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Evaluation of the degradation capacity of WWTP sludge enrichment cultures
towards several organic UV filters and the isolation of octocrylene-degrading
microorganisms

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Abstract

Organic UV filters are present in wastewater treatment plants (WWTPs) due to the
use of these compounds in many personal care products (PCPs) and their
subsequent release into the wastewater system from showering/bathing. Once in
the wastewater system, organic UV filters generally partition into the solid phase
but might also undergo other processes, such as degradation by microorganisms. To
further understand the fate of organic UV filters in WWTPs, the degradation of 7 UV
filters by WWTP sludge was investigated The UV filters 2-ethylhexyl salicylate (ES),
homosalate (HS), butyl methoxydibenzoylmethane (BM) and octocrylene (OC) were

degraded after 20-60 days. The rest of the filters tested, namely, bis-ethylhexyloxyphenol methoxyphenyl triazine (BEMT), methylene bis-benzotriazolyl tetramethylbutylphenol (MBBT) and diethylhexyl butamido triazone (DBT), did not degrade even after 120 days of incubation. The microbial community from the microcosms degrading ES, HS, OC and BM was transferred every 30 days into new microcosms to enrich for microorganisms capable of utilizing the individual UV filters for growth. The enrichment cultures continued to degrade throughout 20 transfers. The microbial community was clearly different between the enrichments degrading ES, HS, OC and BM, meaning that the microbial community was strongly influenced by the UV filter present. Furthermore, several strains were isolated from OC-degrading cultures and two of these strains, *Gordonia sp.* strain OC_S5 and *Sphingopyxis sp.* strain OC_4D, degraded OC with and without other carbon sources present. These experiments show that several organic UV filters can be degraded by a specific set of microorganisms. The lack of degradation observed for BEMT, MBBT and DBT is probably due to limited bioavailability. Indeed, this is the first biodegradation study of these filters, in addition to being the first description of ES and HS degradation in microcosms experiments.

Keywords: UV filters, Degradation, Wastewater sludge, Pure bacterial strains

1. Introduction

UV filters used in personal care products include organic compounds of varying size and characteristics, as well as mineral UV filters, like titanium dioxide (TiO₂) and zinc oxide (ZnO). UV filters are important ingredients in sun protection products (e.g., sunscreens), personal care products, plastics, paints and textiles (Ramos et al., 2015). Due to their increasing use, environmental contamination by UV filters has become an increasing concern since UV filters are now widely distributed in the environment (Sánchez-Quiles and Tovar-Sánchez, 2015; Huang et al., 2021). The main sources of UV filters in the environment can be direct via recreational activities such as bathing but also indirect via wash-off, showering and laundry that end up in wastewater treatment plants (WWTPs). As a consequence, influents from WWTPs contain varying concentrations of UV filters (Ramos et al., 2016). For example, BP3, a widely studied organic UV filter, has been found in concentrations over 1,000 ng/L in influents (Kasprzyk-Hordern et al., 2009; Liu et al., 2012a; Biel-Maeso et al., 2019) with a maximum measured concentration of 7,800 ng/L (Balmer et al., 2005). To understand the fate of UV filters and their ultimate effect on the environment, it is important to understand the processes UV filters are subjected to in WWTPs. Mineral and organic UV filter undergo completely different processes and while it is important to understand the behavior of both types of UV filters, the focus of this study is on organic UV filters. Generally, WWTPs are not equipped to remove all organic pollutants from the wastewater stream. Thus, incomplete removal of organic UV filters in WWTPs represents a major route of input of organic UV filters into the environment

The present study focused on a group of organic UV filters with varying molecular weights and solubility, including 2-ethylhexyl salicylate (ES), homosalate (HS), butyl methoxydibenzoylmethane (BM), octocrylene (OC) bis-ethylhexyloxyphenol methoxyphenyl triazine (BEMT), methylene bis-benzotriazolyl tetramethylbutylphenol (MBBT) and diethylhexyl butamido triazone (DBT) (see Table 1). From this list, OC is the UV filter that has been detected at the highest frequency in WWTPs and the highest influent and effluent concentrations (Table 1). HS concentrations have often been below the detection or quantification limit in WWTP influents and/or effluents, but some concentrations from approximately 600 ng/L to over 1,600 ng/L have been reported in influents (Biel-Maeso et al., 2019; Tsui et al., 2014) (Table 1). ES has been measured at concentrations of over 1,100 ng/L in influents but is generally present at lower concentrations in effluents. BM has only been detected in a few studies, with influent concentrations up to 2,935 ng/L, effluent concentrations up to 1,018 ng/L (Rodil et al., 2009a; Tsui et al., 2014; Cunha et al., 2015) and sludge concentrations up to 510 ng/g (dw) (Rodil et al., 2009b). DBT has only been measured and detected in sludge from Belgium at concentrations up to 136 ng/g (dw) (Rodil et al., 2009b), while BEMT and MBBT have never been measured in WWTPs to our knowledge.

The fact that UV filters are found at lower concentrations in effluents than in influents (Ramos et al., 2016) (Table 1) does not mean that UV filters are degraded. Indeed, once in the WWTP system, the UV filters can either i) stay sorbed to the sludge and thus be removed with the solid phase, ii) stay solubilized and be present

in the effluents or iii) be degraded by microorganisms in the WWTP (or the anaerobic sludge digester). Most organic UV filters are hydrophobic substances with log K_{ow} above 4, and thus, their elimination is strongly influenced by sorption processes. Indeed, sorption into solids is often the main factor for the elimination of organic UV filters (Kupper et al., 2006;Liu et al., 2012a). For the complete understanding of the removal processes of organic UV filters in WWTPs, it is necessary to perform mass balance calculations, which can be difficult to achieve. However, this approach has been successful in indicating the degradation of several organic UV filters in WWTPs (Biel-Maeso et al., 2019;Kupper et al., 2006;Liu et al., 2012a).

An alternative way one can prove that the organic UV filters are indeed degraded is to perform *ex situ* experiments. The addition of the fungus *Trametes versicolor* to sludge or slurry has been shown to be beneficial for the degradation of several UV filters (Badia-Fabregat et al., 2012;Rodríguez-Rodríguez et al., 2012;Gago-Ferrero et al., 2012). Similar results were found with the addition of a yeast species to sludge (Fujii and Kikuchi, 2005). Furthermore, BP3 has been shown to degrade in microcosms of WWTP sludge under different conditions (Liu et al., 2012b). Another important step forward to the understanding of the removal processes is to characterize and identify UV filter-degrading microorganisms. To this end, a *Methylophilus* sp. strain FP-6, capable of BP3 degradation, has been identified from WWTP enrichment cultures (Jin et al., 2019). Furthermore, a *Mycobacterium agri* strain capable of OC degradation has been identified through “reverse discovery”

(Suleiman et al., 2019). However, other carbon sources were available, and thus, the degradation is most likely cometabolic. Finally, our group recently identified a BP3-degrading microorganism in WWTP sludge, *Sphingomonas wittichii* strain BP14P, that degraded BP3 in a growth-dependent manner. The identification and isolation of this strain was made possible by the addition of relatively high concentrations of BP3 to WWTP sludge microcosms followed by multiple serial transfers (Fagervold et al., 2021). These factors put pressure on the microbial community to enrich for microorganisms capable of BP3 degradation.

The goal of this study was to investigate i) whether several UV filters with different structures and characteristics could be degraded by WWTP sludge microorganisms, ii) to investigate the microbial community in degrading enrichment cultures, and iii) to isolate degrading microorganisms from at least one of the cultures. To this end, the same process that successfully resulted in the identification and isolation a BP3 degrading microorganisms (Fagervold et al., 2021) was applied to an expanded range of UV filters.

