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Revision of OECD TG 305 "Bioaccumulation in fish" to improve the identification of PBT substances and to reduce the number of experimental animals involved

by

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Abstract

Solid-phase microextraction (SPME) is capable of extracting total water concentrations as well as the freely dissolved fraction of analytes in the water phase, which is available for bioconcentration in fish. However, SPME is still a rather unusual method in regulatory ecotoxicological research. The potential of SPME as an analytical routine approach for aquatic bioconcentration studies according to OECD TG 305 was investigated with a special focus on the testing of hydrophobic organic compounds (HOCs) characterized by log K_{OW} > 5.

In the first part of this project sorption of eleven HOCs (log K_{OW} 5.5 – 7.8) from different substance classes to organic matter (OM) was systematically investigated with water containing defined concentrations of total organic carbon (TOC) and dissolved organic carbon (DOC). Matrices were fish feed, filter residue of test vessels from OECD TG 305 studies (containing remaining feed, tissue and feces), and Aldrich-humic acid as reference. Water samples were processed by the commonly applied liquid-liquid extraction procedure (LLE) and SPME and measured with GC/MS. Results from SPME extractions were used to estimate partition coefficients (K_d, K_{OC}, and K_{DOC}) and to predict the bioavailable fraction of the test substances at defined concentrations of organic carbon in the test system. Extraction by LLE approximately results in total analyte concentrations, independent from TOC/DOC. However, the studies showed that the bioavailable fraction of a test substance may be significantly reduced due to sorption to OM already at concentrations below 2 mg L^{-1} TOC.

During the second part of the project the effect of different extraction procedures (LLE vs. SPME) on the result of BCF studies was investigated. The bioconcentration potential of two highly lipophilic test substances (hexachlorobenzene, o-terphenyl) was investigated in a flow-through fish test with rainbow trout according to OECD TG 305. Column generated concentrations were applied to continuously deliver a test solution below the solubility limit. Total analyte concentrations in the water phase were measured by LLE and SPME. Both extraction procedures were leading to comparable results.

A further flow-through fish test was carried out with PCB 153 and Dibenz[a,h]anthracene (DBA)in the third part of the project. Dissolved organic carbon (DOC) and total organic carbon (TOC) measurements in the test water were carried out throughout the study and the bioavailable fraction of both test items was estimated by SPME. Results show that TOC concentrations should be kept as low as possible in BCF tests on HOCs to avoid the underestimation of the true uptake when total aqueous concentrations are measured. Tissue concentrations in fish samples collected throughout the study were measured. Based on the results obtained steady state and kinetic BCFs were estimated.

Kurzbeschreibung

Die Festphasenmikroextraktion (solid-phase microextraction, SPME) eignet sich zur Bestimmung der Gesamtkonzentration ebenso wie der frei gelösten Fraktion einer Substanz in der Wasserphase, die von Fischen über die Kiemen aufgenommen werden kann. SPME gehört jedoch zu den bislang weitgehend ungenutzten Methoden, die im Rahmen von ökotoxikologischen Studien zur regulativen Bewertung von chemischen Substanzen zum Einsatz kommen. Das Potential der SPME als analytisches Routineverfahren für aquatische Biokonzentrationsstudien nach Richtlinie OECD TG 305 wurde im Hinblick auf den Einsatz von hydrophoben organischen Substanzen (HOCs), die durch einen hohen log KOW- Wert(log KOW > 5) charakterisiert sind, untersucht.

Im ersten Projektabschnitt wurde die Sorption von elf HOCs (log KOW 5.5 – 7.8)aus unterschiedlichen Substanzklassen an organisches Material (OM) systematisch untersucht, wobei Wasserproben mit definierten Konzentrationen an Gesamtkohlenstoff (TOC) und gelöstem organischen Kohlenstoff (DOC) zum Einsatz kamen.

Als organisches Material kamen dabei Fischfutter, Filterrückstände aus Versuchsanlagen für OECD TG 305 Studien (enthalten hauptsächlich Futterreste und Fischkot) und Aldrich-Huminsäure als Referenzsubstanz zum Einsatz. Wasserproben wurden durch die herkömmliche Flüssig-Flüssig-Extraktion (LLE)und SPME aufbereitet und anschließend mit GC/MS auf ihren Gehalt an Testsubstanzen untersucht.

Die Ergebnisse der Extraktion durch SPME wurden verwendet um Verteilungskoeffizienten (Kd, KOC, and KDOC) zu bestimmen und die bioverfügbare Fraktion der Testsubstanzen bei definierten Konzentrationen an organischem Kohlenstoff im Testsystem abzuschätzen. Die Extraktion durch LLE ermöglicht jedoch weitestgehend nur die Bestimmung von Gesamtkonzentrationen von Testsubstanzen in Wasserproben, unabhängig von den enthaltenen TOC- bzw. DOC-Konzentrationen. Die Studie zeigte, dass die bioverfügbare Fraktion einer Testsubstanz durch Sorption an OM bereits bei Konzentrationen unter 2 mg L-1 TOC signifikant reduziert werden kann.

Der Einfluss unterschiedlicher Extraktionsverfahren (LLE vs. SPME) auf das Ergebnis von BCF Studien wurde im zweiten Projektabschnitt untersucht. Dafür wurde das Biokonzentrationspotential von zwei hochlipophilen Testsubstanzen (Hexachlorbenzol, o-Terphenyl) in einer Durchflussstudie mit Regenbogenforellen nach OECD TG 305 untersucht. Der Einsatz einer Säulenelutionstechnik gewährte die kontinuierliche Produktion von Testlösungen mit Lösungskonzentrationen stets unterhalb der substanzspezifischen Wasserlöslichkeit. Die Gesamtkonzentration der beiden Testsubstanzen in der Testlösung wurde durch LLE und SPME ermittelt. Beide Verfahren führten zu vergleichbaren Ergebnissen.

