iii1-15	RB40
Acidobacteria-6_OTU144	FPLS01035838.16.1549	4.08	Acidobacteria	Acidobacteria-6	iii1-15	
Chloracidobacteria_OTU124	JN869200.1.1518	4.11	Acidobacteria	Chloracido-bacteria	RB41	Ellin6075
Geobacter_OTU163	Y19190.1.1552	3.96	Proteobacteria	Delta-proteobacteria	Desulfuro-monadales	Geobacteraceae
Legionellales_OTU183	EU937840.1.1449	4.24	Proteobacteria	Gamma-proteobacteria	Legionellales	Legionellaceae
				TRICLOSAN		
Chitinophagaceae_OTU4	FPLS01059649.14.1514	5.11	Bacteroidetes	Saprospirae	Saprospirales	Chitinophagacea
Chitinophagaceae_OTU82	FJ592637.1.1398	4.19	Bacteroidetes	Saprospirae	Saprospirales	Chitinophagacea
Chitinophagaceae_OTU127	New.ReferenceOTU85	3.94	Bacteroidetes	Saprospirae	Saprospirales	Chitinophagacea
Sphingobacteriaceae_OTU49	KM035962.1.1433	4.32	Bacteroidetes	Sphingo-bacteriia	Sphingo-bacteriales	Sphingobacteriac
Cytophagaceae_OTU68	FJ479179.1.1483	4.18	Bacteroidetes	Cytophagia	Cytophagales	Cytophagaceae
Cytophagaceae_OTU97	HQ119896.1.1493	3.98	Bacteroidetes	Cytophagia	Cytophagales	Cytophagaceae
Caulobacter_OTU14	FPLS01017875.18.1484	4.46	Proteobacteria	Alpha-proteobacteria	Caulobacterales	Caulobacteracea
Phenylobacterium_OTU72	FJ891027.1.1339	4.1	Proteobacteria	Alpha-proteobacteria	Caulobacterales	Caulobacteracea
Phenylobacterium_OTU92	AY957905.1.1474	3.96	Proteobacteria	Alpha-proteobacteria	Caulobacterales	Caulobacteracea
Agrobacterium_OTU15	HE589815.1.1408	4.25	Proteobacteria	Alpha-proteobacteria	Rhizobiales	Rhizobiaceae
Betaproteobacteria_OTU269	FPLS01017319.10.1531	4.02	Proteobacteria	Beta-proteobacteria	Procabacteriales	Procabacteriacea
Pseudoxanthomonas.mexicana_OTU60	KP232911.1.1556	4.37	Proteobacteria	Gamma-proteobacteria	Xantho-monadales	Xanthomonadac
				MIX		
Cyclobacteriaceae_OTU12	FOPC01000030.107.1632	4.74	Bacteroidetes	Cytophagia	Cytophagales	Cyclobacteriacea
Cyclobacteriaceae_OTU46	AB255108.1.1489	4.51	Bacteroidetes	Cytophagia	Cytophagales	Cyclobacteriacea
Sphingobacteriia_OTU61	FNZR01000023.491.2016	4.25	Bacteroidetes	Sphingo-bacteriia	Sphingo-bacteriales	Sphingobacteriac
Bacteroidetes_OTU108	New.ReferenceOTU99	3.93	Bacteroidetes	VC2_1_Bac22		
Hyphomicrobiaceae_OTU21	JF825500.1.1486	4.39	Proteobacteria	Alpha-proteobacteria	Rhizobiales	Hyphomicrobiac
Caulobacteraceae_OTU49	FPLL01003727.10.1475	3.97	Proteobacteria	Alpha-proteobacteria	Caulo-bacterales	Caulobacteracea
Alphaproteobacteria_OTU64	FPLS01019797.11.1467	3.96	Proteobacteria	Alpha-proteobacteria	Rhodo-bacterales	Rhodobacteracea

Table S2. Quantification by qPCR of the gene copy number per nanogram of DNA of the different taxa from non-contaminated (control) and contaminated BPS microcosms with ibuprofen, diclofenac and triclosan, applied separately and in mixture at 0, 7 and 50 days of the incubation period. Standard errors (in parentheses) are given. Letters indicate significant differences among samples of different incubation times of each treatment and taxa (ANOVA homogeneity test; $p < 0.05$).

		Taxa				
Treatment	**Days**	**Total bacteria**	***α-Proteobacteria***	***β-Proteobacteria***	***γ-Proteobacteria***	***Acidobacteria***
	0	2.93×10^5 (0.65)a	1.92×10^5 (0.42)a	1.73×10^3 (0.57)a	1.47×10^5 (0.53)a	1.12×10^3(0.34)a
Control	7	7.28×10^5 (1.05)a	$4.08 \times 10_5$ (0.76)a	9.35×10^3 (2.14)a	3.27×10^5 (0.32)a	1.56×10^4 (0.26)a
	50	5.16×10^5 (0.35)a	3.03×10^5 (0.19)a	7.47×10^3 (2.25)a	9.18×10^4 (2.46)a	5.84×10^4 (1.07)b
Ibuprofen	7	1.06×10^6 (0.01)b	1.93×10^5 (0.50)a	3.30×10^3 (0.25)a	2.39×10^5 (0.23)a	5.27×10^3 (1.27)a
	50	4.65×10^5 (0.34)a	3.11×10^5 (0.21)a	1.26×10^4 (0.01)b	1.44×10^5 (0.06)a	7.37×10^4(0.29)b
Diclofenac	7	9.05×10^5 (0.10)b	2.64×10^5 (0.17)a	2.01×10^3 (0.04)a	1.26×10^5 (0.27)a	2.85×10^3 (0.34)a
	50	5.44×10^5 (0.09)a	3.92×10^5 (0.28)a	1.73×10^4 (0.31)b	1.65×10^5 (0.04)a	8.84×10^4(0.82)b
Triclosan	7	1.19×10^6 (0.12)b	3.76×10^5 (0.78)a	1.06×10^4 (0.57)a	3.46×10^5 (1.16)a	1.83×10^4 (0.69)a
	50	4.93×10^5 (0.15)a	3.90×10^5 (0.32)a	1.07×10^4 (0.07)a	1.95×10^5 (0.21)a	2.46×10^4 (0.29)a
Mix	7	6.41×10^5(0.57)a	3.34×10^5 (0.26)a	4.78×10^3 (0.33)a	1.83×10^5 (0.06)a	1.82×10^4 (0.37)a
	50	5.05×10^5 (0.47)a	2.72×10^5 (0.24)a	5.04×10^3 (1.08)a	1.34×10^5 (0.34)a	1.18×10^4 (0.22)a

CHAPTER 2

Novel and affordable bioaugmentation strategy to increase the removal of pesticides and PPCPs in biopurification systems and in contaminated aqueous media with autochthonous microorganisms

CHAPTER 2.1

Improvement of pesticide removal in contaminated media using aqueous extracts from contaminated biopurification systems

The results of Chapter 2.1 have been published in the journal *Science of The Total Environment*,

Aguilar-Romero, I., van Dillewijn, P., Nesme, J., Sørensen, S. J., Romero, E. 2019. Improvement of pesticide removal in contaminated media using aqueous extracts from contaminated biopurification systems. *Science of The Total Environment*, 691, 749–759.

https://doi.org/10.1016/J.SCITOTENV.2019.07.087

Resumen

A pesar de ciertas limitaciones, la bioaumentación mejora la eficiencia de los sistemas de biorremediación. En este estudio, se demuestra que tres extractos acuosos (APE, ACE y AVE) procedentes de biomezclas residuales envejecidas de tres sistemas de biopurificación (BPSs) expuestos a plaguicidas a escala piloto mejoran la eliminación de herbicidas fenilurea. La aplicación de los extractos ACE y AVE a soluciones acuosas que contienen diuron como contaminante modelo, aumentó las tasas de eliminación de este herbicida 6 y 17 veces, respectivamente, en comparación con el extracto APE. Estos extractos también incrementaron la eliminación del metabolito 3,4-dicloroanilina, mientras que el extracto AVE, en particular, eliminó todos los pesticidas en 9 días. Mediante el análisis SPME/GC/MS se identificaron tres metabolitos menos peligrosos que la 3,4-dicloroanilina. El extracto AVE, también mejoró la eliminación del linurón en medios líquidos, y aumentó la disipación del diurón 6 veces en el BPS. Por último, el estudio de las comunidades bacterianas reveló un aumento en la abundancia relativa de taxones bacterianos como *Chloroflexi*, *Acidobacteria*, *Gemmatimonadetes*, *Firmicutes*, *Deinococcus-Thermus* y especialmente *Proteobacteria* (10%), en la biomezcla SVP tras la contaminación con diversos pesticidas, así como un enriquecimiento de *γ-proteobacteria* y el género actinobacteriano *Dokdonella* en el extracto AVE con respecto a la biomezcla inicial no contaminada. Este estudio demuestra que los extractos que contienen un microbioma aclimatado a contaminantes podrían usarse como parte de una estrategia de bioaumentación para mejorar el funcionamiento de los BPSs en granjas, así como en otros sistemas contaminados.

Abstract

Despite certain limitations, bioaugmentation enhances the efficiency of bioremediation systems. In this study, three aqueous extracts (APE, ACE and AVE) from aged residual biomixtures in three biopurification systems (BPSs) exposed to pesticides at a pilot scale were found to improve pesticide removal. The addition of ACE and AVE to solutions containing the model compound diuron increased removal rates 6- and 17-fold, respectively, as compared to APE. These extracts also increased the removal of the metabolite 3,4-dichloroaniline, while AVE, in particular, were found to remove all pesticides within 9 days. Three metabolites less hazardous than 3,4-dichloroaniline were identified by SPME/GC/MS. AVE, which also enhance linuron removal in liquid media, were found to increase diuron removal 6-fold in BPSs. We observed an increase in the relative abundance of bacterial taxa, such as *Chloroflexi*, *Acidobacteria*, *Gemmatimonadetes*, *Firmicutes*, *Deinococcus-Thermus* and especially *Proteobacteria* (10%), in SVP biomixtures, as well as an enrichment of *γ-proteobacteria* and the actinobacterial genus *Dokdonella* in AVE with respect to initial non-contaminated SVP biomixture. We demonstrate that extracts containing a pollutant-acclimatized microbiome could be used as part of a bioaugmentation strategy to improve the functioning of on-farm BPSs and contaminated systems.

1. INTRODUCTION

The removal of organic contaminants, such as pesticides, pharmaceuticals and personal care products from wastewater is of great concern worldwide due to their impact on aquatic ecosystems and the scarcity of clean water resources (Batista-Andrade et al., 2018; Castillo Diaz et al., 2016; Delgado-Moreno et al., 2017a). Thus, it is important to develop new affordable technologies to clean up or prevent water resource pollution. Biopurification systems (BPSs), also known as biobed or biofilter systems, which are used on-farm for bioremediation purposes, play a highly effective role in removing pollutants from wastewater and in preventing point source contamination. However, when recalcitrant pollutants are slow to degrade in these systems (Delgado-Moreno et al., 2019, 2017a), the addition of pesticide-primed soil containing adapted microbial communities improved the functioning of on-farm BPSs (Sniegowski et al., 2012, 2011; Sniegowski and Springael, 2015). The inoculation of biomixtures with successfully adapted cultivable indigenous microorganisms also have been shown to boost the pesticide degradation rate (Castillo Diaz et al., 2016; Castillo et al., 2016; Dealtry et al., 2016; Dunon et al., 2013; Nour et al., 2017). Current bioremediation technologies also provide a bioaugmentation technique that uses microbial consortia with complex metabolic networks to mineralize pollutants (Alvarez et al., 2017; Castillo Diaz et al., 2016; Karas et al., 2016; Villaverde et al., 2017). However, the strains, either individually or as consortia, with specific catabolic capabilities, can be difficult and time-consuming to isolate and select in bioaugmentation systems (Castillo Diaz et al., 2016), and most microorganisms cannot be cultivated using currently available techniques (Pinto et al., 2015). In addition, degradative phenotypes are often eliminated when strains are cultured due to the genetic instability of catabolic markers under non-selective conditions (Gillian A. Turnbull et al., 2001). Furthermore, bioaugmentation systems, which use exogenous strains, often fail, while multiple inoculations can be expensive and impractical (Couto et al., 2010). To overcome these limitations, we have designed a new, simple sustainable bioaugmentation system that uses aqueous extracts from aged polluted BPS biomixtures which are enriched in adapted microbial populations to metabolize pesticide residues and their metabolites. These extracts could be used to boost the depuration capability of other

bioremediation and contaminated systems. This alternative bioaugmentation technique resembles the pesticide-primed materials approach, which harbors enriched adapted microbial consortia to boost pesticide degradation in BPSs (Sniegowski et al., 2012, 2011; Sniegowski and Springael, 2015). Nevertheless, the extracts contain fewer traces of persistent recalcitrant pesticide residues than primed-materials. These aqueous extracts are also easy to handle and can be used in vertical and other biobed systems and even in effluents before their release into the environment. Aqueous extracts from compost are actually routinely used in sustainable organic farming to control pests and diseases (Salter and Edwards, 2010). Thus, this technique could constitute a new viable option for farmers to augment the biodepuration capability of bioremediation systems.

The widespread use of phenylurea herbicides, such as diuron (a photosynthesis inhibitor), in urban and rural areas and as an antifouling biocide has increased the detection of phenylurea and its metabolites in soil and water (Batista-Andrade et al., 2018; Horemans et al., 2014; Rico et al., 2019; Sørensen et al., 2008). Although diuron, whose use is restricted in vulnerable areas, is classified as a priority hazardous substance by EU water regulations (Water Framework Directive 2013/39/EU), it continues to be used due to its effectiveness as an antifouling biocide (Batista-Andrade et al., 2018). Diuron and its metabolites, which are recalcitrant compounds present in the environment (Sørensen et al., 2013), act as endocrine disruptors in aquatic organisms (Felício et al., 2018; L. B. Moreira et al., 2018).

The aim of this study is to determine the potential of extracts from aged polluted biomixtures to boost pesticide removal. Diuron, which shows limited biodegradation in BPSs (Delgado-Moreno et al., 2017a) and is a priority contaminant of global concern, whose main metabolite 3,4-dichloroaniline (3,4-DCA) is even more persistent and toxic (Castillo et al., 2014), was used as a model pesticide. Diuron solutions were inoculated with aqueous extracts from aged BPS biomixtures treated with pesticides, as described in a previous study (Delgado-Moreno et al., 2017a), and with extracts from the untreated initial biomixture in order to determine their biodegradation capacity to decontaminate water. These solutions were analyzed with the aid of HPLC and solid-phase microextraction/gas chromatography–mass spectrometry (SPME/GC-MS) to

identify metabolites. The degradation potential of these extracts was also tested in solutions using another phenylurea herbicide. Finally, the degradation potential of microbial biomass in extracts was tested to enhance the biodegradation of diuron in BPS microcosms. Potential degraders were isolated from the most active aged contaminated biomixtures. Bacterial diversity was analyzed in the untreated initial biomixture, the aged herbicide-treated biomixture and in the corresponding extract. This study describes the preliminary practical stages of a new, rapid and affordable bioaugmentation system.

2. MATERIAL AND METHODS

2.1. Chemicals

Diuron and linuron were supplied by Sigma Aldrich (Madrid, Spain). 3,4-dichloroanaline (3,4-DCA) was provided by Chemservice. 3,4-dichloroacetanilide (3,4-DCAN) was obtained from MP Biomedicals, LLC (Illkirch, France), 3,4-dichloronitrobenzene (3,4-DCNB) from ACROS Organics and 2,4,5-trichloroaniline was supplied by Fluka (Germany). Standard chemical solutions were prepared in acetone, while experimental solutions were prepared in MilliQ water at 20 mg L^{-1}. HPLC-grade solvents and chemicals were used.

2.2. Preparation of aqueous extracts in a lab-scale bioreactor

Different aqueous extracts were prepared from non-contaminated biomixtures at initial time before the pesticide treatment (IPE, ICE and IVE) and after a period of time when these biomixtures were contaminated and aged respectively in three biopurification systems developed at a pilot scale (APE, ACE and AVE). Table 1 summarizes the nomenclature of the different biomixtures and extracts used in this study. The first pilot scale BPSs had been constructed with an initial biomixture containing **S**oil, **P**eat and **S**traw (i-SPS; 25:25:50, v:v:v). The second was constructed with a biomixture containing **S**oil, wet olive **C**ake and olive tree **P**runing (i-SCP; 25:25:50, v:v:v), and the third with a biomixture containing **S**oil, **V**ermicompost of wet olive cake and olive tree **P**runings (i-SVP; 25:25:50, v:v:v). These biopurification systems were treated with high 50 mg k^{-1} loading doses of

commercial formulations used in olive tree growing systems. These BPSs were first treated with imidacloprid, dimethoate and tebuconazole, incubated for 5 months and then treated with diuron and oxyfluorfen and incubated for an additional 5 months, as described by Delgado-Moreno et al. (2017b). Depending on their composition, the resulting aged biomixtures (a-SPS, a-SCP and a-SVP) have been shown to remove pesticides including diuron (Delgado-Moreno et al., 2017a). The principal properties of initial biomixtures are described in the General Material and Methods section. The aged polluted biomixtures were homogenized and their main properties were determined (Table 2). Although both initial and aged polluted biomixtures had similar pH and EC values, the initial biomixtures contained relatively higher levels of organic carbon, (16.1%, 21.3% and 21.6%, respectively) and WSC (1.5, 7.3 and 13.7 g k^{-1}, respectively) as reported by Delgado-Moreno et al. (2017b).

Table1. Nomenclature of the biomixtures and extracts used in chapter 2.1.

Biomixtures	Initial biomixtures	Extracts from initial biomixtures	Aged biomixtures	Extracts from aged biomixture	Extracts from sterile aged biomixture
SPS	i-SPS	IPE	a-SPS	APE	SAPE
SCP	i-SCP	ICE	a-SCP	ACE	SACE
SVP	i-SVP	IVE	a-SVP	AVE	SAVE

The extracts were obtained according to the procedure described in the General Materials and Methods section. To estimate the number of viable cells present in each extract, 1 mL samples were serially diluted in M9 minimal medium (Sambrook et al., 1989) and then plated in triplicate onto 1/5 diluted LB (Sambrook et al., 1989) media supplemented with 5 μg mL^{-1} cycloheximide (Sigma Aldrich). Colony forming units (CFUs) were determined after three days of incubation at 30 ºC. In the case of biomixtures, 1 g of each sample was serially diluted and plated as described above in order to determine CFU per g^{-1} biomixture. To determine the effect of inoculum size on bioaugmentation efficiency, biomass from volumes of 75, 150 and 300 mL of each extract was concentrated by centrifugation at 6000 rpm for 1 h. The pellet containing the inoculant biomass was resuspended in 10 mL MilliQ water and then added to the solution or BPS. Although analysis of the supernatants revealed no

dehydrogenase (DHS) activity, water-soluble carbon (WSC) and electrical conductivity (EC) values were similar to those obtained in the non-centrifuged extracts. These findings confirm that the pellet contained total microbial biomass but low levels of soluble carbon and salts.

2.3. Pesticide degradation in aqueous solutions inoculated with extracts

Aqueous pesticide solutions (10 mL in MilliQ water) at 20 mg L^{-1} were mixed in Erlenmeyer flasks of 100 mL with 10 mL of IPE, ICE and IVE, from the initial uncontaminated biomixtures, or with 10 mL of APE, ACE and AVE, from the aged polluted biomixtures. The same pesticide solution in other flasks was mixed with 10 mL of MilliQ water and was run as abiotic control. In other flasks, the diuron solution (10 mL) was mixed with 10 mL of sterilized extracts (SAPE, SACE and SAVE) and was run in parallel. The extracts were sterilized by autoclaving at 121 ºC for 20 min. To determine the inoculum biomass effectiveness in the degradation of diuron in the solutions, pellets containing microbial biomass collected from 75 or 150 mL extracts were obtained by centrifugation and then resuspended in 10 mL of MilliQ water. Thus, the initial concentration of diuron in all flasks was 10 mg L^{-1}, which exceeds the limit for water quality (Directive 2013/39/EU) and enables diuron and its metabolites to be accurately quantified by HPLC. The solutions were inoculated in triplicate and then incubated on a shaker at 145 rpm in darkness at 30 ºC. Sampling was carried out twice a week for 28 days and analyzed as described below.

To determine whether microorganisms from extracts can metabolize other phenylurea herbicides (Sørensen et al., 2008), linuron solutions were treated with AVE under conditions similar to those described above for diuron.

2.4. Pesticide degradation in biobed BPS microcosms inoculated with extracts

To determine whether the extracts can be used in the bioaugmentation of biobed BPSs, two BPSs were constructed at a microcosm scale with 30 g dry weight of i-SPS and i-SVP biomixtures. Each microcosm-scale BPS (mBPS) was contaminated with diuron up to a final concentration of 20 μg g^{-1}, equivalent to a

concentration of 26 kg ha^{-1} at a soil depth of 10 cm, which exceeds the recommended agronomic dosage of 0.3-5 kg ha^{-1}. The BPSs were then moistened to 75% of their field capacity and inoculated with the pellet containing 300 mL of APE and AVE up to a cell concentration of 2 x 10^8 and 9 x 10^8 CFU per g^{-1} biomixture, respectively. Two additional BPSs, non-inoculated with extracts and inoculated with SAPE and SAVE extracts, were run in parallel as controls. All systems were prepared in triplicate and incubated in darkness in a thermostatic chamber at 20 ºC, and samples were collected at day 0, 17 and 32.

2.5. Analytical methods

Total organic carbon content and nitrogen were determined with the aid of a LECO TruSpec CN Analyzer (LECO Corporation, St. Joseph, USA) using 0.2 g samples of ground solid particles from the biomixtures or from particles remaining after liquid extract evaporation at 60 ºC. Electrical conductivity (EC) and pH were measured with a glass electrode at a 1:10 sample:water (dw:v) ratio. Water soluble carbon (WSC) and dehydrogenase (DHS) activity as a measure of total microbial activity were determined in triplicate from 0.5 mL of each extract or from 0.2 g per biomixture as described by (Delgado-Moreno et al., 2017a).

In the liquid solution experiments, diuron, linuron and the 3,4-DCA metabolite were analyzed with the aid of high-performance liquid chromatography (HPLC) as described in the General Materials and Methods section. Aliquots (1 mL) from the solutions were mixed with acetonitrile (1:1), centrifuged at 13000 rpm for 1 min at 20 ºC and passed through a 0.45 μm PTFE filter prior to analysis. The linear calibration curves were in the 0.2 to 10 mg L^{-1} range ($r > 0.999$). The detection and quantification limits for each compound were 0.05 mg L^{-1} and 0.1mg kg^{-1}, respectively.

To determine the inoculum effect on the degradation of diuron in the mBPS, samples (3 g dry weight) from mBPS were extracted with 5 mL acetonitrile and vortexed for 1 min. One gram of QuEChERS (Agilent Technologies, Santa Clara, CA, USA) was added to each sample and vortexed again for 1 min. Samples were centrifuged for 5 min at 3500 rpm and analyzed using HPLC (Agilent Technologies) with 40% acetonitrile as the mobile phase and the diode array detector (DAD) was set at 254 nm. The diuron recovery rates in the i-SPS and i-

SVP biomixtures were 96 ± 1% and 92 ± 3%, respectively as measured previously (Delgado-Moreno et al., 2017a, 2017b).

2.6. Assessment of bacterial populations and diuron degraders

2.6.1. Isolated bacterial strains from aged polluted biomixture a-SVP

a-SVP samples (1 g) were resuspended in 100 mL of phosphate buffer solution and incubated for 1 h at 30 ºC with shaking (200 rpm). Screening was carried out by plating dilutions onto M9 minimal medium/noble agar plates (Sambrook et al., 1989) with 100 mg L^{-1} diuron as sole carbon source. Identification of bacterial isolates, DNA extraction and PCR amplification are described in the General Materials and Methods section. The gene sequences were submitted to GenBank, EMBL and DDBJ under accession numbers MH645844-MH645850.

2.6.2. Total DNA isolation and amplicon sequencing of the rRNA gene

To determine the effect of diuron on bacterial communities in biomixtures, total genomic DNA from 0.5 g of initial unpolluted biomixture (i-SVP) or aged polluted biomixture (a-SVP) was extracted in triplicate using the NucleoSpin® Soil kit (Macherey-Nagel, Duren, Germany). Total genomic DNA from samples (0.5 g pellet) of AVE extract inoculant biomass were also obtained in triplicate using the same method.

To amplify ~500 bp fragments of the 16S rRNA gene (V3-V4) from the samples, the DNA concentration in all samples was adjusted to 5 ng μL^{-1} using water and the 341F (CCTAYGGGRBGCASCAG) and 806R (GGACTACNNGGGTATCTAAT) primers were used. The PCRs were conducted in a volume of 25 µL using 0.5 u PCRBIO HiFi DNA polymerase (PCR Biosystems, United Kingdom) and 400 nM primers. The thermal program consisted of an initial 95 °C denaturation step lasting 1 min followed by 30 cycles at 95 °C for 15 s 56 °C for 15 s, 72 °C for 30 s and a final elongation step at 72 °C for 5 min. PCR products were purified using Agencourt AmpureXP (Beckman Coulter, USA).

Adapters were added with a second PCR, in which 2 µL of purified PCR product was used as a template for a 25 µL reaction. The conditions were similar to those of the first PCR, except that the number of cycles was reduced to 15. The resulting PCR products, whose concentration was normalized with the aid of the SequalPrep™ Normalization Plate Kit (Invitrogen, Carlsbad, USA), were purified using AMPure XP beads. The normalized libraries of samples were pooled and adjusted to 16 pM before proceeding to paired-end sequencing using a MiSeq Reagent Kit v2 (500-cycles) on the University of Copenhagen MiSeq sequencing platform (Illumina, San Diego, USA).

Statistical and bioinformatic data analysis are described in the General Materials and Methods section.

3. RESULTS AND DISCUSSION

3.1. Characteristics of extracts from aged polluted biomixture

The physicochemical properties of both biomixtures and extracts differed significantly ($p<0.05$) (Table 2). Though not compared statistically, the pH and electrical conductivity of the extracts showed tendencies similar to those of the aged biomixtures. Water soluble carbon (WSC) content in the extracts resembled that in the aged biomixtures (ACE > AVE > APE), which is attributable to the abundance of hydrophilic organic compounds, as demonstrated by aqueous extraction efficiencies (64, 61 and 52%, respectively). However, the total carbon and nitrogen percentages in the aqueous extracts do not share the same trend as in the aged biomixtures due to the extraction process (Table 2). DHS activity, an indicator of overall microbial metabolism, was significantly higher in AVE followed by ACE and APE. However, levels of WSC, an assimilable organic carbon source for microorganisms often associated with DHS activity, varied in the different extracts (ACE > AVE > APE). These variations could be due to the presence of phenolic compounds in ACE derived from the wet olive cake component in the a-SCP biomixture which could inhibit microbial activity (Romero et al., 2006). The bacterial colony forming units (CFUs) recorded in the extracts from uncontaminated biomixtures (IPE, ICE and IVE) contained 1.0 x

10^6, 1.5 x 10^7 and 3.2 x 10^7 CFUs per mL^{-1}, respectively. In APE, ACE and AVE, CFUs per mL^{-1} numbered 3.3 x 10^6, 6.5 x 10^6 and 9.0 x 10^6, respectively. These results suggest that, on the whole, lower bacterial abundances are found in IPE and APE obtained from SPS biomixtures as compared to the other biomixtures.

Table 2. Physicochemical properties and dehydrogenase (DHS) activity in aged polluted biomixtures from purification systems (BPSs) composed of soil containing peat and straw (SPS), wet olive cake, or olive cake vermicompost, and olive tree prunings (SCP and SVP), as well as in their extracts.

Aged polluted biomixture	pH	EC dSm^{-1}	OC %	N[Y] %	WSC $g\ Kg^{-1}$	DHS $\mu g\ INTF\ g^{-1}h^{-1}$
a-SPS	7.4[a]	3.0[c]	12.8±0.0[a]	0.64±0.0[a]	1.13±0.25[a]	24.0±1.2[a]
a-SCP	8.7[b]	1.3[a]	17.3±0.2[c]	0.98±0.01[b]	8.55±0.08[c]	61.2±6.2[b]
a-SVP	8.2[b]	1.4[b]	15.9±0.5[b]	1.36±0.0[c]	5.23±0.01[b]	65.9±0.6[b]
Extracts			OC [Y] %	N [Y] %	WSC $mg\ L^{-1}$	DHS $\mu g\ INTF\ mL^{-1}\ h^{-1}$
APE	7.6[a]	2.47[c]	1.08±0.02[a]	0.85±0.04[b]	59.5±1.2[a]	0.05±0.01[a]
ACE	8.5[c]	1.45[a]	15.55±0.21[c]	1.48±0.01[c]	547.6±6.3[c]	0.15±0.01[b]
AVE	8.0[b]	1.69[b]	10.05±0.07[b]	0.37±0.01[a]	319.8±8.5[b]	0.41±0.03[c]

EC, Electrical conductivity; OC, organic carbon; N, total nitrogen; WSC, water soluble carbon. [Y] OC and N content analyzed in liquid particles collected after evaporation of extracts. Letters in the same column indicate significant differences in properties among biobed biopurification system biomixtures and extracts (ANOVA homogeneity test; $p < 0.05$).

3.2. Potential of extracts to enhance the removal of diuron in aqueous solutions

To determine their effect on diuron removal, different amounts of the extracts were added to the liquid solutions. In solutions treated with 10 mL extracts from the uncontaminated biomixtures (IPE, ICE and IVE) (Figure 1), very little diuron was removed after 28 days as compared to initial levels (1.7 ± 0.2%, 2.7 ± 0.3% and 7.0 ± 4.2%, respectively). This indicates that most microorganisms extracted from the initial biomixtures have little or no capacity to remove diuron.

