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Efficient degradation of the organic UV filter benzophenone-3 by *Sphingomonas wittichii* strain BP14P isolated from WWTP sludge

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Abstract

Benzophenone-3 (BP3) is a widely used organic UV filter present in many environmental compartments. One way BP3 is released into the environment is through effluents from wastewater treatment plants (WWTPs). These plants are possible sources for degradation activity as WWTP sludge can potentially degrade BP3. Our goal was to identify the BP3 degrading microorganism(s) in WWTP sludge and to investigate whether the degradation was co-metabolic. Initial WWTP sludge microcosms spiked with BP3 showed 100% degradation after 20 days. Multiple

transfers of these microcosms, while maintaining a strong selective pressure for BP3 degradation capabilities, resulted in the dominance of one bacterial strain. This strain, *Sphingomonas wittichii* BP14P was subsequently isolated and shown to degrade BP3 in a growth dependent manner. Strain BP14P utilized BP3 as the sole energy and carbon source and completely degraded BP3 after 7 days in minimal media. We tested the capability of BP14P to degrade nine other UV filters, but the degradation ability seemed to be restricted to BP3. However, whether this specificity is due to the lack of degradation genes, cellular transport or low bioavailability of the other UV filters remained unclear. The efficient degradation of BP3 by a group of bacteria well known for their potential for xenobiotic degradation is an important step forward for a complete risk assessment of the long-term environmental impact of BP3.

Keywords: Sunscreens, BP3 degradation, growth-dependent, *Sphingomonas wittichii*

1. Introduction

Benzophenone-3 (BP3) is an organic UV filter commonly used in sunscreen formulations and other cosmetic products. Due to its widespread use, BP3 is now found in many environmental compartments such as freshwater and seawater, benthic sediments and aquatic biota (see Mao et al. (2018) for a recent review). Further, BP3 has been found in bird eggs (Molins-Delgado et al., 2017) and even in human breast tissue (Barr et al., 2018) suggesting that BP3 can biomagnify. Also, it

47 has been demonstrated that BP3 can be absorbed through the skin but the effects of
48 elevated systemic BP3 concentrations in humans are still unclear (Suh et al., 2020).
49 BP3 has been shown to be toxic in several species used for toxicological testing
50 including fish, corals and algae (see recent review and references therein (Lozano et
51 al., 2020)). Thus, the toxicity of BP3 combined with the possibility of
52 bioaccumulation indicates that BP3 might pose an ecological risk (Díaz-Cruz et al.,
53 2019).
54
55 Wastewater treatment plants (WWTPs) are a major source of BP3 to the
56 environment, in addition to recreational water activities and industrial wastewater
57 discharges (Mao et al., 2018). Indeed, BP3 has been found in influents and effluents
58 of WWTPs (Ramos et al., 2016; Mao et al., 2018) in relatively high concentrations.
59 Often, the concentrations of BP3 are lower in the effluents of WWTPs than the
60 influents. This difference can be attributed to sorption to activated sludge and/or
61 biodegradation.
62
63 Biodegradation of BP3 has been shown to occur in different systems. Liu and
64 collegues observed degradation of BP3 in activated sludge microcosms (Liu et al.,
65 2012) and in microcosms using aquifer materials as an innoculum (Liu et al., 2013).
66 The data from this latter study was used as input in a model to better understand
67 the fate of UV filters, including BP3, in aquifers (Rodríguez-Escales and Sanchez-Vila,
68 2020). Among others, this model took into account sorption processses, both to
69 sediments and biomass, but also assumed that the UV-filters were degraded in a co-

metabolic manner with the oxidation of sediment organic matter as the main process.

The microorganisms responsible for the degradation in the studies described above were not identified (Liu et al., 2012; Liu et al., 2013). So far the only bacterial strain shown to be capable of BP3 degradation is *Methyliphilus* sp. strain FP-6, which degraded BP3 co-metabolically when grown with several other carbon substrates (Jin et al., 2019). The white root fungus *Trametes versicolor* can also degrade BP3 (Badia-Fabregat et al., 2012; Gago-Ferrero et al., 2012) but it is not known if this degradation was a co-metabolic process since dried and sterilized sludge was used as solid support in these experiments.

The objective of our study was to investigate the potential degradation of BP3 in microcosms with WWTP sludge, to determine whether the BP3 degradation is indeed co-metabolic and whether any bacterial species exist that can completely mineralize BP3.

2. Materials and methods.

2.1 Chemicals

The UV filters used for biodegradation studies, 2-hydroxy-4-methoxybenzophenone (BP3) and methylene bis-benzotriazolyl tetramethylbutylphenol (MBBT) were

purchased from Sigma-Aldrich (Lyon, France), while 2-ethylhexyl salicylate (ES), homosalate (HS), butyl methoxydibenzoylmethane (BM), octocrylene (OC), diethylamino hydroxybenzoyl hexyl benzoate (DHHB), diethylhexyl butamido triazone (DBT), and ethylhexyl triazone (ET) were kindly provided to us by Pierre Fabre Pharmaceuticals. Analytical standards for the above-mentioned UV filters were purchased from Sigma-Aldrich (Saint-Quentin-Fallavier, France). Analytical-grade dichloromethane (DCM), 1-methyl-2-pyrrolidinone (NMP), methanol, formic acid (98 %) and molecular grade formaldehyde were obtained 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, initial culture and enrichment culture setup

The sludge was sampled from the Argelès-sur-mer (France) wastewater treatment plant (WWTP) on August 24th, 2018, from the bottom of a clarifying tank. The sludge was collected in glass mason jars and stored at 4 °C until utilized for degradation experiments. The sludge contained 2765 (+/- 310) mg/L total suspended solids (TSS) and the initial BP3 concentration was below the detection limit for our analysis (4.6 ng/g, see Fagervold et al. (2019)). For the initial degradation experiments, 100 mL sludge was mixed with 500 mL freshwater mineral media (OECD301) in a 1-L glass bottle (live). BP3 was added in methanol (2 mg/mL) to a final concentration of 10 µg/ml. A sterile control (SC) was prepared by autoclaving twice (48 hours apart) a 1-L bottle containing sludge and media at 120

°C for 20 minutes, followed by the addition of sodium azide (1 %) and then BP3. The two bottles (one live and one SC) were incubated at 25 °C in the dark and were systematically shaken and opened inside a fume hood twice per week to allow aeration.

