Universitat de Lleida

Effect of compounds from agricultural biodegradable plastics on the environment and on plant development

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TESI DOCTORAL

Effect of compounds from agricultural biodegradable plastics on the environment and on plant development

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- Serrano-Ruiz, H., Martin-Closas, L., Pelacho, A.M., 2018. Application of an in vitro plant ecotoxicity test to unused biodegradable mulches. *Polym. Degrad. Stab*. 158, 102–110. Doi: 10.1016/j.polymdegradstab. 2018.10.016. (CHAPTER II).

- Serrano-Ruiz, H., Eras, J., Martin-Closas, L., Pelacho, A.M., 2020. Compounds released from unused materials after contact with water biodegradable mulch. *Polym. Degrad. Stab.* 178, 109202. Doi: 10.1016/j.polymdegradstab.2020.109202. (CHAPTER III).

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SUMMARY

Agricultural plastic mulches are an essential part of the agricultural system, contributing to face the food demand for the growing world population. Its use increases crop production, earliness and quality, reduces water consumption and pesticide delivery and prevents weed development. Mulches are mostly made of polyethylene (PE), non-biodegradable. Although they must be removed after harvesting, many fragments remain and accumulate in the field, reducing soil and crop quality. Biodegradable plastic mulches (BDM) have been fostered as a sustainable alternative preventing this accumulation. After harvest they will be biodegraded by the soil microorganisms in which they are integrated. However, this entails the various compounds (polymers and additives) present in the fragments are supplied to the soil, but their effects on cultivated plants and on soil organisms have hardly been studied.

The objective of this PhD thesis is to evaluate the effect of eight BDM of different formulation, and their components, on the agricultural soil microbiome and on plants. For this purpose two plant species commonly cultivated with mulches which are among the main horticultural products were targeted, lettuce (*Lactuca sativa* L.) and tomato (*Lycopersicon esculetum* Mill.). One PE mulch was included as control mulch.

Firstly, it was evaluated whether BDM can release compounds by contact with an aqueous environment before the onset of their biodegradation, and whether the released compounds can affect plant development. It was found that all the BDM tested released a diversity of compounds, which in several cases (Bioplast SP4 and SP6, Mirel and Biofilm) inhibited germination, root morphology or the development and physiology of both plant species, while those from other BDM caused minor (Ecovio, Mater-Bi) or non-significant (Bioflex) effects.

Next, the released compounds were identified, which were eventually diverse, both components of its polymeric structure (1,4-butanediol, lactic acid, terephthalic acid, etc.) and additives (fatty acids, glycerol, etc.). Among those identified, the ones having previously shown to affect tomato and lettuce plant development (1,4-butanediol, lactic acid and adipic acid) were quantified. They were found to be in substantially lower concentrations than the ones responsible for causing effects on plants, which does not allow establishing a direct relationship between their release from BDM and the effects they may have on plants.

Thirdly, the effect of the accumulation of BDM fragments in the soil on tomato and lettuce germination and plant development was studied. For most BDM, the presence of their fragments did not affect germination but it reduced plant growth and chlorophyll content in tomato and especially in lettuce. In general, the identified effects were consistent with those of compounds released from BDM previously found, and PE fragments caused no effects. Altogether, results suggest that the BDM chemical composition plays a relevant role in its interaction with the plant root system, and that the consequences of the presence of BDM fragments in the soil is related to this composition, likely due to the release of components, rather than to their physical presence.

Finally, the impact of the BDM fragments' accumulation in the soil on the structure and functions of the agricultural soil microbial communities was studied. After incubation for three months, this accumulation had a low impact on the soil microbial communities' diversity and structure. However, some materials caused significant changes in the abundance and diversity of selected bacterial (Mater-Bi), fungi (MIMGreen paper) and protists (Ecovio) groups. Although the total microbial activity was not altered, the chitinase activity, involved in the nitrogen cycle, was significantly decreased by both BDM and PE presence.

The results obtained in this doctoral thesis provide new knowledge on the potential effects of BDMs on cultivated plants and soil microorganisms. They mainly show that BDM (1) easily release several compounds soon before their biodegradation starts, after contact with water, (2) the solution containing the released compounds, depending on its composition, may have effects on plants and (3) the accumulation of BDM fragments in the soil has the capacity to affect plant development and to modify the abundance and diversity of soil microorganisms depending on the composition of the BDM. The results will contribute to the design and development of biodegradable plastic mulches that have a low impact on cultivated plants and the environment.

RESUMEN

Los acolchados plásticos agrícolas son una pieza fundamental del sistema agrícola, contribuyendo a hacer frente a la demanda de alimentación de la creciente población mundial. Su uso incrementa la producción, precocidad y calidad de las cosechas, reduce el consumo de agua y la aplicación de pesticidas y previene el desarrollo de malas hierbas. Los acolchados son mayoritariamente de polietileno (PE), no biodegradables, y aunque se deben retirar tras la cosecha, muchos fragmentos permanecen en el campo y se van acumulando, disminuyendo la calidad del suelo y de las cosechas. Los acolchados de plástico biodegradable (BDM) se han presentado como una alternativa sostenible que evita este acúmulo; tras la cosecha serán biodegradados por los microorganismos del suelo en el que se integran. Sin embargo, ello implica el aporte al suelo de los diversos compuestos (polímeros y aditivos) presentes en los fragmentos, pero apenas se han estudiado sus efectos en las plantas cultivadas y en los organismos del suelo.

El objetivo de esta tesis es evaluar el efecto que tienen ocho BDM de diferente formulación y sus componentes en el microbioma del suelo agrícola y en plantas cultivadas. Para ello se eligieron dos especies comúnmente cultivadas con acolchados que están entre los principales productos hortícolas a nivel mundial, lechuga (*Lactuca sativa* L.) y tomate (*Lycopersicon esculetum* Mill.). Como control se incluyó un acolchado de PE.

En primer lugar, se evaluó si los BDM pueden liberar compuestos por contacto con un medio acuoso antes de iniciar su biodegradación, y si los compuestos liberados pueden afectar al desarrollo de las plantas. Se encontró que todos los BDM ensayados liberaron una diversidad de compuestos, que en varios casos (Bioplast SP4 y SP6, Mirel y Biofilm) afectaron negativamente a la germinación, la morfología de las raíces o el desarrollo y fisiología de ambas especies, mientras que los de otros BDM causaron efectos menores (Ecovio, Mater-Bi) o no significativos (Bioflex).

A continuación, se identificaron los compuestos liberados, que resultaron ser diversos, tanto componentes de su estructura polimérica (1,4-butanediol, ácido láctico, ácido tereftálico, etc.) como aditivos (ácidos grasos, glicerol, etc.). De entre los identificados se cuantificó principalmente los que anteriormente habían mostrado afectar al desarrollo de plantas de tomate y de lechuga (1,4-butanediol, ácido láctico y ácido adípico). Las concentraciones en que se encontraron resultaron ser sustancialmente menores que las

responsables de causar efectos en las plantas, lo que no permite establecer una relación directa entre su liberación de los BDM y los efectos que puedan tener en las plantas.

En tercer lugar, se estudió el efecto del acúmulo de fragmentos de BDM en el suelo sobre la germinación y desarrollo de plantas de tomate y de lechuga. La presencia de fragmentos de la mayoría de los BDM no afectó a la germinación pero si redujo el crecimiento y el nivel de clorofila en tomate y especialmente en lechuga. En general, los efectos identificados fueron consistentes con los de los compuestos liberados de los BDM encontrados anteriormente, y los fragmentos de PE no causaron efectos. En conjunto, los resultados sugieren que la composición química del BDM tiene un papel relevante en su interacción con el sistema radical de las plantas, y que las consecuencias de la presencia de fragmentos de BDM en el suelo se relaciona con esta composición, probablemente debido a que liberan componentes, más que a su presencia física.

Finalmente, se estudió el impacto del acumulo en el suelo de fragmentos de BDM en la estructura y funciones de las comunidades microbianas del suelo agrícola. Tras tres meses de incubación, este acúmulo tuvo un bajo impacto en la diversidad y estructura de las comunidades microbianas del suelo. Sin embargo, algunos materiales provocaron cambios significativos en la abundancia y diversidad de determinados grupos bacterianos (Mater-Bi), fúngicos (papel MIMGreen) y protistas (Ecovio). Aunque la actividad microbiana total no se vio alterada, la actividad quitinasa, implicada en el ciclo del nitrógeno, disminuyó significativamente por la presencia tanto de BDM como de PE.

Los resultados obtenidos en esta tesis doctoral aportan nuevo conocimiento sobre los potenciales efectos de los BDM en las plantas cultivadas y los microorganismos del suelo. Principalmente evidencian que los BDM (1) liberan con facilidad diversos compuestos mucho antes de que se inicie su biodegradación, tras el contacto con el agua, (2) la solución que contiene los compuestos liberados, en función de su composición, puede tener efectos sobre las plantas, (3) que el acúmulo de fragmentos de BDM en el suelo presenta capacidad de afectar al desarrollo de las plantas y de modificar la abundancia y diversidad de los microorganismos del suelo en función de la composición del BDM. Todo ello resulta relevante para el diseño y desarrollo de acolchados plásticos biodegradables que tengan un bajo impacto sobre las plantas cultivadas y sobre el medio ambiente.

RESUM

Els encoixinats plàstics agrícoles són una peça fonamental del sistema agrícola, contribuint a fer front a la demanda d'alimentació de la creixent població mundial. El seu ús incrementa la producció, precocitat i qualitat de les collites, redueix el consum d'aigua i l'aplicació de pesticides i prevé el desenvolupament de males herbes. Els encoixinats són majoritàriament de polietilè (PE), no biodegradables, i encara que s'han de retirar després de la collita, molts fragments romanen en el camp i es van acumulant, disminuint la qualitat del sòl i de les collites. Els encoixinats de plàstic biodegradable (BDM) s'han presentat com una alternativa sostenible que evita aquesta acumulació; després de la collita seran biodegradats pels microorganismes del sòl en el qual s'integren. Tanmateix, això implica l'aportació al sòl dels diversos compostos (polímers i additius) presents en els fragments, dels que a penes s'han estudiat els seus efectes en les plantes conreades i en els organismes del sòl.

L'objectiu d'aquesta tesi és avaluar l'efecte que tenen vuit BDM de diferent formulació i els seus components en el microbioma del sòl agrícola i en plantes conreades. Per a això es van triar dues espècies comunament conreades amb encoixinats que estan entre els principals productes hortícoles a nivell mundial, l'enciam (*Lactuca sativa* L.) i el tomàquet (*Lycopersicon esculetum* Mill.). Com a control es va incloure un encoixinat de PE.

En primer lloc, es va avaluar si els BDM poden alliberar compostos per contacte amb un mitjà aquós abans d'iniciar la seva biodegradació, i si els compostos alliberats poden afectar el desenvolupament de les plantes. Es va trobar que tots els BDM assajats van alliberar una diversitat de compostos, que en diversos casos (Bioplast SP4 i SP6, Mirel i Biofilm) van afectar negativament la germinació, la morfologia de les arrels o el desenvolupament i fisiologia de totes dues espècies, mentre que els altres BDM van causar efectes menors (Ecovio, Mater-Bi) o no significatius (Bioflex).

A continuació, es van identificar els compostos alliberats, que van resultar ser diversos, tant components de la seva estructura polimèrica (1,4-butanediol, àcid làctic, àcid tereftàlic, etc.) com a additius (àcids grassos, glicerol, etc.). D'entre els identificats es va quantificar principalment els que anteriorment havien mostrat afectar el desenvolupament de plantes de tomàquet i d'enciam (1,4-butanediol, àcid làctic i àcid adípic). Les concentracions en què es van trobar van resultar ser substancialment menors que les responsables de causar efectes en les plantes, la qual cosa no permet establir una relació directa entre el seu alliberament dels BDM i els efectes que puguin

tenir en les plantes.

En tercer lloc, es va estudiar l'efecte de l'acumulació de fragments de BDM en el sòl sobre la germinació i desenvolupament de plantes de tomàquet i d'enciam. La presència de fragments de la majoria dels BDM no va afectar la germinació però si va reduir el creixement i el nivell de clorofil•la en tomàquet i especialment en enciam. En general, els efectes identificats van ser consistents amb els dels compostos alliberats dels BDM trobats anteriorment, i els fragments de PE no van causar efectes. En conjunt, els resultats suggereixen que la composició química del BDM té un paper rellevant en la seva interacció amb el sistema radical de les plantes, i que les conseqüències de la presència de fragments de BDM en el sòl es relaciona amb aquesta composició, probablement pel fet que alliberen components, més que a la seva presència física.

Finalment, es va estudiar l'impacte de l'acumuació en el sòl de fragments de BDM en l'estructura i funcions de les comunitats microbianes del sòl agrícola. Després de tres mesos d'incubació, aquesta acumulació va tenir un baix impacte en la diversitat i estructura de les comunitats microbianes del sòl. No obstant això, alguns materials van provocar canvis significatius en l'abundància i diversitat de determinats grups bacterians (Mater-Bi), fúngics (paper MIMGreen) i protistes (Ecovio). Encara que l'activitat microbiana total no es va veure alterada, l'activitat quitinasa, implicada en el cicle del nitrogen, va disminuir significativament per la presència tant de BDM com de PE.

Els resultats obtinguts en aquesta tesi doctoral aporten nous coneixements sobre els BDM i els seus potencials efectes. Principalment evidencien que els BDM (1) poden alliberar amb facilitat diversos compostos molt abans que s'iniciï la seva biodegradació, (2) que la solució que conté els compostos alliberats, en funció de la seva composició, pot tenir efectes sobre les plantes i (3) que l'acumulació de fragments de BDM en el sòl presenta capacitat d'afectar el desenvolupament de les plantes i de modificar l'abundància i diversitat del microbioma del sòl en funció de la composició del BDM. Tot això, resulta rellevant per al disseny i desenvolupament d'encoixinats plàstics biodegradables que tinguin sota impacte sobre plantes conreades i sobre el medi ambient.

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CHAPTER I

BIODEGRADABLE PLASTIC MULCHES: IMPACT ON THE AGRICULTURAL BIOTIC ENVIRONMENT

This chapter reproduces the text of the following published literature review:

Serrano-Ruiz, H., Martin-Closas, L., Pelacho, A.M., 2021. Biodegradable plastic mulches: Impact on the agricultural biotic environment. Sci. Total Environ. 750, 141228. https://doi.org/10.1016/j.scitotenv.2020.141228

Abstract

The increasing use of plastic films for agricultural mulching continues worldwide. Mulching improves crop yield, decreases pesticide' inputs to the field, saves irrigation water and contributes to tackle the food demand for the growing world population. However, plastic mulching results in polyethylene residues that contaminate agricultural soils and contribute to the massive worldwide plastic pollution, a serious environmental concern. Biodegradable plastic mulches (BDM) have emerged as a promising alternative to alleviate polyethylene pollution. BDM, made of different polymers and compositions, are designed to biodegrade *in situ*, into the agricultural soil. Their use may entail environmental impacts for the agricultural system that deserve to be explored on the short and on the long-term. This review discusses emerging findings on the impact of BDM on agroecosystem organisms, with special emphasis on cultivated plants and on soil organisms. The relevance of the material composition is highlighted by some reports evidencing specific BDM to alter development of cultivated plant species and to modify soil microbiome on the short-term (spanning a few months); model organisms may also be affected. Long-term studies have not yet been attempted. In-depth studies focused on the effects of the diversity of BDM on agroecosystem organisms are urgently required to identify low-impact BDM materials and to guarantee advanced agriculture in a sustainable environment.

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1. Plastic films for agricultural mulching

1.1. Plastic mulch use: current status

Plastics are widespread in our world and have a profound impact in all human activities and in our lives; in recognition, the current Anthropocene period has been proposed to be remembered as “The Plastic Age” (Thompson et al., 2009; Giaimo, 2016). Technological developments on plastic production have led to massive production of synthetic polymers and plastics (Andrady and Neal, 2009), which are used for virtually any purpose and market: packaging, construction, automotive, electronics, household items, leisure, agriculture, etc. (Plastics Europe, 2018). Plastics receive the name from Plastikos: “it can be shaped”, the main characteristic which makes them so versatile. However, plastic physical properties may change dramatically depending on the polymers and additives they are composed of.

In agriculture, plastics contribute to meet the growing demand for food to sustain the escalating population (Orzolek, 2017; Mormile et al., 2017). The use of plastic films for mulching, a technique that consists of covering the soil to improve microclimate for crop growth, has led to a revolution by increasing yield and allowing cultivation on lands where water and environmental conditions are limiting (Lamont, 2005; Kasirajan and Ngouajio, 2012; Kader et al., 2017; Gao et al., 2019). Plastic mulches retain humidity and heat, prevent soil erosion and weed development. They favor plant development and fruit earliness and quality while decreasing water demand and herbicide and fertilizer requirements, a valuable contribution to sustainable agriculture (Kasirajan and Ngouajio, 2012). Most plastic mulches are made of low-density polyethylene (LDPE), a low cost and easy processing material fulfilling optical and physical properties mulches require: high puncture resistance, mechanical stretch, long durability and water impermeability (Espí et al., 2006). Reported to be used from the 60's, global consumption of LDPE mulches continues to grow worldwide, with an increase of 35% between 2006 and 2017, up to over 2 Mt (Espí et al., 2006; LeMoine and Ferry, 2019). Asia Pacific, the main consumer, is expected to drive the global market in the forthcoming years (Transparency Market Research, 2013; Le Moine and Ferry, 2019). Only in China, a 5-fold increase in plastic mulch consumption between 1991 and 2014 has been reported (Wenqing et al., 2017).

1.2. Plastic mulches: a source of pollution in agroecosystems and for the environment

Massive use of non-degradable LDPE mulches, valued for their high stability, is contributing to aggravating the generation and accumulation of high amounts of plastic wastes, an environmental concern for the agricultural ecosystem. Estimations account for 80% of all plastic waste ever generated to be presently accumulated in natural ecosystems or in landfills (Geyer et al., 2017). Plastic wastes spread out along ecosystem compartments; they have reached wild remote regions far from the areas where they are produced and used, denoting the ease at which they are transported along biogeochemical cycles (Li et al., 2016; Bergmann et al., 2019). Plastic fragments and compounds released from plastic wastes are ubiquitous all over the world, and can be found in the atmosphere, in water resources, as well as into soils and organisms, including humans (Gregory, 2009, Dris et al., 2017, Huerta Lwanga et al., 2017, Schwabl et al., 2019, Wong et al., 2020), with effects on the environment and on human health (Li et al., 2016; Rodrigues et al., 2019; Peng et al., 2020).

Plastic waste release into land systems is estimated to be 4 to 23-fold higher than that which is released into marine environments (Horton et al., 2017). Yet, terrestrial contamination has received less attention than ocean pollution. Risk perception in terrestrial systems, where plastic waste is mostly hidden to the naked eye, often buried underground, is usually less evidenced than in aquatic environments, where plastics may be seen floating and transported over long distances. Yet, the surface plastic waste on waters is estimated to account for only 1% of the total plastic waste in the ocean (Van Sebille et al., 2015, Jambeck et al., 2015). Meanwhile, terrestrial studies face associated technical difficulties in estimating the true amount of plastic waste pollution, such as lack of efficient procedures to detect, separate and quantify plastic wastes in the complex particulate soil matrix (Bläsing and Amelung, 2018; Gangadoo et al., 2020). Research on plastic pollution and on their environmental effects in terrestrial environments is limited and insufficient (Horton et al., 2017; Wong et al., 2020). However, following the worldwide growing environmental concern for plastic pollution, methods are being developing and studies on the environmental impact of plastics on terrestrial ecosystems are emerging (Rillig et al., 2017; Erni-Cassola et al., 2017; Bläsing and Amelung, 2018; Piehl et al., 2018; Corradini et al., 2019; Nelson et al., 2019; Gangadoo et al., 2020).

The fate of plastic waste deposited into aquatic and into soil systems is comparable. In both systems, plastic materials suffer degradation and fragmentation at surface level

before sinking or being buried, respectively. Fragments may then be transported to new ecosystem compartments (i.e. from soil to fresh water, or conversely) or remain and accumulate over time (Van Cauwenberghe et al., 2013; De Souza Machado et al., 2017; Chae and An, 2018; Wong et al., 2020). Once plastics are buried, low temperatures and nutrient and oxygen availability slow down the (bio)degradation rate. As a result, persistence of the plastic fragments in water and soil environments is considerably greater than initially expected (Corcoran et al., 2015; Li et al., 2016).

Recycling rates for mulches are substantially lower than the already low global plastic recycling rate, estimated below 30% (Plastics Europe, 2018). Mulches aggravate the plastic pollution mainly due to (1) the inability to recover all mulch fragments from the soil after use, releasing them and their plastic components to the agricultural soil, and (2) mismanagement of plastic mulch removal and low value of recovered mulch fragments. Plastics remaining at the end of the crop are to be collected and to enter an established waste management system. They can be recycled, used for energy recovery or accumulated in landfills. However, the recycling of agricultural plastic films faces specific difficulties. The cleaning steps required to eliminate soil, plants and agrochemicals adsorbed by the films increase complexity and are costly, and the agrochemicals they may release are harmful to the environment. High quantities of organic chemicals from fertilizers, pesticides and herbicides are traced in soils where plastic mulches have been used (Ramos et al., 2015). All these burdens lead to substantial mismanagement of the used mulches, being thrown to non-controlled environments, ending up in natural ecosystems or being burnt under uncontrolled conditions, releasing organic pollutants into the atmosphere (Levitan, 2005; Briassoulis et al., 2013). On the other hand, plastic mulches incorporated into the soil are continuously exposed to repeated fragmentation, and small fragments are easily dispersed (Ramos et al., 2015; Wong et al., 2020). The sum of plastic fragments in soils, increasing crop after crop, has been reported to affect soil health and decrease crop yield in the medium and long term (Wenqing et al., 2014; Liu et al., 2014; Chae and An, 2018; Gao et al., 2019; Zhang et al., 2020; Hu et al., 2020).

Mulch fragments affect soil density and water infiltration (Dong et al., 2013; Liu et al., 2014; Jiang et al., 2017), and make a significant input of micro and nanoplastics into agricultural soils (Hurley and Nizzetto, 2018, Chae and An, 2018, Qi et al., 2019, He et al., 2018). Repeated mechanical fragmentation of LDPE mulches results in the accumulation of plastic fragments, eventually in the micro and nanoscale (Rillig, 2012), which may be adsorbed and/or absorbed by biological membranes and alter biological functions (De Souza Machado et al., 2017; Ng et al., 2018). Recent studies have shown

potential for plastic fragments to enter the terrestrial food web (Huerta Lwanga et al., 2017) and to inhibit plant growth (Qi et al., 2018).

The chemical composition of plastic films may also affect the soil environment. When in contact with water (rainfall, irrigation, liquid fertilizers), plastic mulch compounds may leach into the soil (Du et al., 2009; Serrano-Ruiz et al., 2020). Special attention has been focused on plastic mulch additives, a diverse group of components incorporated to the polymer backbone that are essential for the final product desired characteristics; additives are easily leached and released into the soil (Clarke and Smith, 2011; Hahladakis et al., 2018). Most common additives in agricultural mulches are plasticizers, dyes, photostabilizers and pro-oxidants (Kyrikou and Briassoulis, 2007; Hayes et al., 2019). Some plasticizers present in many plastics, including mulches (e.g. phthalate esters -PAEs), are hazardous to the environment and for human health (Gómez-Hens and Aguilar-Caballos, 2003; Meeker et al., 2009; Talsness et al., 2009; Sandeep and Rowdhwal, 2018). PAEs are able to migrate from plastic mulches to the soil and then to plants. High levels of PAEs have been reported to accumulate in agricultural fields under continuous plastic mulching, as well as their subsequent absorption and accumulation in cultivated plants (Zeng et al., 2008; Wang et al., 2013; He et al., 2015; Wang et al., 2016). Inhibition of plant development in several cultivated species has been reported (Du et al., 2009; Ma et al., 2014; Wang et al., 2016). They can also migrate into water resources, entailing risks for ecosystems and for human health (Net et al., 2015).

To sum up, to date repeated application of plastic mulches is resulting in the release and accumulation into the soil of a complex mixture of fragments and chemicals with potentially harmful effects. As the world is comprised of interdependent dynamic systems, consequences of using plastic mulches are not restricted to agricultural soils but also threaten natural ecosystems, as elements from one system, including plastic wastes, may migrate from agroecosystems to natural ecosystems (Jambeck et al., 2015; Chae and An, 2018; Wong et al., 2020). Despite the evidence for this, the fate of released plastic mulch fragments, compounds and other contaminants they carry has been scarcely monitored (Steinmetz et al., 2016; Wong et al., 2020). Overall, repeated application of LDPE mulches is leading to a scenario of persistent plastic fragments and chemicals, accumulating year after year into agricultural soils and compromising agricultural soil health, food security and environmental sustainability (Zhang et al., 2020).

1.3. Biodegradable plastic mulches to alleviate plastic pollution

Biodegradable plastics have been proposed to decrease the accumulation of LDPE and

other persistent plastic wastes in the environment. Worldwide, governments and companies are promoting the development of biodegradable plastics, including mulches, while taxing or banning non-degradable plastic utilities (European Commission, Horizon, 2020). Biodegradable mulches must also fulfil properties similar to LDPE ones during their service. BDM are designed to be later tilled into the agricultural soil, where native microorganisms are to break down and use the mulch polymers (Kasirajan and Ngouajio, 2012). They are aimed to save time and cost in collecting and managing plastic fragments and to avoid waste generation.

A variety of biodegradable plastic mulches composed of different polymers and additives are available in the growing market (Miles et al., 2017) and improvements equivalent to LDPE mulches have been already reported for the yield of many crops (Kasirajan and Ngouajio, 2012; Martin-Closas et al., 2017; Briassoulis and Giannoulis, 2018). The use of BDM to reduce agrochemicals in organic farming is also considered in Europe and in the United States. However, their requirement in the United States to be 100% biobased is not accomplished to date by any commercial plastic film, a situation impeding their implementation in organic farming. Their higher cost as compared to LDPE is also limiting expansion (Goldberger et al., 2015; Brodhagen et al., 2017).

The need for substitution of LDPE plastic materials by biodegradable ones has already been sustained, but some concerns are also emerging. Similarly to LDPE mulches, biodegradable ones will undergo fragmentation, and fragment accumulation may have similar physical effects to that of LDPE (Bandopadhyay et al., 2018). Effects of biodegradable micro and nanoplastics on terrestrial environments have scarcely been addressed, and insufficient effort has been focused on in-soil biodegradable microplastic surface functionalities (Shruti and Kutralam- Muniasamy, 2019). In addition, additives incorporated can leach into the soil, together with monomers and intermediates from biodegradation. Consequently, repeated use of biodegradable mulches results in the input to the soil of a wide diversity of compounds accumulated over time, with unknown effects on living organisms (Miles et al., 2017; Chae and An, 2018).

Plastic mulches have revolutionized and improved crop yields, but the environmental impact of plastics is to be mitigated to allow preservation of agricultural and natural ecosystems. While the biodegradation process of BDM has been investigated (Kyrikou and Briassoulis, 2007, Weng et al., 2013, Ardisson et al., 2014, Barragan et al., 2016, Zumstein et al., 2018), long-term effects on plants and on the soil ecosystem have received little attention (Steinmetz et al., 2016). To anticipate and prevent potential

undesired effects on the environment, especially on crops, a comprehensive understanding of the environmental impact and sustainability of continuous incorporation of BDM into the soil is urgently needed (Sintim and Flury, 2017; Shen et al., 2020). The safety of the new materials and of their degradation and biodegradation by products is to be addressed. Focus on the agricultural ecosystem is especially required (Bandopadhyay et al., 2018).

This review is focused on the interaction of biodegradable plastic mulches with the agroecosystem environment and its organisms, the first receptors of the plastic mulches. It updates and discusses the current and yet limited knowledge of the impact of biodegradable plastic mulches on agricultural soils, with emphasis towards their effects on the organisms naturally living in agricultural systems, especially on cultivated plants and on soil microorganisms. Since compounds released from biodegradable plastic mulches are highly susceptible to migrate across ecosystems, including aquatic ecosystems, and of being transported along the food chain (Huerta Lwanga et al., 2017; De Souza Machado et al., 2017; Chae and An, 2018), the assessment of BDM impact on a wide range of organisms from terrestrial and aquatic ecosystems is also required.

2. Properties and composition of biodegradable plastic mulches

2.1. Polymers in biodegradable plastic film production

Plastics films result from the combination of monomers in a polymer backbone, along with low amounts of additives incorporated to meet processing requirements and achieve desired properties in the final product. The monomers building the polymer backbone provide for the main properties of the mulches, such as physical resistance and low water vapor transfer, and for their biodegradability.

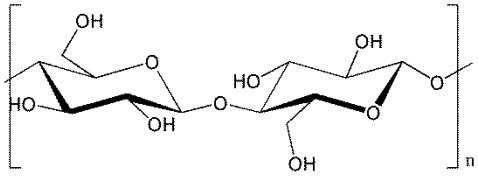
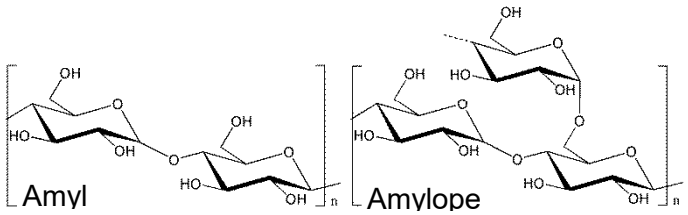
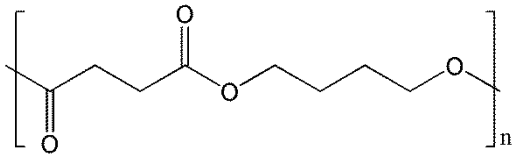
For a plastic polymer to be biodegradable, extracellular enzymes from microorganisms are to break the monomer bonds of the polymer chain, and the released monomers are to be used by microorganisms to growth, eventually resulting in the mineralization of the polymer molecules to their basic compounds, CO_2 (CH_4 under anaerobic conditions), H_2O and minerals, increasing microbial biomass and with no plastic waste remaining into the soil (Luckachan and Pillai, 2011; Kyrikou and Briassoulis, 2007). Agricultural biodegradable plastic mulches must accomplish biodegradation by native soil microorganisms and in the agricultural soil.

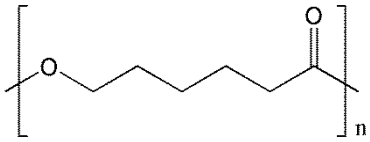
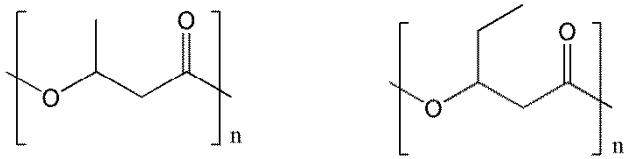
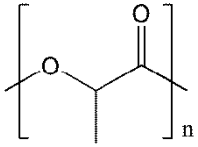
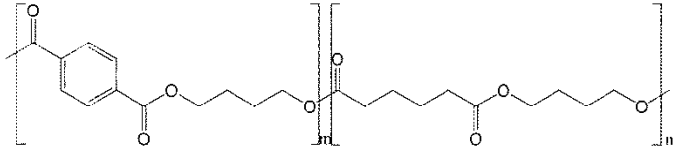
LDPE is a highly stable material estimated to last over years in the environment, with no significant degradation of the polymeric chain (Hurley and Nizzetto, 2018). Hydrophobic

non-polar C\\C single bonds between ethylene molecules highly restrict hydrolysis (Roy et al., 2011). Furthermore, the macromolecular semi crystalline structure of LDPE chains prevents water and oxygen diffusion and hydrolysis, either abiotically or by microorganisms. Some attempts to increase LDPE degradability aim to facilitate oxidation and biodegradation by additivation of prooxidants (Abrusci et al., 2011; Vázquez-Morillas et al., 2016). However, this has only resulted in loss of LDPE mulch physical properties following fragmentation to persist as non-degradable smaller pieces over time (Feuilloley et al., 2005; Briassoulis et al., 2015).

To achieve biodegradation, hydrolysable bonds between the polymer monomers are required, and the released monomers are to be used as energy and carbon source by soil microorganisms to grow. The biodegradation process involves three main steps: (1) microbial colonization of the polymer surface, mainly bacteria and fungi, (2) depolymerization by extracellular microorganism enzymes and, (3) microorganism consumption of the hydrolysis products (Sander, 2019). Factors driving microbial metabolism and affecting biodegradation rate (e.g. oxygen, water and temperature) have already been addressed and reviewed (Kyrikou and Briassoulis, 2007; Brodhagen et al., 2015; Zumstein et al., 2018; Ahmed et al., 2018). Several polymers accomplishing the properties BDM require have been identified, together with their main characteristics, and are already used for production of biodegradable plastic mulches (Table 1). The main commercial biodegradable plastic mulches, together with their performance in crops, have been reviewed by Martin-Closas et al. (2017).

Table 1. - Classification, chemical structure and characteristics of the main biodegradable polymers used in production of agricultural biodegradable plastic mulches.

Polymer	Chemical origin	Biodegradation in soil [a]	Characteristics [b]
Polysaccharides			
Cellulose 	Natural (Plants, some Bacteria)	Moderately high	One of the most abundant naturally occurring organic polymers. Soil bacteria and fungi are the main cellulolytic microorganisms. Obtaining cellulose from plants requires removing other compounds (e.g. pectin, lignin, resins, etc.). Chemical transformation to cellulose derivatives is required to be used for plastic production.
Starch 	Natural (Plants)	High	Major carbohydrate reserve in higher plants. Used in blends with other polymers to enhance biodegradability. Native starch is moisture-sensitive and brittle. Chemically modified into thermoplastic starch to gain hydrophobicity for plastic films production.
Aliphatic polyesters			
Poly(butylene succinate) (PBS) 	Synthetic	Moderate	Mechanical properties similar to LDPE. Crystallinity restricts the action of degrading enzymes. Often blended with other polymers (i.e. starch, PLA) or copolymers (i.e. polybutylene succinate adipate) to decrease crystallinity and enhance biodegradability.

Poly(ε-caprolactone (PCL) 	Synthetic	Moderate	Partially crystalline and hydrophobic. Mostly used in blends with biopolymers (e.g. starch, PHB or PLA), to increase biodegradability.
Poly(3-hydroxybutyrate) (PHB)/Poly(3-hydroxyvalerate) (PHV) 	Natural (mainly Bacteria)	Moderate	Energy reserve for certain microbial groups. PHB is most abundant. To avoid fast thermal degradation and brittleness, PHB is often blended with PHV or other biodegradable polymers (e.g. PLA). Use limited by high production costs.
Poly(lactic acid) (PLA) 	Natural or Synthetic	Low	Main source: glucose fermentation from corn starch hydrolysis. As homopolymer, it is brittle and highly stiff. Used in blends with other polymers due to its strength and low cost. Low in-soil (bio)degradation due to high temperature and humidity conditions required.
Aromatic polyesters			
Poly(butylene adipate terephthalate) (PBAT) 	Synthetic	Moderately low	The most used copolymer for agricultural plastic mulch production. Comparable to LDPE films; aromatic groups enhance mechanical properties, but are not prone to biological degradation and have to be limited to allow full biodegradation Aliphatic chains contribute to biodegradability. Often blended with starch or PLA to reduce stiffness and gain hydrophilicity.

Adapted from: Brodhagen *et al*. 2015, Bastioli 2014, Niaounakis 2015, Künkel *et al*. 2016.

2.2. Additives in plastic mulch production

Plastic mulch additives are mainly intended to facilitate mechanical installation of the material (e.g. elasticity), to achieve an efficient functional performance during the crop cycle (e.g. mulch stability), to provide specific mulch characteristics (e.g. color), and to facilitate the *in situ* (bio)degradation, into the soil, following the mulch use. Mulch additives, a diverse group of chemicals (Table 2) that do not chemically bind to the plastic polymer, retain a high potential to migrate when in contact with water.

Table 2. - Additives most commonly used in the production of plastic film mulches.

Additives	Use
Slip agents Fatty acid amides [a] Glycerol oleates/stearates [a] Saponified fatty acids [a]	Reduce the polymer surface friction to facilitate processing. Lubrication to avoid mulch films adhesion to surfaces and to itself when rolled during storage.
Stabilizers HALS (hindered amine light stabilizers) Phenolics Organophosphites Benzophenone [a]	Protection of mulch films to UV radiation and to atmospheric conditions. UV radiation generates highly reactive free radicals on polymers, which may lead to the film breaking due to the incorporation of atmospheric oxygen atoms. Most common photoprotective mechanisms are UV radiation screening and absorption.
Dyes Carbon black (black) TiO_2 (white) Fe_2O_3 (red) $CaCO_3$ (white)	Carbon black, TiO_2 or Fe_2O_3 are also stabilizers due to their action as UV radiation screeners. Dark colors inhibit weed growth under the plastic mulch. White or clear colors provide greater soil warming than dark films, but poor inhibition of weed growth. Other colors or color combinations are used for specific purposes (e.g. green colors for aesthetic purposes).
Fillers Clays Carbon black Silicates Glass $CaCO_3$ and talcs Polymers (e.g. starch) [a]	Increase bulk at low price and enhance a diversity of properties of the plastic mulch, mainly stiffness, thermal and photo stability, and abrasion resistance. Examples of use: carbon black as an aid in cross-linking, talc to improve stiffness and tensile strength.
Plasticizers Phthalate esters Glycerol [a] Sorbitol [a] Tri-ethyl citrate [a] Oligomers	Improve processability by reducing brittleness of some polymers. Improve flexibility and enhance impact resistance of the plastic films.
Nucleating agents and clarifiers Sodium benzoate TiO_2 $CaCO_3$ Amide compounds Phosphate metal salts Basic inorganic aluminium compounds Sorbitol derivatives [a]	Improve mechanical properties by promoting crystallization of the polymer in many small nuclei (spherulites). Few and large spherulites exhibit inter-spherulithic cracks.

Adapted from Vieira et al. 2011, Ambrogi et al. 2017, Hahladakis et al. 2018 and Hayes et al. 2019. [a] Bio-based additives.

After biodegradable plastics were introduced into the market, interest was raised in regards to biobased and biodegradable additives with low toxicity and good compatibility with biodegradable plastics. They have been reviewed for substitution of additives questioned for their toxicity potential (Vieira et al., 2011) (Table 2) and they are attracting interest for the manufacturing of biodegradable plastics as an alternative to the oil-based, synthetic additives (Ambrogi et al., 2017). However, although these additives are presently incorporated to mulches, additives are frequently similar both for biodegradable and for nonbiodegradable films; specific additives used for the manufacturing of the diversity of biodegradable plastic mulches remain mostly insufficiently identified.

Additives make a small contribution to the final mulch composition. When they comprise <1% (w/w) of the biodegradable mulch composition there is no requirement for proving their biodegradability (EN 17033, 2018), thus passing mostly unattended from the environmental safety perspective. Additionally, under this norm, even substances listed as of “Very High Concern” are allowed at 0.1% maximum as part of the biodegradable plastic mulch final weight, some mulch additives remaining under this threshold limit. Because biodegradable mulches are intended to (bio)degrade and to release their compounds *in situ*, into the agricultural soil, assessment of the additives environmental impact needs to be addressed.

3. Environmental dynamics in the use of biodegradable plastic mulches

Additives and monomers from all plastic mulches can migrate to soil during their use (Fig. 1). LDPE films are to be retired from the soil surface at the end of the crop cycle, and the many plastic residues left will remain stable for decades (Briassoulis et al., 2015). Conversely, the tilling of biodegradable mulches into the agricultural soil to facilitate biodegradation is an open door to potential environmental impacts. During and after their use, they may release compounds entering in close contact with soil organisms and plants. Within the agricultural plastic mulch cycle, three differential stages can be established (Fig. 1).

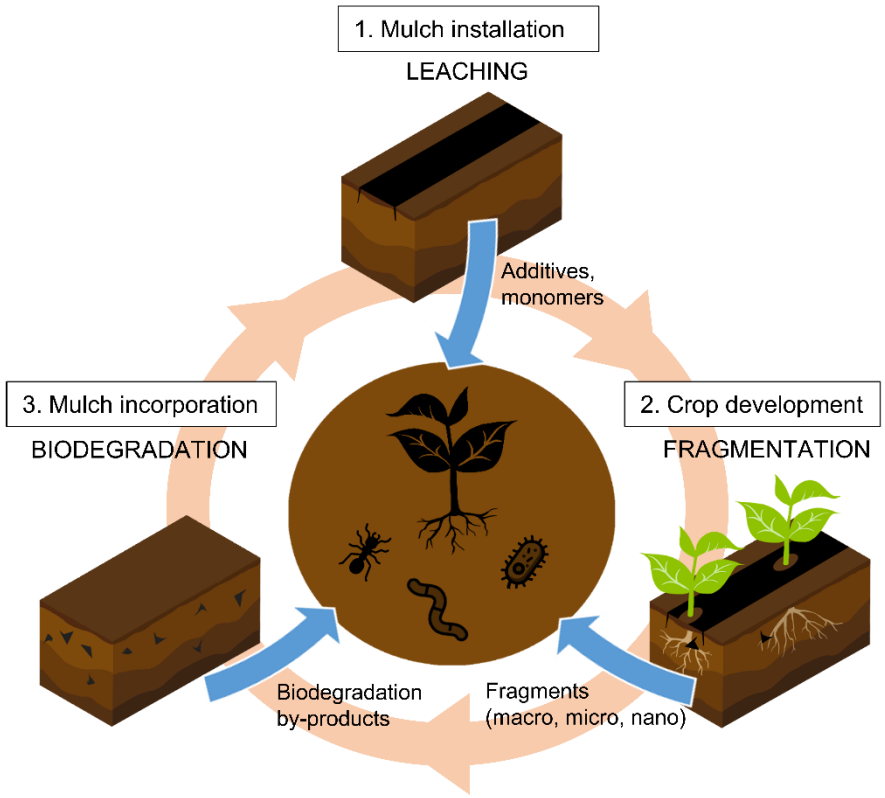


Figure 1. - Agricultural cycle of biodegradable plastic mulch films during time of use.