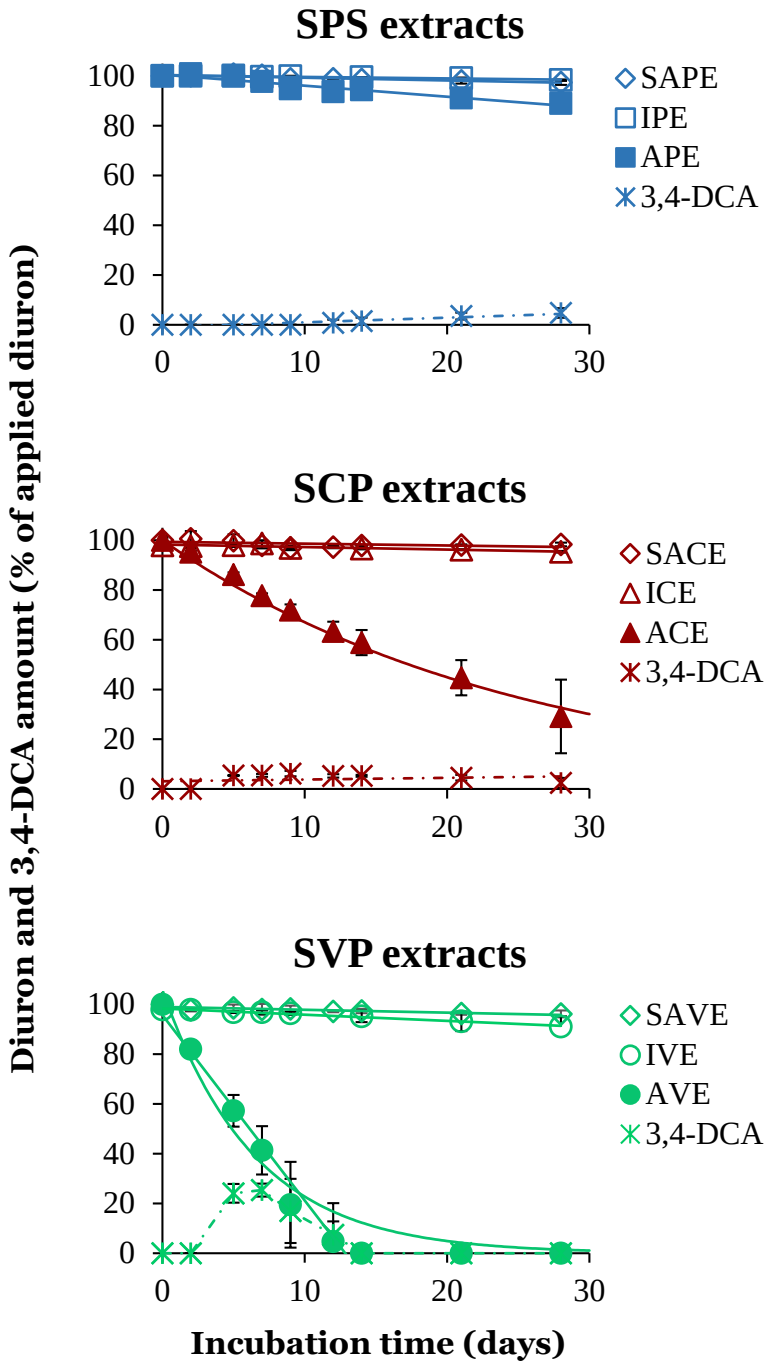


Figure 1. Diuron removal in aqueous solutions inoculated with 10 mL extracts from initial and aged biomixtures, as well as with aged sterile extracts from the SPS biomixture (IPE, APE, and SAPE) from the SCP biomixture (ICE, ACE, and SACE) and from the SVP biomixture (IVE, AVE and SAVE). Formation and elimination of 3,4-dichloroaniline (3,4-DCA) in solutions inoculated with non-sterile extracts (a, b and c). Symbols are experimental data; solid lines represent theoretical kinetics. Values are means of three replicates. Error bars represent the standard deviation.

The kinetics of the diuron control solutions, with indistinguishable curves, resembled those observed following the addition of sterilized extracts (SAPE, SACE and SAVE), in which only 2.6 ± 1.9%, 1.7 ± 1.7% and 3.8 ± 0.8%, respectively, of the initial diuron was removed, thus indicating that no significant abiotic degradation occurs. However, in the solutions inoculated with 10 mL of extracts from aged polluted biomixtures (ACE and AVE), 71 ± 15% and total removal of initial diuron was observed within 28 days and within <14 days, respectively. By contrast, diuron removal following the addition of APE extracts was not significant (4.3 ± 0.4%). Thus, the biodegradation capacity observed in ACE and AVE extracts may have been acquired by microbial populations enriched in the BPS during previous pesticide exposure (Delgado-Moreno et al., 2017a) and may be associated with biomixture composition (Delgado-Moreno et al., 2017b). In solutions inoculated with 10 mL of extracts from aged polluted biomixtures, the single first-order (SFO) equation fitted the experimental data more closely ($R^2 \geq 0.929$) than the zero-order kinetic/linear models (Table 3). While the fit of both these models to the data did not differ greatly, the SFO model showed the lowest Chi square and scaled error values ($\chi^2 \leq 14.067$, $err_{scaled} \leq 2.1$). Nevertheless, in the solutions inoculated with AVE, err_{scaled} (4.42) in the linear model was lower than in the SFO model (5.65). As shown in Figure 1, the SFO model appears to be less appropriate in this specific case. Thus, we used the *k* constant from linear equations to compare degradation rates, which showed an approximate 6- and 17-fold increase, respectively, in diuron removal, especially following the addition of ACE and AVE as compared to the solutions treated with APE (Table 3). The kinetic parameters measured after adding 10 mL extracts correlated with the properties of the extracts: a significant correlation was observed between degradation *k* values, DHS activity (r=0.98, *F*=132.25, *p*=0.000) and C/N ratios (*r*=0.905, *F*=31.726, *p*=0.001). No correlation was observed between DHS activity and number of bacterial CFUs (*r*=0.544, *F*=2.945, *p*=0.130) or between *k* values and number of bacterial CFUs (*r*=0.633, *F*=4.687, *p*=0.067). Given that DHS activity in AVE was 8.2- and 2.7-folds higher than in APE and ACE, respectively (Table 2), the extracts with high DHS levels might be expected to be more effective for bioaugmentation due to their considerable overall metabolic potential. However, WSC content (5 and 9 times lower than in AVE and ACE, respectively) and the C/N ratio (1.3 as compared to 10.5 and 27.2

for the ACE and AVE) of APE could limit microbial activity and consequently diuron removal in solutions inoculated with these extracts. Ellegaard-Jensen et al. (2013) have reported that diuron degradation by *Mortierella* occurs more rapidly at a C/N ratio of 7.6 in the medium. Similarly, diuron mineralization by isolated bacterial strains requires additional growth substrates and alternative carbon sources (Sørensen et al., 2008). In summary, bioaugmentation with AVE had a greater impact on diuron removal than ACE and APE.

Table 3. Single first-order (SFO) and linear kinetic parameters for diuron removal in solutions inoculated with 10 mL of extracts from aged polluted biomixtures and in solutions treated with suspended pellets of 75 and 150 mL of each extract, respectively.

Solutions (model)	C0 ± SE %	*k* ± SE days^{-1}	DT_{50}/DT_{90}	R^2	Diuron degraded % (days)
APE 10 (SFO)	100 ± 0.6	0.005 ± 0.00	147/488	0.929	4.3 ± 0.4 (28)
APE 10 (Linear)	101 ± 0.5	0.44 ± 0.41		0.824	
ACE 10 (SFO)	102 ± 2.1	0.04 ± 0.00	17/57	0.994	71 ± 14.0 (28)
ACE 10 (Linear)	97.5 ± 1.8	2.55 ± 0.13		0.941	
AVE 10 (SFO)	106 ± 7.3	0.15 ± 0.02	4.5/15	0.957	100 ± 0.0 (14)
AVE10 (Linear)	95.7 ± 3.6	7.45 ± 0.42		0.943	
APE 75 (SFO)	103 ± 1.3	0.02 ± 0.00	33/109	0.981	41.9 ± 9.5 (28)
APE 150 (SFO)	103 ± 1.5	0.02 ± 0.00	34/113	0.976	41.4 ± 0.9 (28)
ACE 75 (SFO)	105 ± 5.9	0.17 ± 0.02	3.5/12	0.914	99.8 ± 0.4 (28)
ACE 150 (SFO)	105 ± 4.9	0.26 ± 0.02	03-sep	0.953	98.2 ± 1.6 (9)
AVE 75 (SFO)	101 ± 3.2	0.44 ± 0.03	1.7/5	0.983	99.7 ± 0.3 (7)
AVE 150 (SFO)	100 ± 1.9	0.72 ± 0.04	0.9/3	0.999	99.8 ± 0.4 (5)

To determine the potential of microbial biomass from the aqueous extracts to accelerate diuron and 3,4-DCA degradation, the solutions were inoculated with 75 and 750 mL pellets from APE, ACE and AVE (Figure 2). In the solution treated with either 75 or 150 mL APE pellets, the biodegradation of diuron increased approximately 10-fold, with 41.6% of initial diuron removed; no increase in the removal of the 3,4-DCA metabolite was observed. On the other hand, in the diuron solutions inoculated with ACE and AVE pellets (75 and 150 mL), *k* values increased and diuron was totally removed within < 28 days and 7-5 days, respectively.

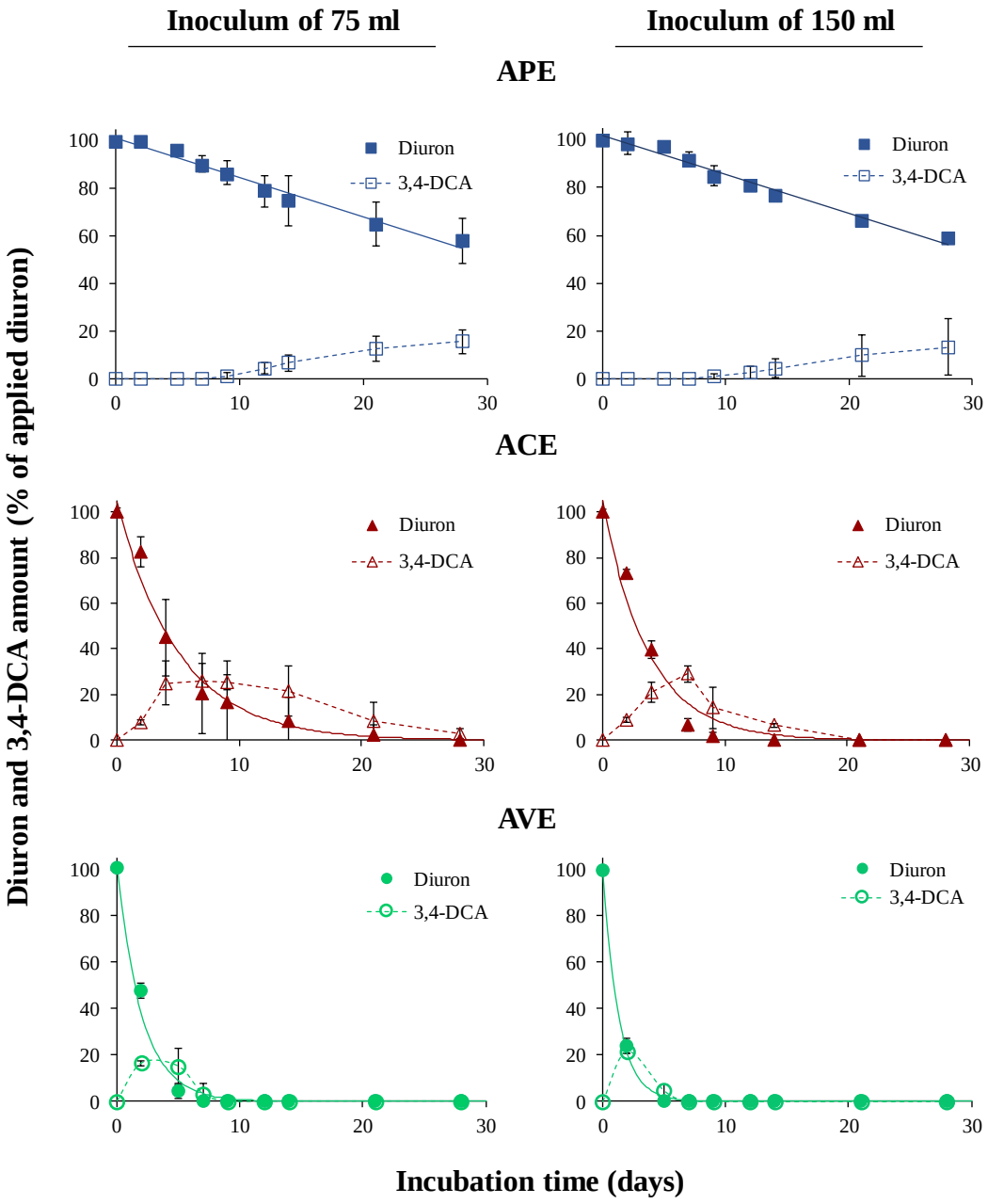


Figure 2. Diuron removal in aqueous solutions inoculated with resuspended pellets containing 75 and 150 mL of extract from aged polluted biomixtures (APE, ACE and AVE). Formation and elimination of 3,4-dichloroaniline (3,4-DCA). Symbols are experimental data; solid lines represent the SFO kinetics. Values are means of three replicates. Error bars represent the standard deviation.

The SFO model fitted the experimental kinetic data at the lowest values for χ^2 and err_{scaled} (Table 3). The AVE inocula of 75 and 150 mL reduced DT_{90} values 4.8- and 6-fold, respectively, while herbicide behaviour changed from persistent

to non-persistent. The quantity of 3,4-DCA, which was removed more rapidly in the solution with 150 mL inoculum, accounted for over 20% of the initial diuron. The largest reduction in DT_{90} took place when the AVE inoculum was increased, which eliminated diuron and 3,4-DCA within less than 7 days (Figure 2, Table 3).

Co-extracted particles in ACE and AVE (7.9 and 4.7 g L^{-1}, respectively) as compared to APE (3.17 g L^{-1}) and water soluble carbon (WSC) may affect the catabolic activity of inoculated microorganisms (Horemans et al., 2014). However, our results on bioaugmentation involving large inoculum sizes in pellets obtained by centrifugation, which have a high biomass density but relatively low WSC and nutrient levels, indicate that the efficiency of this type of bioaugmentation is due to the catabolic potential of microbial populations in extracts (Sørensen et al., 2013). This bioaugmentation technique could therefore be used not only for liquid solutions but also to boost diuron removal in other contaminated environments and even those with low soluble organic carbon levels.

3.3. Metabolite identification

HPLC analysis of the diuron solutions during incubation detected the formation of 3,4-DCA (Figure 1) in all inoculated solutions but not in sterile controls. The diuron metabolites, N-(3,4-dichlorophenyl)urea (DCPU), and N-(3,4-dichlorophenyl)-N-methylurea (DCPMU), were not detected, which suggests that 3,4-DCA was the principal metabolite. This metabolite disappeared more rapidly in the diuron solution inoculated with AVE. This indicates that cleavage of the urea bridge, which could be mediated by hydrolase enzymes that attack phenylurea herbicides, is the principal strategy used by microbial consortia present in the extracts to degrade diuron, especially in AVE (Bers et al., 2013, 2011; Khurana et al., 2009; Gillian A. Turnbull et al., 2001). Using SPME-GC/MS, we identified, but did not quantify, three 3,4-DCA metabolite derivatives in solutions treated with non-sterile extracts (Supplementary figures S1-S3). The first metabolite derivative 3,4 dichloroacetanilide (3,4-DCAN) was identified only in the solution treated with APE, while 3,4-dichloronitrobencene (3,4-DCNB) was identified in the solution inoculated with ACE and AVE but not in that inoculated with APE. The third metabolite derivative, 3,4-dichlorobenzene (3,4-

DCB), was identified only in solutions inoculated with AVE and APE. The disappearance of 3,4-DCA and the appearance of other less toxic metabolites (3,4-DCAN, 3,4-DCNB and 3,4-DCB) suggest that, once formed from diuron, 3,4-DCA may be degraded *via* two routes. 3,4-DCA could be transformed into a less toxic metabolite (3,4-DCAN) *via N*-acetylation or be degraded by sequential oxidation of aniline amine groups to nitro groups. These nitro groups could then be reduced by denitrification, with the resulting 3,4-DCB metabolite mineralized *via* the 3,4-dioxygenase pathway (Bers et al., 2011; Castillo et al., 2014). The appearance of different metabolites suggests that several metabolic routes play a role in diuron degradation, which may, in turn, be attributed to the activity of functionally different microbial communities and/or their cooperation in the degradation pathway.

3.4. Enhanced linuron degradation in solutions supplemented with extracts from a-SVP biomixture

In order to determine their bioaugmentation effectiveness, AVE extract was applied to linuron solution, another phenylurea herbicide. The degradation kinetic in Figure 3 shows that linuron removal, which was most effective and complete in diuron-contaminated solutions, was enhanced following inoculation with non-sterile AVE. A small amount of linuron dissipated after 15 days in the solution inoculated with 10 mL of SAVE, possibly due to a loss of sterile conditions (Figure 3), while the herbicide concentration remained stable in the non-inoculated control solution. The solutions inoculated with AVE showed a 3-day lag and the degradation kinetic then went through two phases. In the first 13-day phase, about 48% of the initial linuron was dissipated, while only 9% was removed in the second phase. The hockey-stick model, consisting of two sequential first-order kinetics, fitted the linuron degradation curve (χ^2 = 12.59, err_{scaled} = 3.58). The breakpoint was 13.7 ± 1.81 and C_0 was 106.2 ± 2.9. The degradation rate constants k_1 and k_2 were 0.05 ± 0.00 (day^{-1}) and 0.01 ± 0.00 (day^{-1}), respectively.

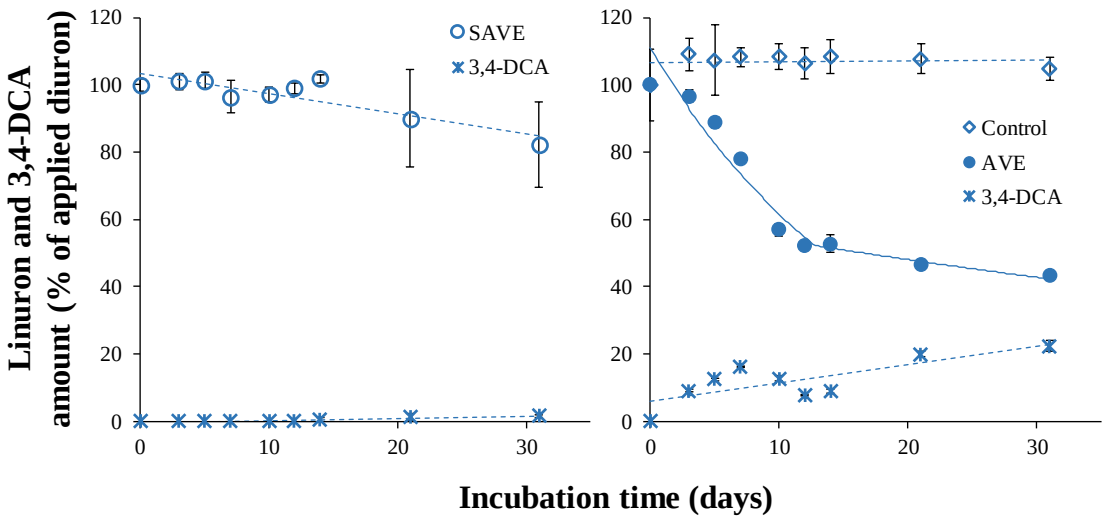


Figure 3. Linuron degradation in solutions inoculated with sterile (SAVE) and non-sterile (AVE) extracts as well as in the non-inoculated solution (control). Formation and elimination of 3,4-dichloroaniline (3,4-DCA). Symbols are experimental data and the solid line represents the Hockey stick kinetic.

We also detected the 3,4-DCA metabolite which increased while linuron content decreased in these solutions, as had previously occurred with diuron. This suggests that, as previously reported for other phenylurea degraders, microbial consortia in AVE contain microorganisms with genes encoding specific amide hydrolase enzymes for either *N*, *N*-dimethyl- or *N*-methoxy-*N*-methyl-substituted compounds (Bers et al., 2011; Sørensen et al., 2008). The lag phase could be due to the time taken by consortia to acclimate to linuron, as the a-SVP biomixture had been pre-exposed to diuron (Sniegowski et al., 2011; Villaverde et al., 2017). The hockey stick model may fit the linuron dissipation curve due to the accumulation of the toxic metabolite 3,4-DCA in the medium, which could inhibit cell growth (Villaverde et al., 2017). On the other hand, the two degradation phases suggest differences in the bioavailability of this herbicide due to possible *N*-methoxy group (-OCH_3) interactions in the linuron molecule with suspended particles present in AVE. The more marked lipophilic character of linuron (K_{ow} = 1010) as compared to diuron (K_{ow}= 700) may facilitate the rapid degradation of linuron free molecules, while the less bioavailable linuron bound fractions slowed down biodegradation.

3.5. Enhanced removal of diuron in biobed biopurification systems with the addition of extracts

Two BPSs were constructed using the unpolluted SPS and SVP biomixtures, to investigate the bioaugmentation efficiency of consortia from APE and AVE, respectively. In the BPSs composed of SPS biomixtures, the total amount of diuron eliminated did not significantly differ (46.5 ± 1.0%) (Figure 4) under either treatment, possibly due to their relatively low C/N ratio (Table 2).

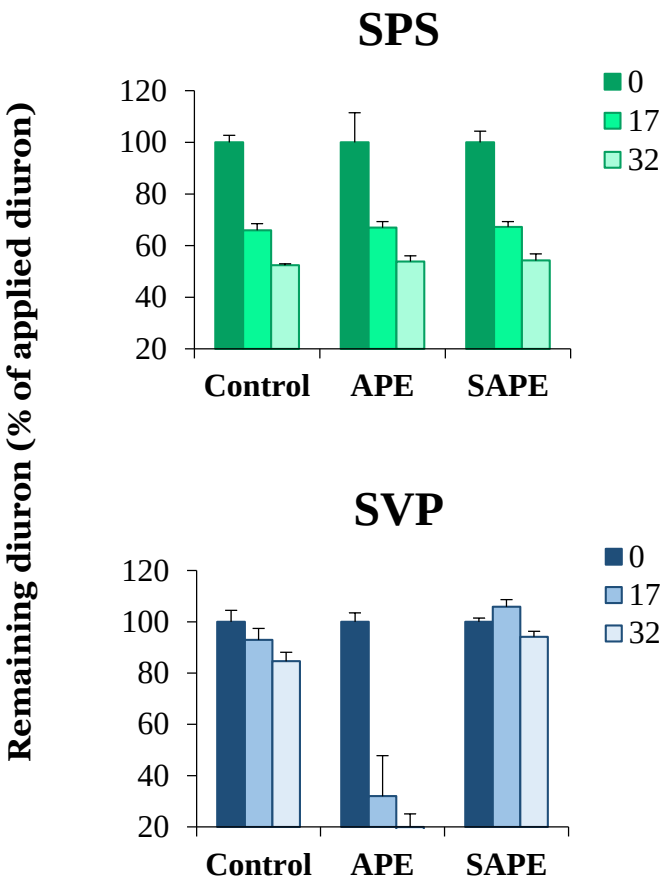


Figure 4. Diuron removal in microcosm biopurification systems SPS or SVP which have been left untreated (control), inoculated with sterilized, SAPE and SAVE, or non-sterilized extracts, APE and AVE, respectively. Values are means of three replicates. Error bars represent the standard deviation.

These results suggest that bioaugmentation with the presence of consortia in APE failed to enhance diuron removal. However, in line with the findings of a previous study (Delgado-Moreno et al., 2017a), the slower degradation of diuron

in the BPS composed of the SVP control led to the removal of only 15 ± 1% (Figure 4). Nevertheless, bioaugmentation involving consortia from AVE resulted in the removal of 89 ± 3% of the initial diuron (Figure 4). The lowest dissipation rate (6 ± 1%) occurred in SPS biopurification systems inoculated with SAPE, indicating that the consortia present in AVE greatly boosted diuron removal.

Despite the similar number of culturable bacteria determined in APE and AVE added to the SPS and SVP biopurification systems (2 x 10^8 and 9 x 10^8 bacterial CFUs per g^{-1} biomixture, respectively), the removal of diuron in the amended SPS system scarcely improved. This resembles the results of APE bioaugmentation liquid assays in which the herbicide was more bioavailable than in the biomixture. Thus, bacterial abundance contributes little to the acceleration of diuron removal. Microorganism activity in APE (Table 2) might also be negatively affected by the relatively smaller amount of easily available organic substrates such as cellulose and hemicellulose, the low WSC and relatively higher EC of the SPS biomixture (Delgado-Moreno et al., 2017a). Therefore, the considerable capacity of the SPS bioremediation system to remove diuron is probably due more to intrinsic factors, such as sorption capability, than to microbial activity (Delgado-Moreno et al., 2017b). On the other hand, the relatively higher level of DHS activity in AVE (Table 2) and the marked capacity of the SVP bioremediation system to remove diuron point to the presence of active consortia.

3.6. Bacterial community structure

Bacterial communities in i-SVP and the a-SVP biomixtures as well as AVE extract were evaluated to determine their role in the effectiveness of these extracts (Figure 5). Moreover, several bacterial strains, which can grow with diuron as sole carbon source in solid medium but not in liquid medium, were isolated from a-SVP biomixture (Supplementary Table S1). In liquid media containing diuron as the sole carbon source, these strains may lose genetic elements (Gillian A. Turnbull et al., 2001) and/or the biodegradation capability can be affected due to culture media conditions as concentration of contaminants, addition of appropriated growth substrates, etc, which has been reported in the literature as requiring conditions to trigger pesticide degradation in liquid media by the

isolated strains (Sørensen et al., 2008). However, these strains may form part of microbial populations responsible for the effectiveness of AVE bioaugmentation.

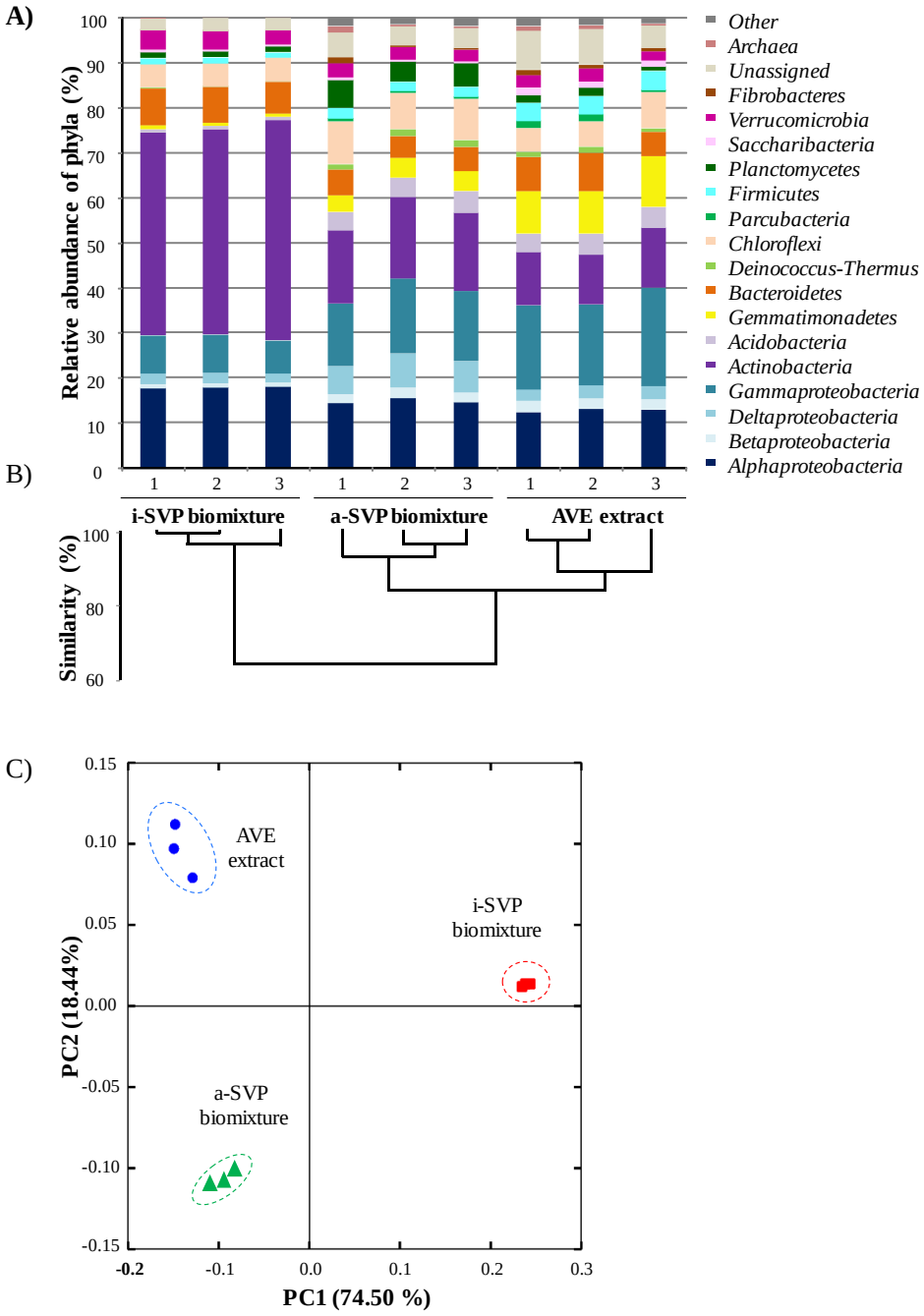


Figure 5. Relative abundance of the most dominant phyla (>1% in any sample) and classes of *Proteobacteria* in the initial unpolluted (i-SVP), in the aged pesticide polluted (a-SVP) biomixtures and in the corresponding AVE extract (A). Hierarchical cluster analysis of the bacterial community composition based on Bray–Curtis distances (B). Principal coordinate analysis of bacterial communities based on weighted UniFrac distances (C).

We used 16S rRNA gene sequencing to analyze bacterial communities in every replicate of each sample type (i-SVP, a-SVP and AVE extracts) after quality filtering and chimera removal, resulting in a mean of 29,574 sequences per sample. According to the rarefaction curves (Supplementary Figure S5), sequencing depth was sufficient to cover the wide diversity of samples. The Chao index and number of species in i-SVP (5689 and 5956, respectively) and a-SVP biomixtures (3110 and 3581, respectively) were both higher than in AVE (3809 and 2542, respectively). On the other hand, the Shannon diversity index increased significantly ($p < 0.05$) from 9.03 in i-SVP to 9.93 in a-SVP biomixtures but decreased in AVE (8.64) due to the extraction process. As expected, hierarchical clustering analysis of the composition of bacterial communities (Figure 5B), which divided the samples into three groups showed that AVE and a-SVP samples clustered more closely than i-SVP samples. Similarly, PCoA analysis (Figure 5C) showed significant grouping of samples (PERMANOVA $p < 0.004$) with respect to sample origin (i-SVP biomixtures, a-SVP biomixtures or AVE). The first principal component (PC1), accounting for 74% of statistical variability, separates aged biomixtures and extract samples from the initial biomixtures, suggesting that pesticide treatment has a significant impact (PERMANOVA $p < 0.02$). Similarly, the second principal component (PC2) separates samples by type (extracts vs. biomixture samples) and accounts for 18% of the variation which, however, was not significant (PERMANOVA $p=0.085$). This indicates that pesticide contamination was an important determinant of bacterial community structure.

Bacterial community OTUs in i-SVP and a-SVP biomixtures as well as AVE samples were grouped into 35 different phyla, 13 of which predominated and accounted for 88-97% of all OTUs detected (Figure 5A). In the i-SVP samples, *Actinobacteria* was the most representative phylum (45.0-49.0%), followed by *Proteobacteria* (28.2-29.4%) and *Bacteroidetes* (7.0-8.3%). However, a significant reduction in the relative abundance of *Actinobacteria* was observed in aged a-SVP biomixtures (16.3-18.2%) and AVE (11.2-13.4%). Of the most abundant genera detected in the samples (Figure 6), those belonging to the *Actinobacteria* phylum, especially *Promicromonospora* and *Streptomyces*, were diminished by pesticides treatment (Figure 6). However, the nitrogen-fixing actinobacterial genus *Frankia* was enriched in the extracts (Figure 6).