Eine weitere Durchflussstudie mit Regenbogenforellen wurde mit den Testsubstanzen PCB 153 und Dibenzo[a,h]anthracen (DBA) im dritten und abschließenden Projektabschnitt durchgeführt. Die Gehalte an DOC und TOC wurden im Testmedium im Verlauf der Studie gemessen und die bioverfügbare Fraktion beider Substanzen durch SPME bestimmt. Die Ergebnisse zeigen, dass TOC Konzentrationen im Testsystem bei der Durchführung von BCF-Studien so niedrig wie möglich gehalten werden sollten, um eine Unterschätzung der wahren Aufnahme von Testsubstanzen auf Basis der gemessenen Gesamtkonzentration zu vermeiden. Die im Verlauf der Durchflussstudien gewonnenen Fischproben wurden auf ihre Gewebskonzentrationen der einzelnen Testsubstanzen untersucht. Auf Basis der erzielten Ergebnisse wurden „Steady state“ BCFs und kinetische BCF-Werte ermittelt.

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AHA	Aldrich humic acid
BCF	Bioconcentration factor
BMF	Biomagnification factor
DBA	Dibenzo [a,h]anthracen
DEHA	Hexanedioic acid bis (2-ethylhexyl) ester
DEHP D4	bis (2-ethylhexyl) phtalate
DOC	Dissolved organic carbon
DOM	Dissolved organic matter
FF	Fish feed
FR	Filter residue
GC-MS	Gas chromatography – Mass spectrometry
HCB	Hexachlorobenzene
HOC	Hydrophobic organic chemicals
IS	Internal standard
$K_{d, OC, DOC}$	Partition coefficients
KOW	n-octanol-water partition coefficients
LLE	Liquid-liquid extraction
LOQ	Limit of quantification
m/z	Mass-to-charge ratio (in mass spectrometry)
NP	4-n-nonylphenol
OCR	Octocrylene
OECD	Organisation for Economic Co-operation and Development
oTP	o-Terphenyl
POC	Particulate organic carbon
POPs	Persistent organic pollutants
REACh	Registration, Evaluation, Authorization and Restriction of Chemicals [Regulation (EC) No 1907/2006]
RSD	Relative standard deviation
SPME	Solid-phase microextraction
TCN	1,2,3,4-tetrachloronaphthalene
TG	Technical guideline
TOC	Total organic carbon
TWA	Time weighted average concentration

1 Summary

The performance of an Aqueous Exposure Bioconcentration Fish Test according to OECD TG 305 requires the possibility of preparing stable, measurable dissolved aqueous concentrations of the test substance. For strongly hydrophobic substances (log KOW > 5 and a solubility below ~ 0.01-0.1 mg/L), testing via aqueous exposure may become increasingly difficult. For these highly hydrophobic substances the dietary test is recommended, provided that the test is consistent with the relevant regulatory framework and risk assessment needs (OECD 305). The dietary approach yields a biomagnification factor (BMF) rather than a BCF. Being able to estimate a BCF from the data generated in the dietary study would be a great advantage and would meet an accepted regulatory need. Therefore, the estimation of BCF values from data generated in the dietary studies is currently under investigation to keep the results of bioconcentration and biomagnification studies comparable [Crookes and Brooke, 2011]. However, this approach seems to be very complicated and might be even impossible due to the clear differences in the biomagnification and bioconcentration processes. In addition, defined regulatory cutoff criteria for the biomagnification potential of chemical compounds are still missing. Under such circumstances, the estimation of BCFs for highly HOCs in flow-through fish tests might remain essential in the future.

Measured bioconcentration factors (BCF) and n-octanol-water partition coefficients (KOW) of hydrophobic organic chemicals (HOC) show a curvilinear relationship up to log KOW 5-6. BCF values of more lipophilic substances tend to level of or decline (hydrophobicity cutoff). Debated reasons are steric effects, but also measurement artefacts. Jonker and van der Heijden (2007) suggested that the overestimation of bioavailable aqueous HOC by the presence of non-bioavailable HOC bound to dissolved organic matter, might lead to an underestimation of the true uptake. However, if bioconcentration factors for HOC are to be generated from the results of dietary studies, correct BCF estimates from aqueous exposure studies are required to verify the results of the suggested conversion procedure.

The aim of this project was 1) to investigate the potential of SPME as an analytical routine approach for aquatic bioconcentration studies according to OECD TG 305 with a special focus on the testing of hydrophobic organic compounds (HOCs) characterized by log KOW > 5; 2) to investigate the freely dissolved analyte concentrations in the test medium by SPME; 3) to investigate the sorption of HOCs (log KOW 5.5 – 7.8) from different substance classes to total and dissolved organic matter; 4) to investigate the effect of different extraction procedures (LLE vs. SPME) on the result of BCF studies and 5) to investigate the effect of total organic carbon (TOC) present in the test water on the result of BCF-studies.

OECD TG 305 (OECD 2012) does not define specific analytical techniques to determine analyte concentrations in the water phase. However, commonly applied is liquid-liquid extraction (LLE) which yields total analyte concentrations. In contrast, solid-phase microextraction (SPME) is capable of additionally analyzing the freely dissolved analyte concentrations. This is relevant due to the binding of hydrophobic organic compounds (HOCs) to organic matter (OM) present in the test system. Organic matter, quantified by total and dissolved organic carbon (TOC, DOC), influences the bioavailability of HOCs, because only the freely dissolved fraction of a test substance is capable to cross an organisms membrane.

During the first part of this project sorption studies were carried to confirm the hydrophobic characteristics of different test substances, and to estimate their affinity to bind to different organic substances. The use of LLE and SPME for the extraction of analyte concentrations in aqueous phases enriched with different sorbent matrices relevant for fish bioconcentration studies was compared and kinetic studies were performed to show the time dependent characteristics of sorption of HOCs within aqueous phases containing OM from different sorbent matrices.

