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D2.1 Technologies and analytical tools for better understanding of factors influencing OMPs biotransformation.

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1 Background

Organic micropollutants (OMPs) comprise a wide variety of organic chemicals such as pharmaceuticals, synthetic hormones and personal care products (PCPs) which can pose an environmental and health risk despite their low concentrations in wastewater, typically within the range of µg L^{-1} to ng L^{-1} (Jiang et al, 2013). A major challenge in relation to OMPs is their sometimes inefficient removal by existing water treatment technologies and that most OMPs have a lack of regulated standard effluent criteria (Kanaujiya et al, 2019).

Biological treatment is conventionally used as secondary step in wastewater treatment plants (WWTPs). Advantages of this treatment technology are its low cost and the potential flexibility of the microbial degrader community for removing a large diversity of OMPs. In fact, certain OMPs are almost completely degraded by such a biological treatment step (Behera et al, 2011). Moreover, OMP biodegradation can involve the initial biotransformation of the parent compound to intermediate metabolites, but sometimes their complete mineralization to innocuous inorganic forms such as CO_2. This is not always the case with alternative water treatment technologies such as Advanced Oxidation (AO) and Reverse Osmosis(RO). AO can generate undefined breakdown products with unknown toxicities (Mcdowell et al, 2005) whereas RO removes untransformed OMPs and up concentrates them for their subsequent disposal.

A main challenge of biological wastewater treatment technologies remains their variable performance for different OMPs. Vuono et al. (2016) reported a removal efficiency lower than 1% for some OMPs. This occurs since biodegradation is dependent on a combination of the physico-chemical properties of the pollutants, external conditions and/or on microbial factors (Tran et al, 2013). Relevant factors include sorption to the sludge limiting availability to the microbial degraders, changes in the speciation of ionic OMPs linked to the pH of the wastewater which limits microbial uptake, OMP structures that are inherently recalcitrant to enzymatic breakdown or in specific cases toxicity to the microbial degrader community (e.g., when OMP mixtures or specific compounds with antimicrobial properties are present), (Rosal et al, 2010; Gómez et al, 2007). Microbial characteristics such as the composition of the sludge community, abundance of suitable degraders and the availability of essential nutrients can also influence OMP removal (Vuono et al, 2016). In some cases, inefficient biodegradation of OMPs can be improved by modifying the activated sludge process in WWTP, for instance by combining anoxic and oxic conditions (Behera et al, 2011) or by increasing sludge residence times (Vuono et al, 2016).

Whether an OMP can be efficiently taken up by the degrading microorganisms has a large impact on its removal efficiency. This comes under the term bioavailability and covers several processes. It is generally accepted that an OMP has to exist in the dissolved state for it to taken up by a degrading microorganism (Patel et al, 2019). This is because molecular diffusion of the dissolved OMP molecules along the concentration gradient existing from outside to inside the degrading cell is a major uptake pathway. When these external dissolved concentrations become depleted due to this biodegradation, then these can be replenished by the desorption of OMPs that are sorbed to the surrounding matrix (Reichenberg and Mayer, 2006). This is particularly relevant for those OMPs with high sorption affinities such as the hydrophobic OMPs, and here the kinetics of desorption play a key role. A compound that is rapidly desorbed can therefore maintain a higher bioavailability and faster biodegradation, whereas a compound that is only slowly desorbed can lead to a reduced bioavailability and thus inefficient microbial removal.

Therefore, understanding this external bioavailability to the degrading microorganisms requires analytical tools that specifically control or measure the dissolved OMP concentrations (Cui et al, 2013). In this regard, one open issue is whether there are thresholds in these external dissolved and bioavailable concentrations, below which no biodegradation occurs. This might explain the common

observation that OMP biodegradation often slows and stops at very low concentrations. Reasons for this could be that:

- a minimum concentration is needed to activate the required catabolic machinery
- too low external dissolved concentrations result in limited diffusive uptake and biodegradation (i.e., a kinetic limitation that is explained by whole cell Michaelis-Menten kinetics)
- low OMP substrate concentrations unfavourably shift the substrate versus product equilibrium reaction in the direction of the former (i.e., a thermodynamic explanation)

Hence, a better understanding of the impact of OMP bioavailability will help to improve existing biological processes in WWTPs by identifying those factors that either limit or promote the microbial uptake and degradation of the pollutants. Additionally, information on bioavailability is relevant to understand the fate and ecotoxicity of OMPs, which could then be used within risk assessment frameworks for the better regulation of organic chemicals (Ortega-Calvo et al, 2015).