2. Materials and methods

2.1. Chemicals

Several of the UV filters used for biodegradation studies, namely, ES, HS, BM, OC, BEMT and DBT, were provided by Pierre Fabre Pharmaceuticals, while MBBT was purchased from Sigma–Aldrich (Saint-Quentin-Fallavier, France). Analytical

standards for the UV filters mentioned above were purchased from Sigma–Aldrich. Analytical-grade dichloromethane (DCM), 1-methyl-2-pyrrolidinone (NMP), methanol, formic acid (98%) and molecular-grade formaldehyde were acquired from Sigma–Aldrich. Pure water was obtained from an Elga Purelab Flex System (Veolia LabWater STI, Antony, France). Glassware was cleaned with DCM and calcinated at 450 °C for 2 hours to remove all traces of organic matter.

2.2. Sampling of sludge and initial culture setup

Sludge sampling and setup of the initial enrichment cultures were performed as described previously (Fagervold et al., 2021) with no modifications except that different UV filters were utilized. Briefly, sludge from Argeles-sur-mer (France) WWTP was collected and used for initial degradation experiments. Sludge (100 mL) was mixed with freshwater mineral media (500 mL) in 1 L glass flasks. UV filters dissolved in methanol were added to the sludge slurries to obtain a final concentration of 10 µg/mL. These live cultures were incubated at 25 °C in the dark in parallel with sterile controls (SC); see (Fagervold et al., 2021) for more details.

2.3. Enrichment cultures and biodegradation assays

Enrichment cultures were set up with inoculum from the flasks that showed degradation of different UV filters, namely, OC, ES, HS and BM, as described previously (Fagervold et al., 2021). The theoretical UV filter concentrations were 100 µg/mL from the second transfer (Enr 2), and the cultures were incubated in 100 mL Erlenmeyer flasks containing 2 g of inert sand and minimal freshwater media

with no other carbon source added except the individual UV filter. The cultures were transferred each month (1% vol/vol).

Biodegradation assays were performed as described previously (Fagervold et al., 2021). Briefly, the assays were performed in 15 mL glass tubes with Teflon lined caps (Pyrex, Analytic lab, France) and contained 0.2 g of inert sand, 3 mL of minimal freshwater media and the target UV filter at a concentration of approximately 100 µg/mL. Inoculate for the biodegradation assays were either 150 µL enrichment culture or 150 µL washed cell suspension for the isolates. The tubes were incubated at 25 °C in the dark on a rotary shaker at 100 rpm, and triplicate tubes were sacrificed for extractions at each time point.

For the degradation assay with single strains, the cell numbers were determined by flow cytometry as described previously (Fagervold et al., 2021).

2.4. UV filter extractions and HPLC analysis

Two different procedures were used for UV filter extractions, one for the initial biodegradation experiments and another for the biodegradation assays for enrichment cultures and isolated strains, while the HPLC analysis remained the same. These procedures were described previously in detail (Fagervold et al., 2021).

2.4.1. Extraction procedure for the initial degradation experiment and biodegradation assays

To determine the degradation potential in initial WWTP sludge microcosms, the UV filter concentration was determined in a live culture and compared with a sterile control (SC) over time. Briefly, duplicate 5 ml samples were taken from each flask at each time point. The slurry was centrifuged (800 g for 20 min), and the aqueous and solid phases were extracted separately. The extracts were dried and dissolved in NMP/water (8:2, v/v) before HPLC analysis. The recovery rate for the slurries ranged from 50% for ES to 95% for OC (data not shown). For the biodegradation assay, the whole tube containing sand and liquid media was sacrificed for extraction at each time point. Here, the solid and liquid phases were directly extracted in DCM; see (Fagervold et al., 2021) for details. As reported previously, the recovery rate of the different UV filters for the degradation assays ranged from 63 % for ES to 93 % for OC (Fagervold et al., 2021).

2.4.2. HPLC analysis

The UV filters were analyzed using an Ultimate 3000™ HPLC system equipped with a DAD detector (Thermo Fisher Scientific) and a Phenomenex Kinetex Biphenyl 2.6 µm, 150 x 4.6 mm column as previously described (Fagervold et al., 2019;Fagervold et al., 2021). Briefly, the solvent carriers were acidified (0.1% formic acid) water and methanol, starting with a 2:8 ratio of methanol:water, held for 10 minutes, followed by a gradual increase of methanol up to 100%, tailed by 100% methanol for 10 minutes. Oven temperature was 50°C, the flow rate was 1 mL min⁻¹ and the injection volume was 5 µL. The data acquisition software was Chromeleon™ 7.2

(Thermo Fisher Scientific). Calibration curves and retention times of all the different UV filters were determined as described by Fagervold et al. (2019).

2.5. Microbial community analysis of enrichment cultures and strain isolation

The microbial community was analyzed by Automated Ribosomal Intergenic Spacer Analysis (ARISA) and Illumina sequencing as previously described (Fagervold et al., 2021). Briefly, DNA from enrichment cultures was extracted using a Maxwell® 16 LEV Blood DNA Kit (Promega, Charbonnières-les-Bains, France) with the Maxwell® 16 MDx Instrument (AS3000; Promega).

ARISA was performed as described by Fisher et al. (Fisher et al., 1999) using the intergenic spacer primers 1406F and 23SRY, with minor modifications as described by (Fagervold et al., 2021). For the analysis of the resulting chromatograms, individual fragment lengths (within a 0.1 bp “error range”) were considered separate operational taxonomic units (OTUs), and the relative abundance of each OTU was calculated by dividing the area of the peak by the total area of the chromatogram after 200 bp. It is also important to note that only peaks contributing to more than 1% of the relative peak area were taken into account.

For details on the Illumina sequencing and sequence processing, see Fagervold et al. (2021). Sequencing was performed by RTL genomics (Lubbock, Texas, USA) using the bacterial primers 341F (CCTACGGGNGGCWGCAG) and 806R (GACTACHVGGGTATCTAATCC) (Herlemann et al., 2011). The sequences were

processed using QIIME 2 (Bolyen et al., 2019). The different samples yielded approximately 27 000 to 56 000 sequences per sample, divided into 244 different OTUs. For further analysis, the focus was on OTUs with a relative abundance over 1% in any of the sequenced samples resulting in 33 OTUs. These OTUs have accession numbers OL468926-OL468958.

OC-degrading strains were isolated on R2B agar, screened and sequenced as described previously (Fagervold et al., 2021). The accession numbers for the isolated strains are: OL457616 for strain OC_4D, OL457617 for strain OC_S5, OL457618 for strain OC_S4 and OL457619 for strain OC_S1.

3. Results and discussion

3.1 Degradation of UV filters in initial sludge microcosms

The potential of WWTP sludge to degrade the different UV filters was assessed in a preliminary experiment. The temporal evolution of the UV filter concentration in amended flasks containing active sludge was compared with the concentration of the UV filters in the amended flasks containing sterilized sludge. Degradation was clearly observed for ES, HS, OC and BM after 20–60 days of incubation (Figure 1) in live cultures. However, the concentration seems to increase in some of the sterile control samples, for example for ES and HS. This phenomenon is probably due to a “matrix effect” caused by the sterilization process (autoclaving) changing the

extraction efficiency with time. This phenomenon has been observed in other experiments, when autoclaving samples with high organic content (data not shown). Nevertheless, degradation is evident for ES, HS and BM with a sharp decrease in live cultures over time. Although less evident, OC decreased significantly in the live culture. These initial microcosms were not analyzed further once degradation was observed because they were selected for more detailed experiments described below.