Results show that SPME is capable of determining reduced bioavailability of hydrophobic test substances even in the presence of very low concentrations of OM as found in fish

bioconcentration studies. Results further indicate that the influence of organic matter inside BCF test systems is highly dependent on the quality of organic matter and interacts with the molecular characteristics of the test substance. These interactions between structural quality of OM and analyte characteristics highly influence the amount of sorption. Furthermore, LLE may capture significant amounts of molecules bound to OM which are not available to fish. OM from BCF studies has a high sorption capacity for HOCs and it is to be expected that the calculation of BCF values based on LLE results may lead to an underestimation of BCFs as the amount of extracted analyte concentrations is higher than for free water concentrations extracted by SPME which represent the bioavailable analyte concentrations in bioconcentration studies. Sorption data for OM relevant to BCF studies (FF and FR) are missing in the literature, because subject of most studies is the interaction of test substances with humic substances as organic matter (natural and reference).

During the second and third part of this project the effect of different extraction procedures on the result of BCF studies carried out under realistic experimental conditions was investigated. To examine if there is an underestimation of fish BCFs caused by measuring artifacts, two flow-through fish tests with HOCs were carried out under realistic exposure conditions. Substances characterized by high (HCB, o-TP) and very high (PCB 153, DBA) lipophilicty were tested in the first and second study, respectively. Part of these studies was the preparation of column generated concentrations of all HOCs over the maximum uptake period of 56 days, the measurement of total organic carbon concentration in the test water during the flow-through study, and the comparison of the results obtained by different extraction procedures (LLE vs. SPME) for water sample preparation.

A column elution method was developed as part of this project to generate stable concentrations of the HOCs which can be maintained even throughout an extended uptake period. The analysis of the test media applied during the BCF studies showed that stable column generated concentrations of HOCs in the test system can be obtained over a period of 8 weeks without using solvents. Test concentrations were measured by SPME and LLE throughout the studies. Internal standard-corrected results obtained by SPME and LLE were equal; therefore, the results of BCF studies should be comparable independent of the method (LLE or SPME) used for the extraction of total water concentrations. Although already well established in environmental analyses to determine and quantify analytes mainly in aqueous matrices, SPME is still a rather unusual method in regulatory ecotoxicological research. Due to the potential benefits (Duering et al, 2012), the use of SPME as an alternative analytical routine approach for aquatic bioconcentration studies should be considered.

The results of the BCF studies show that Aqueous Exposure Bioconcentration Fish Tests according to OECD 305 can be also carried out with HOCs up to a logKOW of 7.8. For three of the four test items it was possible to estimate kinetic and steady-state BCF values. However, as shown in the first part of this project natural organic matrices have a high sorption potential and can thus influence the bioavailability of HOC in BCF studies. DOC and POC concentrations in the test system were therefore monitored during the uptake periods. The results show that the experimental conditions are characterized by low concentrations of TOC. TOC levels can be kept below the threshold concentrations defined in the OECD TG 305 provided that the experimental tanks are kept as clean as possible. The results presented here were obtained from two flow-through bioconcentration studies on rainbow trout. Due to differences in feeding behavior and the stability of fecal material, flow-through tests on other fish species such as common carp may lead to different results regarding TOC concentrations.

SPME (without internal standard correction) can be used to measure bioavailable analyte concentrations in bioconcentration studies and help to assess the presence of non- bioavailable molecules bound to OM in the test system. SPME measurements carried out during the second BCF study showed that natural organic matrices present in the test system caused only a slightly reduced bioavailability of the HOCs. The recalculation of the estimated BCF values based on the bioavailable (freely dissolved) fraction showed that the presence of non-bioavailable molecules

only caused a slight underestimation of the BCFs which cannot explain the hydrophobicity cutoff as suggested by Jonker and van der Heijden (2007). For the substance with the highest lipophilicity (PCB153, logKOW 7.8) still a lower BCF was estimated compared to the less lipophilic HOCs tested in this study, however, the decrease of the bioaccumulation potential must be caused by other factors, e.g. stearic effects, than measuring artifacts.

For the other test substance with a very high logKOW (DBA) no BCF could be determined. Obviously caused by the rapid metabolism of the test item no bioconcentration of the test substance could be observed. During the first BCF study animals were exposed for a longer uptake period (56 days) than required according to OECD 305 to reach the steady-state concentration. It was observed that tissue concentrations of oTP reached the steady state level already in the middle of the uptake period followed by a clear decrease of tissue concentrations toward the end of exposure. The results indicate that fish metabolism may adapt to a contaminant leading to a stronger elimination during the bioconcentration process. In contrast, tissue concentrations of HCB remained at a constant level after reaching the steady state. The BCF estimates provided in this study are of high quality and can be used to verify the results of BMF to BCF conversion procedures. The project has shown that BCF studies can be carried out with HOCs reducing the need to run BMF studies which still do not fulfill the risk assessment requirements and thus challenge the use of experimental animals for such studies.

2 Zusammenfassung

Die Durchführung von Fisch-Biokonzentrationsstudien nach OECD TG 305 erfordert die Herstellung stabiler wässriger Konzentrationen einer Testsubstanz. Für stark hydrophobe Substanzen (log KOW > 5 und einer Löslichkeit unter ~ 0.01-0.1 mg/L) stellt sich die Herstellung wässriger Lösungen jedoch als zunehmend problematisch dar. Für diese Substanzen wird in der revidierten OECD TG 305 (2012) die Durchführung einer Fütterungsstudie zur Ermittlung des Biomagnifikationspotentials empfohlen, soweit die regulatorischen Anforderungen der Risikobewertung erfüllt sind. Im Gegensatz zur BCF-Studie werden durch Fütterungsstudien Biomagnifikationsfaktoren (BMF) ermittelt. Die Möglichkeit der Umwandlung von BMF in BCF-Werte wäre ein großer Vorteil für die regulatorische Bewertung chemischer Verbindungen. Die Ableitung von BCF-Werten auf Basis von Fütterungsstudien wird momentan untersucht, um die Ergebnisse von Biokonzentrations- und Biomagnifikationsstudien vergleichbar zu halten [Crookes and Brooke, 2011]. Dieser Ansatz stellt sich jedoch als sehr kompliziert heraus und könnte sogar unmöglich sein aufgrund der klaren Unterschiede zwischen den Prozessen der Biomagnifikation und Biokonzentration. Hinzu kommt, dass definierte regulatorische Ausschlusskriterien für das Biomagnifikationspotential chemischer Substanzen bislang fehlen. Unter diesen Umtänden könnte sich die Bestimmung von BCF-Werten für hydrophobe organische Chemikalien (HOCs) auch in Zukunft als notwendig erweisen.

