Environmental persistence assessment of heterocyclic polyaromatic hydrocarbons - Ultimate and primary biodegradability using adapted and non-adapted microbial communities

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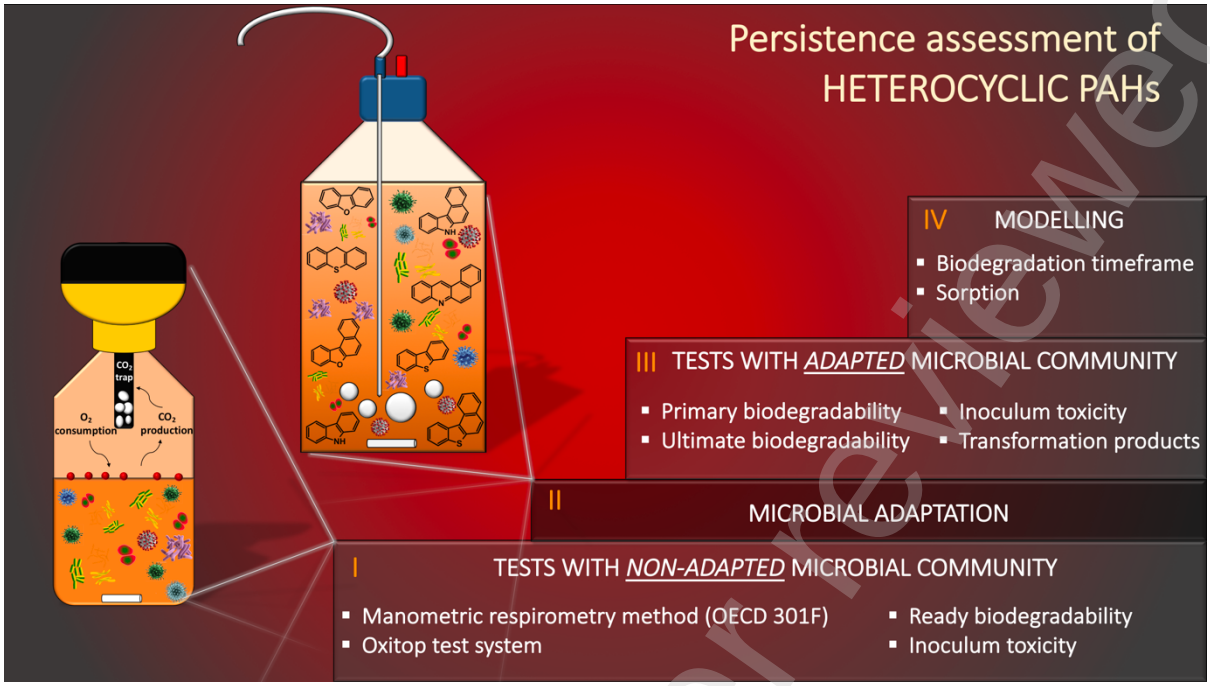
† Supporting information (SI) available.

Abstract

Heterocyclic polyaromatic hydrocarbons (heterocyclic PAHs) are of increasing interest to environmental and human impact assessments due to their abundance and potential persistence in the environment. This study investigated the ultimate and primary biodegradability of ten heterocyclic PAHs, nine of which were found to be non-readily biodegradable. To generate a community capable of degrading such compounds, a bacterial inoculum, isolated from a wastewater treatment plant (WWTP), was adapted to a mixture of heterocyclic PAHs for one-year. Primary biodegradation, ultimate biodegradation, and inoculum toxicity tests were conducted with bacteria sampled at different stages of adaptation. Interestingly, the one-year-adapted community developed the ability to mineralize carbazole, while simultaneously becoming gradually more sensitive to benzo[c]carbazole. In two consecutive primary biodegradation experiments, degradation of four heterocycles was observed. For five compounds, no biodegradation was detected in any of the tests. The findings of this work were decisively compared with those predicted by *in silico* models for biodegradation timeframe and sorption, and it was found that the models were only partially successful in describing these processes. In brief, this study provides insights into the aquatic persistence of a group of representative heterocyclic PAHs, which is highly relevant for the hazard assessment of this class of substances.

Keywords: NSO-heterocyclic compounds, microbial adaptation, inoculum toxicity, ready biodegradability

Graphical abstract



Environmental Implication

Heterocyclic polyaromatic hydrocarbons (heterocyclic PAHs) pose a threat to the environment and to human health and are thus of increasing concern, but they have been somewhat neglected in hazard and fate assessments. Here, the biodegradability of heterocyclic PAHs is reported using ultimate biodegradation, primary biodegradation, microbial adaptation and inoculum toxicity tests. The findings highlight that heterocyclic PAHs have a high risk of persistence which cannot be reliably predicted using models. Further action is therefore urgently needed to closely monitor these chemicals in the environment.

Highlights

- None of the heterocyclic PAHs (≥ 3-rings) studied were readily biodegradable.
- Mineralization of carbazole was facilitated by microbial adaptation.
- Through adaptation, bacteria gradually become more sensitive to benzo[c]carbazole.
- Primary biodegradation of four heterocycles was observed.
- Heterocyclic PAHs have a high risk of persistency.

1. Introduction

In recent decades there has been an increasing interest in understanding the hazard potential of heterocyclic polyaromatic hydrocarbons (heterocyclic PAHs) substituted with nitrogen, sulphur or oxygen heteroatoms. Recently, more attention has been devoted to the monitoring of heterocyclic PAHs, which disperse in the environment concurrently with their homocyclic analogs, originate from the same sources (coal, tar, asphalt, gas plants, etc.) [1,2], and have been detected in surface waters, groundwater, sediments and soils [3–6]. Given their abundance and occurrence, even in areas far from the sources of contamination [5], heterocyclic PAHs are of particular importance for long-term soil and water monitoring programs. Importantly, the heterocycles indole, quinoline and xanthene have been identified as mutagenic [7,8], carbazole as clastogenic [9], acridine, dibenzothiophene, and dibenzofuran as genotoxic [10], and benzonapthothiphene and dibenzocarbazole as bioaccumulative [11,12], suggesting that these compounds pose a high risk, especially if they are also persistent in the environment.

The focus on homocyclic PAHs has, over the years, overshadowed the evaluation of the potentially hazardous heterocycles in this group. For instance, only homocyclic PAHs are included in the list of substances of very high concern for authorisation under the REACH Regulation [13]. More recently, the German Federal Soil Protection and Contaminated Sites Ordinance mandated the monitoring of heterocyclic PAHs, but without specifying the extent of such an analysis (no specific thresholds or clearly defined list of compounds), while 15 heterocyclic PAHs have been identified as priority substances of environmental concern that need to be legally assessed due to their potential to be Persistent, Bioaccumulative and Toxic (PBT) to biota and humans [2].

Persistence is a globally established hazard criterion for chemicals, along with bioaccumulation, toxicity and mobility. In fact, high persistence alone is a significant concern because persistent chemicals will continue accumulate in the environment for as long as they are released. Whether or not they cause immediate adverse effects, the long-term consequences are difficult to predict or even test. Nevertheless, if detrimental effects occur, they can be extremely difficult to reverse. For this reason, there has been recent advocacy for accepting persistence alone as a sufficient criterion for trigerring action [14].

The results of standard OECD biodegradation tests (see SI-S1 for more information on different tests) are often used to assess persistence, and compounds are considered ‘non-persistent’ if they are rapidly AND extensively biodegraded. However, for heterocyclic PAHs, hardly any standard tests have been performed. The results of the few standard tests showed

that the heterocyclic PAHs quinoline, carbazole, dibenzofuran and dibenzothiophene were "not readily biodegradable" [15]. Most of the available biodegradation studies of small heterocyclic PAHs (with 2 or 3 rings) have been carried out using specific strains or mixed bacterial communities isolated from PAH-contaminated sites (and thus adapted to the test compounds) to stimulate biodegradation [16–20]. These studies have often shown that N/O-PAHs are generally more susceptible to biodegradation than S-PAHs and homocyclic PAHs of similar size [3]. The degradation pathways of prevalent NSO-heterocycles such as carbazole, dibenzothiophene and dibenzofuran are well documented [21,22]. However, little is known about larger heterocyclic PAHs and the degradation of heterocycles by non-adapted communites. Thus, the data that could be used to assess their persistence in non-heavily polluted environments or in a regulatory context is missing.