It should be noted that the solid and aqueous phases of the sludge was extracted separately (Fagervold et al., 2021), and that the UV filters partitioned strongly into the solid phase. In fact, the UV filters could barely be detected in the aqueous phase (data not shown). Thus, as previously shown for BP3 (Fagervold et al., 2021), ES, HS, OC and BM are clearly bioavailable for degradation even when partitioned into the solid phase, as the concentration decreased with time in live slurries but not in the sterile controls.

Several of the UV filters did not degrade even after 120 days of incubation, namely, BEMT, MBBT and DBT. These UV filters have log P values over 10 (Table 1), and thus, the lack of degradation in the sludge might be due to strong sorption to the solid phase and hence poor bioavailability for the degrading microorganisms. Another reason might be the high molecular weight of the target compounds. Indeed, it is thought that molecules have to be less than 600 Daltons to pass through the bacterial cell wall through porin proteins (Weiss et al., 1991), and BEMT, MBBT

and DBT are very large molecules with molecular weights of 600 g/mol. Since no degradation of BEMT, MBBT and DBT was detected in sludge slurries after 120 days, no further enrichments or incubations were initiated using these filters.

3.2 Enrichment cultures

Slurry from the flasks that showed biodegradation activity (OC, ES, HS and BM) was transferred to enrich putative UV filter-degrading microorganisms as described previously (Fagervold et al., 2021). The enrichments were set up in 100 mL Erlenmeyer flasks that contained 50 mL of minimal media, inert sand, and one of the UV filters. The UV filter concentration was relatively high, with theoretical concentrations of 100 mg/L for the second transfer “enrichment 2”. Indeed, this concentration is above the predicted/measured water solubility for OC, HS and BM (see Table 1). It should be noted that the UV filter (BM, HS, ES or OC) was the only available carbon source for the microorganisms in the enrichment cultures. The cultures were transferred every month to further enrich UV filter-degrading microorganisms.

3.2.1 Degradation assays of enrichment cultures

After 5 consecutive transfers, the degradation activity of the enrichment cultures was verified (“Enr 5”) by performing biodegradation assays in separate tubes as described in Fagervold et al. (2021). Briefly, biodegradation assays involve the extraction of entire tubes with both the sand phase and the liquid phase. The same procedure was performed on Enr 9 cultures.

298

299 ES, OC and HS were degraded over 70% in only 5–7 days in Enr 5 and Enr 9 cultures
300 and were approximately 90% degraded after 12–14 days (Figure 2). BM was slower
301 to degrade, with approximately 35% degradation after 7 days in Enr 5 cultures and
302 approximately 50% degradation after 12–14 days. However, with an extended
303 incubation time, the degradation of BM was more complete, with 80% degradation
304 after 28 days in Enr 9 cultures. It was thus clear that the enrichment cultures
305 retained their degradation capability throughout several serial transfers. This
306 degradation activity was observed after more than 20 transfers (data not shown).

307

308 *3.2.2 Stability of the enrichment culture microbial community through transfers*

309 ARISA was performed to investigate the diversity and stability of the microbial
310 communities in enrichment cultures throughout the transfers. ARISA exploits the
311 fact that different bacteria have different sized intergenic spacers between the 16S
312 rRNA gene and the 23S rRNA gene on their chromosome. Theoretically, one peak in
313 an ARISA profile will correspond to one “OTU”, but some bacteria have several
314 copies of the 16S in their genome and give rise to two or more peaks. This analysis
315 was performed on “Day 4” samples of the enrichment cultures degrading ES, HS, BM
316 and OC and after 7, 11, 12 and 20 transfers (Enr 7, Enr 11, Enr 12 and Enr 20).

317

318 Generally, 15–28% of the peaks in the enrichment cultures were “prevalent peaks”,
319 meaning peaks were detected in all 4 enrichments (Table 2). This means that the
320 microorganism responsible for that peak would be strongly enriched for through 20

transfers over nearly 2 years. Thus, the “prevalent” peaks clearly had a major role in the enrichment cultures. Furthermore, approximately 55–60% of the peaks were found in only one or two of the transfers and generally in smaller amounts. Thus, one can assume that the microorganisms responsible for these peaks do not have a central role in the degradation of the target UV filter.

ES-degrading cultures were the most diverse, containing 29 peaks in total. Eight peaks were prevalent (Figure 3A and Table 2), and the stability of the cultures was relatively high. One prevalent peak stood out as dominant, namely, peak 908, which was present up to 40% in Enr 11. HS-degrading cultures contained 18 peaks, and only 3 peaks were prevalent (Figure 3B). The dominant peak changed with the different transfers; thus, the stability of the microbial community was low. BM-degrading cultures contained 20 peaks, with 5 of these peaks being prevalent (Figure 3C). Although some of the peaks changed with time, the most predominant peak did not seem to change significantly through the different transfers. One can therefore consider the microbial community in BM-degrading microcosms as relatively stable. OC-degrading cultures contained 27 peaks between the four transfers (Figure 3D). Of these peaks, 4 peaks were prevalent. Many of the peaks were present only in small relative abundances, and there was no one dominant peak in any of the enrichments, although several peaks had a relative abundance between 10 and 20%.

3.2.3. Identity of major microorganisms in enrichment cultures

Day 4 samples of Enr 11 and 12 were sequenced to identify the major microbial players in the enrichment cultures (Figure 4). Overall, 33 OTUs with a relative abundance over 1% in any of the sequenced samples were identified. The different samples contained 8-12 OTUs with a relative abundance over 1%, and only one OTU was common in all enrichment cultures, namely, OTU 28, a *Betaproteobacteria* belonging to the genus *Metylophilus*. This OTU was only one bp different from *Metylophilus luteus* Mim^T (FJ872109), a restricted facultative methylotroph capable of utilizing both methanol and glucose (Gogleva et al., 2010). However, the exact role of this OTU in the degradation of the various UV filters is not known.

It was evident that the microbial community composition in the different UV-degrading enrichment cultures was very dissimilar (Figure 4). Thus, the presence of an individual UV filter strongly influences the microbial community when transferred multiple times. ES cultures were dominated by two *Pseudomonas* OTUs within the *Gammaproteobacteria*, with a total relative abundance of over 70% for the two OTUs. The more abundant of these two OTUs, OTU 33, is one bp different from *Pseudomonas benzenivorans* strain DSM 8628 (NR_116904). This strain belongs to the *Pseudomonas aeruginosa* group and is able to grow on benzene, toluene or *para*-cresol as the sole source of carbon and energy (Lang et al., 2010). The other *Pseudomonas* OTU, OTU 32, was only one bp different from several strains, one being *Pseudomonas indoloxydans* strain IPL-1 (NR_115922), which can oxidize indole (Manickam et al., 2008). Enrichments degrading the other filters contained fewer *Pseudomonas* OTUs, and thus, the dominance of *Pseudomonas* OTUs

367 seemed to be specific for ES-degrading cultures. Indeed, the other enrichments
368 contained more OTUs within the *Alphaproteobacteria* and/or the *Betaproteobacteria*.
369

370 The most dominant group in the BM-degrading enrichment cultures was
371 *Betaproteobacteria*, with relative abundances of 55% and 65% in Enr 11 and 12
372 cultures, respectively. The OTUs within the *Betaproteobacteria* consisted mainly of 4
373 OTUs, *Comamonas* OTU 23, *Hydrogenophaga* OTU 27, *Methylophilus* OTU 28 and
374 *Thauera* OTU 29, and they were present in significant amounts in both the Enr 11
375 and 12 samples. *Methylophilus* OTU 28 was described above. The nearest published
376 strain to *Comamonas* OTU 23, which is 97.8% identical (418 of 427 bp), is
377 *Comamonas nitrativorans* strain 23310 (NR_025376), a strain that can perform
378 nitrate reduction in the absence of oxygen and can use many different fatty acids as
379 carbon sources but cannot use sugars (Etchebehere et al., 2001). One can thus
380 envisage that this OTU can be important in the intermediary metabolism of BM and
381 perhaps is not involved in the initial degradation or benzene ring oxidation.