3.1. Stage 1. Mulch storage and installation in the field

Biodegradable plastic mulches remain stable under convenient storage conditions, including dry conditions, low temperature, and indoors storage in the dark. An impermeable cover further protects BDM from photo, oxo and biodegradation. PBAT-PLA (polybutylene adipate terephthalatepolylactic acid) film integrity and properties proved to be stable after one (Hayes et al., 2017) and two years storage (Künkel et al., 2016).

Plastic films can be installed manually in small vegetable cultivated areas, but mechanical installation is the norm for cultivated fields. Mulches are pulled and stretched while the film edges are buried into the soil. Biodegradable films hold equivalent mechanical properties to LDPE films but they are usually more sensitive to stretching forces; equipment for LDPE mulch installation is amenable to be used for biodegradable plastic mulches by adjusting tension for the film's optimal performance. Excessive tension during their installation may entail stress increasing the likelihood of tears later affecting the prospective deterioration pattern, while loose films are susceptible to wind breaks (Martin- Closas et al., 2017).

3.2. Stage 2. Mulch during the crop cycle

After installation, plastic mulches are exposed to conditions affecting their structure and properties, which includes climate (rainfall, wind, solar radiation), irrigation, agrochemicals (fertilizers, herbicides, pesticides), laboring, soil organisms, cultivated plant growth, weed development, etc. Both biodegradable and LDPE mulches may release fragments and chemicals into the soil any time during their use.

Water from rainfall, irrigation or from agrochemical' solutions may leach additives and polymer compounds into the soil. Migration of additives and monomers from biodegradable mulches is significant even after short exposure to water solutions (Serrano-Ruiz et al., 2020), while LDPE mulches only release glycerol derived molecules or other plasticizers (Du et al., 2009; He et al., 2015; Lü et al., 2018). Additionally, radiation and atmospheric oxygen affect the carbon structure of the backbone polymer, increasing the film brittleness and susceptibility to fragmentation (Ammala et al., 2011). Eventually, mechanical stress (labor, rain, etc.) produces breaks and tears, the onset of fragmentation and the beginning of in-soil accumulation of fragments. Concurrently, agrochemicals commonly sprayed on mulched fields may be adsorbed and/or absorbed to the plastic fragments, increasing pesticide residue mixtures already present in the soil and toxic to the soil biota (Ramos et al., 2015; Silva et al., 2019).

In addition, plastic mulches are non-sterile; they bear and release microbes into the agricultural soil system. Specific microorganisms associated to a diversity of chemically different plastic films have been already suggested (Kirstein et al., 2019; Zhang et al., 2019). On its turn, immediately after installation, native soil microorganisms may colonize the mulch surface and initiate biodegradation, releasing monomers and by-products into the soil. While LDPE mulch fragmentation and degradation is an extremely slow process that is estimated to take hundreds of years (Ohtake et al., 1998; Briassoulis et al., 2015), a few months is enough for biodegradable plastic mulches to exhibit substantial deterioration (Touchaleaume et al., 2018).

Over time, weathering of plastic mulches intensifies. As a significant share of the additives are leached into the environment, the film brittleness increases. Moreover, parallel to crop development, weed growth contributes to tears in the mulch, which concurrently with other agents (e.g., wind, plant growth), stripes it into fragments. All factors together contribute to further mulch breaking into smaller pieces. The higher the fragmentation, the higher the mulch exposure to abiotic degrading factors, to soil microorganisms and to oxidation processes facilitating progressive release of mulch

components.

3.3. Stage 3. Mulch after the crop harvesting: incorporation into the soil

Biodegradable mulches are tilled into the soil shortly after the crop cycle ends; later on, a new crop and mulch cycle will start. Mulch biodegradation by soil microorganisms becomes massive and the buried mulch macro, micro and nanofragments continuously release additives, monomers, and by-products from the biodegradation process. These components accumulate and continue to biodegrade until their complete mineralization to CO_2 and H_2O. Iterative field mulching, a common practice occurring several times a year in many intensive cultivation areas with mild-winter temperate climates, results in repeated mulch input to the soil. Thus, high *in situ* biodegradation rate is crucial to prevent accumulation of biodegradation intermediates.

For plastic mulches to be considered biodegradable, criteria from international standards (EN 17033, 2018) require films or their base material in its primary form(i.e. powder) reach at least 90% biodegradation in < 2 years in natural topsoil from an agricultural field or forest, in aerobiosis at 20–28 °C. However, even when BDM accomplish these requirements, a fraction of the BDM may accumulate in soils when continuously applied (Miles et al., 2017). Several studies addressing the in-soil biodegradation under controlled laboratory conditions demonstrate the process to be strongly dependent on the material nature, fragment size and on the incubation conditions, such as the temperature, pH, humidity, oxygen level, and nutrient availability (Ardisson et al., 2014; Barragan et al., 2016; Al Hosni et al., 2019; Tosin et al., 2019). However, in natural settings these conditions vary strongly depending on location, climate, soil type and depth (Li et al., 2014a; Haider et al., 2019).

Field studies are scarce. In one of them <4% of the initial weight of a biodegradable starch-based mulch film was found in the soil after being buried for one year (Kapanen et al., 2008). Contrasting results were obtained by Sintim et al. (2020), who after 3 years registered between 26 and 83% degradation depending on the nature of the biodegradable material, soil type and climate. Climatic conditions are suggested to play a major role, with higher biodegradation rates in warmer climates in contrast to cooler environments (Sintim et al., 2020). After four years of repeated application of BDM, Ghimire et al. (2020) reported recovering macroscopic fragments; the amount recovered was decreasing as compared to the total amount of mulch applied in the whole period, indicating macroscopic fragments were not accumulating, but smaller fragments were not collected. There is a need of methodologies to assess the in-soil field mulch

biodegradation and to develop sampling methods able to efficiently collect fragments smaller than the macrofragments reported in Kapanen et al. (2008), Sintim et al. (2020), and Ghimire et al. (2020).

Research on the persistence of micro and nanoplastics in agricultural soils is still substantially limited (Kumar et al., 2020). Nevertheless, biodegradable plastics undergo fragmentation more intensively than conventional plastics in the field, and due to high surface exposition to microorganisms, their micro and nanofragments are expected to be fast biodegraded (Tosin et al., 2019). However, to our knowledge, field studies about micro and nanoplastics accumulation from biodegradable mulches in agricultural soils have not been reported. In summary, the in-soil persistence of fragments and compounds released from biodegradable mulches needs to be addressed, taking into account the soil characteristics and the climate conditions of the agroecosystems where a specific biodegradable polymer is used.

4. Ecotoxicity assessment of biodegradable plastic mulches

The ecotoxicity assessment of a marketable product is essential to prevent environmental and health risks. The assessment is specifically relevant for biodegradable plastic mulches, materials completely and repeatedly incorporated into the agricultural soil (Fig. 1). In contrast with LDPE films, which after their end-of-life cycle are to be retired from the field and enter the waste management system, BDM, i.e. all their compounds, are released into the soil and put in direct contact with native soil organisms and with cultivated plants.

Use and mismanagement of LDPE mulches is polluting agricultural soils with plastic fragments buried into them, with an overall impact that differs from that of biodegradable materials. Nevertheless, since effects associated with the presence of plastic fragments are mostly independent from the nature of the mulch, LDPE films' physical impact evaluation is considered; results from research on additives used in agricultural LDPE plastic mulches are also integrated because they are most frequently analogous to the ones in the biodegradable mulches (Table 2). Impacts from individual chemicals participating in the mulch composition are relevant, but also those coming from the end product; both together are aimed to elucidating chemical sources associated with potential harmful effects, and to contributing in the design and selection of safe biodegradable, low-impact materials.

The research on ecotoxicity assessment of biodegradable plastic mulches and of their

components has typically been evaluated by their adherence to biodegradation and ecotoxicity standards (Fritz et al., 2003; Rychter et al., 2006; Ardisson et al., 2014; Muroi et al., 2016). In the last six years, along with the escalation in diversity, availability, commercialization and use of biodegradable plastic mulches, there has been a rising interest towards deeper understanding of the effects of biodegradable plastic mulches on agroecosystem organisms (Li et al., 2014a; Sintim and Flury, 2017; Bandopadhyay et al., 2018), but research in this area is still in its infancy. Barely a few publications have tested for effects of biodegradable mulches on a small number of plant species and on soil microorganisms. To date, studies have been limited to those conducted by Fritz et al. (2003) and Sforzini et al. (2016), which tested, as recommended by ecotoxicity standards, a variety of model organisms representative of soil and aquatic ecosystems. Yet, most of these assays report biodegradable plastic mulches or some of their components to produce specific effects on living beings (Tables 3 and 4). Main findings are discussed in the following sections.

4.1. Plants

Analyses of toxicity from biodegradable mulches on plants have been mainly carried out by monitoring plant growth in soils containing biodegradable plastic film fragments (Fig. 1. Stage 3) (Fritz et al., 2003; Rychter et al., 2006; Muroi et al., 2016; Sforzini et al., 2016; Qi et al., 2018) or on aqueous extracts from soils containing biodegradable plastic films (Palsikowski et al., 2018; Souza et al., 2020). Tests based on ecotoxicity standards, sowing seeds in soils where ca. 1% (w/w) of plastic fragments were previously buried for 6–7 months, did not find significant effects of the material degradation on barley, cress, rape and sorghum germination rate, nor on plantlet dry mass (Rychter et al., 2006; Sforzini et al., 2016, and Muroi et al., 2016). However, Fritz et al. (2003) found that cress, millet and rape decreased plant biomass by 20–50% when in soils with 2% (w/w) film fragments from a polyesteramide mulch (Table 3).

Other comprehensive studies have widened the scope for plant growth and development evaluation in interaction with biodegradable mulches, some of them reporting on significant effects (Table 3). Through a comprehensive experiment, Qi et al. (2018) demonstrated wheat vegetative and reproductive growth to be affected by soils containing 1% (w/w) mulch fragments, both LDPE and BDM. The effects varied depending on plastic composition and on fragment size, with stronger inhibitory effects from biodegradable plastics than from LDPE mulches. Microfragments (50 to 1000 μm) produced slightly stronger effects than macrofragments (ca. 4–10 mm), while

earthworms in the soil alleviated the overall effects of the plastic fragments. In contrast, Palsikowski et al. (2018) did not observe inhibitory effects of biodegradable films on onion plant growth, nor cytotoxic, genotoxic or mutagenic effects in the meristematic cells of plants exposed to soil aqueous extracts containing 2% (w/w) Ecoflex® (PBAT) film fragments previously biodegraded in soil for 6 months. However, upon exposure to a 25/75 blend of Ecoflex® with PLA (polylactic acid), one of the two tests performed identified significant chromosomal aberrations in the onion cells, which deserves further in-depth research. With a similar approach, Souza et al. (2020) found soil aqueous extracts of Ecoflex® mulch, either alone or in combination with UV radiation stabilizers, and both before and after 6 months of biodegradation buried in the soil, did not affect lettuce germination and early growth, nor did they produce genotoxic or mutagenic effects on onion; however, the tested concentration for the mulch was significantly low, 0.04% (w/w), and it does not allow to progress further.

It is worth noting that Fritz et al. (2003), Sforzini et al. (2016) and Qi et al. (2018) tested the final commercially available product, while Rychter et al. (2006), Muroi et al. (2016) and Palsikowski et al. (2018) tested polymer blends without the incorporation of additives, and thus their results exclusively refer to the polymer (bio)degradation, while the effects of the complete final products as used in real conditions cannot be inferred.

Potential impacts of biodegradable plastic mulches on plant growth have been addressed not only after burying the used mulch fragments into the soil but also from the beginning of the mulch installation (Serrano-Ruiz et al., 2018) (Fig. 1. Stage 1). Compounds from unused biodegradable plastic mulches are released into water solutions which, depending on the nature of the mulch, resulted in substantial effects on plants, including abnormal and limited lettuce and tomato root growth and morphology subsequently modifying aerial part development. The plant physiology was also affected in both species, as shown by the increase in proline, a plant stress marker. Similar to the previous findings of Qi et al. (2018) in wheat, the effects of LDPE were minor as compared with those of the biodegradable mulches: LDPE mulch aqueous extracts did not alter lettuce plant growth and had little effect on tomato plants.

An alternative approach to identify effects of biodegradable mulches on living organisms is to test the ecotoxicity of targeted individual polymers, the compounds and additives constituting the mulch, as well as the biodegradation by-products. Following this strategy, exposure of *in vitro* tomato and lettuce plants to adipic, succinic and lactic acids, and 1,4-butanediol monomers (5 to 500 mg L−1), resulted in dose response effects, with

adipic acid showing the strongest inhibitory effects on both plant species; all compounds increased proline both in tomato and in lettuce plants, demonstrating on the effect to be the alteration of plant physiology (Martin-Closas et al., 2014). All these four monomers are readily released into water, not only during the biodegradation of the already used mulch when buried in soil, but also from unused biodegradable mulches (Serrano-Ruiz et al., 2020) from the beginning of the plastic mulch installation (Fig. 1, Stage 1). However, concentrations identified in the corresponding water extracts were substantially lower to the ones producing effects on plants, and interactive effects of binary or ternary combinations have not been attempted.

Studies on microplastics released from biodegradable plastic films after their first contact with water (Shruti and Kutralam-Muniasamy, 2019) and continuing throughout their use and burial have only recently been tested for phytotoxicity. Boots et al. (2019) reported perennial ryegrass germination and plant height to decrease in soils with buried PLA microplastics (0.1% w/w), and there was evidence for alterations on the photosynthetic system by changes in the chlorophyll a/b ratio. Similarly, maize plants decreased shoot biomass and chlorophyll content, and arbuscular mycorrhizal fungi communities associated with the plant roots were altered when grown in soils with PLA microplastics (10% w/w) (Wang et al., 2020). In both plant species, inhibitory effects exerted by LDPE microplastics were lower, suggesting microplastic effects being mainly associated to the chemical composition of the biodegradable plastics, but also partly to the physical presence of microplastic fragments, biodegradable or not. Smaller fragments, nanoplastics, have the potential to permeate biological membranes, thus entering plant tissues and subsequently transported along the food chain (Ng et al., 2018; Rillig et al., 2019). Although we have not identified phytotoxicity studies from biodegradable mulch nanoplastics, there is evidence that nanoplastics may be internalized by plant roots and transported to shoots, stressing plants and altering their growth (Giorgetti et al., 2020; Lian et al., 2020).

Hahladakis et al. (2018) extensively reviewed chemical additives on plastics, including migration from plastic mulches to the soil when in contact with water. Among them, phthalate esters plasticizers have attracted the most attention. They may act as endocrine disruptors and are suspected of promoting genetic mutations and cancer (Sandeep and Rowdhwal, 2018); some companies avoid their use in biodegradable mulches (Novamont, 2018; Ambrogi et al., 2017) and several countries (Europe, Canada, US, China) (European Commission, 2017, US Environmental Protection Agency, 2019) consider them high priority pollutants. However, their low cost and broad application

range as compared to biobased alternatives, together with lack of strict regulations in some countries, results in the continued use of phthalates (Ghosh, 2017). In addition, the fate and toxicity of additives that substitute phthalates are still largely uncovered. Although we are not aware of papers on effects from PAEs migrating from biodegradable plastic mulches to leachates; studies from migrating LDPE mulch additives, frequently analogous to those added to biodegradable materials, can provide an adequate ecotoxicological insight for additives. Biodegradable plastic mulches are expected to leachate additives to the soil, presumably undergoing biodegradation to a higher extent than those of LDPE mulches, simply because BDM are not retired from the agricultural soil.

A few ecotoxicity studies on PAEs additives in agroecosystems have been reported. DEHP (di-(2-ethylhexyl) phthalate) proved to migrate from LDPE plastic films covering the soil to the soil itself and then to 10 cultivated vegetable species (Du et al., 2009). The level of DEHP uptake was dependent on the species and on the part of the plant in question, and DEHP accumulation in the edible parts of Chinese cabbage and wax gourd was close to daily intake threshold recommended by the US Environmental Protection Agency. Uptake of PAEs additives has also been reported in other plant species, including turnip, eight maize cultivars and three forage plant species (Li et al., 2014b; Kong et al., 2018), with concomitant limitations in plant growth. Increased nitrate levels in plants exceeding thresholds established by standards determining safe human vegetable consumption (AQSIQ, 2001) have also been associated to PAEs exposure (Kong et al., 2018). DEHP and DBP (di-n-butyl phthalate) likewise altered plant growth and development in a diversity of crops (rape, wheat, alfalfa, perennial ryegrass, radish, cucumber, oat and onion) (Ma et al., 2013; Ma et al., 2014). In addition, PAEs (DBP) have been proposed as disruptors of plant development through inducing changes in endophytic bacteria from leaves and roots (Kong et al., 2018; Kong et al., 2019), and in the bacterial and fungal communities in the phyllosphere, the bacterial habitat on the plant surface, where DBP specifically increased saprotrophs and plant pathogens (Kong et al., 2019).

Overall, the biodegradable plastic mulches together with their different compounds (monomers and additives) have shown potential to affect plant growth and physiology. Studies on the impact of biodegradable plastic mulches on plants are still scarce, but available research suggests their effects on plant development are likely greater than those of LDPE mulches. The BDM composition plays a major role; on a given plant species effects are strongly dependent on the mulch composition. The size of the mulch

fragments into the soil also plays a significant role, with smaller fragments showing stronger effects than macrofragments. There is also evidence that cultivated plants experience an inhibition of growth and alter their physiology due to additives used in the plastic mulches, such as PAEs. Presence of PAEs in biodegradable mulches remains an undisclosed possibility for many of the available products. Furthermore, uptake of PAEs by cultivated plant species has been associated to decreasing crop yields, and consumption of vegetables grown with plastic mulches containing PAEs may entail risks for humans.

Table 3. - Effects of biodegradable plastic mulches and components on plant growth and development.

Material (polymer blend / compound), concentration	Assay	Plant Species	Exposure time	Main effects reported	References
Mulch fragments (poly ester-amide) 2 % w/w	Pots, plastic buried in soil	Rape Cress Millet	ca. 2 months	Decreased cress, millet and rape plant biomass.	Fritz *et al*. 2003
Mulch fragments (Macro: 4 – 10 mm / Micro: 1 mm – 50 µm) (Pullulan, PET, PBT) 1 % w/w	Pots, plastic buried in soil	Wheat	2 and 4 months	Decreased plant biomass, fruit biomass, leaf nr., leaf area, stem diameter. Microfragments had stronger effects.	Qi *et al*. 2018
Mulch fragments aqueous extracts 1x1 cm (PBAT, PBAT-PLA, PBAT-starch, PHB) 1.6 % w/v	*In vitro* culture	Lettuce Tomato	3 and 4 weeks (tomato and lettuce, respectively)	Lettuce: some decreased germination and shoot biomass, and modified root development. Increased chlorophyll and proline in leaves. Tomato: some decreased plant biomass, chlorophyll content, modified root development. Increased proline in leaves.	Serrano-Ruiz *et al*. 2018
Lactic acid, adipic acid in aqueous solution 5-500 mg L^{-1}	*In vitro* culture	Lettuce Tomato	3 and 4 weeks (tomato and lettuce, respectively)	Lettuce: decreased germination (adipic ac., 500 mg L^{-1}); decreased growth (adipic and lactic acids). Tomato: decreased growth (adipic ac., 50 and 500 mg L^{-1}); increased growth (lactic ac. and 1, 4-butanediol).	Martin-Closas *et al*. 2014
Microfragments (PLA) 0.1 % w/w	Pots, plastic buried in soil	Perennial ryegrass	1 month	Decreased germination, shoot length. Increased chlorophyll a-b ratio.	Boots *et al*. 2019
Microfragments (PLA) 0.1 – 10 % w/w	Pots, plastic buried in soil	Maize	1 month	Decreased plant biomass. Decreased chlorophyll content.	Wang *et al*. 2020
Mulch films (LDPE), 32 cm diameter disks	Pots, plastic film covering the soil surface	Wax gourd Cucumber Pumpkin Chinese cabbage Bitter gourd Lettuce	6 weeks	All plant species: uptake of DEHP released from the film to the soil. Wax gourd, cucumber, pumpkin and Chinese cabbage accumulated the highest DEHP concentrations.	Du *et al*. 2009

		Hot pepper Towel gourd Water spinach Tomato			
DEHP 117 ± 5.2 mg Kg^{-1}	Pots, soil spiked with DEHP	Alfalfa Ryegrass Teosinte Maize (7 cvs.)	40 days	All plants species: uptake DEHP.	Li *et al*. 2014b
DEHP and DBP 1-500 mg Kg^{-1}	Petri dish, soil spiked with PAEs	Rape	14 days	Both PAEs decreased plant biomass, root and shoot elongation. Increased proline and ascorbate peroxidase activity.	Ma *et al*. 2013
DEHP and DBP 5-500 mg Kg^{-1}	Petri dishes, soil spiked with PAEs	Radish Cucumber Onion Alfalfa Perennial ryegrass	14 days	All species: DBP inhibited root elongation, seedling growth and biomass. Alfalfa: DEHP inhibited root elongation, seedling growth and biomass. Both PAEs increased MDA content and altered chlorophyll content of all the species except alfalfa and perennial ryegrass.	Ma *et al*. 2014
DBP 50-500 mg Kg^{-1}	Pots with spiked DPB in soil	Rapeseed	1 month	DBP accumulated in plant tissues. All concentrations decreased plant height and weight. Physiological alterations: nitrate increased and soluble sugars decreased with increasing of DPB concentration. Changes in phyllosphere microorganisms (bacteria and fungi)	Kong *et al*. 2018; Kong *et al*. 2019

PET, polyethylene terephthalate; PBT, polybutylene terephthalate; PBAT, poly(butylene adipate terephthalate); PLA, poly(lactic acid); PHB, poly(3-hydroxybutyrate); LDPE, low-density polyethylene; DEHP, di-(2-ethylhexyl)phthalate; DPB, dibutyl phatalate; PAEs, phthalate esters; MDA, malon-dialdehy

4.2. Soil microorganisms

As for plants, the analysis of biodegradable plastic mulch effects on soil microorganisms has been linked to biodegradation of plastic fragments tilled into soil (Fig. 1. Stage 3). The biodegradation of these materials is intended to proceed through being consumed by soil microorganisms; consequently, they have potential to alter soil microbial communities. However, biodegradable plastics and microbial soil communities may readily start interacting significantly earlier, shortly after the film installation process (Fig. 1. Stage 2), at the area of the mulch edges buried and also at the underside of the mulch surface that is in direct contact with the soil. Mulch additives can also migrate from the films to interact with soil microorganisms. Consequently, the assessment of the impact of biodegradable plastic mulches and their compounds on soil health is to be monitored not only after tilling the mulches into the soil but from the onset of the mulch installation.

Biodegradable plastic mulches have been shown to interact with the agricultural plant-soil system and to change soil microbial communities both when they are in use and following their incorporation into the soil after the crop is harvested. In both cases, short-term changes (weeks to months) appear (Table 4). After only two weeks of a PBSA (polybutylene succinate adipate) plastic film covering on the soil surface, drastic changes occurred in fungal soil populations, with increased abundance of *Aspergillus* spp. and *Penicillium* spp., and of the protozoan *Acanthamoeba* spp. (Koitabashi et al., 2012). Equally, in two different soils, a PBAT-PLA mulch on a cotton crop for seven months not only changed soil bacteria abundance but also the specific species distribution (Zhang et al., 2019). The changes were soil-dependent, where one of the soils became enriched in bacterial groups able to degrade exogenous substances, such as aromatic esters, while the other soil sample decreased the abundance of these bacterial groups.

Changes in the agricultural soil microbiome have also been reported to occur after the integration of mulch into the soil. Four weeks after burying PBSA film fragments into soil, soil fungi degraders and the soil enzymatic activity for film degradation increased (Yamamoto-Tamura et al., 2015). Qi et al. (2020) demonstrated that bacterial communities in the rhizosphere, the soil in close contact with plant roots, are modified after four months of growing plants in a soil containing buried biodegradable plastic mulch fragments. Several bacteria genera were promoted, including *Bacillus*, *Variovorax* and *Clostridium*, while others decreased; effects also depended on the size of the fragments. Similarly, Muroi et al. (2016) found Ecoflex® (PBAT) mulch fragments buried for seven months increased the abundance of soil fungal and bacterial communities,

specifically that of the fungal phytopathogen *Setophoma terrestris*. Substantial changes also occurred in soil that was in direct contact with the film surface. Fungal diversity changed drastically and enriched in members of the Ascomycota phylum. Two not formerly present bacteria genera that are known to grow on plastic films and to form biofilms were detected in soil, *Caenimonas* and *Hyphomicrobium*. While specific effects of BDM depend on a wide array of factors, including the nature of the mulch, general effects of biodegradable plastic mulches on the agricultural environment may be considered. A diversity of commercial biodegradable plastic mulches has been shown to increase soil microbial activity when their fragments were buried in soil for 6 months (Barragan et al., 2016).

Soil and soil-plant system microbial communities are also modified by components demonstrated to be released from plastic mulches to the soil in a timeframe of only a few months. Poly-vinyl chloride plastic mulch fragments buried into the soil for 2 months results in migration of PAEs, decreasing soil microbial activity and diversity (Wang et al., 2016). Bacterial soilα-diversity decreased and the community structure was altered from the first day after DBP spiking in the soil (Kong et al., 2018). DBP also extensively affected soil fungal communities by changing α and β diversity, decreasing mutualistic relationships among fungal species and causing the destabilization of the ecological network structure (Kong et al., 2019).

Research on the impact of biodegradable mulches and their components on soil microorganisms beyond a year is scarce, but significant. Long-term effects have analyzed microbial biomass and extracellular enzymatic activities. One year after Mater-Bi® (PBAT-starch) mulch had been tilled into the soil Kapanen et al. (2008) did not find changes in the nitrification potential of soil ammonia-oxidizing bacteria, a soil health indicator. Equally, soil bacterial abundance and community structure did not change after two-year of PBSA and poly-caprolactone mulching, as compared to either LDPE or no mulch (Masui et al., 2011). In contrast, other reports showed that one and two year use of biodegradable mulches alter the soil microbiome. One year after biodegradable mulching, Moreno and Moreno (2008) identified higher soil microbial biomass carbon and soil organic matter mineralization than when LDPE films were used. Similarly, after two years of comprehensive field studies, both Li et al. (2014a) and Sintim et al. (2019), reported biodegradable mulches to affect soil quality indicators, including microbial growth and activity, with effects varying among locations, season and production systems. In a two year experiment, Bandopadhyay et al. (2020) found only minor effects of biodegradable and LDPE mulch treatments on soil microbial and fungal community

enrichment and on their functions, with location and season being more relevant drivers of the microbial communities than mulch type. However, soil communities were screened only twice per year, before and after harvest, while effects during time following harvest were not monitored. To our best understanding, long-term impact studies exceeding two years have not been reported.

In summary, knowledge on the impact of biodegradable plastics on microbial communities is mostly based on a few short-term (months) experiments. Results suggest biodegradable mulches may change soil microbial communities, both fungal and bacterial, not only after they are buried into the soil but also previously, when covering the soil surface around the crop. Further research is required to determine whether specific microbial groups are preferentially promoted against others as a result of BDM use. Long term effects on agricultural soil health, after iterative use of biodegradable plastic mulches throughout crop seasons and years, remain largely undisclosed. Deeper insight into the effects of biodegradable mulches on soil microbial communities' diversity and functions (i.e. nutrient cycling), which contribute to a large extent to the sustainability of agricultural ecosystems, is also urgently required (Bender et al., 2016; Bandopadhyay et al., 2018).

Table 4. - Effects of biodegradable plastic mulches and components on microorganisms.

Material (polymer blend / compound), concentration	Assay	Microorganisms	Exposure time	Main effects reported	References
Mulch fragments (PBSA) 2x2 cm 4 pieces/40 g soil	Petri dishes, plastic buried in soil	Soil Fungi	4 weeks	Increased PBSA degrading fungi and esterase activity.	Yamamoto-Tamura *et al*. 2015
Mulch fragments (PBSA and PBS) 10x14 cm 1 piece/70 g soil	Plastic film covering the soil surface	Soil Fungi	1 month	Increased soil population of filamentous soil fungi and *Acanthamoeba* spp.	Koitabashi *et al*. 2012
Mulch fragments (Macro: 4 – 10 mm / Micro: 1 mm – 50 µm) (Pullulan, PET, PBT) 1 % w/w	Pots, plastic buried in soil	Rhizosphere soil Bacteria	2-4 months	Changes in the relative abundance of several bacterial genera (e.g. *Bacillus*, *Variovorax*).	Qi *et al*. 2020
Mulch fragments (PBAT, PBAT-PLA, PBAT-starch, PHB) 7x7 cm 3 pieces/400 g	Jars, plastic buried in agricultural soil	Soil microbial hydrolytic activity	6 months	Increased microbial activity.	Barragán *et al*. 2016
Film fragments (PBAT) 2x2 cm 0.6 % w/w	Pots, plastic buried in agricultural soil	Bacteria and Fungi, from bulk soil and from soil on plastic surface	7 months	Bacteria: emergence of *Caenimonas* and *Hyphomicrobium* groups in soil plastic surface. Fungi: plastic surface enriched in Ascomycota phylum and with lower fungal diversity than bulk soil. Phytopathogens detected.	Muroi *et al*. 2016
Mulch film (PBAT-PLA) 1 film	Plastic buried in agricultural field	Soil Bacteria	7 months	Changes in the abundance of genera *Sphingomonas*, *Bacillus* and *Streptomyces*.	Zhang *et al*. 2019
DBP 50-500 mg Kg^{-1}	Pots, soil spiked with DBP and plants growing	Soil Bacteria	1 month	Increased abundance, changes in α-diversity and community structure.	Kong *et al*. 2018
DBP 50-500 mg Kg^{-1}	Pots, soil spiked with DBP and plants	Soil Fungi	1 month	Changes in α and β diversity and community composition.	Kong *et al*. 2019

	growing			Molecular ecological network structure destabilized, detrimental to mutualistic relationships.	
PAEs released by plastic fragments (PVC) 2 x 2 cm 67.5-337.5 kg ha^{-1}	Pots, plastic buried in soil	Soil microbial hydrolytic activity	2 months	Reduced microbial activity and diversity.	Wang *et al*. 2016
Mulch fragments (PBAT-starch, PLA) 103 cm^2 in 2x2 cm	Plastic buried in field	Soil quality index, including microbial biomass and activity	18 months	Increased microbial biomass. Reduced soil quality index.	Li *et al*. 2014a
Mulch film 4 m x 1,5 m 1 film/year	Plastic used for mulching and then buried in field	Soil microbial biomass and mineralization activity	12 months	Increase of soil microbial biomass carbon and organic matter mineralization as compared with LDPE mulch.	Moreno and Moreno 2008
Mulch film 1 film/year	Plastic used for mulching and then buried in field	Soil bacteria and fungi	24 months	Increased abundance in bacteria (in two locations) and Fungi (only in one location). Decreased NAG enzymatic activity (in one location).	Bandopadhyay *et al*. 2020

PBSA, poly(butylene succinate adipate); PBS, poly(butylene succinate); PET, polyethylene terephthalate; PBT, polybutylene terephthalate; PBAT, poly(butylene adipate terephthalate); PVC, poly(vinyl chloride); PLA, poly(lactic acid); PHB, poly(3-hydroxybutyrate); DEHP, di-(2-ethylhexyl)phthalate; DBP, dibutyl phatalate; PAEs, phthalate esters; NAG, N-acetyl-β-glucosaminidase.

4.3. Other organisms

In addition to the ecotoxicological assessment of biodegradable plastic mulches on plants and on soil microorganisms, considering that (1) the agroecosystem sustainability is supported by an ecological network comprised of a diversity of organisms, (2) compounds released from mulches into soils may migrate to other ecosystems, and (3) the use of model organisms contributes to the understanding of mechanisms underlying toxicity on living beings, addressing impacts of biodegradable plastic mulches and of their components on other organisms is also required. Literature is still scarce and, as far as the authors are aware to date, there are only two papers that have tested the effects of biodegradable plastic mulch fragments buried into the soil on a battery of organisms.

Fritz et al. (2003) reported that poly(ester-amide) film inhibited the growth of three plant species (rape, cress and millet), *Daphnia magna* crustacean and *Vibrio fischeri* bacteria, while it increased the growth of earthworms. In contrast, Sforzini et al. (2016) found no significant ecotoxic effects for Mater-Bi® (PBAT-corn starch) on *Vibrio fischeri* bacteria, slime mold protozoa, green algae, sorghum and cress plants, and on *Daphnia* and earthworm invertebrates. Likewise, a previous field study revealed no significant effects of one-year tilling Mater-Bi® into the soil on *Vibrio fischeri* bacteria and on an enchytraeid worm (Kapanen et al., 2008).

Among terrestrial organisms, earthworms are key species for agricultural soil health. Acting as ecosystem engineers, mainly improving soil structure and nutrient cycling, they benefit crops (Bertrand et al., 2015). Together with plants and with soil microorganisms' activity, they are included in the European standard norm for BDM ecotoxicity assessment. Boots et al. (2019) reported that PLA microplastics buried in soil (0.1% w/w) decreased earthworm biomass but did not cause mortality. Similarly, Zhang et al. (2018) found there were interactions between BDM fragments and earthworms, and mortality was not affected. Only composted and soil buried starch-based BDM were eaten, while other biodegradable mulches or LDPE ones were not. Earthworms were also found to move fragments from the soil surface, burying them and thus favoring their in-soil biodegradation. Since they promote conditions for microorganism proliferation and contribute to burying and fragmentation of plastic fragments, earthworms have been proposed as organisms helping and enhancing BDM biodegradation (Sanchez-Hernandez et al., 2020).

Recently, the nematode *Caenorharbditis elegans*, a model organism widely used for

ecotoxicological studies in contaminated soils, was used to report on terrestrial ecotoxicity of plastic mulches. Exposure of *C. elegans* to mulch microplastics led to the nematodes ingestion of the microplastics, followed by decreased growth and reproduction, with minor differences between LDPE and biodegradable (Ecoflex®-PLA blends) mulch microplastics suggesting a physical effect (Schöpfer et al., 2020). In another *in vitro* assay on hepatocarcinoma human cells, Ecoflex® mulch soil extracts caused no cytotoxic, genotoxic and mutagenic effects (Souza et al., 2020). These initial works and their compatible but differing results highlight the uncertainty on the limited existing knowledge of the effects these materials may have and the different sensitivities among species. There remains a need for further investigating and testing the diversity range of biodegradable plastic mulches on different types of organisms.

The potential ecotoxicity of certain identified components that may be released from biodegradable plastic mulches on terrestrial invertebrates was reviewed by Ma et al. (2017), which reported that low DEHP concentrations in the soil (1 $mg \cdot kg^{-1}$) can alter earthworm physiological functions, including changes in oxidative enzymatic content and activity, critical protein concentration decrease, and DNA and cell membrane damage. In general, stronger inhibitory effects correlated with increasing DEHP concentration. Similarly, although exposure of *C. elegans* to DEHP containing soils (0.02–2.0 $mg \cdot L^{-1}$) for 24 h caused no mortality, stress-related gene expression increased, along with an increase in growth alterations and reproduction (Roh et al., 2007). Yin et al. (2018) also reported that exposure to DEHP (0.01 to 100 $mg \cdot L^{-1}$) altered *C. elegans* reproductive function, decreasing the number of oocytes and increasing that of apoptotic germ cells.

To sum up, the effects of biodegradable plastic mulches and of their components on the diversity of organisms in the agroecosystem and in other natural ecosystem environments remain largely unexplored. Nevertheless, available results stress a potential for some biodegradable plastic mulches and for some of the compounds they may release in altering growth and reproductive functions of several living organisms, including bacteria, crustaceans, earthworms and nematodes. The sustainability of agricultural soils is highly dependent on the relationships among the diversity of organisms they host, to be taken into account in the assessment of biodegradable plastic mulches environmental impact (Kibblewhite et al., 2008; Bender et al., 2016).

5. Conclusions and future trends

The increasing use of biodegradable plastics for agricultural mulching as an alternative to conventional non-degradable LDPE film mulching is highly valued to alleviate plastic

pollution, but still entails iterative incorporation of these mulches into the agricultural soil. Dynamics of the biodegradation process involve mulch fragmentation, release of compounds and consumption of plastic constituents by the soil microbiome. Migration of mulch compounds to the environment may start from the time the mulch is placed on the soil at the beginning of the crop season. Impacts of biodegradable mulches on cultivated plants and on soil organisms remain mostly uncovered, the mulch composition playing a major role, as well as the diversity of the environmental conditions in which they are used. Plastic fragments themselves have also an impact, with effects of their size poorly known but pointing to higher impact of microplastics on plants than on other soil organisms. To our knowledge, no reports have been produced on the impacts of nanoplastics from biodegradable mulches on plants or on soil microorganisms.

Sensitivity of plants to a mulch material is species-dependent. A few studies have shown that some BDM and specific mulch components may alter plant development, while a few others show certain mulches to be likely safer for use in the agricultural environment. However, very few BDM have been intensively tested on a diversity of organisms, and studies on long-term effects exceeding a few months are still required. Additives constitute a small portion of the mulch and they frequently pass unnoticed, both in biodegradation and ecotoxicity studies. Substantial effects of some plastic additives recommend additives to be identified in the biodegradable mulch composition and to test them for their safety. Further research on the impact of individual mulches on the growth and development of plant species in which the mulches are normally used will contribute in designing sustainable mulch formulations.

Similar to studies with plants, the impact assessment of biodegradable plastic mulches on soil microorganisms is yet to be thoroughly investigated. Research carried out to date suggests soil microbiome may be altered by mulches and their components. However, as for plants, there is a need for comprehensive and long-term studies addressing the influence of these mulches on the dynamics of soil microbial communities and their functions. Comprehensive studies, on other organisms in the agroecosystem and their ecological interactions are also needed to uncover the full potential impact of biodegradable plastic mulches on soil health.

Finally, migration of compounds and chemicals released from the films beyond the agricultural system to other compartments, including natural ecosystems, should be considered. Special attention is also to be paid to avoid the incorporation of chemicals and other components susceptible of being transported and accumulated within the food

web that could jeopardize and risk human health. In-depth research on the effects on living organisms emerging from using biodegradable plastic mulches is required to guarantee the environmental safety and sustainability of these materials.

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AIMS AND OBJECTIVES

The general aim of the thesis is to determine whether biodegradable plastic films used for agricultural mulching (BDM) may affect horticultural plant development and agricultural soil microbiome.

The specific objectives are:

1. To determine whether BDM fragments and the compounds released during their (bio)degradation may affect plant development of two species commonly cultivated with plastic mulches, lettuce and tomato. Chapters II and IV.

2. The chemical characterization and quantification of the compounds released by the BDM. Chapter III.

3. To determine whether the burial of BDM may alter the agricultural soil microbiome. Chapter V.

4. Identification of key factors for BDM to constitute a sustainable alternative to the non-biodegradable polyethylene mulches and their innocuousness to the agricultural environment. Chapter VI (General discussion).

CHAPTER II

APPLICATION OF AN *IN VITRO* PLANT ECOTOXICITY TEST TO UNUSED BIODEGRADABLE MULCHES

This chapter reproduces the text of the following published manuscript:

Serrano-Ruiz, H., Martin-Closas, L., Pelacho, A.M., 2018. Application of an *in vitro* plant ecotoxicity test to unused biodegradable mulches. Polym. Degrad. Stab. 158, 102–110. https://doi.org/10.1016/j.polymdegradstab. 2018.10.016.

Abstract

Biodegradable plastics have emerged as an encouraging alternative to reduce the production of plastic waste, especially for agricultural mulches. However, degradation of these plastics in the field may involve the release of products from the mulch into the soil, before and during the in-soil biodegradation. The present work aims to assess the potential effects on two agricultural plant species (*Lactuca sativa L*.-lettuce, and *Lycopersicon esculentum Mill*. -tomato) of extracts from biodegradable (BDMs: Mater-Bi, Bioplast-SP4 and SP-6, BioFilm, BioFlex, Ecovio, Mirel, Paper) and Polyethylene mulch films. A previously designed highly sensitive *in vitro* ecotoxicity test was used.

Some of the extracts from the biodegradable plastics had effects on plant development. Germination was reduced by Bioplast films treatments, both in lettuce (B-SP4) and in tomato (B-SP4 and B-SP6). In lettuce, root development was notably reduced by all treatments except for Paper and Polyethylene. Plant aerial growth was also limited with Bioplast and BioFlex, but enhanced with Paper extracts. At a whole, tomato plants showed higher sensitivity than lettuce in the test. Tomato aerial plant part and root growth were reduced by all treatments with the exception of BioFlex and Polyethylene. For both plant species, inhibitory effects on development were associated to proline increases, a physiological marker for some plant stresses.

It can be concluded that the contact of unused biodegradable films with a water solution may result in changes in plant development that depend on the nature of the biodegradable film. The *in vitro* used test revealed to be a highly sensitive tool for ecotoxicity studies. These results are to contribute to design safe materials for agricultural applications.

1. Introduction

The disposal of plastic residues has arisen as an urgent issue to manage. Only in Europe, the total production of plastics is over 60.000 t per year, and less than 50% of the plastics end up in the official waste stream [1]. In the case of agricultural mulching, plastics have to be removed from the field and properly managed, but available recycling systems are poorly satisfactory and plastic mulches are frequently mishandled after use. In several agricultural environments mulch films, mostly polyethylene-based (PE), are more frequently than expected systematically ploughed into the soil, with adverse deteriorating effects on the soil physical properties; they also release toxic substances, decreasing soil quality [2,3]. Biodegradable mulches (BDMs) have proven to provide equivalent agricultural advantages than conventional PE mulches [4]. In addition, they biodegrade into the agricultural soils under environmental conditions [5] and have thus arisen as an encouraging solution to overcome the constraints of conventional plastics.

As relevant as biodegradability, it is to ensure that BDMs are not to cause harmful effects to the soil environment. Accordingly, several European [6] and national [7, 8], standards foresee requirements for ecotoxicity and environmental safety. Among other requirements, threshold limits are established for heavy metals and for potential toxic substances. In-soil BDMs have to pass a set of ecotoxicity tests on living organisms to become certified and allowed to be used. Recent reviews for determining ecotoxicity of BDMs in soil can be found in Fritz [9] and in Briassoulis and Degli- Innocenti [10].