Die in Biokonzentrationsstudien ermittelten BCF-Werte und die n-Oktanol-Wasser-Verteilungskoeffizienten (KOW) hydrophober organischer Chemikalien zeigen eine kurvengerade Beziehung bis zu einem log KOW 5-6. BCF-Werte höher lipophiler Substanzen zeigen jedoch ein abflachendes oder gar abnehmendes Niveau (hydrophobicity cutoff). Als Grund dafür werden sterische Effekte aber auch Messartefakte diskutiert. Jonker und van der Heijden (2007) postulierten, dass die Präsenz physiologisch nicht verfügbarer, an gelöster organischer Substanz gebundener Moleküle zu einer Überbewertung der bioverfügbaren wässrigen Konzentration von HOCs führen kann. Die Ableitung von BCF-Werten für HOCs aus den im Rahmen von Fütterungstudien ermittelten Ergebnissen erfordert jedoch die Verfügbarkeit korrekter BCF-Werte aus OECD 305 Studien, die eine Verifizierung der Umrechnung von BMF in BCF-Werte zulassen.

Ziel diese Projekts war es: 1) das Potential von Festphasenmikroextraktion (SPME) als analytisches Routineverfahren für Biokonzentrationsstudien nach OECD TG 305 im Hinblick auf den Einsatz von HOCs, die durch einen log KOW > 5 charakterisiert sind, zu untersuchen; 2) die Untersuchung frei gelöster Konzentrationen im Testmedium durch SPME; 3) die Untersuchung der Sorption von HOCs (log KOW 5.5 – 7.8) unterschiedlicher Substanzklassen an partikuläre und gelöste organische Substanz; 4) die Untersuchung des Effekts unterschiedlicher Extraktionsverfahren (LLE vs. SPME) auf das Ergebnis von BCF-Studien; und 5) die Untersuchung der Wirkung von Gesamtkohlenstoffgehalten (TOC) im Testmedium auf die Ergebnisse von BCF-Studien.

OECD TG 305 (OECD 2012) definiert keine spezifische analytische Technik zur Bestimmung von Testsubstanzkonzentrationen in der Wasserphase. In der Regel kommt jedoch die Flüssig-flüssig-Extraktion (LLE) zum Einsatz, die zur Bestimmung von Gesamtkonzentrationen führt. SPME kann darüber hinaus auch zur Analyse frei gelöster Substanzkonzentrationen eingesetzt werden, die nach Bindung hoch lipophiler Testsubstanzen an organische Substanzen (OM) im Testsystem verbleiben. Organische Substanz, die als Gesamt- und gelöster organischer Kohlenstoff (TOC, DOC) quantifiziert wird, beeinflusst die Bioverfügbarkeit von HOCs, da nur die frei gelöste Fraktion einer Testsubstanz in der Lage ist, Membranen von Organismen (z.B. Kiemen) zu passieren.

Im ersten Abschnitt des Projekts wurden Sorptionsstudien durchgeführt, um die hydrophoben Eigenschaften unterschiedlicher Testsubstanzen zu bestätigen und ihre Bindungsaffinität bezüglich unterschiedlicher organischer Matrizes zu bestimmen. Der Einsatz von LLE und SPME für die Extraktion von gelösten Substanzen in wässrigen Phasen mit einem hohen Gehalt an unterschiedlichen sorbierenden Matrizes, die für Fisch-Biokonzentrationsstudien relevant sind, wurde verglichen. Kinetische Studien wurden durchgeführt, um die zeitabhängigen Sorptionseigenschaften von HOCs in aquatischen Phasen, die mit OM aus unterschiedlich sorbierenden Matrizes angereichert wurden, zu untersuchen.

Die Ergebnisse zeigen, dass SPME die Möglichkeit bietet, die reduzierte Bioverfügbarkeit hydrophober Testsubstanzen unter realistischen Expositionsszenarien, d.h. sehr niedrigen OM-Konzentrationen, wie man sie in BCF-Studien findet, zu bestimmen. Desweiteren weisen die Ergebnisse eindeutig darauf hin, dass der Einfluss organischer Substanz im BCF-Testsystem sehr stark von der Qualität der organischen Substanz abhängt, die mit den molekularen Eigenschaften der Testsubstanz interagiert. Interaktionen zwischen der Qualität organischer Matrix und den Eigenschaften chemischer Substanzen haben einen starken Einfluss auf den Sorptionsprozess.

Durch LLE wird ein signifikanter Anteil OM-gebundener Moleküle, der für Fische nicht verfügbar ist, erfasst. Gerade die für BCF-Studien charakteristische OM ist durch eine hohe Sorptionskapazität gekennzeichnet. Die Berechnung von BCF-Werten auf Basis von Analyseergebnissen, die durch LLE erzielt wurden, könnte daher zu einer Unterschätzung der Bioakkumulation führen, da die durch LLE extrahierten Analytkonzentrationen vermutlich höher sind als die frei gelösten Konzentrationen, die durch SPME erfasst werden und die den bioverfügbaren Anteil einer Testsubstanz ausmachen. Sorptionsdaten zu OM aus BCF-Studien (Fischfutter und Filterrückstände) fehlen bislang in der Literatur, die sich vorwiegend auf die Interaktion von Chemikalien mit Huminsäure als organischer Substanz bezieht.

Im zweiten und dritten Abschnitt des Projekts wurde die Wirkung unterschiedlicher Extraktionsverfahren auf das Ergebnis von BCF-Studien mit HOCs untersucht, die unter realen Studienbedingungen durchgeführt wurden. Der Einfluss möglicher Messartefakte auf das Ergebnis von Fisch-BCF-Studien wurde bestimmt. Vier Testsubstanzen, die durch eine hohe (HCB, o-TP) bzw. sehr hohe (PCB 153, DBA) Lipophilität charakterisiert sind, wurden in zwei BCF-Studien getestet. Der Fokus dieser Studien lag auf der Herstellung der Testlösungen mit konstanten HOC-Konzentrationen unter Verwendung einer Säuleneluationstechnik über die maximale Aufnahmeperiode von 56 Tagen, der Messung der TOC-Konzentrationen im Testwasser im Verlauf der Durchflussstudien sowie dem Vergleich der Analyseergebnisse, die durch unterschiedliche Extraktionsverfahren (LLE vs. SPME) erzielt wurden.