2 Objectives

This deliverable aims to explain the working principle of passive dossing and passive sampling as technologies to obtain a better understanding of the key factors determining the bioavailability of OMPs during biological wastewater treatment and how this impacts their biotransformation and/or mineralization. Additionally, it is described how analytical tools complement the use of passive sampling and dosing to monitor OMPs degradation. Finally, a general overview of ESR2 project is presented for a possible application of the tools and analytical methods.

3 Technologies to control and measure OMP bioavailability

The bioavailability of OMPs can be controlled or measured using the related techniques of passive dosing and passive sampling, respectively. Other techniques such as filtration or centrifugation are also commonly applied for measuring dissolved OMP concentrations via separation of the matrix-sorbed and dissolved phases. However, particularly for hydrophobic OMPs these can be accompanied by methodological artefacts such as sorption to the filters or to dissolved sorbing matrices (e.g., dissolved organic carbon) that are not fully separated from the liquid phase using these techniques.

Passive sampling allows determination of the dissolved concentrations in complex matrices such bioreactor media via measurement of the OMP concentrations accumulated in an inert polymer reference phase. This can be done either during the uptake phase (kinetic passive sampling) or when the passive sampling polymer reaches an equilibrium with the dissolved concentration in the medium (equilibrium passive sampling). Passive sampling specifically targets the bioavailable dissolved concentrations, since both uptake and equilibrium are directly related to the chemical activity or chemical potential of the compound in the medium. These in turn can be related to the OMP dissolved levels (Mayer et al, 2003). The up-concentration of the OMP in the sampling polymer also allows for accurate detection of even low dissolved concentrations, which are environmentally more relevant.

Passive dosing can be regarded as a type of “inverted” passive sampling. Here, a polymer phase is pre-loaded with the OMP and maintains a constant dissolved concentration of the compound in the medium via partitioning. By pre-loading the polymer with decreasing OMP concentrations, corresponding decreasing dissolved concentrations can be produced in the aqueous phase according to partitioning considerations. Passive dosing avoids spiking co-solvents and facilitates the introduction of even low concentrations of hydrophobic compounds into aqueous environments. Furthermore, the large OMP reservoir in the dosing polymer can compensate for depletion due to the biodegradation process. This allows for a high compound turnover even though the dissolved concentrations are kept

low and constant, which facilitates the measurement of relevant biodegradation endpoints (e.g., metabolite production for biotransformation or CO_2 production for mineralization) (Smith et al, 2012). In addition, other depletion processes like sorption to the test vessel or volatilization are compensated by this further desorption from the dosing polymer (see Figure 1). Therefore, with passive dosing the controlled and constant supply of low dissolved OMP levels in the medium facilitates study of the impacts of OMP bioavailability on biodegradation and the reasons behind these.

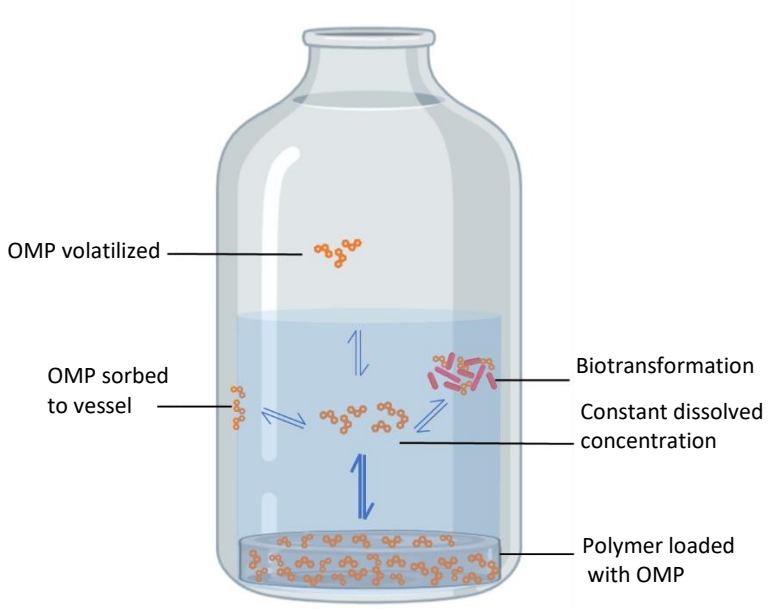

Figure 1. Principles of passive dosing. The polymer works as an OMP reservoir that keeps a constant dissolved concentration despite possible lossess through volatilization, OMP turnover and sorption to biomass, medium or the vessel.