An enrichment culture was initiated with inoculum from the initial flask that showed degradation. The final theoretical BP3 concentration was about 10 µg/mL for Enrichment 1 (the first transfer), but this concentration was increased to a theoretical concentration of 100 µg/mL for Enrichment 2 and subsequent enrichments. To obtain a concentration of 100 µg/mL, 2 mL of a solution of BP3 in acetone (2.5 mg/mL) was added to 100-mL Erlenmeyer flasks containing 2 g of inert sand. The acetone was left to evaporate overnight. Mineral media (50 mL, as above) was added and the flasks were autoclaved. Each month, the cultures were transferred (1% vol/vol) into new flasks with sand and BP3 (see Figure 1 for diagram of the experimental setup). The cultures were incubated at 25 °C in the dark on a platform shaker at 100 rpm.

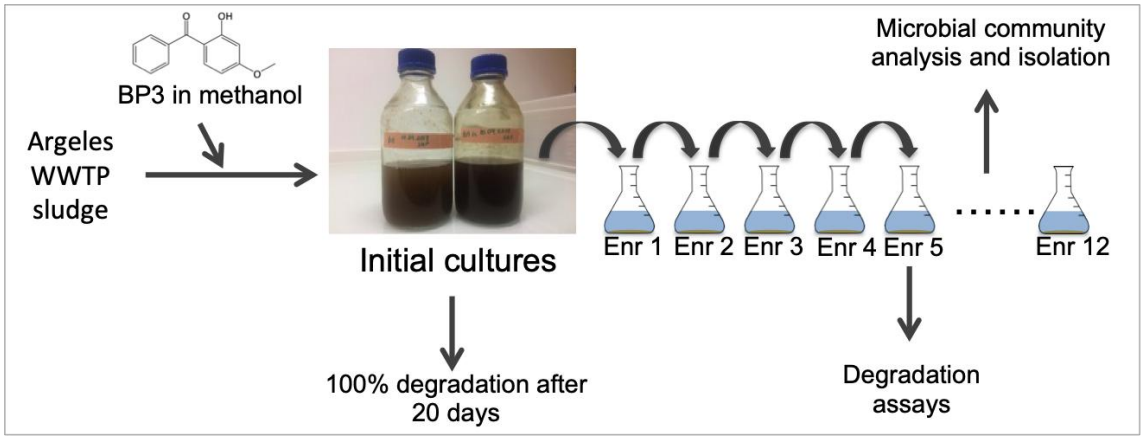
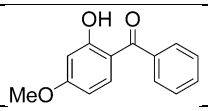
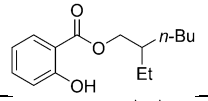
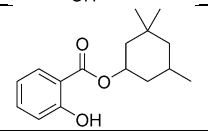
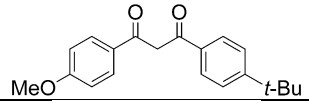
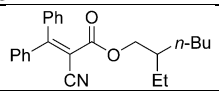
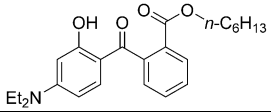
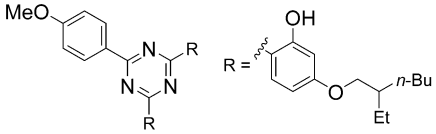
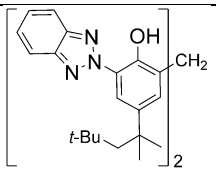
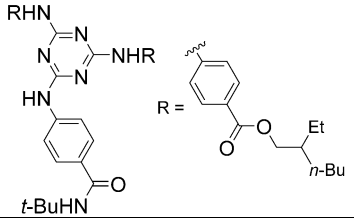
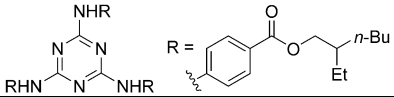


Fig. 1. Schematic of the experimental design.

Table 1

Selected chemical properties and structure of the UV filters used in this study (ordered by increasing molecular weight). Note the abbreviations that are used throughout.

Compound name (abbreviation) [CAS number]	MW	Biodegradation rate[a]	Freshwater solubility at 25 °C (mg/L)[b]	LogP[c]	Chemical structure
Benzophenone-3 (BP3) [131-57-7]	228.2	100 %	130	3.514	
2-Ethylhexyl salicylate (ES) [118-60-5]	250.3	15 % 28 % with R2B or BP3	137	5.335	
Homosalate (HS) [118-56-9]	262.3	18 % with R2B or BP3	< LOD (LOD = 0.85)	5.431	
Butyl methoxydibenzoylmethane (BM) [70356-09-1]	310.4	n.d.	19	5.391	
Octocrylene (OC) [6197-30-4]	361.5	n.d.	9.1	7.083	
Diethylamino hydroxybenzoyl hexyl benzoate (DHHB) [302776-68-7]	397.5	n.d.	Not measured	7.078	
bis-Ethylhexyloxyphenol methoxyphenyl triazine (BEMT) [187393-00-6]	627.8	n.d.	0.3	10.627	
Methylene bis-benzotriazolyl tetramethylbutylphenol (MBBT) [103597-45-1]	658.9	n.d.	< LOD (LOD = 0.04)	15.451	
Diethylhexyl butamido triazone (DBT) [154702-15-5]	766.0	n.d.	0.2	12.004	
Ethylhexyl triazone (ET) [88122-99-0]	823.1	n.d.	Not measured	14.589	

[a] Degradation rate at day 14, when no degradation is seen in SC, and when $p < 0.05$ compared to SC with a Student t-test. n.d. stands for no degradation.