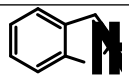
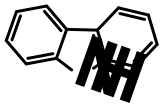
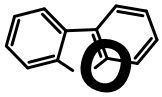
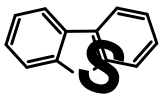
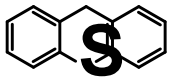
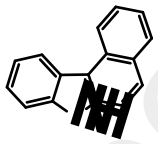
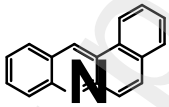
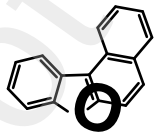
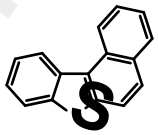
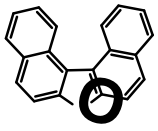
At this point, it should be mentioned that extrapolating data from laboratory tests to real environmental conditions is a challenge due to the difficulties in mirroring environmental complexity in *in vitro* tests, and the bacterial inoculum often remains a "black box" [23], regularly leading to poor reproducibility of test results [24]. Recently, multigenomic (and other –omics) analyses and flow cytometry, have been proposed as tools that can improve the mechanistic understanding of biodegradation processes by allowing in-depth characterization of a community's diversity and function [25,26]. Although microbial adaptation is explicitly not permitted in standard biodegradation testing protocols (e.g., OECD 301, 303), the formation of specific degraders in a community is important for the remediation of contaminated sites (through active indigeneous communities) or as an argument against persistence (*weight of evidence* in chemical hazard assessment).

Microbial communities can exhibit remarkable metabolic plasticity, sometimes developing the ability to degrade xenobiotics after prolonged exposure. Such adaptation of microbial communities has been demonstrated in the laboratory [27] or even on a much larger scale in the example of WWTP communities developing the metabolic capability to degrade artificial sweeteners [28,29] or pharmaceuticals [30]. The mechanisms involved in adaptation may cover population shifts (e.g., preferential growth of specific degraders) or changes in individual organisms (e.g., induction of enzymes or propagation of metabolic capacity by horizontal gene transfer). Induction of metabolic capacity for xenobiotics at the organism or community level may be caused by exposure to the specific xenobiotic or a structurally analogous compound, i.e., cross-adaptation [31]. This is particularly relevant for NSO-PAHs, which usually occur in the environment as mixtures.

Taking into account the significance of persistence for hazard assessment (and also for the regulatory context), the scarcity of data, and the volume of chemicals in circulation, approaches to screening potentially persistent compounds that are more pragmatic than even the lowest-tier biodegradability test are needed to fill the data gap. This can theoretically be rectified by the use of mathematical models, as is the case of other environmental endpoints, i.e., toxicity, bioaccumulation and mobility. As part of the *weight of evidence* approach, QSAR predictions can be used for a preliminary identification of substances with respect to their potential for persistence using information on their degradation half-life. However, the size of the calibration set of existing models is often limited to specific chemical classes, such as hydrocarbons [32], aromatics [33], pesticides [34], herbicides [35], ultimately limiting the application domain and thus the regulatory use of the predicted data.

In our recent study, we conducted a hazard screening for a group of N-, S- and O-containing PAHs made of two to five fused rings [36]. In that investigation, many compounds were classified as potentially (very) persistent based on BIOWIN mathematical models. Here, we experimentally determined the biodegradability of ten heterocyclic PAHs (Table 1) in a comprehensive battery of tests - involving ultimate and primary biodegradation tests at different concentrations and using both adapted and non-adapted inocula from a WWTP. The course of degradation was followed manometrically or by GC/MS. Subsequent LC-MS/MS analysis was performed to screen transformation products. Changes in inoculum composition during the adaptation period of 365 days were observed by flow cytometry analysis. Additionally, toxicity tests were performed with adapted and non-adapted inoculum to investigate whether the chemicals could have caused toxic inhibition of microbial activity. Sorption to biomass was also evaluated as another mechanism of removal from aqueous samples. Lastly, the experimental results were compared with the outcomes of mathematical models predicting biodegradation and sorption to sludge. The results obtained in this work were used to evaluate the persistence potential of heterocylic PAHs in aquatic systems.

Table 1. Environmentally relevant physicochemical properties of heterocyclic PAHs.

Name (abbreviation) **Formula** **MW [g mol^{-1}]**	**Chemical structure**	**log K_{OW}** [a] **log K_{OC}** [a] **S_W** [a]	**VP** **[mm Hg]** [b]
indole (IND) C_8H_7N 117.1		log K_{OW} = 2.14 [c] log K_{OC} = 2.36 [d] S_W = 3560 mg L^{-1} [c]	1.22x10^{-2}
carbazole (CRB) $C_{12}H_9N$ 167.2		log K_{OW} = 3.72 [c] log K_{OC} = 3.35 [c] S_W = 1.5 mg L^{-1} [c]	7.50x10^{-7}
dibenzofuran (DBF) $C_{12}H_8O$ 168.2		log K_{OW} = 4.12 [c] log K_{OC} = 3.49 [c] S_W = 3.48 mg L^{-1} [c]	2.48x10^{-3}
dibenzothiophene (DBT) $C_{12}H_8S$ 184.3		log K_{OW} = 4.61 log K_{OC} = 3.98 S_W = 0.96 mg L^{-1}	2.05x10^{-4}
thioxanthene (TXT) $C_{13}H_{10}O$ 198.3		log K_{OW} = 5.05 log K_{OC} = 4.06 S_W = 0.27 mg L^{-1}	*1.99x10^{-5}*
benzo[c]carbazole (BCRB) $C_{16}H_{11}N$ 217.3		log K_{OW} = 5.22 log K_{OC} = 4.84 S_W = 0.80 mg L^{-1}	*2.38x10^{-7}*
benz[a]acridine (BACR) $C_{17}H_{11}N$ 229.3		log K_{OW} = 4.48 [c] log K_{OC} = 4.39 [c] S_W = *1.12* mg L^{-1} [e]	*5.34x10^{-7}*
benzo[b]naphtho[1,2-d]furan (BNF) $C_{16}H_{10}O$ 218.3		log K_{OW} = 5.60 log K_{OC} = 4.98 S_W = 0.25 mg L^{-1}	*2.35x10^{-6}*
benzo[b]naphtho[1,2-d]thiophene (BNT) $C_{16}H_{10}S$ 234.3		log K_{OW} = 5.75 log K_{OC} = 5.50 S_W = 0.026 mg L^{-1}	1.14x10^{-7}
dinaphtho[2,1-b:1',2'-d]furan (DNF) $C_{20}H_{12}O$ 268.3		log K_{OW} = 6.89 log K_{OC} = 6.61 S_W = 0.0015 mg L^{-1}	*1.47x10^{-8}*

Abbreviations: MW – molecular weight, K_{OW} – n-octanol-water partition coefficient, K_{OC} – organic carbon-water partition coefficient, S_W – water solubility, VP – vapor pressure.

Predicted values are given in *italics.*

[a] Measured data for log K_{OW}, log K_{OC} and S_W obtained from our previous work [36], if otherwise is not indicated, i.e., sources: [c] [37], [d] [38], [e] [39].

[b] Measured data for VP were taken from EPA Comptox chemicals dashboard database [37] if available, otherwise predicted by the MPBPVP model [40].