The ecotoxicological risk of biodegradable materials is usually considered as inherent to the in-soil biodegradation process. This conception comes from the view of the biodegradable plastics waste composting process, where the mature compost is intended to be applied to the soil as fertilizer. Ecotoxicity is consequently usually assessed after the biodegradable material has started to disintegrate; new materials are presumed to be biologically inert and safe before being submitted to biodegradation [11, 12]. It is also to consider that some intermediate compounds recalcitrant for biodegradation, together with plastic additives and fillers, can remain in the soil. These intermediates are to be taken into account for ecotoxicity testing [11].

BDMs are laid on the soil, where they exert their effects on the intended crop and remain for months. From the moment the BDMs are installed, their physical and chemical degradation begins through the interaction of the materials with the environment (rainfall and irrigation water, light, etc.). For the fraction of the BDM into the soil, biodegradation may start shortly after installation, and it is massive after the crop is harvested and the

BDMs are ploughed into the soil. The compounds released before and during biodegradation may be transformed through the soil environment or be absorbed by the plant root system. Methodologies used for studying ecotoxicity on plants come across some limitations in sensitivity for identifying potential effects at the medium and long term. Among others, it is relevant the shortcoming to identify effects on the hidden plant system, roots, determinant for plant growth [13]. The sensitivity of the test methods and the timing for the assessment of potential risks are key factors [14]. Ecotoxicity studies of BDMs in soil are scarce and difficult to be compared [15-19]. Consequently, the establishment of their ecotoxic potential and thresholds remains under discussion.

Recently, an *in vitro* plant ecotoxicity test was suggested [13]; the system demonstrated high sensitivity of key plant species to chemicals frequently included in the formulation of BDMs. The present study aims to assess if water extracts from unused BDMs may exert effects on the development of *in vitro* cultivated plants.

2. Materials and methods

2.1. Test materials

Four commercial (Mater-Bi, Ecovio, Bio-Flex and BioFilm) and three experimental (Mirel, B-SP4, B-SP6) biodegradable plastics, one paper (MIMgreen) and one non-biodegradable polyethylene (PE) mulching films were chosen for the experiments. Biodegradable plastics were composed of blends of polybutyrate adipate terephthalate (PBAT) with other polymeric compounds of different compositions: thermoplastic starch (TPS), polylactic acid (PLA), polyhidroxybutyrate (PHB) and cereal flour, or a mixture of them (Table 1). All these materials represent the majority of BDMs available for agriculture. All plastic films were black and samples were obtained from rolls of 1.2 m width; film thickness was 15-17 mm except for Mirel, that was 40 mm. Paper mulch was made of virgin cellulose fibers and was black upwards and brown downwards, with a grammage of 85 gm^2.

Table 1. - Mulch materials assayed in the treatments: name, manufacturer, grade and composition.

Product name	Manufacturer	Grade	Blend composition
Mater-Bi®	Novamont (Italy)	CF-04P	PBAT, Corn TPS, vegetable oils
Ecovio®	Basf (Germany)	M2351	PBAT, PLA (~7 %)
Bioplast® (B-SP4)	Group Sphere Ibérica Biotech (Spain)	GF106	PBAT, Potato TPS
Bioplast® (B-SP6)	Group Sphere Ibérica Biotech (Spain)	GF 106 + GS2189	PBAT, Potato TPS, PLA
Bio-Flex®	FkuR (Germany)	F1130	PBAT, PLA (~ 30 %)
BioFilm®	Limagrain/Carbios / G. Barbier (France)	BF3012	PBAT, Cereal flour
Mirel®	Metabolix (USA)	P5001-4	PBAT, PHB
MIMgreen® (Paper)	MimCord (Spain)	-	Cellulose fibre
Polyethylene (PE)	Solplast (Spain)	-	LLDPE

PBAT: polybutyrate adipate terephthalate; TPS: thermoplastic starch; PLA: Polylactic acid; PHB: polyhidroxybutyrate; LLDPE: lineal low density polyethtylene.

2.2. Extract preparation from mulches

New unused films were frozen with liquid nitrogen and immediately ground by using a mechanical mill, to pass an 8 mm mesh sieve (Retsch SM300; Germany). Ground materials were stored in glass bottles in the dark at room temperature during 1-2 days until use. Extracts were obtained by mixing 32 g of ground films with 400 ml of the sterile mineral fraction of MS [20] culture medium at 5 concentration. Sterile glass bottles with the corresponding mixtures were incubated in an orbital shaker (Heidolph Unimax 2010, Germany) for 7 days at room temperature. Resulting extracts were sterilized through a 0.20 µm filter and used to perform the tests. An extract without mulch was made in parallel as control.

2.3. In vitro culture test

Extracts were diluted to obtain the MS standard mineral solution and pH was adjusted to 5.7. Sucrose (3%), vitamins and Gelrite™ gelling agent were added to complete the MS culture medium. Two sets of experiments with different sterilization procedures for the culture media were carried out. In one set, sterile conditions required for *in vitro* plant culture were obtained through the standard procedure of autoclaving at 121°C for 15 min; in the other set of experiments, autoclaving was substituted by filter sterilization of the medium through a 0.2 µm membrane (Millipore™). The media were distributed in culture tubes. For lettuce, the test with the different extracts was performed with both autoclave

and filter sterilized culture media. For tomato, filter sterilization was carried out. Seeds of *Lactuca sativa L.* cv. Trocadero Ribera (lettuce) and of *Lycopersicon esculentum Mill*. cv. Red Cherry (tomato), were surface sterilized by soaking with 2% sodium hypochlorite solution. For each experiment, a total of 100 seeds per treatment were sown in culture tubes. Plants were grown in a climatic chamber at 21 ± 2° C, under 90-110 mmol $s^{-1}m^2$ light intensity (Sylvania Gro-Lux F58W/GRO-T8) and 16 h light photoperiod. Germination was monitored and Relative Germination Inhibition (RGI) calculated by the following equation:

Relative Germination Inhibition (%) = [(Germination Control * Germination Treatment) / Germination Control] *100

The plants, including the root system, were visually monitored weekly. After 4 (lettuce) or 3 (tomato) weeks, fresh and dry weight of aerial plant parts and roots, chlorophyll and proline content of leaves were recorded for both species. For lettuce, with no visible shoot, the aerial plant part is referred in the figures as leaves.

2.4. Chlorophyll content

For every treatment and repetition, five leaves from different plants were randomly selected and chlorophyll content was measured with a SPAD 502 chlorophyll meter (Minolta, Japan).

2.5. Proline content

Proline content in leaves was measured according to Bates et al. [21]. Briefly, 0.1 g of leaf fresh weight was homogenated with 2 ml of 3% sulfosalicylic acid. A mixture of the homogenate, acetic acid and acidic ninhydrin reagent (1:1:1) was incubated at 90°C for 30 min. Then, 2 ml of toluene was added to the mixture and vigorously vortexed. The absorbance of the resulting upper phase was measured at 520 nm in a spectrophotometer (Helios Gamma, Thermo Electron Corporation, England). Proline concentration was calculated using L-Proline for the standard curve. Results are expressed as mg of proline per g of fresh weight.

2.6. Statistical analysis

The *in vitro* test was performed using a randomized complete block design with two blocks. Measurements recorded in the plants correspond to two independent experiments. Statistically significant differences were determined with SAS software (JMP Pro 12.0.1) by analysis of variance (ANOVA) and treatment means were compared

by Dunnett's test, where the control group were plants grown in culture medium without mulch extract (α = 0.05).

3. Results and discussion

3.1. Germination

The use of BDMs on lettuce and tomato crops is a worldwide spread agricultural technique [4]. Tomato is the main vegetable for human consumption, while lettuce is also relevant as a vegetable and it is also one of the most recommended and sensitive species for testing ecotoxicity to a diversity of chemicals [22, 23]. In the present work, germination of seeds in control culture medium was over 98% for lettuce and over 85% for tomato. Lettuce seeds showed sensitivity to the extracts, with significant 18% inhibition for seeds cultured in B-SP4 treatments. Tomato seed germination was also significantly reduced in B-SP6 and B-SP4 treatments, with RGI of 19 and 23%, respectively (Fig.1).

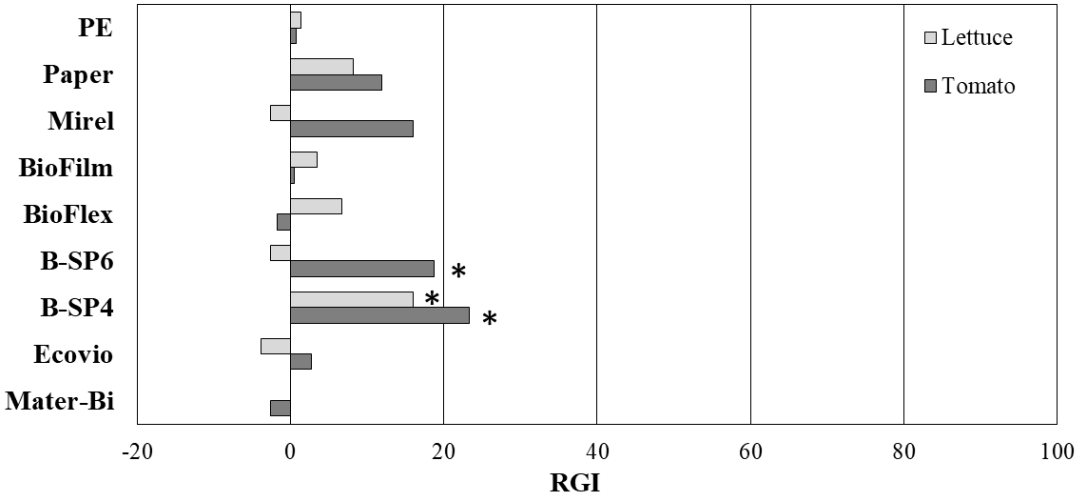

Figure 1.- Germination index, RGI, (Relative Germination Inhibition, percentage) of lettuce and tomato seeds *in vitro* after 10 days in MS medium with biodegradable plastic or paper extract incorporated, as compared to seeds in control medium.

Germination tests are routinely accepted for assessing the biotoxicity of chemicals and of biodegradable plastics as potential releasers thereof [6, 8, 24-26], which may be involved in essential plant physiological processes. However, as pointed out previously, ecotoxicity tests based on germination are limiting and unsatisfactory for identifying effects on plant development; relevant alterations may go unnoticed [27]. A few papers have studied the effects of the corresponding monomers from plastic polymers on seed germination. Arfsten et al. [28] and Ma et al. [29] found no effect of plastic monomers in germination, although depending on the compound and concentration considered, plant

development was later affected. Martín-Closas et al. [13] reported that relatively high concentrations of adipic acid limited germination, while other monomers and lower concentrations of adipic acid had no effect on germination but plant development was limited. Recently, Sforzini et al. [26] identified no effects of degraded Mater-Bi on seed germination, but plant development was unfortunately not further monitored.

To our knowledge, there are no previous reports on the effect of unused BDMs on plant germination. In the present experiment, extracts from B-SP4 and B-SP6 had proven inhibitoring effects on germination, while no significant differences in germination were identified for other materials.

3.2. Lettuce plant growth

Lettuce plant development and plant visual aspect were monitored during and at the end of the culture time, four weeks. Since under some of the treatments the growth of the plantlets was limited and exhibited stress symptoms, chlorophyll content and the physiological status of the plants in terms of proline content were also determined.

The extracts from the mulch materials had effects on lettuce plant development when the culture media were autoclaved. As found for germination, development of both leaves and roots was limited by B-SP4 and also by BioFlex and B-SP6 extracts, leading to significantly lower plant dry weight (Fig. 2a). The B-SP4 and B-SP6 mulch extracts also caused shorter and smaller plants with significantly higher chlorophyll in the leaves (Figs. 2c and 3). Leaves and roots were differently affected by the treatments. Overall, root development was more sensitive, and it was notably limited by Mirel, BioFlex, B-SP6, and Ecovio, but Ecovio and Mirel had no parallel effects on leaf development and B-SP4 had more effect on leaf than on root development (Fig. 2a and b). It was also observed that BioFilm caused no apparent visually effects on leaf plant development, nor was leaf and root dry weight affected; however, it markedly altered the structure of the root system. Plants growing in BioFilm treatment had shorter primary and secondary roots than control plants (Fig. 3). In contrast to the above results, Paper treatment promoted lettuce plant growth and development, both in leaves and especially in roots, where the dry weight increase was outstanding (Fig. 2a and b). The contribution of roots to total dry weight shifted from ca. 15% in control plants to ca. 40%. Moreover, Paper treatment extensively altered the root system structure and colour (Fig. 3). Roots in control plants were thin, white or creamy and scarcely branched, while in the Paper treatment the oldest roots were thick and dark yellow, with callus-like outgrows and highly branched. Paper biodegradable mulch main matrix consists mostly of cellulose fibres, but it also

includes a complex mix of lignin, lignin monomers, pectins and other organic compounds, together with additives for the black colour. These plant compounds, or its derivatives, are likely acting as growth factors, with plant-hormone mimicking effect.

Although PE is considered very stable and non-biodegradable, plantlets growing in PE extracts also exhibited higher root development than control plants (Fig. 2b), with allocation of assimilates also higher for roots than in control plants. The PE mulch is composed not only from PE but it also incorporates several additives and plasticizing agents able to migrate from the mulch to the aqueous phase, and ultimately being absorbed by plants. Migration of plasticizing agents from non-biodegradable PE films to the soil and further to plants has been recently reported [30-33]. Filtered extract effects on plant development were mostly equivalent to those observed with the autoclaving procedure, indicating no substantial overall changes in the media. For all treatments, plantlet growth was only slightly higher than when autoclaving (Fig. 2a). Also, as through autoclaving, root development was promoted in PE and Paper culture medium and limited by Mirel, B-SP6 and Ecovio. BioFilm and Mater-Bi filtered extracts had no effect on aerial plant development but however they decreased root development (Fig. 2b), pointing to some degradation during autoclaving in the extracts from these materials. In general, it can also be assumed that effects on root dry weight, as compared to control plants, were more remarkable when the extracts were not heated but filtered, while effects on the aerial part of the plant were not affected by the sterilization system. When the heat stability of the compounds in an *in vitro* medium cannot be established, filtering is the procedure to follow. However, this introduces a methodological complexity for routine testing. Results obtained demonstrate that effects of the extracts from the materials tested remain after autoclaving the media. Thus, the test may be performed also under these routine and simple conditions.

A critical aspect for plant development is nutrient availability, which, among others, is dependent on the pH of the soil. When pH was measured in the prepared culture media, it did not differ between Mater-Bi, BioFilm and control media, while it increased between 1 and 2 units for the remaining media prepared with the other leachates. To ensure that effects of the materials are not associated to pH, the pH of all culture media was adjusted to 5.7 before seeding, the common procedure routinely followed for maximizing *in vitro* nutrient intake and plantlet development. Plantlets growing *in vitro* utilize the mineral nutrients in the media, and this is usually accompanied by a slow but continuous decrease in the pH during time. The *in vitro* autoclaved and filter sterilized lettuce culture media were monitored for pH changes, which dropped 1.0-1.5 units during culture for all

treatments, including control ones, except for B-SP6 medium, where it remained unchanged. The stability of pH in the B-SP6 medium was associated to reduced growth, without intake of nutrients from the medium. Also, to track the parallel nutrient consumption in the media, conductivity was recorded at the beginning and at the end of the culture time. For most treatments, the limited decrease in conductivity was indicative of no limitations in nutrient availability during the essay; only in the Paper medium, where plant growth was strongly promoted, a decreased of 50% from the initial conductivity was recorded. Our results provide evidence of some of the plastic mulch films tested, Ecovio, B-SP4, B-SP6, BioFlex, Mirel, Paper and Polyethylene, potential to alter pH conditions in their leachates. A few authors have also reported limited increases in soil pH associated to the use of biodegradable plastic film mulches [14, 15, 34]. Agricultural mulches remaining in the field are expected to release some of the compounds to the soil environment, with potential long term effects on soil pH [35]. On its turn, small pH changes may not only affect plant growth but also alter soil microbiota environment [11].

Some treatments were found to limit plant growth, including reduced root development, and to increase leaf chlorophyll content, pointing out to plants undergoing stress. Proline has relevant roles in major plant physiological processes associated to overcoming stress, and it is widely accepted as a marker for several plant stress conditions [36]. Proline increase in plants is extensively documented when they face several biotic or abiotic stress: salinity, high radiation levels, heavy metals, oxidative stress or biotic stress [37]. Since markers associated to stress responses for plants in interaction with BDMs have not yet been developed, proline content was analysed as a potential marker. Proline was found to significantly increase in plants growing with BioFilm, B-SP6 and especially with B-SP4 autoclaved and filtered extracts (Fig. 4a). Interestingly, these extracts lead to abnormal lettuce plants and/or to decreased biomass (Figs. 2 and 3), suggesting a relationship between proline accumulation and the stress situation caused by the extracts. Conversely, for plantlets that increased growth, in autoclaved Paper and PE treatments, proline significantly decreased (Fig. 4). Other results require further analysis: proline also decreased in visually normal plants growing in Mater-Bi, and BioFlex treatments. The *in vitro* culture controlled system used allowed precise control and monitoring of the development of the lettuce plants well after seed germination. Most remarkable, it is a unique system to allow close 3D monitoring of the root system development, the plant organ directly interacting with the soil. Root development is crucial for plant survival, biomass production and crop productivity. When lettuce plants grew *in vitro* in the BioFilm extract medium, with unrestricted access to water and

nutrients, shorter and fewer roots were produced, but the aerial plant development was not compromised. However, in the field, where plants are frequently exposed to periods of water and nutrient scarcity, shorter and fewer roots may become a decisive handicap for plant development; under these circumstances, crop yield may be limited.

A set of variables is associated to the release of compounds from the BDMs during the degradation and biodegradation processes. Little is known on the nature, diversity and properties of these compounds, but it is considered that most of them are rapidly transformed by soil microbiota into CO_2 and biomass. However, concomitant to the increasing use of plastic mulches, they may persist for longer periods in the soil and directly or indirectly [35] affect plant growth and development. Effects on the soil and on plants of compounds migrating from the plastic mulch films, and especially from unused materials, have received little attention. Equally, the interaction between plastic mulches, soil and plant is only recently being addressed due to the increasing concern about worldwide plastic contamination that has led to growing use of BDMs. Although there are yet very few reports dealing with this uprising subject in the literature, some initial findings have already emerged, adipic acid, a compound in BDMs, has proven to alter and limit plant growth [13, 31, 38], and extracts from biodegradable plastics proven safe and not affecting germination may have thresholds over which plant development is affected [13, 28]. A few authors have reported on the migration of chemicals from agricultural plastic mulches to water solutions and plants and the effects on plant development thereof [28, 32, 33]. The physical stability of the compounds released from the BDMs is equally significant, both for studies in the field and for the *in vitro* test proposed. The culture medium is usually autoclaved to get sterile *in vitro* conditions for the explants to grow. The heating of the culture media with the mulch leachates, whose nature has not yet been determined, could break down the potential compounds leached from the mulches, to undergo unknown modifications rendering them inactive, or on the contrary to increase their toxicity and ultimately affect plant development. Autoclaving temperatures are far from those found in agricultural soils; however, soils may easily reach peaks of 50°C in the hot season, altering the BDMs and affecting the processes for degradation and biodegradation. The first set of results shown above for lettuce has been obtained following the autoclaving routine procedure. To test the effect of high temperatures on potential toxicity of the extracts, these results were compared with those from the second analogous assay where sterile conditions were obtained by filter sterilization of the media. Both autoclaving and filtering procedures led to similar results. The results of the *in vitro* culture controlled system are consistent and the system constitutes a reliable tool for

determining the effect of extracts from the BDMs on germination and plant growth, which are to be further validated in the crop environment, in the field, where myriad variables may contribute. In-depth studies on the potential effects of mulch leachates in the soil environment, and particularly in root development, are complex, and to our knowledge they have not been attempted yet.

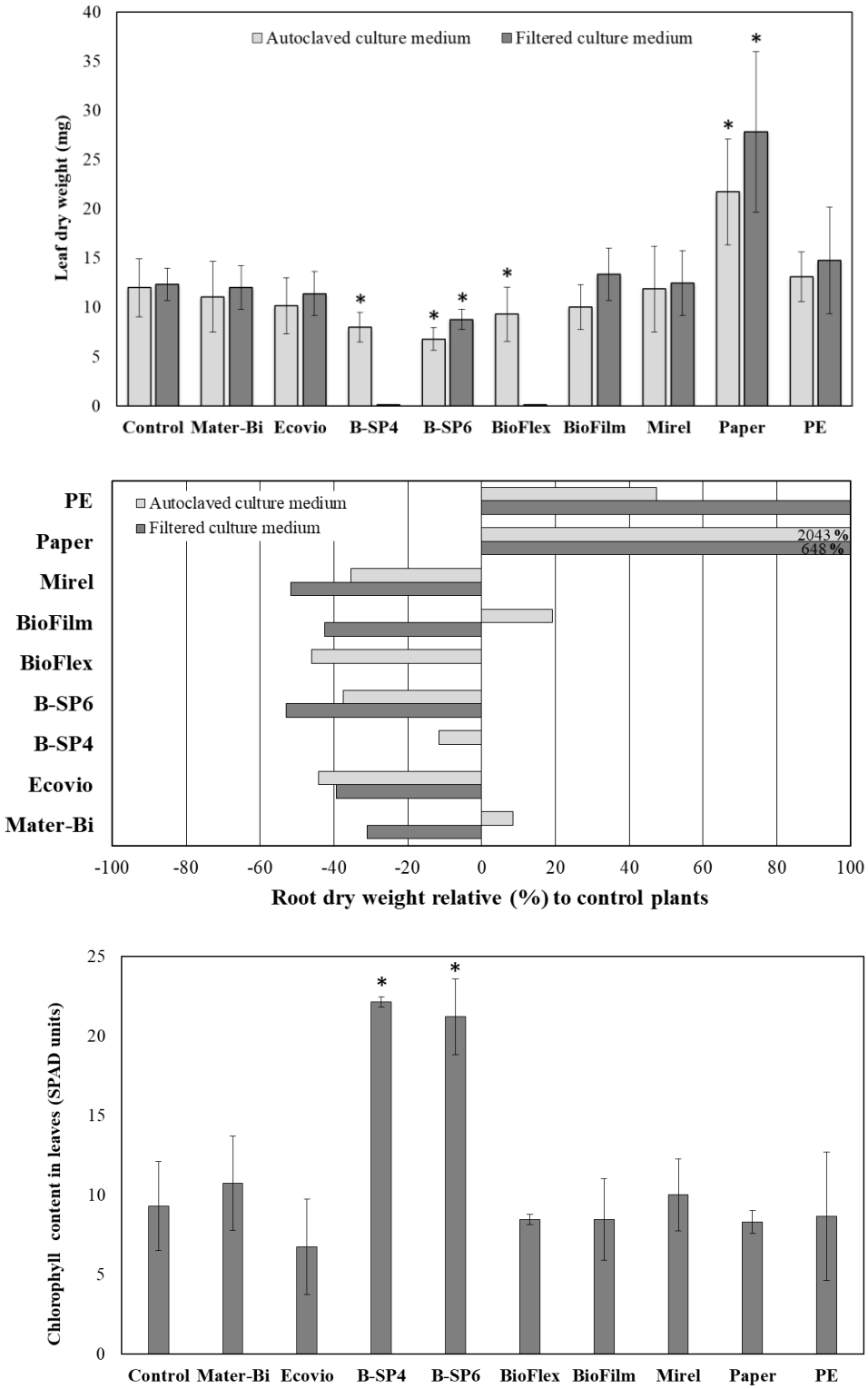

Figure 2. - Lettuce plant growth after 4 weeks in MS media with biodegradable plastic extracts. a) Leaf dry weight. b) Percentage of promotion/inhibition of root dry weight relative to control plants. c) Leaf chlorophyll content (SPAD units). Bars show standard deviation. *Treatments statistically different from control (P ≤ 0,05).

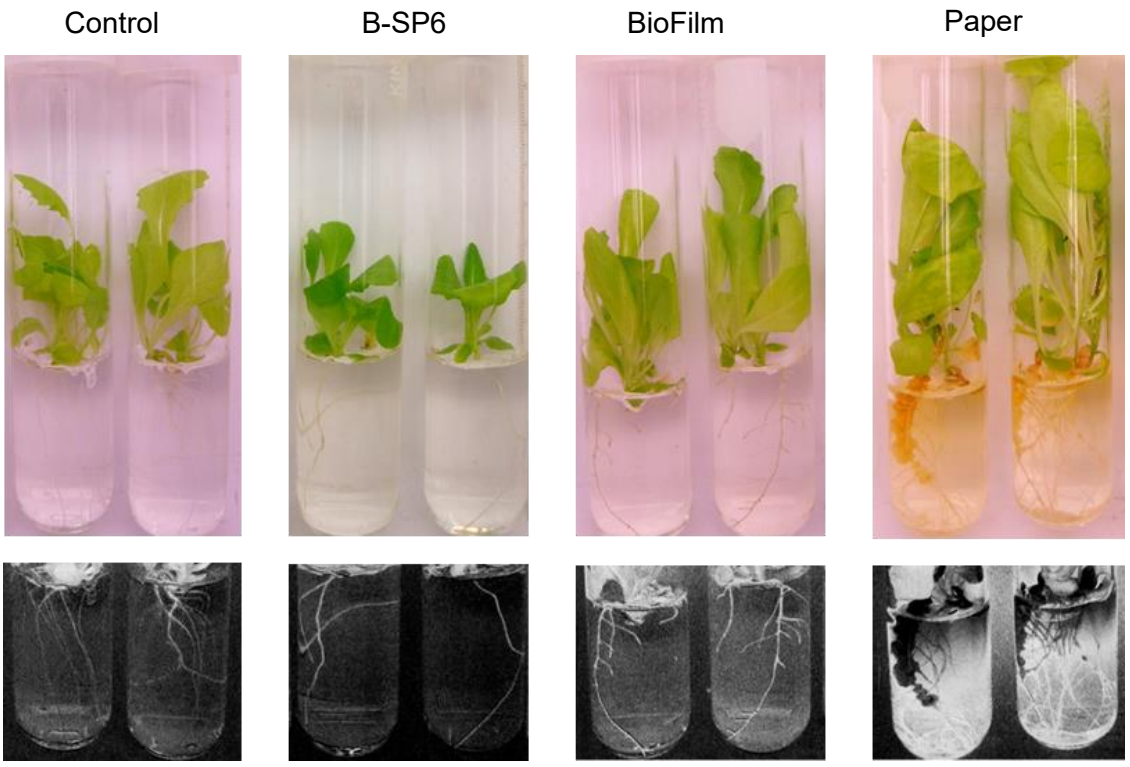

Figure 3. - Lettuce plants after 4 weeks of *in vitro* growth in control media or with B-SP6, BioFilm or Paper mulch extracts. a) Complete plants, b) Roots; transformed image to black and white. Exposure and contrast has been increased to facilitate visual identification of the root system structure.

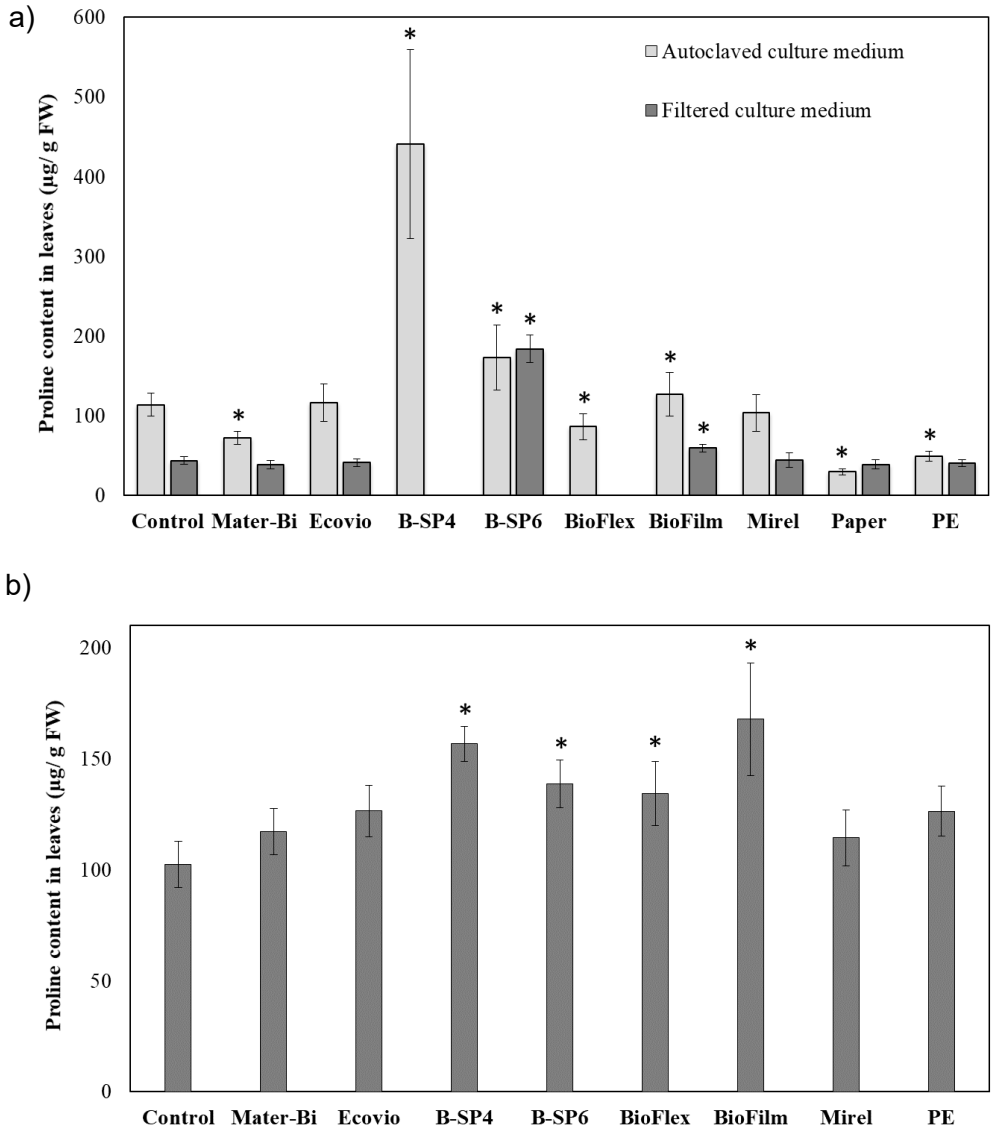

Figure 4. - Proline content in the *in vitro* plants grown with autoclaved (light color bars) or filtered (dark color bars) treatments. a) Lettuce, b) Tomato. Bars show standard deviation *Treatments statistically different from control (P ≤ 0,05).

3.3. Tomato plant growth

Tomato plant development was limited by most mulch extracts *in vitro*. All treatments except PE and BioFlex, significantly decreased the dry weight of the aerial part of the plants (Fig. 5a). Root development was strongly inhibited in most treatments and correspondingly dry weight sharply decreased (Figs. 5b and 6). Root growth was almost totally repressed by the BioFilm treatment (Fig. 6). However, the visual look of the tomato plants differed between treatments. Whereas Paper, Ecovio and Mater-Bi grown plants were visually indistinguishable from control plants, B-SP6, B-SP4, Bio-Film and Mirel plants were smaller, and chlorophyll in leaves of BSP6, B-SP4, and BioFilm treatment

plantlets was significantly decreased (Figs. 5c and 6). Furthermore, the stems of plants grown with the BioFilm extract exhibited intense red colour, indicative of anthocyanin synthesis and accumulation (Fig. 6). The stems of several other treatments were also slightly reddish. Anthocyanins are secondary metabolites with antioxidant properties, synthetized by some plant species, such as tomato, when undergoing environmental stress [39]. Together with proline, anthocyanin synthesis is associated to oxidative stress. In accordance, plants from the BioFilm treatment had significantly more proline than control plants (Fig. 4b). Proline was also significantly increased in plants growing in media with B-SP6, B-SP4 and BioFlex leachates, suggesting stress situations obvious for the naked eye for B-SP6 and BSP4 plants (Figs. 4b and 6). The changes in pH and conductivity in the different media after the growth of the tomato plantlets were in parallel to those already described in the lettuce experiment; most relevant the maintenance of the initial levels in the B-SP6 medium. Tomato plants showed more sensitivity to several of the extracts from the biodegradable agricultural mulches than lettuce plants. While different sensitivity to stressors between plant species is widely acknowledged, certain extracts largely and similarly altered both plant species. Results from these *in vitro* tests, which showed significant and remarkable inhibitory effects in shoot and root development for both species and increased proline, prove that both tomato and lettuce plant development is sensitive to the extracts of B-SP4 and B-SP6 agricultural mulches. The nature of the putative compounds released from the mulches into the extract aqueous solution remains unknown and deserves to be investigated. In parallel, the results from the *in vitro* test require to be scaled up; whether the effects of these mulch materials found *in vitro* prevail on plants growing in the soil environment is to be determined. Progress may be also followed on the associated mechanisms, promoting or inhibiting growth, and to the corresponding physiological and biochemical markers in plants.

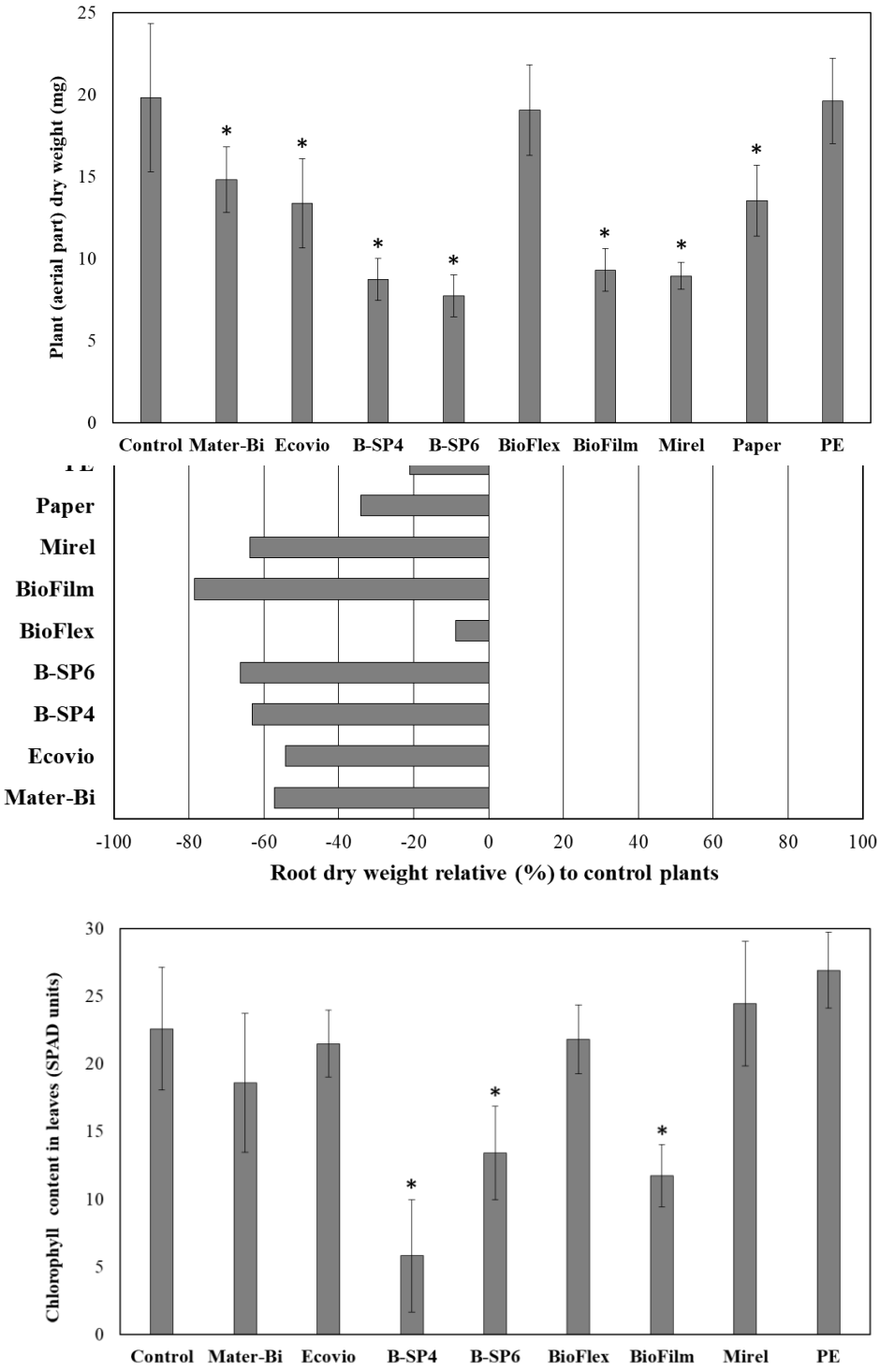

Figure 5. - Tomato plants growth after 3 weeks in MS media with biodegradable plastic extracts. a) Leaf dry weight. b) Percentage of promotion/inhibition of root dry weight relative to control plants. c). Leaf chlorophyll content (SPAD units). Bars show standard deviation. *Treatments statistically different from control (P ≤ 0,05).

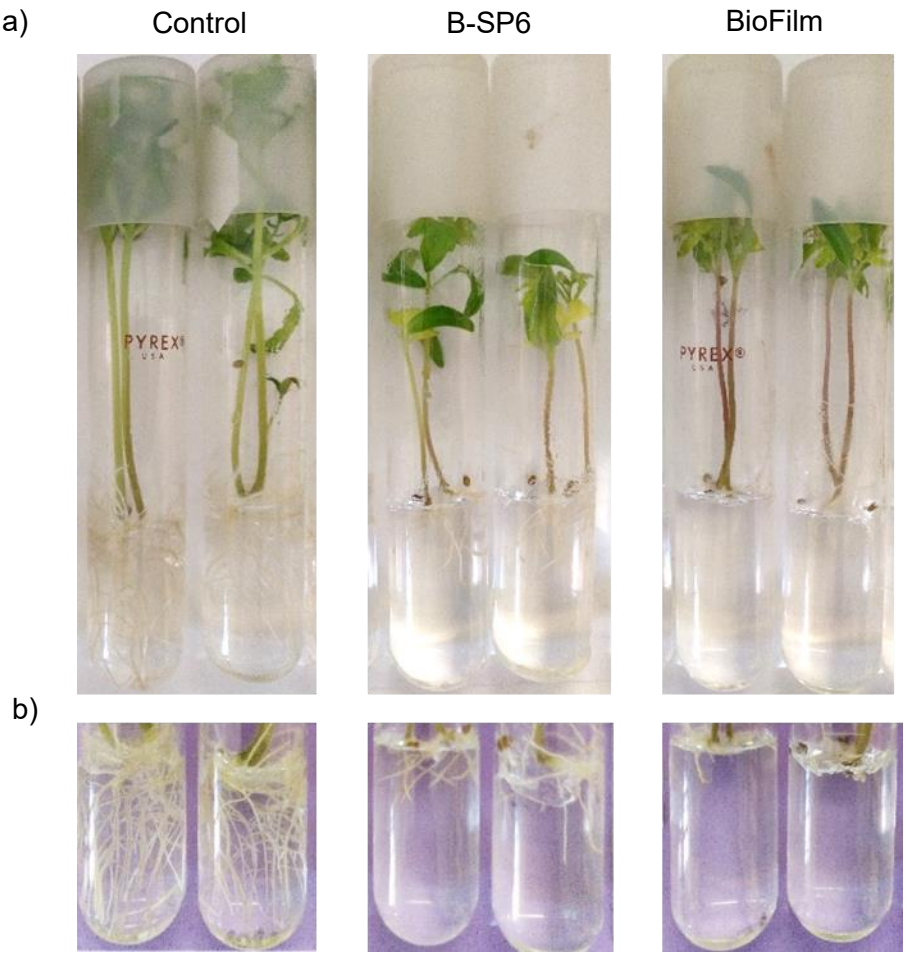

Figure 6. - Tomato plants after 3 weeks of *in vitro* growth in MS media with biodegradable plastic extracts. a) Complete plants. b) Roots.

4. Conclusions

The *in vitro* system used with lettuce and tomato had previously proven effectiveness for testing plant sensitivity to compounds that can be released from biodegradable plastic films [13]. The test carried out has revealed the potential of extracts from unused BDMs to affect lettuce and tomato seed germination and plant development. Overall, some of the extracts (B-SP6, B-SP4 and Bio-Film) significantly compromised plant growth, both in lettuce and in tomato, while others resulted in no effect. These findings denote the pertinence to invest in selecting and identifying the mulches to prevent potential middle or long term effects from their repeated use. The *in vitro* test is suggested as a simple and convenient tool to gather detailed knowledge on the effects of the different materials in plants. The test allows close monitoring of plants during culture time under tight control of the environmental conditions and in a controlled artificial nutrient medium, granting

conditions to analyse the potential effects in a diversity of situations, and it is highly reliable. It is especially valued for monitoring changes in the root system, a situation likely to proceed unnoticed in other ecotoxicity assays (e.g. in plant pots). In the *in vitro* tests performed, the materials with inhibitory effects on plant growth mostly affected the root system. Proline has also emerged as a valuable marker for plant stress associated to the mulch materials. To our best understanding, this is the first report revealing inhibitory and stress effects on plant development of extracts from unused agricultural mulches. Complementary *in vitro* and in-the field ecotoxicity tests for BDMs are needed; this knowledge will lead to approach the complete scenario ultimately required to allow selection of the biodegradable mulches, to substitute PE ones with best benefits and no impact on plants and on the environment.

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CHAPTER III

COMPOUNDS RELEASED FROM UNUSED BIODEGRADABLE MULCH MATERIALS AFTER CONTACT WITH WATER

This chapter reproduces the text of the following published manuscript:

Serrano-Ruiz, H., Eras, J., Martin-Closas, L., Pelacho, A.M., 2020. Compounds released from unused biodegradable materials after contact with water. *Polym. Degrad. Stab*. 178, 109202.https://doi.org/10.1016/j.polymdegradstab.2020.109202.