[b] 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)

2.3 Biodegradation assays

Biodegradation assays were conducted using 15-mL glass tubes with Teflon lined caps (Pyrex, Analytic lab, France). The tubes were set up as for the enrichment cultures, but with 0.2 g sand, 3 mL media, and care was taken so that for each batch of degradation assays, the exact same amount of BP3 (around 100 µg/mL) was added to the tubes. Three tubes were sacrificed for each time-point. The tubes were incubated at 25 °C after inoculation. For the enrichment cultures, the inoculum for the assay was 150 µL of supernatant after rigorous mixing of the enrichment culture. Inoculum preparation for the biodegradation assay of strain BP14P was done as follows: The strain was grown in 100 % R2B broth (Thermo Fisher Scientific, Illkirch-Graffenstaden, France) for about 40 h until the culture had reached an optical density at 600nm of approximately 1.0. The cell suspension was centrifuged at 4200 g for 10 min and subsequently washed 2 times with minimal media before a final resuspension in minimal media (4% v/v). The cultures were incubated at 25 °C in the dark on a rotary shaker at 100 rpm.

2.4 BP3 extraction

Two different protocols were used for the extraction of BP3, one for the initial biodegradation experiments and another for the biodegradation assays for enrichment cultures and isolated strains.

For the initial cultures containing WWTP sludge, 5 mL slurry was taken in duplicate from each flask for each timepoint, both live and SC. The slurry was centrifuged (950

x g for 20 min) to separate the aqueous and solid phases. The aqueous and solid phases were then extracted and analyzed separately. The supernatant (aqueous phase) was transferred to a new tube and subjected to liquid/liquid extraction with acidified (0.1 % formic acid) DCM. This was achieved by adding 5 mL acidified DCM to the aqueous sample, mixing by vortexing and shaking overnight at 25 °C in the dark. The DCM fraction was decanted off and dehydrated with Na_2SO_4. The DCM was then evaporated using the HT-4X system (Genevac, Biopharma Technologies, Diemoz, France) and the residue was dissolved in 1 mL of NMP/water (8:2, v/v) before analysis.

The solid phase was extracted as described by Fagervold and coworkers (Fagervold et al., 2019) with modifications. Briefly, the pellet was frozen at –80 °C, lyophilized and extracted twice with 2 ml acidified (0.1% formic acid) DCM/methanol mix (1:1, vol/vol) and sonicated for 10 min. The supernatants were combined, evaporated and re-dissolved in NMP/water (8:2, v/v) before HPLC analysis.

For the biodegradation assays, the whole tube containing sand and liquid media was sacrificed for extraction at each timepoint. DCM (3 mL) containing an exact known amount of MBBT (internal standard) was added to each tube. The tubes were mixed by vortexing twice for 10 seconds, 1 h apart and shaken overnight at 100 rpm at 25 °C in the dark. After a brief sonication, 1 mL of the DCM phase was transferred to a HPLC vial and diluted 20X in DCM before HPLC analysis.

187 *2.5 HPLC analysis*

188 The UV filters were analyzed using an Ultimate 3000™ HPLC system, equipped with
189 a DAD detector (Thermo Fisher Scientific) and a Phenomenex Kinetex Biphenyl 2.6
190 µm, 150 × 4.6 mm column as previously described (Fagervold et al., 2019) with
191 some minor modifications. The injection volume was 5 µl and the samples were
192 diluted in 100% DCM. We expanded the analysis described in Fagervold et al.
193 (2019) to include all targeted UV-filters. The calibration curves and retention times
194 of all the different UV filters (Table 1) were determined as described by Fagervold
195 and coworkers (Fagervold et al., 2019). The recovery rate was above 85 % for the
196 extraction of BP3 in the initial sludge microcosms. The recovery rate for the
197 degradation assays were variable for the different UV filters, ranging from 63 % for
198 ES to 93 % for OC. However, the extraction efficiency was repeatable for each UV
199 filter over time, which is the most important factor for the detection of degradation
200 activity.

201

202 For determination of possible BP3 degradation products, we also performed a
203 UHPLC-MS/MS analyses as described by Stien and coworkers (Stien et al., 2019).
204 The data were acquired using the Trace Finder™ 3.1 software and were processed
205 with FreeStyle™ and Compound Discoverer™ (all from Thermo Fisher Scientific).

206

207 *2.6 Microbial community analysis of enrichment cultures*

208 DNA was extracted using a Maxwell® 16 LEV Blood DNA Kit (Promega,
209 Charbonnières-les-Bains, France) with the Maxwell® 16 MDx Instrument (AS3000;

Promega) following the manufacturer's instructions and with an initial lysing step. Briefly, 2 mL of supernatant was transferred to a microcentrifuge tube and centrifuged at 14 500 x g for 10 minutes. The pellet was resuspended in 300 µL MilliQ water and 300 mL of the lysing buffer from the Maxwell® 16 LEV Blood DNA Kit was added to "lysing matrix B beads" (MP Biochemicals, Illkirch, France) and homogenized for 20 s at 6 m/s on a FastPrep-24™ 5G Instrument (MP biochemicals). After extraction, the DNA was stored at –80 °C until further analysis.

The composition and diversity of the bacterial communities in enrichment cultures were determined by Automated Ribosomal Intergenic Spacer Analysis (ARISA) and by Illumina sequencing (RTL genomics, Texas, USA). The ARISA was performed as described by Fisher et al. (Fisher MM and Triplett EW, 1999) with minor modifications. Briefly, for the PCR we utilized the intergenic spacer primers 1406F and 23SRY (Fisher MM and Triplett EW, 1999) and the KAPA2G Fast HotStart ReadyMix (Roche, Analytic Lab, St Mathieu de Treviers, France) with the following PCR cycling parameters: 94 °C for 3 minutes, 30 cycles of 94 °C for 15 s, 55 °C for 15 s and 72 °C for 30 s, then a final elongation of 72 °C for 1 min. The products were purified, denatured and injected into a 16 capillary Applied Biosystems Sequencer 3130XL (Thermo Fisher Scientific) together with the internal standard MapMarker® X-Rhodamine Labeled 50-1000 bp (Bioventures Inc., Tennessee, USA) for the determination of peak lengths. For the analysis, individual fragment lengths (within a 0.1 bp "error range") were considered as 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. Furthermore,

we only took into account peaks contributing to more than 1 % of the relative peak area. Concerning the Illumina sequencing analysis, the DNA was sent to RTL genomics where paired end, 2×300 bp MiSeq Sequencing was performed using standard conditions of the sequencing provider. We opted for dual indexing and used the bacterial primers 341F (CCTACGGGNGGCWGCAG) and 806R (GACTACHVGGGTATCTAATCC) (Herlemann et al., 2011). The sequences were processed using Qiime2 (Bolyen et al., 2019). Briefly, the sequences were quality filtered, stitched, denoised and clustered using dada2. Representative sequences were classified using the Naive Bayes classifier and the Silva 132 database as a reference. Our two samples (Enr 11 and Enr 12) yielded about 41000 and 47000 sequences, of which 39000 and 44000, respectively, belonged to a single OTU. There were a total of 37 OTUs in the two samples, but most were present at very low relative abundances.