382 *Hydrogenophaga* OTU 27 is 100% identical (427 bp) to *Hydrogenophaga borbori*
383 strain LA-38 (NR_170459). This strain was also isolated from sludge, and contrary
384 to what its name suggests, it cannot grow autotrophically on hydrogen gas but can
385 use various organic molecules for growth (Choi et al., 2020). This OTU seems to be
386 essential for BM degradation, as it is present in over 25% relative abundance in both
387 enrichments sequenced. *Thauera* OTU 29 is 1 bp different (427 of 428 bp identical)
388 from *Thauera humireducens* strain SgZ-1 (NR_109534), a facultative anaerobic

389 bacterium isolated from a microbial fuel cell capable of nitrate reduction and the use
390 of various organic molecules as electron donors (Yang et al., 2013).
391
392 The BM-degrading enrichment cultures also contained approximately 20-30%
393 *Alphaproteobacteria* within the family *Sphingomonadaceae*. The two main OTUs
394 within this family in BM enrichments were OTU 13 and OTU 15. Their relative %
395 varied between the different samples, but OTU 13 was clearly important in both
396 samples, with relative abundances of over 10% and 16% in Enr 11 and 12,
397 respectively. This OTU was 1 bp different (of 402 bp) from *Rhizorhabdus*
398 *dicambivorans* strain Ndbn-20 (NR_151992), a dicamba-degrading strain (Yao et al.,
399 2016), and very similar (400/402 bp identical) to *Sphingomonas wittichii* RW1
400 (NR_074268). Indeed, the BP3-degrading activity of *Sphingomonas wittichii* strain
401 BP14P isolated from BP3 enrichment cultures has recently been described
402 (Fagervold et al., 2021). This strain is “OTU 16” here (Figure 4). Thus, it seems that
403 different but very closely related *Sphingomonas* strains are enriched and are
404 perhaps responsible for the initial degradation of some UV filters.
405
406 The microbial community compositions of the two HS samples, Enr 11 and 12, were
407 apparently quite dissimilar in regard to the dominant OTUs present. Although both
408 belonged to the order *Sphingomonadales* in the *Alphaproteobacteria*, the dominant
409 OTU in Enr 11 was *Sphingopyxis* OTU 17 (44% relative abundance), while
410 *Erythrobacteraceae* OTU 20 dominated in Enr 12, with 50% relative abundance. This
411 kind of “instability” of the microbial community in HS-degrading cultures was also

observed in the ARISA experiments (discussed above). Perhaps the ability of HS degradation is less specific and more widespread, meaning that the enzymatic functioning necessary for HS degradation is present in many genera within the *Sphingomonadales*. OTU 17 was very similar (399/402 bp identical) to several described strains, one of which was *Sphingopyxis indica* strain DS15 (NR_108185), which was isolated from a hexachlorocyclohexane (HCH)-contaminated site (Jindal et al., 2013). OTU 20 was similar (398/402 bp identical) to several strains within the genera *Altererythrobacter*/*Erythrobacter*.

There were some differences between the samples from the OC-degrading microcosms but not as much difference as for HS. Some OTUs, such as OTU 1, OTU 24 and OTU 28, were present in both Enr 11 and 12 at approximately the same relative abundance, while other OTUs were present at different ratios in the two samples. For example, OTU 18 (*Sphingopyxis* sp.) was dominant in the Enr 11 sample, with a relative abundance of 35%. OTU 18 is 100% identical to the described strain *Sphingopyxis terrae subsp. ummariensis* strain UI2 isolated from a hexachlorocyclohexane dump site in India (Sharma et al., 2010). In Enr 12, another *Alphaproteobacterial* OTU, OTU11 was most predominant, with almost 40% relative abundance. This was 394/402 bp (98%) identical to a *Ferrovibrio xuzhouensis* strain isolated from cyhalothrin-contaminated wastewater (Song et al., 2015).

3.3 Perspectives on degradation capacities of enrichment cultures/slurries towards organic UV filters

This is the first report of aerobic microcosm degradation experiments for ES, HS BEMT, MBBT and DBT to our knowledge and the first report of ES and HS degradation. OC has been shown to degrade in aquifer materials (Liu et al., 2013) and WWTP microcosms (Suleiman et al., 2019; Badia-Fabregat et al., 2012). Furthermore, there were some indications of OC degradation in marine sediment microcosms (Azaroff et al., 2021) and in lake water microcosms (O'Malley et al., 2021). BM has also been incubated in a sediment/water system, but it was unclear if the removal was due to biodegradation or sorption processes (Li et al., 2016). BM was also tested in lake water microcosms, but no biodegradation was detected (O'Malley et al., 2021)

Moreover, this is the first time that the microbial communities responsible for the degradation of the different UV filters were investigated. Indeed, the fact that the microbial communities were clearly different in the different enrichment cultures suggest that the each different UV filters requires a specific set of microorganisms with a specific set of enzymatic activities for degradation. However, there might be some redundancy in the microbial community since an enrichment of only one specific microorganism was not observed, as seen in BP3 degrading enrichment cultures where one specific strain comprised over 85% relative abundance in enrichment cultures (Fagervold et al., 2021).

Three of the organic UV filters tested, namely, BEMT, MBBT and DBT, clearly did not degrade. The reasons why some of the organic UV filters are degraded while others

are not are undetermined. These molecules have different characteristics, such as solubility and size, and thus, the bioavailability may be a determining factor. Another possibility is that the appropriate microorganisms/enzymes are not present in WWTPs. This might be the case since UV filters apparently require a specific set of microorganism, thus the degradation capability is not universal. One way to determine if this is the case is to screen other environments for degradation activities.

Nevertheless, one might have to come to terms with the idea that some organic UV filters, such as BEMT, MBBT and DBT, are not biodegradable. The question then becomes whether these nonbiodegradable organic UV filters are at all bioavailable to cause toxic effects and/or can be bioaccumulated and biomagnified. Filters such as BP3 and OC have been shown to be bioaccumulative and have been shown to cause toxic effects (Peng et al., 2020;Lozano et al., 2020), but there are few data to suggest that this is the case for UV filters such as BEMT, MBBT and DBT. These are important factors to keep in mind for the full assessment of risk associated with the use of these organic UV filters.

3.4. Isolation of putative degrading microorganisms from OC-degrading cultures

Putative OC-degrading microorganisms were isolated on R2A agar as described previously for BP3-degrading microorganisms (Fagervold et al., 2021). The focus was on OC because this filter has been detected in WWTP influents, effluents and sludge at relatively high frequencies and at high concentrations (Balmer et al.,

2005;Biel-Maeso et al., 2019;Kupper et al., 2006;Li et al., 2007;Rodil et al., 2009a;Moeder et al., 2010;Liu et al., 2012a;Magi et al., 2013;Langford et al., 2015;Cunha et al., 2015;Plagellat et al., 2006). Enr 7 cultures were used as inoculum for isolation attempts. After several restreaks on R2A agar, colonies were screened by ARISA to investigate whether the isolates were the same and if they were present in the enrichment cultures. Several strains were successfully isolated from the enrichment cultures, but it should be noted that it was challenging to obtain pure cultures. This difficulty in purifying isolates was previously reported to be a problem (Suleiman et al., 2019). 11 bacterial types were isolated. Of these 11 initial types, only four isolates could be found in the enrichment cultures (as shown either by ARISA or Illumina sequencing) (see Table 3).