Die Analyse der Testmedien im Verlauf der BCF-Studien zeigte, dass stabile Testkonzentrationen für die einzelen HOCs durch die Säuleneluationstechnik über einen Zeitraum von acht Wochen erzielt werden können, ohne dass Lösungsvermittler zum Einsatz kommen. Obwohl SPME in der Umweltanalytik bereits für die Bestimmung von Analytkonzentrationen in vorwiegend wässrigen Matrizes etabliert ist, kommt diese Methode in ökotoxikologischen Studien im Rahmen der Regulation von Chemikalien bislang kaum zum Einsatz. Aufgrund zahlreicher potentieller Vorteile (Duering et al, 2012) sollte der Einsatz von SPME als alternative Methode für die Analyse wässriger Konzentrationen in BCF-Studien nach OECD 305 jedoch berücksichtigt werden. Wie in dieser Studie gezeigt wurde, konnten durch den Einsatz von SPME Gesamtkonzentrationen im Testsystem ermittelt werden, die mit den Ergebnissen des LLE-Verfahrens vergleichbar waren, wenn die SPME-Ergebnisse durch interne Standards korrigiert wurden. Die Ergbnisse der BCF-Studien zeigten, dass Fisch-BCF-Tests nach OECD 305 auch mit HOCs durchgeführt werden können, die durch einen logKOW von bis zu 7.8 gekennzeichnet sind. Für drei der vier Testsubstanzen war es möglich, neben den kinetischen BCF-Werten auch Steady state BCF-Werte

zu ermitteln. Wie im ersten Teil des Projekts gezeigt wurde, können die für BCF-Studien charakteristischen OM-Belastungen im Testsystem jedoch zu einer hohen Sorption von HOC führen und somit potentiell deren Bioverfügbarkeit für die Versuchstiere herabsetzen. Die Gehalte an gelöstem (DOC) und partikulärem Kohlenstoff (POC) im Testsystem wurden daher im Verlauf der Studien aufgezeichnet. Die Ergebnisse zeigten, dass die Versuchsbedingungen nur durch relativ niedrige Konzentrationen an Gesamtkohlenstoff (TOC = DOC + POC) gekennzeichnet waren. Die in der OECD TG 305 beschriebenen kritischen Schwellenwerte wurden nie überschritten, was den hohen Reinigungszustand der Testsysteme bestätigte. Die im Rahmen dieser Studie erzielten Ergebnisse wurden in Durchflussstudien mit Regenbogenforellen erzielt. Aufgrund des unterschiedlichen Fütterungsverhaltens und der abweichenden Stabilität des Fischkots, können vergleichbare Studien mit anderen Fischarten, wie z.B. Spiegelkarpfen, zu unterschiedlichen Ergebnissen bezüglich der Gesamtkohlenstoffgehalte im Testsystem führen.

Neben der Bestimmung von Gesamtkonzentrationen im Testmedium unter Verwendung interner Standards kann SPME auch zur Messung der frei verfügbaren, d.h. nicht an OM gebundenen Analytkonzentrationen, eingesetzt werden. In dieser Weise im Rahmen der zweiten BCF-Studie durchgeführte Messungen zeigten, dass die im Testsystem natürlich vorkommende OM-Belastung nur einen geringen Einfluss auf die Bioverfügbarkeit von HOCs ausübt. Die Berechnung von BCF-Werten basierend auf den frei verfügbaren Substanzkonzentrationen zeigte dementsprechend, dass Sorptionseffekte nur zu einer geringen Unterbestimmung von BCF-Werten führen und somit nicht als Erklärung für den “Hydrophobicity Cutoff”, wie von Jonker and van der Heijden (2007) vorgeschlagen, dienen können. Für die im Rahmen dieser Studie getestete Substanz mit der höchsten Lipophilität (PCB153, logKOW 7.8) wurde ein BCF-Wert ermittelt, der vergleichsweise niedriger war als die BCF-Werte der weniger lipophilen Testsubstanzen HCB und oTP. Als Erklärung für das niedrigere Bioakkumulationspotential von PCB153 kommen somit eher andere Faktoren, wie sterische Effekte, in Frage, da Messartefakte weitgehend auszuschließen sind. Im Gegensatz zu PCB153 konnte für die zweite höchst lipophile Testsubstanz (DBA) kein BCF-Wert bestimmt werden. Offensichtlich bedingt durch die schnelle Metabolisierung der Testsubstanz, konnte keine Biokonzentration beobachtet werden.

Im Rahmen der ersten Durchflussstudie (Exposition mit HCB und oTP) wurden die Versuchstiere über einen Zeitraum von 56 Tagen und somit länger als für die Erreichung der Gleichgewichtskonzentrationen im Fischgewebe erforderlich exponiert. Die Gewebskonzentrationen an oTP nahmen dabei nach Erreichen des Gleichgewichts zur Mitte der Studie wieder ab, was auf eine Adaptation des Fischmetabolismus an die akkumulierte Testsubstanz und damit auf eine stärkere Elimination gegen Ende der Studie schließen lässt. Die Biokonzentration von HCB führte hingegen zu konstanten Gewebskonzentrationen nach Erreichen des Gleichgewichts. Die in dieser Studie ermittelten BCF-Werte für HOCs sind von hoher Qualität und können für die Verifizierung der Ergebnisse aus der BMF/BCF-Umrechnung eingesetzt werden. Das Projekt hat gezeigt, dass BCF-Studien nach OECD 305 mit HOCs durchgeführt werden können. Auf den Einsatz von BMF-Studien, für die bislang keine eindeutigen Schwellenwerte für die Risikobewertung vorliegen und die somit zu einem fragwürdigen Einsatz von Versuchstieren führen, kann daher verzichtet werden.