2.7 Strain isolation and sequencing

The supernatant of an actively degrading enrichment culture transferred 7 times (Enrichment 7, or Enr 7) was diluted 100 fold and spread on R2B agar and incubated for 3 days at 25 °C in the dark. Several colony morphologies were observed and each morphotype was re-streaked on R2B agar to ensure purity. These strains were tested for BP3 degradation activity and one positive strain, BP14P, was further characterized. DNA was extracted as described above and sequenced on the same sequencer used for ARISA. Here, we used universal bacterial primers 27F and 1492R in the first PCR and then the internal primers 907R, 804F

and S8 for the sequencing reactions realized by the dideoxy reaction Sanger sequencing using the BigDye™ Terminator v3.1 Cycle Sequencing Kit (Thermo Fischer) and the manufacturer's protocol.

2.8 Flow Cytometry

Strain BP14P cells were counted throughout the degradation assay. The glass tubes were vortexed and 1 mL of cell suspension was transferred to a microcentrifuge tube before adding glutaraldehyde (1% v/v final concentration). The tubes were incubated at 4 °C in the dark for 15 minutes before flash freezing with liquid N_2. The samples were kept at –80 °C until analysis. Samples were defrosted at room temperature and incubated with the nucleic acid stain SYBRGreen I (Molecular Probes) for 15 min at room temperature in the dark before enumeration (Marie et al., 1997) using a FACSCanto flow cytometer (BD-Biosciences) equipped with optics fiber emitted light (407, 488 and 633 nm). Fluorescent 1.002 µm beads (Polysciences Inc., Europe) were added to each sample as an internal standard to normalize cell properties and to compare cell populations. Accurate analyzed volumes and subsequent estimations of cell concentrations were calculated using Becton-Dickinson TrucountTM beads. Total bacterial cells (TBA) were enumerated according to the variations of light scatter properties (relative cell size) and green fluorescence related to nucleic acid content.

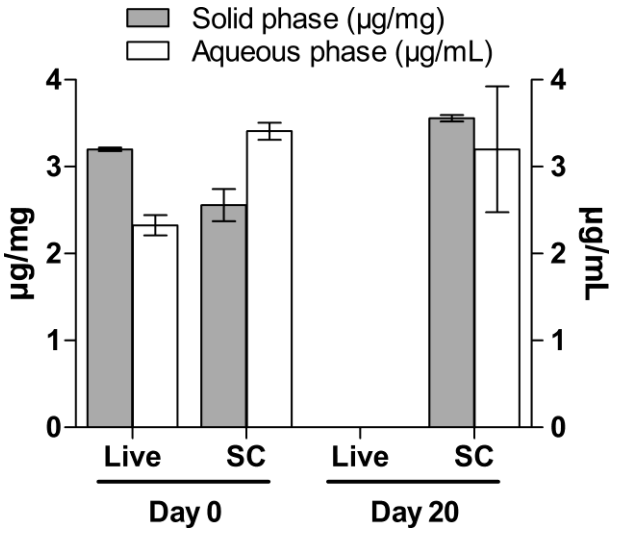
3. Results and discussion

279

280 *3.1 Degradation of BP3 in initial sludge microcosms and enrichment cultures*

281 To detect BP3 degradation, the concentration of BP3 in the amended flask
282 containing active sludge was compared with the amended flask containing sterilized
283 sludge. The theoretical added total BP3 concentration was 10 µg/mL slurry and the
284 slurry contained approximately 2 mg/mL dry matter. We analyzed the aqueous and
285 the solid phase separately and it was clear that some BP3 partitioned onto the solid
286 phase in the slurry (Figure 2). This fraction was bioavailable since we could not
287 detect any BP3 in either the solid or the aqueous phase after 20 days of incubation.
288 The concentration in the SC remained approximately the same after 20 days,
289 showing that this is not an artifact due to abiotic sorption processes. Thus, we
290 observed a complete degradation of BP3 in live cultures compared to the SC after 20
291 days.