3.4.1. Phylogenetic description of isolated microorganisms

Strain OC_S5 exhibited small orange/pink, dry, well-defined colonies. The 16S rRNA gene sequence was 99.9% identical to *Gordonia alkanivorans* strain HKI 0136 (NR_026488), a strain isolated from tar-contaminated soil (Kummer et al., 1999). This strain degrades alkenes and is also orange colored. Strain OC_S5 could not be found in the ARISA chromatograms in significant relative amounts but could be detected with a relative abundance of approximately 7% in both Enr11 and 12 in the Illumina sequencing data (OTU 1 in Figure 4).

Strain OC_S4 was 99.9% identical to *Hydrogenophaga electricum* sp. strain AR20 (NR_132676) isolated from an anodic biofilm in a fuel cell (Kimura and Okabe,

2013). This strain can oxidize hydrogen and can also use alternative electron acceptors (nitrate and anode) for anaerobic growth. *Hydrogenophaga electricum* sp. strain AR20 colonies are glistening and moderate yellow. Isolate OC_S4 was also moderate yellow and glistening with diffuse colonies. Strain OC_S4 was detected in the ARISA chromatograms, and it was shown to be present in approximately 20% relative abundance in the OC-degrading cultures (OTU 24, Figure 4).

Strain OC_4D was over 99% identical to *Sphingopyxis terrae subsp. ummariensis* strain UI2 (NR_116018), a bacterium isolated from a hexachlorocyclohexane (HCH) dump site (Sharma et al., 2010). This strains was metabolically relative versatile, but the authors did not report on the degradation ability of any pollutants (Sharma et al., 2010). Strain OC_4D was also yellow, circular with entire margins, as the described strain. This strain is 100% similar to OTU18 that is present in significant amounts in the enrichment cultures (Figure 4 and Table 3).

Strain OC_S1 was 99.4% identical to *Pseudomonas delhiensis* strain RLD-1 (NR_043731), isolated from a fly ash dumping site in India (Prakash et al., 2007). This strain can use phenantrene as a sole carbon source, but this ability was lost after several streaks on Luria-Bertani (LB) agar. Strain OC_S1 was present in small relative amounts (Table 3) in the OC-degrading enrichment culture.

Interestingly, both *Gordonia* sp. and *Hydrogenophaga* sp., in addition to *Mycobacterium* sp., have been previously reported to be major players in OC-

degrading enrichment cultures (Suleiman et al., 2019). These enrichment cultures also started from WWTP sludge, and the major microbial players were identified by denaturing gradient gel electrophoresis (DGGE) and subsequent sequencing of the major bands in the gel. However, the authors were not able to isolate these strains but used a “reverse discovery approach”. This approach involves using deposited strains phylogenetically close to the strains identified by DGGE and sequencing to test their biodegradation ability.

3.4.2 OC degrading activity of isolated strains

The isolated strains were tested for their degradation capacities in minimal media and in media amended with 10% R2B, in addition to surveying the growth by cell counts (Figure 5). The strains were also combined in a mixture to determine any syntrophic effect this would have on biodegradation activity.

Only two of the isolates clearly degraded OC, with or without the presence of other carbon sources. OC_S5 (*Gordonia alkanivorans*) and OC_4D (*Sphingopyxis terrae*) degraded approximately 50% of the OC present after only 4 days and over 90 and approximately 80%, respectively, after 20 days. Interestingly, the degradation kinetics seemed to be the same in minimal media and 10% R2B, even though growth was higher in cultures amended with R2B media. Strain OC_S1 (*Pseudomonas delhiensis*) clearly did not degrade OC and the degradation capacity of strain OC_S4 (*Hydrogenophaga electricum*) was ambiguous. This strain did not degrade OC in the presence of R2B, but OC seemed to decrease in quantity in some but not all of the

550 replicate cultures after 14 days (Figure 5). Thus, one should consider that strain
551 OC_S4 does not degrade OC until additional verification of these results.
552 Unsurprisingly, clear growth was detected for all the strains when grown on more
553 rich media (minimal media with 10% R2B added). However, some increase in cell
554 numbers in minimal media were also detected for all the strains (Figure 5), even
555 those strains that did not degrade OC. This increase in cell number is probably due
556 to some nutrients being left in the inoculum after the washing procedures. However,
557 this also means that one cannot determine whether the degradation of OC by OC_S5
558 (*Gordonia alkanivorans*) and OC_4D (*Sphingopyxis terrae*) is co-metabolic or growth
559 linked.

560

561 These results are in contrast to previous results from (Suleiman et al., 2019), which
562 showed that *Gordonia cholestiolivorans* did not degrade OC, but *Mycobacterium agri*
563 showed some degradation activity; however, this was with other carbon sources
564 present. Interestingly, the authors reported biofilm formation around droplets of
565 OC, and the same could be observed here. The enrichment culture was not uniform;
566 there were aggregates of bacteria in the media. This observation, together with the
567 fact that we, as Suleiman and coworkers reported (Suleiman et al., 2019), also had
568 difficulties in obtaining pure cultures, suggests that optimal OC degradation is
569 performed by mixed cultures. One can then hypothesize that *Sphingopyxis* sp. strain
570 OC_4D and perhaps *Gordonia* sp strain OC_S5 could degrade OC directly while
571 *Hydrogenophaga* sp. strain OC_S4 and *Pseudomonas* sp. strain OC_S1, which did not

degrade OC directly, have a role in degrading intermediary products in the degradation pathway.

4. Conclusions

This is the first time the degradation is shown for several of the UV filters (HS and ES) and the first time the microbial community responsible for the degradation is explored with molecular techniques in concurrence with the isolation of OC degrading microorganisms. It is clear that WWTP sludge has the capability to degrade some organic UV filters, such as ES, HS, OC and BM. The degradation activity is stable since the activity can be sustained for over 20 transfers in minimal media. The microbial communities responsible for the degradation of the different UV filters were specific to each UV filter. Thus, the addition of high concentrations of the target UV filter in addition to multiple serial transfers was a successful strategy to enrich specific degrading microbial communities. However, the exact dynamics and the specific role of the major OTUs in the degrading enrichment cultures remain to be determined, except for OC where two strains were confirmed to degrade OC in isolation.

It was also clear that some of the UV filters, namely BEMT, MBBT and DBT did not degrade in these WWTP slurries. The reason for the non-degradation is unclear but is most probably due to poor availability. In this regard one should take into account the seemingly non-bioavailability, in addition to the non-biodegradability of these compounds when performing environmental risk assessments.