Abstract

Biodegradable plastics (BDP) are an alternative to environmental and economical drawbacks of PE agricultural mulches. They are intended to biodegrade into the soil or in compost. BDP materials certified as biodegradable in soil or in compost have passed ecotoxicity biotests after biodegradation, but most BDP ecotoxicity studies do not provide insights on the components these materials may release. The present work aims to characterize the compounds released from BDP to water solutions before biodegradation. New unused BDP, one paper mulch for agricultural use, a control without any material, and one non-degradable PE mulch, were incubated in a water solution for identification of the released compounds through GC-MS, NMR and UPLC-MS/MS. All BDP released compounds and modified the pH of the solution, with differences depending on the composition of the material. A diversity of compounds used in the formulation of the materials, and their derivatives, were identified in most solutions; mostly, but not exclusively, adipic acid, 1,4-butanediol, lactic acid, glycerol, terephthalic acid, mono and disaccharides, or fatty acids; some were also quantified. Results prove BDP to interact with the environment and to release compounds well before biodegradation may be acknowledged. They are also a step forward towards identifying the links between reported changes in plant development and specific compounds or compound mixtures, and towards a more sustainable and targeted selection and use of BDP for mulching specific plant species in agriculture.

1. Introduction

Agricultural biodegradable plastics (BDP) have proven to be a qualified alternative to polyethylene (PE) mulching, technically and agronomically [1]. BDP overcome some undesired environmental and economical drawbacks of PE mulch, such as the need to remove and manage the residue at the end of the crop, and the risk of plastic pieces remaining into the soil or of being delivered to the surrounding environment. Polyethylene accumulation into the soil alters soil biota and decreases soil fertility [2], with an impact on plant development. During the BDP life cycle in the field, the material undergoes an early degradation from laying on. It starts by interacting with weathering agents (UV radiation, rain and irrigation water) before a significant biodegradation begins, then with plants and with the above ground fauna, and finally, after plowing, with soil weathering agents and soil biota. When into the soil, the bio-deterioration, depolymerisation, bio-assimilation and mineralization sequential process follows [3, 4]. However, all along this biodegradation pathway, a pool of intermediates and molecules are released.

Available biodegradable plastics for agricultural use are commonly certified as biodegradable in soil or in composting environments [1]. The recent standard EN 17033 [5] considers environmental safety and ecotoxicity requirements. It includes threshold limits for heavy metals and also for potential toxic substances, the later through overcoming biotests in plants, invertebrates and microorganisms. The biotests are focused on identifying the hazards, on the tested organisms, imposed from putative substances released to the environment any time during the biodegradation process, or from degradation products derived thereof. Most ecotoxicity studies [6-8] have been performed, after BDP degradation in soil or in compost, in accordance with the standard and presuming that potential ecotoxic products are released only after this process. BDP contact with the natural-agricultural environment before being buried into the soil, and abiotic degradation may occur (e.g. hydrolysis) [3]. It is thus likely that products start to be released previously to the onset of in soil biodegradation. Most biodegradable plastics are water insoluble [9]; it has been considered that they are expected not to release compounds and this scenario has previously received little attention. A recent report on the risk of plastic debris in the environment recommends assessing the risk covering all exposure pathways for organisms [10], with water being one of the most essential. Soil and compost are experimental environments entailing recognized associated limitations [11]; the products discharged on them are difficult to be extracted, and the obtained extracts are usually unsuitable for further chemical analysis [12]. Products released from BDP have been scarcely identified in the literature, either from compost [12, 13] or from

in soil degradation [14]. All three reported cases were performed only after the biodegradable material started biodegradation and only a few specific compounds expected to be released from the materials studied were targeted. Furthermore, the biodegradable materials tested were noticeably different from the ones presently used for agricultural applications. An *in vitro* plant culture procedure in the laboratory has been proposed as a simple and reliable approach to test ecotoxicity of bioplastic constituents in plants [15]. The system allows identifying effects on plants of compounds released from BDP with several advantages over other procedures. Among others, the test is carried out under strict control of environmental conditions, thus overcoming constrains of using other traditional soil or compost-based biotests. In addition, the test allows to elucidate the presence of putative substances affecting plant development in the testing medium coming from the (bio)degradation of the materials, even though the specific compounds responsible for the reported effects remain unknown and require to be further identified. The test has successfully proven to uncover potential ecotoxic effects of water leachates from unused biodegradable mulch films [16]. The present work aims to characterize chemical compounds released from biodegradable films into water solutions, associated to reported effects of these solutions on plant development.

2. Materials and methods

2.1. Reagents and solvents

The aqueous phase for the extraction was MS mineral solution, composed of the macro and micronutrients routinely used for Plant Tissue Culture [17] (Duchefa Biochemie, Amsterdam) (Table 1) dissolved in milliQ-quality water (Millipore, Madrid, Spain; conductivity = 0.055 mS cm^{-1} 25°C). The pH of the solution was 4.83 (±0.05) and the conductivity 5.98 (±0.09) mS cm^{-1} at 25° C.

Derivatization was performed with methoxylamine hydrochloride (MEOX, purity 98%, Sigma-Aldrich, Madrid), pyridine (purity 99.8%, Sigma-Aldrich, Madrid) and N-methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA, purity 97%, Sigma-Aldrich, Madrid). Nuclear magnetic resonance (NMR) analyses were performed using dimethyl sulfoxide (DMSO, purity 99.7%, Sigma-Aldrich, Madrid). The following standards were used for quantification: lactic acid (98%, Alfa Aesar, Landau), adipic acid (99,5%, Sigma-Aldrich, Madrid), terephthalic acid (>99%, Acros Organics, Madrid), and 1,4- butanediol (99%, Alfa Aesar, Landau).

Table 1. - Composition of the MS mineral solution used for the extraction, based on Murashige and Skoog basal salt mixture formulation (Murashige and Skoog, 1962).

Macronutrients	Concentration ($mg \cdot L^{-1}$)	Micronutrients	Concentration ($mg \cdot L^{-1}$)
NH_4NO_3	1650.0	$MnSO_4 \cdot H_2O$	16.900
KNO_3	1900.0	$ZnSO_4 \cdot 7H_2O$	8.600
$CaCl_2$	332.0	H_3BO_3	6.200
$MgSO_4$	180.5	$Na_2MoO_4 \cdot 2H_2O$	0.250
KH_2PO_4	170.0	KI	0.830
		$CuSO_4 \cdot 5H_2O$	0.025
		$CoCl_2 \cdot 6H_2O$	0.025
		FeNa-EDTA	36.700

2.2. Test materials

Mulching films selected for the experiments were 4 commercial (Mater-Bi®, Ecovio®, Bio-Flex®, BioFilm®) and 3 experimental (Mirel®, B-SP4®, B-SP6®) biodegradable plastics, one paper (MIMgreen®) and a non-biodegradable polyethylene (PE). Biodegradable plastics were composed of blends of polybutyrate adipate terephthalate (PBAT) with other polymeric compounds of different composition: thermoplastic starch (TPS), polylactic acid (PLA), polyhydroxybutyrate (PHB) and cereal flour, or mixtures of them (Table 2). All these materials represent the majority of biodegradable mulches available for agriculture. All plastic films were black and samples were obtained from rolls of 1.2 m width; film thickness was 15-17 mm except for Mirel®, which was 40 mm. Paper mulch was made of virgin cellulose fibres and was black upwards and brown downwards, with a grammage of 85 g m^2.

Table 2. - Characteristics of the plastic and paper mulches selected.

Mulch	Manufacturer	Grade	Blend composition
BioFilm®	Limagrain/Carbios/G. Barbier (France)	BF3012	PBAT, Cereal flour
Bio-Flex®	FkuR (Germany)	F1130	PBAT, PLA (~30%)
Bioplast® (B-SP4)	Group Sphere Ibérica Biotech (Spain)	GF106	PBAT, P-TPS
Bioplast® (B-SP6)	Group Sphere Ibérica Biotech (Spain)	GF 106+GS2189	PBAT, P-TPS, PLA
Ecovio®	Basf (Germany)	M2351	PBAT, PLA (~7%)
Mater-Bi®	Novamont (Italy)	CF-04P	PBAT, C-TPS, Vegetable Oils
MIMgreen® (paper)	MimCord (Spain)	–	Cellulose fibre
Mirel®	Metabolix (USA)	P5001-4	PBAT, PHB
Polyethylene (PE)	Solplast (Spain)	–	LLDPE

PBAT: Polybutyrate adipate terephthalate; PHB: Polyhydroxybutyrate; TPS: Thermoplastic starch; C-TPS: Corn thermoplastic starch; P-TPS: Potato thermoplastic starch; PLA: Polylactic acid; LLDPE: Linear low density polyethylene.

2.3. Extract preparation from mulches

New unused films were cut into 1 cm2 pieces with scissors. Extracts were obtained by mixing 2.00 g of cut films with 100mL of the sterile mineral solution (Table 1) in glass bottles and under sterile environment. Bottles were previously rinsed three times with milli Qquality water and autoclaved (20 min, 121°C). The corresponding mixtures were incubated in a bottle roller at 8 rpm (Thermo Fisher Scientific™, Shangai) for 7 days at room temperature. The mixtures were paper-filtered and centrifuged at 2000 rpm for 5 min to obtain the extracts. A sterile MS mineral solution without mulch was incubated as control. All extract solutions were then frozen at 80°C before lyophilisation in a Cryodos-50 lyophiliser (Tel-star, Terrassa, Spain) and stored in a desiccator at 4°C until further analysis.

2.4. GC analysis of extracts

The extracts were analysed by GC-MS for qualitative analysis. Samples were derivatized by suspending 10 mg of the lyophilized extracts in 300 mL of MEOX dissolved in pyridine (20 mg mL1). The samples were sealed with a cap, vortexed until the residues were completely solved and incubated in a ThermoMixer at 40°C for 60 min. Then the samples were removed and 80 mL of MSTFA were added. Finally, they were vortexed and incubated again at 40°C for 60 min.

The samples were injected in a chromatograph (AgilentGC7890 N, Agilent Technologies, S.L., Las Rozas, Spain) coupled to an electronic impact-single mass quadrupole mass spectrometer (5973 N MSD, Agilent Technologies, S.L., Las Rozas, Spain). The column used was: DB5MS UI (30m0.25mmx 0.25 mm) (J&W122-3832 UI). The split/ splitless injector worked at splitless mode at 50°C. The split purge flow was 20 mL min1 for 0.3 min. Helium was used as a carrier at constant flow of 1 mL min1. Oven conditions were 80°C for 2min initial temperature increasing at a slope of 6°C min^{-1}until 310°C and remaining at this condition for 5 min. Mass spectra were recorded in electronionization (EI) mode at 70 eV. The transfer line, the ion source and the quadrupole were set at 280°C, 230°C, and 200°C, respectively. Mass spectra were scanned in the range m/z 35e700 amu. The software for analysis was ChemStation D.03.00.611 and the spectra data base was NIST17. Quantitative analyses of 1,4-butadiene in lyophilized extracts were carried out by GC-FID using an external calibration curve. Previous derivatization, the standards and the samples were injected in a GC chromatograph (Agilent GC7890A) working at the same conditions as above.

2.5. NMR analysis of extracts

Lyophilized extracts, 15 mg, were dissolved in 700 ml DMSO and analysed in a MERCURYplus NMR Spectrometer Systems VARIAN. The acquisition parameters were: 25°C temperature, 128 scans with 2.559 s acquisition time, 6402.0 Hz spectral width, and 1 s relaxation delay with 45° pulse angle.

2.6. UPLC-MS/MS analysis of extracts

Quantitative analysis of adipic, lactic and terephthalic acid in the lyophilized extract were carried out by UPLC-MS/MS using external standards. The samples were dissolved with the injection solvent prior to analysis with a Waters ACQUITY UPLC™ system (Waters, Milford, MA, USA) consisting of ACQUITY UPLC™ binary solvent manager and ACQUITY UPLC™ sample manager, coupled to a tandem quadrupole mass spectrometer Xevo TQS (Waters, UK). Compounds were separated with an ACQUITY UPLC® HSS T3 column (1.8 mm, 2.1 x 150 mm). The injection volume was 2.5 mL. The temperature of the injector was 5°C and the column was heated at 35°C. To allow proper separation, a gradient system consisting of solvent A, ACN-iPrOH (80:20), and solvent B, pure water with 10 mM ammonium formiate and 0.15% of formicacid solvent A (95:5), was used. The linear gradient was 0-1 min 60% B, 0.35 mL min^{-1} (isocratic); 1-3 min 0% B, 0.40 mL min^{-1} (gradient); 3-5 min 0% B, 0.7 mL min^{-1} (isocratic); 5-5.1 min 60% B, 0.35 mL min^{-1} (gradient); and 5.1-6 min 60% B, 0.35 mL min^{-1} (isocratic). The average maximum pressure in the chromatographic system was 62.053 kPa (9.000 psi). The MS equipment used an electronic spray ionization (ESI) source in negative ion mode. The ESI parameters were: 2.5 kV capillary voltage; 30 V cone voltage; 150°C source temperature; 500°C desolvation temperature; and 800L h^{-1} desolvation gas flow. Flow injections of each individual standard were used to optimize cone voltage and Multiple Reaction Monitoring (MRM) parameters. Collision induced dissociation was achieved by using argon at 0.15 mL min^{-1} flow rate in the collision cell. Instrument control and data acquisition and processing were carried out by using MassLynx™ software (version 4.1; Waters, USA). Repeatability of the procedure was determined by triplicate analysis of every sample.

3. Results and discussion

3.1. Effects of biodegradable mulches on water solution

The direct contact of the unused biodegradable plastic mulches with the mineral water solution resulted in pH changes and in components of the materials being released to

the solution (Table 3), as determined after lyophilisation, while conductivity remained basically unaffected. Among biodegradable plastic mulches, the ones richer in carbohydrates (TPS, cereal flour) or PHB released more components, while those with PLA released fewer components. Concurrently, BioFilm® and Mater-Bi® acidified the solution, whereas the other materials increased pH, especially Bioplast® (B-SP4, B-SP6).

These results evidence that BDP are not strictly water-insoluble [9]; thus they are not to be considered as inert as polyethylene plastics. Even though BDP are manufactured to biodegrade into the soil, they start actively interacting with the environment soon after entering in contact with the water solution, well before beginning biodegradation. This situation is not to be neglected; biodegradable plastics may be used in areas where sprinkle irrigation is the predominant watering system, in fields under frequent flooding, or in rainy areas. Changes in physicochemical properties in the soil or in compost have been reported after the mulch biodegradation has started [8, 13, 14], but, to our knowledge, early changes before biodegradation have not been described in the literature. Nonetheless, a recent study on compostable plastic bags, mainly manufactured with Mater-Bi® [18] also found a decrease in the pH of leachates from unexposed Mater-Bi® samples. Equivalent results were obtained in weathered samples [19, 20].

Table 3. - Physicochemical characteristics of extracts from black mulches after 7 days of incubation in MS solution and the amount of dried solids leached from mulches to the extracting solution. Mean ± standard deviation (n = 3).

Mulch	pH	Conductivity (mS/cm 25 °C)	Plastic components leached ($mg \cdot g^{-1}$)
Control (no mulch)	4.83 ± 0.032	6.02 ± 0.121	
Biofilm®	3.79 ± 0.012	6.08 ± 0.058	68.5 ± 1.32
Bio-Flex®	5.99 ± 0.012	5.89 ± 0.095	23.3 ± 4.86
Bioplast® (B-SP4)	6.43 ± 0.289	5.81 ± 0.078	-
Bioplast® (B-SP6)	6.14 ± 0.015	6.12 ± 0.058	37.8 ± 2.47
Ecovio®	5.66 ± 0.278	6.03 ± 0.012	27.5 ± 2.18
Mater-Bi®	3.77 ± 0.025	6.07 ± 0.118	60.5 ± 2.65
MIMGreen® (paper)	5.69 ± 0.260	6.02 ± 0.098	19.7 ± 3.18
Mirel®	5.60 ± 0.032	5.98 ± 0.185	41.7 ± 2.25
Polyethylene	5.76 ± 0.023	5.98 ± 0.081	16.3 ± 0.76

3.2. Compound identification in the extracts

The identification of the compounds delivered from the films into the leachates was carried out by GC-EI-MS and ^{1}H-NMR. For GC-MS identification, to obtain the maximum number of volatile compounds, an aliquot of ca. 10 mg of each lyophilized extract was derivatized with MEOX and MSTFA in pyridine. Compounds with hydroxyl groups were identified as its corresponding trimethylsilyl ethers (TMS), and compounds with carboxylic groups as TMS esters. Compounds with functional groups susceptible to be silanised were obtained as the corresponding TMS derivatives.

Previously to silanisation, for the simplification of chromatograms, MEOX was used to block the anomeric carbon of aldoses and ketoses. Chromatographic profiles obtained (Table 4) reveal the presence of non-polar and polar organic compounds with low and medium molecular mass in the extracts. The profiles and the complexity of the chromatograms obtained with GC-MS analysis evidenced substantial differences among control, PE, and BDP samples.

The ^{1}H-NMR spectra of lyophilized extracts supported GC-MS results (Table 4). As compared with GC-MS, ^{1}H-NMR is valued for contributing to identify compounds in complex samples and for allowing characterization of non-volatile compounds, components and polymeric short chains, where GC is ineffective. Thus, ^{1}H-NMR uncovered PBAT components remaining unnoticed through GC-MS, very likely coming from small non-volatile polymer fragments but soluble enough in dimethylsulfoxide (DMSO), the solvent used in ^{1}H-NMR.

Table 4. Main compounds and components found in the mulches extracts, as identified by GC-MS and ^{1}H-NMR.

Mulch	GC-MS[a]	^{1}H-NMR
Biofilm®	1,4-butanediol, mono and disaccharides, adipic acid, glycerol monostearate	Butylene-adipate chains, carbohydrates, terephthalate
Bio-Flex®	Lactic acid, 1,4-butanediol, pentaerythritol	Lactate, butylene-adipate chains, terephthalate
Bioplast® (B-SP4)	Lactic acid, 2-oxopropanoic acid, 1,4-butanediol, 3-hydroxybutyric acid, glycerol, 1,4-butanedioic acid, mono and disaccharides, adipic acid, fatty acids, terephthalic acid, glycerol monostearate	Lactate, butylene-adipate chains, fatty acids chains, carbohydrates, terephthalate, glycerol
Bioplast® (B-SP6)	Lactic acid, 1,4-butanediol, 3-hydroxybutyric acid, glycerol, 1,4-butanedioic acid, mono and disaccharides, adipic acid, fatty acids, terephthalic acid, glycerol monostearate	Lactate, butylene-adipate chains, carbohydrates, glycerol
Ecovio®	Lactic acid, 1,4-butanediol	Lactate, butylene-adipate chains
Mater-Bi®	Lactic acid, 1,4-butanediol, glycerol dimers and trimers, 4-hydroxybutyric acid, mono and disaccharides, terephthalic acid, adipic acid	Lactate, butylene-adipate chains, carbohydrates, terephthalate, glycerol
MIMGreen® (paper)	Glycerol, diethylene glycol, fatty acids, disaccharides	Glycerol/polyethylene glycol
Mirel®	4-hydroxybutyric acid, 3-hydroxybutyric acid, tributyl acetylcitrate, glycerol monopalmitate, glycerol monoestearate	Hydroxybutyrate chains, fatty acids chains
Polyethylene	Fatty acids, 1,10-decanedioic acid bis (2-ethylhexyl) ester	No NMR signals

[a]: Compounds with hydroxyl and carboxylic groups were identified as TMS derivatives.

Chromatographic profiles of control samples showed very few signals (Fig. 1a), with some of them from compounds in the mineral solution (e.g. boric or phosphoric acid) or as artifacts from the derivatization reagents. Polyethylene plastic mulch, traditionally used in agriculture, displayed a low profile chromatogram (Fig. 1b), with the compounds from the liquid extraction solution and artifacts and only a group of small signals. These were identified as fatty acids (C16:0 and C18:0) and the bis(2-ethylhexyl) ester of 1,10-decanedioic acid, likely used as additives in film manufacturing. The NMR spectrum of

PE film did not show significant peaks. On the contrary, the samples from biodegradable mulches resulted in dense chromatograms rich in a diversity of compounds (Fig. 1). Depending on the polymeric composition of the biodegradable mulch, different compounds were prevailing. Nevertheless, many of the compounds were common to most samples: dicarboxylic acids, hydroxyacids, diols, triols, glycerol dimers and trimers, monosaccharides, disaccharides, and terephthalic acid were present regardless of the biodegradable material. These compounds are regularly used in the formulation of biodegradable mulch materials, added either as structural components of the backbone polymers or as auxiliary compounds contributing to the required properties of the final product (colourings, additives, plasticizers, etc.). The BioFilm® chromatographic profile included 1,4-butanediol, mono and disaccharides and glycerol monostearate. The NMR spectrum revealed the presence of components of the PBAT chain and carbohydrates, the later likely coming from cereal flour, a basic component of this blend. Bioplast® (B-SP4) and Bioplast® (B-SP6) are products of the same manufacturer and share the PBAT and P-thermoplastic starch polymeric basis; B-SP6 also contains PLA. Chromatograms of samples from both materials displayed abundant signals, including the PBAT derivatives 1,4-butanediol and adipic and terephthalic acids, and 3-hydroxybutyric acid, 2-oxopropanoic acid, 1,4-butanedioic acid, fatty acids, monosaccharides, disaccharides and glycerol monostearate (Fig. 1c). Lactic acid, also present in both Bioplast® samples with different intensities, exhibited a marked signal in the B-SP6 sample consistent with the blend composition, and a weak signal in the B-SP4 sample, very likely a cross-contamination from B-SP6 associated to the sharing of machinery in the production process. NMR analyses of both samples were consistent with GC analyses and validated the identification of all compounds. Mater-Bi® chromatograms (Fig. 1d) were as rich in signals as Bioplast® (B-SP4 and B-SP6), revealing a complexity and variety of compounds released in the mineral solution. Many PBAT components were identified, mainly 1,4-butanediol and adipic acid. Moreover, huge peaks of glycerol and glycerol dimers and trimmers were characterised. Mono and disaccharide peaks also identified were consistent with the thermoplastic corn starch in the Mater-Bi® composition. Additionally, small signals of 4-hydroxybutanoic acid and fatty acids were found, likely from the vegetable oils included in the blend composition. The NMR spectrum correlated the GC peaks for all components except for the fatty acid ones; the low sensitivity of the NMR analysis and the low solubility of free fatty acids and oils in the aqueous media may be responsible. Bio-Flex® is composed of a mixture of PBAT and ca. 30% PLA. The chromatogram of the corresponding extract identified 1,4- butanediol, pentaerythritol and

a small lactic acid signal. In addition to the PBAT components, the NMR spectrum displayed a d 1.35 ppm Hz signal attributed to lactate chains. Chromatograms from Ecovio® were poor in signals; the main peak was from 1,4-butanediol, together with small lactic and fatty acid peaks. The NMR spectrum revealed very small lactate and butylene adipate derivatives peaks. MIMgreen® (paper) extract chromatogram also exhibited very few peaks, a prominent one for glycerol, and diethylene glycol, fatty acids and disaccharides smaller ones (Fig.1e). The NMR spectrum only showed glycerol. Mirel® GC chromatogram exhibited a medium-size 3- hydroxybutyric acid peak. Other peaks identified 4-hydroxybutyric acid, tributyl acetylcitrate, glycerol monopalmitate, and glycerol monoestearate (Fig.1f). The corresponding NMR spectrum was rich in signals and corroborated GC-MS identification.

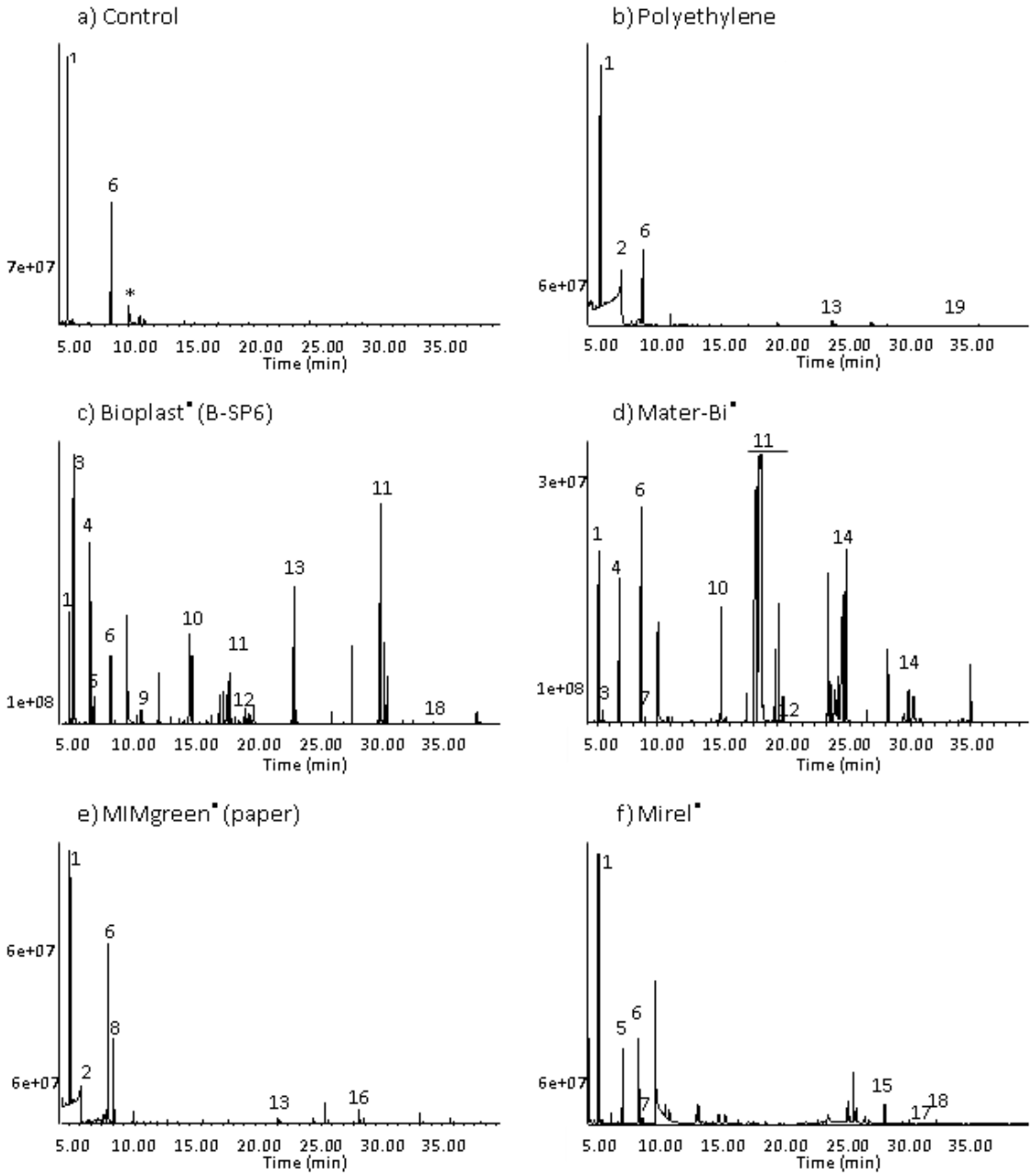

Figure 1.- GC-MS chromatograms after incubation in the extracting solution of control and mulch extracts (PE, Bioplast® B-SP6, Mater-Bi®, MIMgreen® paper, and Mirel®). (1 and 2) Derivatization reagents, (3) lactic acid, (4) 1,4-butanediol, (5) 3-hydroxybutyric acid, (6) glycerol, (7) 4-hydroxybutyric acid, (8) diethylene glycol, (9) 1,4-butanedioic acid, (10) adipic acid, (11) mono and disaccharides, (12) terephthalic acid, (13) fatty acids, (14) glycerol dimers and trimmers, (15) tributyl acetylcitrate, (16) disaccharides, (17) glycerol monostearate, (18) glycerol monopalmitate, (19) 1,10-decanedioic acid bis (2-ethylhexyl) ester. (*) phosphate groups from the extracting solution, present in all chromatograms.

3.3. Quantification of compounds in the extracts

After identification, selected compounds were quantified in the leachates (Table 5). Their concentration was mostly low. Hydrolysis of the prevailing PBAT polymer in the plastic mulches and the higher water-solubility of 1,4-butanediol, as compared to adipic and terephthalic acids, resulted in higher levels for 1,4-butanediol. PBAT is a well-known aliphatic aromatic copolyester consisting of two types of dimers: BT, an ester repeat unit of 1,4-butanediol and terephthalic acid monomers, and BA, an ester unit of 1,4-butanediol and adipic acid monomers. Consequently, primary hydrolysis of PBAT in contact with water solution releases 1,4-butanediol units, preferentially coming from the BA dimer, which is more susceptible to hydrolysis than the BT one [21]. Furthermore, the higher concentration of adipic acid found to have been released from the mulches, mainly in Mater-Bi® leachates, as compared with the ones of terephthalic acid, may be associated to adipic acid being more water soluble than terephthalic acid. In contrast with the high water solubility, the lactic acid concentration was low, likely due to low hydrolysis. Considering that ca. 7% PLA is present in Ecovio®, GC lactic acid in the corresponding extract was unexpectedly low; however this compound was clearly visible in the NMR spectrum. It may be speculated that despite the presence of short fragments of the polymer in the extract, under the conditions assayed, hydrolysis was low. In fact, blending PLA with PBAT is used to decrease PLA hydrolysis to toughen the material [22]. Concentration of lactic acid was highest in B-SP6 leachates (Table 5); B-SP6 blend includes PLA and TPS, the later likely weakening the compatibility of the PBAT/PLA blend and facilitating PLA hydrolysis.

Table 5. Quantitative analysis ($mg \cdot L^{-1}$) of specific compounds in the mulch materials extracts previously reporting effects on lettuce and tomato *in vitro* plant tissue culture. Mean ± standard deviation (n = 3).

Mulch	Adipic acid[a]	Lactic acid[a]	1,4-butanediol[b]	Terephthalic acid[a]
Biofilm®	0.009 ± 0.002	0.0004 ± 0.00001	0.59 ± 0.119	0.0003 ± 0.0001
Bio-Flex®	NQ	0.002 ± 0.0003	24.31 ± 0.548	0.0005 ± 0.0001
Bioplast® (B-SP4)	0.003 ± 0.0004	0.001 ± 0.0001	22.01 ± 0.517	0.001 ± 0.0002
Bioplast® (B-SP6)	0.008 ± 0.002	0.049 ± 0.011	28.82 ± 0.188	0.003 ± 0.001
Ecovio®	0.002 ± 0.0001	0.001 ± 0.0001	20.87 ± 1.343	0.001 ± 0.0002
Mater-Bi®	0.040 ± 0.0004	0.001 ± 0.0001	35.65 ± 2.447	0.003 ± 0.0003
MIMGreen® (paper)	NQ	NQ	ND	ND
Mirel®	NQ	NQ	NQ	NQ
Polyethylene	NQ	NQ	ND	NQ

Not quantified, NQ < 0.03 $\mu g \cdot g^{-1}$; Not detected, ND < 0.01 $\mu g \cdot g^{-1}$. Analysis by [a] UPLC-MS/MS; [b]: GC-FID.

The identification and quantification of compounds proves BDP mulches may release small fractions of the components from their blend composition to a water solution before their installation in the field, only through contact with a sterile mineral water solution. It has been previously reported lactic and adipic acids, and 1,4-butanediol, to have effects on *in vitro* plant development, although the concentrations reporting effects are mostly above the ones in the present leachates (Table 5). When provided at 5-500 mg L1 to tomato and lettuce *in vitro* cultured plantlets, these compounds increased proline, a plant stress marker [15]; adipic acid reduced plant development, inhibiting lettuce and tomato root and shoot growth, and lactic acid inhibited plant growth in lettuce [15]. Adipic acid, 7-60 mg L1, has also showed to reduce growth in taro, bean, strawberry and tobacco plants, mostly limiting root development [23-26]. Water leachates from the same biodegradable plastics as the ones hereby analysed have been already tested for their effects on *in vitro* cultured tomato and lettuce plants [16]. In this system, all BDP leachates reduced lettuce root growth; Bioplast® and Bio-Flex® leachates limited overall plant growth, and germination was also inhibited by Bioplast® leachates. *In vitro* growing tomato plants were more sensitive than lettuce, and all BDP leachates, except the one from Bio-Flex®, reduced root and shoot growth. The sensitivity of plant species to different environmental agents in nature is widely known (e.g. salinity, pH, cold, etc.); equally sensitivity to chemicals and to manufactured products varies among plant species. Knowledge on compounds readily released by BDP mulches to water may contribute to optimize and target their formulation for the cultivation of specific plant species. The present work using the UPLC-MS/MS technique, reports concentrations of the identified monomers in the BDP leachates to be in several orders of magnitude under those previously reported to have inhibitory effects on tomato and lettuce *in vitro* growing plants [15]. However, the low sensitive NMR technique reveals the presence of these compounds in the leachate. Thus, the effects reported for the leachates [16] cannot be attributed to individual effects of specific compounds; whether they may be attributed to the presence of short chain oligomers or polymeric fragments, to the complex mixture of a few or several of the compounds identified hereby in the water solutions, or to unknown compounds in them, requires to be further investigated. It has also to be acknowledged that the composition of plastic mulches includes small amounts of many organic and inorganic compounds used as additives whose effects are largely unexplored [27].

4. Conclusions

In this paper compounds from unused biodegradable plastic mulches were released to a water mineral solution closely resembling the soil water mineral composition or transferred from continental water to the sea. Our results demonstrate that, before biodegradation starts, BDP release compounds when in a water environment. Furthermore, these compounds have been qualitatively identified and several of them have been also quantified in the water solution. Concentrations found are low, and far for the ones previously reported to influence plant growth, which does not allow establishing direct relationship between specific compounds quantified and effects on plants. BDP mulches also release a wide diversity of other compounds, likely short chain oligomers or polymeric fragments, remaining to be identified and studied.

Appendix A. Supplementary data Supplementary data to this article can be found online at https://doi.org/10.1016/j.polymdegradstab.2020.109202.

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CHAPTER IV

EFFECTS OF BURIED AGRICULTURAL BIODEGRADABLE PLASTIC MULCHES IN TWO HORTICULTURAL SPECIES: TOMATO AND LETTUCE

This chapter reproduces the text of manuscript in preparation for send it to publication.

Abstract

Biodegradable plastic mulches (BDM) are a valuable resource in horticulture, saving film removal time and costs and avoiding plastic accumulation in agricultural soil.They are tilled into soil, where their fragments release polymeric and additive compounds thereafter throughout their biodegradation. The present work aims to evaluate the effect of buried BDM fragments on two plant species commonly cultivated with BDM, tomato

(*Lycopersicon esculentum* Mill., cv. Red Cherry) and lettuce (*Lactuca sativa* L., cv. Trocadero Ribera). A mesocosm experiment was performed sowing seeds in plant pots containing pieces from seven different BDM formulations (BP1 to BP7), one biodegradable paper mulch and one non-biodegradable, low density polyethylene plastic mulch (PE). To test whether the weathering of the BDMs during their use in the field influences the effects, the tests were performed with both BDM pieces from new unused mulches and from mulches collected after their service life in the field.

Germination of both tomato and lettuce seeds was unaffected by the presence of any of the mulch fragments, while the growth of both plant species was severily inhibited by BP7 and several other BDM caused detrimental effects on both plant species. In lettuce, all BDM fragments caused significant retardation in plant development. In tomato, plants developed better than in lettuce, but growth retardation was also somewhat evidenced with several BDM, including a reduction in the leaf chlorophyll content. For most of the biodegradable mulches, the field weathered ones caused stronger adverse effects than the new unused mulches.On the contrary, buried PE mulch, either unused or weathered, had no effects on plants.

Overall, the results demonstrate that buried BDM interact with plants and may alter their development. They also suggest that the BDM effects depend on the chemicals they release, rather than to the physical presence of the BDM fragments.

1. Introduction

Agricultural plastic mulching consists of covering the soil with plastic films to provide cultivated plants a microclimate favouring their development, increasing productivity, hastening earliness and enhancing fruit quality. Due to the material properties, mulches increase water-use efficiency and prevent weed development (Kasirajan and Ngouajio, 2012; Kader et al., 2017; Gao et al., 2019). However, plastic mulches made of low-density polyethylene (PE), a very stable and non-biodegradable material, are massively used, contributing to the global plastic waste accumulation. In particular, fragments released during their use often end up buried into the agricultural soil, where they increasingly accumulate and threaten crop productivity and food safety (Wenqing et al., 2014; Liu et al., 2014; Gao et al., 2019; Zhang et al., 2020; Hu et al., 2020). Biodegradable plastic mulches (BDM) have arisen as a promising alternative to PE use (Martin-Closas et al., 2017). They are intended to be tilled after the crop harvest and eventually to biodegrade by native soil microorganisms, avoiding plastic waste accumulation into the agricultural soil. Yet, the soil is continuously fed with BDM materials to biodegrade, fragments and chemicals (polymers, oligomers, monomers and additives), whose environmental impact is currently one of the main concerns for BDM adoption in agriculture (Sintim and Flury, 2017; Serrano-Ruiz et al., 2021). The BDM first and more likely impact on the biotic environment is on cultivated plants. Particularly, they interact with plant roots, from the initial laying of films on the soil to the BDM after-use service, and up to their biodegradation. Plants are the feed and food resource for living organisms, including humans; consequently, the putative BDM effects on growth of cultivated plants are to be investigated.

BDM are required to pass ecotoxicity tests according standard norms (EN 17033, 2018), including phytotoxicity testing on plants in pots (OECD 208, 1984) after significant BDM in-soil biodegradation. Establishing the time period of the BDM cycle in which to run the test for assessing BDM ecotoxicity on plants is a key issue (Degli-Innocenti, 2014) and different approaches have been followed. Overall, most of the ecotoxicity tests on plants have been performed after burying the BDM in soil for several months. The first report of BDM ecotoxicity testing on plants was on pepper (*Capsicum annuum L.*) germination in a substrate where one new non-degraded BDM had been buried (Olsen and Gardner, 2001). Subsequently, Fritz et al. (2003), following OCDE 208 and DIN (German Institute for Standardisation) standards, tested the effects of a poly(ester-amide) BDM after exposure to soil degradation for 0 to 160 days on plant growth. Rychter et al. (2006) determined ecotoxiciy of BDM made of 3-hydroxybutyrate blends with poly lactic acid

(PLA), after film degradation in sandy soil for 1 and 6 months, on plant growth. Other authors have also allowed PBAT (polybutylene adipate terephthalate) and PBAT-PLA BDM to biodegrade into soils for time periods ranging 6 to 12 months to run ecotoxicity tests on plants of these soils (Sforzini et al., 2016; Muroi et al., 2016; De Souza et al., 2021) or of extracts from these soils (Palsikowski et al., 2018; Souza et al., 2020) by running germination tests in Petri dishes or a pot assay (Rychter et al., 2006, Muroi et al., 2016). Recently, two authors have tested phytotoxicity of unbiodegraded PBAT-starch (Qi et al., 2018) and PBAT-PLA (Meng et al., 2021) fragments in contact with plants thourgh a pot assay.

While several of the mentioned studies did not find evidence of BDM phytotoxicity effects (Olsen and Gardner, 2001; Rychter et al., 2006; Muroi et al., 2016; Sforzini et al., 2016; Palsikowski et al., 2018; Souza et al., 2020; De Souza et al., 2021), some others reported BDM fragments buried into soil and compounds leached from a variety of BDM to alter growth, physiology and morphology of a diversity of plant species soon after their burial (Frizt et al., 2003; Qi et al., 2018; Boots et al., 2019; Wang et al., 2020; Meng et al., 2021). A recent review on the effects of biodegradable plastics on the biotic environment, including plants, can be found in Serrano-Ruiz et al. (2021).

Most assays for BDM ecotoxicity testing on plants have used species not commonly cultivated with BDM, including S*orghum bicolor*, *Lepidium sativum*, *Pennisetum* sp., *Brassica* sp., *Hordeum vulgare*, *Avena sativa*, *Triticum aestivum*, *Raphanus sativa*, *Allium cepa* and *Lolium perenne*. Plant species commonly cultivated with BDM, *Lactuca sativa* or *Phaseolus vulgaris,* have been tested only scarcely (Souza et al., 2020; De Souza et al., 2021; Meng et al., 2021). Meanwhile, preliminary *in vitro* assays found evidence of growth and physiology of two plant species widely cultivated with BDM, *Lycopersicon esculentum* (tomato), and *Lactuca sativa* (lettuce) being altered by monomers commonly present in BDM, and also by leachates from different BDM formulations (Martin-Closas et al., 2014; Serrano-Ruiz et al., 2018). Whereas testing BDM on diverse plant species will contribute to the understanding of the phtyotoxicity potential of BDM, testing on especies cultivated with BDM will shed light on their impact on the agricultural environment.

BDM are made of different polymer blends mixed with additives, and the specific combinations may affect their interaction with plants. Previous research found evidence of BDM composition to be a relevant factor in the interaction with *in vitro* grown tomato and lettuce plants (Serrano-Ruiz et al., 2018); however, there is a gap in the knowledge

of the mulch composition role on the interaction of BDM fragments buried into soil with plants. As shown in the above paragraphs, research has been mostly carried out by assessing one sort of BDM, with or without modifications on one base polymer. Besides, only the pure polymer is often assessed, without the additives, whose effects on plants may be relevant (Rychter et al., 2006; Muroi et al., 2016; Palsikowski et al., 2018; Wang et al., 2020; Meng et al., 2021). While the ecotoxicity of several blends has been addressed, the different methodologies and test conditions used impedes comparisons. In addition, during their service life BDM are exposed to environmental and agricultural agents (sunlight, rainfall, wind, agrochemicals, etc.) that alter their physicochemical properties before they are buried into soil (Touchaleaume et al., 2018; Anunciado et al., 2020, 2021). The consequences of these changes on the ecotoxicity potential of buried BDM have neither been explored.

Overall, the present study aims to assess the effects of BDM fragments buried into soil, in direct contact with plant roots, on germination, growth and development of lettuce and tomato plants. In order to understand the role of the BDM composition on their interaction with plants, several commercial BDM of different composition were included. Furthermore, it was explored whether changes induced by environmental factors influence BDM ecotoxicity potential. The effects were investigated by burying BDM fragments, both new and field weathered, into pots where lettuce and tomato plants grew and developed.

2. Materials and methods

2.1. Mulches

New and field weathered mulches were tested for their effects on lettuce and tomato plants. In a first assay, seven fresh-roll plastic mulches not previously used in the field were tested. Six BDM were PBAT-based films (polybutylene adipate terephthalate) blended with starch (BP1, BP2), starch and PLA (polylactic acid) (BP3), PLA (BP4, BP5) and cereal flour (BP6), and one BDM was PHB-based (BP7) (polyhydroxy butyrate) (Table 1). In addition, a biodegradable paper mulch made of virgin cellulose fibres, black upwards and brown downwards, and a non-biodegradable low density polyethylene (PE) plastic mulch were included. All plastic mulches were black and 15 -17 μm thick, except BP7, which was 40 μm thick. Paper mulch was of 85 g m^{-2} grammage.