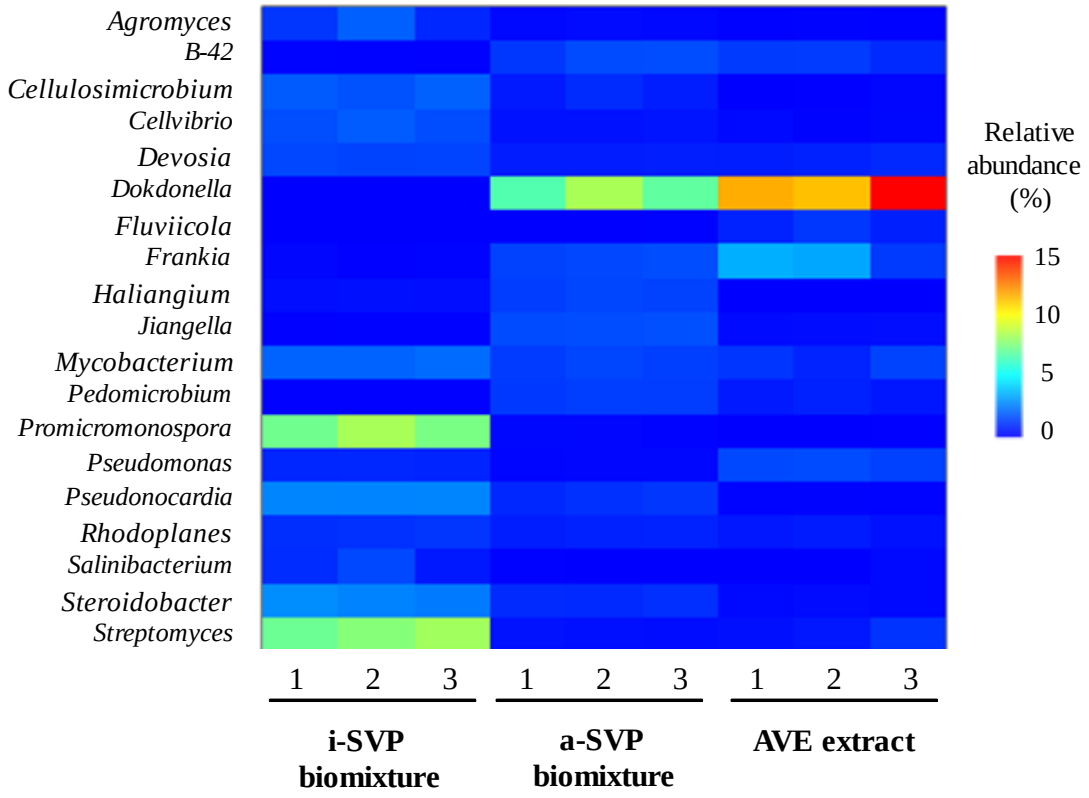


Figure 6. Heatmap of the most abundant genera in the initial unpolluted (i-SVP), aged polluted (a-SVP) biomixtures and in extract from the aged polluted biomixture (AVE).

One of the microorganisms isolated from the a-SVP biomixture, which grows on diuron on solid media also belongs to this phylum and presented 99% identity with *Arthrobacter nitroguajacolicus* sp. (Supplementary Table S1), a 4-nitroguaiacol-degrading actinobacterium (Kotoučková et al., 2004). Some *Arthrobacter* species have been reported to transform diuron into 3,4-DCA (Sørensen et al., 2008). It was related with the phenylurea hydrolase (*puh*) genes as was described in *Arthrobacter globiformis* (Gillian A. Turnbull et al., 2001) and *Mycobacterium brisbanense* (Khurana et al., 2009). OTUs which could be classified as belonging to *Arthrobacter* were only present at relatively low abundances of 0.1% in i-SVP biomixture and even less in the a-SVP biomixture and AVE. Abundant *Mycobacteria* were observed in the initial biomixture, although fewer were found in the aged contaminated biomixture and the extracts (Figure 6). On the other hand, *Proteobacteria* (36.3-41.8%), *Chloroflexi* (7.9-9.4%), *Acidobacteria* (4.1-4.7%), *Gemmatimonadetes* (3.8-4.5%), *Firmicutes*

(2.2-2.6%) and *Deinococcus-Thermus* (1.1-1.5%) were found to have higher relative abundance levels in the a-SVP than in i-SVP biomixture. This pattern is similar to that observed in an on-farm BPS to which pesticides such as diuron were added (Holmsgaard et al., 2017). Holmsgaard et al. (2017) reported an increase in the relative abundance of *Proteobacteria* (especially γ-proteobacteria), *Chloroflexi* and Candidate phylum *Saccharibacteria* (formerly TM7) and a decrease in that of *Firmicutes* and *Bacteriodetes*, while other phyla such as *Actinobacteria* and *Acidobacteria* remained largely unchanged. In the present study, all classes of *Proteobacteria* showed significant changes in abundance between treatments (Figure 5A). In the aged polluted a-SVP biomixture, the relative abundance of gamma-, delta- and *beta-proteobacteria* was found to increase, while that of *alpha-proteobacteria* declined with respect to the initial i-SVP biomixture. *Proteobacteria*, which are known toxic compound degraders, were also found to predominate in pesticide-sprayed soil (Paul et al., 2006) and in biopurification systems (Dealtry et al., 2016; Holmsgaard et al., 2017). In the present study, the most abundant *Proteobacteria* found in the bacterial communities of a-SVP biomixtures (13.7-16.5%) and AVE samples (17.8-21.7%) belonged to the class gamma-proteobacteria, in which *Dokdonella* was the most dominant genus in a-SVP biomixture (5.9-7.6%) and was even 2-3-fold more abundant in AVE samples (10.0-14.0%) (Figure 6). Recently, species belonging to this genus were identified as degraders of paracetamol metabolites (Palma et al., 2018) and pesticides (Qi and Wei, 2017), indicating that the genus *Dokdonella* detected, though not easily cultured, may also be involved in diuron biotransformation. The relative abundance of the genus *Pseudomonas* (another gamma-proteobacterium) varied from 0% in the a-SVP biomixture to approximately 1% in AVE (Figure 6). *Pseudomonas* strains have been reported to degrade the diuron metabolite 3,4-DCA (Egea et al., 2017). Four of the isolated strains from a-SVP biomixtures belong to the genus *Pseudomonas* (Supplementary Table S1).

The increase in the relative abundance of *Gemmatimonadetes* (9.5-11.5%), *Bacteroidetes* (5.3-8.6%), *Firmicutes* (4.0-4.3%) and *TM7* (1.2-1.7%) in AVE with respect to a-SVP samples is also noteworthy (Figure 5A). Despite the more limited alpha biodiversity observed in AVE (Supplementary Table S2), these data confirm that the extraction process promotes the enrichment of certain bacterial

strains in AVE. *Bacillus* strains, which belong to the phylum *Firmicutes*, have also been found to degrade diuron (Egea et al., 2017), and two isolated strains from the a-SVP biomixtures, which grow with diuron as sole carbon source, also belong the genus *Bacillus* (Supplementary Table S1.).

Supplementary material

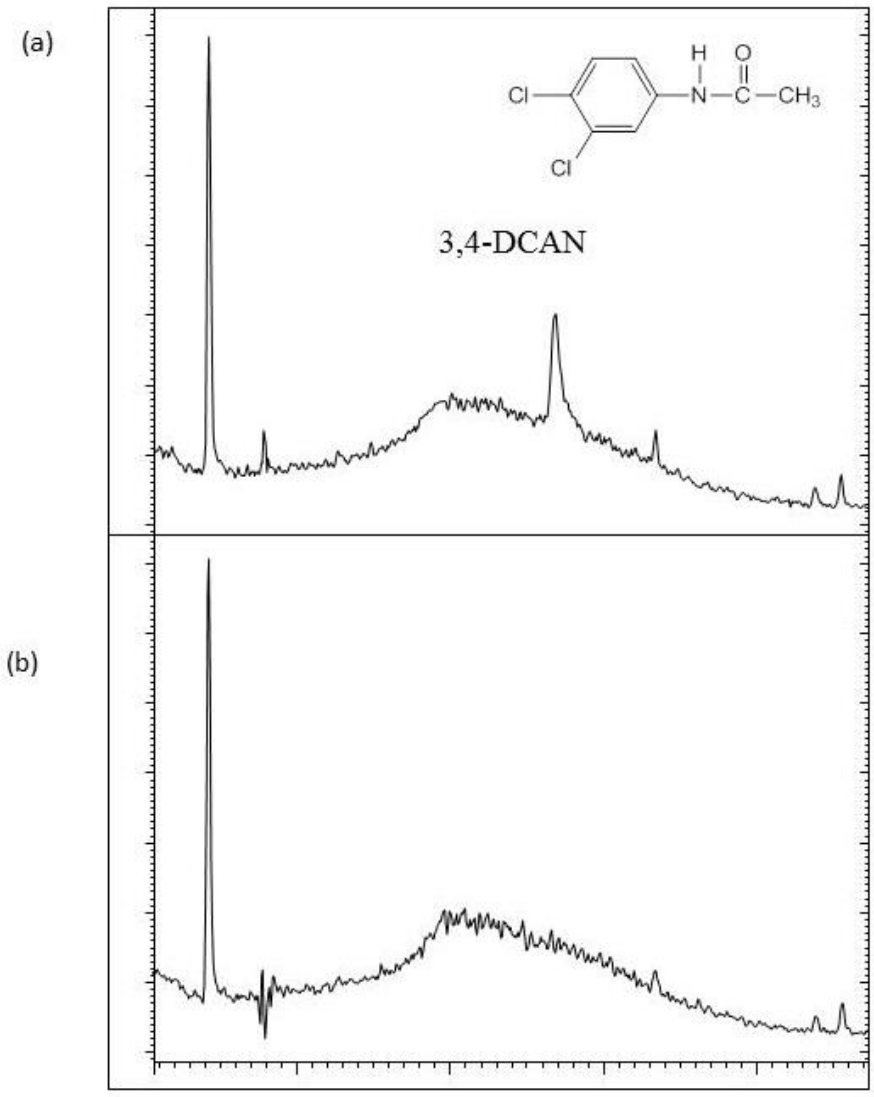


Figure S1. SPME-GC/MS chromatograms showing the peak corresponding to 3,4-dichloroacetanilide (3,4 DCAN) after 28 days of incubation of diuron aqueous solutions inoculated with APE extract (a) but which is absent in the sterilized SAPE extract (b).

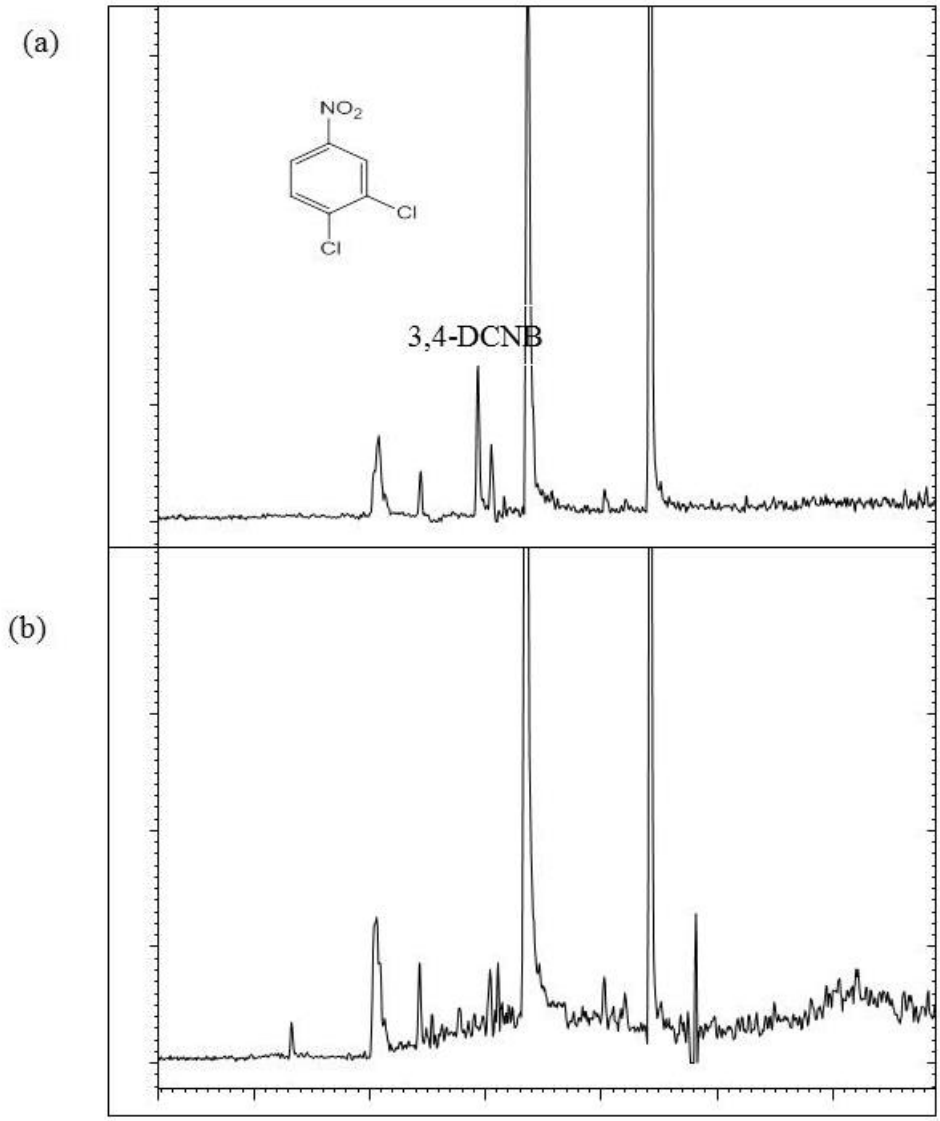


Figure S2. SPME-GC/MS chromatograms showing the peak corresponding to 3,4-dichloronitrobencene (3,4 DCNB) after 28 days of incubation of diuron aqueous solutions inoculated with ACE extract (a) but which is absent in the sterilized SACE extract (b).

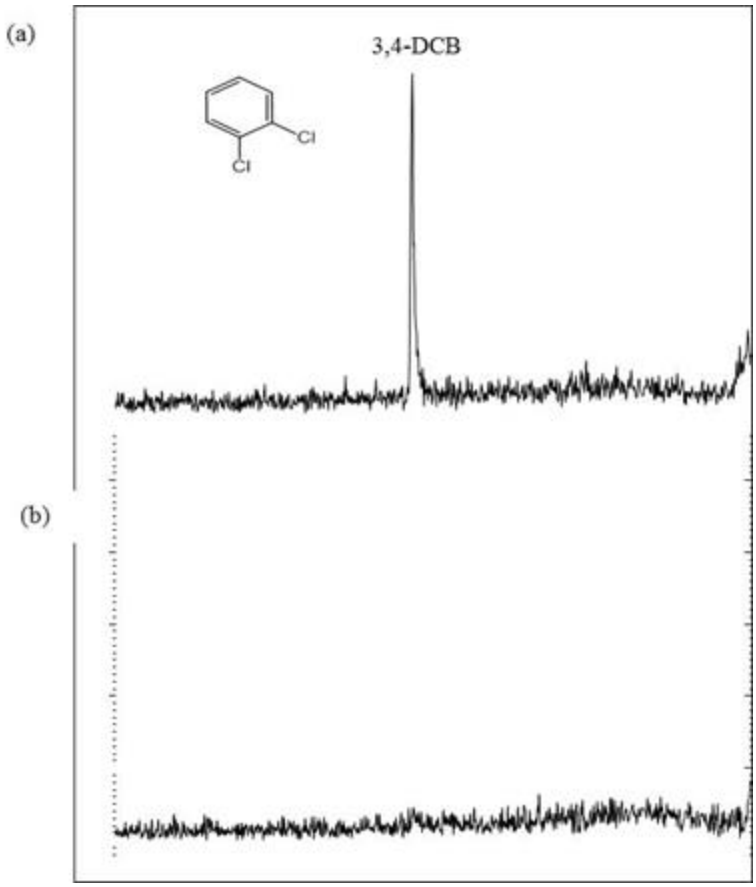


Figure S3. SPME-GC/MS chromatograms showing the peak corresponding to 3,4-dichlorobencene (3,4 DCB) after 28 days of incubation of diuron aqueous solutions inoculated with APE extract (a) but which is absent in the sterilized SAPE extract (b).

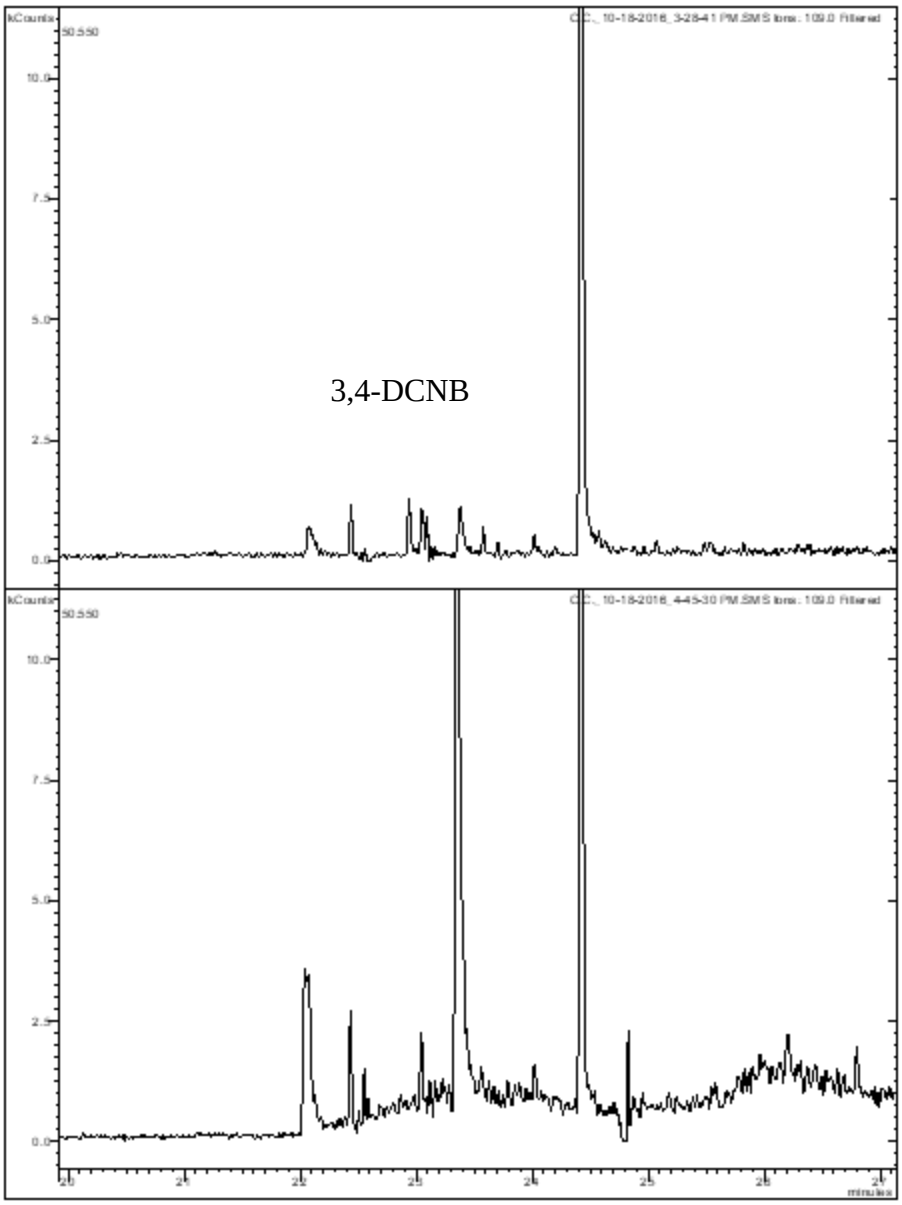


Figure S4. SPME-GC/MS chromatograms showing the peak corresponding to 3,4-dichlonitrobencene (3,4-DCNB) after 28 days of incubation of diuron aqueous solutions inoculated with AVE extract (a) but which is absent in the sterilized SAVE extract (b).

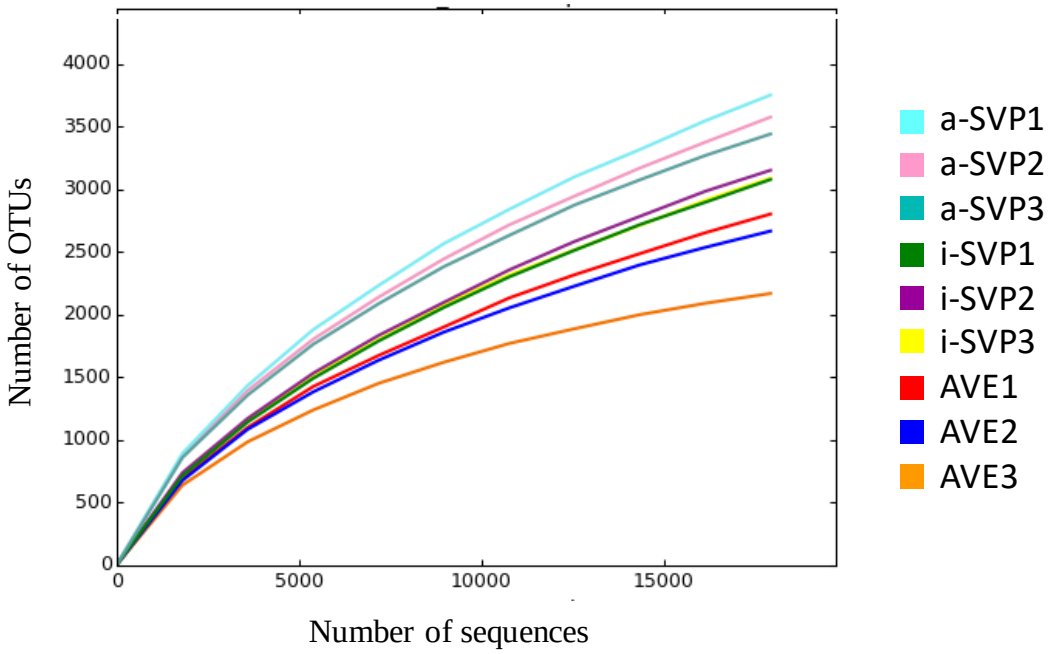


Figure S5. Rarefaction curves for the Operational Taxonomic Units (OTUs) of the initial un-contaminated (i-SVP), the aged contaminated (a-SVP) biomixtures and of extracts from the aged contaminated biomixture (AVE).

Table S1. GenBank accession numbers and percentage similarity with the closest match of the 16s rRNA gene sequences of isolates obtained from the aged contaminated a-SVP biomixture which grow in solid medium with diuron as sole carbon source obtained with BLASTN (Altschul et al., 1990) against NCBI 16S ribosomal RNA database.

Strain	GenBank accession number	% Identity	Closest relative
110A2	MH645844	99	*Arthrobacter nitroguajacolicus*
210C1	MH645845	98	*Pseudomonas songnenensis* strain NEAU-ST5-5
210C2	MH645846	99	*Bacillus simplex* strain LMG 11160
25C1	MH645847	99	*Pseudomonas mosselii* strain CFML 90-83
25C2	MH645848	99	*Pseudomonas mosselii* strain CFML 90-83
112A	MH645849	99	*Bacillus pacificus* strain MCCC 1A06182
24C	MH645850	99	*Pseudomonas soli* strain F-279,208

Table S2. Diversity indices (Chao1, Shannon and Simpson) and Observed OTUs of the initial unpolluted SVP biomixture (i-SVP), aged polluted biomixture (a-SVP) and extract from the polluted biomixture (AVE). Each value is the mean of three replicates ± standard deviation. Different letters indicate significant differences among the biomixtures or tea extracts ($p<0.05$).

Samples	Chao1	Observed OTUs	Shannon	Simpson
i-SVP	5689 ± 81^{b}	3110 ± 45^{b}	9.03 ± 0.06^{b}	0.989 ± 0.000^{b}
a-SVP	5956 ± 212^{b}	3581 ± 137^{b}	9.93 ± 0.08^{c}	0.995 ± 0.001^{b}
AVE	3809 ± 1005^{a}	2542 ± 330^{a}	8.64 ± 0.24^{a}	0.980 ± 0.006^{a}

CHAPTER 2.2

A promising new bioaugmentation strategy to accelerate the biodegradation of emerging contaminants in different media

The results of Chapter 2.2 have been submitted for publication,

Aguilar-Romero, I., van Dillewijn, P., Delgado-Moreno, L., Nogales, R., Romero, E. A promising new bioaugmentation strategy to accelerate the biodegradation of emerging contaminants in different media.

Resumen

Las técnicas de biorremediación tienen factores importantes que deben mejorarse para aumentar su eficiencia de descontaminación. En este estudio, un extracto acuoso, obtenido a partir de un sistema aclimatado tras su exposición a productos farmacéuticos y de cuidado personal (PPCPs) se ensayó como inóculo en sistemas de biopurificación (BPSs) contaminados y en soluciones acuosas para mejorar la eliminación de ibuprofeno (IBP), diclofenaco (DCF) y triclosán (TCS). La bioaumentación con extractos procedentes del BPS aclimatado incrementó significativamente la disipación de DCF y TCS, alcanzando un 90% de eliminación 69 y 108 días antes que con el extracto procedente del BPS control no inoculado, respectivamente. La aplicación de este extracto a una solución acuosa de IBP aumentó la tasa de degradación eliminándose el 99.2% de este contaminante tras 21 días de incubación. El análisis GC/MS reveló niveles bajos de acumulación de metiltriclosán y de metabolitos hidroxilados del IBP tras la inoculación con el extracto procedente de la biomezcla aclimatada en comparación con los sistemas no inoculados. Por último, el análisis de la comunidad bacteriana de BPS reveló un enriquecimiento de algunos taxones bacterianos en la biomezcla aclimatada y la existencia de varios OTU dominantes en el extracto, como los OTU relacionados con *Flavobacterium*, *Thermomicrobia*, *Nonomuraea* y *Fluviicola*, los cuales pueden estar involucrados en la degradación de los PPCPs. Este estudio muestra por primera vez la idoneidad de este extracto como una nueva estrategia de bioaumentación asequible para mejorar la eliminación de los PPCPs y de sus metabolitos en distintos medios contaminados.

Abstract

Bioremediation techniques have important factors which must be improved to increase their decontamination efficiency and application. In this study, an aqueous extract, obtained from a pharmaceutical and personal care product (PPCP) acclimated system was assayed as an inoculum in contaminated biopurification systems (BPSs) and aqueous solutions to improve the elimination of ibuprofen (IBP), diclofenac (DCF) and triclosan (TCS). Bioaugmentation with extracts of BPS significantly accelerated the dissipation of DCF and TCS compared to the non-inoculated control BPS reaching 90% removal earlier in 69 and 108 days, respectively. The application of this extract to an IBP aqueous solution increased the degradation rate by eliminating 99.2% after 21 days. GC/MS analysis revealed low concentration levels or accumulation of methyl triclosan and hydroxylated metabolites of IBP compared to non-inoculated systems. The bacterial community analysis of BPSs revealed an enrichment of some bacterial taxa in the acclimated biomixture and of several dominant OTUs in the extract, such as *Flavobacterium*-, *Thermomicrobia*-, *Nonomuraea*- and *Fluviicola*-related OTUs, which may be involved in the degradation of PPCPs. This study shows for the first time the suitability of this extract as an affordable novel bioaugmentation strategy to enhance the elimination of PPCPs and their metabolites from contaminated media.

1. INTRODUCTION

The increased production and consumption of pharmaceutical and personal care products (PPCPs) has resulted in their occurrence in different environments and occasionally in drinking water, raising worldwide concern (Houtman, 2010; Jurado et al., 2012; Pai et al., 2020; Wilkinson et al., 2017). Currently, these contaminants are not efficiently removed by conventional wastewater treatment processes (Deblonde et al., 2019; Jelic et al., 2011; Rivera-Utrilla et al., 2013). Consequently, both PPCPs and their biotransformation products are continuously released into the environment and can cause harmful effects on non-target organisms, mainly interfering with the endocrine system (Farré et al., 2008; Nilsen et al., 2019). Non-steroidal anti-inflammatory drugs such as ibuprofen (IBP) and diclofenac (DCF), as well as the anti-microbial agent triclosan (TCS) are some of the PPCPs most commonly detected in water at concentrations around of ng L^{-1} (Kim et al., 2014; Wilkinson et al., 2017). IBP biotransformation products, such as carboxyibuprofen and hydroxylated metabolites originating from human and animal metabolism or by the activity of microorganisms, and the main metabolite of TCS generated by microbial methylation, methyl triclosan (M-TCS), have been found in WWTP effluents, superficial water and biotic samples (Bester, 2005; Brausch and Rand, 2011; Chen et al., 2011; Ferrando-Climent et al., 2012; Macherius et al., 2014). Unfortunately, M-TCS is more lipophilic (log Kow = 5.4) and more persistent than the parental compound TCS (log Kow = 4.8), and thus, more harmful for organisms due to its bioaccumulation potential (Brausch and Rand, 2011; H. Wang et al., 2016). Therefore, the development of advanced wastewater treatment technologies is required to improve the removal efficiency of PPCPs and its biotransformation products prior to effluent disposal or wastewater reuse (Grassi et al., 2013; Jelic et al., 2011).

Biopurification systems (BPSs) are sustainable bioremediation techniques used worldwide to prevent point-source contamination, eliminating pesticides from polluted water generated by agricultural activities (Castillo et al., 2008; De Wilde et al., 2007; Dias et al., 2020). These systems are also widely developed for the decontamination of effluents from industries, or to degrade other types of

contaminants such as PPCPs (Delgado-Moreno et al., 2019; Jiménez-Gamboa et al., 2018; Karanasios et al., 2010; Papazlatani et al., 2019). The efficiency of these systems depends on the biologically active biomixture used, which are typically composed of soil, humified material and lignocellulosic wastes (Castillo et al., 2008; Castro-Gutiérrez et al., 2019). In this study, a biomixture containing soil, vermicompost of wet olive-mill cake and olive tree prunings was used due to its high retention capacity, removal efficiency of PPCPs and high microbial activity (Aguilar-Romero et al., 2020; Delgado-Moreno et al., 2019, 2017a). Autochthonous bioaugmentation strategies are suitable for accelerating the dissipation of recalcitrant organic pollutants in BPS (Castillo Diaz et al., 2016; Castro-Gutiérrez et al., 2019; Saez et al., 2018). However, this strategy is very complex and time consuming. Moreover, most microorganisms with specific catabolic capacities are not easily cultivable (Pinto et al., 2015). Recently, to overcome these disadvantages, a novel and affordable bioaugmentation strategy based on aqueous extracts from aged residual biomixtures which have been enriched with degrading microorganisms has proven to be highly successful for enhancing the removal of residues from phenylurea pesticides (Aguilar-Romero et al., 2019).