Passive dosing and passive sampling are therefore both suitable techniques to investigate, for example, whether thresholds in OMP bioavailability exist since they both specifically target the dissolved OMP levels. As mentioned above, in terms of biodegradation these dissolved concentrations are important given that diffusive mass transfer of dissolved molecules across the cell membrane is the first step in the microbial degradation process.

4 Analytical tools for measuring OMPs and their biodegradation

Biological degradation of OMPs normally occurs via co-metabolism, meaning that the microorganisms metabolize the OMPs in the presence of other energy and nutrient sources (Alvarino et al, 2018). Here, OMP removal varies under different co-substrate conditions. OMP biodegradation can also occur via metabolism, where the microorganisms use the compound as the main source of energy and carbon.

During metabolism or co-metabolism, OMPs are typically broken down in steps following one or more specified metabolic pathway and lead to the production of different metabolites. This is defined here as biotransformation. In some cases, this involves the complete breakdown of the OMP leading to a release of inorganic CO_2, and here is termed mineralization.

Analytical techniques such Gas Chromatography (GC) or High Performance Liquid Chromatography (HPLC) coupled to Mass Spectrometry (MS) are suitable methods to quantify the concentrations of the OMPs and their metabolites. These can be complemented by using ^{14}C radiolabeled OMPs, which is particularly useful for measuring the production of $^{14}CO_2$ via mineralization. For this, the radioactivity collected in CO_2 traps is measured with a Liquid Scintillation Counter (LSC). In addition, transformation

products can be more easily identified and quantified when using radiolabeled compounds via radio HPLC or Thin Layer Chromatography (TLC). Targeting the ^{14}C activity in the resulting chromatograms allows the link between parent and metabolite to be unambiguously determined. Metabolites quantified using these different approaches can then be compared to those described for literature metabolic pathways of each OMP for the corresponding bacterial degrader community. All these analytical tools are particularly suited for analyzing OMP degradation given their high specificity and sensitivity, meaning that low concentration ranges that can be investigated that are environmentally more relevant.

5 ESR2 project as an example

Passive dosing and the described analytical tools will be applied in the project of ESR2 to obtain a better understanding of the bioavailability, biotransformation, and mineralization of OMPs. The obtained results aim to clarify the impact of bioavailability in OMPs persistence during wastewater treatment. In this context, the following hypothesis will be researched:

> For an OMP to be degraded by a pure bacterial culture a series of threshold bioavailable concentrations exist which need to be exceeded to trigger (i) the onset of responses at the cellular level (e.g., increases in the abundance and activity of relevant enzymes), (ii) their biotransformation to metabolites and(iii) their mineralization to CO2.

To investigate the hypothesis, the specific objectives are:

1.Develop a passive dosing method for the precise experimental control of the dissolved concentrations of polar and non-polar OMPs to investigate whether there are thresholds in bioavailability that are required for the onset for microbial degradation.

2. Apply the passive dosing method to investigate whether there are bioavailability threshold concentrations of different model OMPs for biotransformation, mineralization and cellular responses (e.g. induction of relevant enzymes).

5.1 Methodology

5.1.1 Selection of the model OMPs

The initial choice of the OMPs to be studied in the project is important to maximize the information gained from the various experiments but also to allow extrapolations to be made as to the fate of other OMPs with similar properties. The selection of the OMPs to be studied in the project therefore takes into account several criteria:

- known biodegradability
- known metabolic pathways and bacterial degraders
- availability in ^{14}C radiolabelled form
- a high specific radioactivity for more sensitive analytics
- a range of relevant physico-chemical properties (particularly with regards to sorption, but also solubility, polarity and no dissociation)

Choosing a compound that is known to be biodegraded is necessary to be able to detect the influence of those chemical and microbial factors that determine the efficiency of the biological treatment step. These may not become evident when investigating toxic or recalcitrant compounds, since either of these characteristics may be the underlying cause for an observed lack of biodegradation. A complementary search in the scientific literature covering the biodegradation of the identified OMPs

was also performed to identify bacterial strains that can grow on them as the sole source of carbon and energy.