2. Materials and Methods

2.1. Chemicals

Carbazole (CRB, 96%, CAS# 86-74-8), dibenzothiophene (DBT, 98%, CAS# 132-65-0), and thioxanthene (TXT, 98%, CAS# 261-31-4) were purchased from Acros Organics (Geel, Belgium). Dibenzo[b,d]furan (DBF, 98%, CAS# 132-64-9), 7H-benzo[c]carbazole (BCRB, 97%, CAS# 205-25-4), benzo[b]naphtho[1,2-d]thiophene (BNT, 97%, CAS# 205-43-6), benzo[b]naphtho[1,2-d]furan (BNF, >98%, CAS# 205-39-0), and dinaphtho[2,1-b:1',2'-d]furan (DNF, 97%, CAS# 194-63-8) were obtained from BLD Pharmatech GmbH (Kaiserslautern, Germany). Benzo[a]acridine (BACR, 99.8%, BCR®-157, CAS# 225-11-6) was purchased from the European Commission Joint Research Center (Geel, Belgium). Indole (IND, CAS# 120-72-9) was also tested as a reference compound. Fluorene (FLR, 98%, CAS# 86-73-7) and benzo[a]anthracene (BANT, 99%, CAS# 56-55-3) were purchased from Sigma-Aldrich (Steinheim, Germany), while pyrene (PYR, 99%) was from Dr. Ehrenstorfer (Augsburg, Germany). D(+)-glucose (anhydrous), sodium benzoate, and sodium azide (≥99%) were obtained from Merck KGaA (Darmstadt, Germany). N-allylthiourea (98%) was obtained from Sigma Aldrich (Steinheim, Germany). Carbazole-d8 (CRB-d8, 98%, in acetone, Dr. Ehrenstorfer), dibenzofuran-d8 (DBF-d8, 96%, in methanol, Neochema), dibenzothiophene-d8 (DBT-d8, 96%, in methanol, Neochema), fluoranthene (98%, Sigma-Aldrich) and benzo[k]fluroanthene (99%, in acetone, Restek) were used as surrogate standards in extraction.

2.2. Biodegradation tests

A timeline of a series of experiments conducted in this study and a detailed test scheme are presented in Figure 1 and Figure S2, respectively.

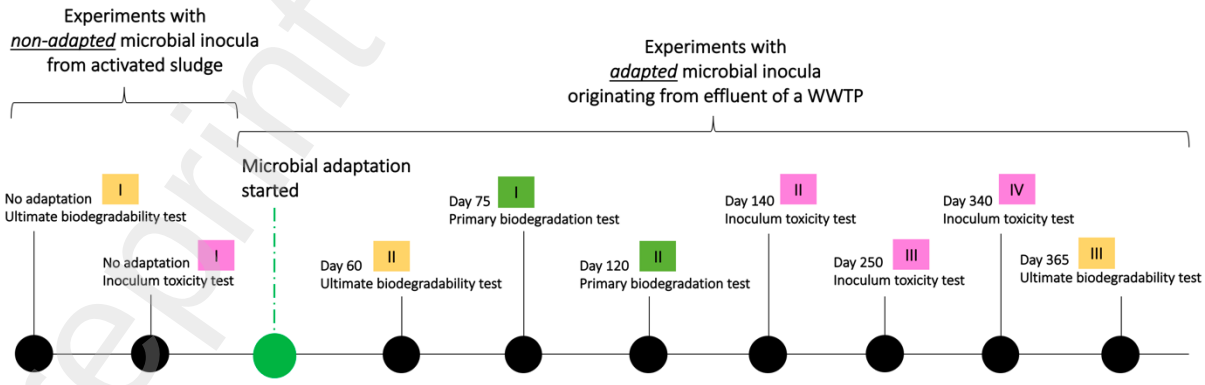


Fig. 1. Timeline of biodegradation experiments conducted in this study. Tests of the same type are marked with the same color, with each test described below or in SI file section S1.

2.2.1. Ultimate biodegradability

The ultimate biodegradability of the heterocyclic PAHs was measured according to OECD guideline 301F (the so-called manometric respirometry method) [41] using the OxiTop® test system (WTW, Weilheim, Germany), which determines the complete biodegradation (mineralization) of organic substances in a closed respirometer filled with aqueous medium and air under automated thermostatic conditions. In the OxiTop® system, biological oxygen demand (BOD) measurements were recorded based on the pressure changes in the test bottles.

Activated sludge from an aeration tank of a municipal WWTP in Dresden, Germany, sampled in December 2020, was the inoculum source. The supernatant - the inoculum - was collected by sequential sedimentation and resuspension of flocs in tap water. The inoculum was then preconditioned by aeration for 3 days to allow the microbial community to consume the residual organic matter, to reduce the blank values and ensure high oxygen levels prior to testing. The total suspended solids (TSS) content of the inoculum was measured, and the inoculum was diluted with tap water to achieve a final concentration of 30 mg TSS L^{-1}. The number of live aerobic bacteria was estimated by counting colonies on agar plates (product# 535102B, VWR). Subsequently, the diluted inoculum was supplemented with minerals to achieve final concentrations of 8.5 mg L^{-1} KH_2PO_4, 21.75 mg L^{-1} K_2HPO_4, 33.4 mg L^{-1} $Na_2HPO_4*2H_2O$, 0.5 mg L^{-1} NH_4Cl, 36.4 mg L^{-1} $CaCl_2*2H_2O$, 22.5 mg L^{-1} $MgSO_4*7H_2O$ and 0.25 mg L^{-1} $FeCl_3*6H_2O$ (OECD 301F). A nitrification inhibitor (n-allylthiourea, 5 mg L^{-1}) was added to avoid possible oxygen consumption by nitrification. The test compounds were added to the test bottles as solids (14.6 to 15.6 mg L^{-1}) to yield a BOD of 40 mg O_2 L^{-1}. Two or three replicates were run for each test substance, accompanied by three blank samples to account for internal cellular respiration and two positive controls containing sodium benzoate. The tests were run for 28 days under constant stirring at 20 ± 1 °C in amber glass bottles.

The tests were accepted as valid if (i) the BOD of the blank samples after 28 days was less than one-quarter of the BOD of the test compounds, i.e., <10 mg O_2 L^{-1}, (ii) the positive control was degraded by more than 60% in the first 14 days, and (iii) the difference in biodegradability between replicates at the end of the test or at plateau was within 20% [42]. It should be noted that the first criterion was more stringent than the criterion suggested by OECD 301F, where the BOD blank consumption was set to a maximum of 60 mg O_2 L^{-1}. However, since a total BOD of 40 mg O_2 L^{-1} was used in our test system, this criterion was not applicable to our biodegradation scenario and was therefore modified. The manometric respirometry test was performed three times, each under different experimental conditions with a concentration of the test substance corresponding to the BOD of:

(1) 40 mg O_2 L^{-1} using non-adapted microbial community from activated sludge
(2) 40 mg O_2 L^{-1} using the adaptation culture sampled on day 60
(3) 10 mg O_2 L^{-1} using the adaptation culture sampled on day 365

All tests met the validity criteria. A schematic representation of the OxiTop® test bottle and the principle chemical reactions is shown in Figure S3.

2.2.2. Microbial community toxic inhibition test

The manometric respirometry method demands relatively high concentrations of test substances, which can lead to inhibition of the microbial community or mass transfer problems, resulting in false negative biodegradability results (biodegradable compounds cannot be identified as such) [43]. This is particularly critical for heterocyclic PAHs, since they were added to the test systems at concentrations above their solubility limit to achieve the sensitivity required by the method. Therefore, the inhibitory effect of the heterocyclic PAHs on the inoculum was determined by performing a toxicity test, namely a glucose biodegradation inhibition test, using the OxiTop® system. The test was performed by adding the test substance at a concentration identical to that used for ready biodegradability testing and an easily biodegradable reference compound (D(+)–glucose) to the same test vessel, providing 40 mg O_2 L^{-1} from the test substance and 40 mg O_2 L^{-1} from the glucose, so that the total BOD of each test vessel was 80 mg O_2 L^{-1}. In some cases, the concentration of the test substance was reduced to 10 mg O_2 L^{-1} to check whether it still had an inhibitory effect on microbes at lower concentrations, while the glucose concentration always remained the same (40 mg O_2 L^{-1}). The oxygen consumption of the test vessels was monitored for 14 days in four experiments, each under different experimental conditions with a concentration of the test substance corresponding to the BOD of:

(1) 40 mg O_2 L^{-1} using a non-adapted microbial community from activated sludge
(2) 40 mg O_2 L^{-1} using the adaptation culture sampled on day 140
(3) 10 mg O_2 L^{-1} using the adaptation culture sampled on day 250
(4) 10 mg O_2 L^{-1} using the adaptation culture sampled on day 340

For each test substance and glucose mixture, two or three replicates were run in parallel, supplemented with four blank samples and three controls containing only glucose. In a toxicity test containing both the test substance and glucose, if 25% inhibition occurred within 14 days compared to glucose controls, the test substance was classified as “inhibitory.”