595

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609

610 **Figures**

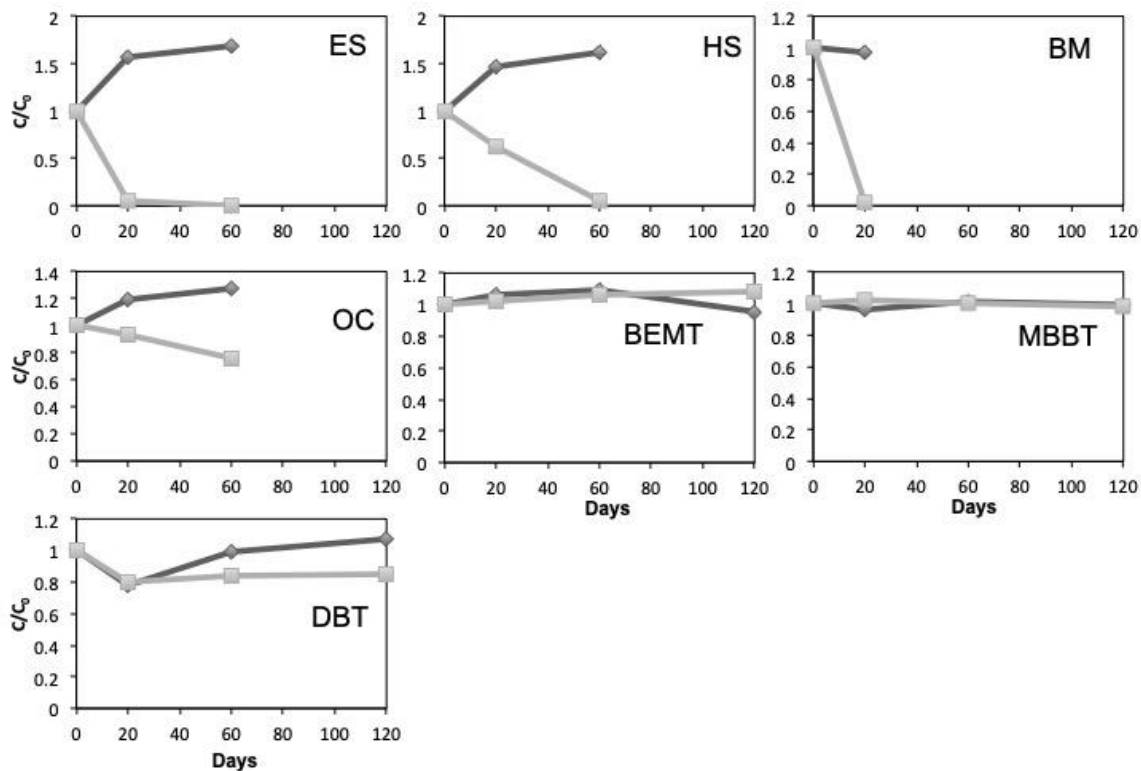
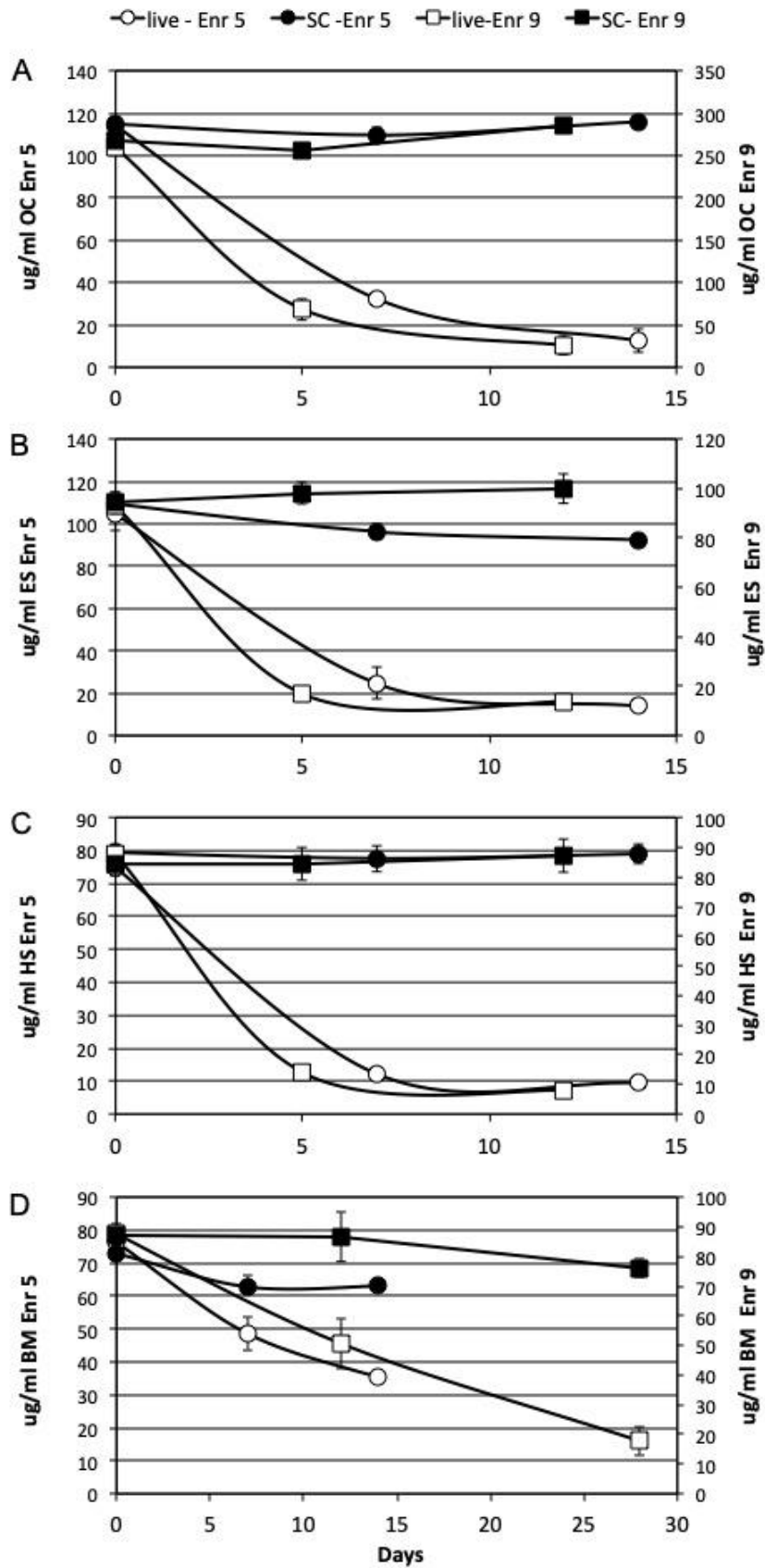


Figure 1. Degradation of UV filters in initial sludge cultures. The y-axis represents the concentration (C) of the UV filter per mL of sludge at the time point in question (x-axis) divided by the concentration of the UV filter at day 0 (C_0). Dark gray diamonds represent sterile controls, and light gray squares represent live sludge.



618 Figure 2. Degradation of UV filters in enrichment cultures. Degradation of OC (A), ES
619 (B), HS (C) and BM (D) in live cultures (white fill) compared to sterile controls (SC)
620 in two enrichment cultures, Enr 5 and Enr 9. Enr 5 live cultures (white circles) and
621 SC (black circles); Enr 9 live cultures (white squares) and SC (black squares).
622