Labelling of the OMP with the ^{14}C isotope is useful for monitoring the biodegradation process and to identify and quantify the various metabolites including any $^{14}CO_2$ produced via mineralization. Additionally, having a high specific activity simplifies the analysis by increasing the analytical sensitivity (more ^{14}C labelled carbons in the molecule ring allow the radioanalytical methods to detect and quantify lower concentrations).

With respect to the physico-chemical properties, there is no simple relationship between polarity, bioavailability and biodegradation. Therefore, the choice of a polar and nonpolar compound allows different scenarios to be studied. For example, hydrophobic compounds may have a limited bioavailability due to sorption to the matrix, whereas polar compounds may be less sorbed and thus have higher dissolved concentrations, but be poorly transported across the lipid microbial membrane.

5.1.2 Passive dosing method for measuring threshold concentrations in pure cultures

The aim of the first set of experiments is to investigate whether there are thresholds in the dissolved concentrations of OMPs which need to be exceeded before these can be either biotransformed or mineralized. As explained above, these external dissolved concentrations serve as a surrogate for OMP bioavailability in the set-up.

As model OMPs, a polar compound and a non-polar compound have been selected to represent OMPs with different fate behaviors. These experiments will be conducted under non-growth conditions using pure bacterial cultures that are capable of metabolizing the respective OMPs as the sole source of carbon and energy. These will therefore not consider co-metabolism.

OMP concentrations in the degradation set-ups will be kept constant at a series of decreasing dissolved concentrations by using passive dosing, and the degradation activity of the pure cultures determined using three different endpoints. A range in endpoints is important since this will further explain the cause of any limited OMP biodegradation. For example, identifying an increase in the expression of relevant enzymes may point to a potential for OMP biodegradation but that the cell numbers remain too low or insufficiently active for significant biodegradation. The general approach of these experiments is shown in Figure 2.

Therefore, for these initial experiments two phases are planned as follows:

a) Characterization of the abiotic kinetic and partitioning characteristics of passive dosing set-ups developed for the polar and non-polar model OMPs.
b) Applying these to investigate thresholds for mineralization, biotransformation and sub-cellular responses during the degradation of the model OMPs.

The polymer used for passive dosing has to have a sufficiently high affinity for the model OMP such that this can sufficiently buffer any decreases in C_{free} in the aqueous medium due to the degrading bacterial cells. The choice of dosing polymer therefore depends on the properties of OMP that will be dosed into the aqueous phase. In general, a polymer to water partition coefficient ($K_{Polymer/water}$) for the OMP that is higher than around 3 allows for a good control of the dissolved concentration at constant levels via passive dosing.

In the case of hydrophobic compounds, polydimethylsiloxane silicone (PDMS) has been successfully applied for passive dosing in various contexts including biodegradation studies (Smith et al., 2012). PDMS is well suited as a dosing polymer since it is well-established, biocompatible, can be cast in various forms and has a high internal mass transfer so that internal concentration gradients do not

develop. For the hydrophilic OMP, PDMS does not have a high enough affinity for successful passive dosing. The affinity is higher to various types of solid phase extraction (SPE) polymers. However, these SPE polymers are in the form of small beads, and for practical reasons require immobilizing in some sort of a solid support before they can be used for passive dosing in biodegradation studies. One potential choice could be the mixed polymer (MP) described in Jeong et al (2018). This consists of OASIS HLB polymer beads as used for SPE embedded in PDMS silicone. This combines the advantages of PDMS listed above, but this mixed polymer has a much higher affinity for polar compounds compared to PDMS silicone alone. A possible alternative to using PDMS to immobilize the SPE polymer beads could be a hydrogel (e.g., polyacrilamyde). This format is used in organic Diffusive Gradient Thin film (DGT) passive samplers, and would simplify characterization of the mixed polymer properties since the hydrogel can to all intents and purposes be treated as an immobilized water phase (rather than as an additional polymer as is the case the when PDMS is used).