Table 1. Mulches used in the experiments.

Mulch	Blend composition
BP1	PBAT, Corn TPS, vegetable oils
BP2	PBAT, Potato TPS
BP3	PBAT, Potato TPS, PLA
BP4	PBAT, PLA (~7 %)
BP5	PBAT, PLA (~ 30 %)
BP6	PBAT, Cereal flour
BP7	PHB
Paper	Cellulose fibre
PE	Low density polyethylene

PBAT: polybutylene adipate terephthalate; TPS: thermoplastic starch; PLA: Polylactic acid; PHB: polyhidroxy butyrate.

In a second assay field weathered mulches were used. Rolls of BP1, BP2, BP3, BP4, BP5, paper and PE were used for mulching an organic pepper crop running for 180 days, from May to November, at Vilanova de l'Aguda (41° 54′ 47″ N 1° 15′ 14″ E, Lleida, Spain). Pepper crop was selected because it is usually grown with mulch films and because the pepper canopy is straight, so that it allows sunlight and other climatic events impacting on the mulch. Climatic data during the crop cycle were registered to characterize the weathering pressure on the mulches (Table 2). After pepper harvest, mulch samples were collected from the soil surface and kept dry in the dark until their use. They were rinsed with distilled water and air dried to remove soil and vegetable debris stacked on their surface. All samples were cut to 1 cm^2 pieces using scissors.

Table 2. Weathering conditions on the mulch materials along the crop cycle duration, 180 days (20th May to 14th November) at Vilanova de l'Aguda (Lleida, Spain).

Global Radiation ($MJ \cdot m^{-2}$)	UV Radiation ($MJ \cdot m^{-2}$)	Rainfall (mm)	Temperature (°C)			Relative Humidity (%)	Wind Speed ($m \cdot s^{-1}$)
			Min.	Mean	Max.	Mean	
3641	158.83	283.7	11.96	19.18	26.89	62.73	1.56

2.2. Plant material

Plant species tested were *Lycopersicon esculentum* Mill., cv. Red Cherry (tomato) and *Lactuca sativa* L., cv. Trocadero Ribera (lettuce). For both plant species and cultivar,

seeds were selected from a single batch exhibiting over 85% germination rate in the substrate used in the experiments.

2.3. Experimental set up and design

To determine the effects of new and of weathered BDM fragments on lettuce and tomato germination and plant development, two greenhouse scale pot assays were carried out from April to August. A substrate with optimized nutrients was used (Traysubstrat, Germany) to avoid nutrient limitation during plant growth. The physicochemical characteristics of the substrate were: pH 5.9, conductivity 1.2 (mS cm^{-1} 25°C), organic matter content 85% (dry weight), density 285 (g L^{-1}), and NPK 14-16-18 (Kg m^{-3}).

For every treatment, four 1.5 L pots (15 cm diameter) were filled with a mixture of air-dried plant growth substrate containing pieces of the corresponding new or weathered mulch, 4.6% (w/w); equivalent control pots with the substrate without mulch were included. The water content of the substrate was adjusted to 70% of water holding capacity (WHC), which was maintained throughout the experiments. All pots were randomly placed in a greenhouse bench and allowed to settle down for four days before seeds of lettuce and tomato, 15 seeds of the corresponding plant species per pot, were sown. Germination rate was monitored until 50% germination was reached in the control treatment, then 5 plantlets per pot were left to develop.

2.4. Plant growth evaluation

Lettuce and tomato plants were allowed to growth for 5 and 7 weeks, respectively. Then, plant height was determined, all plants were collected and fresh weight was separately determined for the aerial part and for roots by using an analytical scale (AND ER-120A, Australia). After drying at 70°C until constant weight, dry weight was also recorded.

2.5. Chlorophyll and proline determination

The leaf chlorophyll content was determined by using a SPAD 502 chlorophyll meter (Minolta, Japan). For every pot, five fully developed leaves from different plants were randomly selected.

The leaf proline content was recorded according to Bates et al. (1973). Briefly, 0.1 g fresh weight samples from five randomly selected leaves per pot were homogenized with 2 mL of a 3% sulfosalicylic acid solution. A mixture of the homogenate, acetic acid and acidic ninhydrin reagent (1:1:1) was incubated at 90°C for 30 minutes. Then, 2 mL toluene was added to the mixture and vigorously vortexed. The absorbance of the resulting upper

phase was determined at 520 nm in a UV/VIS spectrophotometer (Helios Gamma, Thermo Fisher, England). Proline concentration was calculated using L-Proline for the standard curve. Results are expressed as mg of proline per g of fresh weight. .

2.6. Substrate pH and microbial activity

The pH of the substrate was determined at the set-up of the two assays, and then on every pot at the end after plant collection. For this, an air-dried substrate with distilled water mixture, 1:10, was stirred for 30 minutes, and after left to settle for 30 minutes. Then, pH was recorded with a pH-electrode (Hach, Germany).

Total microbial activity in the pots' substrate was determined after collecting the plants, following Green et al. (2006) procedure based on FDA (fluorescein diacetate) enzymatic hydrolysis. Briefly, substrate samples, 2.0 g, were mixed in a sterile Erlenmeyer flask with 50 mL of 60 mM potassium phosphate buffer (pH 7.6). Then, 0.5 mL of a FDA 4.9 mM stock solution was added to start the reaction. The flasks were incubated at 37°C for 90 min and acetone was added to stop the reaction. Aliquots of the mixture were centrifuged for 3 minutes at 8000 rpm and optical density of the eluates was determined at 490 nm in a UV/VIS spectrophotometer (Helios Gamma, Thermo Fisher, England). Fluorescein concentration was calculated by a calibration curve using fluorescein disodium salt standard solutions in 60 mM potassium phosphate buffer (pH 7.6). Results are expressed as µg of fluorescein released per g of dry soil per hour. Three replicates per treatment were analysed.

2.7. Statistical analyses

For every plant species and assay (new, weathered mulches), statistically significant differences were determined by analysis of variance (ANOVA) with SAS software (JMP Pro 14.0.0). Where corresponding, treatment means were compared by Dunnett's test; with the control group being the plants grown in pots without mulch fragments ($\alpha = 0.05$).

3. Results and discussion

3.1. Effects of new unused mulches

The growth of tomato and lettuce plants in the substrate with buried new unused BDM was monitored for 7 and 5 weeks, respectively, and the growth and physiological *status* of the plants was finally evaluated.

For both plant species, 50% seed germination was reached 7 and 9 days after lettuce

and tomato sowing, respectively, regardless of the treatment, but both plant species were later distinctly affected by the BDM. In particular, plant development, plant weight (both fresh and dry weight showed equivalent reductions) and leaf chlorophyll content were afected by some of the BDM.

In tomato, all during the 7 weeks of the assay, the visual aspect of the plants developing on the BP1, BP4, BP5 and PE treatments was basically equivalent to that from control plants, without changes in plant height, size or leaf number, nor did they exhibited any other changes. However, in pots with BP2, BP3 and BP6, the plant leaf coverage was somehow lower than in control plants. More outstanding, plant height and size were considerably reduced in the BP7 and paper treatment pots (Fig. 1), where plant development retardation was evidenced from the second week after sowing, with plantlets being only at their cotiledonary stage, while the first two leaves had already emerged and started development in the control plants. Moreover, in contrast with control plants where only the basal part of the stems were reddish, their stems were reddish-purple most along them, and in plants grown in the paper treatment a marked purple coloration was also evidenced in the leaves' adaxial side. Reddish colours result from the synthesis and accumulation of anthocyanin red pigments, exerting antioxidant functions and indicators of oxidative stress induced by abiotic (e.g. UV radiation) and biotic agents (e.g. bacteria) (Chalkter-Scott, 1999; Winkel-Shirley, 2002; Pourcel et al., 2007).

In line to the eye observations, BP1, BP4, BP5 and PE treatments did not affect tomato plant growth, while BP2, BP3 and BP6 fragments in the substrate significantly reduced the plant aerial dry weight by ca. 28-38% (Fig. 2). BP7 and paper treatments drastically inhibited plant growth, reducing dry weight by almost 90% (Fig. 2).

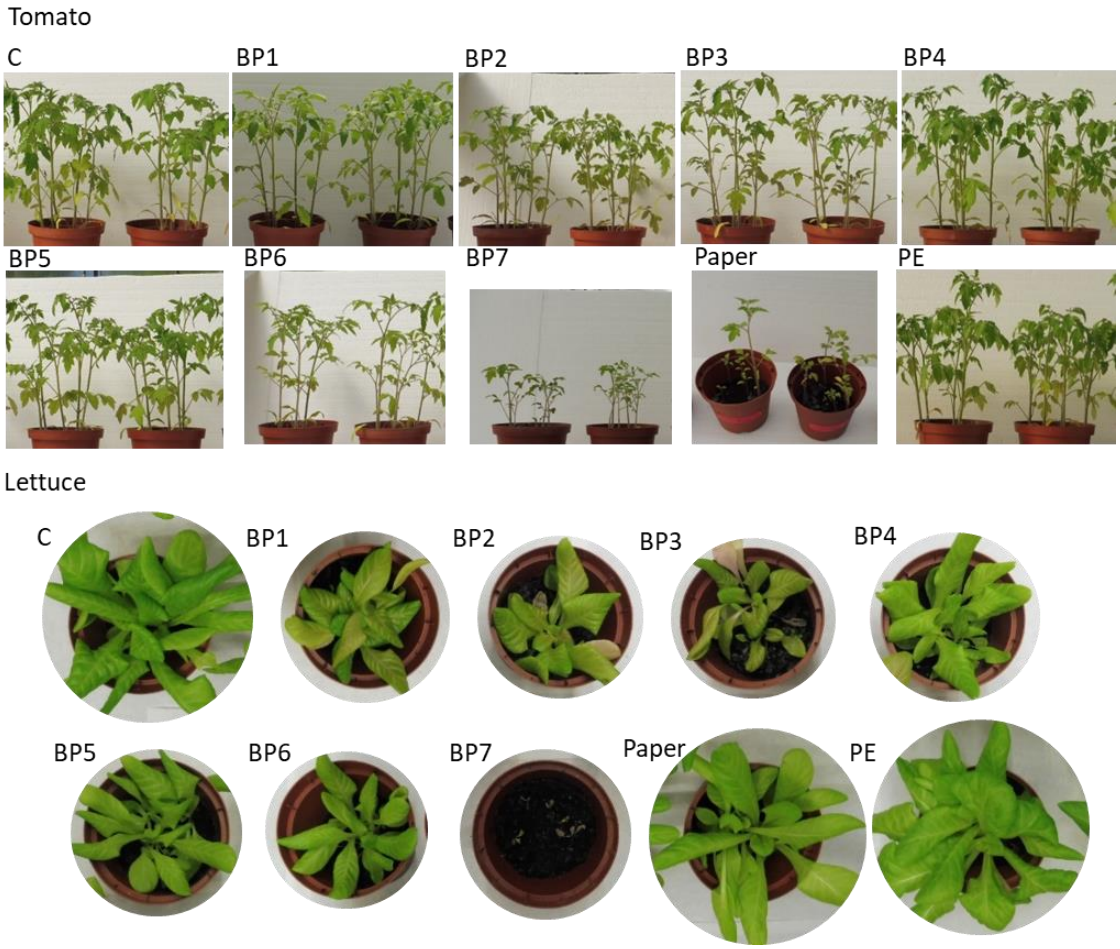

Figure 1. - Tomato (up) and lettuce (down) plants grown for 7 and 5 weeks in substrates containing new unused plastic mulch fragments.

To get knowledge on the plant physiological *status*, chlorophyll and proline content of leaves were determined at the end of the assay. They hold key roles in photosynthesis and on plant protection against abiotic stress, respectively, and changes in their levels are considered indicators of plant stress (Kaur and Asthir, 2015). In accordance with the previous effects on tomato severely limiting plant growth, BP2, BP3, BP6, and especially the BP7 and paper treatments, significantly decreased the leaf chlorophyll content (Fig. 2), while no significant changes were identified in plants from BP1, BP4, BP5 and PE treatments, the ones previously shown not to alter tomato plant growth. Proline was significantly decreased in BP3 treated plants (Fig. 2), while plant development limitations impeded proline determination for BP7 and paper treatments.

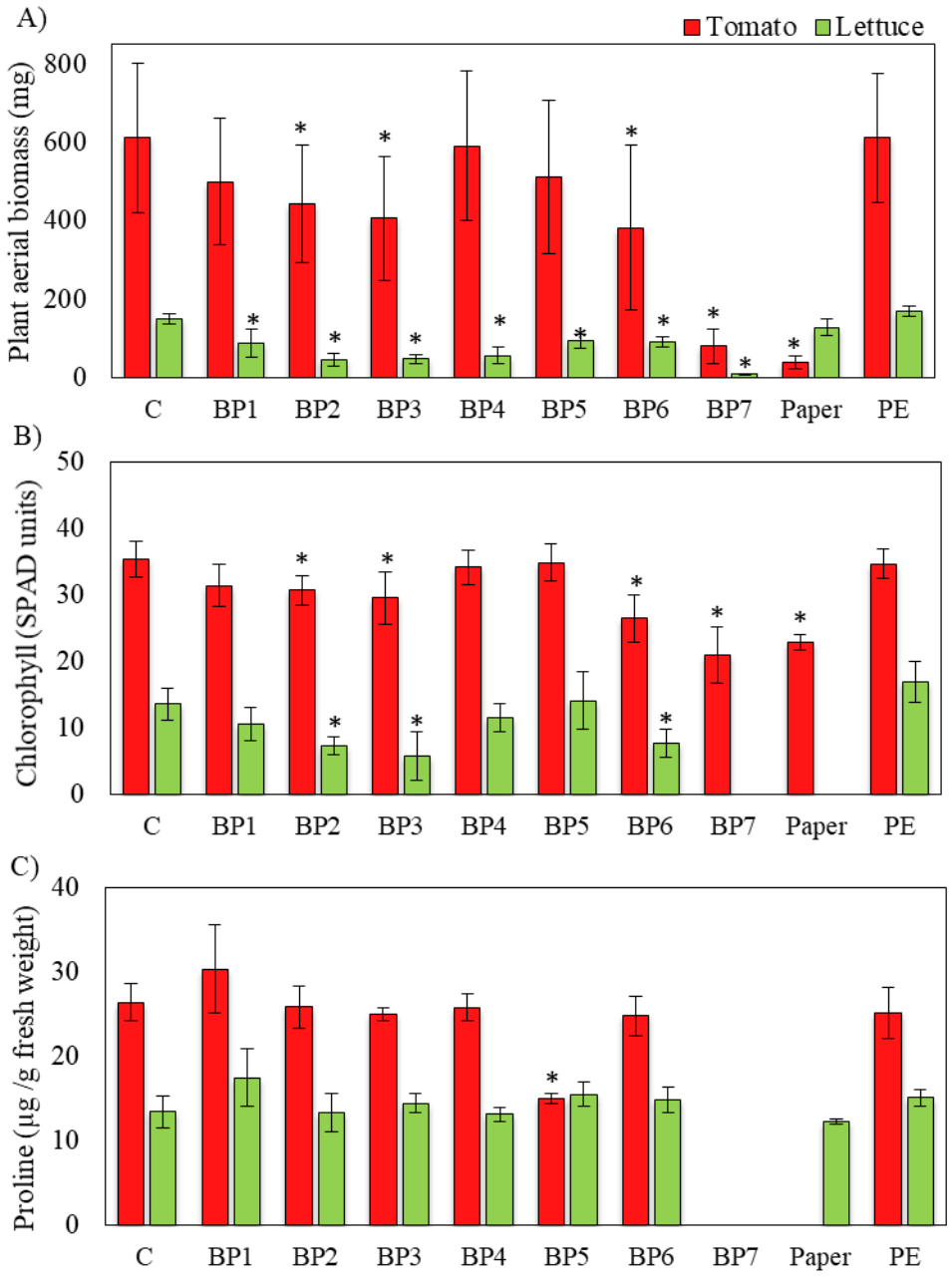

Figure 2. – Growth and physiology of tomato (red) and lettuce (green) plants grown in substrate with buried new unused mulch fragments. C=control treatment without mulch. A) Aerial part plant dry weight (n=20). B) Leaf chlorophyll content (n=20). C) Leaf proline content (n=4). Bars represent average and standard deviation. *Indicate statistical difference from control plants ($P \leq 0.05$).

In lettuce, all plastic BDM limited plant development evidenced three weeks after sowing and plant stress was denoted by the reduced size of plants compared to the control ones. At the end of the assay, plants grown on these treatments were smaller than the control ones (Fig. 1), with a significant reduction in the plant dry weight ranging from 40% for the BP1, BP6 and BP5 treatments to ca. 70% in the BP2, BP3, and BP4 ones (Fig. 2).

Overmore, leaves from BP1, BP2 and BP3 treated plants developed a reddish coloration. Similarly to that found for tomato plants, the BP7 treatment severely inhibited plant growth, (Fig. 1) and decreased plant weight by 95%. On the other hand, no visual effects on plant growth and development were identified for lettuce plants grown on paper and on PE containing pots (Fig. 1, Fig. 2). As in tomato, BP2, BP3 and BP6 treatments significantly decreased the leaf chlorophyll content (Fig. 2), while none of the treatments, BP1 to BP6, paper and PE, changed proline in lettuce leaves (Fig. 2).

Both in tomato and in lettuce, BDM fragments in the substrate affected plant growth, which was most severely inhibited when exposed to the PHB based BDM, BP7, but also to several PBAT based BDM, BP2, BP3 and BP6. The pot assay approach has been followed in a few studies to determine potential BDM effects on plant growth, reporting biodegradable fragments of different composition and concentration to alter development and physiology of several plant species. Polyamide BDM, 2% w/w, inhibited cress, millet and rape plant biomass (Fritz et al., 2003). A starch-based BDM, 1% w/w, altered wheat growth from the early plant growth to the end, decreasing aerial and root biomass (Qi et al., 2018). PLA decreased shoot biomass and chlorophyll in corn, 1-10% w/w, (Wang et al., 2020) and germination and plant height in *Lolium perenne*, 0.1% w/w, also modifying chlorophyll a/b ratio, an indicator of plant stress (Boots et al., 2019). More recently PBAT-PLA mulch micro-fragments, 1.5 - 2.5% w/w, have been reported to decrease common bean shoot, root and fruit biomass, shoot to root ratio, leaf area and leaf chlorophyll content (Meng et al., 2021). All these results underline the potential of BDM fragments to interact with plant roots and to interfere in plant growth and physiology, which may have consequences in plant performance.

The fate of BDM is the *in situ* biodegradation into the soil, mainly driven by the hydrolytic activity of the soil microorganisms feeding on them. The introduction of BDM fragments could result in changes in the substrate biophysicochemical properties, further affecting plant development; therefore, the effects of the BDM on the substrate microbial activity and pH were investigated. The microbial activity was evaluated through FDA hydrolysis determination, representative of the soil microorganisms total hydrolytic enzyme activity (extracellular and membrane-bound) and an estimation of microbial decomposing activity (Green et al., 2006). A general trend towards increased microbial activity after tomato and lettuce plant growth with the different BDM fragments was evidenced, although it was only significant for tomato, ca. 35%, with paper and BP3 treatments and for lettuce in paper treatment ca. 45%. The microbial activity increase has been correlated to BDM biodegradation rate (Barragán et al., 2016), thus pointing to BDM

biodegradation in the pots.

At the end of the assay, (bio)degradation was evident only for the paper treatment; paper fragments were scarcely visible, whereas plastic fragments kept their initial size, shape and aspect. The span of the assay, 5-7 weeks, is likely sufficient for the paper mulch to have undergone significant biodegradation, while biodegradation of plastic BDM takes longer. Other authors have reported paper fragments being undetected or mostly biodegraded in the range of months, while plastic fragments may remain in the field even after 3 years (Moreno et al., 2017; Ghimire et al., 2020; Sintim et al., 2020; Anunciado et al., 2021). The massive degradation of the paper fragments observed is likely associated to the microbial activity increase in the substrate. Paper and BP3 treatments increased microbial activity similarly, but tomato plant growth was severely inhibited only when in the pots with the paper fragments, denoting a relationship between the paper degradation and the strong inhibition in tomato plant development (Figure 2) that remains to be investigated.

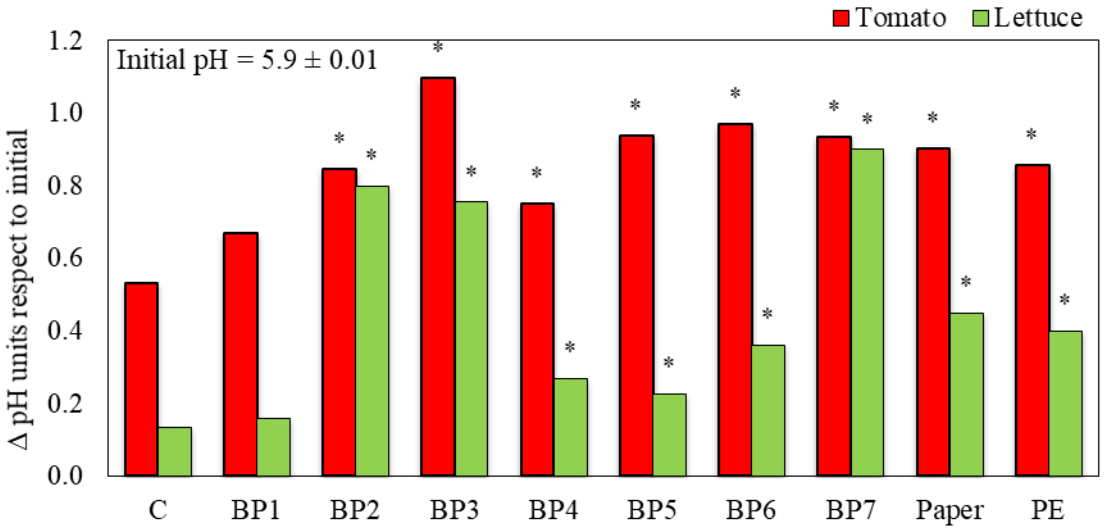

Figure 3. – Change of substrate pH form the initial value at the beginning of the assay to after growth of tomato (red) and lettuce (green) with buried new unused mulch fragments (n=4). C=control treatment without mulch. *Indicate statistical difference from control plants ($P \leqslant 0.05$).

The mobility and absorbance of minerals within the soil, growth of soil microorganisms, and soil structure depend on the soil pH (Perry, 2003; Neina, 2019), mineral availability being essential for plant growth. The substrate in the assay had a slightly acidic pH, 5.9, appropriate for plant growth. At the end of the assay, pH in the substrate was higher in all pots, including control ones (Fig. 3). The increase was higher for the pots with tomato

plants than for the ones with lettuce, reaching 6.4 and 6.0, respectively, at the end for the culture time for the control pots. This difference may be likely due to the shorter duration of the lettuce growth, 5 weeks vs. 7 weeks for tomato. In addition, all treatments in both plant species, with the exception of BP1, significantly increased the substrate pH as compared to the control one (Fig. 3), All BDM and the PE mulches used in the assay have proven to release compounds when in contact with a mineral solution equivalent to that in the soil and to change the pH of the solution (Serrano-Ruiz et al., 2020). Even though soils and substrates are more complex matrices than water solutions, and the biogeochemical processes within them contribute to buffering pH (Neina, 2019), biodegradable plastic fragments buried into soil have been also reported to cause some changes in soil pH (Ardisson et al., 2014 ; Li et al., 2014 ; Qi et al., 2020; Wang et al., 2020). The changes in the substrate pH hereby produced are moderate and keep within the threshold levels for tomato (5.5 – 7.5) and lettuce (6.0 -7.0) cultivation; thus, it is unlikely directly related to the inhibition of tomato and lettuce plant growth in the BDM containing pots. Nevertheless, indirect effects through changes on soil physical properties and biogeochemical processes cannot be discarded. Gaining knowledge on the compounds BDM release to the soil along time and how they may affect these processes is required to understand the potential impact of BDM on soil and plants.

3.2. Effects of field weathered mulches

While in service on the field, BDM are exposed to the environmental and crop labouring conditions (solar radiation, rainfall, wind, irrigation, fertilization and pesticide treatments, etc.), which weather them and alter their physicochemical properties (Hayes et al., 2017; Anunciado et al., 2020). Thereafter, BDM are fragmented and buried into the agricultural soil. The effects on the weathered mulch fragments on plant growth may differ from those previously found for the unused BDM.

In the assay with weathered fragments, collected after used on an organic farming field, germination of 50% was reached after 7 days, at the same time in all control and mulch treatments of both tomato and lettuce. Treatment effects were identified later on plant development, as reported above for the new unused BDM fragments.

Two weeks after sowing, tomato plantlets grown in any of the BDM treatments were somehow smaller and showed some retardation in plant growth. Retardation in plant development was still somehow visually evident at the end of the assay for all BDM treatments, especially for BP1 and paper (Fig. 4). Plants grown on BP1 and paper containing pots were the shortest. Correspondingly, plant weight was significantly lower

when grown on BP1, BP2, BP4, BP5 and paper weathered mulches (Fig. 5), and the decrease in weight for BP3 treated plants was close to the standardized significant level (P-value 0.0532). BP1 exhibited the strongest inhibitory effect, followed by paper, BP2, and BP4, with weight decreases ranging from 76 to 37%. The leaf chlorophyll content decreased significantly by all the BDM tratments, and, as reported above with new unused fragments, it was lowest when plant weight decreased most. Proline significantly decreased only in plants grown in the presence of BP3 (Fig. 5).

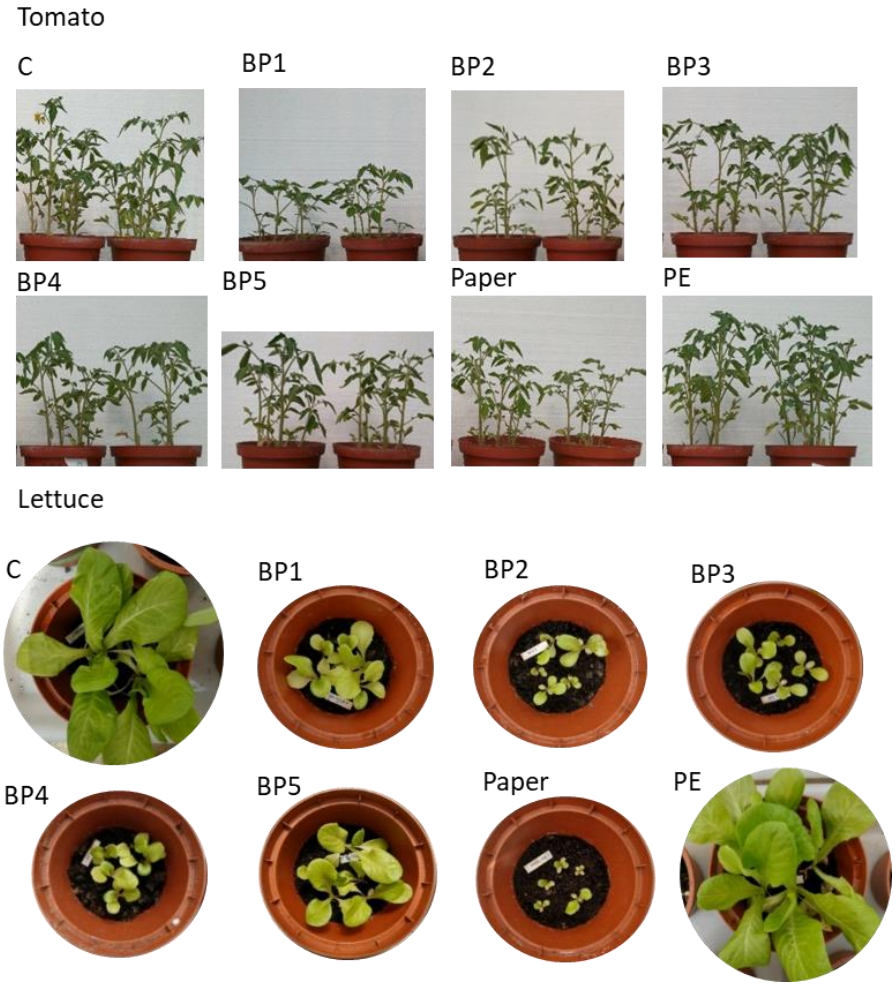

Figure 4. - Tomato (up) and lettuce (down) plants grown for 7 and 5 weeks in substrates containing weathered mulch fragments.

Lettuce growth retardation in all pots with weathered BDM was obvious for the naked eye (Fig. 4), and highest for plants grown on paper mulch, which at the end of the assay, at week 5th, were alike to control plants grown for three weeks. While lettuce plants grew normally in the presence of unused paper mulch, weathered paper was strongly inhibitory for their growth. Plant weight was also strongly and significantly inhibited, over

75%, by all weathered BDM (Fig. 5), which also decreased chlorophyll in leaves except with BP5 (P-value 0.175) (Fig. 5). However, none of the decreases in proline were significant (Fig. 5).

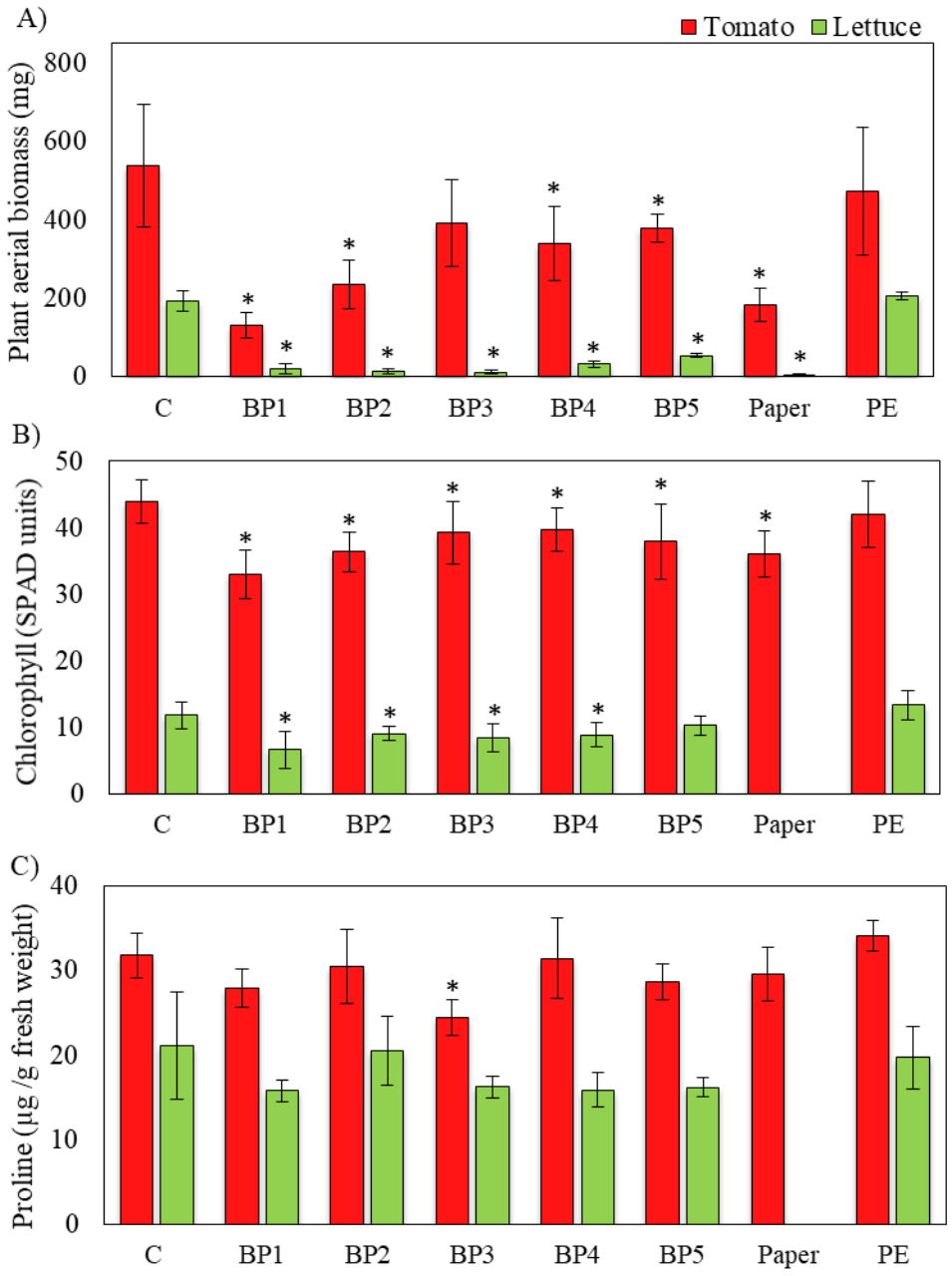

Figure 5. - Growth and physiology of tomato (red) and lettuce (green) plants grown in substrate with buried weathered mulch fragments. C=control treatment without mulch. A) Aerial part plant dry weight (n=20). B) Leaf chlorophyll content (n=20). C) Leaf proline content (n=4). Bars represent average and standard deviation. *Indicate statistical difference from control plants ($P \leq 0.05$).

The substrate microbial activity increased in pots with weathered BDM, but changes

were low and not significant for any of the treatments after tomato plant growth. However, it significantly increased after lettuce plant growth, by ca. 60%, in the BP2 and BP3 treatments.

As reported above for the assay with unused mulches, pH rose from the beginning to the end of the assay in all treatments, including the control and the PE ones, both after tomato and lettuce plant growth. However increases were significantly higher for all BDM treatments but not for the PE one (Fig. 6), the pH remaining within the optimal threshold levels for the cultivation of tomato (5.5 – 7.5) and slightly over the optimal maximum for lettuce (6.0 – 7.0) in BP2 and BP3 treatments, that increased it up to 7.2.

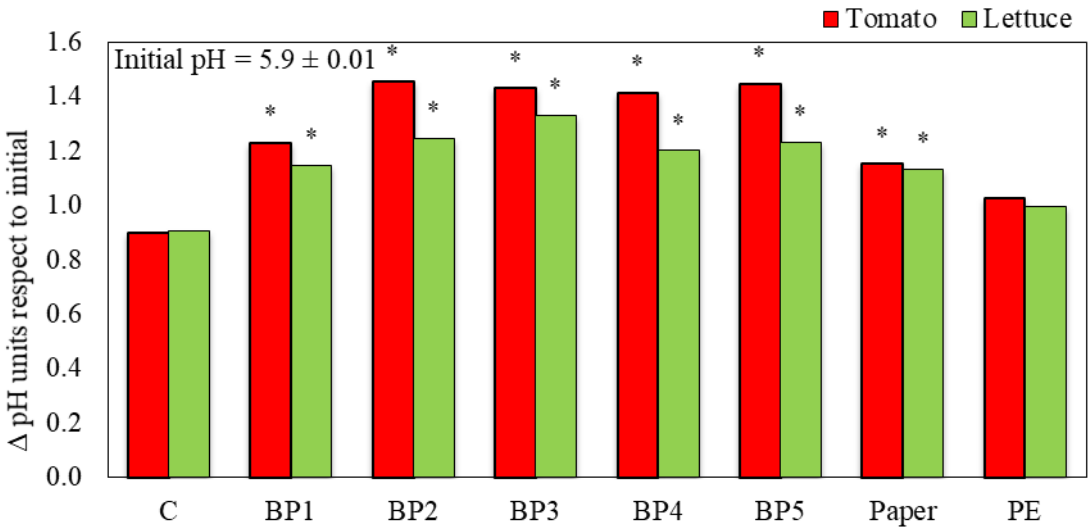

Figure 6. – Change of substrate pH form the initial value at the beginning of the assay to after growth of tomato (red) and lettuce (green) with buried weathered mulch fragments (n=4). C=control treatment without mulch. *Indicate statistical difference from control plants ($P \leqslant 0.05$).

To the best of our knowledge, this is the first report on the effects of field weathered BDM fragments on plant development. To gain knowledge on whether the weathering process may change the BDM interaction with plants, their effects were compared to the ones caused by new unused BDM fragments; when the differences were not visually evident, significance was tested through one-way anova.

Overall, weathered BDM fragments exerted more adverse effects on lettuce and tomato plant development than the new unused ones. In tomato, where differences were smaller than in lettuce, plant weight was significantly more inhibited by weathered BDM than by the new unused ones; chlorophyll inhibition was also significantly higher inhibited by weathered BDM than by the new unused ones except for BP2 and BP3, where the

weathering caused equivalent inhibition. In lettuce, the greater plant growth inhibition from the weathered BDM was fully evident to the naked eye; however, the weathering of the mulches did not caused significant differences in chlorophyll decrease. These results suggest assays for testing potential BDM effects on plant growth using new non-weathered BDM instead of weathered ones may be underestimating their effects on plants.

Differences on the effects of new unused and weathered BDM fragments on substrate pH and microbial activity changes were also evaluated. In both tomato and lettuce, microbial activity was found not to be modified by the weathering of the mulches, with a few exceptions: in tomato, it was significantly higher with new unused than with weathered BP3, while on the contrary in lettuce microbial activity increased more with weathered than with new unused BP3 and BP2 fragments. The increase in the substrate pH over that in control pots with most of the BDM was higher with weathered than with the new unused BDM fragments, with exceptions in pots with lettuce plants, where pH increase was higher with new than with weathered BP2 and BP3.

The paper mulch effects were not alike to those from the plastic BDM. In tomato, the effects caused by the new unused paper fragments were significantly higher than the ones from weathered one, while on the contrary new unused paper did not inhibited lettuce plant development and weathered fragments highly restrained it. New unused paper fragments caused higher pH substrate and microbial activity increases in both tomato and lettuce than weathered paper mulch. These findings are likely related to the paper chemical nature, composed of cellulose, hemicelluloses and lignin together with pectines and other organic compounds, most of them readily biodegradable by microorganisms, especially fungi. It is reasonable to expect the compounds most readily biodegradable within the new unused paper are firstly consumed by soil microorganisms, the significant pH and microbial activity increase being related with this consumption. A share of these easily biodegradable compounds would be lost in the weathered paper fragments, while paper compounds more recalcitrant to biodegradation, such as lignin, would stay and (bio)degrade later at a slower rate, allowing minor not significant increase of microbial activity.

The differences found due to the weathering process are hypothesized to be related to the BDM chemical structure. BDM are designed to remain stable for a few months after their installation on the field and to suffer little changes, and to undergo (bio)degradation once their mulching role on a crop has been fulfilled, usually ca. 3-6 months later (EN-

17033:2018; Kasirajan and Ngouajio, 2012). Upon exposure during their service life to the environmental factors in the field (rainfall, fertilizers, sunlight), the initially slow BDM deterioration speeds up (Touchalaume et al., 2018; Moreno et al., 2017; Anunciado et al., 2021); additives protecting from degradation being gradually released, further favouring deterioration of the film structure and leaching the mulch compounds once buried into the soil.

BDM fate and effects into soil are expected to be highly associated to the specific conditions they undergo. Even the dissimilarities with the procedures hereby followed, effects of a starch-PBAT based film on three plant species have been reported to change upon exposure to the environment (Menicagli et al., 2019; Balestri et al., 2019), with germination and early growth being dependent on the material weathering and also on the specific environmental conditions they were exposed to.

3.3. General implications

Overall, the results found proof the tested BDM have effects on tomato and lettuce plant development, while the PE mulch produces any. Similarly, Wang et al. (2020) found no effects from PE microfragments, whereas Qi et al. (2018) and Meng et al. (2021) reported effects of PE fragments on plant development to be substantially minor than the ones from biodegradable plastics, suggesting the physical presence of the mulch fragments is not linked to their effects. The underlying mechanisms of the BDM on plant effects have not been addressed in the present experiment, nor in the literature identified by the author; nevertheless, the impact of BDM on plant development, as compared with the lack of effects from the PE mulch, is likely associated to their sensitivity to interact with the surrounding biotic and abiotic environment, also depending on their chemical structure. Consistent with this hypothesis, the BDM used in this study have been proven to release a diversity of compounds when in contact with water, while PE mulch only released a small fraction of fatty acids, used as additives (Serrano-Ruiz et al., 2020).

Interestingly, an *in vitro* culture system assay containing the bulk of compounds released from several BDM has shown them to alter tomato and lettuce plantlet growth, physiology and morphology, in a way mostly consistent with that found in the present pot assay (Serrano-Ruiz et al., 2018). It is hipothesized that the BDM fragments continuously leached compounds, easily reaching the plant roots in the pots, being absorbed by them and altering plant development. Further research is needed to get knowledge on this interaction.

The species used in this paper are two highly economic relevant vegetable species,

ranking among the most consumed vegetable products worldwide. They are also among the most commonly mulched cultivated plant species with BDM (Kasirajan and Ngouajio, 2012; Martín-Closas et al., 2017). The lettuce and tomato crop cycle, 1-2 months and ca. 3 months, respectively, allows for several crops a year, especially in lettuce, with the consequent repeated soil mulch covering (Cirujeda et al., 2012). Every time a crop cycle ends, BDM films are buried into soil, and a new crop and mulch cycle may start. However, the mulch biodegradation is not likely accomplished before starting the new cycle and the plants are to grow on soil with BDM fragments accumulated from previous crop cycles. Commercial BDM must be certified to reach 90% biodegradation in soil in less than two years at ambient temperature (EN-17033:2018), while 10% of the material it is allowed to remain. However it is worth to highlight that biodegradation is highly dependent on the environmental conditions and presumably slower in the field than under laboratory conditions (Ghimire et al., 2020; Sintim et al., 2020). The persistent BDM use may lead to their buildup into agricultural soils, entering in contact with the roots of the subsequent crops.