2.2.3. Microbial adaptation

The effluent from a WWTP in Dresden, Germany was collected in January 2021 and the coarse particles were allowed to settle after several washing steps. The dry mass of the supernatant containing the formerly suspended microbial community was measured (0.7 g L^{-1}). The inoculum was transferred to three reactors, each equipped with magnetic stirring and aeration with air pumps. Subsequently, the inoculum was fed with synthetic sludge (160 mg L^{-1} peptone, 110 mg L^{-1} meat extract, 30 mg L^{-1} urea, 7 mg L^{-1} NaCl, 4 mg L^{-1} $CaCl_2.2H_2O$, 2 mg L^{-1} $MgSO_4.7H_2O$ and 28 mg L^{-1} K_2HPO_4, in deionized water, pH 7.5) prepared according to OECD guideline 209 to boost microbial growth [44]. A mixture of heterocyclic PAHs, including CRB, DBF, DBT, TXT, BACR, BCRB, BNF, and BNT, was prepared in methanol. DNF was excluded from this mixture due to its very low water solubility (1.5 µg L^{-1}), which requires a large sampling volume for the extractions. Since heterocyclic PAHs sorbed significantly to the organic matter in the microbial reactors, the sorptive capacity had to be exceeded first to allow for reliable measurement in liquid phase. Therefore, the mixture containing 30 µg L^{-1} of each test compound in methanol, was spiked into each adaptation culture reactor every 4 days until the concentrations of the test compounds exceeded the LOQ of the GC/MS method (approximately 5-9 µg L^{-1}), which occurred on day 30. Subsequently, the flocs were resettled, and supernatant was collected again as the microbial density increased significantly after the addition of synthetic sludge that could further limit the bioavailability of test compounds. From day 30 onwards, the reactors were spiked with 30 µg L^{-1} test substance mixture in methanol once a week. The microbial community was sampled for the tests one week after the final feeding to lower the BOD in the blanks. Samples were taken periodically from each reactor to check pH, conductivity, DOC and concentration of heterocyclic PAHs. Adaptation cultures were fed with synthetic sludge at low concentrations when the DOC in the reactors fell below 40 mg L^{-1} (generally once a month). The microbial communities were adapted to heterocyclic PAHs over 365 days, with sampling of the microbial community at various stages of the adaptation period in order to conduct the biodegradation tests.

2.2.4. Primary biodegradation

The microbial community adapted to the test compounds was sampled at days 75 and 120 and used as the inoculum source for the two consecutive primary biodegradation experiments. The dry mass of the adapted inoculum was measured (2 g L^{-1}), then diluted with tap water to obtain a concentration of 0.5 g TSS L^{-1} and supplemented with the mineral medium described in section 2.2.1. Two replicates were run for the blank and positive control (sodium benzoate), accompanied by three replicates for the mixture of the test compounds (CRB, DBF, DBT, TXT,

BCRB, BACR, BNF, and BNT), each with a final volume of 200 mL. The test compounds were added as a mixture in methanol, resulting in a theoretical concentration of each compound equal to 0.16 mg L^{-1}, at which all compounds remained below their water solubility, except BNT (S_W=0.26 µg L^{-1}), which exceed its solubility with insoluble solid/liquid fractions. The concentration of the test compounds was chosen to allow reliable measurement of a 90% reduction in concentration during the test. The concentration of sodium benzoate in the positive control samples was 1.6 mg L^{-1} (close to the chemical concentrations at which it can also be measured by HPLC/DAD). Blank samples contained medium and inoculum, but no test substance or benzoate. Each type of sample (test samples, positive controls, and blanks) contained 0.8% methanol as a result of spiking with test compounds (for test samples and positive controls) or added for the sake of consistency (the blanks). Samples were stirred continually and were loosely covered to allow gas exchange while limiting evaporation.

The primary biodegradation experiment was performed twice in the same way: the first primary degradation test was started using inoculum sampled from the adaptation reactors on day 75, and the second primary degradation test used inoculum sampled from the same vessel on day 120. Additionally, abiotic controls were added in the second test to quantify concentration losses that do not occur from microbial degradation but from abiotic processes such as sorption to biomass. Sodium azide (15 g L^{-1}) was added to each abiotic vessel to kill bacteria [45]. Both first and second primary degradation tests lasted five weeks. During this time, 10 mL samples were collected twice a week from each vessel and the pH and conductivity of each sample were measured. In addition, the DOC content of the collected samples was measured every week. Samples containing test compounds were centrifuged and 2 mL of the supernatant was immediately extracted for GC/MS analysis, while positive controls were first centrifuged, filtered, and then frozen at -18 °C until analysis by HPLC/DAD.

2.3. Analytical methods

2.3.1. Liquid-liquid extraction and GC/MS analysis

Heterocyclic PAHs were quantified by liquid–liquid extraction followed by GC/MS analysis using gas chromatography (GC system 7890A) and a mass selective detector (MS 5975C, Agilent, Waldbronn, Germany). Aqueous 2 mL samples were collected from the supernatant after centrifugation and transferred to an extraction vial. Surrogate standards were spiked into the samples and then extracted twice with 1 mL of hexane. The hexane extracts were combined and dried with Na_2SO_4. Afterwards, 900 µL of the hexane extract was transferred to a GC vial and 50 µL of internal standards (fluorene or pyrene, 1 mg L^{-1} in hexane) were added. For

GC/MS analysis, samples (1 µL) were injected using an autosampler in pressure-pulsed splitless mode. The capillary column (Restek Rxi-5ms (5% diphenyl/95% dimethyl siloxane, 30 m x 0.25 mm; 0.25 µm film thickness) was run at a flow rate of 1.3 mL min^{-1} with helium as the carrier gas. The parameters of the GC method were as follows: Inlet temperature: 80 °C; oven program: 100 °C, hold for 1.8 min, ramp to 320 °C at 50 °C min^{-1}, hold for 1.4 min. The results were analysed using Chemstation software (Agilent Technologies, Germany). The concentrations of each component in the extract were determined using the peak area normalized with the corresponding internal standard (fluorene or pyrene) and a five-point calibration series. The list of surrogate standards, internal standards, limit of detection (LOD) and limit of quantification (LOQ) of the GC/MS method is given in Table S3 for each main compound.

2.3.2. HPLC/DAD analysis

Degradation of sodium benzoate in biotic positive controls of the primary biodegradation test was quantified by HPLC/DAD using a Gemini-NX 3u C18 (110A, 150 x 2 mm) column. Samples were first prepared for analysis by centrifugation followed by filtration (0.45 µm). Isocratic elution was performed with 80% eluent A (950 mL water + 50 mL acetonitrile + 150 µL formic acid) and 20% eluent B (1000 mL acetonitrile + 150 µL formic acid) at a flow rate of 0.5 mL min^{-1}. The compound was monitored at 230 nm and analysis was begun following the loop injection of a 100 µL sample. A calibration curve was constructed with eleven concentration points (0.1 – 1.1 mg L^{-1}) prepared in the test medium.

2.3.3. Transformation product analysis with LC-MS/MS

Analysis of transformation products was conducted using the QTRAP 6500^{+} LC-MS/MS system (AB Sciex Instruments). The samples were analysed with a Kinetex EVO C18 column (100 x 2.1 mm I.D., 1.6 µm) at 40 °C and a flow rate of 0.3 mL min^{-1} using a binary mobile phase (A–water and B–acetonitrile, both containing 0.04% acetic acid). The elution gradient consisted of 100% A from 0 to 1 min, 95% A from 1 to 4.5 min, 2% A from 4.5 to 7.2 min, and 95% A from 7.2 to 8.3 min. The analysis was initially performed in scan mode for m/z between 90 and 350 with a scan rate of 200 Da s^{-1}. The electrospray ionization (ESI) source was used in positive and negative ion modes at an ion source temperature of 500 °C and an ion spray voltage (IS) of 5.5 kV. The nebulizing gas pressure was 50 psi, while the declustering potential and entrance potential were 100 and 10 volts, respectively. Standards of each parent compound (five-point calibration series) prepared in methanol and sample matrix (1:1, v/v) were injected to determine their retention times and adduct ions. Target analysis was then

performed to identify selected product and precursor ions that differed from the parent compounds using 46 eV collision energy and 10 eV cell exit potentials.