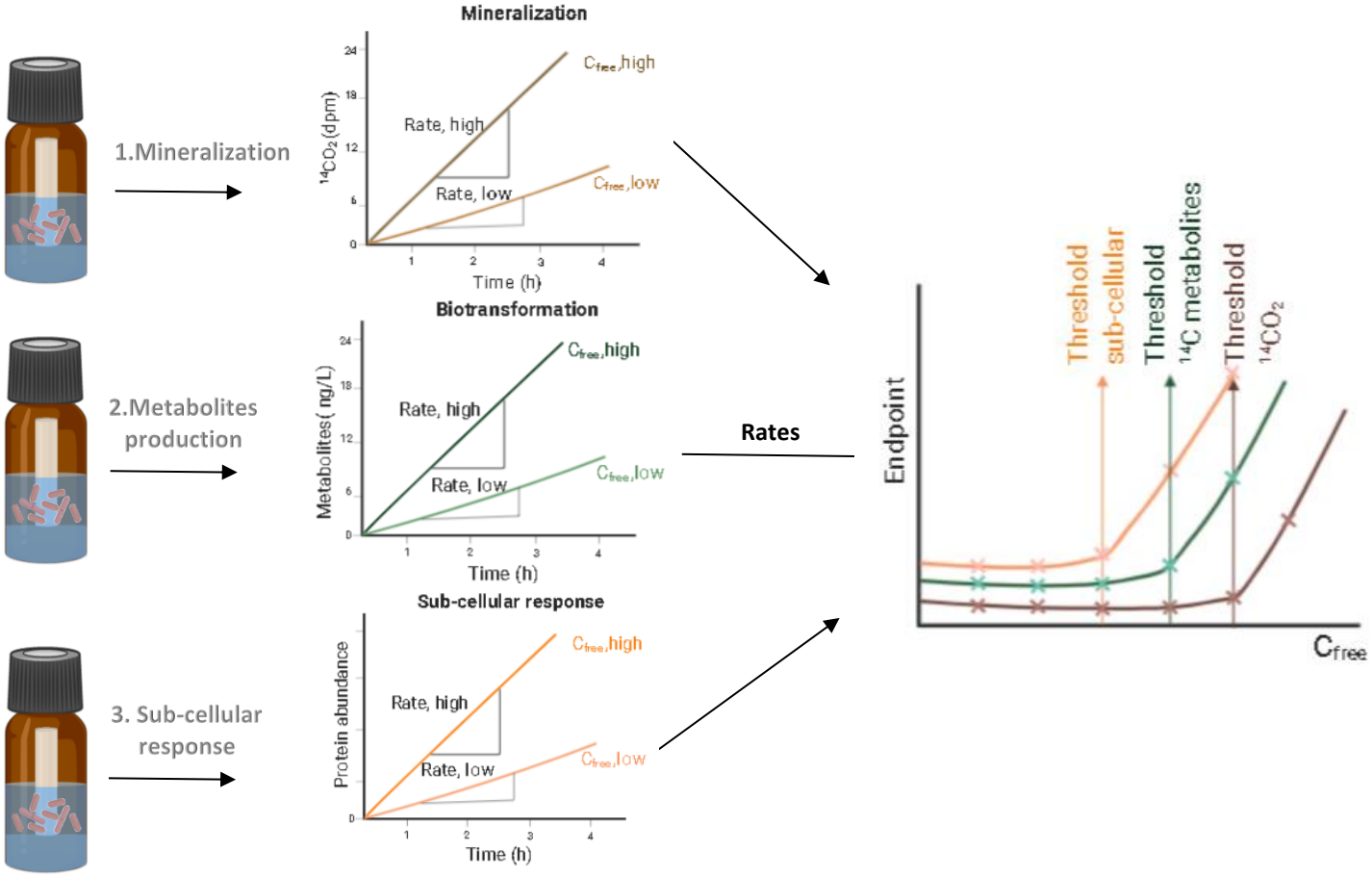

Figure 2. Bacterial degradation activity over time will be measured at different OMP dissolved concentrations (C_{free}) by considering the production of $^{14}CO_2$, metabolites, as well as changes in cellular response (e.g. enzyme induction) as endpoints. The measured rates for each endpoint will then be plotted against C_{free} to investigate if there is a threshold above which there is a change in the rates. An example of output would be increasing thresholds in OMP C_{free} starting from a cellular response towards mineralisation.

Similar to Smith et al (2012), the passive dosing set-up will consist of small 4 mL amber glass vials with the appropriate dosing polymer cast in the base (see Figure 3). Such a miniaturized set-up has advantages in that it reduces the amount of expensive radiolabeled OMP that is needed and also allows for more treatments and replicates per tested endpoint.