The purpose of this study is to develop an efficient and sustainable strategy to obtain microorganisms that degrade hazardous compounds and /or metabolites to be applied in bioaugmentation techniques. Thus, our study investigates, to the best of our knowledge for the first time, the effectiveness of this strategy to enhance the elimination of PPCPs and their main metabolites in biopurification systems at a microcosm scale and in polluted aqueous solutions. We have investigated the shifts in bacterial communities in a sample submitted to continuous and reiterated exposures to several PPCPs. The main bacterial taxa in the aqueous extracts from these samples, which were used as an inoculum in bioaugmentation processes to decontaminate different media, in order to determine which taxa can be potentially involved in the elimination of these PPCPs were also investigated.

2. MATERIALS AND METHODS

2.1. Chemicals

Ibuprofen (IBP), 1-hydroxyibuprofen (1-OH IBP), 2-hydroxyibuprofen (2-OH IBP), 3-hydroxyibuprofen (3-OH IBP) and carboxyibuprofen (CBX), diclofenac sodium salt (DCF) and triclosan (TCS) all with purity >97%, were supplied by Sigma-Aldrich (Steinheim, Germany). Methyl-triclosan (M-TCS, >99% purity) was purchased from Fluka (Germany). HPLC grade solvents from Scharlau (Barcelona, Spain) and MilliQ water were used to prepare standards and experimental solutions.

2.2. Acclimatization of the biomixture with PPCPs

The initial biomixture selected to construct the microcosm biopurification systems was also selected to obtain an acclimated biomixture (ac-SVP) with an autochthonous microbial community resistant or adapted to contaminant exposure. For this purpose, the initial biomixture (i-SVP) composed of soil, vermicompost of wet olive cake and olive tree prunings (1:1:2, v:v:v) which had not previously been exposed to any pollutant, was treated four times with a mixture of IBP, DCF and TCS at a concentration of 20 μg g^{-1} at 0, 40, 70 and 125 days of the incubation period. For a homogeneous PPCP application in the biomixture, silica sand was spiked with a solution of acetone containing the three PPCPs and, after solvent evaporation, it was mixed with 500 g of i-SVP in duplicate. The moisture content of the biomixture was adjusted to 75% of its field capacity and incubated in a chamber at 30 ºC in darkness for 166 days. The moisture content was controlled by weighing. The physicochemical properties of the i-SVP are shown in table 2 in the General Materials and Methods section.

2.3. Preparation of aqueous extracts from biomixtures

Aqueous extracts from both i-SVP and ac-SVP were prepared in a lab-scale bioreactor as described in the General Materials and Methods section. To determine the number of cultivable cells, 1 mL of extract was serially diluted in M9 minimal medium (Sambrook et al., 1989), plated in triplicate onto LB media (Sambrook et al., 1989) supplemented with 5μg mL^{-1} of cycloheximide to prevent

fungal growth and incubated at 30 ºC for 3 days. Finally, the bacterial colony forming units (CFUs) were counted.

2.4. Dissipation of PPCPs in microcosm biopurification systems (BPSs) inoculated with extracts.

In order to study the potential of aqueous extracts from acclimated BPSs to improve PPCP dissipation, nine BPSs were constructed at microcosm scale with the initial biomixture (i-SVP) described above. These microcosm-scale BPSs (mBPS) were contaminated with 100 μg g^{-1} of IBP, 20 μg g^{-1} of DCF and 20 μg g^{-1} TCS. For this purpose, 1 g of silica sand was placed in a glass beaker and spiked with a solution of acetone containing IBP, DCF and TCS. After organic solvent evaporation, silica sand was mixed with 70 g (dry weight) of i-SVP in an end-over-end rotary shaker for 15 min at room temperature. Thereafter, 230 mL of extract from ac-SVP was centrifuged at 6,000 rpm for 15 min. The pellet was collected and resuspended in 41 mL of distilled water, the volume necessary to reach 75% of biomixture field capacity, and distributed in the mBPS, increasing the number of CFUs in each microcosm by 1.8 x 10^7 CFUs per g^{-1}. As controls, mBPS moistened with 41 mL of distilled water, and mBPS inoculated with resuspended pellets from sterilized extracts were run in parallel. Sterilized extracts were obtained by autoclaving at 121 ºC for 20 min. All the mBPS were incubated in triplicate in a thermostatic chamber at 20 ºC in darkness for 109 days, and the moisture content was maintained weekly by adding distilled water. PPCP residues were extracted at 0, 7, 14, 21, 59, 79 and 109 days. For this, 6 mL of acetonitrile acidified with 1% acetic acid was added to 3 g (dry-weight equivalent) of biomixture sample and vortexed for 1 min. Then, 1 g of QuEChERS (Agilent Technologies, Santa Clara, CA, USA) was added to each sample and vortexed again for 1 min. Finally, the samples were centrifuged for 5 min at 3,500 rpm, passed through a 0.45 μm PTFE filter and analysed by high-performance liquid chromatography (HPLC, series 1100, Agilent Technologies), following the conditions described in the General Materials and Methods section. The recovery rates determined for IBP, DCF and TCS were 89%, 87% and 92%, respectively.

2.5. Dissipation of ibuprofen in aqueous solution inoculated with extracts.

Aqueous solutions of ibuprofen at 20 mg L^{-1} MilliQ water in Erlenmeyer flasks were inoculated respectively, with extracts (E) or concentrated extracts (CE) from aBM at a 1:1 (v:v) ratio, to obtain experimental solutions with an initial concentration of 10 mg IBP L^{-1}. As abiotic controls, the IBP solution was mixed with distilled water (AC) or with sterile extracts from ac-SVP (SE). The IBP solution was also inoculated with extracts from the untreated biomixtures i-SVP (IE) to control if the previous exposure to these PPCPs could stimulate degrader indigenous bacteria communities. The concentrated extract contains the pellet from the centrifugation of 75 mL extract from ac-SVP and resuspended in 10 mL, which increases by ten-fold the number of CFUs of the inoculum. Sterile extracts were obtained by autoclaving at 121 ºC for 20 min. All the experimental solutions were inoculated in triplicate and incubated on a shaker at 145 rpm at 30 ºC. After 0, 1, 4, 11 and 21 days, aliquots from different solutions were mixed with acetonitrile (1:1), centrifuged at 13,000 rpm for 1 min, filtered with 0.45 μm PTFE filters and then analysed by HPLC following the conditions described in the General Materials and Methods section to determine the IBP concentration.

2.6. Analysis of physicochemical properties, dehydrogenase activity and fluorescein diacetate hydrolysis of aqueous solutions.

All the analyses were performed in triplicate using validated methods as described briefly below. The pH and electrical conductivity (EC) were measured with a glass electrode by using a digital pH meter (Metrohm). The water-soluble carbon (WSC) content was determined with potassium dichromate and sulphuric acid at 160 °C for 30 min. Subsequently, the amount of Cr^{3+} produced by the reduction of Cr^{6+} was quantified spectrophotometrically at 590 nm (Sims and Haby, 1971). The dehydrogenase activity was determined from 5 mL of extract by quantifying spectrophotometrically at 490 nm the iodonitrotetrazolium formazan (INTF) produced after the reduction of INT (2-p-iodophenyl-3-p-nitrophenyl-5-phenyltetrazolium chloride), according to the method published by García et al. (1997). The total hydrolytic enzyme activity was estimated from

10 mL of extract by using the fluorescein diacetate (FDA) method as reported (Perucci et al., 2000).

2.7. Metabolite analysis

The metabolites derived from the transformation of IBP and TCS were analyzed by gas chromatography/mass spectrometry (GC/MS) according to the conditions described in the General Materials and Methods section. Ibuprofen metabolites (1-hydroxyibuprofen, 2-hydroxyibuprofen, 3-hydroxyibuprofen and carboxyibuprofen) were analysed in aqueous solutions inoculated with different extracts and also in the control samples. For this purpose, 6 mL of aqueous solution samples, previously acidified with formic acid (pH 3), were vortexed with 12 mL of ethyl acetate and then frozen until analysis by GC/MS.

Methyl triclosan (M-TCS) was extracted from biomixture samples using the PPCP extraction procedure described in section 2.4, but an aliquot of the supernatant (3-4 mL) was concentrated under an N_2 stream at 55 °C, dissolved in 1 mL of acetonitrile: water (1:1) and filtered through 0.45 μm (PTFE filters) to be analysed by HPLC. The recovery rate determined for M-TCS was 82%. To confirm the identity of this metabolite, the samples extracted from mBPS were also subjected to GC/MS analysis.

2.8. Total DNA isolation and amplicon sequencing analysis

In order to determine the shifts in the bacterial community structure of the biomixture after treatment with PPCPs and to study the bacterial populations enriched in the extracts, amplicon sequencing of the 16S rRNA gene was performed. For this purpose, total genomic DNA was extracted in triplicate from 0.5 g of i-SVP, ac-SVP and the inoculant biomass from ac-SVP extract using the NucleoSpin® Soil kit (Macherey-Nagel, Duren, Germany). Primers for the V3-V4 variable regions of the 16S rRNA gene (341F *5'*-CCTAYGGGRBGCASCAG-*3'* and 806R *5'*-GGACTACNNGGGTATCTAAT-*3'*) were used and the amplified products were sequenced in the University of Copenhagen using the MiSeq sequencing platform (Illumina, San Diego, USA) as described by Aguilar-Romero et al. (2019). Statistical and bioinformatic data analysis are described in the General Materials and Methods section.

3. RESULTS AND DISCUSSION

3.1. Bioaugmentation of biopurification systems with microbial consortia from aqueous extracts

The dissipation curves of IBP, DCF and TCS in the control biopurification system (C), and in the system inoculated with non-sterile (E) and sterile aqueous extract (SE) are shown in Figure 1.

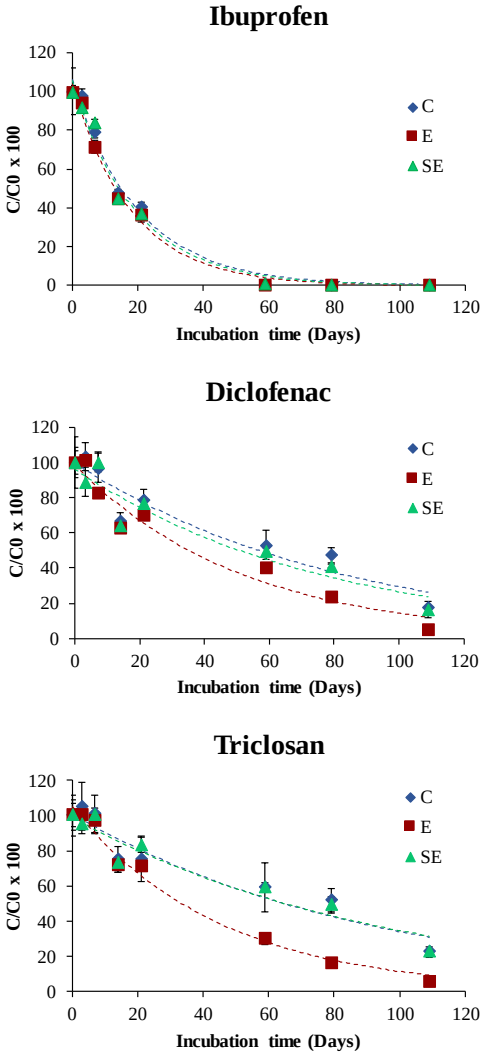


Figure1. Dissipation of ibuprofen, diclofenac and triclosan in microcosm biopurification systems: non-inoculated (C), inoculated with the aqueous extract from acclimated biomixture (E) and with sterilized extract (SE). Symbols represent experimental data and the lines the single first-order kinetic model. Error bars represent the standard deviation (n=3).

The single first-order model accurately fitted the experimental data with determination coefficient values (R^2) higher than 0.91 in most cases (Table 1). This was corroborated by the χ^2 test and the scaled error ($\chi_{7,0.05}^2 = 14.067$, $err_{scaled} < 6.77$). The dissipation rate constant *k* for IBP was the highest of the three PPCPs tested, becoming undetectable after 59 days of incubation. No significant differences were observed between these treatments suggesting that the microbial activity of the extract had a negligible effect on the elimination of IBP in mBPS. However, the dissipation of DCF and TCS was significantly ($p \leq 0.012$) accelerated after the inoculation with the extract from the acclimated biopurification system (E). The *k* value for TCS was 2-fold faster in E systems compared to C and SE, requiring 108 and 115 days less to reach 90% removal (DT_{90}), respectively (Table 1).

Table 1. Single first-order kinetic parameters, determination coefficient (R^2) and scaled errors for ibuprofen, diclofenac and triclosan removal in microcosm biopurification systems: non-inoculated (C), inoculated with aqueous extract from acclimated biomixture (E) and inoculated with sterilized extract (SE).

	Ibuprofen			Diclofenac			Triclosan		
	C	E	SE	C	E	SE	C	E	SE
$C_0 \pm$ sd (%)	105.9 ± 2.2	103.7 ± 2.5	105.2 ± 2.6	99.8 ± 3.3	98.5 ± 3.0	96.3 ± 3.2	101.0 ± 3.7	105.2 ± 2.5	99.1 ± 2.6
$K_t x10^2 \pm$ sd (d^{-1})	5.0 ± 0.3	5.5 ±0.3	5.2 ± 0.3	1.2 ± 0.1	1.9 ± 0.2	1.3 ± 0.1	1.1 ± 0.1	2.2 ± 0.2	1.1 ± 0.1
R^2	0.99	0.98	0.98	0.88	0.94	0.89	0.84	0.97	0.91
Err_{scaled}	3.55	2.85	4.11	6.65	5.53	6.77	5.99	3.51	5.53
DT_{50}	14	13	13	57	36	54	64	31	66
DT_{90}	46	42	45	189	120	179	212	104	219
D ± sd (%)	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	82.2 ± 0.6	95.1 ± 1.1	83.5 ± 4.7	77.3 ± 0.9	94.7 ± 2.3	77.5 ± 3.1

Data represent the mean (± standard deviation, sd) of three independent experiments. DT_{50} and DT_{90} represent the time required for the 50% and 90% dissipation of the initial concentration, respectively.

In the case of DCF, 90% was removed in 120 days in the E samples, whilst it took 189 and 179 days in the C and SE samples, respectively. These results suggest that the microorganisms present in the extract from the acclimated biomixture have a high degradation potential for TCS and DCF. In previous studies, this technique was also found to be effective in improving phenylurea

herbicide removal in BPS, increasing diuron removal 6-fold as well as the removal of its most hazardous metabolite 3,4-dichloroaniline (Aguilar-Romero et al., 2019). Thus, in agreement with other authors (Holmsgaard et al., 2017), the BPS may constitute a suitable media for the proliferation, selection and genetic adaptation of degrading microorganisms during their exposure to contaminants.

The analysis by HPLC of the mBPS samples during the incubation time revealed the presence of a new peak. This peak was determined and identified by GC/MS as methyl triclosan (M-TCS). The amount of M-TCS determined in samples from C and SE systems increased during the incubation period as the TCS dissipated, peaking at 109 days with M-TCS concentrations of 7.3 μg g^{-1} (Figure 2).

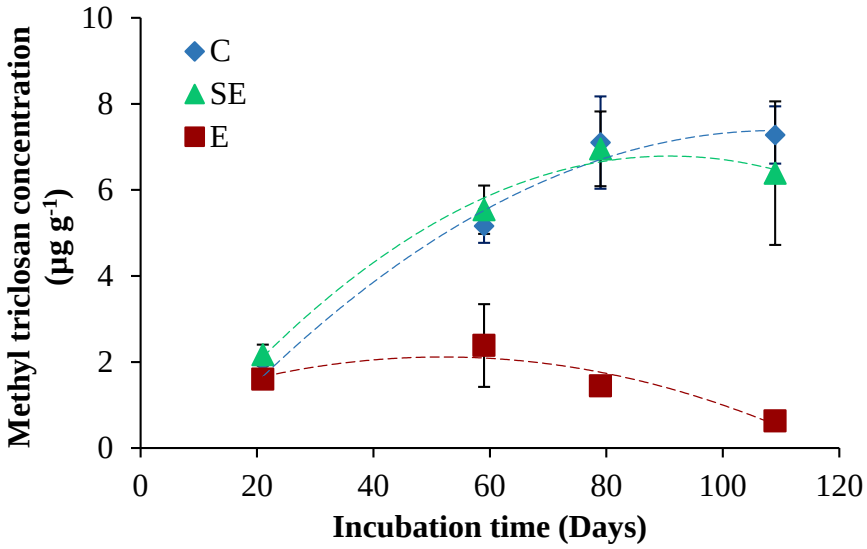


Figure 2. Amount of methyl triclosan in microcosm biopurification systems: non-inoculated (C), inoculated with aqueous extract from the acclimated biomixture (E) and inoculated with sterilized extract (SE). Symbols and lines indicate experimental data and theoretical fit, respectively. Error bars represent the standard deviation (n=3).

No significant differences were observed between C and SE treatments. In contrast, in the biopurification systems inoculated with the extract from the acclimated biomixtures (E), M-TCS did not accumulate and the recorded amount steadily decreased during the incubation period to values 12-fold lower compared to the control systems. This metabolite is formed under aerobic conditions by

microbiological methylation of TCS (Chen et al., 2011). It is commonly detected in WWTP effluents and can be released to ecosystems (Bester, 2005; Kantiani et al., 2008; Lozano et al., 2013). This fact has raised concern due to its toxicological properties, especially as an endocrine disruptor (Dann and Hontela, 2011; Hinther et al., 2011). These results support the degradation potential of the microorganisms present in this extract and suggest that it is also capable of eliminating the most hazardous metabolite of triclosan.

The lack of efficiency observed in the IBP removal in the mBPS inoculated with the acclimated extracts may be due to the intrinsic capacity of the indigenous microorganisms in the mBPS to degrade IBP. Therefore, in order to demonstrate the degradation potential of the extracts for IBP, the study was carried out in liquid solutions.

3.2. Bioaugmentation of ibuprofen solutions with extracts

Figure 3 shows that the application of the extract from acclimated biomixture (E) led to faster elimination of IBP after an adaptation period of 11 days, with 72.4% of the initial IBP removed after 21 days.

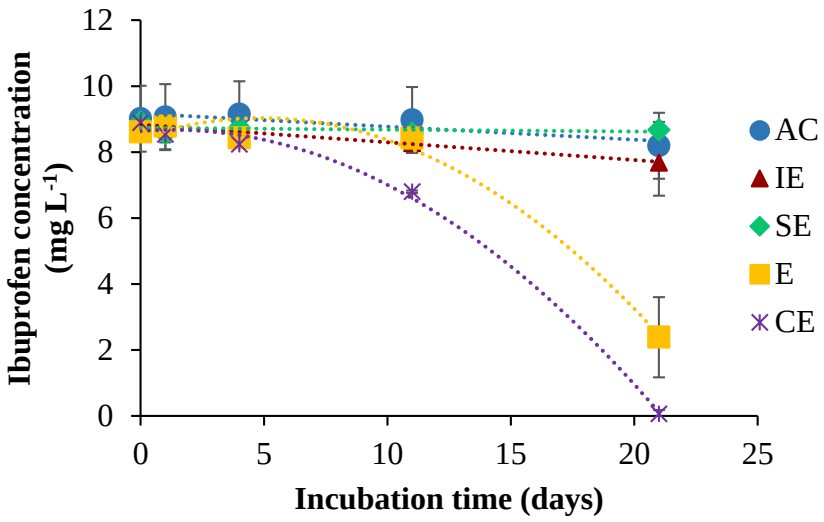


Figure 3. Ibuprofen removal in aqueous solutions: non-inoculated (AC), inoculated with extract from initial (IE) and acclimated biomixture (E) as well as with sterilized (SE) or concentrated extract (CE) from acclimated biomixture. Symbols and lines indicate experimental data and theoretical fit, respectively. Error bars represent the standard deviation (n=3).

The IBP dissipation was further enhanced by applying the concentrated extract (CE), with a 99.2% of the initial amount of IBP removed after 21 days of incubation and with a reduced adaptation period (Figure 3). In contrast, the solutions inoculated with sterilized extract (SE) or with extract from initial unpolluted biomixture (IE) showed a limited amount of IBP elimination so that only 2.6% and 12% of the initial amount of IBP had dissipated, respectively. Both percentages are not significantly different from the abiotic control (AC) solutions, which showed removal of 9.1% of IBP. These results suggest that the acceleration in IBP dissipation was caused by the microbial activity of the extract from acclimated biomixture.

Table 2. Physicochemical properties, dehydrogenase activity (DHS) and fluorescein diacetate hydrolysis (FDA) determined in an ibuprofen aqueous solution inoculated with the extract from initial (IE) or the acclimated biomixture (E) or with sterilized (SE) or the concentrated extract (CE) from the acclimated biomixture.

Samples	pH	EC dS m^{-1}	WSC mg L^{-1}	DHS activity µg INFT mL^{-1} h^{-1} ($x10^2$)	FDA hydrolysis µg FDA mL^{-1} h^{-1}
E	8.01	1.07	65.06 ± 4.81^{c}	1.58 ± 0.68^{b}	1.06 ± 0.00^{c}
CE	8.07	1.13	87.28 ± 3.85^{b}	2.66 ± 0.17^{a}	10.96 ± 1.22^{a}
SE	8.48	1.05	72.83 ± 10.4^{bc}	0.57 ± 0.10^{c}	0.74 ± 0.32^{c}
IE	8.34	0.61	243.94 ± 8.39^{a}	3.59 ± 0.29^{a}	5.26 ± 0.00^{b}

CE: electrical conductivity; WSC: water soluble carbon. Data represent the mean of triplicate measurements ± standard deviation. Letters indicate significant differences between treatments (ANOVA homogeneity test; $p < 0.05$).

Table 2 shows the physicochemical properties of these extracts as well as their corresponding dehydrogenase (DHS) and fluorescein (FDA) activities which are indicators of overall microbial metabolism. The pH values of the extract samples were quite similar and the electrical conductivity was low, especially for IE (Table 2). The highest water soluble carbon (WSC) content was determined in the extract from the untreated initial biomixture, IE. On the other hand, CE solutions showed DHS and FDA values 1.7- and 10.3-fold higher than E solutions, respectively.

Therefore, the efficiency to enhance the IBP dissipation may be attributed to the increase in microbial activity after inoculation with the concentrated extract. As expected, DHS activity was significantly reduced after sample sterilization, however no significant differences were observed in FDA values with E solutions. According to Delgado-Moreno et al. (2017) the application of a high dosage of contaminants such as pesticides did not affect the overall microbial activity of the biomixtures. Nevertheless, in this study, both enzyme activities were found to be significantly higher in IE compared to the E treatment. This higher microbial activity in IE may partially be related with its high WSC content (Table 2). On the other hand, it cannot be ignored that in the acclimatization period of the biomixtures with PPCPs, the antimicrobial agent, TCS, could cause a decrease in the number of microorganisms (Aguilar-Romero et al., 2020). According to Butler et al. (2011) although the application of TCS inhibits soil respiration, this contaminant causes subsequent acclimatization of the microbial community. Consequently, despite the microbial activity being relatively lower in E, this does not mean that it does not contain active microorganisms with catabolic capabilities for degrading IBP. The enrichment in degrading microorganisms also occurs in other systems continuously exposed to PPCPs such as WWTPs (Ferrando-Climent et al., 2012). Moreover, several authors have described the biotransformation of IBP by microorganisms isolated from different contaminated environments (Żur et al., 2018). Therefore, BPSs under continued exposure to PPCPs appears to have enriched resilient degrading microorganisms which could be collected by this simple and low cost aqueous extraction process.

Several new peaks were detected in the experimental solutions analysed by HPLC. At the end of the incubation period, four ibuprofen biotransformation products, 1-hydroxyibuprofen (1-OH IBP), 2-hydroxyibuprofen (2-OH IBP), 3-hydroxyibuprofen (3-OH IBP) and carboxyibuprofen (CBX IBP) were identified by GC/MS analysis at different levels (Supplementary Figure S1). Carboxyibuprofen was the most abundant biotransformation product identified in all the samples, suggesting that hydroxylation at C3 of IBP followed by oxidation to form the carboxyl group could be a common process in aqueous solutions (Borges et al., 2011; Żur et al., 2018). In fact, ibuprofen metabolites can be generated by both human metabolism and microorganisms present in natural water as well as in WWTPs, soils and sediments. Consequently, these

biotransformation products have been widely detected, not only in WWTP effluents, but also in water resources (Ferrando-Climent et al., 2012; Lolić et al., 2015). The three hydroxylated metabolites were found at the highest levels in IE. 1-OH IBP and 2-OH-IBP were also detected in SE and at still lower levels in E. However, in the CE samples, 1-OH IBP was the only hydroxylated metabolite detected but at the lowest intensity. Hence, the microorganisms present in the extract from ac-SVP, especially when added in increased numbers as in the CE treatment, could be able to prevent the accumulation of hydroxylated metabolites or have the genetic machinery necessary to eliminate them. An IBP degradation pathway has been described by Marchlewicz et al. (2017) for the *Bacillus thuringiensis* B1 (2005b), a soil isolate, which hydroxylates the aliphatic chain of IBP by the activity of an aliphatic monooxygenase, generating 2-OH IBP which in turn is hydroxylated by phenol and hydroquinone monooxygenases. Since IBP was easily removed in the mBPS, as was observed in the assays described above, these IBP transforming microorganisms from the soil or the other biomixture components may have easily developed in the BPS.

3.3. Bacterial community structure and composition in biomixtures and extracts

Bacterial communities were analysed by amplicon sequencing of the 16S rRNA gene in order to determine which groups of microorganisms could be involved in the effectiveness of the extract. A total of 240382 high quality sequences were obtained. Rarefaction curves indicate that the sequencing depth was sufficient to cover the full range of diversity of these samples (Supplementary Figure S2).

Alpha diversity analyses of the bacterial community in i-SVP, ac-SVP and aqueous extract from ac-SVP is shown in supplementary figure S3. According to the Chao1 index, the bacterial richness was significantly lower in the PPCP-treated biomixture (ac-SVP) than in the initial biomixture (i-SVP). Several authors have reported that TCS alters the composition and structure of bacterial communities in different ecosystems (Peng et al., 2019; Phandanouvong-Lozano et al., 2018; Richmond et al., 2017), and reduces respiration and richness in microbial populations exposed even at low concentrations (Butler et al., 2011; Clarke et al., 2019). In contrast, the diversity and evenness, according to the

Shannon and Simpson indices, respectively, were significantly higher in ac-SVP. Recently, TCS has been described as an environmental stressor decreasing species diversity in active sludge (Oh et al., 2019). However, IBP and DCF can induce an increment of alpha diversity and compensate part of the TCS negative effects (Jiang et al., 2017). In a previous study, no significant differences were observed in alpha diversity indices between control samples (no PPCPs) and biomixtures with a single treatment with IBP, DCF and TCS (Aguilar-Romero et al., 2020). Thus, the repeated applications and prolonged exposure to PPCPs may cause a decrease in species richness and an increase of resilient microbial communities in the BPS. On the other hand, the number of observed OTUs and alpha diversity indices in extract samples, in general, were significantly lower compared to the biomixtures, indicating a reduction in richness and diversity during the extraction process. Similar tendencies were observed for aqueous extracts from biomixtures contaminated with pesticides (Aguilar-Romero et al., 2019).

Hierarchical clustering analysis and PCoA (Supplementary Figure S4) of the bacterial communities grouped samples into three distinct groups, but the extract samples clustered more closely with the ac-SVP than with the i-SVP. Moreover, 48.13% of the diversity variations in the bacterial community structure (PC1) were influenced by PPCP-exposure. Similarly, the permutational multivariate analysis of variance (PERMANOVA) from sequencing data reveal that the PPCP-treatment had a stronger effect on the structure of the microbial community ($p < 0.009$) than the sample type ($p < 0.03$).

The bacterial community in the i-SVP and ac-SVP, as well as extract samples, were composed of 13,328 different OTUs which could be grouped into 27 phyla, 12 of which were predominant and included 88-97% of detected OTUs (Figure 4 and supplementary Figure S5). The relative abundance of *Actinobacteria*, *Verrucomicrobia* and *Alphaproteobacteria* was significantly reduced in ac-SVP and extract samples compared to the initial untreated system i-SVP. Hence, when NMDS ordination was constrained with data of the main phyla (Figure 4), the former taxa showed more influence in i-SVP, whereas *Beta-* and *Deltaproteobacteria, Gemmatimonadetes, Acidobacteria, Deinococcus-Thermus* and *Planctomycetes* were constrained to ac-SVP, indicating an

enrichment after PPCP-treatment. Nevertheless, the phyla *Bacteroidetes, Chloroflexi, Firmicutes, Chlorobi* and *TM6* mainly influenced the bacteria community structure of the extract samples. Similar trends could also be observed in the relative abundance (RA) of the dominant phyla (Supplementary Figure S5). Accordingly, Zhao et al. (2015) reported that PPCP dosing promotes the dominance of *Betaproteobacteria* and *Bacteroidetes* in an aerobic granular sludge bioreactor. Furthermore, *Bacteroidetes*, *Acidobacteria* and *Chloroflexi* are among the most abundant groups in river sediment biofilm exposed to WWTP effluents containing PPCPs (Nega et al., 2019), whilst *Firmicutes* was the most dominant phylum in wetland mesocosms receiving IBP-enriched wastewater (Zhang et al., 2016). Moreover, *Acidobacteria* may contribute to the effective removal of halogenated carboxylic PPCPs such as DCF (He et al., 2020).

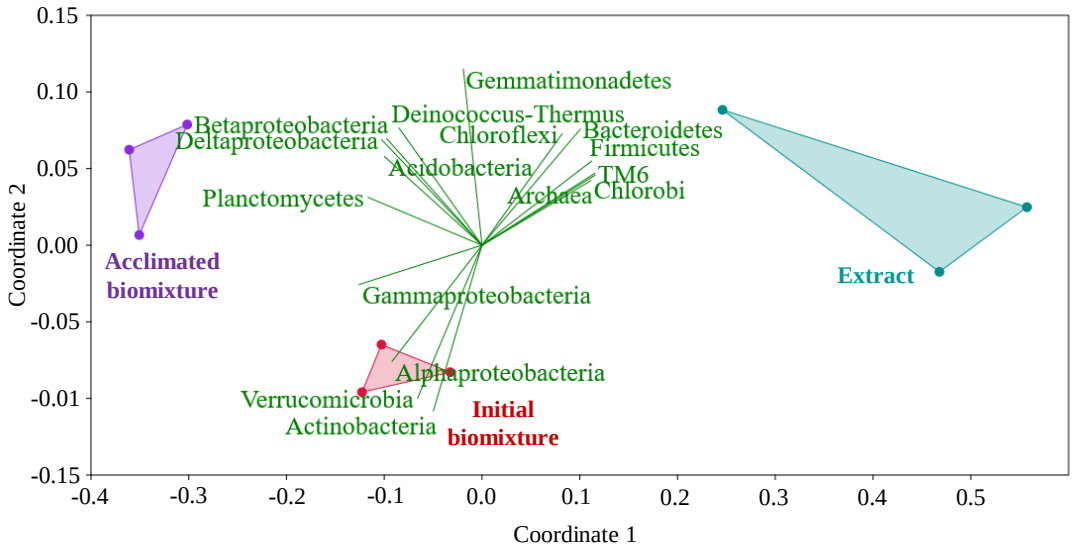


Figure 4. Non-metric multidimensional scaling (NMDS) ordination of the bacterial community composition in the initial and acclimated biomixtures as well as in the extract from the acclimated biomixture constrained by the most dominant phyla and the main classes of *Proteobacteria*.