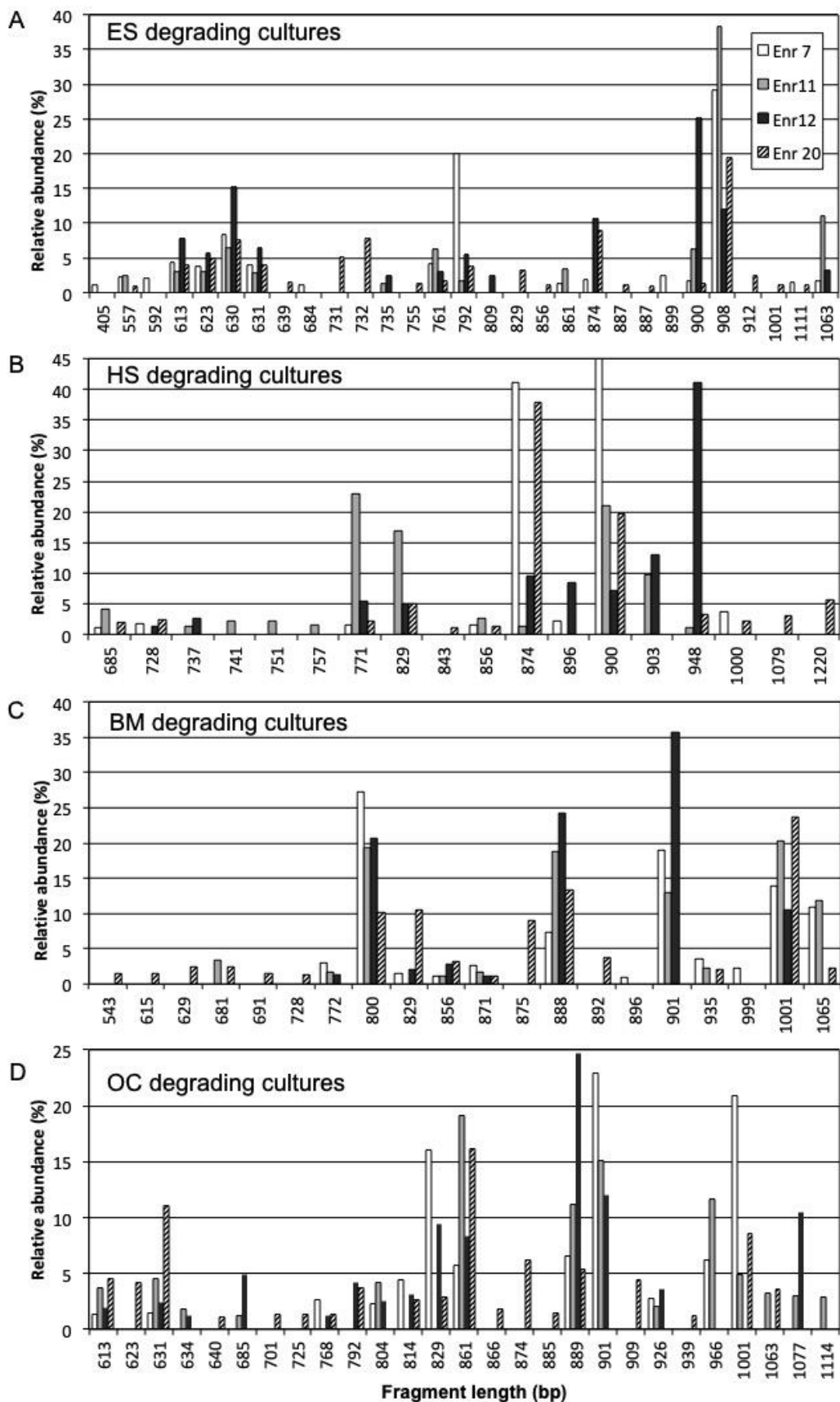


Figure 3. Analysis of the microbial community diversity in enrichment cultures degrading ES (A), HS (B), BM (C) and OC (D). Each bar represents one “OTU”, and the relative abundance is calculated from the area of each peak; see the description in the Materials and Methods section for more details. “Enr 7” (white bars), “Enr 11” (gray bars), “Enr 12” (black bars) and “Enr 20” (striped bars).

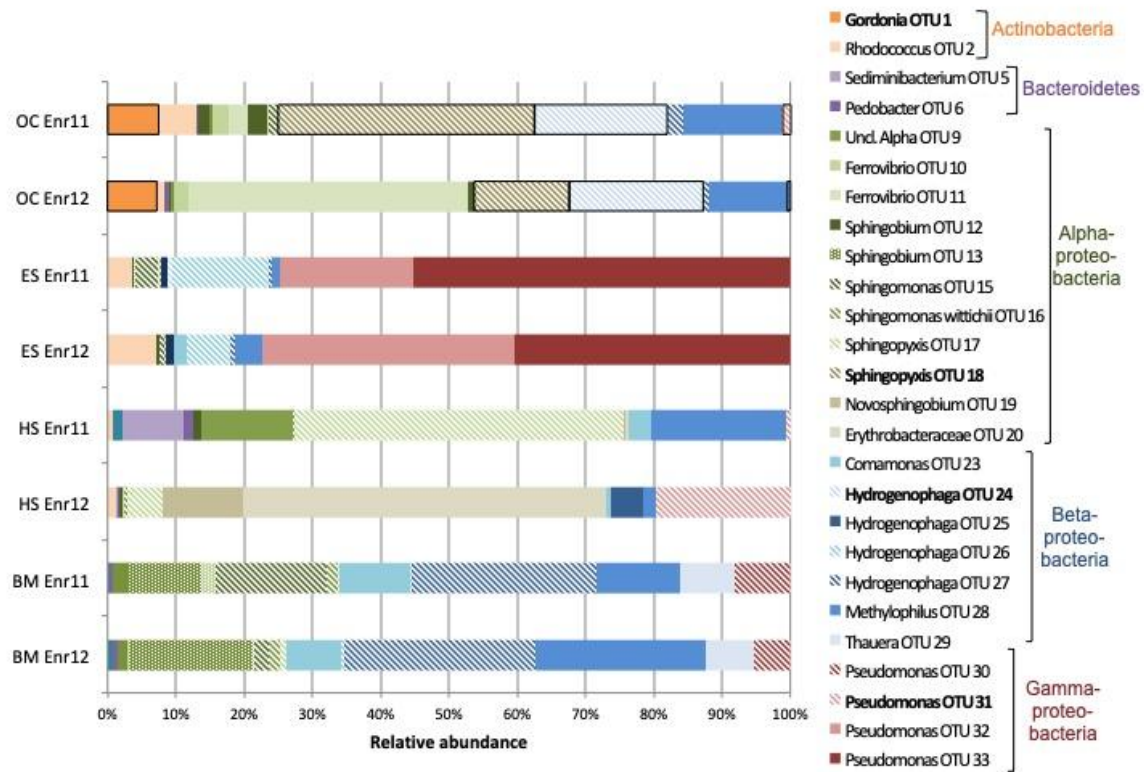


Figure 4. Bar graph of OTUs present in the different enrichment cultures and their taxonomic affiliation. Orange-colored OTUs belong to *Actinobacteria*, purple to *Bacteroidetes*, green to *Alphaproteobacteria*, blue to *Betaproteobacteria* and red to *Gammaproteobacteria*. The OTUs in bold are those that have been isolated, these OTUs have also been encased with black rectangles in the bar graph.