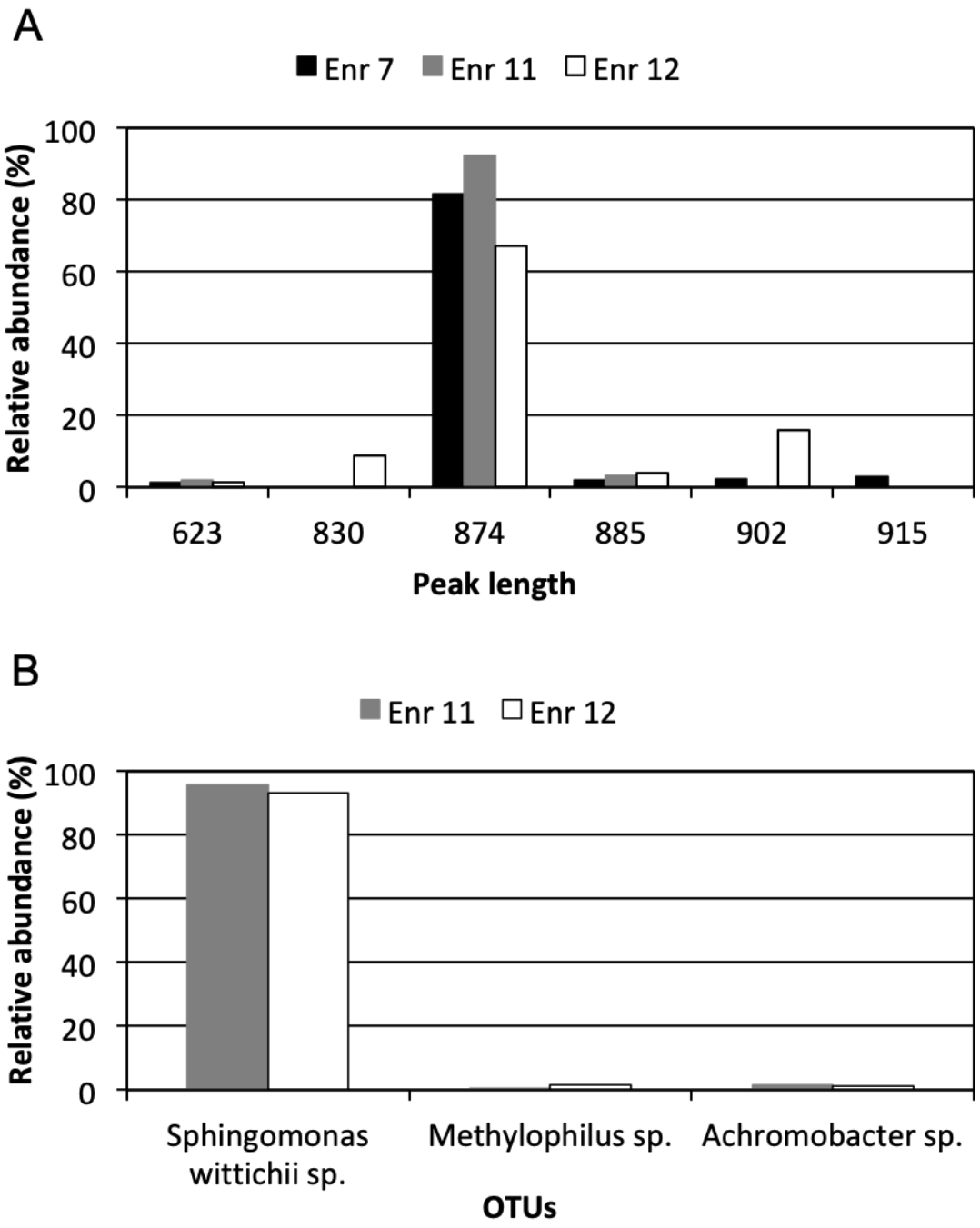
292



293

294 **Fig. 2.** BP3 concentrations in initial microcosms with WWTP sludge. BP3
295 concentrations in the aqueous phase (µg/mL, white bars) and in the solid phase (µ

296 g/mg, grey bars) at day 0 and after 20 days of incubation. Live, active degrading
297 slurries and SC, sterile control.
298
299 The live culture actively degrading BP3 was transferred into an Erlenmeyer flask
300 containing 10 μg/mL BP3 (Enrichment 1, Enr 1). After 30 days, the Enr 1 culture
301 was transferred into a flask containing a higher BP3 concentration (100 μg/mL)
302 (see figure 1), Enr 2. We added sand to the flasks with the hypothesis that it may
303 provide a solid support for the microorganisms. The cultures were transferred
304 every 30 days to further enrich BP3 degrading microorganisms. To maintain a
305 selective pressure on the microbial community, the only available carbon source for
306 the microorganisms was BP3. We could clearly see growth in the Erlenmeyer flask
307 based on the turbidity of the medium.
308
309 After 5 consecutive transfers, we verified the degradation activity of the Enr 5
310 enrichment culture by performing biodegradation assays in separate tubes. After 7
311 days of incubation, we could only detect trace amounts BP3 in the live
312 biodegradation assays, while the SC showed no decrease compared to day-0
313 samples. This was also observed in another biodegradation assay from Enr 9 (after
314 9 transfers) after 5 days. Thus, it was clear that these enriched cultures degrade BP3
315 in a relatively fast manner.
316
317 *3.2 Microbial communities in enrichment cultures*
318



319

320 **Fig. 3.** Analysis of the microbial community diversity in BP3 degrading enrichment

321 cultures. (A) ARISA analysis of enrichment cultures "Enr 7" (black bars), "Enr 11"

322 (grey bars) and "Enr 12" (white bars) where each peak is one "OTU" and the relative

323 abundance is based upon area of each peak. (B) Relative abundance of different

OTUs based upon Illumina sequencing in “Enr 11” (grey bars) and “Enr 12” (white bars).

In order to estimate the diversity and stability of the enrichment cultures degrading BP3, we performed ARISA of several enrichments and confirmed these results with Illumina sequencing. We extracted DNA of cultures at day-4 after transfer and this was done for different transfers (Enr 7, 11 and 12). Briefly, ARISA is based upon the concept that different bacteria have different sized intergenic spacers between the 16S rRNA gene and the 23S rRNA gene on their chromosome. An ARISA profile will give an approximation of the microbial diversity of a sample, with one peak representing one “OTU”. From the ARISA we could see 5 different main peaks (Figure 3A) and it was clear that the enrichment cultures were dominated by one specific bacterium corresponding to a peak length of 874 bp. This was confirmed with the results from the Illumina sequencing, where we detected only 3 major OTUs, with a clear dominance of an OTU identified as *Sphingomonas wittichii* with a relative abundance of about 95 % in both Enr 11 and 12 (Figure 3B). We hypothesized that this strain was responsible for the degradation of BP3 in these enrichment cultures.

3.3 Isolation and identification of the BP3 mineralizing strain BP14P

The most predominant strain in the enrichment cultures, BP14P, was isolated and its degradation capability tested in minimal media (OECD301) and in minimal media

amended with 10 % R2B media. This was done to investigate whether strain BP14P would degrade BP3 when other carbon sources were present.