3.3.1. Analysis of specific taxa potentially involved in improving PPCP dissipation

The heatmap of the most dominant families and the radar chart of the most abundant operational taxonomic units (OTUs), in initial and acclimated

biomixtures as well as in extracts from acclimated biomixtures (Figures 5 and 6) allow us to discriminate which groups of bacteria from i-SVP were affected by PPCP treatments and possibly involved in PPCP degradation.

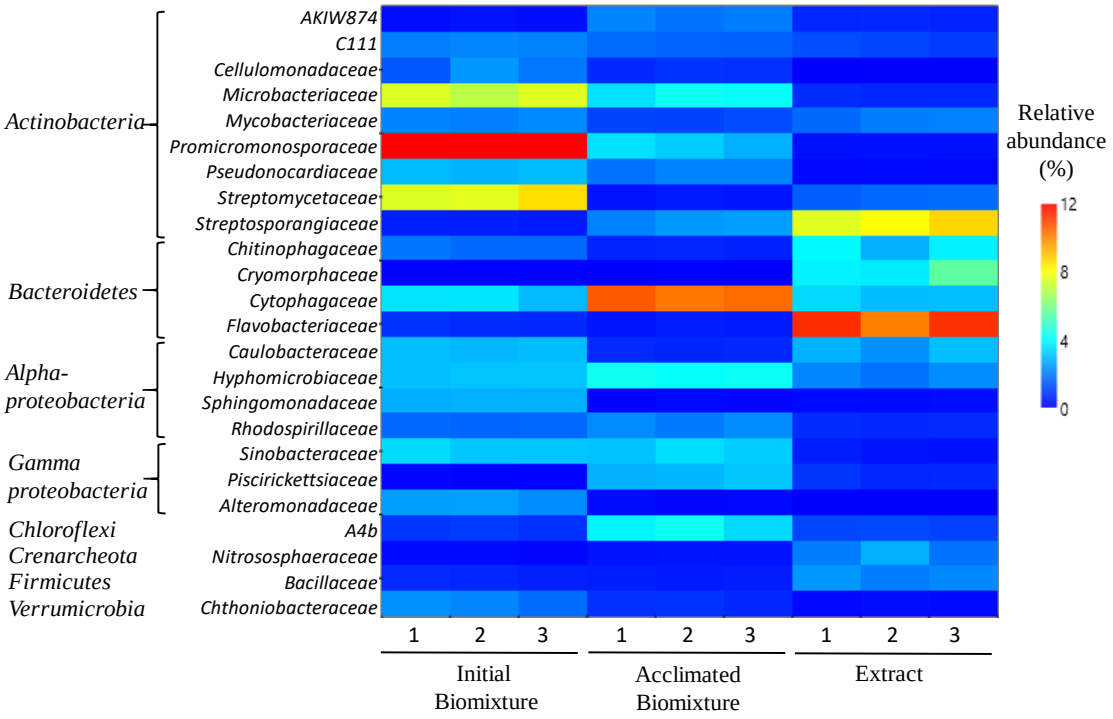


Figure 5. Heatmap of the most dominant families in initial and acclimated biomixtures as well as in the extract from the acclimated biomixture. The relative abundance of the represented taxa was greater than 2% in all the samples.

The PPCP exposure mainly favoured the enrichment in the ac-SVP of the bacteroidetal family *Cytophagaceae* as well as of the alphaproteobacterial family *Hyphomicrobiaceae*, the gammaproteobacterial family *Piscirickettsiaceae* and the Chloroflexi family *A4b* (Figure 5). Interestingly, bacterium strains belonging to the *Cytophagaceae* family have been related to successful PPCP biodegradation (Kim et al., 2014; Li et al., 2016). In a previous study, one of the biomarkers identified in PPCP-treated microcosm BPS belongs to the *Hyphomicrobiaceae* family (Aguilar-Romero et al., 2020). Despite the reduction in *Actinobacteria* and *Alphaproteobacteria* with PPCP application, Promicronosporaceae_OTU10 and Alphaproteobacteria_OTU12 were dominant in ac-SVP (Figure 6).

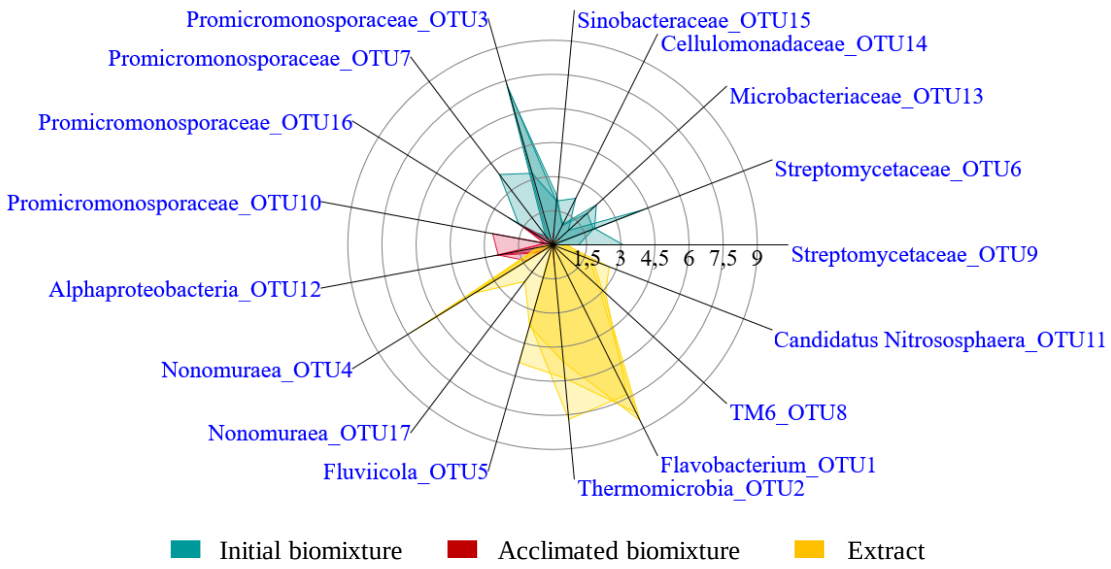


Figure 6. Radar chart showing the most abundant operational taxonomic units (OTUs) in initial and acclimated biomixtures as well as in the extract from the acclimated biomixture. The relative abundance of the represented taxa was greater than 2% in all the samples.

During the extraction process, a strong enrichment occurs in some families and, specially, in some OTUs. The family *Flavobacteriaceae* was the most dominant and includes the Flavobacterium_OTU1, whose RA increased between 7.3 and 8.5% in extract samples compared to ac-SVP (Figure 5). The abundance of *Flavobacterium* was statistically related to the biotransformation of IBP, DCF and TCS in biotreatment reactors (Rossmassler et al., 2019). This genus was also enriched in TCS-exposed soils (Phandanouvong-Lozano et al., 2018) as well as in a membrane bioreactor used for the treatment of synthetic wastewater containing pharmaceuticals (Tiwari et al., 2019). Furthermore, the RA of *Streptosporangiaceae* increased by 1.5-2.1% in the biomixture after acclimatization with PPCPs and up to 6.1% in the extract samples (Figure 5). This family includes two of the most abundant OTUs in extract samples, Nonomuraea_OTU4 and Nonomuraea_OTU17 (Figure 6). Interestingly, a strain belonging to this genus, *Nonomuraea recticatena* NBRC 14525S, harbouring actinomycete cytochrome P450 monooxygenase, catalyzes the hydroxylation of aromatic compounds such as DCF (Kabumoto et al., 2009). In addition, the RA of Thermomicrobia_OTU2, increased up to 7.7% in the extract samples (Figure

6). Both Thermomicrobia_OTU2 and one of the enriched families after PPCP exposure, *Ab4*, belongs to the *Chloroflexi* phylum. The RA of this phylum was 2-fold higher in the biomixture after acclimatization with PPCPs and increased by 2.2-6.6% in extract samples. Several authors reported that *Chloroflexi* is one of the most dominant phyla in different environments exposed to PPCPs such as sediments and wetland soils (Nega et al., 2019; Peng et al., 2019; Zhang et al., 2016). Furthermore, Mcnamara and Krzmarzick (2013) hypothesized that triclosan enriches Dehalococcoides-like *Chloroflexi* since these bacteria respire organochlorides and are probably more resistant than others to the antimicrobial effects of this biocide.

A similar trend was also observed for the families *Chitinophagaceae*, *Cryomorphaceae*, *Caulobacteraceae*, *Nitrososphaeraceae* and *Bacillaceae* (Figure 5). Within the *Cryomorphaceae* family, Fluviicola_OTU5 was considerably enriched in extract samples increasing in RA by 3.6-5.4% (Figure 6). According to Song et al. (2016), the *Fluviicola* genus could be involved in the enhanced degradation of a persistent organic pollutant since its abundance was exponentially correlated with hexachlorobenzene dissipation. The alphaproteobacterial family *Caulobacteraceae* was considered resistant or even capable of biodegrading TCS in an aerobic water sediment system and in BPS (Aguilar-Romero et al., 2020; Guo et al., 2016). Extract samples were enriched in Candidatus Nitrososphaera_OTU11 belonging to *Nitrososphaeraceae* family (Figure 6). This genus was dominant in soil contaminated with heavy metals and polybrominated diphenyl ethers (Wu et al., 2019). Furthermore, the *Bacillaceae* family includes microorganisms such as *Bacillus thuringiensis* B1 (2015b), which efficiently degrades ibuprofen in the presence of an additional carbon source (Marchlewicz et al., 2016).

Another dominant OTU in extract samples was TM6_OTU8 (Figure 6). The RA of the candidate phylum TM6 was between 13-15 fold higher in extract samples than in ac-SVP samples (Supplementary Figure S5). TM6 has not previously been related to PPCP degradation. However, Meng et al. (2016) reported that this candidate phylum was enriched in seawater exposed to petroleum hydrocarbons, including cycloalkanes and polycyclic aromatic hydrocarbons, when dispersants are applied to improve the dissolution of these compounds into water. Thus,

dominant OTUs of extract samples may also be involved in the improvement of PPCP removal and/or its biotransformation products observed upon bioaugmentation of i-SVP.

Supplementary material

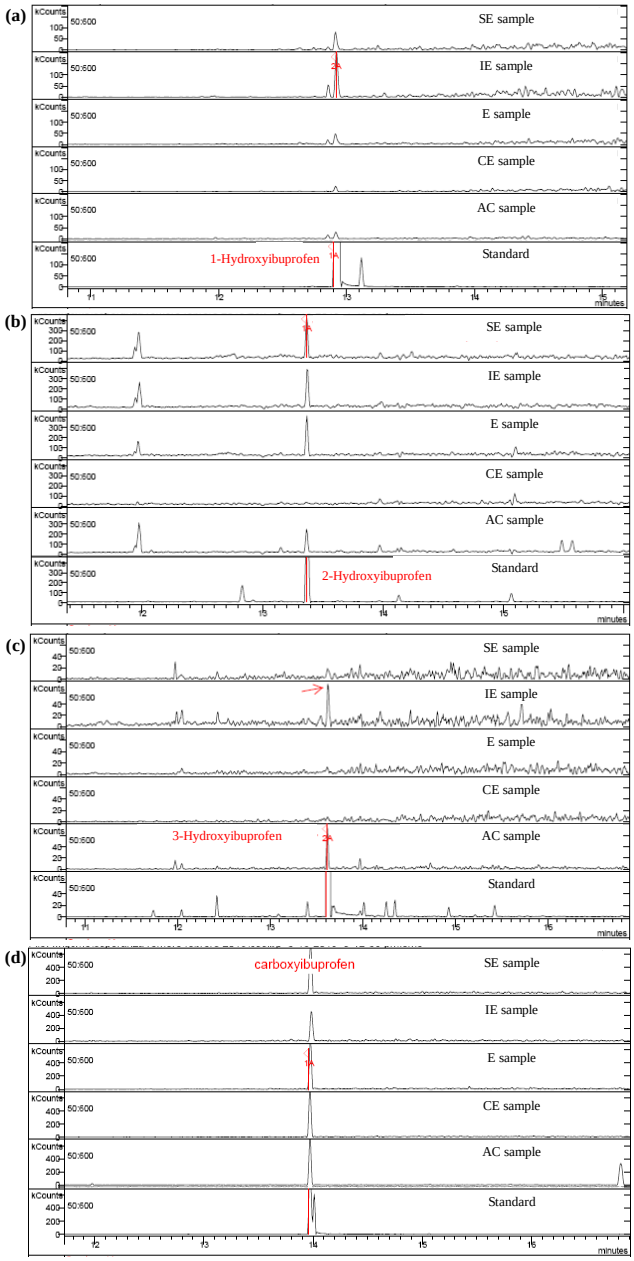


Figure S1. GC/MS chromatograms showing the peaks corresponding to 1-hydroxyibuprofen, 2-hydroxyibuprofen, 3-hydroxyibuprofen and carboxyibuprofen (a, b, c, and d, respectively) in non-inoculated ibuprofen aqueous solutions (AC), inoculated with extract from the initial (IE) or the acclimated biomixture (E) or inoculated with sterilized (SE) or concentrated extract (CE) from the acclimated biomixture. Standard samples of ibuprofen metabolites were analysed as references.

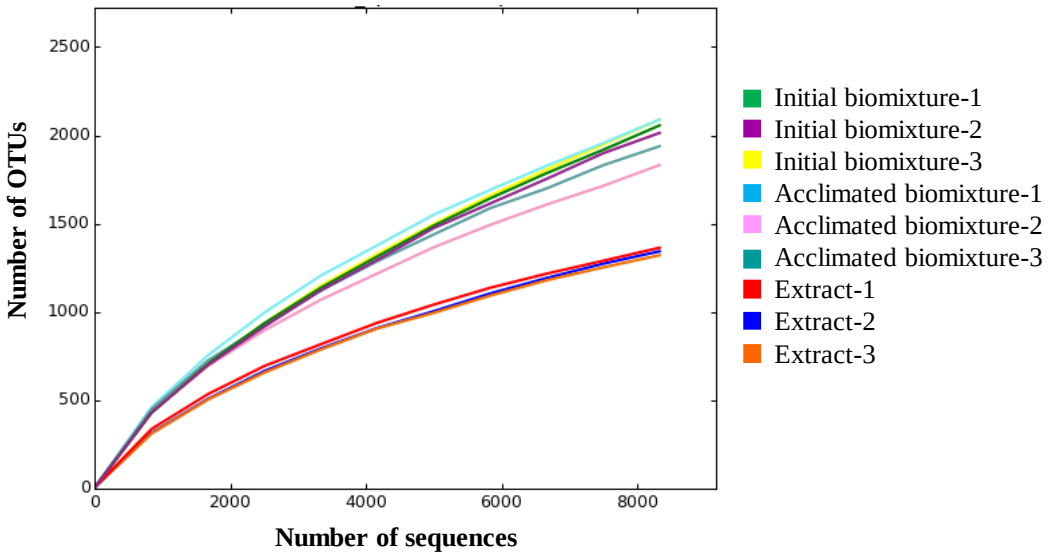


Figure S2. Rarefaction curves for the operational taxonomic units (OTUs) of the initial and acclimated biomixtures as well as of the extract sample from the acclimated biomixture.

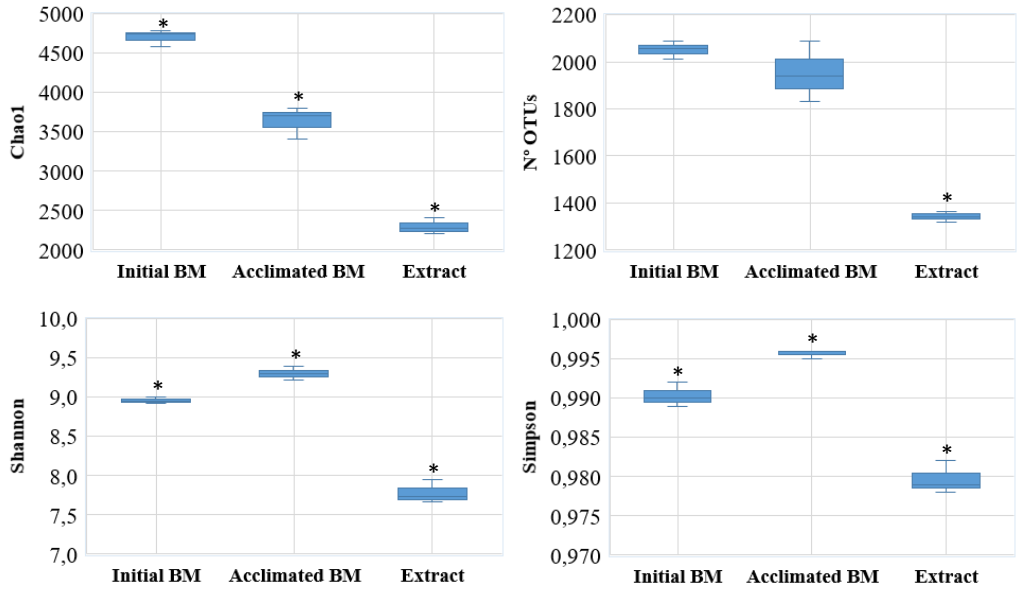


Figure S3. Alpha diversity indices (Chao1, Shannon and Simpson evenness) and observed OTUs of the initial biomixture (initial BM), acclimated biomixture (acclimated BM) and extract from the acclimated biomixture. Error bars represent the standard deviation (n=3). * Significant differences between the initial biomixture, acclimated biomixture and the extract (ANOVA homogeneity test; $p < 0.05$).

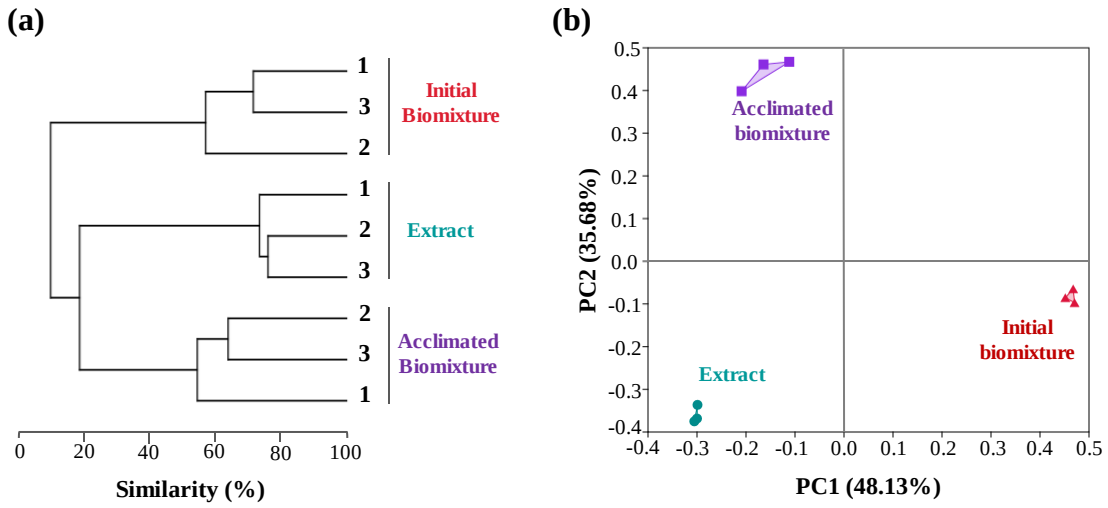


Figure S4. Hierarchical cluster (a), and principal co-ordinate (b) analyses of the bacterial community structure based on Bray-Curtis distances in the initial and acclimated biomixtures as well as in the extract from the acclimated biomixture.

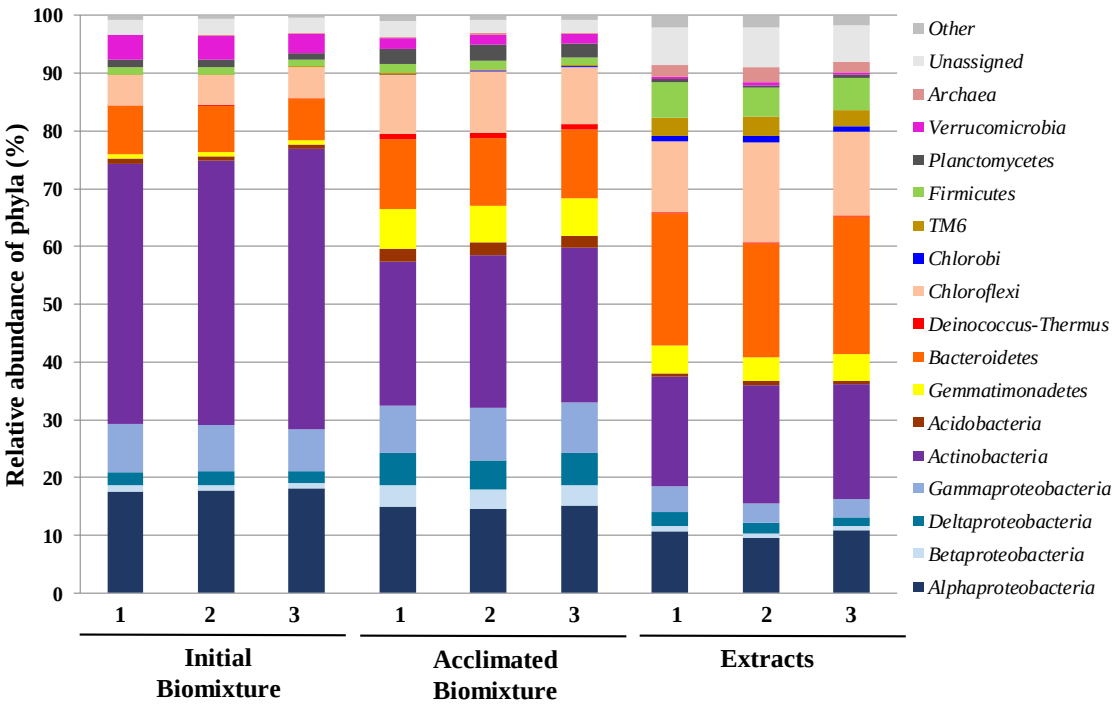


Figure S5. Relative abundance of the most dominant phyla (>1% in any sample) and classes of *Proteobacteria* in the initial and acclimated biomixtures as well as in the extract samples.

CHAPTER 3

Improvement of PPCPs removal in contaminated media by bioaugmentation with exogenous bacterial strains

CHAPTER 3.1

Draft genome sequences of isolates from sediments of the river Elbe that are highly tolerant to diclofenac

The results of Chapter 3.1 have been published in the journal *Microbiology Resource Announcements*,

Quesada, J. M., Aguilar, I., de la Torre, J., Wittich, R. M., Van Dillewijn, P. 2018. Draft Genome Sequences of Isolates from Sediments of the River Elbe That Are Highly Tolerant to Diclofenac. *Microbiology Resource Announcements*, 7:e00849-18.

https://doi.org/10.1128/MRA.00849-18.

Diclofenac, an over-the-counter medication used in many countries, has a yearly production volume of about 90 tons in Germany and 800 tons in India. Insufficient removal by wastewater treatment plants has led to an increasing occurrence of diclofenac, as well as that of other pharmaceuticals and personal care products (PPCPs), in surface waters such as the River Elbe in Germany (Wiegel et al., 2004). Moreover, its toxic effects toward microbes as well as higher organisms (Jiang et al., 2017; Lonappan et al., 2016) have converted diclofenac into an emerging contaminant.

Isolates were obtained from enrichment cultures of sediment samples from the River Elbe downstream of the Hamburg harbor (Wittich et al., 1999) with 0.5 mM diclofenac as the sole carbon and energy source. Bacteria growing in the presence of diclofenac were isolated and identified by sequencing the corresponding 16S rRNA genes. A MIC(s) study with various concentrations of diclofenac in lysogeny broth (LB) medium showed that the isolates RW405 and RW409 grew at concentrations greater than 1,500 mg L^{-1}, but RW407, RW408, and RW410 grew only in a range between 750 and 1,200 mg L^{-1}.

To characterize each isolate, pure cultures of each strain were grown overnight at 30 °C in LB medium under agitation. Total genomic DNA was extracted using the Wizard genomic DNA purification kit (Promega). The quality and quantity of the DNA were assessed with the Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific). To obtain the draft genome of each isolate, 300-bp paired-end sequencing libraries were prepared using the Illumina Nextera XT DNA library version 3 sample preparation kit, and sequencing was performed with the Illumina MiSeq platform at the Center for Scientific Instrumentation of the University of Granada (Spain). Sequence reads were assessed for quality using FastQC (Babraham Bioinformatics, Q > 30), filtered using Trimmomatic (Bolger et al., 2014), and assembled de novo with Velvet (version 1.2.10) and VelvetOptimiser (version 2.2.5) (Zerbino and Birney, 2008) within a customized workflow on Galaxy (http://galaxy-mel.genome.edu.au/galaxy/). The contigs obtained were further annotated with the Rapid Annotations using Subsystems Technology (RAST) server version 2.0 (Aziz et al., 2008), and for submission to GenBank, gene annotation was performed using the NCBI Prokaryotic Genome Annotation Pipeline (PGAP) (Tatusova et al., 2016).

Genome sizes vary between 5,862 and 7,346 Mbp and GC contents from 61.8 to 67.6% (Table 1). Taxonomy was determined by comparing 16S rRNA gene sequences in EzBioCloud (Yoon et al., 2017a) and the *rpoB*, *rpoD*, and *gyrB* genes (Pascual et al., 2010) with a BLAST search (Altschul et al., 1990). As possible mechanisms for diclofenac tolerance, the isolates harbored between 35 and 46 genes related to efflux mechanisms for multidrug resistance and between 143 and 240 genes related to the metabolism of aromatic compounds, which constitute between 0.6 and 0.7% and 2.4 and 3.8% of the total number of genes, respectively. Pairwise similarity between the genomes as determined by average nucleotide identity (ANI) with the OrthoANIu tool (Yoon et al., 2017b) ranged between the pseudomonad genomes from 76.6 to 82% and between the pseudomonads and the *Achromobacter* strain from 69.2 to 70.7%, which are all well below the 95 to 96% species threshold (Richter and Rosselló-Móra, 2009).

Table 1. Characteristics and accession numbers of genomes of the diclofenac-tolerant bacterial isolates.

Isolates	Bacterial species[a]	Genome size (bp)	No. of contigs	N_{50} (bp)	Total no. of genes	G+C content (%)	No. of genes related to		GenBank accession number
							Efflux systems[b]	Metabolism of aromatic compounds[b]	
RW405	*Pseudomonas putida*	5,862,946	33	127,964	5,612	61.8	35	152	QHJD00000000
RW407	*Pseudomonas citronellolis*	7,346,097	108	38,667	6,724	67.4	37	256	QGSL00000000
RW408	*Achromobacter xylosoxidans*	6,532,250	49	57,962	6,06	67.6	44	143	QHHO00000000
RW409	*Pseudomonas chlororaphis*	7,154,805	42	130,324	6,555	62.5	46	171	QHHP00000000
RW410	*Pseudomonas aeruginosa*	6,529,102	74	120,648	6,289	66.2	42	166	QGSM00000000

[a]Strain identification by ≥ 99% similarity with 16S rRNA and ≥ 97% with the *rpoB*, *rpoD*, and *gyrB* genes.

[b]According to annotation with the RAST server.

Data availability.

The whole-genome sequences have been deposited in DDBJ/ENA/GenBank under the accession numbers listed in Table 1. The versions described in this paper are the first versions.

CHAPTER 3.2

Metabolic and genetic features of the ibuprofen-mineralizing strain *Sphingopyxis granuli* RW412 and its effectiveness for the decontamination of effluents and biopurification systems

The results of Chapter 3.2 have been prepared for publication.

Resumen

El éxito de las bacterias para mineralizar contaminantes orgánicos debe estar respaldado por su capacidad catabólica para hacer frente a diferentes ecosistemas. La detección de ibuprofeno y sus metabolitos en sistemas acuáticos, debido al alto consumo de este analgésico en todo el mundo, y su limitada eliminación por las plantas de tratamiento de aguas residuales (PTAR), así como su efecto sobre el medio ambiente y la salud pública, ha suscitado preocupación. En este trabajo, se caracterizó y ensayó una cepa bacteriana capaz de mineralizar ibuprofeno aislada de sedimentos del río Elba para bioremediar diferentes medios contaminados con este fármaco. Esta cepa, identificada como *Sphingopyxis granuli*, tiene un tamaño total de genoma de 4,48 Mbp que incluye tres secuencias plasmídicas de 193 kbp, 138 kbp y 44 kbp. Su secuencia genómica contiene los genes *ipf* los cuales codifican enzimas implicadas en la ligación inicial de coenzima A al ibuprofeno, probablemente seguido de la activación del anillo aromático por una escisión de dioxigenasa y retroaldol para producir inequívocamente 4-isobutilcatecol que sufre una posterior degradación. En medio líquido, la cepa crece eliminando más de 2 mM de ibuprofeno en 74 horas con un tiempo de duplicación de 16 horas. Tras su inoculación en un sistema de biopurificación, la cepa RW412 eliminó dosis repetidas de ibuprofeno en pocos días. En el efluente secundarion de una planta de tratamiento de agua residuales (PTAR) enriquecido con ibuprofeno, la eliminación por esta cepa fue 7 veces más rápida que la realizada por la microbiota autóctona del efluente, lo que sugiere que esta cepa tiene una alta resistencia a condiciones ambientales relevantes y puede ser útil para eliminar este contaminante emergente de los sistemas de tratamiento de las PTAR antes de su liberación al medio ambiente.

Abstract

The success of bacteria to mineralize organic contaminants must be supported by its catabolic capability to face different environmental media. The detection of ibuprofen and their metabolites in aquatic systems, due to the high consumption of this analgesic throughout the world, and its limited elimination by wastewaters treatment plants, as well as its potential effect on the environment and public health has raised concern. In this work, an ibuprofen-mineralizing bacterial strain isolated from sediments of the River Elbe was characterized and assayed to remediate different ibuprofen-polluted media. The strain identified as *Sphingopyxis granuli* has a genome total size of 4.48 Mbp which includes three plasmid sequences of 193 kbp, 138 kbp and 44 kbp. Its genomic sequence contains the *ipf* genes which we show to encode enzymes which initiate CoA ligation to ibuprofen, likely followed by aromatic ring activation by a dioxygenase and retroaldol cleavage to unequivocally produce 4-isobutylcatechol which undergoes further degradation. In liquid medium, the strain grows eliminating more than 2 mM ibuprofen within 74 hours with a doubling time of 16h. Inoculated in biopurification systems, it eliminated repeated doses of ibuprofen within a few days. In ibuprofen spiked secondary effluent from a municipal wastewater treatment plant (WWTP), the removal by this strain was 7 times faster than the indigenous microbiota did, suggesting that this strain has a high resistance to environmental relevant conditions and can be useful for eliminating this emerging contaminant from WWTPs before release into the environment.