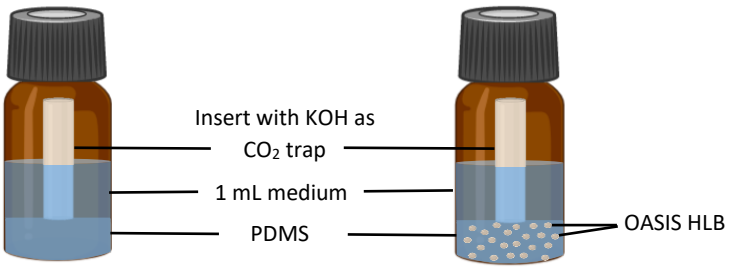

Figure 3. Representation of the two different set ups to be used in the experiments based on passive dosing. The vial on the left contains PDMS cast at the bottom, whereas the vial on the right contains a MP (OASIS HLB embeded in PDMS). A glass insert containing 200 uL KOH is placed in each set up to serve as a CO_2 trap in the mineralization experiments with radiolabelled compound. 1 mL of aqueous medium will be added at the beginning of the experiments.

Further details of the experiments are described below:

Abiotic characterization of polar and non-polar OMPs by passive dosing. Initial characterization of the passive dosing vials is necessary to interpret the results of the degradation experiments. This requires determination of the OMP release rate ($k_{release}$) from the polymer to the aqueous media and the equilibrium partition coefficient ($K_{polymer/water}$). Both parameters contribute to keeping a certain stable dissolved concentration of OMP (C_{free}) in the case of losses due to degradation (see figure 4).

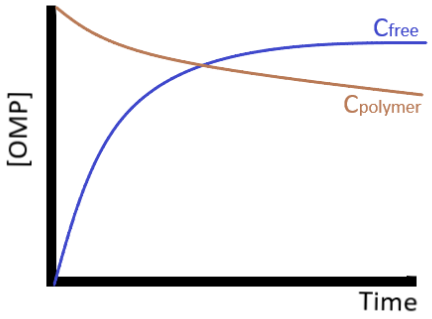

Figure 4. Upon contact with the aqueous phase, the OMP in the dosing polymer ($C_{polymer}$) is released into the aqueous media at a specific rate ($K_{release}$). The dissolved concentration (C_{free}) of OMP increases until the equilibrium partitioning concentrations are reached. These can be buffered for a period of time that is determined by the mass of dosing polymer as well as partitioning affinity of the polymer for the OMP.

The OMP concentration in the passive dosing polymer is directly reflected in the bioavailable dissolved concentrations in the medium according to partitioning considerations. Hence, it is important to dimension the dosing polymer so that any depletion due to biodegradation is sufficiently buffered by the OMP reservoir in the polymer. In this way, the dissolved concentrations remain approximately constant. In general, a higher $K_{polymer/water}$ implies a more efficient buffering against losses since the OMP reservoir in the polymer is larger. In parallel, the buffering capacity of the dosing polymer can be increased by including a larger mass of polymer in the set-up. The mass of OMP required for loading the polymer in order to achieve the required dissolved concentration therefore depends on (i) $K_{polymer/water}$ and (ii) the mass of polymer. Therefore, dimensioning of the passive dosing set-up requires consideration of two contrasting aspects: ensuring that there is sufficient buffering whilst minimizing the amount of OMP that is required for loading to the required levels. The latter is particularly relevant for radiolabeled compounds which are costly. As shown in the figure 5, for the

selected set-up with 1 mL of medium, 100mg polymer is sufficient to buffer the dissolved concentration of all OMP with a log $K_{polymer/water}$ of 3 or higher.