Whether the buried BDM fragments from previous mulched crops may affect growth and development of next generation vegetable crops in the field, including tomato and lettuce, remains mostly undisclosed. Germination and dry weight of lettuce plantlets was not inhibited when grown for 4 weeks in a soil having contained PBAT mulch fragments for one year under laboratory conditions (De Souza et al., 2021), but the plastic fragments were removed prior to the plant testing and no control with non- biodegradable mulch as PE nor without mulch was included. Other studies testing phytotoxocity of BDM on vegetables, including lettuce and onion have exposed seeds to aqueous extracts from BDM containing soil, without allowing the plants to interact with BDM fragments (Souza et al., 2020; Palsikowski et al., 2018). Although germination is a key phase in plant development, it is worth noting BDM are commonly used in crops starting from seedlings, thus germination tests provide limited information on potential effects that may arise in subsequent phases of plant development. Our results provide the first evidence BDM fragments did not affected germination but have the potential to interact with tomato and lettuce plants and alter plant development. Besides, to the best of our knowledge, this is the first report on tomato plants having been used to test BDM ecotoxicity.

Both tomato and lettuce plant species were affected by BDM; however, lettuce was overall more sensitive to the BDM fragments. Lettuce is especially acknowledged in BDM testing, not only for being highly sensitive to a wide diversity of contaminants and chemicals and one of the main plant species used to test phytotoxicity in contaminated

soils (EPA Test OCSPP 850.4230, 2012; UNE ISO 17126, 2009), but also because it is commonly cultivated with BDM. The lettuce assay provides relevant information within the agricultural context where BDM are used. Tomato is not considered as sensitive to chemicals as lettuce, however it was also found to be affected by BDM fragments, exhibithing similar or different responses than lettuce, depending on the BDM nature, and highlighting the need to including representative plant species on which BDM are used, together with soil pollution indicator species.

4. Conclusions

The presence of buried BMD fragments into plant pots altered tomato and lettuce plant growth and physiology. Effects varied depending on the mulch composition, with the specific formulation of every material playing a relevant role in its interaction with plants. No effects were found on plants grown with the non-biodegradable PE mulch, indicating the effects are to be related to chemicals released from the BDM rather than to physical effects of the mulches' fragments. Overall, field weathered fragments exerted stronger effects than new unused materials, not always coincident with those from unused new BDM; thus, considering the BDM weathering process previous to its burial is essential for ecotoxicity testing. The sensitivity to the BDM fragments varied between the two species tested; lettuce being more sensitive than tomato, it is a reliable species to identify potential ecotoxicity from BDM. Tomato was more sensitive to some specific BDM, stressing the need to assess the effects of BDM materials on a wide broad of cultivated plant species, especially on the ones routinely mulched with BDM. This research provides the first knowledge on the potential interaction of BDM fragments with tomato and lettuce plants, and may assist in the development of low environmental impact BDM formulations.

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CHAPTER V

EFFECTS OF BURIED BIODEGRADABLE PLASTIC MULCHES ON AGRICULTURAL SOIL COMMUNITIES

This chapter reproduces the text of manuscript in preparation for send it to publication.

Abstract

Biodgradable plastic mulches (BDM) are an alternative to the use of non-biodegradable polyethyelene films to avoid soil and environment pollution. Once the crop ends, BDM are tilled into soil, where native soil microorganisms biodegraded them. However, a variety of chemically diverse BDM are commercialized. Little is known yet on how the continuous input of BDM into soil affects the agricultural soil microbiome. The objective of the present work is to determine whether the burial of BDM, depending on their composition affects the diversity and community structure of the soil microbiome and its functions. For this, a mesocom experiment was performed by burying three commercial BMD of different composition, (Bioflex, Ecovio and Mater-Bi) for three months at 3 concentrations (0.5-1-4% w/w). Besides, one paper and one polyethylene mulch were included. The soil microbial community structure was analyzed by amplicon sequencing and the microbial functions were evaluated through the activity of extrecullar enzymes.

The soil microbial community structure differed in soil with BDM compared to control soil without mulch and with PE mulch, especially after the third month. It also differed among BDM depending on the mulch composition. Mater-Bi at 4% concentration caused the stronger bacterial effects, decreasing diversity and the relative abundance of *Acidobacteria Gp6* and of *Planctomycetes*, while the other BDM casued minor effects on bacterial communities. In eukaryotic communities, Ecovio and paper had a strong significant effect, increasing the abundance of *Tubulinea* protist and *Ascomycota* fungi, respectively. The total hydrolytic activity was not affected by mulch burial, however, enzymatic activity related to the nitrogen cycle decreased in the presence of BDM and PE mulches. Overall, most of the changes were limited and occurred the highest concentration treatments, suggesting the tested BDM have low impact in soil microbial communities.

1. Introduction

The use of plastic films for agricultural mulching is a cornerstone technique to meet the food demand of a growing population, due to their benefits increasing crop yield and use-efficiency of water, herbicides and fertilizers (Lamont, 1993; Kasirajan and Ngouajio, 2012; Kader et al., 2017; Le Moine et al., 2019). Nevertheless, the massive use of non-biodegradable polyethylene (PE) mulches has led to a scenario of highly environmental persistent fragments accumulated in the agricultural soil, which threatens soil health and crop yield in the medium and in the long term (Wenqing et al., 2014; Steinmentz et al., 2016; Gao et al., 2019; Zhang et al., 2020). Furthermore, PE fragments' migration entails a source of plastic pollution to other environments (Kumar et al., 2020). Biodegradable plastic mulches (BDM) are proposed as an alternative to PE to avoid plastic waste generation and accumulation (Kasirajan and Ngouajio, 2012; Mormile et al., 2017). Once the crop ends, BDM are tilled into soil where the native soil microorganisms, mainly bacteria and fungi, use them as source of carbon and energy to grow. Consequently, BDM burial has the potential to alter microorganism's abundance and activity (Bandopadhyay et al., 2018). However, how the burial of BDM affects the soil microbiome and essential soil processes regulated by microorganisms, as nutrient cycling and plant-microbes relation (pathogens, plant growth promoters, etc.), remains largely unexplored.

Field studies suggest that in the short and medium term, 1 and 4 years, the BDM impact on agricultural soil microbiome is low, but longer term effects have not yet been targeted. Some papers report BDM use caused no significant differences in soil microbial community composition, diversity and activity (Masui et al., 2011, Kapanen et al., 2008, Bandopadhyay et al., 2020ab), whereas others have found evidence of increased microbial activity and abundance (Moreno and Moreno 2008, Li et al., 2014, Sintim et al., 2019). While field studies provide essential real knowledge, they also entail several limtations for evaluating potential BDM effects in soil microbiome. On the one hand, the plastic to soil ratio it uses is low and it does not allow infererring potential BDM use and accumulation effects on the long term. On the other hand, environmental factors and agricultural labours may introduce noise and variability in the measurements taken, which may hinder the BDM effects. Mesocosm studies allow for the homogenization of environmental factors and facilitate the evaluation of potential BDM effects. Mesocosm studies have shown that BDM in the soil (1% w/w) may increase soil microbial hydrolityc activity (Barragán et al., 2016) and alter the composition of rhizospheric soil communities (Qi et al., 2020). However, there is still a knowledge-gap on which specific soil microbial groups and functions may be affected by BDM burial.

Once buried, before being consumed by soil microorganisms BDM presence may induce physical changes in soil properties, comparable to that of PE, which could affect the soil microbiome. Although BDM are expected to (bio)degrade faster than PE (with decades showing negligible biodegradation), their biodegradation rate is highly dependent on environmental conditions, soil type, burial depth and material composition, and may proceed over years (Haider et al., 2019; Sintim et al., 2019). Studies on buried PE fragments have shown the presence of plastic fragments in soil (in a range of 0.5 to 2% w/w) had effects in soil bulk density, water holding capacity and microorganism activity (De Souza et al., 2018; Qian et al., 2018). Thus, to distinguish whether BDM effects are associated to their biodegradation by-products or due to their physical presence, PE mulch is required to be included in BDM testing.

A variety of polymer blends of different compositions are available on the market for use in BDM manufacturing (Miles et al., 2017). They comprise natural occurring polymers, like starch, poly-3-hydroxybutyrate or poly (lactic acid) (PLA); and synthetic polymers, like poly (butylene adipate terephthalate) (PBAT) and poly (butylene succinate) (PBS). The BDM biodegradability studies have shown the type of polymer conditions their biodegradability (Brodhagen et al., 2014). Similarly, the microorganisms that biodegrade the BDM and consequently the BDM effects on the soil microbiome, are conditioned by the type of polymer. Studies on the BDM surface colonization have shown that mulches of different composition differential enrich in specific microorganisms (Bandopadhyay et al., 2020b).

In this study, we aimed to understand how buried BDM may influence agricultural soil bacterial and eukaryotic diversity, community structure and microbial activity depending on their composition. A microcosm experiment was performed by burying mulch fragments on agricultural soil under different conditions. The microbial communities were characterized and microbial activity was evaluated. We hypothesize that (i) the introduction of BDM fragments in the soil may affect the agricultural soil microbiome structure and function; (ii) the changes caused would be dependent on the mulch composition, and (iii) there would be greater changes with increasing fragment concentrations.

2. Materials and methods

A mesocosm experiment was conducted to study the effect of burying biodegradable plastic mulches on soil microbial communities.

2.1. Agricultural film mulches

The materials tested were all commercially available fresh-roll agricultural plastic and paper films for mulching (Table 1): three biodegradable plastic films: Mater-Bi®, Ecovio® and BioFlex®; a non-biodegradable plastic made of polyethylene; and one biodegradable paper film: MIMgreen®. All plastic mulches were black and 15-17 µm thick. The paper mulch was black upwards and brown downwards and had 85 g m^{-2} grammage.

Table 1. - Plastic and paper agricultural mulches product name and main composition.

Product name	Manufacturer	Grade	Blend composition
Mater-Bi®	Novamont (Italy)	CF-04P	PBAT, Corn TPS, vegetable oils
Ecovio®	Basf (Germany)	M2351	PBAT, PLA (~7 %)
BioFlex®	FkuR (Germany)	F1130	PBAT, PLA (~ 30 %)
MIMgreen® (Paper)	MimCord (Spain)	-	Cellulose fibre
Polyethylene (PE)	Solplast (Spain)	-	LLDPE

PBAT: polybutyrate adipate terephthalate; TPS: thermoplastic starch; PLA: Polylactic acid; LLDPE: lineal low density polyethylene.

2.2. Soil

The soil was collected from an ecological agriculture crop field placed in the Agronomic Campus of the University of Lleida, Spain (41° 37′ 45″ N, 0° 35′ 55″ E). Prior to the experiment, it was air-dried at the laboratory for 4 days and passed through a 0.5 cm mesh sieve. The main physical and chemical properties were determined (Table 1S). Texture clay loam (16.3 % silt coarse-grain, 25.7 silt fine-grain, 30.6 clay, 27.4 sand); pH 8.46.; electrical conductivity 0.218 (dS m^{-1}); bulk density 1124 (kg m^{-3}).

2.3. Experimental set-up

The experimental design consisted on the burial of five types of mulches (Table 1) at three different concentrations: 0.5, 1 and 4 % (w/w dry soil) into agricultural soil. Pots were incubated under green-house conditions for three months. Mulches were previously cut to 1 cm^2 fragments with scissors and mixed manually with previously air-dried soil to a total weight of 500 g. A control without mulch was included. Pots used were 1.5 L (15 cm top diameter), polyethylene and brown. Three replicates were made for

each treatment and all pots were randomly distributed in a green-house bench. A total of 48 pots were incubated. During the experiment, tap water was added daily to maintain 60% soil water holding capacity (WHC). The greenhouse temperature and relative humidity were recorded with a data logger, Testo 175 H1 (Table 2S). In-soil temperature and moisture were monitored with sensors RT-1 and ECH_2 EC-5, respectively, (Meter Group) placed randomly in two pots (Table 2S).

Soil samples were taken monthly from each pot to determine physicochemical and microbiological properties. Samples were collected from the central part of each pot, in the first 5 cm depth from surface, then kept at 4°C until their physicochemical and microbial characterization. For DNA extraction, soil samples were immediately frozen, lyophilised and kept at -80° C until further analysis.

2.4. DNA extraction and quantification

Soil DNA was extracted from lyophilised soil samples taken at first and third months by the MoBio™ PowerLyzer™ Power Soil DNA isolation kit (Qiagen™), following the manufacturer´s instructions. DNA samples were kept at -20°C until analysis. DNA in the extracted samples was quantified using the Quant-It™ PicoGreen™ dsDNA Quantification Kit (ThermoFisher Scientific) following the manufacturer´s instructions.

2.5. DNA sequencing and analysis

DNA extracts were sequenced by Genomic Services Laboratory (GSL) at Hudson Alpha (Huntsville, AL, USA) for characterization of microbial community composition, using Illumina Miseq platform. The V4 region of DNA in the 16S rRNA gene was amplified with primers 515F (GTGCCAAGCAGCCGCGGTAA) and 806R (GGACTACHVGGGTWTCTAAT) and 250 bp paired end reads sequenced. Eukaryotic communities were characterized by amplification of V4-V5 regions of DNA in the 18S rRNA gene with primers 574F (CGGTAATTCCAGCTCYV) and 1132R (CCGTCAATTHCTTYAART) and 300 bp paired end reads sequenced.

Sequencing data was processed with Mothur 1.42.3, following the MiSeq SOP published by Scholss et al. (2009). Ambiguous bases were removed and sequences trimmed to 275 and 552 pb for 16S and 18S analysis, respectively. Reads were aligned with SILVA 132. Pre-cluster was set to up to two differences in nucleotides. Chimeras were detected with VSEARCH and removed. The total sequences of 16S V4 region generated were: 13061053. Among them, 362681 were unique sequences. In 18S V4-5 region sequencing, a total number of 10823689 sequences were generated. The number of unique sequences was 3852282. Sequences were clustered in OTUs (operational taxonomic untis). For 16S V4 region analysis, sequences were classified by using

taxonomic information from the RDP training set 16_022016 (Ribosomal Database Project), using cut off value of 80%. Then, cluster.split was run at genus level by 97% of similarity. For 18S V4-5 region, sequences were binned into phylotypes using SILVA 132 taxonomic information. For bacterial analysis, Chloroplast-Mitochondria-unknown-Archaea-Eukaryota sequences were removed. In eukaryotic analysis, Chloroplast-Mitochondria-unknown-Archaea-Bacteria assigned sequences were removed. Shared and taxonomy files were generated. Shared files were filtered in order to include only sequences present in at least two samples, and subsampled to the smallest library size before downstream analysis. The smallest library size was 73756 for 16S sequences and 46605 for 18S sequences. Both shared and taxonomy files were then imported to R for further analysis.

Alpha-diversity estimates Chao1 for richness and inverse Simpson's index for diversity were calculated in Mothur by using summary.single.

2.6. Microbial activity

Total soil microbial activity was determined by the method based on the enzymatic hydrolysis of FDA (fluorescein diacetate) described in Green et al. (2006). Briefly, 2 g of soil was mixed with 60 mM potassium phosphate buffer (pH 7.6). Then, 4.9 mM FDA stock solution was added to start the reaction. The mixture was incubated for 90 min h at 37°C. Acetone was added to stop the reaction. Aliquots of the mixture were centrifuged for 3 minutes at 8000 rpm. Optical density of the eluates was measured at 490 nm in a UV/VIS spectrophotometer (Helios Gamma, Thermo Fisher, England). Fluorescein concentration was calculated by a calibration curve using standard solutions of fluorescein disodium salt dissolved in 60 mM potassium phosphate buffer (pH 7.6). Results are expressed as μg of fluorescein released g oven dry soil $^{-1}$ h^{-1}.

At the end of the third month of incubation, the activity of enzymes for chitin degradation, phosphorus mineralization and cellulose degradation, N-acetyl-β-glucosaminidase (NAG), phosphatase (PHOS) and β-D-cellubiosidase (CB), respectively were determined at 25°C through the fluorometric enzyme assay described in Bell et al. (2013). The method is based on the addition of a substrate labelled with a synthetic fluorescent dye (4-methylumbelliferone, MUB) emmiting fluorescence when released by an enzyme-catalyzed reaction. Soil slurries were prepared in 50 mM buffer mixed in a blender. A standard curve was prepared for each sample. Substrate (MUB), 200 μM was added into microplates to start the reaction, microplates were incubated for 3 hours, then centrifuged at 1500 rpm and aliquots (100 μl) transferred to flat-bottomed black 96-well plates to register fluorescence in a BioTek® Synergy plate reader, with 365 nm excitation

wavelength and 450 emission wavelength.

2.7. Soil pH

Air-dried soil with a 1:5 substrate-to-distilled water ratio (w/w) was stirred during 30 minutes. The mixture was left to settle for 30 minutes and pH was recorden in a pH-meter (Hach, Germany).

2.8. Statistical analysis

For soil pH, microbial activity and alpha diversity, statistical significant differences were tested by ANOVA and *post hoc* Tukey´s test performed with JMP Pro 5.1. Previous to ANOVA, normality of data distribution was tested with Shapiro-Wilk. All data sets showed normal distribution. Beta-diversity analyses were performed with R. Bray-Curtis dissimilarity matrix of microbial community's composition were calculated with vegan package. The Bray-Curtis dissimilarities metrics were visualized by nMDS ordination method using the phyloseq package. To test for dissimilarities' statistical significant differences, PERMANOVA (permutational multivariate analysis of variance) analyses were performed (number of permutations = 999). The relative abundance values of taxa were arcsine transformed previous to ANOVA and *post hoc* Tukey´s test with JMP Pro 15.1. Data normal distribution were tested with Shapiro-Wilk test. When normality distribution in the data set was not met, Kruskal-Wallis non-parametric test was used to check for significant differences among treatments. In treatments where PERMANOVA analysis detected significant differences due to mulch concentration, SIMPER (Similarity Percentages) analyses were performed using the "simper" function of vegan R package, to determine the OTUs that most contribute to the variability between mulch concentrations.

3. Results

3.1. Alpha-diversity of microbial communities

Bacterial and eukaryotic communities' richness were estimated by Chao1 index and diversity by the inverse Simpson index. One-way ANOVA was performed to test for differences between mulch treatments and control without mulch, followed by a *post hoc* Tukey test to detect differences due to mulch concentration at each of the two time points. In bacterial communities, no significant differences in richness were found between soil with mulches and control soil (Figure 2S). Diversity was not affected by Bioflex, Ecovio and PE as compared to control (Figure 1A). On the contrary, 4% Mater-Bi decreased bacterial diversity after one month ($p = 0.0208$); but it was not significantly different from control after the third month ($p = 0.3073$).

Eukaryotic communities showed no significant changes in richness between soils with mulches and control soil (Figure 2S). In the 4% paper treatment, diversity showed a trend to decreased after the first month, although the change was not significantly from that in the control treatment ($p = 0.11$); after the third month the decreasing effect persisted and it was significant in 1% and close to it ($p = 0.0029$ and 0.053 respectively) (Figure 1B). Among the plastic mulches, no significant changes in eukaryotic diversity were observed.

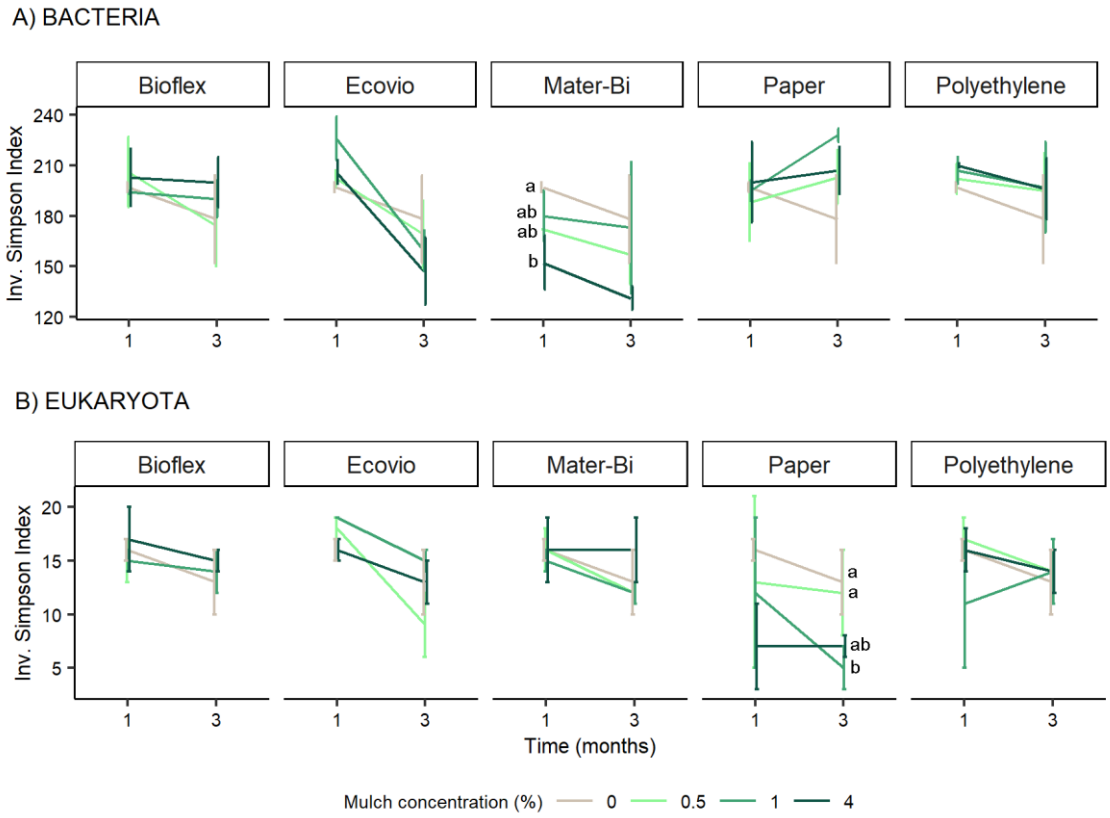

Figure 1.- Diversity (inverse Simpson index) of soil bacterial (A) and eukaryotic (B) communities after one and three months of mulch fragments buried in soil at different mulch concentrations. Mean and standard deviation (n=3). Letters indicate significant differences between mulch concentrations based on *post hoc* Tukey following a one-way ANOVA.

3.2. Beta-diversity

3.2.1. Bacterial community

The nMDS ordination of bacterial community dissimilarities showed differential grouping based on the mulch composition and concentration (Figure 2) and PERMANOVA analyses indicated there were significant differences due to thses two factors (Table 2A). Besides, there was an interaction between the two factors, indicating the changes due to mulch concentration being dependent on the material tested. Therefore, PERMANOVA analyses of mulch concentration levels for each material were performed to test in which materials there was a concentration-dependent response. Results

indicated significant changes due to mulch concentration buried for the Mater-Bi and paper treatment after the first and third month, and also in Bioflex one after the third month (Table 2B). The nMDS ordination showed 4% concentration of Mater-Bi was clearly differentiated from any of the the remaining treatments and concentrations after the first month of mulch burial (Figure 2A). On the contrary, Ecovio and Bioflex, the two mulches with PLA were grouped close to control (no mulch) independently of their concentration. Paper and PE treatments were also differentiated from the control one, but not between each other. In paper, 0.5% concentration was differentiated from other paper concentrations, whereas PE did not show concentration-dependent clustering.

After three months, the clustering of treatments with plastic BDM was different from the control, paper and polyethylene ones (Figure 2B). Among the plastic BDM, Ecovio and Mater-Bi exhibited similar trends and were markedly dispersed from the others, whereas Bioflex had lower dispersion and was closer to the control treatment. As observed after the first month, 4% Mater-Bi also grouped separately from the rest of the mulch concentrations. The same distinction from other concentrations was found for paper and Bioflex at 4%. Regarding the buried mulch quantity, no distinct separation based on mulch concentration in the rest of the materials was evidenced. Polyethylene showed low dispersion and it was the closest treatment to control. Both PE and paper were differentiated from BDM plastic treatments.

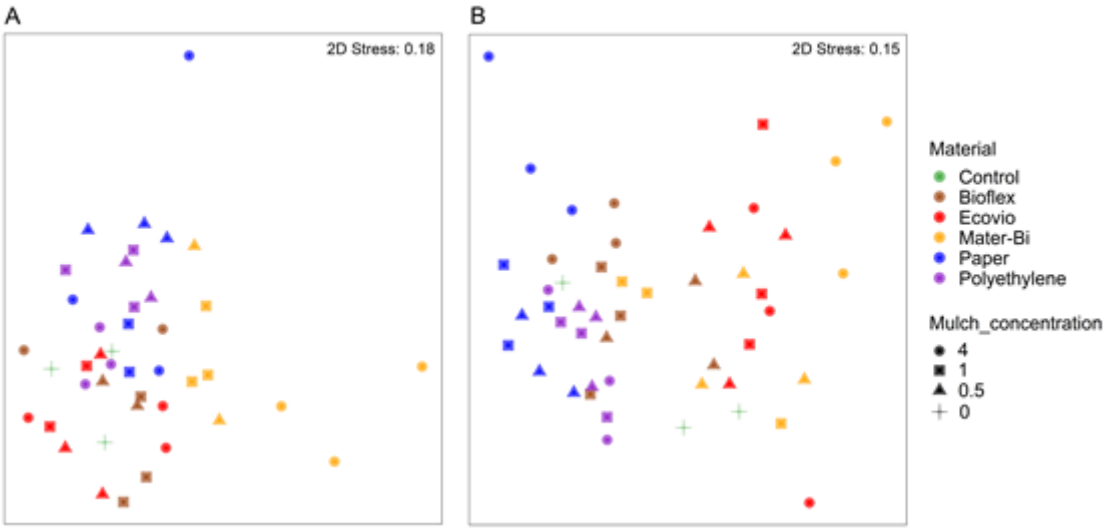

Figure 2. - nMDS ordination of Bray Curtis dissimilarities of bacterial OTUs identified based on the material and concentration tested after one (A) and three (B) months of mulch in soil incubation. 2D stress was 0.18 one month dataset and 0.15 in the three months dataset.

Table 2. – F values from PERMANOVA test based on Bray-Curtis dissimilarity distance matrix of bacterial and eukaryotic OTUs identified after one and three months of mulch in soil incubation. A) Tested factors: material (control, Bioflex, Ecovio, Mater-Bi, paper and polyethylene), mulch concentration (0, 0.5, 1 and 4%) and their interaction. B) Tested factor: mulch concentration within each material. Significant differences are in bold. Asterisks indicate p values: ***p<0.001, **p<0.01, *p<0.05.

	Bacteria		Eukaryota	
	One month	Three months	One month	Three months
A) Factors				
Material	**2.6050*** **	**4.9916*** **	**4.5282** **	**15.2993*** **
Mulch concentration	**1.3876** *	**2.4069** **	1.0958	1.8906
Material x Mulch concentration	**1.2800** *	**1.3081** *	1.1386	1.6335
B) Mulch concentration within each material				
Bioflex	1.1192	**1.5918** *	0.72253	1.3848
Ecovio	1.0453	0.96094	1.2443	0.90283
Mater-Bi	**1.5894** *	**1.9951** *	1.1809	**1.8938** *
Paper	**1.4062** *	**2.4596** **	1.0228	**3.4309*** **
Polyethylene	1.2354	0.90657	1.2577	0.64195

The relative abundance of taxa at over 2% was analysed by one-way ANOVA to test for significant differences between control and mulch treatments, followed by *post-hoc* Tukey's test to determine for differences among them. In bacterial communities, the dominant phylum in control soils were *Actinobacteria* and *Acidobacteria* (18-16%), *Proteobacteria* (10-9%), *Chloroflexi* (8%), *Planctomycetes* (9-6%) and *Candidate_division_WPS-1* (3%) (Figure 3S). *Firmicutes* (3%) was also found after the third month. After the first month of the mulches burial, no significant differences in relative abundance at phylum level were found between control and mulch treatments. An exception was found for the relative abundance of phylum *Planctomycetes*, which decreased to 5.8% in soil with 4% Mater-Bi as compared to 9.4% in control soil. After the third month, *Planctomycetes* relative abundance increased to 9-10% in pots containing 4% Bioflex, 1 and 4% Paper and 0.5% PE, compared to 6.4% in control pots. In *Chloroflexi* 4% paper produced higher relative abundance (9.3%) compared to 1% paper (7.9%).

At the bacterial class level, *Actinobacteria* was the dominant taxa (20%), followed by *Planctomycetia* (6-9%), *Alphaproteobacteria* (7-8%), *Acidobacteria Gp6* (7%), *Thermomicrobia* (4%) and *Acidobacteria Gp16* (4%) (Figure 3S). *Actinobacteria*, *Planctomycetes* and *Chloroflexi* phyla were mainly composed by the classes *Actinobacteria*, *Planctomycetales* and *Thermomicrobia*, respectively, and results for these taxa at the phylum were found equally at class level (Figure 3S). *Acidobacteria Gp6* relative abundance decreased in 4% Mater-Bi, to 4.5% compared to 6.6% of control pots after both one and three months of burial. Besides, after the third month,

Alphaproteobacteria increased significantly to 8.5% with 4% paper, compared to 0.5% paper, 6.4%. The taxa close to the limit detection, *Acidobacteria Gp4*, *Bacilli* and *Betaproteobacteria*, were also analysed, although their relative abundance was below 2.0% in some treatments and their values are not shown in the barplot. Together with *Actinobacteria* and *Acidobacteria Gp16*, no significant changes in relative abundance were found for these taxa (Figure 3S).

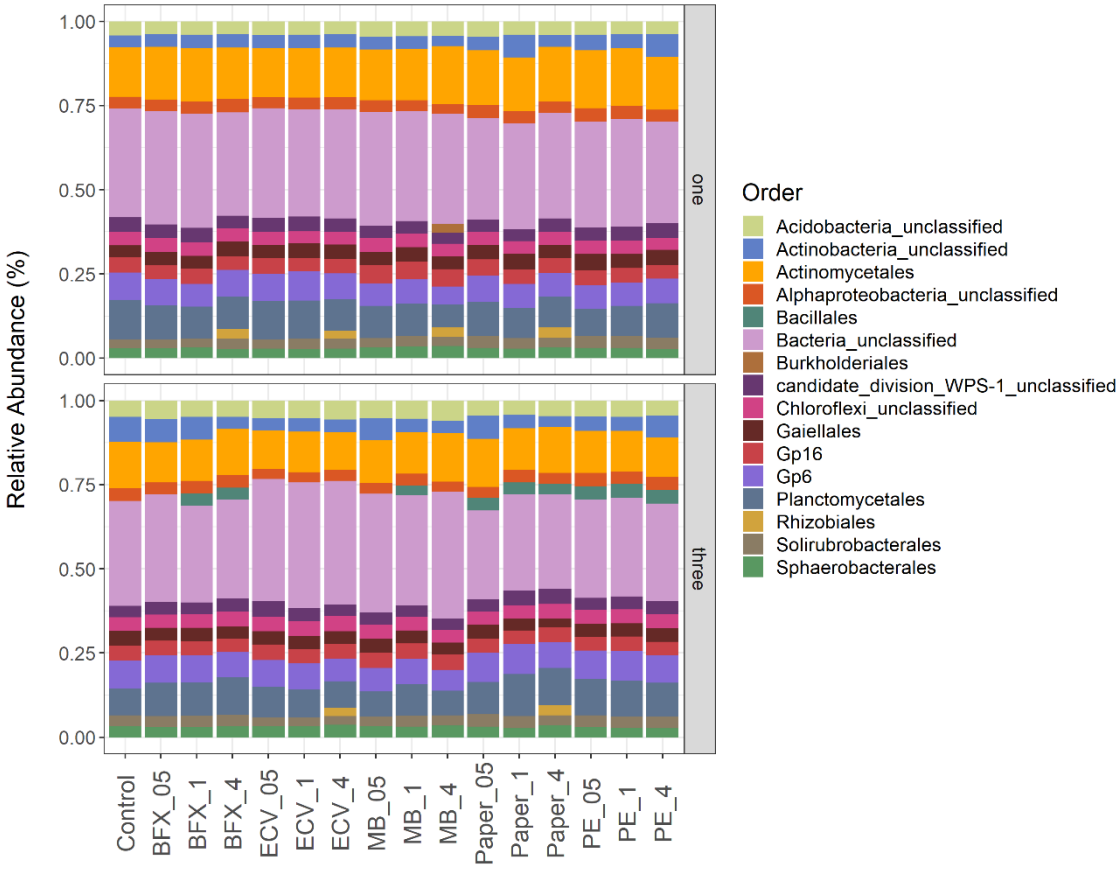

Figure 3. – Mean relative abundance of soil bacterial communities at order level after mulch fragments buried for one (upper panel) and three months (lower panel). Taxa included had at least 2% of relative abundance.

At the order level, the predominant taxa were *Actinomycetales* (12%), *Planctomycetales* (8%) and *Acidobacteria Gp6* (7%), followed by *Acidobacteria Gp16* (3.5%), *Gaiellales* (3%), *Solirubrobacterales* (2.5%) and *Sphaerobacterales* (2.5%) (Figure 3 and 4S). The taxa at over 2% but close to the detection limit, for some treatments, were: *Rhizobiales*, *Bacillales* and *Burkholderiales*. Significant changes, equal to the ones previously reported at class level in *Planctomycetia* and *Acidobacteria Gp6* classes were found in orders *Planctomycetales* and *Acidobacteria Gp6* (Figure 4S).

To determine the taxa driving variation in the community structure in treatments that

exhibited significant differences due to mulch concentration (Table 3S, Table 2), SIMPER analysis was performed within each material. The highest mulch concentration, 4%, was compared to 1 and 0.5%. Then, the first ten OTUs that contributed most to the community structure variability were identified. In the analyses, the first ten OTUs contributed 23-37% of cumulative variability between the mulch concentrations compared. In Mater-Bi, after one and three months, members of the phylum *Actinobacteria*, *Proteobacteria*, *Acidobacteria* and *Chloroflexi* were identified. Among these groups, the most predominant taxa belonged to the *Actinomycetales* order*,* which increased in 4% treatments relative to the others. In Bioflex after three months, taxa driving variability included several groups from phylum *Acidobacteria*, *Actinobacteria* and *Candidate division WPS-1,* which decreased in 4% treatments, and *Chloroflexi*, *Planctomycetes*, *Proteobacteria* which increased in 4% treatments. For the paper treatment, taxa driving variation included those belonging to *Bacteroidetes*, which decreased in the 4% treatment, *Actinobacteria*, which did not showed a clear trend, some orders decreased while other increased. Other taxa contributing to variation were *Proteobacteria*, *Chloroflexi*, *Planctomycetes*, *Candidate division WPS-1* and *Acidobacteria*, which increased in the paper 4% treatment.

3.2.2. Eukaryotic community

The nMDS ordination of eukaryotic community dissimilarities showed that communities were affected by the material, but not by its concentration (Figure 4) and PERMANOVA analysis confirmed it statistically (Table 2). After one month, soil from the paper treatment had the most distinct communities (Figure 4). Eukaryotic communities with plastic mulches were little distincted from the control or polyethylene treatments. Only Ecovio produced a clear clustering apart from the control one.

After three months treatment, Ecovio treated soil increased its differentiation from the remaining treatments, while no differentiation between Bioflex, Mater-Bi and the control treatments was found. Paper highly increased the differentiation effects identified previously at the first month treatment. Despite the absence of main effects, mulch concentration effects were significant for paper and Mater-Bi ones according to PERMANOVA analysis within each material (Table 2).

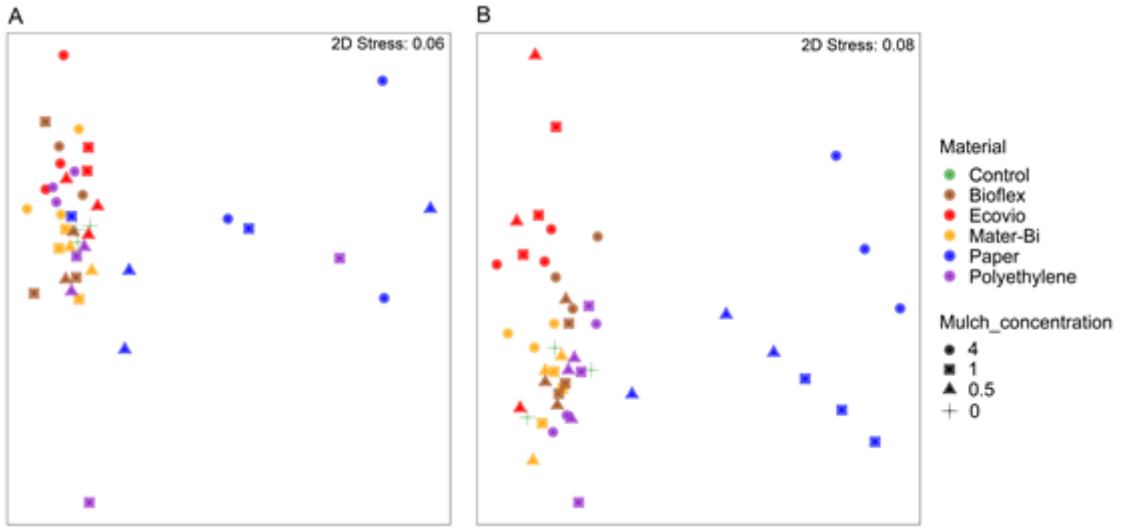

Figure 4. - nMDS ordination of Bray Curtis dissimilarities of eukaryotic OTUs identified based on the material and mulch concentration tested after one (A) and three (B) months of mulch in soil incubation. 2D stress was 0.06 for the one month dataset and 0.08 in the three months dataset.

The relative abundance of eukaryotic communities that were above 3% was analysed equally as for the bacterial ones. The dominant eukaryotic phylum in control soil were *Ascomycota* (24-22%), *Cercozoa* (25-21%), *Mucoromycota* (19-12), *Basidiomycota* (8-9%), *Chytridiomycota* (5-6%) and *Tubulinea* (5-3%) (Figure 5). After the third month, communities additionally found were *Chlorophyta* (3%) and *Schizoplasmodiia* (3%). The relative abundance of certain eukaryotic groups in soil with mulches changed significantly from the ones in the control soil. At the phylum level, after one month, the 4% paper treatment significantly enrichmed in the fungal phylum *Ascomycota*, to 63% *vs*. 24% in the control soil. Although mean values were also higher with 0.5 and 1% paper soil (36-50%), their standard deviation were high and thus differences were not statistically significant. After the third month, in 4% paper treated soils the enrichment increased to 72%, and in 0.5 and 1% treatments the relative abundance also increased significantly, to 50 and 63%, respectively, compared to 22% in control soil (Figure 5). In parallel, the relative abundance of phylum *Cercozoa* in 4% paper treatment decreased after one month, and after the third month, *Cercozoa* and *Mucoromycota* decreased in 1% and 4% paper treatments, while the remaining taxa were below 3% relative abundance. In plastic treatments after the first month the relative abundance of phylum *Ciliophora* increased with 4% Ecovio and PE to 4 and 7%, respectively *vs*. a 2% increase in control soil but no changes were found after the third month. After the third month, 0.5% Ecovio increased *Tubulinea* to 20% relative abundance *vs.* 5% increase in control soil.

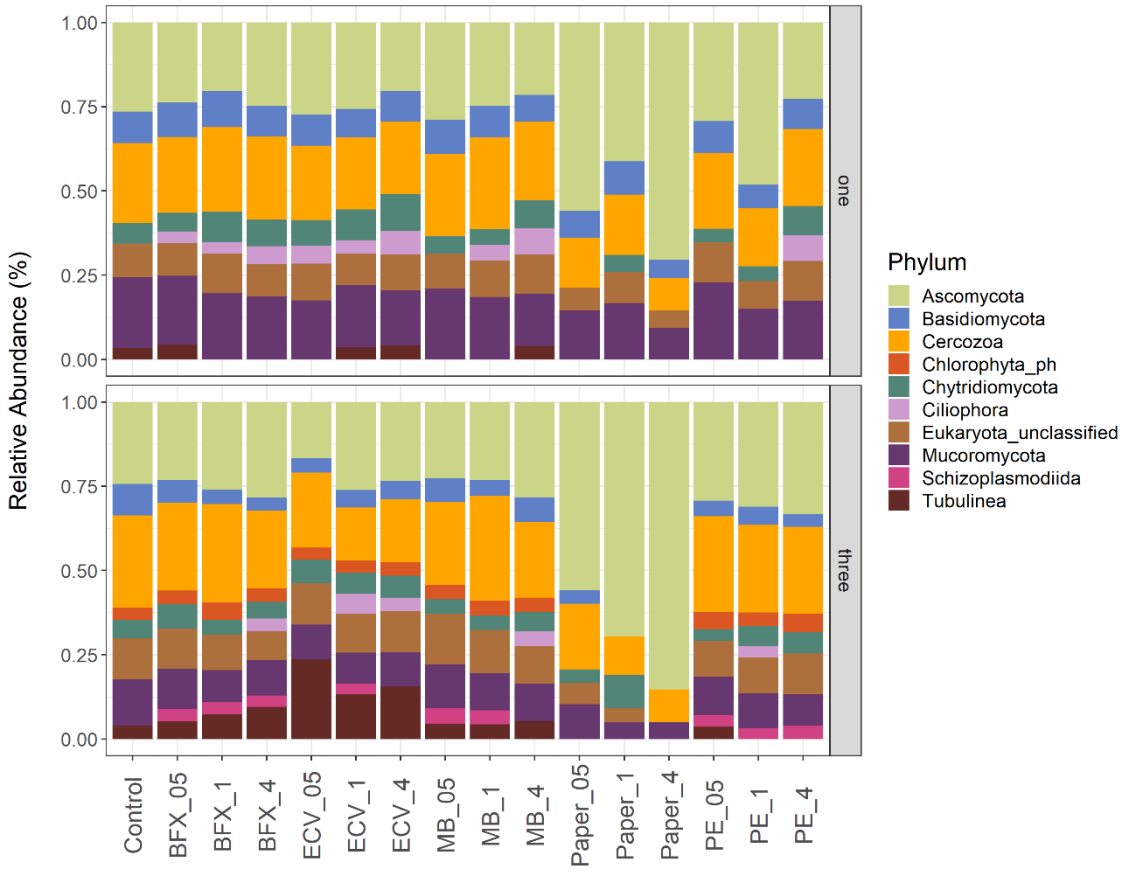

Figure 5. – Mean relative abundance of soil bacterial communities at phylum level after mulch fragments buried for one (upper pannel) and three months (lower panel). Taxa included had at least 3% of relative abundance.

At the class level, the main taxa in all treatments were *Sordariomycetes* (10%), *Eurotiomycetes* (10%), *Tremellomycetes* (8-6%) and *Arcellinida* (3%). The taxa *Intramacronucleata*, *Schizoplasmodiida* and *Techofilosea* were at 3% in some treatments and they were also analysed. The significant enrichment in *Ascomycota* in paper treatments, as described above, was mainly in *Sordariomycetes*. After the third month, *Sordariomycetes* increased in the 1 and 4% paper treated soils to 40 and 50% of relative abundance compared to 10% in the control soil. All soils treated with Ecovio for three months were enriched in *Arcellinida*, whose relative abundance increased significantly to 21, 12 and 14% with mulch concentrations of 0.5, 1 and 4%, respectively.