1. INTRODUCTION

Ibuprofen (IBP), 2-(4-isobutylphenyl)propionic acid, is one of the most widely consumed non-steroidal anti-inflammatory drugs (NSAIDs) in the world. Incomplete elimination in wastewater treatment plants (WWTPs), as well as direct sewage discharges has led, worldwide, to the detection of IBP and several of its metabolites in effluents, sediments and water bodies at concentrations between ng L^{-1} to μg L^{-1} (Archer et al., 2017; Couto et al., 2019; Evgenidou et al., 2015; Fekadu et al., 2019). IBP is partially metabolized (70%) by humans and animals to hydroxylated, carboxylated metabolites and conjugates of these metabolites (De Oliveira et al., 2005; Evgenidou et al., 2015; Waraksa et al., 2018). Most of these metabolites have been identified in WWTP effluents (Evgenidou et al., 2015) and as a consequence, are released into the environment. IBP and its metabolites appear not to be toxic at the concentrations generally detected in surface waters (Parolini, 2020; Verlicchi et al., 2012; Żur et al., 2018), however, the continuous introduction into aquatic ecosystems and long-term exposure even at low concentrations are causes for concern. Both ibuprofen and its metabolites have adverse effects on human cell lines (Liu et al., 2020; Ruggeri et al., 2013), fish (Han et al., 2010; Zhang et al., 2010); water invertebrates (De Lange et al., 2009; Parolini, 2020; Quinn et al., 2008); algae (Cleuvers, 2004; Ding et al., 2017), bacteria (Elvers and Wright, 1995; Quintelas et al., 2020; Veach et al., 2012), and fungi (Kasonga et al., 2019; Marco-Urrea et al., 2009). Therefore, strategies to remove ibuprofen and its metabolites from effluents are required.

These contaminants may be removed by biofilm reactors (Zwiener et al., 2002), wetlands (Matamoros et al., 2008), or activated sludge (Ferrando-Climent et al., 2012; Quintana et al., 2005). Recently, biopurification systems based on biomixtures composed of agro-industrial waste material have been proposed as a promising sustainable bioremediation technique to eliminate NSAIDs present in the effluents of WWTPs (Delgado-Moreno et al., 2019).

Various advanced remediation techniques have been studied to remove IBP residues from water such as chemical oxidation, reverse osmosis, adsorption on activated carbon (Delgado-Moreno et al., 2021). Abiotic technologies, such as

phototransformation eliminates ibuprofen and other contaminants from water but, produces hydroxylated metabolites as well as other products, some of which are more toxic than ibuprofen itself (Ellepola et al., 2020; Miranda et al., 1991; Rubasinghege et al., 2018; Ruggeri et al., 2013; Vione et al., 2011). However, microbial degradation is one of the most effective and environmentally friendly approaches. Bioremediation with microorganisms, such as fungi remove ibuprofen but release the same hydroxylated metabolites as those detected in sewage influents and water bodies (Kasonga et al., 2019; Marco-Urrea et al., 2009). Nevertheless, bacteria have been shown to biotransform or even mineralize IBP by different routes. So far, it seems that the complete mineralization of IBP using this compound as the sole carbon and energy source to form biomass and CO_2 at high yield, is limited to the family of the *Sphingomonadaceae* (Balciunas et al., 2020; Murdoch and Hay, 2005). From the strain *Sphingomomas* sp. ibu-2, the *ipf* genes were described which encode enzymes for a CoA ligase, a dioxygenase and a putative thiolase which first ligate CoA to ibuprofen, followed by ring activation and propionyl-CoA liberation to produce isobutylcatechol as an intermediate, which is susceptible to meta ring cleavage and almost complete degradation (Murdoch and Hay, 2013). Although genes showing similarity to the *ipf* genes seem to be distributed within a few other taxonomic groups of bacteria (Żur et al., 2018), the implication of those taxa for IBP degradation remains still unknown. *Variovorax* sp. Ibu-1 was reported to use ibuprofen as sole carbon and energy source via the formation of trihydroxyibuprofen but the implicated genes were not determined (Murdoch and Hay, 2015). Other bacterial species degrade ibuprofen co-metabolically, using secondary compounds or mixtures for their growth and generation of energy. One of these species such as *Nocardia* reduces the carboxylic group of the chemical structure of IBP to an alcoholic derivative as an end product (Chen and Rosazza, 1994); while most of the other strains oxidize the aliphatic groups of ibuprofen to the corresponding hydroxyl and even carboxyl derivatives and/or perform further biotransformations, as has been shown for the strains *Patulibacter medicamentivorans* I11 (Almeida et al., 2013; Salgado et al., 2020), *Bacillus thuringiensis* strain B1 (Marchlewicz et al., 2017, 2016); *Micrococcus yunnanensis* (Sharma et al., 2020, 2019); *Serratia marcescens* strain BL1 (Xu et al., 2018), and *Pseudoxanthomonas* sp. DIN-3 (Z. Lu et al., 2019). In most of the

latter cases, authors suggested possible catabolites mainly based on mass spectral data but in several cases these were proposed arbitrarily as authentic reference compounds were not available, or not generated at amounts sufficient for NMR-based elucidation to unequivocally confirm the proposed structures.

Studies involving bioaugmentation with bacteria to remove ibuprofen from water are limited. Xu et al. (2018) observed an increase (32-44%) in the removal of ibuprofen in aerated biological filters bioaugmented with *Serratia marcescens* BL1. Recently, Balciunas et al. (2020) reported that a constructed wetland planted with *Juncus effuses* when bioaugmented with a *Sphingobium yanoikuyae* strain no longer showed the 80-day lag phase for ibuprofen removal observed under control conditions.

The aim of this work was to find a bacterium that ensures the complete mineralization of IBP under diverse conditions. The genome of the isolate is investigated to determine which genes are implicated in the degradation process. We evaluated the efficacy of the strain in liquid medium, in a biopurification system used to detoxify polluted wastewaters, as well as in the effluent of a WWTP. The byproducts released during the IBP degradation were identified and the pathway was investigated using the incorporation of labelled oxygen in the IBP molecule and different analytical techniques.

2. MATERIALS AND METHODS

2.1 Chemicals

Ibuprofen sodium salt (≥98% purity) and ibuprofen metabolites, 1-hydroxy-, 2-hydroxy-, and 3-hydroxyibuprofen were purchased from Sigma-Aldrich (Steinheim, Germany). The other chemicals used were: ibufenac, diclofenac, ketoprofen, naproxen, benzoic acid, 2-phenylpropanoic acid, 3-phenylpropanoic acid, phenylacetic acid, 2-methyl-, 3-methyl-, and 4-methylphenylacetic acid, 2-(4-methylphenyl)propionic acid, isobutylbenzene, catechol, 3-methylcatechol, 4-methylcatechol, 4-ethylcatechol, as well as 18O2, were obtained from Sigma-Aldrich (Steinheim, Germany). 4-isobutylbenzoic acid, 4-isobutylbenzaldehyde, and 4-tert-butylcatechol were obtained from TCI (Tokyo Chemical Industry,

Tokyo, Japan). 3-fluorocatechol was from Lancaster Synthesis. All other chemicals were of the highest purity commercially available. 4-Isopropylcatechol and 4-tert-octylcatechol were synthesized in the laboratory by A. Haïdour, starting from the corresponding phenols. Standard solutions of ibuprofen and other chemicals were prepared in ethanol or water, while ibuprofen metabolites were dissolved in ethyl acetate. HPLC-grade solvents were supplied by Scharlau (Barcelona, Spain).

2.2 Enrichment cultures and isolation of ibuprofen degrading bacteria

A strain was isolated from an enrichment culture inoculated with upper oxic sediment samples from the River Elbe taken downstream of the Hamburg harbor (Germany) as described previously (Wittich et al., 1999). The enrichment culture consisted of a modified mineral salts medium (MSM) (Sander et al., 1991) at neutral pH and supplemented with 1 mM (206 µg g-1) ibuprofen as sole carbon and energy source. Cultures were incubated at 30 °C on a rotary shaker at 100 rpm, Subcultures were made monthly to fresh medium with ibuprofen as sole carbon source. After 4 months and 4 transfers, a pure culture called strain RW412 was obtained by isolating colonies on solid minimal media with Difco-Noble agar (BD Biosciences) and 1 mM (206 µg g-1) ibuprofen as sole carbon and energy source.

2.3 Growth and biodegradation of ibuprofen by RW412 in liquid medium

Strain RW412 was cultivated in 250 mL Erlenmeyer flasks containing 50 mL of MSM with 2 mM (412 µg g^{-1}) of ibuprofen as sole carbon and energy source at 30ºC on a shaker at 200 rpm. The bacterial growth was determined by optical density (A_{600nm}) on a Shimadzu UV-2401PC spectrophotometer and by colony forming units (CFU) of serial dilutions on LB medium (Sambrook et al., 1989) plates. The consumption of ibuprofen in the medium was monitored by HPLC as described in the General Material and Methods section.

2.4 Study of potential substrates for RW412 growth and metabolism

To determine the substrates used by the bacteria RW412 for growth and metabolism, a spectrum of different compounds with structural similarity with ibuprofen or its possible degradation products were tested. Since substrates included potentially toxic mono- and dihydroxylated compounds, agar diffusion plates were used. For this assay, RW412 was homogenously plated onto solid MSM agar medium and crystals of the target substrate applied at the rim of the plate so that the compounds could diffuse from this point into the agar medium, thereby forming a gradient. The formation of growing colonies in this concentration gradient at a distance from the loaded substrate was scored.

In order to determine whether RW412 metabolized substrates, oxygen uptake rates were measured polarographically of washed cell suspensions with a DW-1 oxygen electrode system (Hansatech Instruments, King's Lynn, Norfolk, UK) as described previously (Hernández-Sánchez et al., 2013).

2.5 Genome sequencing and assembly

Total genomic DNA was obtained from 2 mL of pure culture of the RW412 strain grown overnight in LB medium at 30 °C on a shaker at 200 rpm using the Wizard Genomic DNA kit (Promega). The quality and quantity of the DNA was assessed with the Nanodrop ND-1000 Spectrophotometer (Thermo Fisher Scientific) and Qubit (Thermo Fisher Scientific). To obtain the genome sequence, multiple 300-bp paired-end sequencing libraries were prepared using Illumina Nextera XT DNA version 3 sample preparation kit and sequencing was performed with the Illumina MiSeq platform at the Center for Scientific Instrumentation of the University of Granada (Spain). For Nanopore sequencing, high molecular weight DNA was size selected using the Pippin Prep (Sage Science, USA). The DNA library was prepared with Ligation Sequencing Kit (Oxford Nanopore Technologies, Oxford, UK) and sequenced on a MinION device (Oxford Nanopore Technologies, Oxford, UK) using flow cell type FLO-MIN106. Real-time basecalling was performed using Guppy version 3.2.10 and the high accuracy basecall model integrated in the MinKNOW software v3.6.5. 260000 of the 1632000 FastQ reads obtained from FAST5 output were concatenated and uploaded together with the triplet sets of sequence reads obtained with Illumina

to the public server at usegalaxy.org.eu (Afgan et al., 2018). Nanopore FastQ files were filtered to obtain reads larger than 10 kb with a minimum quality of Phred 2. The 93256 resulting reads were included together with the raw Illumina for assembly with SPAdes version: 3.11.1 (Bankevich et al., 2012). The assembly resulted in 4 circular contigs which were annotated with the Rapid Annotations using Subsystems Technology (RAST) server version 2.0 (Aziz et al., 2008). For submission to GenBank, gene annotation was performed using the NCBI Prokaryotic Genome Annotation Pipeline (PGAP) (Tatusova et al., 2016). The genome and plasmid sequences are available in BioProject PRJNA662464. The average nucleotide identity (ANI) was calculated using tools in JSpecies Web Server http://jspecies.ribohost.com/jspeciesws, (Richter et al., 2016).

2.6 Study of *ipf* homologue activity: Protein purification and activity of IpfF

To purify the *ipfF* gene homologue of RW412, the sequence was amplified by PCR from genomic DNA with the primers IpfFfor2 5'-TTTCATATGTTGGCAAGAGACCTGG-3' and IpfFrev3 5'-ATATGGATCCTGCTTGACGGGGCACTAC-3' and introduced into protein expression vector pET28b(+) (Novagen). For protein-His6 purification, the resulting plasmid was transformed into E. coli BL21 pLysS (Novagen), cultures were grown and induced with 1 mM IPTG and after harvesting, disrupted by French press (1000 psi) in a buffer containing 50 mM Tris-HCl pH 7.5, 500 mM NaCl, 1 mM DTT, 10 mM imidazole, 10% (v/v) glycerol and protease inhibitor cocktail (Complete®, Roche). The His6-tagged protein was purified by nickel affinity chromatography by FPLC (ÄKTA, GE Healthcare) with a continuous imidazole gradient to almost homogeneity. Fractions eluting purified protein were pooled and dialyzed in buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM DTT, 10% (v/v) glycerol) and the final protein concentration determined by the Bradford method. To detect IpfF activity, assays with ibuprofen were performed consisting of 2 mg purified IpfF in 50 mM Tris-HCl pH7.5, with 0.5 mM (103 μg g^{-1}) ibuprofen, 0.5 mM coenzyme A, 1 mM ATP, 2 mM $MgCl_2$, and 0.2 mM DTT. Products were analyzed by HPLC (high performance liquid chromatography) and HPLC-MS under the conditions described in supplementary Table S1.

2.7 Bioaugmentation study in BPS microcosms

To determine if the IBP degrader strain was capable of improving the disappearance rate of ibuprofen in a more complex media, this bacterium was inoculated in a biopurification system (BPS) constructed at microcosm scale. The BPS contained a biomixture composed of agricultural silty clay loam soil, vermicompost of wet olive cake and olive tree prunings (1:1:2, v:v:v). The physicochemical properties of this biomixture were described in the General Materials and Methods section. For a homogenous distribution of the IBP in the system, 1.5 g of silica sand placed in a 200 mL glass container was spiked with a solution of ibuprofen in ethanol. After ethanol evaporation, 70 g of the biomixture was added and mixed in an end-over-end rotary shaker for 15 min at room temperature. The BPS consisted of the contaminated biomixture in a container and initially contained 80 μg g^{-1} (0.39 mM) of ibuprofen. Then, the BPS was inoculated with 2 x 108 CFU g^{-1} of RW412. To prepare the inoculum, the strain was grown under the conditions described above (Chapter 3.2, Section 2.3). Then, 30 mL of the culture containing 2 x 10^9 CFU mL^{-1} were centrifuged during 10 min at 6000 rpm. The pellet was resuspended in 41 mL of fresh minimal medium and added to the microcosms to obtain the biomixture moistened to 75% of their field capacity. During the incubation time, the inoculated system was repeatedly contaminated with 80 μg g^{-1} (0.39 mM) of ibuprofen after 7 days and 120 μg g^{-1} (0.58 mM) after two weeks post inoculation. For the other two applications of ibuprofen, contaminated sand was added to the microcosms and homogenized in an end-over-end rotary shaker for 20 min at room temperature. Non-inoculated BPS microcosms were run in parallel as a control. All BPS microcosms were incubated in triplicate in a thermostatic chamber at 20 ºC and the moisture content was maintained by adding sterile distilled water before each sampling period. Ibuprofen dissipation was determined at different times during the incubation period by analyzing 3 g (dry-weight) samples of biomixture from each BPS replicate as described in section 2.10. The ibuprofen content was also determined before and after each contamination process. In order to determine ibuprofen metabolites, 6g (dry-weight) samples of biomixture were analyzed by GC/MS as described in the General Materials and Methods section.

2.8 Bioaugmentation study in WWTP secondary effluent

In order to determine the potential application of RW412 on ibuprofen elimination from secondary effluents, a bioaugmentation assay was performed. For this purpose, samples from the secondary effluent of a municipal WWTP located in Granada (Spain) were transferred to the laboratory and stored at 4 ˚C. The effluent had a pH of 7.05, a conductivity of 644 μS cm^{-1}, total suspended solids of 10 mg L^{-1}, a chemical oxygen demand of 66 mg L^{-1} and a biochemical oxygen demand of 8.06 mg L^{-1}. The bioaugmentation assays were conducted in 1L flasks with 250 mL of WWTP effluent previously contaminated with 0.5 mM (103 μg g^{-1}) of ibuprofen. Then, each flask was inoculated with 2 x 10^8 CFU mL^{-1} of the strain RW412. Controls consisting of non-inoculated effluent, or effluent sterilized by autoclaving at 121 ºC for 20 min which were either inoculated with the same amount of RW412 or not, were run in parallel. All treatments were performed in triplicate and incubated at 30 ºC in a shaker at 200 rpm for 7 days. To monitor the ibuprofen concentration, aliquots of 1 mL were analyzed by HPLC as described in the General Materials and Methods section. In order to determine ibuprofen metabolites, 3 mL aliquots were analyzed by GC/MS as described in the General Materials and Methods section.

2.9 Quantitative PCR (qPCR) to trace the survival of RW412

To trace the strain RW412 in bioaugmentation assays, quantitative PCR analyses were performed on a Bio-Rad MyiQ2 Real-Time PCR system using specific primers targeting the ipfF gene (ipfFfor 5´-GTTATGTTGGCAAGAGACCTGG-3´ and ipfFrev4 5´-CTTTCTTGCGTCAGGATGGC-3´) which encodes the aromatic compound-targeted CoA ligase implicated in the biodegradation pathway of ibuprofen. Primers were designed following the requirements for the SYBR-Green qPCR assay and the specificity of each was tested in silico using BlastN. Primers 341F and 534R for the 16S rRNA gene as described by (Philippot et al., 2011) were used to estimate total bacteria. Total genomic DNA was obtained from 0.5 g of biomixture using the NucleoSpin Soil Kit (Macherey-Nagel, Duren, Germany). In the bioaugmentation assay of the secondary effluents, biomass was collected from 200 mL of secondary effluent samples by centrifugation at 6000 rpm for 10 min

from which total DNA was obtained with the aid of the Wizard Genomic DNA kit (Promega). DNA samples were quantified by using the Qubit dsDNA BR Assay kit (Live Technologies, Invitrogen, USA). Quantitative PCR assays were conducted in 12.5 µL of reaction volume using 6.25 µL of iQ™ SYBR® Green Supermix (Bio-Rad Laboratories, Hercules, CA, USA), 400 nM of each primer and 1 ng of DNA. The cycling conditions applied were 95 °C for 5 min, followed by 40 cycles of denaturation at 95 °C for 15 s, annealing at 60 ºC for 30 s and extension at 72 ºC for 20 s with a final extension step at 72 °C of 1 min. Non-template controls were also assessed for the two target genes, and all reactions were run in triplicate. Standard curves were obtained using serial dilutions of linearized pCR2.1-TOPO® containing cloned *ipfF* and 16S rRNA genes. The PCR efficiency values were always higher than 93%.

2.10 Analytical techniques

Analysis of ibuprofen consumption in culture media and effluents was determined by mixing samples with acetonitrile (1:1, v:v), centrifuging at 13000 rpm for 1 min, and filtering the supernatant through a 0.45 µm PTFE syringe filter. Samples were then analyzed by HPLC under the conditions shown in the General Materials and Methods section. To analyze samples from BPS microcosms, 3 g (dry-weight) samples of biomixture were extracted with 6 mL of acetonitrile acidified with 1% acetic acid and vortexed for 1 min. Then, 1 g of a mixture of salts (QuEChERS, Agilent Technologies, Santa Clara, CA, USA) was added and vortexed again for 1 min. Samples were centrifuged for 5 min at 3500 rpm and aliquots of the supernatant were diluted with ultra-pure water (1:1), filtered through a 0.45 µm PTFE syringe filter and analyzed by HPLC according to the conditions indicated in the General Materials and Methods section.

The microbial incorporation of $_{18}O^2$ into bacterial cells was performed in liquid medium as described by Wilkes et al. (1992) by incubating cells of RW412 growing in the presence of $_{18}O^2$ with ibuprofen. After an appropriate time, the reaction was stopped by adding 3-fluorocatechol, a known inhibitor for the cleavage by catechol dioxygenases (Bartels et al., 1984). Cells were removed by centrifugation and the supernatant filtered through a 0.45 µm PTFE syringe filter

to be analyzed by HPLC-MS according to the conditions indicated in supplementary Table S1.

For separation and isolation of enough amount of the accumulating intermediate for further identification of its structure by NMR, RW412 was grown in MSM with 1 mM ibuprofen till late exponential phase. 3-fluorocatechol was added and after an appropriate time cells were removed by centrifugation and the supernatant filtered through a 0.45 µm PTFE syringe filter prior to be separated by repeated semipreparative HPLC runs under the conditions described in supplementary Table S1. Fractions containing the intermediate were pooled, and extracted with ethyl acetate. The organic phase was removed by evaporation, and the partially purified product was subjected to Nuclear magnetic resonance (NMR) spectroscopy. Spectra were acquired on a Varian 500 MHz spectrometer at room temperature in deuterated chloroform.

2. 11 Statistical analyses

One-way analysis of variance (ANOVA) at a significance level of 0.05 followed by post-hoc tests were calculated with the aid of the IBM Statistical Package for Social Sciences (SPSS statistics). Standard deviation was calculated using the Excel program from Microsoft Office 2019 (Microsoft).

3. RESULTS AND DISCUSSION

3.1 Isolation and growth of RW412 with ibuprofen and structurally similar substrates

Strain RW412 was isolated from an enrichment culture and is capable of growth in liquid minimal medium with ibuprofen as sole carbon and energy source. A growth curve with ibuprofen shows its capacity to grow at the expense of this compound (Figure 1) with a doubling time of 16 h during the exponential growth phase and depletion to below the detection limit (<1 µM) after 74 h.

In order to determine whether RW412 grows with and metabolizes compounds which are structurally similar to ibuprofen or its putative transformation products, agar diffusion plates were used and relative oxidation

rates measured. Of the four other NSAIDs tested only ibufenac, which lacks the methyl group at position 2, showed growth and a relative oxidation rate of 46% (Table 1). Although all of other NSAIDs bear an isopropylic or acetic acid side chain, the structure of the aromatic systems differs significantly, probably impeding enzymatic attack and thus further catabolism.

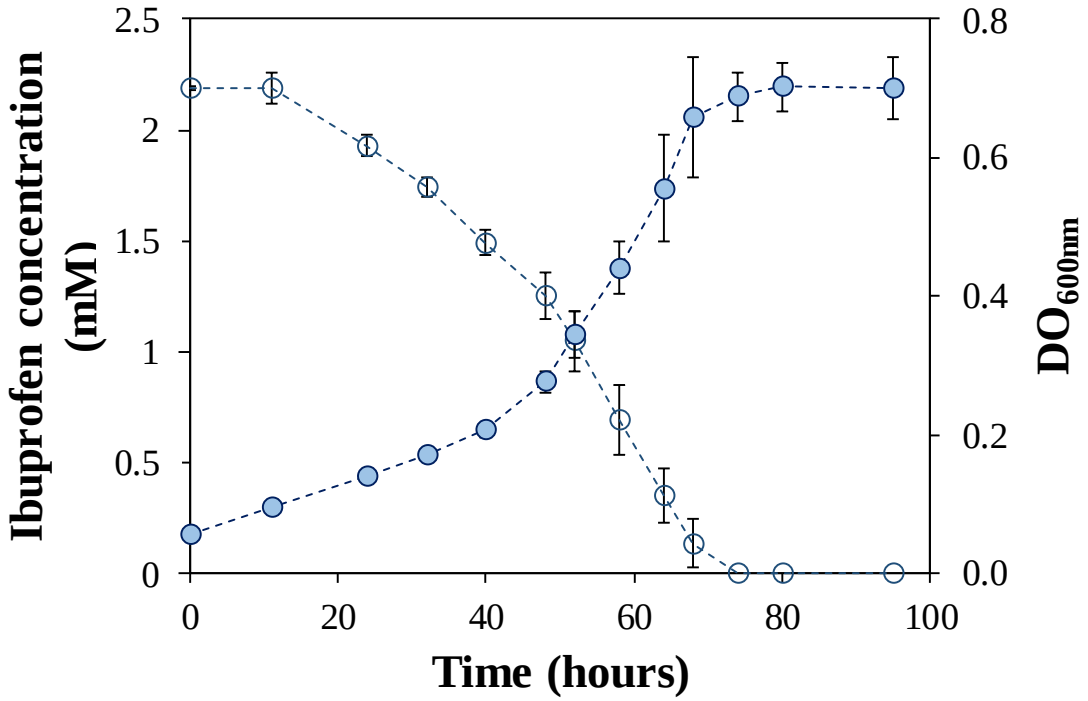


Figure 1. Growth of RW412 as indicated by the depletion of ibuprofen (open circles) over time and the increase in biomass (solid circles), as determined by measuring Optical Density (A_{600}) of the culture. Error bars show standard deviation of three replicates.

According to the revised bibliography, only *Pseudoxanthomonas* sp. DIN-3 has been described to grow with the additional NSAIDs, diclofenac and naproxen, as sole C-sources but without complete elimination (Z. Lu et al., 2019). With regard to structurally similar substrates to ibuprofen, RW412 showed growth and relative oxidation rates above 36% with phenylacetic acid, 3-phenylpropanoic acid, 4-methylphenylacetic acid, and 2-(4-methylphenyl)propionic acid (Table 1). Within the derived catecholic substrates tested, RW412 showed good growth

and relative oxidation rates with catechol and 4-methylcatechol, but lesser growth with 4-ethylcatechol (Table 1).

Table 1. Growth on solid media by the diffusion technique and percent relative oxidation rates of resting cells of RW412 of NSAIDs and structural analogs compared to ibuprofen and catecholic substrates compared to catechol.

Tested compound [a]	Growth on solid medium [b]	Relative oxidation rate [c]
Ibuprofen	++	100
Ibufenac	++	46
Diclofenac	-	< 1
Ketoprofen	-	< 1
Naproxen	-	< 1
Benzoic acid	-	ND
Phenylacetic acid	++	36
2-Phenylpropanoic acid	-	17
3-Phenylpropanoic acid	++	47
2-Methylphenylacetic acid	-	< 1
3-Methylphenylacetic acid	-	6
4-Methylphenylacetic acid	++	46
2-(4-Methylphenyl)propionic acid	++	57
Isobutylbenzene	-	< 1
4-Isobutylbenzoic acid	ND	< 1
4-Isobutylbenzaldehyde	+/-	8
Catechol	++	100
3-Isopropylcatechol	-	ND
4-Isopropylcatechol	-	61
3-Methylcatechol	-	ND
4-Methylcatechol	++	98
4-Ethylcatechol	+/-	97
4-*tert*-Butylcatechol	-	< 1
4-*tert*-Octylcatechol	-	< 1

a The inability for growth may also be due to toxicity, the lack of appropriately specific catabolic pathways or to the lack or very high selectivity of the required transport systems of RW412 to internalize those compounds.

b ++: growth; +/-: little growth; -: no growth; ND: not determined.

c Data represent means of three independently performed experiments, SD up to 16 %.

Poor or no growth was observed with the other substrates tested including isobutylbenzene. Therefore, it appears that the methyl group in position 4 with respect to that of the acetic or (iso)propionic acid residue on the phenyl ring or with respect to catecholic hydroxyl groups, is determinant.

Compared to other bacteria which can use ibuprofen as sole carbon source, only *Sphingomonas* sp. Ibu-2 has been studied for growth and metabolism of some of the above compounds (Murdoch and Hay, 2005). Compared to this strain, RW412 shows some resemblance in substrate preferences except that *Sphingomonas* sp. Ibu-2 was capable of growing with 2-phenylpropionic acid.

3.2 Genetic characterization

In order to identify and determine the genetic repertoire of RW412, its genome was sequenced. The genome assembly from Illumina reads and long-length nanopore reads revealed the genome of RW412 to have a total length of 4,487,867 bp, encodes for 4000 coding sequences, has a GC content of 66.12% and is organized into a chromosome sequence of 4.1 Mb and three plasmid sequences of 193 kb, 138 kb and 45 kb denominated RW412a, RW412b, and RW412c, respectively. Long length nanopore reads confirm the circulization of the chromosome and plasmid sequences.

3.2.1 Taxonomic identification of RW412

In order to identify RW412, the 16S rRNA sequence was analyzed which shares up to 100% similarity with *Sphingopyxis granuli* strains. Comparisons with other housekeeping genes, *gyrB* and *rpoD,* also gave best hits with the species *Sphingopyxis granuli,* specifically with strain TFA (98.2% and 98.4% similarity, respectively) (García-Romero et al., 2016). For further identification, the average nucleotide identity based on BLAST+ (ANIb) (Richter et al., 2016) was determined between RW412 and the genomes of the seven most similar strains according to the Tetra Correlation Search. These analyses revealed that RW412 shares 96.46% ANIb similarity with the *Sphingopyxis granuli* type strain NBRC 100800, and 95.9% ANIb similarity with *Sphingopyxis granuli* TFA (Supplementary Figure S1). Both are above the 95% threshold proposed for

species level similarity (Richter and Rosselló-Móra, 2009), suggesting strongly that RW412 belongs to the *Sphingopyxis granuli* species.

3.2.2 Metabolic repertoire of RW412

The genome sequence was analyzed for genes and possible operons which could be related to its capacity to grow with ibuprofen or related substrates. Using the RAST determined annotation a total of 67 putative gene sequences were identified (Supplementary Table S2) to be related aromatic compound metabolism. A similarity search with the *ipf* genes involved in ibuprofen degradation found in contig 1 of *Sphingomonas* sp. Ibu-2 (Genbank accession EF090268.1; Murdoch and Hay, 2013) revealed almost complete (99.98% at the nucleotide level) similarity along 11.6 kb. This DNA fragment is located in the RW412a plasmid and shares the same genetic organization of *ipfABDEF* and the associated genes (1, 2, 3, 9 and *ipfI*) described in *Sphingomonas* sp. Ibu-2 (Supplementary Figure S2). A search for other genes reported by Murdoch and Hay (2013) revealed another DNA sequence located approximately 40 kb upstream from the *ipf* gene cluster which shares 98.03% similarity with the 6.2 kb contig 2 of *Sphingomonas* sp. Ibu-2 (Risen, 2012) which includes *ipfH* and ORFs 5-9 (Supplementary Figure S3).

These similarities suggest that RW412 uses the same catabolic approach as *Sphingomonas* sp. Ibu-2 to degrade ibuprofen. In the Ipf pathway, the CoA ligase encoded by *ipfF* was proposed to ligate coenzyme A to ibuprofen which is then attacked by a dioxygenase encoded by *ipfAB*, together with the supportive ferredoxin reductase and ferredoxin subunits of the electron transport chain encoded by *ipfHI*. The putative *cis*-1,2-dihydro-2-hydroxyibuprofenyl-CoA product was then suggested to be attacked by the putative thiolase encoded by *ipfD* and the DUF35-motif containing IpfE to finally result in isobutylcatechol (Murdoch and Hay, 2013). The *ipf* genes shared by RW412 and *Sphingomonas* sp. Ibu-2 were not found in the genomes of the two closest relatives of RW412, *S. granuli* TFA or *S. granuli* NBRC 100800, or other *Sphingopyxis* strains and, thus, are unlikely to degrade ibuprofen.