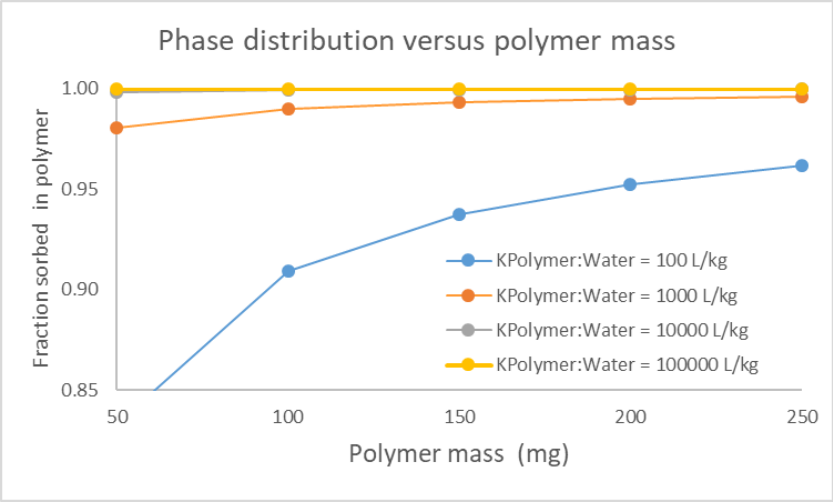

Figure 5. In a passive dosing set-up containing a medium volume of 1mL, all compounds with a polymer:water partition coefficient greater than 1000 L/kg will be retained to more than 99% in the polymer provided its mass is at least 100 mg. Such as set-up can thus maintain a controlled and constant dissolved OMP concentration in the medium. If a lower mass of polymer or a polymer with a lower $K_{polymer/water}$ are used, depletion starts to become significant and control over the dissolved concentrations is lost.

Therefore, a fixed amount of 100 mg of PDMS and 100 mg of the MP containing OASIS HLB will be cast into the base of 4 mL brown glass vials. The procedure for casting PDMS will be done according to Reichenberg et al (2008), with the exception that the PDMS solution will be pipetted into the base of the vial and left to cure. For the MP composed of OASIS HLB embedded in PDMS, the procedure of Jeong et al (2018) will be adapted. All polymers are left to cure for at least 72 hrs, washed twice overnight with methanol, rinsed twice overnight with MilliQ water to remove residual methanol and finally left to dry. Loading of the polymer with the OMP will follow the method of Birch et al, 2010. In for additionally vials, the amount of OMP loaded into the polymer will be immediately extracted with methanol and analyzed to determine the initial loaded concentration for comparison to the nominal concentration added in the loading solution.

The experiments will be done in triplicate per sampling time and under aerobic conditions at room temperature (ca.20°C). The vials will be placed on a shaker set at 150 rpm. Vials will be sacrificially sampled at 8 time points and the medium analysed to follow the increase in dissolved concentrations. After sampling, any remaining medium will be poured off and the concentration of OMP in the polymer determined after solvent extraction and analysis by HPLC-MS. Hence, the initial loading concentration can be verified using a mass balance approach by considering the OMP contained in the extract from the polymer, and adding this to the final medium concentrations.

The OMP release rate ($k_{release}$, h^{-1}) from the dosing polymer can be determined by fitting a one compartment with first order kinetics to the increase in medium concentrations (Equation 1). The equilibrium partition coefficient ($K_{polymer/water}$, L kg^{-1}) can be determined from the final measured medium and polymer concentrations (Equation 2)

$$k_{release} = \frac{A}{V} v \quad \text{(Eq 1)} \qquad K_{polymer/water} = \frac{C_{polymer}}{C_{free}} \quad \text{(Eq2)}$$

where in Eq 1 A stands for the surface area of the polymer (ca. 1.70×10^{-4} m^2), V is the aqueous volume (1×10^{-6} m^3) and *v* is the polymer to water mass transfer velocity (m h^{-1}). In Eq 2, $C_{polymer}$ corresponds to the OMP concentration in the polymer (µg kg^{-1}) and C_{free} (µg L^{-1}) to the OMP dissolved concentration after equilibrium has been reached.

Abiotic loss controls will be performed in parallel. Here, the set up will be prepared as above but without any polymer. As the release rate of an OMP from the polymer under abiotic conditions can differ when including bacteria in the test, a second abiotic control will include inactive bacteria degraders in the MSM with a similar optical density (OD_{600}) at 600 nm than the one in the biotic experiments.

Investigation of thresholds for mineralization and biotransformation. An initial set of experiments has been planned to test the passive dosing set-up and to obtain an initial insight into the kinetics of the OMP mineralization. This information will then be used to optimize the sampling schedule for the main experiments investigating mineralization and biotransformation endpoints. Mineralization will be tracked by means of $^{14}CO_2$ produced from the complete conversion of the ^{14}C radiolabeled forms of the two model OMP. Biotransformation products will be tracked with radioanalytic chromatography and identified with reported metabolites.