The analyses at lower classification levels revealed the fungal enrichment found in the paper treated soils was mainly in the *Chaetomiaceae* family (order *Sordariales*) (Figure 5S). The enrichment in the Ecovio treated soils was in the *Echinamoebida* family, genus *Vermamoeba*.

SIMPER analyses of materials for which PERMANOVA indicated significant differences

due to mulch concentration were performed (Table 4S). In the Mater-Bi treatment, *Ascomycota* and *Mucoromycota* contributed to 65% of accumulated variability observed between 4% and 0.5% treatments. *Ascomycota* and *Cercozoa* were found to drive most of the variability between 4% and 1%. In both concentration comparisons, in 4% treatment *Ascomycota* increased while *Mucoromycota* and *Cercozoa* decreased.

In paper treatment, *Ascomycota* and *Cercozoa* contributed to 60% of the variability between 4% and 0.5% and *Ascomycota* contributed to 67% variation between 4% and 1%. *Ascomycota* was found to increase in 4% while *Cercozoa* decreased.

3.3. Soil microbial activity

In control soils, FDA activity, used as a proxy for microbial activity, released fluorescein per gram of dry soil and hour increased significantle from the first month to the third one (Figure 6). Although none of the treatments significantly increased the soil microbial activity compared to control treatment, either after one or after three month' treatment, it somehow increased after Bioflex, Ecovio and PE one-month treatments and also after Ecovio three-month treatment.

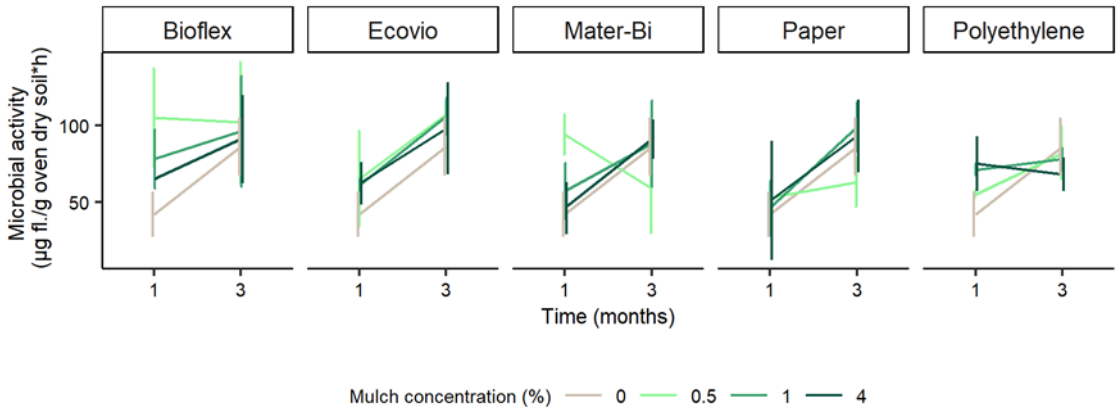

Figure 6. - Soil microbial activity (FDA hydrolisys) after incubation of mulch fragments for one and three months (n=3). Mean and standard deviation (n=3). There were no significant differences between treatments with mulches and controls without for any material or concentration.

After three months of mulch burial, the activity of enzymes potentially participating in mulch biodegradation was determined for the 1% mulch concentration treatments, since it is the one recommended by the European norm regulatingthe BDM ecotoxicity assessment (EN 17033, 2018) and correspondingly, also the one mostly considered in BDM ecotoxicity studies of BDM. Enzymatic activities were tested at 25 and 35°C, representative of the temperature range registered during the experiment and also of warm climate conditions. Changes driven by the treatments in N-acetyl-β-

Glucosaminidase (NAG) activity, for chitin degradation were significant at the two temperatures tested. Barely all plastic mulch treatments decreased itwhile thethe paper one increased it (Figure 7). The mulch presence did not significantly change phosphatase activity, while β-D-cellubiosidase activity was highly variable among treatments impeding identifying significant effects among treatments. Significant effects were only found for the Mater-Bi treatment, which decreased activity at 35°C.

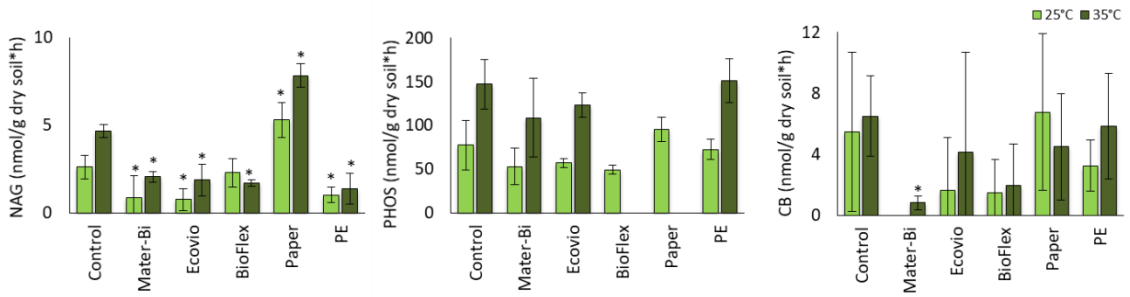

Figure 7. - Enzymatic activity of N-acetyl-β-Glucosaminidase (NAG, chitin degradation), phosphatase (PHOS, phosphorus mineralization) and β-D-cellubiosidase (CB, cellulose degradation) after three months of mulch in soil incubation at 1 % of mulch concentration. Mean and standard deviation. *Statistically significant difference from control. (All significances = P < 0.05).

3.4. Soil pH

Control soil pH increased significantly from the first to the third month of the assay (Figure 8). After the first month, Mater-Bi and paper at 4% increased pH over control treatment. On the contrary, after the third month, the soil pH was significantly decreased by 0.5% Mater-Bi and 1% paper compared to control soil. In 4% Bioflex and paper the pH increased compared to the 1% treatment. Overall, the changes were limited, ranging between 0.4-0.2 pH units over control soil.

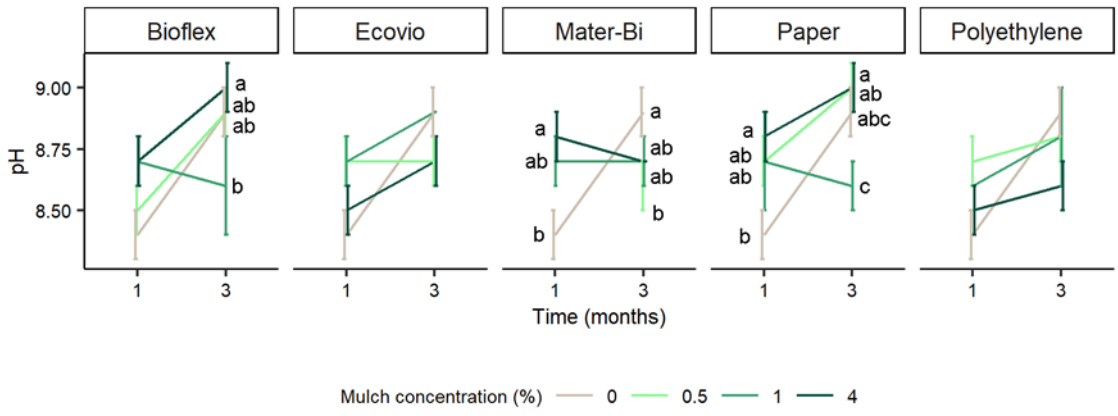

Figure 8. - Soil pH after in soil incubation of mulch fragments for one and three months at different mulch concentrations. Mean and standard deviation (n=3). Letters indicate the result from *post hoc* Tukey following a one way ANOVA.

4. Discussion

4.1. Effect of mulch composition on soil microbial diversity and community structure

In our study, the mulch burial did not significantly alter microbial richness but some of the mulches decreased diversity and had a significant effect on the differentiation of the microbial community structure. The differences found were mostly dependent on the mulch material (Table 2, Figures 2 and 4). The microbial community structure in soil with BDM treatments was distinct from the one in soil with PE. In bacterial communities, the distinction was apparent after the first month and was accentuated after the third one (Figure 2). In eukaryotic communities, plastic burial had little effect on the community structure after the first month but after the third one, the BDM treatments showed differentiation compared to PE (Figure 4). In all cases, after the third month, the changes induced by BDM in the differentiation of community structure compared to control soil were more pronounced than with PE. These results are in line with previous studies that have found the bacterial community of wheat rhizospheric soil was distinct in pots containing PE compared to pots with PBAT-starch when mulches were buried in the soil at 1% w/w for 2 and 4 months (Qi et al., 2020). Similarly, Wang et al. (2020) reported arbuscular mycorrhizal fungi community of maize was differently affected by PLA microplastics than by PE ones. In both studies, biodegradable fragments caused stronger differentiation of microbial communities from control soil than PE fragments. A possible explanation may be related to the difference between the chemical structure of BDM and PE. While the physical presence of BDM and PE fragments would cause comparable effects, BDM may also cause a chemical effect due to the release of their compounds and the formation of biodegradation by-products with the potential to interact with soil microorganisms (Qin et al., 2021). In contrast, not significant compounds are expected to be released from PE, a material that shows negligible biodegradation even after 8 years of burial in soil (Briassoulis et al., 2015).

Among plastic BDM, the mulch composition showed to have a relevant role in their interaction with soil microorganisms. Mater-Bi was the material that caused stronger effects in the bacterial communities. After the first month, the structure of the bacterial communities in the soil with Mater-Bi (PBAT-starch) was differentiated from the ones in pots with Bioflex and Ecovio (PBAT-PLA) (Figure 2). Besides, in contrast to treatments

with PLA containing mulches, the one with starch highly differentiated bacterial community from control soil, especially when buried at 4% of mulch concentration. A hypothesis that would explain the higher differentiation from control is the presence of starch, a naturally occurring polymer, was readily consumed by bacteria during the first month, in contrast to PLA-PBAT components. There are previous studies that have reported in PBAT-starch mulch buried in soil for 3-6 weeks, the starch is preferentially biodegraded to the polyester (Bandopadhyay et al., 2020b). Even before their burial, during BDM performance the crop, starch shows evidence of being (bio)degraded (Touchaleaume et al., 2018). In contrast, PBAT-PLA fragments exhibit low biodegradability in soil, thus it would exert less changes in the microbial community structure (Moore-Kucera et al., 2014; Brodhagen et al., 2014; Touchaleaume et al., 2018).

Interestingly, the 4% Mater-Bi treatment was the only one that significantly decreased bacterial diversity over control soil (Figure 1). It specifically decreased the relative abundance of two bacteria with oligotrophic mode of nutrition: Acibobacteria Gp6 and Planctomycetia. The comparison of the bacterial taxa driving differences of 4% treatment compared to the other concentrations revealed the 23-18% of the accumulated variation was due to the increase of bacteria belonging to the orders Burkholderiales (phylum Alphaproteobacteria) and especially Actinomycetales (phylum Actinobacteria) in the treatment at 4%. Actinobacteria are reported to rapidly degrading starch (Kausar et al., 2011). Besides, both phyla are among the main taxa including multiple species with genes encoding putative cutinase enzymes, able to depolymerize BDM structure and a diversity of plastics (Sander et al., 2019; Gambarini et al., 2021; Lear et al., 2021). It is hypothesize the starch was firstly consumed by these efficient decomposers in rich nutrient conditions, favoring the increase in those degradative organisms belonging to Actinobacteria phylum, while oligotrophic microorganisms would be outgrown.

After the third month of burial, 4% Mater-Bi increased the differentiation of the community structure from control soil but bacterial diversity was not significantly decreased. The variability in the 4% treatment compared to the other concentrations, was not explained by Actinomycetales and Alphaproteobacteria (they only explained 11% of accumulated variability) but by the accumulated little contribution of diverse orders from several phyla. It suggests Actinobacteria may be the first Mater-Bi colonizers and potentially degraders due to their rapid ability to biodegrade starch. It could be also speculated that Actinobacteria may facilitate the colonization and biodegradation by diverse bacterial taxa by increasing the polyester surface exposed due to starch biodegradation. It is worth noting that after the third month, the structure of bacterial communities in soil with Mater-Bi and Ecovio became more similar. Following the reasoning presented above, the starch

may be partially or mostly consumed in Mater-Bi fragments after the third month and the polymeric structure would be similar to the Ecovio one, which is mostly made of PBAT (it has a low content in PLA, 7%, compared to 30% in Bioflex).

Whereas the promotion of potential plastic degraders as Actinobacteria is desirable to avoid mulch persistence in agricultural soil, the decrease of Acibobacteria Gp6, a bacterial group suggested to be a keystone taxa in agricultural soil involved in the decomposition of soil organic matter and denitrification, may have undesirable consequences in the agroecosystem (Kalam et al., 2020). Planctomycetia are known for their role in nitrogen fixation (Delmont et al., 2018). Besides, both have been found in the rhizosphere of cultivated plants and their decrease may affect crop performance (Maul et al., 2014; Qiao et al., 2017; Kalam et al., 2020). However, due to the difficulties of culturing, their ecological functions are not well studied and the consequences of their decrease are hard to predict (Lage and Bondoso, 2012; Kalam et al., 2020). Nevertheless, the decrease in bacterial diversity could lead to loss of ecosystem multifunctionality, essential to preserve agricultural sustainability in the agroecosystem (Bender et al., 2016).

In contrast to Mater-Bi, the PBAT-PLA based Bioflex and Ecovio did not alter significantly bacterial diversity. However, after three months, 4% Bioflex, the BDM blended with 30% PLA, showed the structure of bacterial community significantly different from other concentrations (Table 2). The variability among mulch concentrations was not driven by specific taxa but by small cumulative changes in abundance of several taxa including *Acidobacteria Gp7*, *Chloroflexi*, *Planctomycetes*, suggesting potential soil degraders of Bioflex, presumably of PLA, are widespread among soil bacterial taxa. Bioflex at the highest concentration increased *Planctomycetes* significantly compared to control. However, this taxa increased similarly with PE and paper mulches, suggesting the change is likely linked to the modification of soil environment by mulch fragments rather than the mulch composition.

Ecovio caused minor effects in bacteria community structure but it significantly altered the eukaryotic one, especially after the third month. At all concentrations, it promoted the abundance of *Arcellinida*, an amoeba widely distributed in the soil environment. In contrast, the presence of the other mulches did not affect their abundance suggesting the Ecovio composition is driving the enrichment in *Arcellinida*. A previous report found a type of amoeba, *Acanthamoeba* spp., increased in PBSA incubated in soil for 3-4 weeks (Koitabashi et al., 2012). Although widely known for feeding on bacteria, Acanthamoeba spp. has been found to feed and grew on fungi (Geisen et al., 2016); thus, the increase reported in Koitabashi et al. (2012) may be linked to a drastic change

also found in the fungal population. In Ecovio treatments, no significant changes in the relative abundance of fungal nor bacterial groups were detected. Even when protists have been reported to have key roles in nutrient cycling and shaping microbial communities, they are often forgotten in soil microbiome studies including the ones evaluating the effects of plastic burial in soil microorganisms (Rillig and Bonkowski, 2018; Oliverio et al., 2020). Further exploration on how the alteration of protist groups by BDM may affect the ecological networks in the agroecosystem is needed.

The rest of the plastic mulches Mater-Bi, Bioflex, and PE had a low impact on the community structure of eukaryotic communities. Only 4% Mater-Bi treatment showed significant differences compared to the other mulch concentrations. In 4% treatment, *Mucoromycota* fungi abundance tended to decline, whereas *Cercozoa* and *Ascomycota* increased according to SIMPER results. The promotion of *Ascomycota* could be related to most of the fungi reported to biodegrade plastics belong to *Ascomycota* phylum (Lear et al., 2021; Gambarini et al., 2021). Their efficiency in plastic degradation may shift the development of *Mucoromycota*. Nevertheless, the relative abundance of these taxa did not significantly changed compared to control soil, indicating the impact on eukaryotic communities of Mater-Bi burial even at 4% of concentration was low.

In contrast to plastic mulches, the paper treatment did not alter bacterial diversity and had a limited effect on community structure. It showed to change significantly community structure at 4% concentration compared to other mulch concentrations. The taxa driving the differences included *Solirubrobacterales*, *Sphingomonadales*, *Alphaproteobacteria*, *Actinomycetales* and *Burkholderiales*, main decomposers of organic matter and also of recalcitrant compounds, found to have high efficiency in cellulose degradation (e. g. *Actinomycetales*) (Kausar et al., 2011). However, there were not detected significant changes in the relative abundance of these taxa compared to control soil. On the contrary, paper decreased drastically eukaryotic diversity and at all concentrations, exerted a strong differentiation of eukaryotic structure community by promoting the massive growth of *Ascomycota* fungi. These results were not surprising due to the cellulosic nature of paper, readily biodegradable by fungi. In particular, the enrichment was mainly in the family *Chaetomiaceae*, previously reported to be isolated from a paper mulch (Moore-Kucera et al., 2014).

The soil pH was monitored due to soil microorganisms, especially bacteria, sensitiveness to soil pH changes. In general soil pH was mostly unaffected by BDM and PE but it significantly increased over control soil occurred in treatments 4% Mater-Bi and 4% paper after the first month. It is likely the pH increase is related to the stronger shifts in microbial community structure compared to the ones caused by the other mulches.

However, even significant, the changes were limited (8.50 in control soil to 8.75 in mulch treatments) and do not allow to establish a strong correlation with changes observed in microbial communities. Similarly, BDM and PE burial studies of 1% mulch fragments for 2 and 4 months found the changes in soil pH were limited and there was no consistency among treatments and over time (Qi et al., 2020).

Overall, the results showed most of the plastic mulches buried at 0.5% and 1% of concentration had low or no impact on the richness and diversity of bacteria and eukaryotes present in bulk soil after three months of mulch burial. These results are in accordance with that previously found in mesocosms experiments. No significant changes were reported in bacterial and fungal diversity of bulk soil after burying BDM made of PBAT in soil for 7 months at 0.6% w/w (Muroi et al., 2016); nor after four 4x4 cm PBAT and PLA film fragments buried for 2 months (Rüthi et al., 2021). In field studies, a low quantity of mulch is buried; although comparisons with mesocosms need to be taken with caution, no changes in bacterial diversity of bulk soil were found after a diversity of BDM used compared to soil with no mulch (Masui et al., 2011; Bandopadhyay et al., 2020a).

It is remarkable that most of the studies previously commented reported changes occurring in the soil near to the plastic surface. In particular, two genera, *Caenimonas* and *Hyphomicrobium* were detected in the soil near to plastic surface but not in bulk soil by Muroi et al., (2016) after mulch incubation. Besides, they found drastic changes in fungal communities, highly enriched in *Ascomycota* phylum. Similarly, other studies have found significant changes occur in microbial communities associated to mulch surface. Colonization of films in field showed differential microbial enrichment depending on the mulch composition (Bandopadhyay et al., 2020b). BDM were enriched in *Actinobacteria* (genus *Arthrobacter*) and *Alphaproteobacteria* (genus *Methylobacterium* and *Sphingomonas*) compared to PE. Besides, differential enrichment was found between PBAT mulches blended with starch compared to the ones blend with PLA (Bandopadhyay et al., 2020b). They also found the paper mulch surface was enriched in *Ascomycota* and *Basidiomycota* fungi. Together, our results and previous research suggest the changes promoted in the BDM plastic surface may be extended at spatial scale reaching bulk soil when fragments are accumulated in the soil.

4.2. Effect of buried mulches in microbial activity

The activity of degradative enzymes was evaluated in order to study whether the input of mulches may alter soil nutrient cycling processes. Our results showed the total hydrolytic activity in soil (measure by FDA), an estimation of total organic matter turnover,

was not significantly affected by any of the buried mulches compared to control without mulch. This is in contrast to other studies that found the microbial activity to increase in soil with buried BDM for one (Yamamoto-Tamura et al., 2015) and six months in mesocosm experiments (Barragán et al., 2016) and after two BDM mulch cycles in the field (Moreno and Moreno, 2008). In Yamamoto-Tamura et al., (2015) and Barragán et al., (2016) the microbial activity was positively related to the biodegradation rate of the BDM after being buried for one and six months respectively. A possible explanation for the lack of increased microbial activity in our study may be the differences in methodologies used. In both previous studies, soil with buried BDM was incubated in close systems (jars and Petri dishes) with constant tightly controlled temperature and humidity levels. Our experiment was carried out at green-house conditions, using open containers where the pots were subjected to daily cycles of oscillating ambient temperature and relative humidity. Given the intermediate methodology nature of green-house experiment, between lab and field conditions, it is reasonable to think ambient variability may cause the slowdown of the biodegradation rate of BDM compared to lab experiments.

After three months of incubation, the activity of specific enzymes involved in carbon, nitrogen and phosphorous degradation and mineralization were also evaluated. The cellulolytic potential and phosphorous mineralization were not significantly affected by any of the mulches buried, with the exception of Mater-Bi that decreased significantly cellulose degradation activity. The loss of cellulose-degrading function may be related to the decline of bacterial diversity observed in soil with Mater-Bi. The decrease of microbial diversity is related to the loss of ecological functions (Bender et al., 2016).

Significant differences were also found in activity of NAG (chitin degradation), an enzyme related to the carbon and nitrogen cycle. The paper mulch increased significantly compared to control soil. The increase is likely related to the high enrichment promoted by paper in *Ascomycota* fungi, in particular in the family *Chaetomium*. Several *Chaetomium* spp. are known for their efficiency in chitinase activity to degrade the wall of fungi phytopathogens (Darwis et al., 2020), which may have help them to biodegrade the cellulosic matrix and also to prevail over the other fungal taxa present in the soil as *Basidiomycota* and *Mucoromycota* phyla.

On the contrary, all the plastic mulches, BDM and PE, decreased chitin degradation activity. These results suggest changes may be due to the physical presence of mulch fragments more than to their composition. Another possible explanation would be both BDM and PE shared additives that exert comparable effects. Phthalate esters, commonly added to plastic mulches, have been found to decrease the soil urease activity, a nitrogen

cycle-related enzyme (Xie et al., 2010). There are previous research of PE residues in soil decreased the activity of urease, an enzyme related to nitrogen carbon-cycle (Qian et al. 2018). NAG activity was reported to decrease in the soil where BDM and PE were used compared to soil with no mulch (Bandopadhyay et al., 2020a). However, in Bandopadhyay et al. (2020a), a realistic lower BDM plastic input than used in the present experiment was added to soil in a field experiment and the PE mulch was not buried in the soil. Thus, it is suggested changes are likely related to changes in soil temperature and humidity due to mulch covering the soil. Whether in our study the effects had a physical or chemical origin deserves to be further studied. On the contrary, previous studies showed other nitrogen cycling process, as the nitrification potential, not to change after BDM and PE burial (Kapanen et al., 2008; Ardisson et al., 2014). Together, these findings suggest even when the total microbial activity is not altered, the turnover of specific nutrients as amino sugar compounds may be affected by the burial of plastic mulches. The NAG is an enzyme involved in carbon and nitrogen cycles due to their role in hydrolyzing chitin to amino sugars, a source of nitrogen and carbon to the soil. Therefore, the decrease of chitin degradation activity affects negatively the potential turnover of nitrogenised organic matter, by decreasing an important source of soil mineralizable nitrogen. In consequence, it may have detrimental effects on soil fertility and crops performance.

4.3. Effect of mulch concentration in soil microbial communities

In the ecotoxicity testing of BDM, 1% is the recommended concentration to ecotoxicity assessment of BDM buried in soil by the European norm regulating BDM and by some researchers (Degli-Innocenti, 2014; EN17033, 2018). Even when such a 4% of accumulation levels of BDM are not expected to occur in the medium-short term in field conditions, still the persistence of BDM fragments and their additives in field due to long-term use of BDM, especially in form of micro and nano fragments, is under debate (Kumar et al., 2020). BDM biodegradation is dependent on climate, soil type, depth of burial (Haider et al., 2019). Besides, local limiting conditions could lead to a high ratio of mulch accumulated to the soil. There is also the risk of mulch fragments migrate to other ecosystem compartments with limiting conditions for BDM biodegradation (i.e. anaerobic conditions along with the burial depth).

In the present research, BDM have shown their potential to cause significant effects in microbial community structure and the relative abundance of specific taxa when buried at 0.5, 1 and 4%. The changes induced in bacterial communities were stronger at 4% concentration than at 1 and 0.5 %. However, in the eukaryotic ones, in some cases like in Ecovio containing pots, no consistency among plastic concentrations occurred; 0.5%

caused a greater increase of *Tubulinea* relative abundance than 1 and 4 % concentrations. It revealed the importance of covering a range of concentrations in the testing of plastic effects in organisms. Inconsistency in the effects of a serial concentration of plastics in microbial activity and in soil properties has been previously reported (De Souza et al., 2018). A recent systematic review of microplastics effects in organisms has underlined often there is not a linear-no-threshold dose-response, which is likely analogous to macroplastic effects (Agathokleous et al., 2021). Together, the results highlight the importance of taking into account a range of plastic accumulation, from low to high levels, as a linear prediction it is not necessarily informative on potential effects in specific organisms.

5. Conclusions

The burial of BDM for three months in agricultural soil showed to caused significant effects in the bacterial and eukaryotic diversity and on the community structure. However, the changes were mostly dependent on mulch composition and mulch concentration buried. The differentiation of community structure in pots with BDM was different from the pots with PE. Among the BDM, the greatest shifts in community structure after the first month occurred with the BDM containing readily biodegradable polymers, Mater-Bi, made of PBAT-starch and the paper mulch, in contrast to PBAT-PLA mulches that caused more limited effects. It suggests the mulch composition plays a key role in their interaction with soil microorganisms. Whereas some BDM caused a dose-response effect in the abundance of specific microbial taxa, others caused effects at all concentrations tested. It highlights the relevance of testing a range of concentrations. BDM, together with PE also showed potential to affect nitrogen cycling processes which may have consequences in crops performance. Further research is needed in order to evaluate the evolution in time of the effects observed in the present study.

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7. Supplementary material

Table 1S. Physical and chemical properties of the soil used for the experiment.

Soil property	
pH	8.46
Electrical conductivity (dS m^{-1})	0.218
Bulk density (kg m^{-3})	1124
Calcium carbonate (%)	29
Calcium (ppm)	7639
Silt coarse-grain (%)	16.3
Silt fine-grain (%)	25.7
Clay (%)	30.6
Sand (0.05 < D < 2 mm) (%)	27.4
Texture-USDA	Clay loam
Potassium (ppm)	609
Nitrogen-Nitric (ppm)	10.1
Magnesium (ppm)	262
Organic matter (%) (Walkley-Black)	2.95
Sodium (ppm)	29
Phosphorous (Olsen) (ppm)	35.1

Table 2S. - Average temperatures in the green-house and moisture in-soil. Monthly average (± standard deviation).

Months	Green-house temperature (°C)			In-soil temperature (°C)			Soil water content (cm^3/cm^3)
	Total	Max.	Min.	Total	Max.	Min.	
June	29.8	45.9	18.6	-	-	-	-
July	31.5	47.9	20.8	30	41	22	0.17
August	30.3	45.5	20.8	28	38	21	0.13

Alpha-diversity

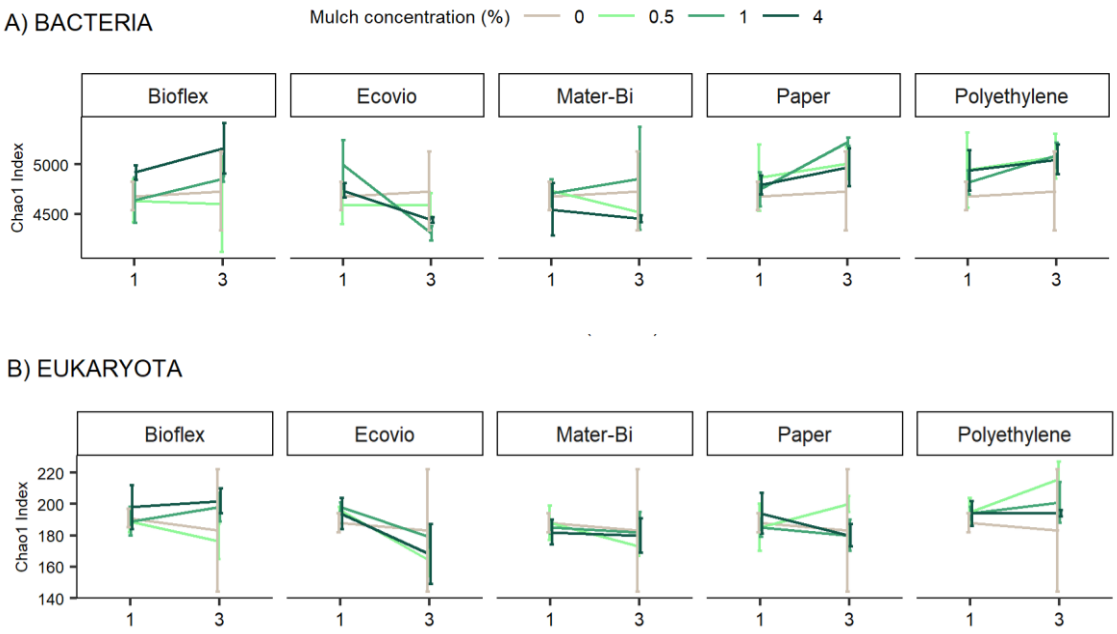


Figure 2S. Richness (Chao1 index) of soil bacterial (A) and eukaryotic (B) communities after one and three months of mulch fragments buried in soil at different mulch concentrations. Mean and standard deviation (n=3). There were no significant differences between treatments with mulches and controls without for any material or concentration.

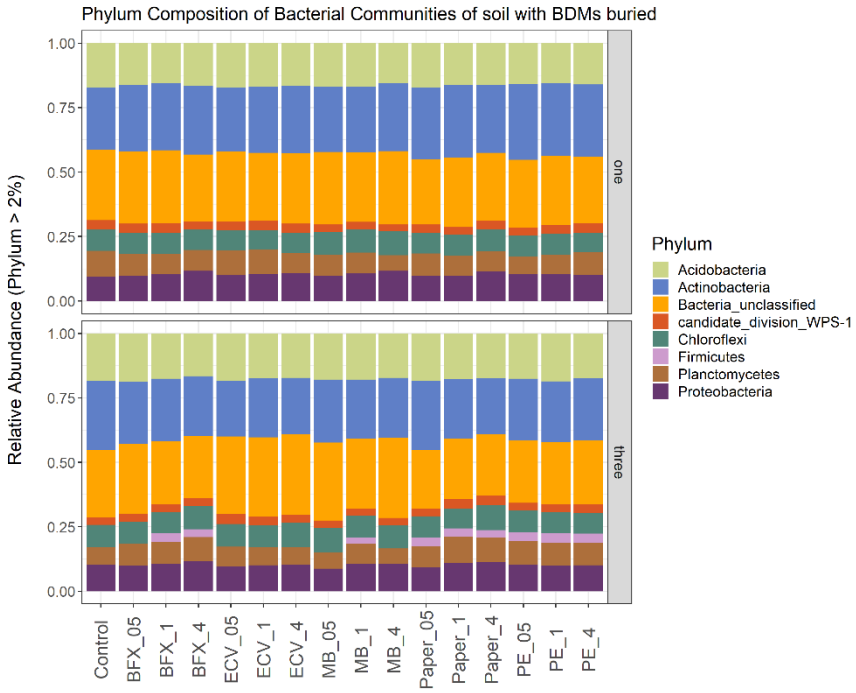
Beta-diversity

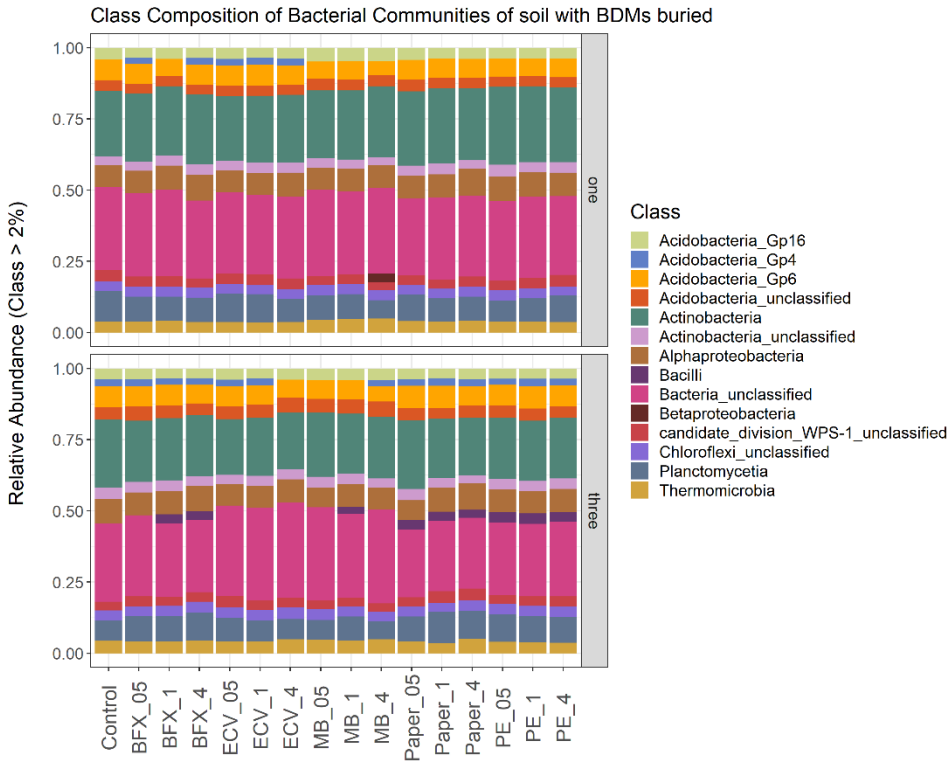
Bacterial communities

Table 3S. SIMPER results for identification of bacteria taxa driving significant differences between the treatments with highest concentration of mulch buried (4%) and the others (1 and 0.5%) detected with PERMANOVA analysis in Mater-Bi, Bioflex and paper. The first OTUs that most contributed significantly to variability between mulch concentrations are shown ($p < 0.05$). Average: average contribution to overall dissimilarity, SD: standard deviation of contribution, Ratio: average to SD ratio, Av.: average abundances per concentrations compared, a-b: mulch concentrations, as indicated in contrast row, Cumsum: cumulative contribution.

	Order	average	sd	ratio	ava	avb	cumsum
One month							
Mater-Bi							
Contrast: 0.5%-4% (a-b)							
	Actinomycetales	0.0055	0.0039	1.4	720	1514	0.03
	Actinomycetales	0.0048	0.0010	4.6	851	1534	0.06
	Burkholderiales	0.0038	0.0014	2.7	246	795	0.09
	Actinomycetales	0.0013	0.0004	2.9	376	194	0.17
	Actinomycetales	0.0009	0.0003	2.7	354	487	0.23
Contrast: 1%-4% (a-b)							
	Actinomycetales	0.0054	0.0038	1.4	743	1514	0.04
	Actinomycetales	0.0011	0.0002	7.5	249	410	0.16
	Actinomycetales	0.0008	0.0005	1.6	704	818	0.18
	Rhizobiales	0.0007	0.0003	2.3	42	146	0.21
Paper							
Contrast: 0.5%-4% (a-b)							
	Solirubrobacterales	0.0011	0.0008	1.4	759	600	0.06
	Sphingobacteriales	0.0010	0.0002	5.8	270	130	0.11
	Alphaproteobacteria_un	0.0009	0.0006	1.5	818	687	0.12
	candidate_division_WPS1	0.0009	0.0005	1.9	233	361	0.13
	Burkholderiales	0.0006	0.0004	1.8	69	162	0.16
	Solirubrobacterales	0.0006	0.0003	1.8	656	566	0.18
	Sphingomonadales	0.0006	0.0002	2.5	136	218	0.19
	Sphingomonadales	0.0006	0.0003	1.8	101	181	0.19
	Actinomycetales	0.0005	0.0003	1.4	99	168	0.20
	Sphingomonadales	0.0005	0.0002	2.1	67	138	0.21
Contrast: 1%-4% (a-b)							
	Actinomycetales	0.0015	0.0004	3.5	702	492	0.02
	Actinomycetales	0.0009	0.0001	17.2	16	142	0.10
	Chloroflexi_un	0.0008	0.0003	3.0	441	556	0.11
	Gaiellales	0.0006	0.0002	2.5	305	223	0.17
	Burkholderiales	0.0005	0.0002	2.9	47	123	0.18
	Gp6	0.0004	0.0002	1.7	40	95	0.22
3 months							
Mater-Bi							
Contrast: 0.5%-4% (a-b)							
	Actinomycetales	0.0028	0.0008	3.3	417	813	0.10
	Actinomycetales	0.0025	0.0014	1.8	291	654	0.12
	Burkholderiales	0.0020	0.0006	3.1	143	427	0.13
	Chloroflexi_un	0.0015	0.0004	4.2	510	296	0.18

	Burkholderiales	0.0015	0.0007	2.0	18	232	0.19
Contrast: 1%-4% (a-b)							
	Actinomycetales	0.0032	0.0006	5.0	359	813	0.11
	Acidobacteria Gp6	0.0020	0.0012	1.7	498	205	0.14
	Bacillales	0.0018	0.0011	1.7	384	122	0.15
	Chloroflexi_un	0.0014	0.0004	3.4	493	296	0.21
Paper							
Contrast: 0.5%-4% (a-b)							
	Acidobacteria Gp6	0.0018	0.0005	3.4	1256	995	0.01
	Solirubrobacterales	0.0017	0.0008	2.0	879	641	0.03
	Rhizobiales	0.0016	0.0011	1.4	73	291	0.04
	Chloroflexales	0.0015	0.0012	1.3	33	249	0.06
	Rhizobiales	0.0015	0.0001	11.0	367	577	0.07
	Solirubrobacterales	0.0013	0.0006	2.4	706	517	0.08
	Gaiellales	0.0011	0.0005	2.3	419	263	0.09
	Gaiellales	0.0010	0.0006	1.7	380	232	0.10
	candidate_division_WPS1	0.0009	0.0004	2.0	215	338	0.14
Contrast: 1%-4% (a-b)							
	Thermomicrobia_un	0.0014	0.0003	4.9	340	546	0.03
	Rhizobiales	0.0014	0.0001	12.9	381	577	0.04
	Sphaerobacterales	0.0012	0.0003	3.9	195	372	0.07
	Solirubrobacterales	0.0012	0.0006	2.0	686	517	0.07
	Sphaerobacterales	0.0011	0.0006	1.8	458	610	0.10
	Actinomycetales	0.0011	0.0006	1.8	383	535	0.11
	Actinomycetales	0.0009	0.0006	1.5	112	247	0.13
Bioflex							
Contrast: 0.5%-4% (a-b)							
	Acidobacteria Gp7	0.0014	0.0009	1.6	427	222	0.11
	Chloroflexi_un	0.0014	0.0005	3.0	557	760	0.12
	Planctomycetales	0.0013	0.0005	2.5	380	563	0.13
	Actinomycetales	0.0011	0.0003	4.3	365	201	0.16
	Rhodospirillales	0.0008	0.0005	1.6	156	271	0.21
	Bacillales	0.0008	0.0005	1.7	117	224	0.22
Contrast: 1%-4% (a-b)							
	Chloroflexi_un	0.0011	0.0004	2.6	596	760	0.09
	Planctomycetales	0.0008	0.0004	2.0	409	528	0.13
	Gaiellales	0.0007	0.0001	5.1	393	299	0.16
	Candidatedivision_WPS1	0.0005	0.0001	5.4	69	0	0.22


Phylum Composition of Bacterial Communities of soil with BDMs buried
Relative Abundance (Phylum > 2%)
1.00
0.75
0.50
0.25
0.00
one
three
Control
BFX_05
BFX_1
BFX_4
ECV_05
ECV_1
ECV_4
MB_05
MB_1
MB_4
Paper_05
Paper_1
Paper_4
PE_05
PE_1
PE_4
Phylum
Acidobacteria
Actinobacteria
Bacteria_unclassified
candidate_division_WPS-1
Chloroflexi
Firmicutes
Planctomycetes
Proteobacteria


Class Composition of Bacterial Communities of soil with BDMs buried
Relative Abundance (Class > 2%)
1.00
0.75
0.50
0.25
0.00
one
three
Control
BFX_05
BFX_1
BFX_4
ECV_05
ECV_1
ECV_4
MB_05
MB_1
MB_4
Paper_05
Paper_1
Paper_4
PE_05
PE_1
PE_4
Class
Acidobacteria_Gp16
Acidobacteria_Gp4
Acidobacteria_Gp6
Acidobacteria_unclassified
Actinobacteria
Actinobacteria_unclassified
Alphaproteobacteria
Bacilli
Bacteria_unclassified
Betaproteobacteria
candidate_division_WPS-1_unclassified
Chloroflexi_unclassified
Planctomycetia
Thermomicrobia

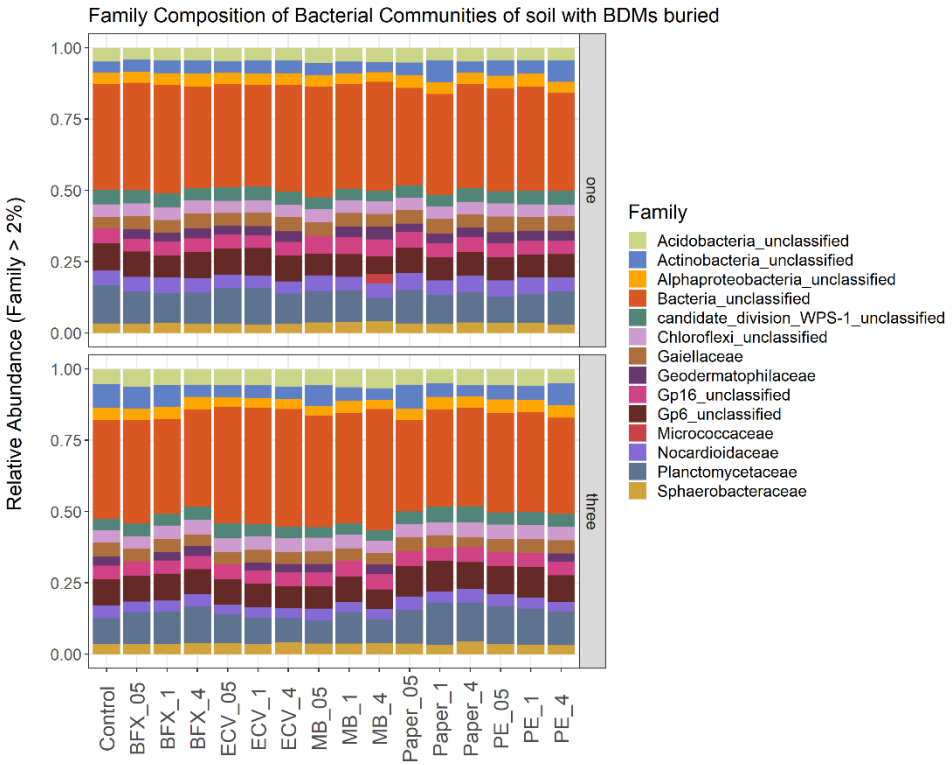


Figure 3S. Mean relative abundance of soil bacterial communities after mulch fragments buried for one (upper panels) and three months (lower panels) at phylum, class and family level. Taxa that were at least 2% relative abundance are shown.