2.3.4. Flow cytometric analysis

A total of six samples, taken at different stages of the adaptation phase were analysed by flow cytometry to monitor the changes in community composition during the adaptation phase. Harvested cells and fixated cells were stored at -20 °C until analysis, and stained with 4',6-diamidino-2-phenylindole (DAPI) for flow cytometric measurement as described by Li *et al.* [46]. Samples were measured with the BD Influx v7 Sorter (Becton, Dickinson and Company, Franklin Lakes, NJ, USA). Monodisperse beads with a size between 0.5 and 1 μm were used to align the flow cytometer and ensure identical daily machine settings. Results were visualized by choosing DAPI fluorescence versus forward scatter (FSC) in 2D plots representing cytometric fingerprints [47]. DAPI provides information on DNA content, and FSC provides information related to cell size. A cell gate was created, which comprised 200,000 cells per measurement. The cell gate excluded calibration beads, unstained particles and instrumental noise.

2.3.5. DOC analysis

The dissolved organic carbon (DOC) content in samples collected during maintenance of the adaptation culture and primary biodegradation tests was measured using a TOC-V_{CPN} analyser (ASI-V, Shimadzu). The aqueous phase samples were first centrifuged, filtered (0.45 μm), diluted 1:30 with MilliQ® water, and then acidified to pH 2 with hydrochloric acid to remove inorganic carbon prior to measurements. Samples collected from the adaptation culture reactors were diluted one to three times to remain within the calibration range (1-100 mg DOC L^{-1}).

2.3.6. Statistical analysis

The experiments were performed in duplicate or triplicate. Data in the graphs were expressed as average values ± standard deviations. The significance of difference between treatments in glucose inhibition tests were determined using Student's t-test.

2.3.7. Predictive models

US EPA EPISuite™ v4.11 was used to estimate the fate of the heterocyclic PAHs in terms of (i) biodegradation under aerobic conditions using the BIOWIN models 3 to 6, (ii) degradation half-lives derived from the BIOWIN3 model results according to the work of Aronson et. al. [48] and (iii) removal by sorption in a typical sewage treatment plant using the STPWIN model. To improve the correctness of the predictions, the S_W, VP and K_{OW} of the test compounds

(given in Table 1) were manually entered into the STPWIN model. The models were explained in detail in SI file Section S2.

3. Results and discussion

The results of the biodegradability tests are shown in Figures 2-4. The initial microbial community conditions of each test are given in Table S4.

3.1. Inoculum toxicity tests at different concentrations using adapted and non-adapted microbial inocula

As relatively high concentrations of the test chemicals are used in the manometric respirometry test, due to the low sensitivity of the method, it is possible that the lack of degradation is caused by toxic inhibition of the inoculum, resulting in false negative biodegradation results [49]. To test this hypothesis, and also to check the health status of the adaptation culture in terms of tolerance to heterocycles, four consecutive inoculum toxicity tests were executed. The first two tests were performed using 15 mg L^{-1} exposure concentrations with non-adapted and 140-day-adapted inocula. The concentrations of the test chemicals were then reduced by a factor of four (to 3.4–3.9 mg L^{-1}) for the third and fourth inoculum toxicity tests performed with the 250- and 340-day-adapted inocula. The glucose concentration was 13.7 mg L^{-1} in all experiments. In addition to the heterocyclic PAHs, FLR and BANT were also tested to investigate the toxic influence of homocyclic PAHs, which are structurally similar to the heterocycles studied. The results of the toxicity tests are presented thoroughly in Figure S4.

In all tests, glucose degradation in the positive controls was very rapid (≥60% within 14 days) and met the validity criteria. None of the compounds were found to be inhibitory to bacteria with no or low levels of inhibition (<25%) being statistically insignificant (p value<0.05), except for BCRB (significant only in the last test). Surprisingly, the adaptation culture became increasingly sensitive to the presence of BCRB, i.e., glucose degradation was inhibited at levels of 11%, 21%, 28%, and 51% at each successive step (Figure 2); this despite the fact that bacteria were exposed to four times lower concentrations of chemicals in the final two tests. The plots shown in Figure 2 indicate that glucose degradation goes through two phases: a rapid initial phase unaffected by BCRB (usually complete after 4 days; the end of this period is characterized by a significant reduction in the rate of glucose degradation) and a second phase, characterized by a further lag phase (particularly noticeable in c) and then a much lower rate of glucose degradation and that does not reach a plateau within 14 days. BCRB appears to inhibit this second phase in all toxicity tests, with inhibition increasing in parallel with

adaptation. It should also be noted that BNF inhibited glucose degradation by approximately 25% in some toxicity tests (Table S5) but, as the inhibitions were not statistically significant, the compound was not labelled as inhibitory.

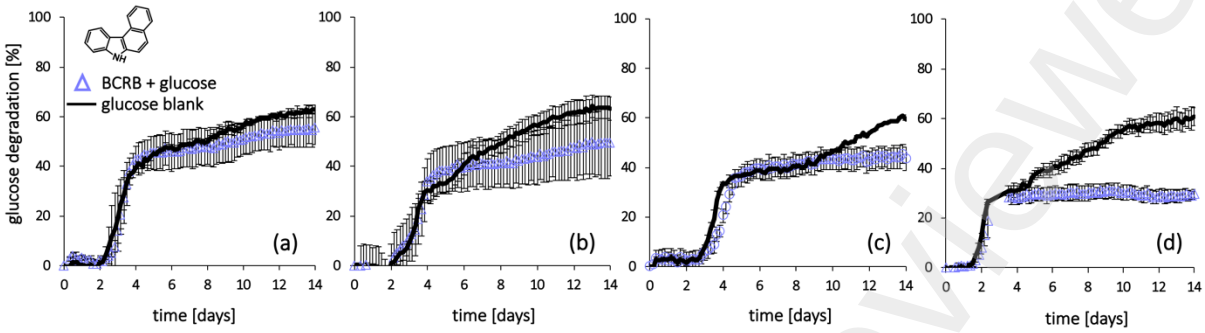


Fig. 2. Inhibition of glucose degradation in the presence of BCRB at exposure concentrations of (**a**) 15 mg L^{-1} with non-adapted inocula (test-1), (**b**) 15 mg L^{-1} with 140-day-adapted inocula (test-2), (**c**) 3.8 mg L^{-1} with 250-day-adapted inocula (test-3), and (**d**) 3.8 mg L^{-1} with 340-day-adapted inocula (test-4). The glucose concentration was 13.7 mg L^{-1} in all experiments. Error bars represent the standard deviation (n=3).

3.2. Ultimate biodegradability tests using adapted and nonadapted microbial inocula

The ultimate biodegradability of heterocyclic PAHs was tested using the manometric respirometry method using the OxiTop® test system with a microbial community of activated sludge. The concentration of the test compounds in the bottles was approximately 15 mg L^{-1}. As poor degradability was expected (due to both the inherent properties of the chemicals and the expected poor mass transfer), the test was extended to a maximum of 46 days to allow degradation to reach a plateau if it had already begun. The inoculum was metabolically active, as evidenced by the fast degradation of sodium benzoate, however, no oxygen consumption was observed in the manometric system in the presence of the heterocyclic PAHs (≥3-rings) and therefore no degradation was measured within 28 days (Figure S5). Accordingly, the compounds were classified as "not readily biodegradable" - similar to the results of the Japanese MITI test for CRB, DBF, and DBT [15]. Following the 30-day incubation period, biodegradation was observed in one of the replicates of TXT and BCRB, reaching 18% and 12%, respectively, on day 46 (Figure S6). Such a slow increase in oxygen consumption could indicate the proliferation of slowly growing degraders. In the same test, conducted with activated sludge, the degradability of DBT was further investigated using an inoculum derived from the effluent of the same WWTP. No degradation of DBT occurred in activated sludge inoculum, but 17% degradation was measured by day 46 in one of the replicates containing effluent inoculum (Figure S6).