Prior to starting the mineralization experiments, the selected pure cultures will be subjected to an enrichment and acclimatization phase to produce enough biomass as well as to avoid OMP toxicity and bacterial inactivity during the test. For use in the mineralization experiments, the bacteria will be washed with MSM to avoid any carryover of OMPs as well as the broth nutrients into the passive dosing set up. The bacterial suspension will be diluted in MSM to reach the optimal OD_{600} prior to inoculating the mineralization test. A portion of this suspension will also be analyzed by HPLC-MS to determine any carryover of the OMP.

The passive dosing vials will be sterilized by autoclaving before use. Loading of the passive dosing polymers will be done with a mixture of unlabeled OMP plus sufficient ^{14}C radiolabeled to act as a tracer. The loading will be done following the approach described for the abiotic experiments above. A $^{14}CO_2$ liquid trap (i.e., 200µL of 1M KOH) inside a glass insert will be added to each passive dosing vial (see Figure 6).

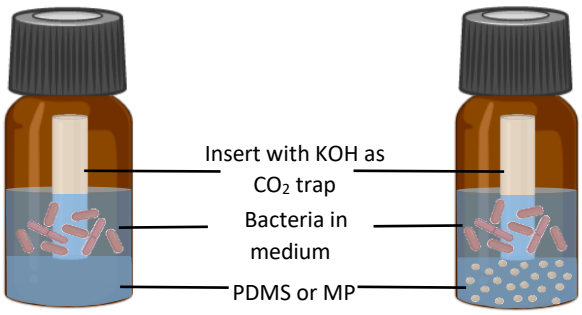

Figure 6. Representation of passive dosing set ups for biotic tests. The vial on the left contains PDMS as reservoir for the non-polar OMP, while the vial on the right includes the MP loaded with the polar OMP. The glass insert and the corresponding aqueous medium containing the bacteria will be added at the start of the experiment.

The dosing polymers will be loaded to the appropriate levels to produce specified equilibrium partitioning dissolved concentrations in the medium. The criteria for selecting the dissolved

concentrations will consider the aqueous solubility of OMPs as the highest concentration, the reported level for degradation tests with the bacteria, and the detection limit of the analytical equipment.

The biodegradation experiments with passive dosing will be performed under identical conditions to the abiotic experiment: aerobic conditions, steady agitation at 150 rpm and room temperature (ca. 20°C). The aerobic conditions of the system are expected to be similar to the experiments of Smith et al (2012), as the setup has the same volume of aqueous medium and headspace. Opening and other handling of the vials will be done under sterile conditions to avoid contamination of the pure cultures.

After loading of the polymers and adding the glass insert containing the KOH as a $^{14}CO_2$ trap, the experiment will be started by adding 1mL of MSM containing the bacteria at the recommended OD_{600}. The KOH in the $^{14}CO_2$ traps will be replaced with fresh KOH at specific time intervals. The sampled KOH will be added to Ready Gel for analysis with LSC. At the end of the experiment, both the medium and the polymer (after solvent extraction) will be analyzed for ^{14}C activity to close the mass balance. In addition, for the highest loading level, the extracts will be screened by radio-HPLC and/or TLC to investigate whether metabolites have been produced. If necessary, these samples can be concentrated and/or combined to increase analytical detection. The metabolites found here will be compared to those identified in the literature collected when searching for the degrader strains applied in these experiments.

Controls for these experiments will include loaded passive dosing vials with mineral salts medium but without bacteria to discount for abiotic losses, plus unloaded vials with mineral salts medium plus bacteria to discount chemical contamination.

Based on the first results investigating mineralization, a full experiment will be performed with focus on a narrower range in C_{free} covering a low concentration level (e.g. ng. L-1). In addition to mineralization, this second set of experiments will also focus on biotransformation by measuring the production of intermediate metabolites found on the first round of mineralization tests. Additionally, controls for the numbers, distribution and activity of the bacteria will be included.

Investigations for thresholds for cellular responses. The aim of this experiment is to verify if there is a cellular response at a similar or lower bioavailable concentration threshold than determined for mineralization or biotransformation above. This experiment will only be performed for one of the OMP. The OMP that shows the clearest metabolite and CO_2 production in the previous experiments will be selected for this.

As biotransformation relies on the enzymatic activity, one case would be to determine whether the enzymes responsible for metabolic pathway are active or increase in abundance. Thus, the possibility to assess enzyme abundance (i.e. protein induction) or enzymatic assays will be further investigated for including in this experiment. This would require either protein extraction from the bacteria at the end of the test followed by peptide identification using analysis in combination with bioinformatics tools, or enzymatic assays with bacteria lysates.

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