The genome sequence was also analyzed for genes which may be involved in the metabolism of the other substrates assayed for growth and metabolism by RW412. The fact that RW412 does not grow with benzoic and 4-isobutylbenzoic acid is very probably due to the lack of a functioning benzoate 1,2-dioxygenase/dihydrodiol dehydrogenase gene system as such genes were not found in its genome, and also due to the fact that the acetyl- or (iso)propionylic acids require esterification with CoA for initializing the breakdown of this class of compounds. On the other hand, RW412 shows the capacity to grow with phenylacetic acid, 3-phenylpropanoic acid, 4-methylphenylacetic acid, and 2-(4-methylphenyl)propionic acid. Phenylacetic acid is readily metabolized by a large number of bacteria mostly through the phenylacetic acid pathway encoded by the *paa* genes (Fuchs et al., 2011). This pathway shows some limited similarity to the Ipf pathway as it also initiates degradation of the substrate with a CoA ligation step. However, in the genome of RW412 no genes with significant similarity (<46%) or genetic organization with *paa* genes were found.

The appearance of colored products was not detected in RW412 cultures grown with ibuprofen. Therefore, neither the Ipf pathway intermediate, isobutylcatechol, nor ring fission products appear to accumulate in the media suggesting that RW412 efficiently metabolizes these intermediates. In the case of catechols, the capacity of RW412 to grow with this metabolite and with 4-methylcatechol (Table 1) suggest that these compounds together with isobutylcatechol, may be subject to aromatic ring cleavage by the activity of the homologs of genes 1 and 4 or by more than 4 other candidate extradiol dioxygenases detected by annotation in RW412. No intradiol dioxygenases were found in the RW412 genome. On the other hand, genes involved in the efficient metabolism of the-ring fission product may be those identified on contig2 of *Sphingomonas* sp. Ibu-2 as genes 5-8 (Murdoch and Hay, 2013), which in RW412 is flanked by three other gene sequences related to the subsystem of biphenyl degradation (Supplementary Figure S3). Genes with more than 49% similarity and belonging to the same subsystem were also detected in the genomes of *S. granuli* TFA and *S. granuli* NBRC 100800 but their implication for isobutylcatechol or catechol degradation remains unknown.

3.2.3 Genetic stability of ipf genes in RW412

The *ipf* gene cluster as well as associated genes are located on plasmid RW412a and, moreover, the *ipf* gene cluster is flanked by mobile elements. This suggests that the ability to degrade ibuprofen could have been attained by RW412 by horizontal transfer. However, the possible mobility of the *ipf* genes also suggests that they may be transferred and lost. To check the rate of such a loss, RW412 was grown in LB medium for 30 generations and the percentage of colonies determined which no longer grew on ibuprofen. In this manner it was found that 2% of the RW412 cells lost their *ipf* genes but not their plasmids, showing that both the plasmids and these genes are stably maintained in RW412 and, therefore, suggest that RW412 could be considered for bioaugmentation applications.

3.3 Elucidation of intermediates of the Ipf pathway for ibuprofen mineralization by RW412

The presence of the *ipf* genes and the metabolic repertoire of RW412 indicate that this strain could metabolize ibuprofen through the same ibuprofen transformation pathway proposed for *Sphingomonas* sp. Ibu-2 (Murdoch and Hay, 2013, 2005). In the proposed catabolic pathway, a postulated ibuprofenyl-CoA adduct is formulated to be the first intermediate followed by attack of a dioxygenase system to form a putative dihydrodiol which, in a subsequent reaction, should be cleaved by the reaction of a putative thiolase into propionyl-CoA and 4-isobutylcatechol. In this route, the formation of 4-isobutylcatechol was proposed based on GC-MS data but the other intermediates were not determined analytically (Murdoch and Hay, 2005). Also, the sequence of enzymatic activities was not unequivocally clear because the proposed ibuprofenyl-CoA could be attacked first by the dioxygenase system and then by the activity of the putative thiolase as proposed by these authors, or vice versa.

Oxygen uptake in crude extracts of RW412 ibuprofen-grown cells was only detectable when CoA and ATP had also been present; no oxygen uptake occurred with ibuprofen alone. This indicates that the initial CoA ligation is absolutely necessary before any oxidation can take place. In order to determine the structure of this initial product, the IpfF protein was purified and the products formed from

ibuprofen in the presence of CoA, ATP an $MgCl_2$ was analyzed by HPLC (Supplementary Figure S4). HPLC-MS analyses of the peak identified as ibuprofenyl-CoA confirmed its identity (Figure 2).

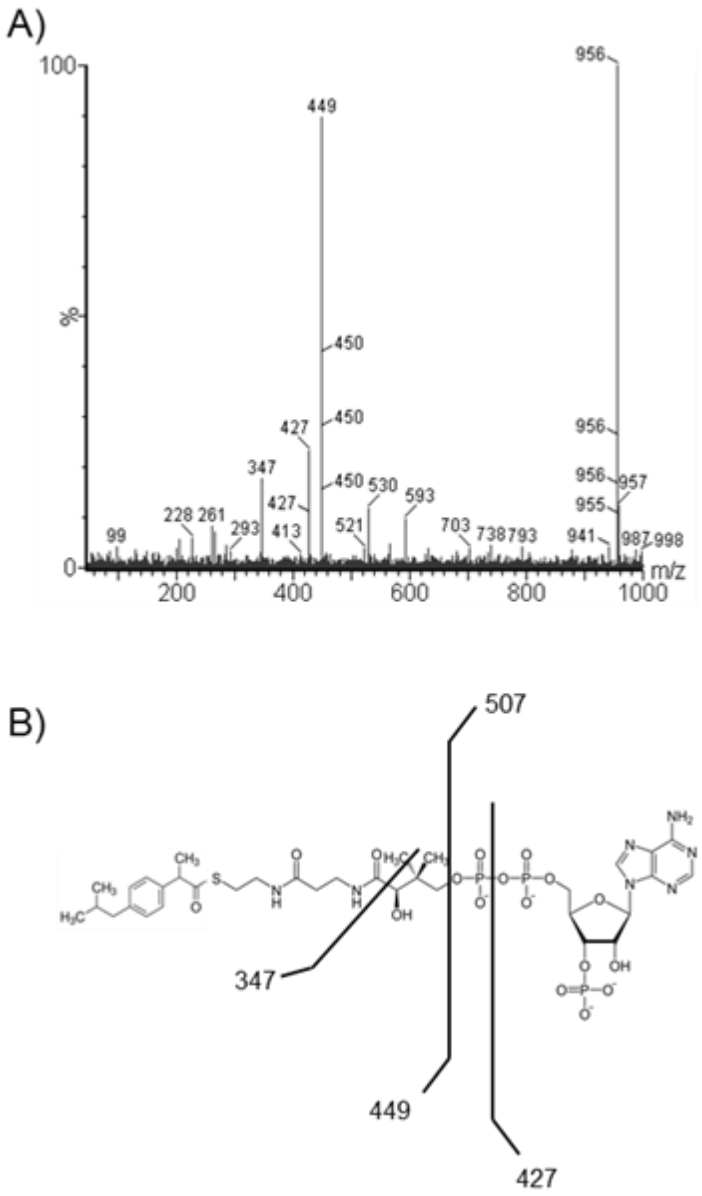


Figure 2. HPLC -MS spectrum (MRM) of the peak corresponding to ibuprofenyl-CoA from the HPLC-chromatogram in ES+ SIR mode at tR= 21.48 and 24.23 min (A). Expected fragment sizes of ibuprofenyl-CoA (parent m/z = 956 in positive mode) (B).

For RW412, isobutylbenzene neither served for growth, nor was it oxidized by resting cells of this strain, suggesting that it is unlikely that the putative thiolase acts before the dioxygenase on ibuprofenyl-CoA. 4-isobutylcatechol was proposed as a central metabolite for ibuprofen degradation (Murdoch and Hay, 2005). However, it was essential to prove experimentally the underlying biochemical reaction mechanism and the unequivocal elucidation of the correct structure of this formed product, in order to exclude two subsequent hydroxylating reactions. $^{18}O_2$ was used to provide experimental proof for the attack by a dioxygenase system onto ibuprofenyl-CoA. Upon incorporation of $^{18}O_2$, the product which accumulated upon inhibition of further dioxygenolytic cleavage, was subjected to analysis by HPLC-MS. Mass spectrometry confirmed the product to be isobutylcatechol and the incorporation of $^{18}O_2$; the correct mass of the product was m/z = 170 (Figure 3), four mass units higher than that expected of $^{16}O_2$ containing isobutylcatechol (m/z = 166).

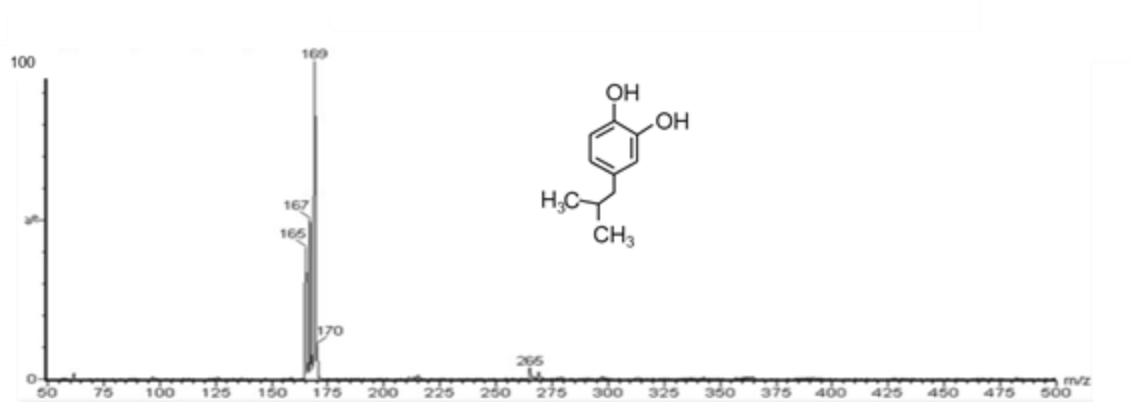


Figure 3. HPLC-MS spectrum of $^{18}O_2$-labelled isobutylcatechol scanned in negative mode.

Next, in order for unequivocal assignment of the position of the two oxygen atoms of the vicinal hydroxyl groups of isobutylcatechol relative to the alkyl substituent, the isobutylcatechol containing peak was partially purified by semi preparative HPLC, concentrated and subjected to NMR analysis which provided clear evidence of the alkyl substituent to be at position 4, relative to the 1,2-dihydroxy substituents (aromatic region in Figure 4, the entire spectrum including the olefinic protons in supplementary Figure S5). The now clarified structure discards the possibility that 3-isobutylcatechol could have been the

product by a possible lateral oxidative attack on ibuprofenyl-CoA. These results confirm how RW412 degrades ibuprofen through the earlier proposed Ipf pathway.

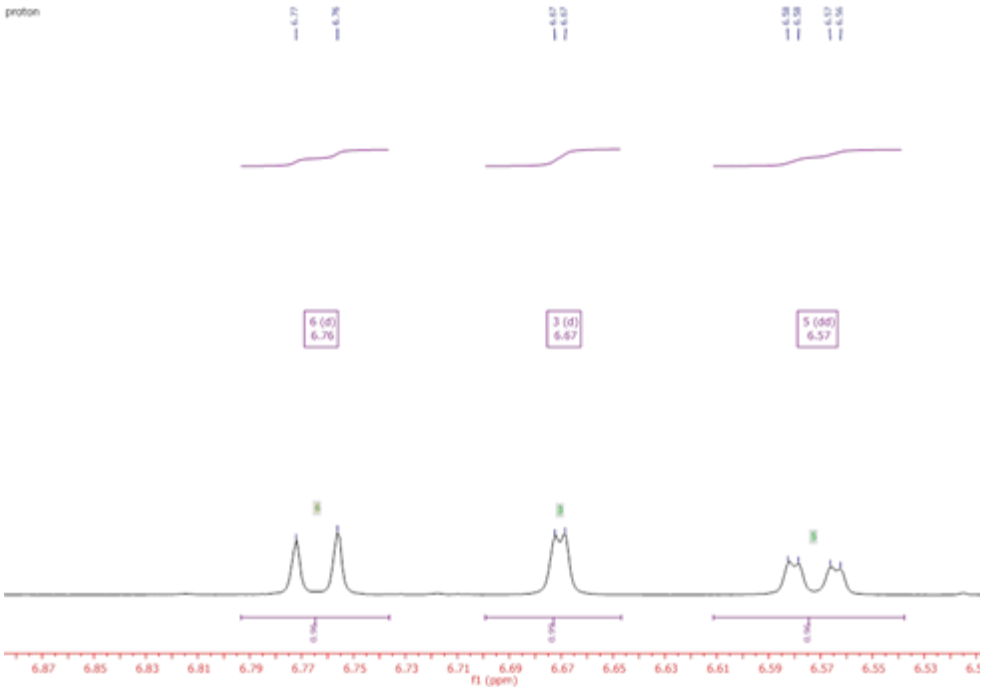


Figure 4. ^{1}H NMR spectrum of the region of the aromatic protons of 4-isobutylcatechol, recorded at 500 MHz in deuterochloroform.

3.4 Bioaugmentation with RW412 in repeteadly contaminated BPS microcosms

Recently, it was found that ibuprofen dissipates in biopurification system microcosms (mBPS) containing biomixtures composed of organic materials such as agro-industrial olive oil waste (Aguilar-Romero et al., 2020; Delgado-Moreno et al., 2019). However, the fate of ibuprofen in BPS is unknown when applied in repeated doses, as could occur upon treatment with contaminated water. Since RW412 efficiently degrades ibuprofen and stably maintains this capacity, this strain was evaluated for bioaugmentation of mBPS treated repeatedly with ibuprofen. The results in Figure 5 show that in 7-day sequences about 68% of ibuprofen dissipates initially, 50% of the amount after the second application and 41% of the amount after the third application in mBPS (Figure 5). However, in the mBPS bioaugmented with RW412, ibuprofen is no longer detected 3 days

after the first two applications and after the third and larger application, 80% is dissipated after 3 days and no longer detected after 7 days (Figure 5). These results indicate that bioaugmentation with RW412 increases the dissipation rate of ibuprofen in mBPS by up to 3 fold even after repeated applications.

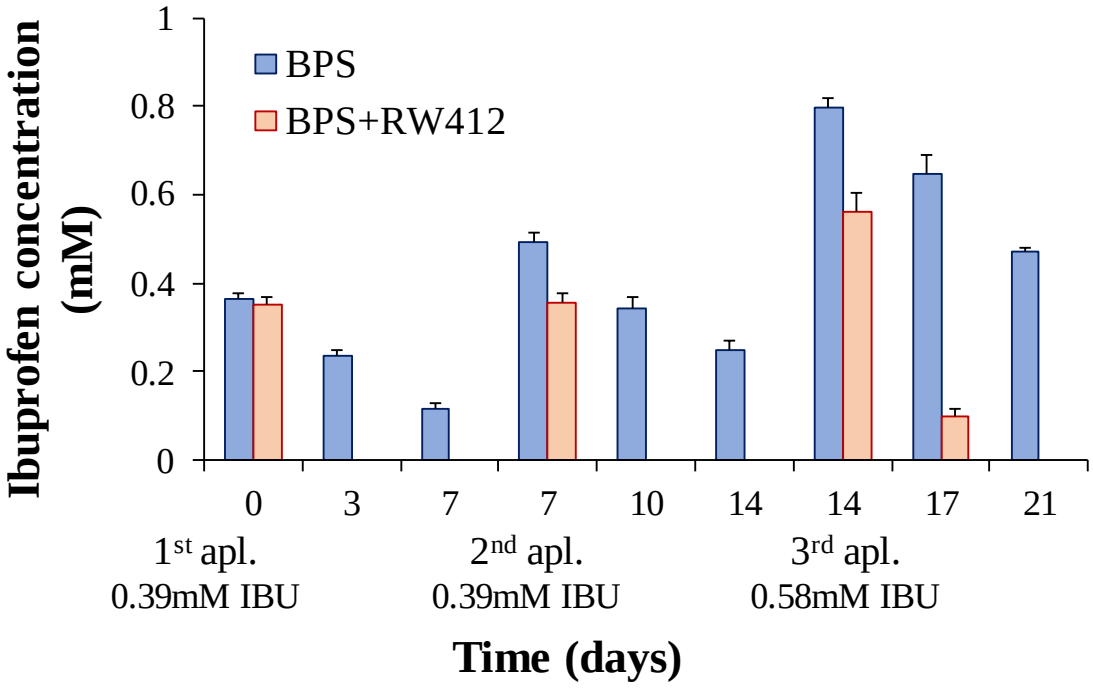


Figure 5. Ibuprofen detected at different time points in BPS biomixtures bioaugmented or not with RW412 after repeated application of ibuprofen. Error bars indicate standard deviation (n=3).

In addition to the dissipation of ibuprofen, also the evolution of hydroxylated or carboxylated metabolites was followed in the mBPS. The results show that primarily 2-hydroxyibuprofen and carboxyibuprofen derivatives accumulate in the BPS however these metabolites appear transiently and at much lower relative amounts in BPS bioaugmented with RW412 (Supplementary Figure S6). In order to determine cell numbers, qPCR was used to quantify 16S rRNA gene copy numbers as an indication for total bacterial numbers and of *ipfF* as an indication of the number of RW412 (Supplementary Table S3). The results indicate that the bacterial population does not change significantly during the course of the experiment. With respect to *ipfF*, there is a low background in the BPS microcosms which only doubles in the presence of ibuprofen. Previously, it was

found that as ibuprofen dissipates in mBPS certain biomarker OTUs belonging to *Sphingomonadales*, *Pseudomonadaceae* and *Verrucomicrobiaceae* proliferate (Aguilar-Romero et al., 2020). *Ipf* genes and similar genes have been identified in strains belonging to *Sphingomonadaceae*, *Xanthomonadaceae*, *Rhodospirilales* and *Comamonadaceae* (Żur et al., 2018) therefore *ipf* genes may become enriched in indigenous bacterial populations upon exposure to ibuprofen but in the BPS biomixtures these populations must be small. On the other hand, the population of RW412 is stably maintained during the incubation period with a decrease of only 10-fold after 21 days of incubation. On the whole the results indicate that RW412 is stably maintained in bioaugmented BPS and its presence enhances the dissipation rate of ibuprofen without the accumulation of hydroxylated or carboxylated metabolites.

3.5 Bioaugmentation study in WWTP secondary effluent

In order to determine whether bioaugmentation with RW412 could accelerate the disappearance of ibuprofen in secondary effluents before their discharge into the environment, a bioaugmentation assay with strain RW412 was performed.

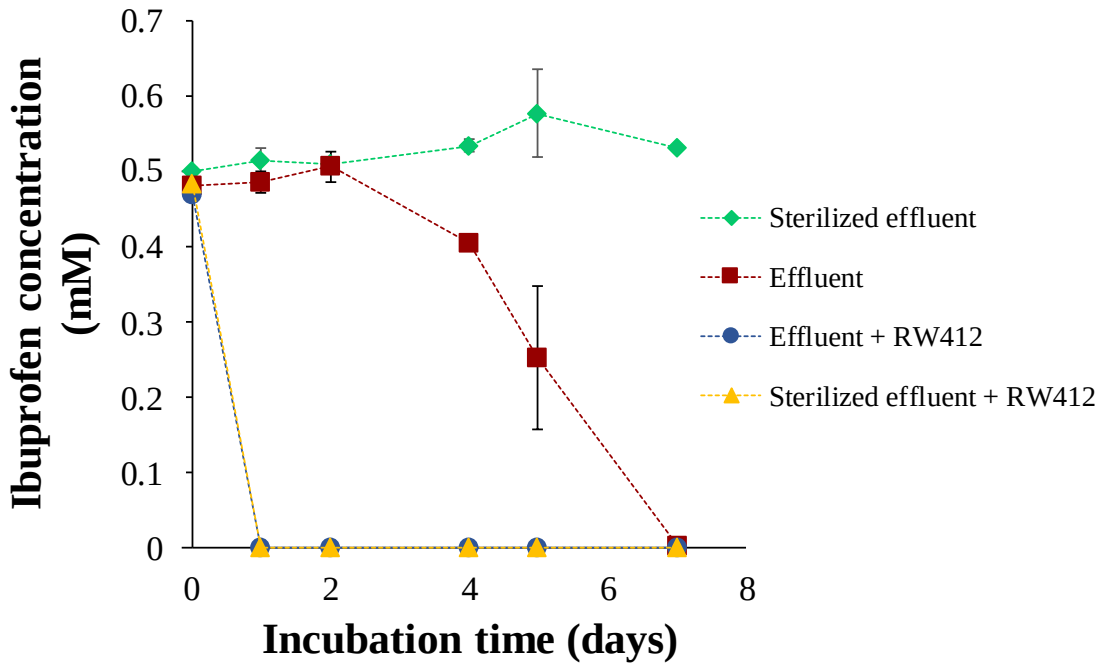


Figure 6. Ibuprofen detected at different time points in effluent from waste water treatment plant bioaugmented or not with RW412. Error bars indicate standard error (n=3).

RW412 was added to secondary effluent collected from a local wastewater treatment plant at the same CFU mL^{-1} as in the BPS assays. In the effluent, after a lag phase of 2 days, the ibuprofen concentration drops to below the detection limit after 7 days of incubation (Figure 6) while in a sterilized control the concentration of ibuprofen remained the same. These results indicate that the microbial community in the effluent is responsible for the ibuprofen dissipation detected and that it requires an adaptation period after which ibuprofen dissipation occurs within a period of 5 days. However, the addition of RW412 either in sterilized or non-sterile effluent permitted the dissipation of ibuprofen to below the detection limit within one day (Figure 6). These results show that bioaugmentation with RW412 increases dramatically the removal of ibuprofen in effluent to above those which could occur through the adaptation of the local microbial community. Unlike in the BPS microcosm assay, neither hydroxylated nor carboxylated derivatives were detected by GS/MS in the effluent or the bioaugmented effluent during the course of the experiment.

To determine the evolution of the number of bacteria in the assays, qPCR was used to quantify 16S rRNA gene copy numbers as an indication for total bacterial numbers and to quantify *ipfF* gene copy numbers as an indication of the number of RW412 (Supplementary Table S4). The results show that generally little change occurs in the gene copy numbers of 16S rRNA gene in effluents along the incubation period except for those inoculated with RW412 which although initially had a 10-fold lower total population, this population doubled during the incubation period (Supplementary Table S4). With respect to the *ipfF* gene copy numbers, a large number could be found in the uninoculated secondary effluent control and this population increased 10 fold after 7 days. Therefore, the effluents contain an autochthonous bacterial population which harbor the *ipf* genes and thus may have the capacity to degrade this compound. Nevertheless, the level of *ipfF* gene copy numbers was initially more than 100-fold higher in inoculated effluents as could be expected by the introduction of RW412. Curiously, the *ipfF* containing population decreased about 4-fold in the inoculated effluent after 7 days of incubation which may indicate that the viability of RW412 in effluents may be compromised especially when ibuprofen no longer is present. Nevertheless, RW412 efficiently eliminated ibuprofen and therefore performed well as an inoculant. Other bioaugmentation studies with ibuprofen degrading

strains also successfully improved ibuprofen removal such as biological aerated filters inoculated with *Serratia marcescens* BL1 (Xu et al., 2018) and constructed wetlands bioaugmentation with a *Sphingobium yanoikuyae* strain (Balciunas et al., 2020). Although both these systems are different from the mBPS and the secondary effluent tested in the current study, RW412 bioaugmentation appears to remove ibuprofen faster than the bacteria in either of the other systems.

Supplementary material

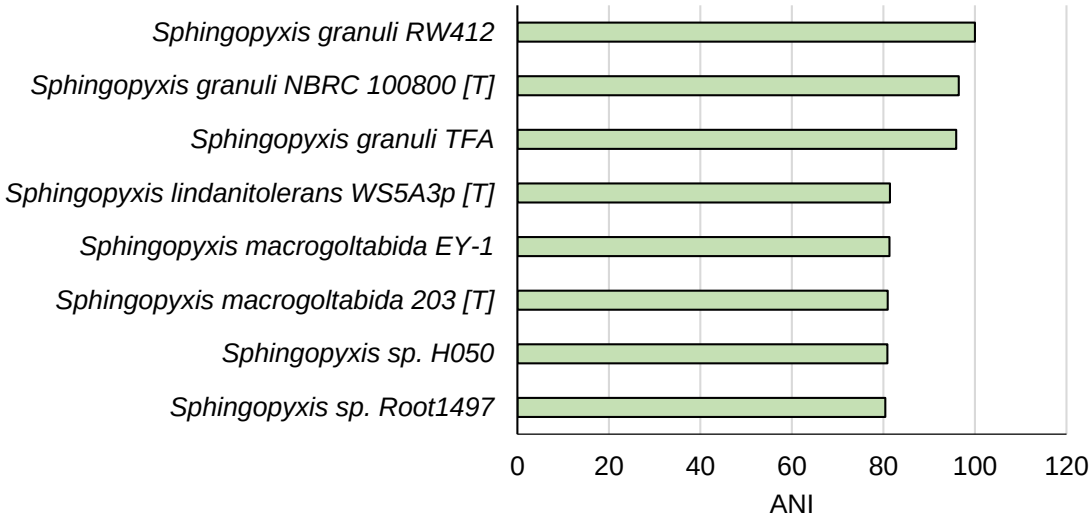


Figure S1. ANIb comparison results between *Sphingopyxis* strains with highest similarity and RW412. [T] indicates Type strains.

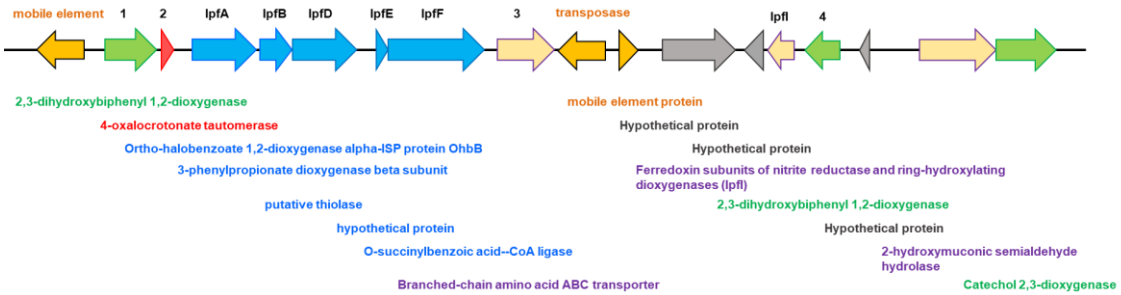


Figure S2. *Ipf* gene homologs and gene organization in RW412. Nomenclature indicated above ORFs as on contig 1 of Murdoch and Hay (2013) nomenclature below according to annotation.

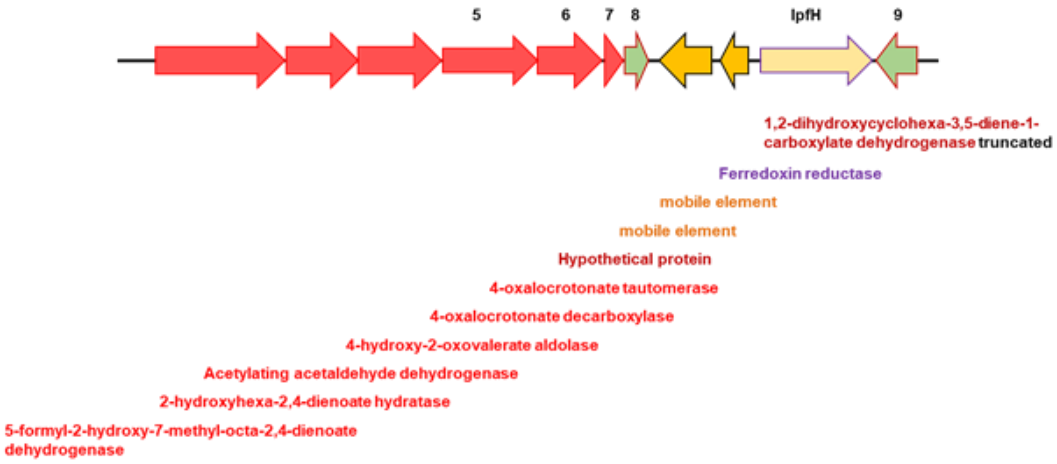


Figure S3. *Ipf* gene homologs and gene organization in RW412 40 kb upstream from *ipfABDEFG* on plasmid RW412A. Nomenclature indicated above ORFs are as as on contig 2 as reported by Murdoch and Hay (2013) nomenclature below is according to annotation.

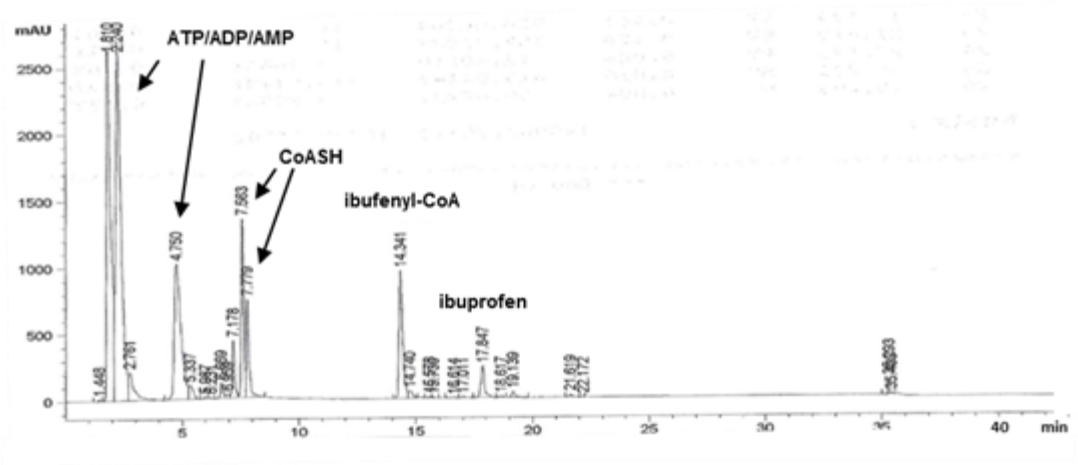


Figure S4. HPLC chromatogram of products produced by IpfF from 0.5 mM ibuprofen after 1.5 h.

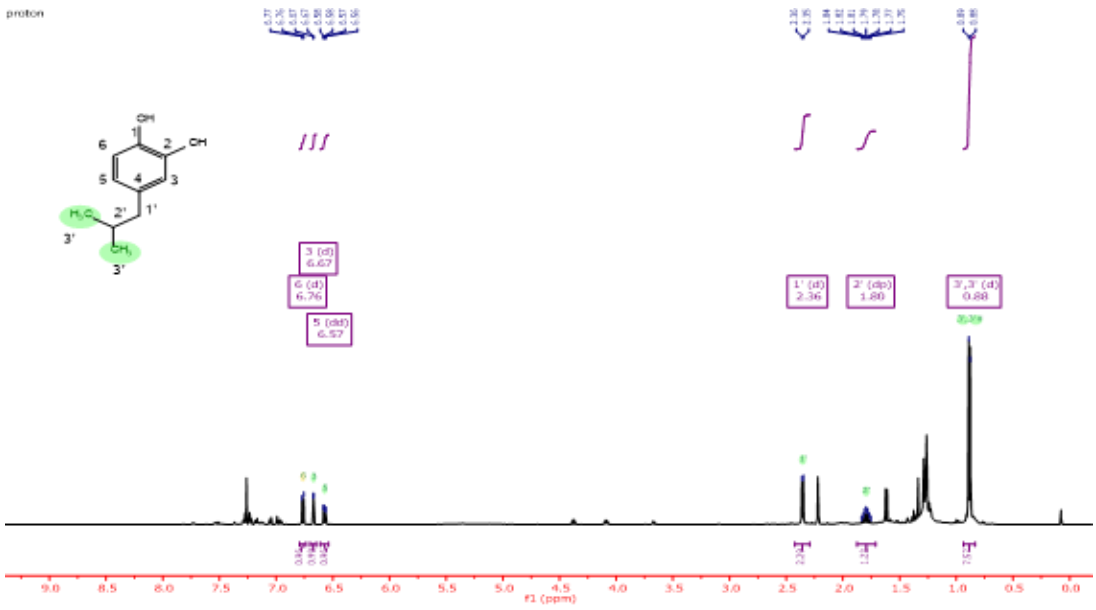


Figure S5. Full range ^{1}H NMR spectrum of 4-isobutylcatechol, recorded at 500 MHz in deuterochloroform.