Microbial adaptation or evolution can be a critical factor, both in the assessment of biodegradation under laboratory conditions and in *in situ* degradation in the environment. To investigate whether pre-exposure allows adaptation and/or cross-adaptation, an inoculum derived from the effluent of a WWTP was adapted to a mixture of heterocyclic PAHs at low concentrations (adaptation culture). Between days 30 and 60, a decrease in chemical concentrations and DOC content was observed in the adaptation culture. Accordingly, a second manometric respirometry experiment was performed with the inocula sampled from the adaptation culture reactor on day 60, the other experimental conditions being identical to those of the first run. In addition to CRB, DBF, DBT, TXT, BCRB, BACR, BNF, BNT and DNF, a readily degradable heterocyclic PAH, indole [15], was also tested as a reference substance. At the end of the test, only indole was found to be degradable, achieving 88%, 77% and 75% mineralization (80% on average) within 28 days (Figure 3-a). None of the other heterocyclic PAHs were found to undergo biodegradation (results not shown).

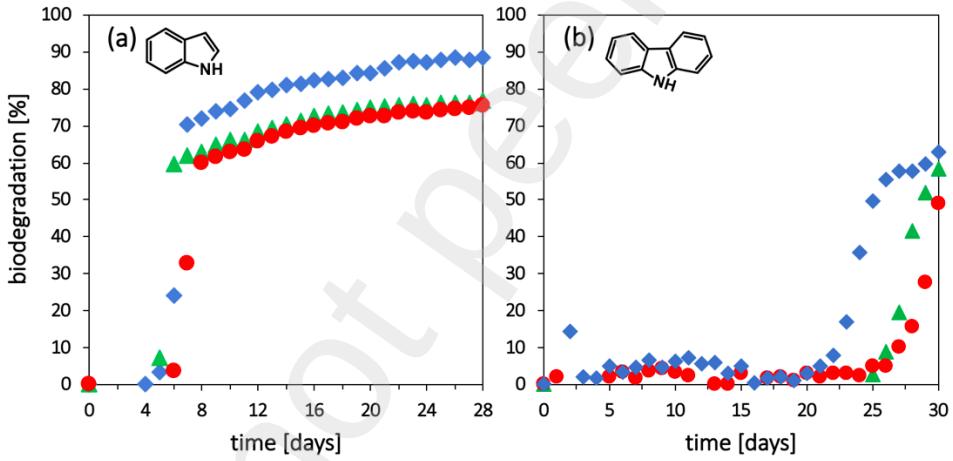


Fig. 3. Mineralization of **(a)** indole (16.3 mg L^{-1}) and **(b)** carbazole (3.9 mg L^{-1}) using an inoculum derived from effluent of a WWTP pre-exposed to test compounds for 60 days and 365 days, respectively. Triplicates are shown in different colours.

Due to the lack of degradation, the adaptation period was extended to one year and the mineralization of heterocycles was re-evaluated by lowering the test concentrations four fold (to 3.4 – 3.9 mg L^{-1}) with the bacteria sampled from the adaptation culture reactor on day 365. The degradation of CRB began after a long lag phase (20–25 days) and quickly reached 57% (average, n=3) by day 30 (Figure 3-b), indicating that CRB can at least be classified as an inherently biodegradable compound. Other heterocyclic PAHs (except indole) were not degraded; moreover, BOD in the presence of TXT, BCRB, BACR and DNF was lower than in the blank samples, suggesting that even the internal cellular respiration of the microorganisms

was marginally hindered, but statistically negligible (Figure S7), meeting the results of the toxic inhibition tests.

In summary, none of the heterocyclic PAHs (≥3-rings) could pass the ready biodegradability criteria and therefore there is a high likelihood that the compounds would not be rapidly and completely degradable. Given the influence of adaptation, and perhaps the somewhat lower chemical concentration (but still exceeding the solubility limit), the adapted bacteria could degrade CRB, which was then considered inherently biodegradable.

3.3. Primary biodegradation

Since no mineralisation occurred during the 28-day ultimate biodegradability test with a non-adapted community, but signs of degradation were evident when the duration of the test was extended, we investigated the primary biodegradability of heterocycles to determine whether the tested heterocycles were degradable at all. Two primary biodegradation tests were performed using microbial communities derived from the adaptation culture (on day 75 for the first test and on day 120 for the second test) exposed to the mixture of heterocyclic PAHs. The second primary biodegradation experiment also included abiotic controls to determine the loss of compounds by abiotic processes such as sorption.

During the primary biodegradation tests, DOC, pH and conductivity were measured periodically (see SI file Section S3 describing the changes in physicochemical parameters and the corresponding discussion added there).

In the abiotic controls, the concentration of test compounds decreased sharply at the beginning of the test (within two hours of spiking). Compared to the theoretical initial concentrations (0.16 mg L^{-1}), the lowest sorption at the beginning of the test was observed for CRB (3%), the least hydrophobic compound under test, while the highest sorption was observed for BNT (88%), the most hydrophobic compound. Overall, the sorption order of the compounds was as follows: CRB (3%) < DBF (25%) < BCRB (38%) < BNF (44%) < BACR (63%) < DBT (69%) < TXT (84%) < BNT (88%), with sulphur-substituted PAHs having a higher affinity to biomass. Although sorption rates did not clearly follow the order of log K_{OC} values (Table 1), the sorption capacity increased systematically with increasing molecular size within the same chemical classes (N-, S- or O-PAHs) as follows: CRB < BCRB < BACR, DBF < BNF, and DBT < TXT < BNT. Thus, sorption was demonstrated to be a key removal mechanism for heterocyclic PAHs, a mechanism that may occur at even higher levels in a real environmental compartment due to the presence of larger amounts of carbonaceous materials or biomass.

Figure 4 shows the results of the primary biodegradation tests, using the measured initial concentrations (t=2 h) as a starting point. Note that BNT may have initially precipitated out of solution as it was added at a concentration above its solubility in water (26 μg L^{-1}). However, the precipitated fraction may still be available as a reservoir in the system [50], since the equilibrium direction of desorption/dissolution is expected to shift as each molecule is degraded.