3 Introduction

The European Community Regulation on chemicals and their safe use dealing with Registration, Evaluation, Authorization and Restriction of Chemicals [REACh] [Regulation (EC) No 1907/2006] defines specific registration requirements for substances that are (very) persistent, (very) bioaccumulative, and toxic (PBT/vPvB substances). As part of other regulatory chemical safety assessments such as for pharmaceuticals, biocides, and pesticides, the identification of PBT/vPvB substances is of increasing importance. With regard to the bioaccumulative properties of substances, bioconcentration factors (BCFs), which are defined as the concentration of a test substance in fish divided by the concentration of the chemical in the surrounding medium exceeding 2,000 and 5,000, are limiting criteria for B and vB -substances, respectively. As a consequence, numerous BCF -tests for hydrophobic organic compounds (HOCs) must be performed according to REACh requirements, and thus, efficient and reliable analytical methods for water and tissue samples are needed to guarantee accurate BCF estimates from flow-through fish tests carried out according to Organisation for Economic Co-operation and Development (OECD) Technical Guideline (TG) 305 [OECD, 2012].

OECD TG 305 describes a procedure for characterizing the bioconcentration potential of substances in fish. The test consists of two phases: the exposure (uptake) and post-exposure (depuration) phases. During the uptake phase, separate groups of fish of one species are exposed to at least two concentrations of the test substance. They are then transferred to a medium free of the test substance for the depuration phase. The concentration of the test substance in the fish is followed through both phases of the test. Where possible, the BCF is calculated preferably both as the ratio of the concentration in the fish and in the water at apparent steady state (BCFss)and as the kinetic bioconcentration factor BCFk, which is the ratio of the rate constants of uptake(k1) and depuration(k2) assuming first-order kinetics [OECD, 2012]. OECD TG 305 does not define specific analytical techniques to determine analyte concentrations in the water phase. Commonly applied are liquid-liquid extraction [LLE] and solid-phase extraction [SPE]. LLE can yield exhaustive extraction of the analytes but is time-consuming, is labor-intensive, and requires the use of considerable amounts of harmful solvents. In contrast, SPE has emerged as a powerful and solvent-saving tool to extract and purify analytes and now plays an important role in a broad range of applications [Liska, 2000]. Compared with LLE, SPE offers reduced processing time and significant solvent saving. However, this method requires multi-steps and is still time-consuming due to limited flow velocity during sample concentration and elution steps. Both LLE and SPE are disadvantageous in terms of losses in the evaporation step. However, higher concentrations can occur due to risks of contamination. Solid-phase microextraction [SPME] combines selective extraction and enrichment of analytes from liquid, gaseous, or solid samples with sample introduction to the analytical device. For HOCs, SPME is preferably coupled to gas chromatography [GC], with a direct thermodesorption of the analytes in the injection system. First reported in 1990, SPME provides solventless extraction of water samples and minimizes problems such as volatilization losses and contamination [Pawliszyn, 1997]. The principle of SPME is the partitioning of analytes from the sample to a coated fiber in the course of an equilibration. This process is highly dependent on the characteristics of the analyte, the sample matrix, and the ambient conditions. Background and applications of SPME have been summarized in textbooks and reviews [Pawliszyn, 1997, 2009; Alpendurada, 2000; Kataoka, 2011]. SPME has been applied in many different areas such as environmental analysis where precise quantification must be validated. This technique has been shown to be suitable and valid for a wide range of analytes from volatile to non-volatile compounds including many HOCs [Llompart & Fingas, 1997; Potter & Pawliszyn, 1994; Nilson et al. 1997; Ferrari et al. 1998] among others. As a consequence, a German DIN standard operational procedure for the analysis of 22 pesticides

of different polarities from water samples was established in 2007[DIN 38407-34:2006-05]. With regard to this standard procedure, SPME can be seen as a well-established and accepted method for the quantitative analysis of organic contaminants from moderate to high hydrophobicity in water samples.

Within bioconcentration tests, it was observed that highly HOCs with log KOW > 5 to 6 bioconcentrate less than what may be expected from their hydrophobicity. Explanations for this hydrophobicity ‘cutoff’ might be a reduced membrane permeation caused by steric effects of larger molecules or measurement artifacts [Jonker & van der Heijden, 2007], possibly because of the presence of organic matter [OM] in the test system. The influence of OM, quantified by the total organic carbon [TOC] in the test system on BCF values, has been noted since the 1980s because the bioconcentration of a substance is mainly influenced by its bioavailability; that is, differentiated from a substance’s total amount, only molecules that are bioavailable or rather bioaccessible can cross an organism’s cellular membrane from the medium the organism inhabits at a given time [Semple et al., 2004]. Increased concentrations of OM in the aquatic phase may reduce bioavailability and calculated BCF values when using total aqueous analyte concentrations [Arnot & Gobas, 2006]. Therefore, the OECD TG 305 requires tanks to be kept as clean as possible and to monitor the TOC content throughout the experiment. Particulate matter and TOC are accepted up to 5 and 2 mg L-1 in the dilution water, respectively, and the concentration should not exceed 10 mg L-1 TOC in the test vessels (without TOC from test substances and solubilizing agents). However, HOCs bind to OM far below a TOC content of 10 mg L-1 [Yabuta et al., 2004]. While the determination of freely dissolved concentrations has long been known to be laborious, SPME is suitable to distinguish freely dissolved and total concentrations and seems to be useful for various environmental and (eco-) toxicological investigations [Ramos et al., 1998].

4 Objectives

The aim of this project was to investigate

- the potential of SPME as an analytical routine approach for aquatic bioconcentration studies according to OECD TG 305 with a special focus on the testing of hydrophobic organic compounds (HOCs) characterized by log K_{OW} > 5.
- the freely dissolved analyte concentrations in the test medium by SPME.
- the sorption of HOCs (log K_{OW} 5.5 – 7.8) from different substance classes to total and dissolved organic matter.
- the effect of different extraction procedures (LLE vs. SPME) on the result of BCF studies.
- the effect of total organic carbon (TOC) present in the test water on the result of BCF-studies.