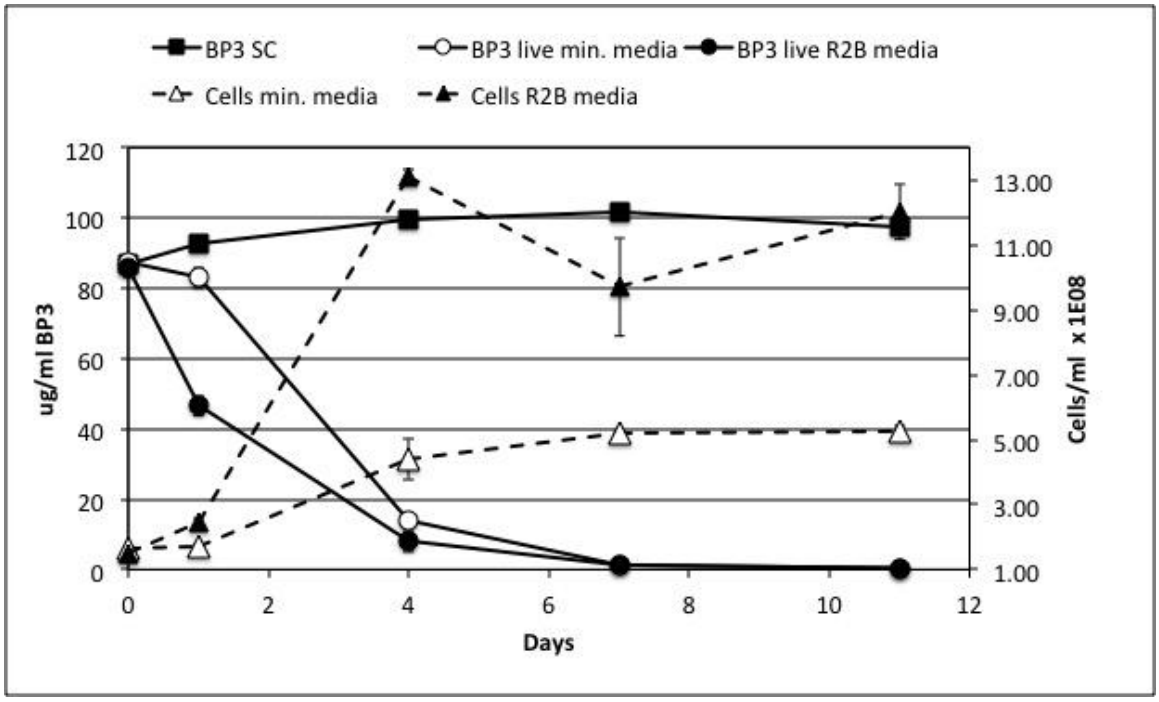


Fig. 4. Degradation of BP3 by *Sphingomonas wittichiii* sp. strain BP14P coupled to growth. BP3 concentrations in sterile controls (black), BP3 concentrations in live cultures with minimal media (open circles), BP3 concentrations in live cultures with minimal media and 10 % R2B media (black circles), cells per ml in cultures with minimal media (open triangles) and cells per ml in cultures with minimal media and 10% R2B media (black triangles).

359 Strain BP14P degraded BP3 relatively fast as we could detect an almost 50%
360 degradation after one day in cultures amended with R2B media, and 90%
361 degradation after 4 days in both cultures with R2B but also in cultures with minimal
362 media (Figure 4). This degradation was clearly linked to growth since the
363 concentration of cells increased with time in cultures where the only carbon source
364 was BP3. We hypothesize that this strain completely mineralized BP3 and used BP3
365 as both carbon and energy source. To the best of our knowledge, this is the first
366 report showing a growth dependent degradation of BP3 in a pure strain.
367
368 A BLAST comparison of the 16S rRNA gene sequence of strain BP14P (1150 bp)
369 showed that it was 100 % identical to *Sphingomonas wittichii* DC-6, isolated from
370 activated sludge (KC410868) and only one base pair different (1149/1150) to the
371 widely studied strain *S. wittichii* RW1 (NR_074268.1). DC-6 can mineralize the
372 herbicides alachlor, acetochlor, and butachlor (Chen et al., 2013) through *N*-
373 dealkylation activity by a Rieske non-heme iron oxygenase system (Chen et al.,
374 2014). *S. wittichii* RW1 was first isolated from an urban river sample (Wittich et al.,
375 1992) and is able to degrade a plethora of aromatic compounds, like chlorinated
376 dibenzo-*p*-dioxins (DDs) and dibenzofurans (DFs) and to grow on DD and DF as sole
377 carbon and energy sources (Wittich et al., 1992). The first step in the degradation of
378 DD and DF is carried out by a dioxygenase and interestingly many of the genes
379 responsible for degradation of aromatic compounds are present on megaplasmids in
380 both strains *Sphingomonas wittichii* RW1 and DC-6 (Armengaud et al., 1998; Cheng

et al., 2019; Miller et al., 2010). Indeed, this feature seems to be common in *Sphingomonas* that degrade xenobiotics (Basta et al., 2004).