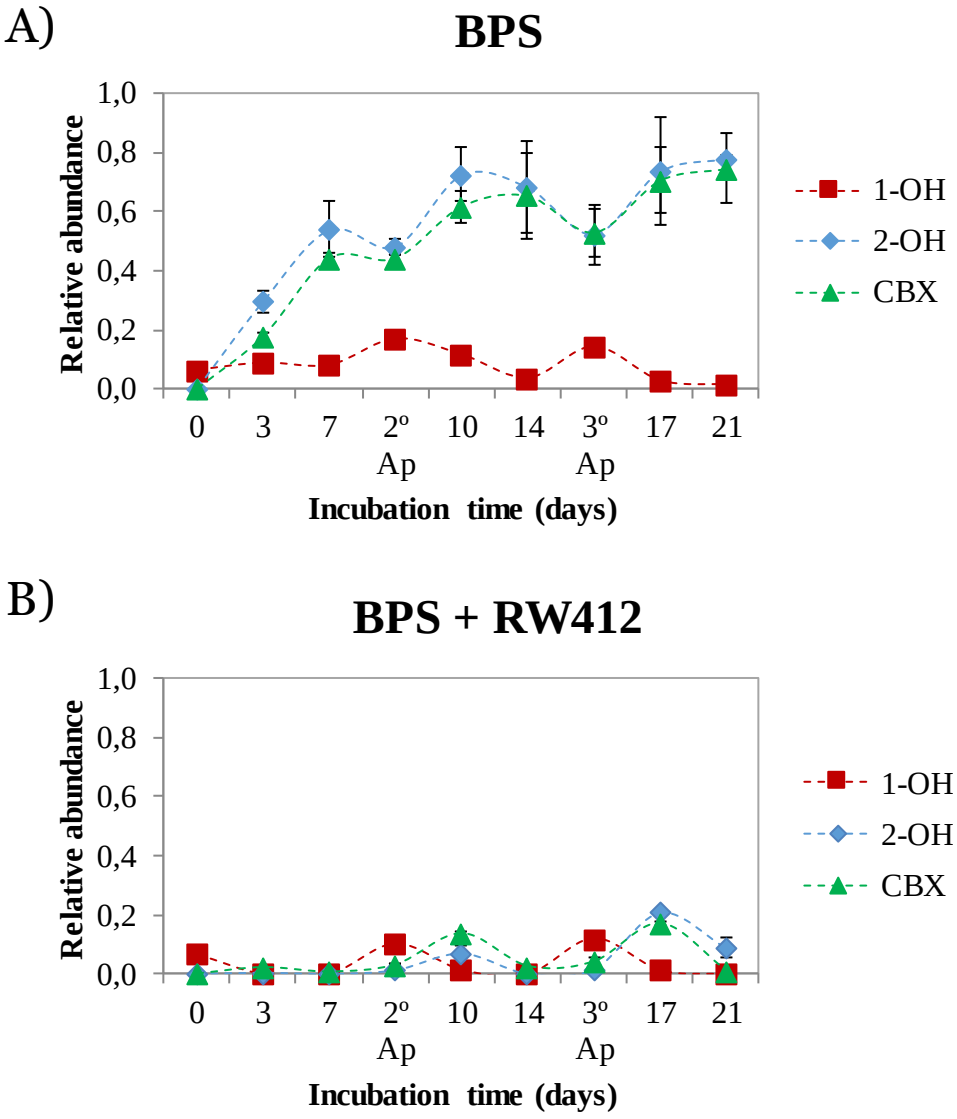


Figure S6. Relative abundance of ibuprofen metabolites compared to internal controls in BPS (A) and BPS bioaugmented with RW412 (B). Error bars indicate standard deviation (n=3).

Table S1. Chromatography and mass spectrometry methods used for the analysis of and structure

ibuprofen, its metabolites and transformation products.

Sample type	Equipment	Chromatography conditions	D
Ibuprofen consumption	HPLC-DAD (series 1100, Agilent Technologies, Santa Clara, CA)	Precolumn: Eclipse XDB-C8 (5µm, 2.1 x 12.5 mm) Column: Zorbax RX-C8 (5µm, 2.1 x 150 mm) Mobile phase: acetonitrile:water at pH3 with H_2SO_4 (50:50) Flow: 0.2 mL min^{-1} Oven temperature: 40ºC and Injection volume: 10 µL	Wave Dete
The separation and isolation of intermediates by semipreparative HPLC	HPLC-DAD (1050 system, Hewlett Packard, Waldbronn, Germany)	Column: Waters Nova-Pak HR C18 (6 µm, 7.8 × 300 mm) Mobile phase: acidified water (1 mL of *ortho*-phosphoric acid per liter, eluent A) and correspondingly acidified (aqueous) acetonitrile (eluent B), isocratic at 45% B Flow: 5 mL min^{-1}	Wave
Separation and detection of IBP and products	HPLC-DAD (1050 system, Hewlett Packard, Waldbronn, Germany	Precolumn: Waters Nova-Pak C18 (4 µm, 3.9 x 20 mm) Column: Waters Nova-Pak C18 (5 µm, 3.9 x 150 mm) Mobile phase: acidified water (1 mL of *ortho*-phosphoric acid per liter, eluent A) and correspondingly acidified (aqueous) acetonitrile (eluent B) Gradient: 3 minutes 100% eluent A followed by a gradient of 0-100% eluent B in 17 min. 100% eluent B was maintained for 15 min and followed by re-equilibration in 100% eluent A for 9 min. Flow: 0.85 mL min^{-1}	Wave range

Ibuprofenyl-CoA detection	HPLC-DAD (1050 system, Hewlett Packard, Waldbronn, Germany	Column: Nova-Pak C18 HPLC column (5 µm, 3.9 x 150 mm) Mobile phase: 25mM NaH_2PO_4 (pH 4.6) (eluent A) and 75% acetonitrile 25mM NaH_2PO_4 (pH 4.6) (eluent B) Gradient: 0-3 min with eluent A at 100%, gradient 3-23 min with eluent B 100%, isocratic 23-33 min with eluent B at 100%, followed by 33-43 min re-equilibration with eluent A at 100%. Flow: 0.85 mL min^{-1} Injection volume: 25 µL	Wave
Ibuprofenyl-CoA and isobutylcatechol-O^{18} mass determination	HPLC (Waters 2695 XE Alliance separation module coupled to the 2996 photodiode array detector), coupled to a tandem quadrupole mass spectrometer (Quattro micro API-ESI)	Column: Nova-Pak C18 narrow-bore column (2.7 µm, 2.1 x 150 mm) Mobile phase: acidified water (1 mL of formic acid per liter, eluent A) and correspondingly acidified acetonitrile (eluent B) Gradient: 5 minutes 100% eluent A, followed by a gradient of 0-100% eluent B in 15 min. 100% eluent B was maintained for 10 min and followed by re-equilibration in 100% eluent A for 10 min. Flow: 0.2 mL min^{-1}	Mode ion el Cone Acqu MS)
Ibuprofen metabolites	Gas chromatography / mass spectrometry (GC-MS) with gas chromatograph Varian 450 with a 240-MS detector (Agilent Technologies, Ca, USA)	Column: J&W Scientific DB 5-MS stationary phase column (30 m x 0.25 mm x 0.25 µm, Agilent Technologies, CA, USA) The temperature gradient was as follows: 90 °C for 2 min, then increased to 280 °C at a rate of 20 °C min-1 and held for 5 min, and finally increased to 310 ºC and held for 20 min. As mobile phase, high purity helium Flow: 1 mL min^{-1} Injector temperature: 280 °C.	Ioniz electr Acqui scan r of 50-

Abbreviations: HPLC: high pressure liquid chromatography; DAD: diode array detector, MRM: multiple reaction m
recording, SIM: Selected Ion Monitoring.

Table S2. Number of genes in RW412 related to the subsystem and subcategories within the Metabolism of aromatic compounds.

Subcategory	Subsytem	Number of genes
Aromatic amino acid synthesis and degradation	Aromatic amino acid synthesis and degradation	26
Metabolism of central aromatic intermediates	Catechol branch of beta-ketoadipate pathway	5
Metabolism of central aromatic intermediates	Salicylate and gentisate catabolism Genistate	9
Metabolism of central aromatic intermediates	Protocatechuate degradation	0
Metabolism of central aromatic intermediates	Homogentisate pathway	0
Peripheral pathways for catabolism of aromatic compounds	Quinate degradation	1
Peripheral pathways for catabolism of aromatic compounds	Biphenyl Degradation	13
-none-	*meta*-cleavage dioxygenases	6*
-none-	4-Hydroxybenzoate degradation	1
-none-	4-Hydroxyphenylacetate catabolic pathway	1
-none-	Benzoate transport	1:
-none-	Chlorobenzoate degradation	1
-none-	Phenol degradation	5
-none-	Vanillate degradation	2
	Total number of genes	67

* 4 *meta*-cleavage dioxygenase genes are included in the genes associated with Biphenyl degradation.

Table S3. qPCR data in BPS. Gene copy numbers ng DNA^{-1} of 16S rRNA as a proxy for the total number of bacteria and *ipfF* as a marker gene for the number of RW412.

	16S rRNA		*ipf*F	
BPS	Initial (T=0 d)	Final (T=21 d)	Initial (T=0 d)	Final (T=21 d)
BPS	9.47×10^5 (4.19)	7.92×10^5 (1.52)	11.4 (5.72)	5.02 (3.85)
BPS + IBP	1.52×10^6 (0.16)	1.36×10^6 (0.12)	30.3 (16.80)	28.3 (14.55)
BPS + RW412	8.12×10^5 (3.90)	1.28×10^6 (0.19)	1.42×10^5 (0.62)	1.80×10^4 (0.61)
BPS + IBP + RW412	1.39×10^6 (0.17)	1.30×10^6 (0.03)	2.07×10^5 (0.31)*	2.05×10^4 (0.45)*

IBP indicates the addition of ibuprofen to the BPS

RW412 indicates bioaugmentation of the BPS with 2×10^8 CFU g^{-1}

Standard error is indicated between parenthesis.

* indicates significant difference between the initial and final times (ANOVA, $p<0.05$).

V. GLOBAL DISCUSSION

The presence of organic pollutants derived from agricultural, industrial and urban activities deteriorates water quality. Incomplete elimination of these compounds by conventional activated sludge treatments in wastewater treatment plants (WWTPs) causes their continuous and uncontrolled entry into the environment through secondary effluents, posing a risk to ecosystems and human health. For this reason, it is important to develop affordable bioremediation techniques to avoid the transfer of polluted waters to the environment. Biopurification systems (BPSs) or "biobeds" comprise an efficient and eco-friendly technology to prevent punctual environmental contamination caused by the disposal of contaminated water from the use and handling of pesticides in agriculture (Castillo et al., 2008). The effectiveness of these BPSs is closely related to their main component, an active biomixture consisting of agricultural soil and organic materials that harbour microorganisms which may be involved in the degradation or co-metabolism of pollutants (Karanasios et al., 2012; Sniegowski et al., 2011). BPSs are being implemented worldwide as low-cost bioremediation techniques (Dias et al., 2020). The introduction of these systems for best management practices in agriculture in a country involves intense research and proposals for modifications to adapt them to regional conditions, practices and needs.

Our research group has designed novel biomixtures composed of organic waste from the olive-oil and wine agro-industries or from greenhouse cultivation which are abundant in Spain. Previous studies have shown that biomixtures with agro-industrial waste efficiently remove mixtures of pesticides with different physicochemical characteristics (Delgado-Moreno et al., 2017a). Recently, we expanded its potential application to remove pharmaceutical and personal care products (PPCPs) present in wastewater (Delgado-Moreno et al., 2019). However, little is known about the effect of PPCPs on the microbial communities of this bioremediation system.

This doctoral thesis delves into understanding the functioning of BPSs formed by a novel biomixture composed of soil, vermicompost of wet olive mill cake and olive tree prunings (SVP) at the microbiological and molecular level. The interactions between three PPCPs, ibuprofen, diclofenac and triclosan, during their degradation in the BPS, as well as their effects on bacterial viability and the

microbial community structure of this system, have been determined (Chapter 1). Dissipation kinetics reveal that diclofenac was affected by the presence of either triclosan or ibuprofen or both (Chapter 1, Figure 1 and Table 1). This could be attributed to the possible negative effect of these compounds on groups of microorganisms involved in diclofenac dissipation, or to the existence of microorganisms that preferentially degrade ibuprofen or triclosan rather than diclofenac (Z. Lu et al., 2019). Bacterial richness and diversity analysis showed that PPCPs have an impact on the bacterial community structure. In the BPSs exposed only to ibuprofen or diclofenac the alpha diversity increased punctually, indicating the high resilience of the bacterial community in response to the concentrations of these two non-steroidal anti-inflammatory drugs (Chapter 1, Table 2). However, the individual treatment with triclosan not only negatively affected the richness and diversity in this system, but also caused an initial negative impact on bacterial viability (Chapter 1, Figure 5). This observation is in general agreement with other studies in which several authors reported that triclosan is an environmental stressor, alters the community structure and reduces species diversity and richness (Clarke et al., 2019; Oh et al., 2019; Peng et al., 2019). However, in our study this antiseptic effect of triclosan was mitigated when the three PPCPs were applied simultaneously in the BPS in which high bacterial community diversity was maintained despite the contamination levels and the biocide agents added. Furthermore, PPCP treatment altered the relative abundance of the most dominant phyla (Chapter 1, Figure 2A). Triclosan had a negative effect on certain bacterial groups such as *Acidobacteria*, *Methylophilales*, and *Legionellales* and could therefore interfere with the enrichment of degrading strains which directly interact with the other PPCPs, especially diclofenac. Although hierarchal clustering analysis grouped bacterial communities according to the contaminant applied, both PERMANOVA and non-metric multidimensional scaling (NMDS) analysis as well as the principal coordinates analysis (PCoA), indicated that time is the main determining factor which affected the bacterial community composition of BPSs (Chapter 1, Figures 3 and S1B). Similar results were observed in other BPSs contaminated with pesticides and even when co-applied with an antibiotic, in which the incubation time had an even stronger impact (Castro-Gutiérrez et al., 2019, 2018, 2017; Diez et al., 2017; El Azhari et al., 2018; Marinozzi et al., 2013). These changes in the

bacterial community and in some specific taxa may suggest the occurrence of an adaptive response of the community to select those members implicated in biodegradation processes of these PCPPs. Linear discriminant analysis (LDA) and effect size (LEfSe) were used to find biomarkers consisting of differentially abundant OTUs in contaminated and uncontaminated BPSs (Chapter 1, Figure 4). Using these analyses, it was possible to identify the main bacterial groups potentially involved in the degradation of PPCPs. Within these biomarkers, *Sphingomonadales*-, *Pseudomonadaceae*- and *Verrucomicrobiaceae*-related OTUs were identified for ibuprofen, *Acidobacteria* Group-6- and *Legionellaceae*-related OTUs for diclofenac, *Chitinophagaceae*-, *Sphingobacteriaceae*-, *Caulobacteraceae*- and *Rhizobiaceae*-related OTUs for triclosan, and a *Cyclobacteriaceae*-related OTU was identified for the PPCP mixture. Previous studies have associated these bacterial taxa, or even specialized bacteria belonging to these groups, with the dissipation of PPCPs or other similar aromatic compounds (C. Lu et al., 2019; Murdoch and Hay, 2005; Wang et al., 2019; Zhou et al., 2013). Hence, the biomarkers identified in contaminated BPSs could be interesting candidates for identifying PPCP resistance and degradation mechanisms. On the other hand, it should be noted that despite the high contamination level of the BPSs, these systems provide an ideal habitat for the survival of resistant and/or degrading bacteria of these compounds.

These results revealed that the SVP biomixture has a high decontamination potential and suggested the presence of potentially PPCP degrading microorganisms. However, it is also important to consider increasing the efficiency of these systems further in order to improve their performance and implementation. For this reason, a simple and sustainable bioaugmentation system based on aqueous extracts from residual biomixtures from BPSs exposed/acclimated with contaminants has been developed, for the first time, as a novel strategy to accelerate the biotransformation of organic pollutants in freshly constructed bioremediation systems. The degradation potential of these extracts was also assayed as an inoculum in other contaminated media (Chapter 2).

In the first bioaugmentation study, different residual and aged biomixtures from different BPSs, previously exposed to pesticides for 5-10 months, were used

to obtain aqueous extracts enriched in adapted indigenous microorganisms. The model pesticides selected for this study were the phenylurea herbicides, diuron, which was previously applied in this systems, and linuron (Chapter 2.1). Inoculation of diuron contaminated aqueous solution with extracts from aged biomixtures composed of olive mill cake and vermicompost of olive mill cake (ACE and AVE, respectively), eliminated this herbicide and its more toxic metabolite 3,4-dichloroaniline more efficiently than the APE extract obtained from a contaminated biomixture composed of the original Swedish biomixture components, soil, peat and straw (SPS). No significant abiotic degradation occurred, and most microorganisms originally present in the initial unpolluted biomixtures showed little or no capacity for removing diuron (Chapter 2.1, Figure 1). Therefore, the biodegradation capacity observed upon bioaugmentation with these extracts may be due to microbial populations enriched in the BPSs during previous pesticide exposure. Interestingly, as the amount of inoculated microbial biomass increased, the degradation rate of these pollutants accelerated (Chapter 2.1, Figure 2 and Table 3). Furthermore, the relatively low water soluble carbon and nutrient levels added together with the biomass in these extracts also indicated that the efficiency of this type of bioaugmentation is due to the catabolic potential of microbial populations in the extracts.

The fact that the main metabolite detected was 3,4-DCA supports the hypothesis that cleavage of the urea bridge, generally mediated by hydrolase enzymes that attack phenylurea herbicides, is the main degradation strategy used by the microbial consortia present in the extracts (Bers et al., 2013, 2011; Khurana et al., 2009; G.A. Turnbull et al., 2001). Likewise, the detection of other less toxic metabolites of 3,4-DCA, such as 3,4-dichloroacetanilide, 3,4-dichloronitrobencene and 3,4-dichlorobenzene, suggested that the 3,4-DCA formed from diuron was subsequently degraded by different metabolic routes which may also be attributed to the activity of different microbial communities and/or their cooperation in the degradation pathways (Bers et al., 2011; Castillo et al., 2014). The AVE extract from the biomixture composed of vermicompost of wet olive mill cake was the most effective. This extract also boosted the degradation of linuron, whose main metabolite was also 3,4-DCA (Chapter 2.1, Figure 3). This result suggests that microbial consortia in the AVE extract contain microorganisms with genes encoding amide hydrolase enzymes specific for either

N, N-dimethyl- or *N*-methoxy-*N*-methyl-substituted compounds (Bers et al., 2011; Sørensen et al., 2008).

Bioaugmentation of the BPS composed of the SPS biomixture with the extract from this polluted SPS biomixture (APE) scarcely improved the already notable diuron removal demonstrated by SPS biomixture on its own (Chapter 2.1, Figure 4). Therefore, the capacity of the SPS biomixture to remove this herbicide is probably due more to intrinsic factors, such as sorption capability. On the other hand, the extract AVE greatly boosted diuron elimination in the BPS composed of SVP biomixture indicating that this extract contains an effective consortium.

The analysis of the bacterial communities found in the biomixtures at the initial moment before contamination (i-SVP) and after an exposure period to the pesticides as well as in their corresponding extract (a-SVP and AVE), revealed an increase in the relative abundance of some taxa, such as *Chloroflexi*, *Acidobacteria*, *Gemmatimonadetes*, *Firmicutes*, *Deinococcus-Thermus* and especially *Proteobacteria*, in the aged biomixture, as well as an enrichment of *γ-proteobacteria* and the actinobacterial genus *Dokdonella* in the extract compared to initial uncontaminated biomixture (Chapter 2.1, Figures 5 and 6). Therefore, these bacterial taxa could be involved in improving the dissipation of both phenylurea herbicides and its main metabolites. In fact, species belonging to the genus *Dokdonella* were identified as degraders of paracetamol metabolites with related structures to diuron and other pesticides (Palma et al., 2018; Qi and Wei, 2017). Moreover, several bacterial strains isolated from the a-SVP biomixture, which grow with diuron as the sole carbon source in solid medium, belong to the genera *Arthrobacter*, *Pseudomonas* and *Bacillus* and could also be involved in the degradation of this herbicide. According to the literature, bacterial species belonging to these genera have been described which have the ability to degrade diuron and even its main metabolite, 3,4-DCA (Egea et al., 2017; Sørensen et al., 2008; G.A. Turnbull et al., 2001).

To confirm the efficiency of this new bioaugmentation alternative, this strategy was used to enhance the removal of ibuprofen, diclofenac and triclosan in two different media, in BPSs and in contaminated aqueous solution (Chapter 2.2). For this aim, a sample of the i-SVP biomixture was acclimatized by means of repeated and consecutive treatments with PPCPs and then, the aqueous extracts were

obtained to inoculate the different media. The extract from acclimated biomixture accelerated the elimination of diclofenac and triclosan in BPSs (Chapter 2.2, Figure 1), and prevented the accumulation of the toxic metabolite methyl triclosan (Chapter 2.2, Figure 2). However, the microbial activity of the extract had a negligible effect on ibuprofen removal, probably due to the high capacity of the indigenous microorganisms of BPSs to degrade this contaminant. Thus, the degradation potential of the extracts to remove ibuprofen was researched in aqueous solutions. In this medium, the extract from acclimated biomixture increased ibuprofen elimination compared to the control solutions (Chapter 2.2, Figure 3). Therefore, as observed in the previous study, PPCP-acclimatization of BPS appears to have enriched the resilient and degrading PPCP microorganisms which could be harvested by the extraction process. Interestingly, the microorganisms present in this extract, also prevented the accumulation of hydroxylated metabolites of ibuprofen which have been widely detected in wastewater treatment plant effluents and in water resources (Ferrando-Climent et al., 2012; Lolić et al., 2015). Therefore, this extract harbours microorganisms that may also have the genetic machinery necessary to eliminate these biotransformation products of ibuprofen (Chapter 2.2, supplementary Figure S1). The analyses of the bacterial communities in the non-contaminated initial biomixture (i-SVP) and PPCP-acclimated biomixture (a-SVP), as well as in the aqueous extract from this acclimated biomixture indicate that repeated PPCP applications cause a decrease in species richness but most of bacterial communities in the BPS were resilient to these contaminants. Bioinformatic analyses reveal that the PPCP treatment affected the structure of the microbial community. Furthermore, PPCP exposition not only altered the relative abundance of the most dominant phyla in the biomixture (Chapter 2.2, Figure 4 and S5) and negatively impacted the families *Microbacteriaceae*, *Promicronosporaceae* and *Streptomycetaceae*, but also enriched mainly the family *Cytophagaceae* (Chapter 2.2, Figure 5). In the extract, the most dominant OTUs detected, *Flavobacterium*-, *Thermomicrobia*-, *Nonomuraea*- and *Fluviicola*-related OTUs (Chapter 2.2, Figure 6) could be involved in PPCP elimination since these bacterial taxa have also been related to the biodegradation of PPCPs and other related organic contaminants (Kabumoto et al., 2009;

Phandanouvong-Lozano et al., 2018; Rossmassler et al., 2019; Song et al., 2016; Tiwari et al., 2019).

Traditional bioaugmentation with cultivable bacteria requires long periods of time for isolation, identification, enrichment and screening. In contrast, our bioaugmentation strategy using the aqueous extract from aged biomixtures exposed to contaminants is a low-cost alternative for the inoculation of indigenous degrading consortia and an affordable technique for its application on farms by non-specialist personal.

Another way of to enhancing the performance of the biopurification systems assayed in this doctoral thesis is by bioaugmentation with exogenous degrading bacterial strains. First, it is necessary to obtain bacteria with the ability to degrade or resist high concentrations of PPCPs as well as with the ability to compete with indigenous microorganisms. Several bacterial strains were isolated from sediments of the River Elbe, a river in Germany with a long history of exposure to industrial and urban pollutants (Chapter 3). Five isolates were found which are highly tolerant to diclofenac and, interestingly, another bacterium was isolated which is able to use ibuprofen as a sole carbon and energy source (Chapter 3, Figure 1). This strain was characterized and identified as *Sphingopyxis granuli* RW412. Genetic characterization revealed that this strain harbours three plasmids, one of which encodes the *ipf* genes described for ibuprofen degradation by (Murdoch and Hay, 2013). The degradation route for ibuprofen proposed by Murdoch and Hay (2013, 2005) was deduced by the authors from the annotation of the *ipf* genes, indirect functional assays and the molecular masses obtained by GC-MS of accumulating compounds by *Sphingomonas* sp. Ibu-2. In this proposed route, coenzyme A is initially ligated to ibuprofen to form a product which is then attacked by a dioxygenase and a putative thiolase to produce an isobutylcatechol which is subsequently degraded by *meta*-cleavage dioxygenases for mineralization. In order to confirm that RW412 degrades ibuprofen via this pathway and whether this pathway leads to 4-isobutylcatechol production, this intermediate was purified from the culture media of RW412. Characterization of this intermediate by LC-MS (Chapter 3, Figure 3) and by NMR confirmed unequivocally the location of the hydroxyl groups to be at the 1 and 2 positions of the 4-isobutylcatechol molecule (Chapter 3, Figure 4). Since RW412 does not

accumulate 4-isobutylcatechol or yellow ring fission products during growth with ibuprofen, this strain probably harbours multiple dioxygenases for *meta*-cleavage and efficient enzymes to degrade the resulting ring fission products. Although a number of candidate *meta*-cleavage dioxygenases were found in the genome, and even in the same plasmid as the *ipf* genes, their implication in ibuprofen degradation will require further studies.

Since the *ipf* genes are located on a plasmid of RW412 and moreover are flanked by mobile elements, suggests that they may have been acquired by RW412 through horizontal transfer. Curiously, the *ipf* genes of RW412 are almost identical both in gene organisation and at the nucleotide level to the *ipf* genes described in *Sphingomonas* ibu-2. Each of these strains was isolated from different continents but both belong to *Sphingomonadaceae*. Therefore, it would be interesting to understand how these genes have moved between microbial populations worldwide and why ibuprofen degradation through the *ipf* route is apparently restricted to this bacterial family. Nevertheless, this gene mobility could facilitate the loss of capacity of RW412 to degrade ibuprofen when other more labile carbon sources are available or in the absence of ibuprofen in the media. However, the *ipf* genes are stably maintained in RW412 even after 30 generations of growth on non-selective media, suggesting that RW412 could have good applicability for the bioaugmentation of contaminated environments. Experimental results have demonstrated that bioaugmentation with RW412 not only drastically improves ibuprofen dissipation in the BPS even after repeated applications of this contaminant (Chapter 3, Figure 5) but also prevents the accumulation of hydroxylated and carboxylated ibuprofen metabolites. Moreover, this strain also dissipated ibuprofen much faster than the autochthonous bacterial community in secondary effluents (Chapter 3, Figure 6). Therefore, RW412 is a robust strain that could be applied to both aqueous and solid matrixes to eliminate this emerging contaminant in the treatment of urban wastewater before their release into the environment.

VI. CONCLUSIONS

1. The biopurification system based on a biomixture composed of soil, vermicompost from olive-oil agroindustrial wastes and olive tree prunings showed high efficiency to simultaneously remove the three PPCPs assayed, ibuprofen, diclofenac and triclosan, maintaining high bacterial community diversity, which demonstrates the high resilience of the microbial population in this system.

2. Triclosan adversely affects bacterial viability and the bacterial community in the biopurification system, but this antiseptic effect may be mitigated when other PPCPs are present. This biocide agent negatively impacts certain bacterial groups such as *Acidobacteria*, *Methylophilales*, and *Legionellales* and could therefore interfere with the enrichment of degrading strains which directly interact with the other PPCPs.

3. The biomarkers *Sphingomonadales*-, *Pseudomonadaceae*- and *Verrucomicrobiaceae*-related OTUs identified for ibuprofen, *Acidobacteria* Group-6- and *Legionellaceae*-related OTUs for diclofenac, *Chitinophagaceae*-, *Sphingobacteriaceae*-, *Caulobacteraceae*- and *Rhizobiaceae*-related OTUs for triclosan, and a *Cyclobacteriaceae*-related OTU identified for the PPCP mixture could be key taxa involved in the degradation of these PPCPs in biopurification systems.

4. Aqueous extracts are a new, affordable and sustainable bioaugmentation strategy which inoculates microbiomes obtained from residual aged biomixtures composed of agro-industrial wastes acclimated to several phytosanitary products to boost the biodegradation of phenylurea herbicides and prevent the formation or accumulation of the biodegradation product, 3,4-dichloroaniline, in biopurification systems or in aqueous solutions.

5. Bioaugmentation with aqueous extracts obtained from PPCP acclimatized biomixtures increase the depuration activity of new biopurification systems and can be an effective alternative to improve the elimination of organic contaminants present in effluents from industry, hospitals or wastewater treatment plants, and thus prevent their entry and accumulation in the

environment. However, additional studies are required to determine its application on a large scale.

6. Bacterial taxa enriched in extracts from the biomixture aged with pesticides such as *Dokdonella,* and the most dominant OTUs detected in the extract from PPCP-acclimatized biomixture, such as *Flavobacterium*-, *Thermomicrobia*-, *Nonomuraea*- and *Fluviicola*-related OTUs, could play a significant role in the biotransformation of phenylurea herbicides and PPCPs, respectively, as well as in the elimination of their main biodegradation products.

7. *Sphingopyxis granuli* RW412 can use ibuprofen as sole carbon and energy source. It can metabolize ibuprofen using the gene products encoded by the *ipf* genes, with ibuprofenyl-CoA and 4-isobutylcatechol being unequivocally identified as intermediate products of this ibuprofen biodegradation pathway.

8. Bioaugmentation with RW412 improves ibuprofen dissipation and avoids the accumulation of hydroxylated and carboxylated ibuprofen metabolites in biopurification systems. Likewise, bioaugmentation with this strain dissipates ibuprofen much faster than the autochthonous bacterial community in secondary effluents. Thus, RW412 can be applied to both aqueous and solid matrixes to accelerate the elimination of this emerging contaminant and to improve the quality of effluents from wastewater treatment plants before their release into the environment.

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