5 Part I: Effect of organic matter on the bioavailability of HOCs

5.1 Background

Bioconcentration factors (BCFs) for regulatory purpose are usually determined in fish bioconcentration studies according to OECD TG 305 (OECD 2012). This guideline does not define specific analytical techniques to determine analyte concentrations in the water phase. However, commonly applied is liquid-liquid extraction (LLE) which yields total analyte concentrations. In contrast, solid-phase microextraction (SPME) is capable of additionally analyzing the freely dissolved analyte concentrations. This is relevant due to the binding of hydrophobic organic compounds (HOCs) to organic matter (OM) present in the test system. Organic matter, quantified by total and dissolved organic carbon (TOC, DOC), influences the bioavailability of HOCs, because only the freely dissolved fraction of a test substance is capable to cross an organisms membrane.

5.1.1 Objectives

- Performance of sorption studies to confirm the hydrophobic characteristics of the investigated test substances, and to estimate their affinity to bind to different organic substances (sorption coefficients (K_d, K_{OC}, K_{DOC}).
- Comparison of LLE and SPME for the extraction of analyte concentrations in aqueous phases enriched with different sorbent matrices relevant for fish bioconcentration studies.
- Performance of kinetic studies to show the time dependent characteristics of sorption of HOCs within aqueous phases containing OM from different sorbent matrices

5.2 Materials and methods

Sorption of eleven HOCs (log KOW 5.5 – 7.8) from different substance classes to OM was systematically investigated with water containing defined concentrations of TOC and DOC. Matrices were fish feed (FF) and filter residue (FR) taken from the experimental tank used for an OECD TG 305 study on rainbow trout (containing feed residues and fish feces), which are the main components of the natural organic matter (particulate and dissolved) occurring in test systems according to OECD TG 305. Aldrich-humic acid (AHA) was used as reference. Samples were extracted by LLE and SPME and measured with GC/MS. Sorption of analytes in a mixture was determined indirectly due to decreased analyte extraction by SPME in presence of OM. Detailed information on the methods applied is given below.

5.2.1 Characterizing parameters

Matrix samples and preparation

Three matrices were used to estimate the reduction of bioavailable concentrations of test substances by sorption (Figure 1). Matrices were chosen according to their appearance in OECD TG 305 test systems:

- fish feed for rainbow trouts (micro pellets)
- filter residue from rainbow trout breeding vessels (containing feed residues and fish feces)

- Aldrich humic acid sodium salt (reference substance, CAS # 68131-04-4)

Fish feed and Aldrich humic acid were used “out of the box”, the filter residue was prepared by drying at 105 °C and grinding by ball mill.

Dry matter of all matrices was determined by heating for 24 h at 105 °C. Elemental composition (total carbon (C_t), total nitrogen (N_t), total sulfur (S_t)) was determined by CNS Analyzer (VarioEL III Elementar, Hanau, Germany), as the content of TOC is most relevant for the dimension of sorption. In absence of inorganic carbon, C_t and TOC are equal.

FF was purchased from Biomar (INICIO PLUS, 0.8mm) and contains 56.0 % crude protein, 18.0 % crude fat, 0.5 % crude fiber, 11.5 % ash, and 1.6 % total P according to the supplier.

Figure 1: Investigated sorbent matrices.

FR: filter residue from rainbow trout breeding vessels, FF: rainbow trout fish feed, AHA: Aldrich humic acid.

For sorption experiments with dissolved organic matter (DOM), stock solutions of approx. 3 g L-1 of each matrix were prepared in water (MilliQ quality). Dissolution of DOM was enhanced by exposing the stock solutions to ultrasonic for 3 h. Stock solutions were filtrated by pressure filtration with cellulose-acetate filters of 0.45 µm pore size (Sartorius, Göttingen, Germany). DOC concentrations in DOM solutions were measured by photometric cuvette tests (Hach-Lange, Düsseldorf, Germany).

5.2.2 Test substances

All test substances were purchased from Dr. Ehrenstorfer GmbH (Augsburg, Germany). Hexachlorobenzene (HCB, 99.5 %), 4-n-nonylphenol (NP, 99.3 %), o-terphenyl (OTP, 99.5 %), Polychlorinated biphenyls 2,2',3,4,4',5'-hexachlorobiphenyl (PCB 138, 98.5 %), 2,2',4,4',5,5'-hexachlorobiphenyl (PCB 153, 99.0 %), and 2,3,3',4,4',5-hexachlorobiphenyl (PCB 156, 99.0 %), hexanedioic acid bis(2-ethylhexyl) ester (DEHA, 99.5 %), bis(2-ethylhexyl) phthalate (DEHP D4, 98.4 %), and 2-ethylhexyl 2-cyano-3,3-diphenyl-2-propenoate (Parsol 340, Octocrylene, OCR, 98.0 %) were purchased as neat substances, polybrominated diphenyl ethers 4,4'-dibromodiphenyl ether (BDE 15) and 2,2',4,4'-tetrabromodiphenyl ether (BDE 47) were purchased in methanolic solution (100 µg mL-1). DEHP was purchased as deuterated compund (DEHP D4) to avoid contamination of the ubiquitary appearing plasticizer. 1,2,3,4-tetrachloronaphthalene (TCN) was purchased from Dr. Ehrenstorfer GmbH (100 mg L-1 in cyclohexane) and used as internal standard for LLE. Molecular structures of the test substances are given in Figure 2, further parameters in Table 1.

A stock solution of the nine neat compounds was prepared in methanol (100 – 500 µg mL-1) and mixed in same amounts with the BDE solutions. The concentration used for spiking the samples was between 30 and 160 µg mL-1 depending on the analyte. The selected test substances are

highly relevant as they belong to the group of persistent organic pollutants (POPs, according to Stockholm Convention) (HCB, PCBs, BDE 47) or rather because of their ubiquity in everyday life and the environment like plasticizers (DEHA, DEHP), and UV filters (OCR). DEHP is on European Chemicals Agency’s candidate list of substances of very high concern for authorization.