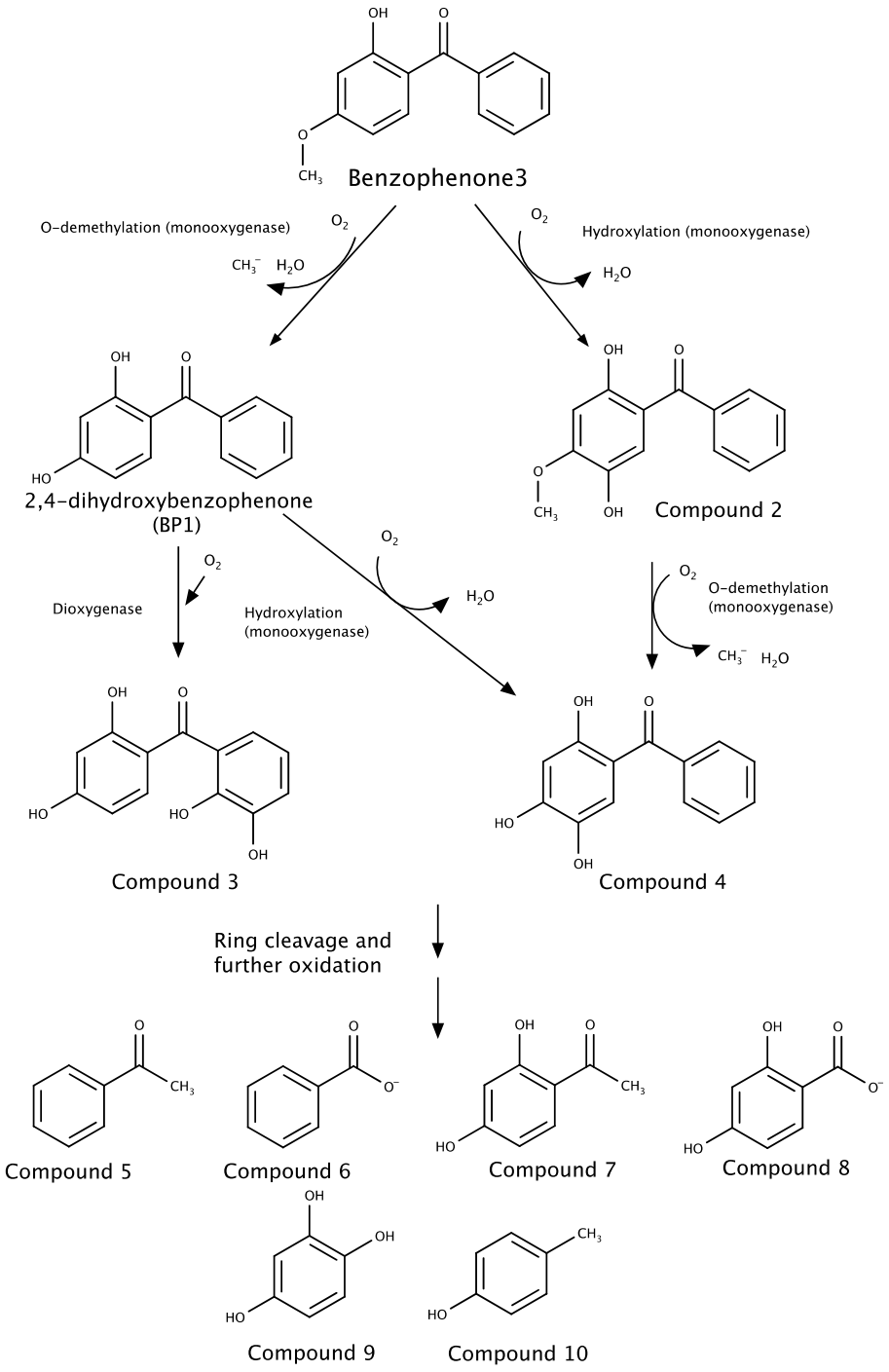
3.4 Putative degradation pathway of BP3

During the HPLC-UV analysis, we could clearly detect an UV absorbing peak at an earlier retention time than BP3 in some samples. To identify this peak and other putative degradation products or intermediates, we analyzed several samples using LC-MS/MS and molecular networks (MN) constructed by means of the Global Natural Products Social Molecular Networking (GNPS) platform (Wang et al., 2016) (https://gnps.ucsd.edu/). GNPS is a MS/MS data community sharing and curation platform. Its MN tool organizes all MS/MS spectra recorded in complex mixtures of compounds by families of analogous compounds, each grouped in one MN. This enables the user to highlight possible degradation products in samples without any presupposition of the chemical structure. In the present MN analysis, the number of similar fragments was set to 2, and the minimum cosine for linking two parent ions was set to 0.7. With these parameters, only one compound clustered with BP3, with a parent mass at *m/z* 215.070 ($[M+H]^+$). This compound was identified as BP1, a BP3 demethylation product (Figure 5). BP1 was detected in high concentrations in day 1 samples of the degradation assay with *S. wittichii* BP14P when R2B was added. At this timepoint, BP3 was already about 50% degraded. This peak was detected only at trace levels in the following timepoints, meaning that BP1 was rapidly degraded. This initial demethylation reaction has been previously reported to be a probable degradation step in activated sludge microcosms (Liu et al., 2012)

404 and as one probable pathway for BP3 degradation by *Methylophilus* sp. strain FP-6

405 (Jin et al., 2019).

406



407

408 **Fig. 5.** Putative degradation pathway of BP3.

409

410 We investigated other possible degradation pathways by submitting the BP3
411 structure to an online pathway prediction tool (Gao et al., 2010) and then searching
412 for particular exact masses of the predicted products (*m/z* of corresponding $[M+H]^+$
413 ions) in all the LC-MS profiles of our samples. Based upon the prediction, another
414 possible first step is a hydroxylation of the benzene ring (Compound **2** in Figure 5).
415 An ion corresponding to this compound was detected in some of our samples, as
416 was compound **4**. However, we did not detect compound **3** in any of our samples.
417 Based upon these results we hypothesize that the main degradation pathway
418 involves an initial demethylation of BP3 to BP1, followed by a hydroxylation to give
419 **4**. However, an initial hydroxylation of BP3 yielding compound **2** and a following
420 demethylation to result in compound **4** might be a minor pathway.
421
422 Jin and colleagues identified other compounds in *Methylophilus* sp. strain FP-6
423 cultures degrading BP3 (Jin et al., 2019). The presence of these compounds, not
424 depicted in Figure 5, suggested a degradation pathway involving hydroxylation of
425 BP3 and ring cleavage, but no demethylation. We did not find any evidence for this
426 pathway in *S. wittichii* BP14P. This might be due to the different nature (co-
427 metabolism versus growth linked) of the degradation process of the two different
428 strains in question.
429
430 Compounds **5**-**9** are examples of possible products after cleavage based upon the
431 prediction system. However, these low molecular weight compounds could not be
432 detected by our LC system that analyzed molecules between 133 Da and 2000 Da.