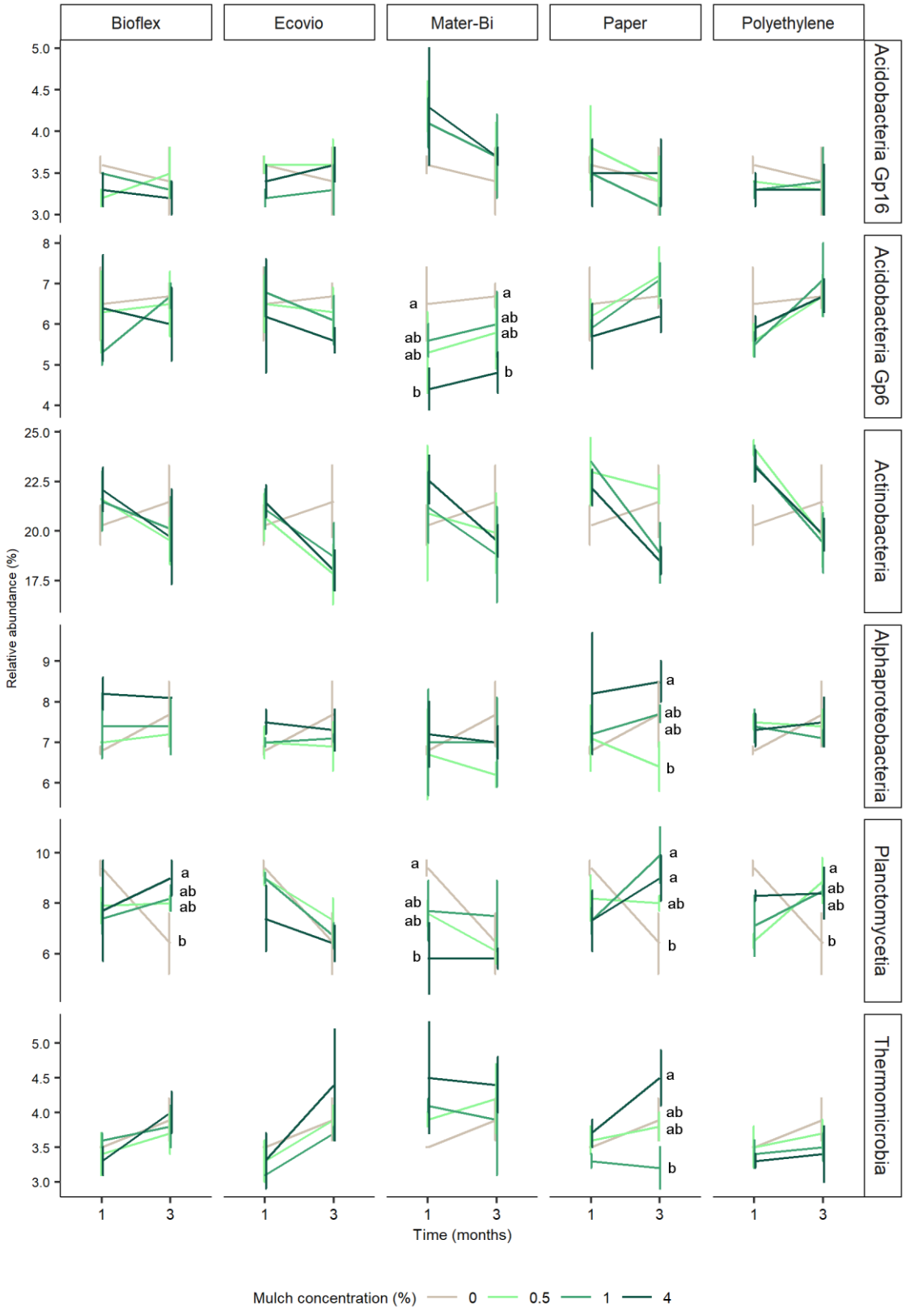


Figure 4S. - Relative abundance of bacterial classes. Mean and standard deviation (n=3). Letters indicate the result from *post hoc* Tukey following a one-way ANOVA comparing mulch concentrations within each material for each time point.

Eukaryotic communities

Table 4S. SIMPER results for identification of bacteria taxa driving significant differences between the treatments with highest concentration of mulch buried (4%) and the others (1 and 0.5%) detected with PERMANOVA analysis in Mater-Bi and paper. The OTUs that most contribute significantly to variability between mulch concentrations are shown ($p < 0.05$). Average: average contribution to overall dissimilarity, SD: standard deviation of contribution, Ratio: average to SD ratio, Av.: average abundances per concentrations compared, a-b: mulch concentrations, as indicated in contrast row, Cumsum: cumulative contribution.

	Phylum	Average	SD	Ratio	Av. a	Av. b	Cumsum
Three months							
Mater-Bi							
Contrast: 0.5%-4% (a-b)							
	Mucoromycota	0.0096	0.0058	1.7	3263	2457	0.37
	Ascomycota	0.0083	0.0041	2.0	923	1692	0.48
	Ascomycota	0.0040	0.0020	2.0	58	434	0.65
	Ochrophyta	0.0039	0.0047	0.8	29	390	0.67
Contrast: 1%-4% (a-b)							
	Cercozoa	0.0348	0.0141	2.5	10185	6949	0.14
	Ascomycota	0.0058	0.0050	1.2	177	713	0.55
Paper							
Contrast: 0.5%-4% (a-b)							
	Ascomycota	0.0576	0.0374	1.5	4462	9818	0.16
	Cercozoa	0.0338	0.0209	1.6	5868	2729	0.39
	Ascomycota	0.0161	0.0029	5.6	87	1584	0.52
	Ascomycota	0.0149	0.0041	3.6	2435	1048	0.60
	Mucoromycota	0.0079	0.0037	2.2	1068	333	0.72
Contrast: 1%-4% (a-b)							
	Ascomycota	0.0642	0.0362	1.8	3842	9818	0.45
	Ascomycota	0.0159	0.0029	5.5	101	1584	0.67
	Ciliophora	0.0041	0.0035	1.2	113	492	0.80
	Ascomycota	0.0027	0.0009	3.1	35	289	0.84

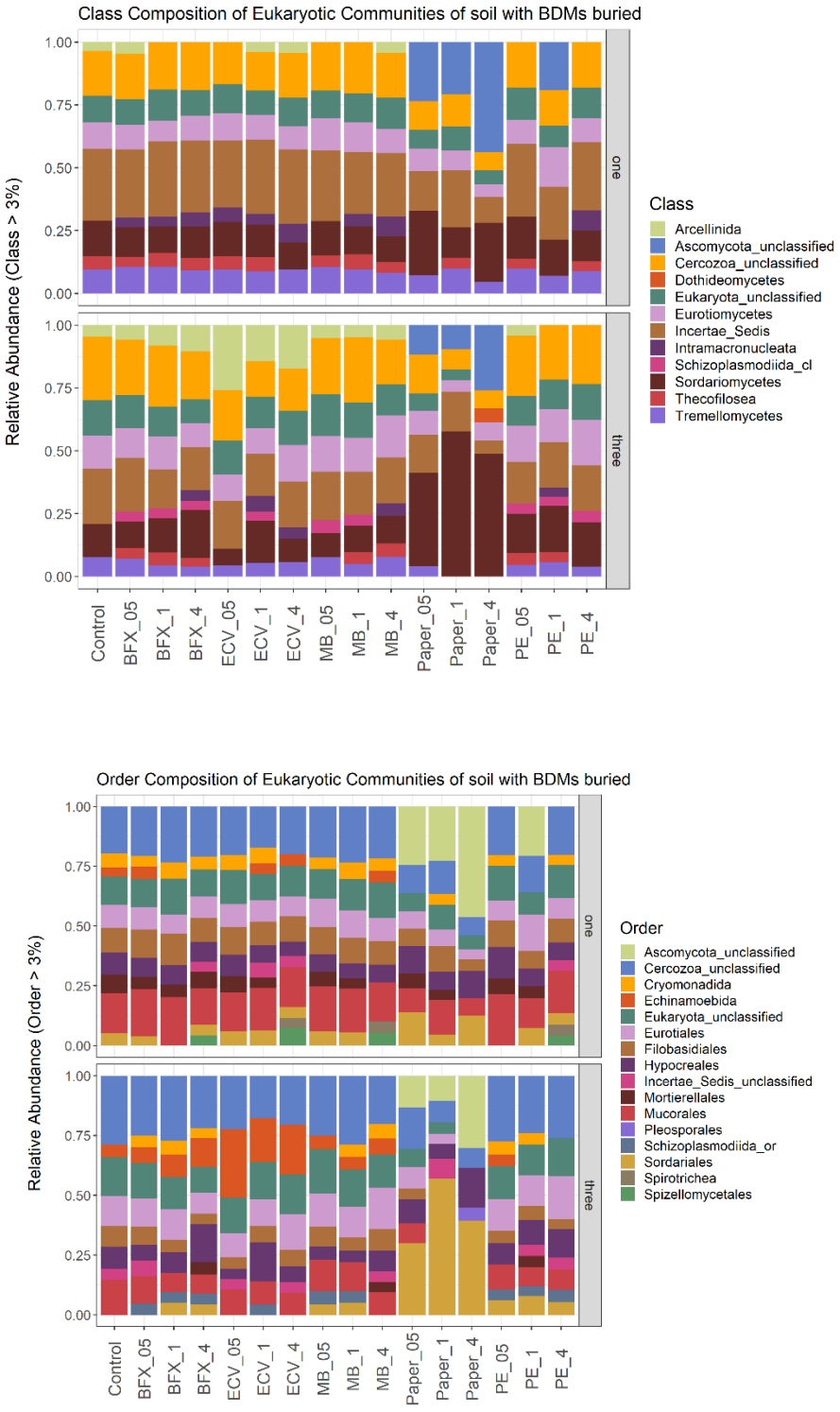


Figure 5S. Mean relative abundance of soil eukaryotic communities after mulch fragments buried for one (upper panels) and three months (lower panels) at class, order and family level. Taxa that were at least in 3% relative abundance are shown.

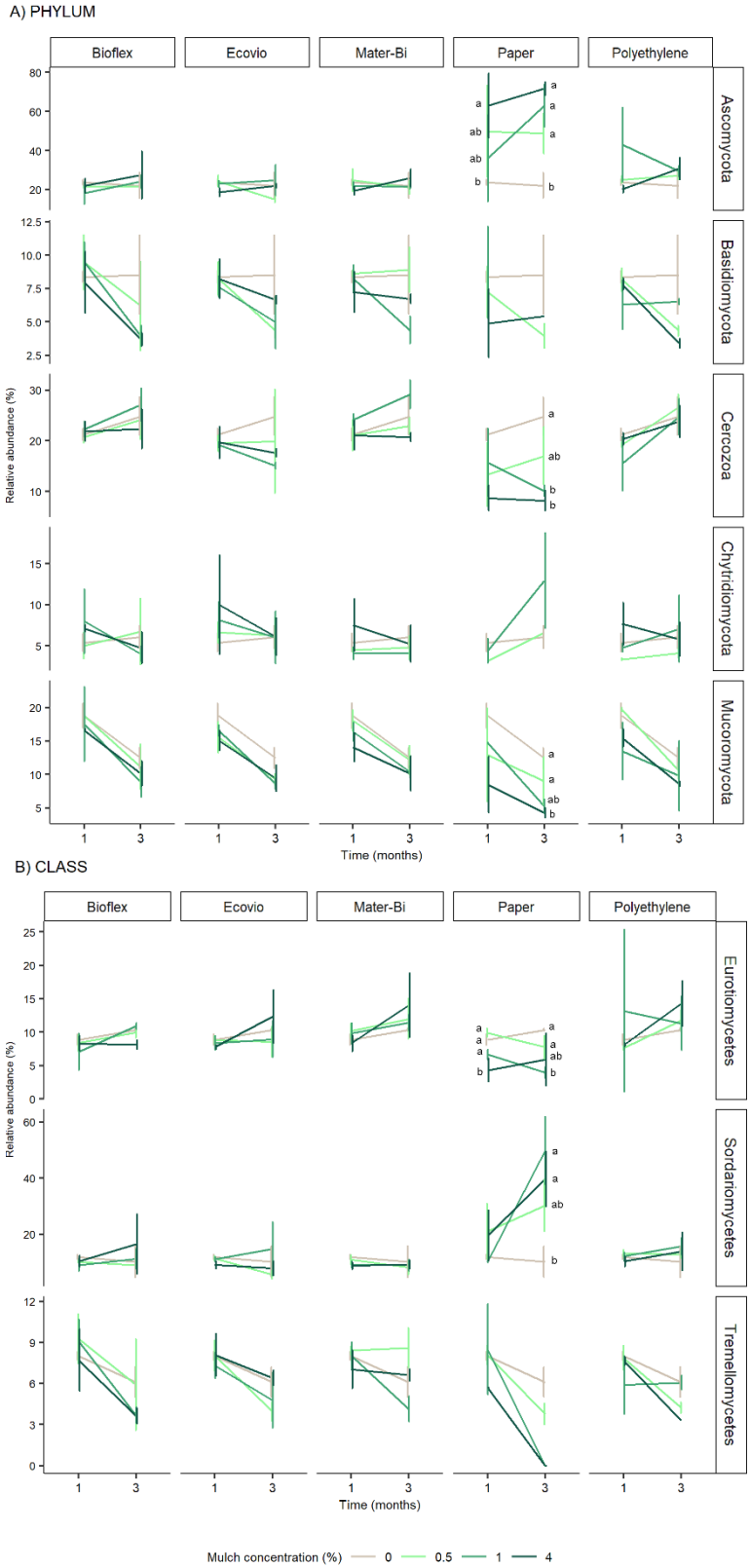


Figure 6S. Relative abundance of eukaryotic phyla (A) and classses (B). Mean and standard deviation (n=3). Letters indicate the result from *post hoc* Tukey following a one-way ANOVA comparing mulch concentrations within each material for each time point.

CHAPTER VI

GENERAL DISCUSSION

GENERAL DISCUSSION

The use of plastic films for agricultural mulching provides several benefits: increases crop yield, earliness and quality, prevents weed development and improves water and agrochemicals-use efficiency (Lamont, 2005; Kasirajan and Ngouajio, 2012). Due to their high effectiveness in controlling soil environment, their use has increased dramatically since their commercialization in the 60's; by 2019, its global consumption is estimated at 2 Mt (Kader et al., 2017; Le Moine and Ferry, 2019). However, most of the films are made of non-biodegradable polyethylene (PE), causing an extending environmental problem due to waste generation and to the accumulation of fragments in the agricultural soil and in the environment, threatening crop production and soil health (Steinmentz et al., 2016; Zhang et al., 2020). This situation has motivated the development and use of biodegradable plastic mulches (BDM) as more environmentally friendly materials. BDM show comparable performance to PE mulches in many crops, and contribute to avoiding plastic soil accumulation while saving time and labour costs because after crop harvest they are tilled into soil, where the native microorganisms biodegrade. Over the last decade, BDM use is showing an increasing trend, expected to growth in the next years (Kasirajan and Ngouajio, 2012; Martin-Closas et al., 2017; Transparency Market Research, 2019).

BDM persistence in soil is shorter than PE due to its polymeric structure susceptibility to be hydrolysed by the soil microorganism's enzymes, in contrast to the durable PE structure, highly resistant to hydrolysis. Whereas PE films show no (bio)degradation signs even after 8-10 years buried into soil, BDM are relatively rapidly (bio)degraded: to be commercialized, at least 90% must biodegrade in up to 2 years buried into soil (Briassoulis et al., 2015; EN17033, 2018). However, until (bio)degradation is completed, the fragments and the released compounds are in the soil, in contact with the root system of cultivated plants and with soil organisms. Soils receive a continuous input of BDM components after repeated BDM, whose impact on organisms needs to be addressed to ensure they are a sustainable alternative to PE in the long term.

Still, knowledge on the impact of BDM compounds on organisms, and particularly on plants is very scarce (Chapter I). Most studies have usually assumed that effects on plants could arise only after significant in soil biodegradation has occurred, ca. 6-12 months after burial (Rychter et al., 2006; Degli-Innocenti, 2014; Sforzini et al., 2016; Muroi et al., 2016; Palsikowski et al., 2018; EN17033, 2018; Souza et al., 2020; De Souza et al., 2021). The effects of BDM from previous stages, including those during

BDM use and early burial, have received less attention and only four authors report on them (Fritz et al., 2003; Qi et al., 2018; Meng et al., 2021). However, in contrast to PE mulches, BDM ones exhibit obvious deterioration during its use, suggesting abiotic degradation, and some of their components may be released even before the mulches are buried into soil. Later on, after the crop ends, fragments are buried, left in contact with water and microorganisms hastening the release of chemicals into soil. To gain comprehensive knowledge on the BDM impact on organisms, their life-cycle needs to be fully addressed, thus to ensure they do not interfere with the cultivated plants and soil organisms' development.

In this PhD thesis, 8 BDM from different composition were tested for its effects on two horticultural species, tomato and lettuce, and on the agricultural soil microbiome. On the one hand, it was studied whether compounds may leach from BDM through an abiotic process when in contact with water, before biodegradation starts, and affect germination and plant development (Chapter II). Furthermore, the leachates were chemically characterized (Chapter III). On the other hand, potential effects from BDM fragments after their burial into soil were evaluated on plants (Chapter IV) and on the soil microbiome (Chapter V).

Two vegetable species, tomato and lettuce, were selected for testing because plastic mulching is a technique mainly used in vegetable crop production (Lamont, 2005; Kasirajan and Ngouai, 2012; Martin-Closas et al., 2017). Moreover, both crops are among the main vegetables consumed worldwide, with production values of 180 Mt in tomato (only surpassed by potato) and 29 Mt in lettuce (FAO, 2019). Use of plastic mulches has been essential for increasing vegetable commercial production to reach the high values shown above, and they are successfully used in intensive vegetable crop production systems (e.g. watermelon, cucumber, pepper, cole, etc.) (Lamont, 2005). Since the last decade, BDM have proven their agronomic performance, equivalent to PE films in many vegetable crops, including tomato, lettuce, pepper, aubergine and cucurbita species (melon, watermelon, cucumber), and their use has suffered an increasing trend expected to growth (Martin-Closas et al., 2017; Transparency Market Research, 2019).

Nevertheless, researches on the effects of BDM compounds on plants are scarce, especially on vegetables. Often, the selection criteria of species for testing their effects is based on recommendations from ecotoxicity norms and standards designed to test the phytotoxicity potential of chemicals (e.g. OCDE 208, 1984). Although the tests based on

available norms provide an estimation of the phytotoxicity potential, they lack the required focus to search for the specific impact the BDM may have on the environment where they are mostly used, as on vegetable crops. The list of tested species include cress, millet, rape, barley, turnip rape, sorghum, wheat, maize, perennial ryegrass and onion; and, lettuce and common bean, the only two commonly cultivated with plastic mulches (Fritz et al., 2003; Rychter et al., 2006; Muroi et al., 2016; Sforzini et al., 2016; Qi et al., 2018; Boots et al., 2019; Wang et al., 2020; Palsikowski et al., 2018; Souza et al., 2020; Meng et al., 2021). Up to our knowledge, this thesis provides the first assessment of BDM impact on tomato plants. Lettuce has been previously tested for BDM ecotoxicity, but its use for BDM ecotoxicity testing has been mostly limited to shortly exposing seeds and resulting plantlets, 10-14 days, to extracts from soils having contained films either before or after suffering significant biodegradation (Souza et al., 2020). Longer term effects have not been studied. On the other hand, the lettuce crop cycle is short and several crop cycles and BDM may be applied on a soil a year, thus the potential of this crop to grow on soils with high BDM compounds accumulation is higher than for longer cycles' crops. Besides, lettuce is a highly sensitive to contaminants, thus a species to detect phytotoxicity through their use by norm standards (ISO 17126, 2009).

From their installation on soil, BDM keep in contact with weathering agents (rain, irrigation, fertilization, radiation, etc.) that interact with the film structure. In particular, migration of compounds from film to rainfall or irrigation water likely constitute an input of chemicals to soils, which might enter in contact with plant roots and be absorbed by them. In this PhD thesis, after 8 fresh-roll BDM had been in contact with water for seven days, the resulting leachates were tested for their effects on tomato and lettuce germination and plant development (Chapter II). The test was carried out by incorporating the leachates to an *in vitro* plant culture media, a system that allowed for close contact of the leachates with the seeds germinating on it and later with the emerging root system, together witha tight control and homogeneity of the environmental factors. Plant development was closely monitored for a month and changes on plant morphology were evidenced, including those on root development, which are difficult to be observed on other cultivation systems where roots are buried and remain unseen. On this system, some of the BDM leachates inhibited growth, altered plant physiology (leaf' chlorophyll and proline content) and changed root morphology of tomato and lettuce plants, in contrast to PE leachate that did not (Chapter II). These findings reveal that even before significant (bio)degradation has occurred, some BDM compounds are released soon after their contact with water and have a potential to alter plant

development, thus highlighting the need to including the first's stages of mulch use in their ecotoxicity assessment.

The results from the *in vitro* test indicate the specific composition of each BDM plays a key role in its effects on the studied plants. Overall, the root dry weight decreased with most BDM leachates. However, its contribution to total plant biomass is very low as compared to the aerial plant part one; thus, even when most BDM leachates decreased root dry weight, the total plant biomass was not necessarily affected. On the one hand, in both lettuce and tomato plant species, B-SP4 and B-SP6 caused strongly inhibitory effects on root and aerial growth, and on proline (a plant stress marker) and leaf chlorophyll content. Biofilm, besides causing strong plant growth inhibition, profoundly altered the root morphology. Mirel strongly decreased tomato plant growth, both in the root and in the aerial part, whereas in lettuce it only decreased root growth while total plant biomass remained unaffected, this denoting the sensitivity to a specific BDM leachate varies between species. On the other hand, Mater-Bi, Ecovio and Bioflex caused none or minor effects on plant growth and physiology. To our knowledge, effects from BDM leached compounds have not been previously targeted; however, in line with our findings, leachates from PBAT biodegradable bags have been reported to inhibit early development of two coastal dune plant species, which showed different sensitivity to the material (Menicagli et al., 2019; Balestri et al., 2019).

With the aim to gain insight on which are the compounds released from the tested mulches to water, resulting leachates were chemically characterized (Chapter III). Few compounds were identified in the PE leachate: fatty acids and 1,10-decanedioic acid bis (2-ethylhexil) ester; in contrast, BDM leachates released a complex variety of chemicals (Chapter III. Figure 1). Interestingly, every material showed a characteristic chromatographic profile. From the 8 BDM tested, the most complex mixtures were released from the PBAT-starch-based mulches, Mater-Bi, B-SP4 and B-SP6. However, the higher complexity was not linked to a higher ecotoxicity, with differences between them: both Bioplast producing strong inhibitory effects and Mater-Bi causing minor effects on plants, thus the main polymer composition not being the key factor for the potential BDM toxicity and pointing to the specificity on the mixture of released compounds being the determinant to their potential toxicity.

Among the compounds BDM released, molecules involved in biological functions were identified: 3 and 4-hydroxybutyric acids, adipic acid, lactic acid, mono and disaccharides, glycerol and its derivates, etc (Chapter III. Table 4). Previous assays revealed lactic and

adipic acids and 1,4-butanediol, released from all PBAT based BDM, limit *in vitro* tomato and lettuce plant growth and increase proline content (Martín-Closas et al., 2014). However, the concentration of these monomers in the leachates was in orders of magnitude below the ones reported to inhibit plant growth, thus none of these compounds can be directly linked to the ecotoxicity reported. NRM analyses of the BDM leachates identified a complex mixture of molecules, including monomers, short-chain oligomers and small polymeric fragments; to the best of our knowledge, the effects of oligomers and short polymeric fragments from BDM remain largely unexplored. There is a need for the development of optimized methodologies to identify and quantify these compounds released to the environment and to test for their effects on plants.

Following the mulch cycle, the next aim of this PhD thesis was to provide knowledge on the effects of BDM on plants after the crop cycle ends, when BDM are tilled into soil to biodegrade (Chapter IV). The mulch biodegradation is strongly dependent on several factors: the material composition, environmental conditions and soil type. The biodegradation rate requested for approving the commercialization of BDM is fixed under artificial conditions, which are not the real ones in the fields; moreover, even if this biodegradation is accomplished, a 10% fraction of the BDM is allowed to remain for longer than 2 years' burial. Thus, the continuous use of BDM may lead to the accumulation of mulch fragments over time. In fact, BDM macrofragments have already been reported to persist into soil in the field up to 1 and 4 years after BDM use (Kapanen et al., 2008; Ghimire et al., 2020). Whether BDM persistence would be in the form of micro and nanofragments has not been addressed. In the present PhD thesis, BDM macro-fragment accumulation effects on tomato and lettuce germination and plant development were evaluated through a microcosm pot assay, by burying mulch fragments into a plant pot substrate where plants developed for ca. 1.5 months. The impact of BDM fragments on the substrate pH and microbial hydrolytic activity was also determined.

The exposition to the field environmental conditions of the BDM during its service life weathers the materials, inducing changes in their physicochemical properties, exerting later effects on their in-soil biodegradability (Touchalaume et al., 2018; Moreno et al., 2017; Anunciado et al., 2020). However, BDM ecotoxicity testing has been only performed with new unused materials and whether field environmental weathering leads to changes in the BDM ecotoxicity potential has only been targeted on PBAT mulch films artificially weathered in an environmental simulator chamber for 16 days (Souza et al., 2020). The pot assay in this PhD thesis, determined the impact on plants of BDM

accumulation into a substrate both with field weathered mulch fragments recovered after a pepper crop usage and with new unused fragments, fully allowing comparing of their effects.

Overall, the pot assay demonstrated the materials, both unused and weathered, did not affect lettuce and tomato germination, but some of them decreased growth and altered physiology of both plant species soon after plantlet emergence; the inhibition persisted to the end of assay, ca. 1.5 months after sowing. As previously evidenced through the *in vitro* assay, the relevance of BDM composition on plants' effects was evidenced. Unused Mirel fragments (referred as BP7 in Chapter IV) produced the strongest effects, almost completely inhibiting tomato and lettuce plant growth. To a lesser extent, B-SP4, B-SP6 and Biofilm (referred as BP2, BP3 and BP6 respectively) also reduced growth and chlorophyll content of both plant species. Lettuce was somehow sensitive to BDM presence of new unused fragments from the other materials, all of them decreasing plant growth, whereas effects on tomato were minor. The weathered fragments that exhibited the strongest effects in both species were Mater-Bi (BP1) and B-SP4, and B-SP6 in tomato. Unfortunately, Mirel and Biofilm field weathered fragments were not available to perform the tests. Overall, field weathered fragments had stronger inhibitory effects in plants then new unused ones in both species. These findings provide evidence for the need of taking into account the materials condition to obtain a close estimate of ecotoxicity potential of the BDM in field.

Paper mulch chemical and physical properties are very different from those of the other BDM tested, which are all plastics. *In vitro*, tomato plant growth was little affected by the paper mulch leachates, but it was drastically inhibited in the pot assay. The *in vitro* test was performed with extracts obtained after one-week incubation in a water solution of mulch fragments, while in the pot assay the mulches remained in the substrate for 7 weeks, all during the plant growth, allowing the material compounds to be altered through time. Paper, made from cellulose fibres, suffers substantial changes when in contact with water and biodegrades fast when buried, being mostly or completely biodegraded after 6 months burial (Ghimire et al., 2020). Indeed, during the pot assay, the paper BDM biodegraded faster than the plastic ones, its components being likely rapidly released to allow contact with plant roots. However in lettuce, the paper BDM was the only one that did not cause effects in the pot assay. Interestingly, the compounds leached by the paper BDM had a stimulatory effect *in vitro*, promoting plant growth, including roots, likely due to mimicking plant hormone effects. These results indicate once more the variability between species sensitivity to a specific mulch, similarly to that found with plastic BDM.

In contrast to effects found in pots for BDM, PE fragments had no effects on plants, either weathered or new unused. Together, the findings from pot assays are in line with that reported in other ecotoxicity studies in plants: PE microfragments had no effects in maize (Wang et al., 2020), or are minor as compared to those from BDM in wheat and common bean (Qi et al., 2018; Meng et al., 2021). The lack of effects from PE indicates the physical presence of mulch fragments do not affect plant growth and development, suggesting the effects of BDM being related to their chemical composition. Whereas PE is a highly stable material exhibiting negligible (bio)degradation due to abiotic and biotic factors (Briassoulis et al., 2015) and no significant compounds are expected to be released from it, it has been proven BDM delivers compounds soon after their contact with water (Chapter III), which are expected to be massively released once they are buried in the soil. From the pot assays findings it is hypothesized that BDM buried fragments leach compounds interacting with plant roots. In line with this, the larger effects from field weathered materials are due to their compounds being easily leached out from them, due to film structure deterioration, than from the new unused materials

Apart from the direct effect of the BDM on plants due to the contact with plant roots of the fragments and the compounds released during their (bio)degradation, plant development may be altered by mulch fragments indirectly, through modifying the plant underground growing environment by changing soil biophysicochemical properties. Some recent studies have reported buried PE and BDM affect the soil environment by changing bulk density, pH, electrical conductivity and C:N ratio, soil microbial activity and rhizospheric bacterial groups abundance (Souza et al., 2019; Qi et al., 2020ab). Our results showed that the pH of the substrate, key for plant nutrient uptake, slightly but significantly increased more in pots with BDM after plant growth, than in control treatment. These results are in line with other greater pH changes reported after burial of mulch fragments in soil compared to soil without buried mulch (Ardisson et al., 2014; Li et al., 2014; Qi et al., 2020; Wang et al., 2020) and evidence buried mulches impact on substrate chemical properties. However, despite the pH changes, the values always remained in the range for optimal lettuce and tomato growth, thus it is unlikely the alterations in plat growth and development are linked to them.

To get knowledge on whether the introduction of BDM on the substrate promotes changes on microbial activity, microbial hydrolytic activity before and after plant growth was recorded, 1,5 months later. Although it showed a trend to increasing, the BDM presence had no significant effect, suggesting changes in microbial activity are not likely linked to the effects found on plants. Actually, Barragan et al. (2016) reported significant

changes in BDM increasing microbial activity compare to control soil after c.a. 2 months of BDM burial, not before. However, microorganisms may hydrolyse the polymer structure and release of BDM compounds at a low rate which could not been detectable in microbial activity assays. There is a need to develop methods to measure components released from BDM into soil complementary to the activity microbial activity measurements.

Considering all the findings on plants together, the *in vitro* test demonstrated roots were the most sensitive plant organ to compounds leached from BDM, their growth being inhibited by all plastic BDM leachates in both plant species. In the pot experiment, roots were fragmented when trying to separate them from the substrate and biomass was lost even when soaked, thus BDM effects on roots in this system was not accounted. Nevertheless, the effects of BDM fragments in this system were mostly consistent with the ones found in the *in vitro* test. The materials that caused the strongest root dry weight decrease *in vitro*, B-SP4, B-SP6, Biofilm and Mirel, significantly affected the growth and the chlorophyll content both *in vitro* and in the pot assay. The decrease in aerial biomass found in the pot assay may be explained by the continuous leaching of compounds with potential to inhibit root growth from BDM to the substrate, which may have lead to a decrease in nutrient uptake and eventually to decreasing aerial biomass. Overall, it is reasonable to assume that the *in vitro* culture assay is a more sensitive system to monitor the BDM phytotoxicity potential than pot assays. It is a powerful tool to be used for predicting effects happening in further plant development phases. However, pot assays are equally required; they are closer to the field cultivation system and thus they may likely be more reliable to evaluate if effects persist in further plant developmental stages.

Soil microorganism's ability to secrete enzymes that depolymerize plastic film structure makes them the main responsible of BDM biodegradation (Sander et al., 2019). Thus, BDM in soils may change their abundance and activity, with consequences on key agroecosystem functions, their interaction with plants (symbiotic, pathogenic, mutualistic, etc.) and the nutrient and energy fluxes regulation between them, eventually impacting plant and crop performance (Bandopadhyay et al., 2018). However, research on the potential changes driven by BDM on soil microbiome is limited yet. Field BDM incorporation into soil has shown not to modify nitrification or other nutrient cycling activities (Ardisson et al., 2014; Kapanen et al., 2008; Bandopadhyay et al., 2020a). However, BDM have been reported to increase microbial biomass (Li et al., 2014; Moreno and Moreno 2008), microbial hydrolytic activity (Barragán et al., 2016; Yamamoto-Tamura et al., 2015), alter community composition of bulk soil and

rhizospheric bacteria (Zhang et al., 2019; Qi et al., 2020) and enrich the soil in contact with the plastic surface in selected fungi and bacterial groups (Muroi et al., 2016; Zhang et al., 2019; Bandopadhyay et al., 2020b; Qi et al., 2020). With the aim of contributing to gaining a deeper understanding on how the soil bacterial and eukaryotic communities may be affected by biodegradable plastic accumulation into soil, a 3-month mesocosm assay buriying BDM fragments into agricultural soil at three different concentration levels was carried out (Chapter V) and the microbial community's diversity and structure and their degrading activities were characterized.

The mulch burial, even at the highest concentration of most of the mulches, caused low impact on bacteria and eukaryotic communities'. For both types of communities, most of the BDM and PE treatments did not significantly changed alpha diversity; only after the first month, 4% Mater-Bi decreased bacterial diversity and 4% paper after the third month. Other studies equally reported BDM burial produced minor or no changes in alpha diversity of bulk soil (Rüthi et al., 2021, Bandopadhyay et al., 2020ab; Moore-Kucera et al., 2014). Thus, BDM are likely accounted to be low-impact materials that do not compromise the diversity of the pool of agricultural soil microorganisms.

The beta-diversity analysis evidenced changes in the bacterial community structure dependent on mulch composition. On the one hand, BDM containing soils had distinct community structure than PE one, which was especially evidenced three months after the materials burial. On the other hand, among the BDM, the mulch containing starch, Mater-Bi, at 4% concentration produced the greater changes compared to the other BDM, likely associated with the presence of starch (deeper discussed in Chapter V). Mater-Bi fragments decreased the abundance of two oligotrophic bacteria, *Acidobacteria Gp6* and *Planctomycetes*, taxa from phyla found in the tomato rhizosphere of tomato, and which may have detrimental consequences on crop performance (Maul et al., 2014). Changes in the composition of the rhizospheric bacteria community by BDM blended with starch fragments buried for 4 months have been reported to be likely responsible for decreaseing wheat growth (Qi et al., 2020).

In eukaryotic communities, plastic mulches had low impact, and most of them did not change significantly the relative abundance of any taxa. Ecovio was the exception, increasing the abundance of protist *Tubulinea*. Due to their role in predation of bacteria, fungi and other microorganisms, changes in protist populations may affect key soil process, such as nutrient cycling (Oliverio et al., 2020). Concomitant to fungal enrichment due to PBSA incubation in soil, the protist *Achanthamoeba* has shown to

increase (Koitabashi et al., 2012). As for the paper mulch, not surprisingly, the soil with fragments with this readily degradable material was highly enriched in fungi, mainly of *Chaetomium* genus. To sum up, the impact of BDM fragments in the soil community structure was low, but some materials, such as Mater-Bi, paper and Ecovio, showed their capability to significantly impact bacteria, fungi and protists, respectively, suggesting the potential of the BDM composition to differentially affect specific microbial groups. All the three kingdoms interact together with cultivated plants, and may eventually affect crop performance; further research on how the ecological networks are impacted by BDM burial is needed.

In conclusion, in this Ph.D. thesis 8 BDM of diverse composition have been found to leache compounds from their first contact with water. The leached compounds have been chemically characterized, revealing not only to be monomers but also complex mixture of oligomers, short-chain molecules and additives. Some of the leachates containing these mixture of compounds have proved to substantially alter tomato and lettuce plant growth and development through an *in vitro* plant growth assay, while other BDM leachates had little or no effect on their development. Buried BDM fragments were also found to affect the growth of both plant species. The field weathered process BDM undergo, had significant effects on their later interaction with plants, causing more inhibitory effects on plant growth than new unused fragments. Finally, the accumulation of some of the BDM in the agricultural soil modified significantly the microbial community structure, diversity and the activity related to nitrogen cycle. Overall, the results provide novel insights into the BDM impact on lettuce and tomato plants, vegetable crops extensively cultivated with plastic mulches, and on the agricultural soil microbiome structure and functions. They render relevant knowledge that contributes to the identification and development of low-impact BDM to plants and to the environment.

Future research to be considered in this area:

Biodegradable mulches are promising materials for substituting polyethylene ones in order to alleviate plastic pollution in agroecosystems and waste generation. However, BDM were introduced commercially in the early 90's and their impact on agroecosystems and on the environment remains largely undisclosed yet, especially long-term impacts. Based on the research fromin this PhD thesis, suggested further studies are outlined:

1. Ecotoxicity evaluation of a wide range of mulch formulations, since the BDM composition showed to have a key role in their interaction with plants and with the soil microbiome.

2. Quantifying micro and nanoplastics released from the BDM during their (bio)degradation and evaluating their effects needs. This is to be included in the ecotoxicity assessesments.

3. TDevelopment of a methodology that allows for identification of specific components or mixtures of chemicals released from BDM to a complex environment like the agricultural soil. It would facilitate identifying the mulch components causing the ecotoxicity exerted by some of the tested BDM.

4. Ecotoxicity testing in different climatic/environmental conditions, with emphasis on different horticultural plant species and soil organisms and evaluating the potential BDM effects on ecological interactions.

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CONCLUSIONS

The main conclusions of this PhD thesis are:

Application of an *in vitro* plant ecotoxicity test to unused biodegradable mulches

1. Leachates from unused new BDM after their first contact with water have the potential to affect tomato and lettuce germination and plant development in an *in vitro* culture system.
2. Some BDM (Bioplast SP6, SP4 and Biofilm) strongly inhibit tomato and lettuce plant growth, while other BDM had minor or no effects. The mulch composition plays a key role in BDM potential ecotoxicity.
3. The plant root system is the most sensitive part to BDM leachates. Most BDM decrease *in vitro* root biomass; some (Biofilm and paper) also drastically alter the root morphology in both plant species.
4. Overall, *in vitro*, tomato is more sensitive to BDM than lettuce. All BDM reduce lettuce root biomass, but only Bioplast (SP4 and SP6) and Bioflex decrease the aerial part weight. However, all the BDM except Bioflex decrease whole tomato plant biomass.
5. The *in vitro* culture system is found to be a reliable tool to monitor effects of BDM compounds in early stages of plant development. It is especially valuable for evidencing changes in root development and morphology.

Compounds released from unused biodegradable mulch materials after contact with water

6. Release of specific BDM compounds after their first contact with water, before biodegradation starts, was acknowledged by gas chromatography (GC) and nuclear magnetic resonance spectroscopy (NMR).
7. BDM release a complex mixture of a diversity of compounds, while PE mulch only releases minor components, which were mulch additives.
8. Combination of GC and NMR techniques reveal BDM leachates contain a complex mixture of additives, monomers, short chain-oligomers and polymeric fragments.
9. The compounds released by most BDM samples include dicarboxilic acids, hydroxyacids, diols, triols, glycerol dimers and trimmers, mono and disaccharides and terephthalic acids. The prevalence of each one varies with the BDM composition.

10. Lactic acid, adipic acid and 1,4-butanediol, monomers in the leachates are in concentrations several orders of magnitude lower than the ones previously published to have effects on *in vitro* grown tomato and lettuce plants. Therefore, their presence in the leachates does not explain the effects observed in plants.

Ecotoxicity of buried agricultural biodegradable plastic mulches in two horticultural species, tomato and lettuce

11. The presence of buried BMD fragments in plant pots alters tomato and lettuce plant growth and physiology.
12. The specific formulation of each BDM material plays a relevant role in the interaction with plants.
13. PE mulch fragments do not alter tomato and lettuce plant growth. This suggests the effects from BDM are related to the chemicals they release rather than to the physical effect of the mulches' fragments.
14. Field weathered BDM have stronger detrimental effects than new unsed BDM, highlighting the need to take into account the past record of the materials in their interaction with cultivated plants.
15. The sensitivity to some of the BDM fragments is not alike for the two species tested, lettuce and tomato, stressing the need to assess the effects of these materials on a wide broad of cultivated plant species, especially the ones that are routinely mulched.

Effects of buried biodegradable plastic mulches on the agricultural soil bacteria and eukaryotic communities

16. The microbial richness is not affected by PE, neither by BDM burial.
17. Burying BDM, either for one or three months, modifies the soil microbial communities' structure; burying PE does not.
18. BDM based on natural occurring readily biodegradable polymers, starch (Mater-Bi) and cellulose (paper), extert greater effects on bacterial and eukaryotic communities, respectively, than the other mulches tested.
19. Soils containing PBAT-starch and PBAT-PLA mulches for one month have different structure of their bacterial communities.
20. In most of the cases, burying BDM causes greater changes when at 4% concentration than when at lower concentrations (0.5 and 1%). However, Ecovio

causes great effect but no dose-response dependent on protists.

21. Total microbial activity does not change after BDM burial, but both BDM and PE descreases the activity involved in chitin degradation (nitrogen compounds recycling).