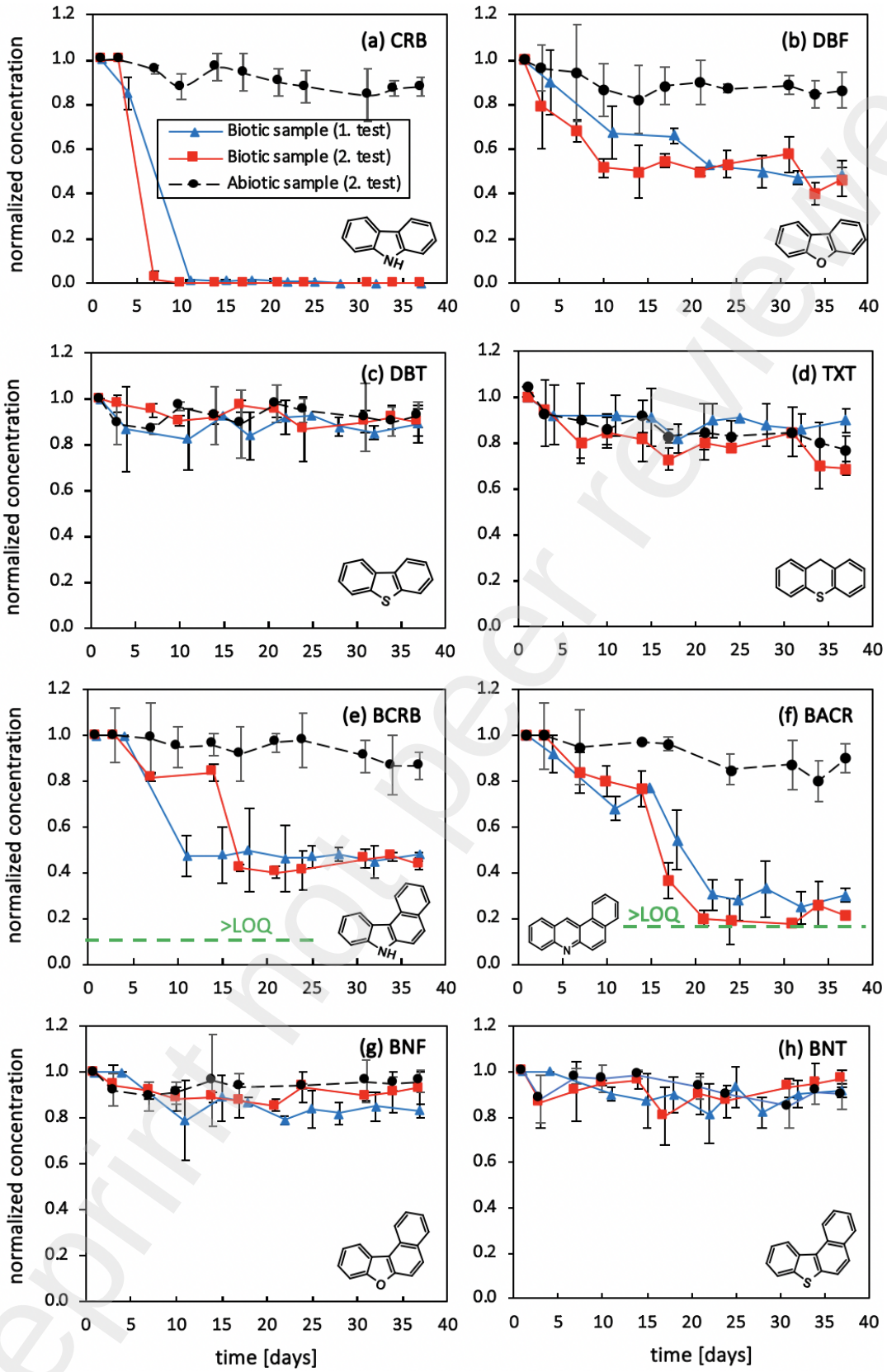


Fig. 4. Primary biodegradation of heterocyclic PAHs after 75 days (first run) and 120 days (second run) of microbial adaptation to the substances under test, expressed as concentration at a given time divided by the measured starting concentration (C/C_0) in biotic and abiotic systems. Mean values are shown ± standard deviation (n=3). The nominal starting concentration for each compound was 0.16 mg L^{-1}.

Four of the eight heterocyclic PAHs tested, CRB (100%), DBF (50-60%), BCRB (50-60%) and BACR (80%), were primarily degraded (percentage degradation shown in brackets). Similar results from two consecutive experiments indicated that the ability of the inoculum to degrade the test compounds did not change between the 75th (1st test) and 120th days (2nd test) of the adaptation period. This assumption was supported by flow cytometry data, which showed a similar composition of the bacterial community sampled on days 75 and 120 (Figure 5). In the first and second tests, CRB was rapidly degraded within 11 and 7 days respectively. The degradation of DBF, BCRB and BACR did not feature a long lag-phase, however, even after 37 days degradation was not complete, indicating a slower degradation compared to CRB. The degradation of BACR appears to have reached a plateau after day 20, with very low concentrations remaining in the samples close to the LOQ. Similarly, biodegradation of BCRB remained incomplete, with 40-50% of the initial bioavailable concentration remaining in solution from day 15 until the end of the test. It is conceivable that BCRB was co-metabolised in the presence of another carbon and energy source.

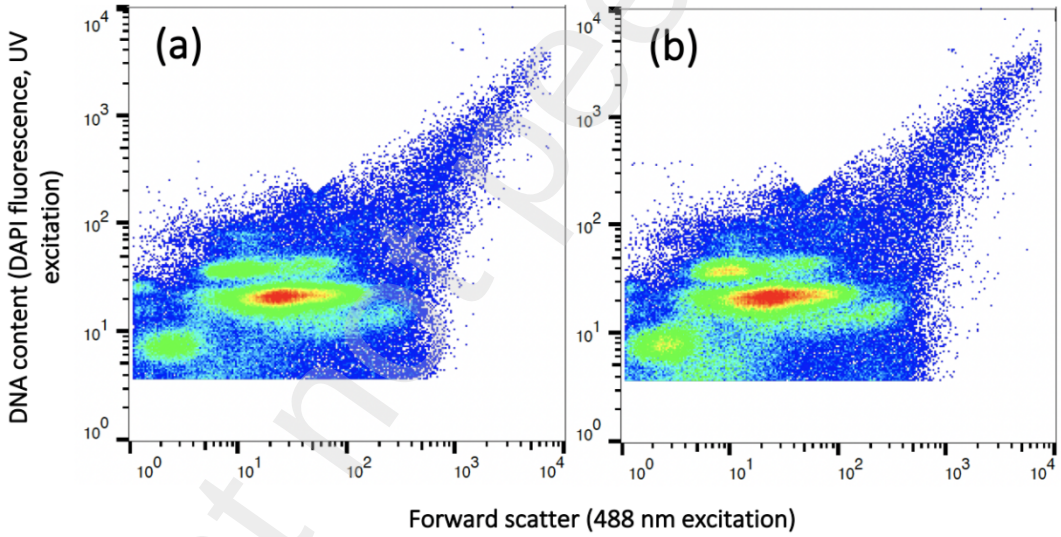


Fig. 5. Flow cytometric fingerprints of microbial communities. Cells were analysed by their DNA content and forward scatter related to cell size. The 2D plots show similar compositions of inocula used in the (**a**) first (day 75) and (**b**) second (day 120) primary biodegradation experiments.

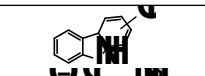
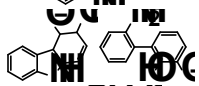
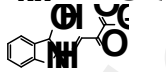
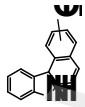
An interesting observation is that some levels of degradation occurred for all tested N-heterocycles – CRB, BCRB, and BACR – following adaptation, but not for other heterocycles - except DBF. The O- or S-heterocycles – DBT, TXT, BNF, and BNT – were not degraded in any of the ultimate or primary biodegradation tests indicating that these compounds might indeed be persistent.

3.4. Analysis of transformation products

Despite it being difficult to identify transformation products in samples containing a mixture of all the test substances (which also have the same adducts), scanning began with analysis of the standards of the parent compounds. LC-MS/MS analysis in positive ion mode produced high intensity signals for three N-PAHs in protonated form $[M+H]^+$, which are primarily biodegradable: CRB, BCRB and BACR. The rest of the compounds, however, could not be reliably detected due to very low intensity signals or no signal at all. In fact, compounds like S-PAHs lacking functional groups for protonation or deprotonation have been reported to be very difficult to detect by electrospray ionization mass spectrometry (ESI/MS) and often require some additives, i.e., charge-transfer reagents, to convert neutral compounds into ionic species by an electron transfer to enable quantification [51,52]. Similarly, O-PAHs (furan derivatives) have been found to be undetectable by the ESI system [53], as was the case in our study either in positive or negative ion modes. We then tentatively gauged the transformation products for N-PAHs.

Two samples taken on days 21 and 37 of the first primary degradation test were scanned. The parent compounds, BCRB and BACR, appeared in the samples, while CRB was not detected in either sample, as expected, due to its complete removal from the sample after 11 days. Apart from the parent compound and matrix ions, the MS analysis identified several clear high intensity signals at m/z 184, 201, 215, 217, 220, 231, 234, 248, and 261. Subsequently, the product and precursor ions were scanned in target screening mode. In five cases a tentative structural assignment was possible on the basis of MS/MS fragmentation behaviour and mass shifts from the parent chemicals, and the projected transformation products are accordingly grouped with respect to the parent compounds in Table 2.