433 Compound **10** was detected by Liu et al. (2012), but was not predicted by the
434 prediction tool.
435
436 *3.5 UV filter degradation capabilities of strain BP14P are limited*
437 We tested the capability of *S. wittichii* BP14P to degrade other UV filters, listed in
438 Table 1. Indeed, Miller et al. (2010) suggest that one of the reasons why *S. wittichii*
439 RW1 is so successful at degrading substrates with low water solubility might be
440 because of the abundance of TonB-dependent receptors, which can act as
441 transporters of aromatic compounds. Further, because the expression of some
442 dioxygenases (like dioxygenases dxnA1A2) have shown to be substrate dependent
443 (Armengaud et al., 1998), we also added BP3 to parallel assays to investigate
444 whether the putative expression of genes involved in degradation of BP3 can
445 stimulate the degradation of other aromatic compounds. Accordingly, we tested the
446 UV filters listed in Table 1 alone in biodegradation assays, as well as with extra
447 carbon (10% R2B) and with BP3.
448
449 We incubated *S. wittichii* sp. BP14P with the 9 different UV filters for 14 days. BP3
450 was over 95% degraded in all the assays where it was added. However, we detected
451 very little degradation of the other UV filters (Table 1). The only other UV filter that
452 was partially degraded with no other carbon sources present was ES (15 % after 14
453 days). *S. wittichii* BP14P degraded ES slightly more when BP3 and other carbon
454 sources were present, namely 28 % after 14 days. Finally, HS was 18 % degraded
455 after 14 days when BP3 and other carbon sources were present.

To summarize, *S. wittichii* BP14P did not degrade other UV filters to a great extent. This specificity could be due to limited bioavailability of the other UV filters, a limitation of putative transporters, or potentially, a limitation in the genes involved in degradation.

3.6 What drives the specificity of BP3 degradation by strain BP14P?

Other researchers have observed that BP3 can be biodegraded. Liu et al. (2012) found that BP3 was completely degraded after 42 days of incubation in both aerobic and anaerobic microcosms also using inoculum from a WWTP. This suggests that several groups of bacteria could be possible degraders, but this was not investigated by Liu and collegues. *Methylophilus* sp. strain FP-6 was shown to co-metabolically degrade BP3, degrading 15 % of BP3 after 8 days when no other carbon source was provided but degrading BP3 to a higher extent when other carbon sources were present (Jin et al., 2019). Based on the data provided, we believe that *Methylophilus* sp. strain FP-6 degraded BP3 co-metabolically. Strikingly, strain BP14P was very specifically enriched in the cultures through many transfers, suggesting there were no other bacteria present in the sludge that could degrade BP3 in a growth dependent manner or in an equally efficient manner as strain BP14P.

Even though the 16S rRNA gene of strain BP14P was 99.99% and 100% identical to *S. wittichii* RW1 and DC-6, respectively, the functional similarity between these strains remains to be investigated through a more polyphasic approach, including

genome sequencing. Interestingly, *S. wittichii* RW1 has a very specific dioxygenase activity that enables it to degrade DD and DF (Armengaud et al., 1998) and thus we hypothesize that *S. wittichii* BP14P degrades BP3 through the activity of at least one specific mono- or dioxygenase responsible for the initial demethylation (see figure 4) or another limiting step in the degradation pathway. We base this hypothesis on the fact that the mono- or dioxygenase activity needed for BP3 degradation did not seem to be widely distributed as no other bacteria were enriched in these cultures. However, whether it uses a homologue of the *S. wittichii* RW1 enzyme, remains to be investigated.

S. wittichii strains seem to be especially suited to degrade xenobiotics. For example *S. wittichii* RW1 has extreme redundancy in its use of aromatic compound metabolism (Coronado et al., 2012). This group of microorganisms generally contains megaplasmids containing degradation genes and horizontal gene transfer seems to be very common. Importantly, it is well known that *S. wittichii* RW1 can survive and retain its degradation capability in soil when amended for bioremediation purposes (Megharaj et al., 1997). All this makes it particularly fascinating to enlarge the degradation repertoire of this group to include UV filters and BP3.

4. Conclusions and perspectives

The extensive use of BP3 has resulted in ubiquitous BP3 contamination in various environments, including in WWTPs and in many aquatic ecosystems (Fagervold et

al, 2019). For a complete environmental risk assessment of BP3, it is important to understand its fate and all the processes the molecule is subjected to. Degradation, though either co-metabolism or mineralization is an important factor in this assessment. We show here that BP3 can be completely degraded in a few days in WWTPs by *S. wittichii* BP14P. Since BP3 can be utilized by a bacterium as an energy source it then ensues that BP3 has the potential to be degraded in other environmental compartments than WWTP sludge. As a consequence, the long-term accumulation of BP3 in the environment could be less likely than previously assumed. However, once released into marine ecosystems, it is as yet unknown whether BP3 can be degraded. This question should be the subject of future studies, as well as performing similar degradation testing on other sunscreen UV filters, with a priority on the most toxic UV filters including octinoxate, octocrylene and homosalate

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Figure captions

Figure 1. Schematic of the experimental design.

Figure 2. BP3 concentrations in initial microcosms with WWTP sludge. BP3 concentrations in the aqueous phase (µg/mL, white bars) and in the solid phase (µg/mg, grey bars) at day 0 and after 20 days of incubation. Live, active degrading slurries and SC, sterile control.

Figure 3. Analysis of the microbial community diversity in BP3 degrading enrichment cultures. (A) ARISA analysis of enrichment cultures Enr 7 (black bars), Enr 11 (grey bars) and Enr 12 (white bars) where each peak is one OTU and the relative abundance is based upon area of each peak. (B) Relative abundance of different OTUs based upon Illumina sequencing in Enr 11 (grey bars) and Enr 12 (white bars).

Figure 4. Degradation of BP3 by *Sphingomonas wittichiii* sp. strain BP14P (left axis) and growth followed by flow cytometry (right axis). BP3 concentrations in sterile controls (SC, black square), BP3 concentrations in live cultures with minimal media (open circles), BP3 concentrations in live cultures with minimal media supplemented with 10 % R2B media (black circles), cell concentrations of cultures with minimal media (open triangles) or minimal media supplemented with 10% R2B media (black triangles).

Figure 5. Putative degradation pathway of BP3. Hypothesized degradation pathway of BP3 based upon the online prediction tool "EAWAG-BBD Pathway Prediction System"(http://eawag-bbd.ethz.ch/predict/)

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