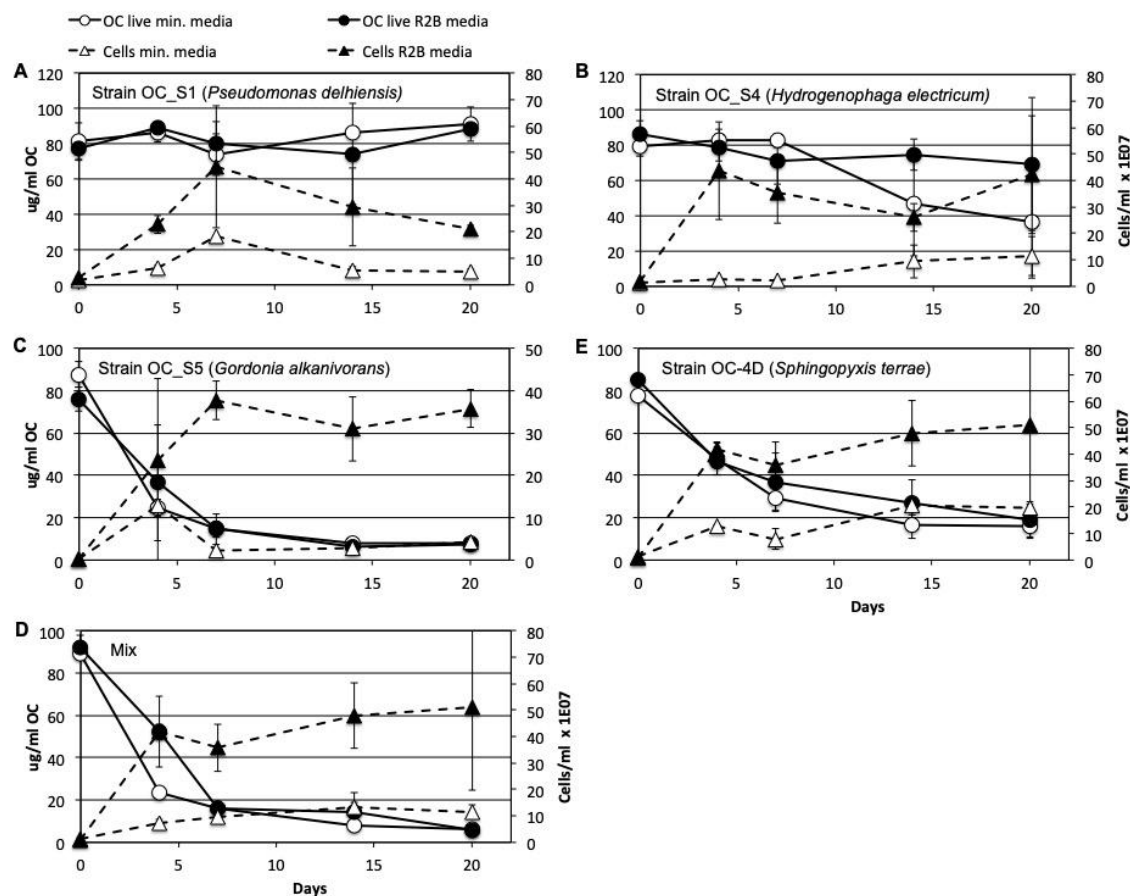


Figure 5. Degradation of OC by different bacterial isolates: strains OC_S1 (A), OC_S4 (B), OC_S5, (C), OC_4D (D) and a mix of all isolates (E). OC concentration (left axis, circles and uninterrupted lines) in degradation assays coupled to cell counts (right axis, triangles and dashed lines) over time (days) in minimal media (open circles and triangles) and with media containing 10% R2B (black circles and triangles).

Tables

Table 1. Chemical properties and concentrations of organic UV filters used in this study in WWTPs. Note the abbreviations that are used throughout.

| | MW ^a | FW sol. (mg/L) ^b | LogP ^c | WWTP influent range (ng/L) [nr. papers] ^d | WWTP effluent range (ng/L) [nr. papers] ^d | WWTP sludge range (ng/g -dw) [nr. papers] ^d |
|--|-----------------|--------------------------------|-------------------|---|---|---|
| 2-Ethylhexyl salicylate (ES) [118-60-5] | 250.3 | 137 | 5.335 | ND – 1,188 ^e [6] | ND - 128.9 ^e [9] | ND – 1,370 ^e [4] |
| Homosalate (HS) [118-56-9] | 262.3 | < LOD (LOD = 0.85) | 5.431 | ND – 1,650.4 ^f [5] | ND – 154.2 ^f [8] | ND – 1,050 ^f [3] |
| Butyl methoxydibenzoylmethane (BM) [70356-09-1] | 310.4 | 19 | 5.391 | 250 – 2935 ^g [3] | ND – 1,018 ^g [4] | 144 – 517 ^g [1] |
| Octocrylene (OC) [6197-30-4] | 361.5 | 9.1 | 7.083 | ND - 13,000 ^h [11] | ND – 6,969 ^h [16] | 30-41,610 ^h [11] |
| bis-Ethylhexyloxyphenol methoxyphenyl triazine (BEMT) [187393-00-6] | 627.8 | 0.3 | 10.627 | - | - | - |
| Methylene bis-benzotriazolyl tetramethylbutylphenol (MBBT) [103597-45-1] | 658.9 | < LOD (LOD = 0.04) | 15.451 | - | - | - |
| Diethylhexyl butamido triazone (DBT) [154702-15-5] | 766.0 | 0.2 | 12.004 | - | - | 54 – 136 ⁱ [1] |

^a MV: molecular weight

^b Freshwater solubility at 25 °C, experimental data. See protocol in Fagervold et al. (2019). LOD: Limit of detection.

^c According to Reaxys database (<https://www.reaxys.com/>, accessed on June 9, 2020)

^d Number of papers reporting

^e References for ES concentrations: (Rodil et al., 2009a; Rodil et al., 2009b; Negreira et al., 2009; Negreira et al., 2011; Kameda et al., 2011; Magi et al., 2013; Wu et al., 2013; Tsui et al., 2014; Cunha et al., 2015; Biel-Maeso et al., 2019)

^f References for HS concentrations: (Rodil and Moeder, 2008; Rodil et al., 2009a; Rodil et al., 2009b; Negreira et al., 2011; Kameda et al., 2011; Magi et al., 2013; Wu et al., 2013; Tsui et al., 2014; Cunha et al., 2015; Biel-Maeso et al., 2019)

^g References for BM concentrations: (Rodil and Moeder, 2008; Rodil et al., 2009a; Rodil et al., 2009b) (Cunha et al., 2015)

^h References for OC concentrations: (Balmer et al., 2005; Plagellat et al., 2006; Kupper et al., 2006; Li et al., 2007; Rodil and Moeder, 2008; Rodil et al., 2009a; Rodil et al., 2009b; Pedrouzo et al., 2010; Moeder et al., 2010; Negreira et al., 2011; Liu et al., 2011; Kameda et al., 2011; Gago-Ferrero et al., 2011; Rodríguez-Rodríguez et al., 2012; Liu et al., 2012a; Rodil et al., 2012; Badia-Fabregat et al., 2012; Magi et al., 2013; Tsui et al., 2014; Langford et al., 2015; Cunha et al., 2015; Biel-Maeso et al., 2019)

ⁱ References for DBT concentrations: (Rodil et al., 2009b)

Table 2. Summary of ARISA results

| | ES (percentage) | HS (percentage) | BM (percentage) | OC (percentage) |
|-----------------------------|--------------------|--------------------|--------------------|--------------------|
| Nr. of peaks ^a | 29 | 18 | 20 | 27 |
| Prevalent ^b | 8 (28%) | 3 (17%) | 5 (25%) | 4 (15%) |
| Semi prevalent ^c | 3 (10%) | 5 (28%) | 4 (20%) | 7 (26%) |
| Spurious ^d | 18 (62%) | 10 (56%) | 11 (55%) | 16 (59%) |

^a Number of peaks total in any of the enrichments above 1% relative abundance

^b Number of peaks present in all 4 enrichments

^c Number of peaks present in 3 of the 4 enrichments

^d Peaks only present in one or two of the enrichments

Table 3. Strains isolated from OC degrading enrichment cultures

| Strain | Closest described strain (accession number) | Ident. ^a | ARISA fragment length (bp) | ARISA ^b | Illumina ^c | Deg. ^d |
|--------|--|-----------------------|----------------------------------|--------------------|-----------------------|-------------------|
| OC_S1 | <i>Pseudomonas delhiensis</i> RLD-1 (NR_043731) | 99.4 % (1249/1256) | 768 | 2.6 % | OTU 31 (1 %) | No |
| OC_S4 | <i>Hydrogenophaga electricum</i> sp. (NR_132676) | 99.9 % (1442/1445) | 861 | 5.7 % | OTU 24 (19%) | ? |
| OC_S5 | <i>Gordonia alkanivorans</i> strain HKI 0136 (NR_026488) | 99.9 % (1408/1409) | 700,723, 728,771 | < 1% | OTU 1 (7%) | Yes |
| OC_4D | <i>Sphingopyxis terrae</i> subsp. ummariensis strain UI2 (NR_116018) | 99.9 % (1404/1406) | 889 | 6.6 % | OTU 18 (25%) | Yes |

^a Identity to the nearest top hit using Basic Local Alignment Search Tool (BLAST) search of describes species (rRNA/ITS databases)

^b Relative abundance in Enr 7 cultures, this number is used here because this culture was the inoculum for isolation attempts

^c OTU number from the sequencing analysis and relative percentage in parenthesis

^d Degradation, verification by in depth biodegradation assay

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