Table 2. Tentatively suggested potential transformation products of N-PAHs by LC-MS/MS analysis.

parent compound characterization			transformation product characterization					
parent compound	$[M+H]^+$ (m/z)	RT (min)	mass shift (Da)	proposed formula	MW [g mol^{-1}]	structural proposal	$[M+H]^+$ (m/z)	RT (min)
CRB	**168**, 139	4.50	+16	$C_{12}H_9NO$	183.207		**184**, 152, 139	3.83
			+33	$C_{12}H_{10}NO_2^-$	200.214		**201**, 184, 152, 139	3.83
			+63	$C_{12}H_8NO_4^-$	230.197		**231**, 197, 213, 184	3.52
BCRB	**218**, 189	4.76	+16	$C_{16}H_{11}NO$	233.265		**234**, 202, 189	4.27
BACR	**230**, 202	6.47 (large peak)	+18	$C_{17}H_{13}NO$	247.291		**248**, 230, 202	3.94

*Precursor ions are given in **bold**, while the rest are product ions.

The biotransformation pathways for CRB have been extensively studied and three degradation pathways have been widely reported (Figure S11): monohydroxylation, lateral dioxygenation and angular dioxygenation [54]. For the biotransformation of BACR, the formation of hydroxy- and epoxy-substituted forms has also been shown [55], nevertheless, no degradation pathways have so far been described for BCRB to the best of the authors' knowledge. We have therefore suggested the simplest theoretically possible path (oxylated or hydroxylated intermediates) for the latter two, while for CRB, the common degradation pathways were shown in detailed in Figure S11.

The product at m/z 231 appeared only in the first sample (day 21) and disappeared in the second (day 37). The rest of the products were present in both sample with different peak intensities. In summary, the identification of transformation products in our test design can only be tentative and may be too notional. A more straightforward investigation can be suggested for future work using standards of the transformation products and possibly using a single substance inclusive test design.

Predicting biodegradability of heterocycles using QSAR models

We compared the experimental results with those predicted by QSAR models (Table 3). According to the outcomes of BIOWIN models 3 (timeframe of complete ultimate biodegradation), 4 (timeframe of complete primary biodegradation), 5 and 6 (probability of ready biodegradability with pass/fail answer), the STPWIN model (%sorption to sludge) and the degradation half-lives, the grey cells in Table 3 show the instances where the predicted data did not match the experimental results, while the white cells show where predictions and

584 experiment matched. The blue category, on the other hand, shows results that are not clearly
585 contradictory, but require longer experiments to be sure of the degradation timeframe.

586 **Table 3.** Measured and predicted biodegradability and sorption data for heterocyclic PAHs. The color codes
587 indicate the correctness of the predicted data compared to the experimental results as follow: white: matches, grey:
588 non-matches, blue: not clearly contradictory, but requires longer experiments for a certain evaluation.

	Experimental degradation and sorption				Predicted degradation and sorption				
Compounds	**Ready**	**Ultimate***	**Primary***	**Sorption**	**Ready [a]**	**Ultimate [b]**	**Primary [c]**	**Half-life [d]**	**Sorption [e]**
IND	yes [f]	80%	–	–	no	weeks	days-weeks	<60 days	2%
CRB	no	57%	100%	3%	no	weeks-months	days-weeks	<60 days	19%
DBF	no	0%	50-60%	25%	no	weeks	days-weeks	<60 days	35%
DBT	no	0%	0%	69%	no	weeks	days-weeks	<60 days	58%
TXT	no	0%	0%	84%	no	weeks-months	days-weeks	<60 days	78%
BCRB	no	0%	50-60%	38%	no	months	weeks	≥60 days	85%
BACR	no	0%	80%	63%	no	months	weeks	≥60 days	54%
BNF	no	0%	0%	44%	no	months	weeks	≥60 days	89%
BNT	no	0%	0%	88%	no	months	weeks	≥60 days	90%
DNF	no	0%	–	–	no	months	weeks	≥60 days	93%

589 * Ultimate and primary biodegradation experiments were conducted with adapted community.
590 [a] BIOWIN5 and 6 (both gave the same results); [b] BIOWIN3; [c] BIOWIN4; [d] half-lives predicted according to the work of
591 Aronson et. al. [48]; [e] STPWIN; [f] reference [15]

592 BIOWIN models 5 and 6 classified all compounds as "not readily biodegradable" and the
593 results were consistent with the experimental data, except for indole. In the latter case, the
594 models seem to deliver a false negative result, which is of course undesired, but can be
595 considered as "overprotective", which is less detrimental than false positives. The BIOWIN3
596 half-lives overestimated the biodegradability of DBF and DBT, which should be degraded
597 within weeks, but showed no signs of ultimate degradation for more than a month. In the cases
598 of BNF, BNT and DNF, we observed only a sorption-dependent decrease in concentration and
599 no evidence of degradation over a month. It is therefore possible that these compounds are
600 indeed persistent and that the BIOWIN3 output of "longer than months" would be expected,
601 but a definitive conclusion on the correctness of the model cannot be made. The BIOWIN4
602 prediction of the primary degradation timeframe often gave false positive results considering
603 that an adapted community was used, e.g., for BCRB about 50-60% of the parent compound
604 was degraded, whereupon degradation ceased, whereas the degradation of DBF proceeded very
605 slowly. The primary degradation of CRB appeared to be faster than suggested by BIOWIN4,
606 but as we used an adapted microbial community, we did not mark it as false to be on the safe
607 side. Overall, the biodegradability predicted by multiple BIOWIN models (3 to 6) agreed with
608 the experimental results only for CRB, while for the rest of the compounds there was no
609 agreement between the data estimated by different models. Therefore, it is difficult to make
610 concrete decisions about the biodegradability of heterocyclic compounds based on the QSAR

outcomes in the absence of experimental data. Our experimental data show that sorption to sludge could be the dominant removal mechanism for DBT, TXT, BACR and BNT. Furthermore, the STPWIN model predicts that sorption also plays a major role in the removal of other large heterocycles (i.e., BCRB and BNF), overestimating their affinity to sludge by a factor of two. This, however, could be a consequence of the rather low amount of biomass in our primary degradation test. Comparisons between the model outcomes and our experimental results confirm that QSAR predictions of the biodegradability of heterocycles are indeed challenging.

4. Conclusions

The removal of heterocyclic PAHs from the environment is a growing concern that needs to be addressed immediately, particularly with regard to persistence assessment. Therefore, this study investigated the biodegradability of ten heterocyclic PAHs under aerobic conditions. All test substances (≥3-rings) were categorized as "not readily biodegradable" and the results were in agreement with QSAR predictions.

To mimic natural degradation processes at contaminated sites, a microbial community from a WWTP was adapted to the mixture of heterocyclic PAHs. CRB was significantly degraded by one-year-adapted bacteria and was thus identified as an inherently biodegradable compound. Although no mineralization was perceived for the other compounds (except indole), their inhibitory effects on bacteria were not particularly notable. Interestingly, however, bacteria became gradually more susceptible to BCRB as the adaptation period was extended (to one year). This suggests that there are trade-offs in bacterial adaptation; a positive outcome (degradation of CRB) is accompanied by a less desirable outcome (increased sensitivity to BCRB).

In the primary biodegradation tests, sorption to biomass was found to be an important removal mechanism for most of the compounds tested and four compounds - CRB, DBF, BCRB and BACR - were identified as primarily biodegradable at concentrations 24 to 98 times lower than those tested by the manometric respirometry method. Nevertheless, no evidence of primary or ultimate biodegradation was found for DBT, TXT, BNF and BNT in any of the tests conducted in this work.

Flow cytometry results indicated that the composition of the bacterial community did not change between adaptation days 75 and 120, as expected from the very similar results of two consecutive primary biodegradation tests. However, the community shifted between days 250

and 365 (Figure S12), which could explain the increasing sensitivity of the bacteria to BCRB and the degradation of CRB over time.

According to the experimental results, only indole and carbazole can clearly be labelled as non-persistent compounds in the environment. However, the compounds that showed no evidence of degradation in any of the tests (DBT, TXT, BNF, BNT, DNF) are likely to be persistent in the environment, while the others that showed some extent of degradation (DBF, BCRB, BACR) require further investigation (e.g., degradation half-life from simulation tests) to evaluate their persistence. The results of this study suggest that the most heterocyclic PAHs pose a high risk of environmental persistency that cannot be reliably predicted using QSAR models, and that urgent action is needed to thoroughly monitor these chemicals in waters and in soils.

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Declaration of competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Credit for author contributions

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at link

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