Figure 2: **Molecular structures of test substances used within sorption studies. Order of test substances according to chromatographic separation.**

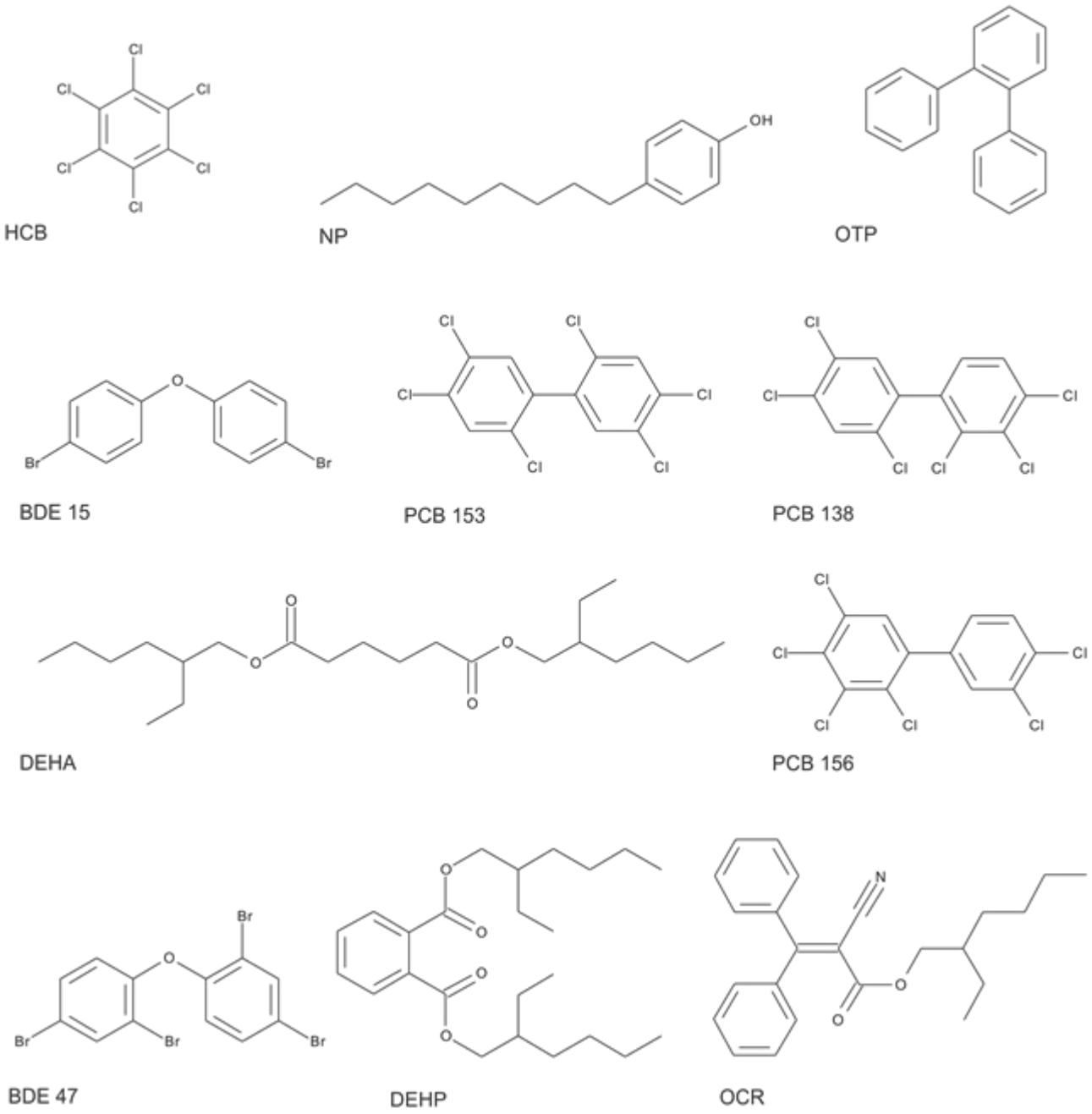


Table 1: Parameters of the test substances.

Analyte	Structure	CAS RN	Molar mass [g mol^{-1}]	Water solubility [µg L^{-1}] [a]	log Kow [a]	log Koc [b]
HCB	C_6Cl_6	118-74-1	284.78	6.2	5.73	[c] 4,11 – 4,90
NP	$C_{15}H_{24}O$	104-40-5	220.35	7000	5.76	[d] 5.44 – 6.18
OTP	$C_{18}H_{14}$	84-15-1	230.30	1240	5.52	-
BDE 15	$C_{12}H_8Br_2O$	2050-47-7	328.00	180	5.83	[e] 4.53 - 5.14
PCB 153	$C_{12}H_4Cl_6$	35065-27-1	360.88	0.95	7.75	[f] 5.3-7.7
PCB 138	$C_{12}H_4Cl_6$	35065-28-2	360.88	1.5	7.44	[f] 5.8-7.4
DEHA	$C_{22}H_{42}O_4$	103-23-1	370.57	780	6.11	-
PCB 156	$C_{12}H_4Cl_6$	38380-08-4	360.88	5.33	7.60	[f] 5.4 – 6.5
BDE 47	$C_{12}H_6Br_4O$	5436-43-1	485.79	1.46	6.77	[g] 6.32 - 6.54
DEHP	$C_{24}H_{38}O_4$	117-81-7	390.56	270	7.60	[h] 5,41 – 5,95
OCR	$C_{24}H_{27}NO_2$	6197-30-4	391.49	3.81	6.88	-

[a] www.syrres.com, [b] experimental data from literature: [c] Yabuta et al. 2004 ; [d] Heemken et al. 2001 ; [e] Olshansky et al. 2011 ; [f] Mackay et al. 2006 ; [g] Jia et al. 2012 ; [h] Williams et al. 1995

5.2.3 Sorption experiments

Experimental design

All sorption studies were performed with test substance concentrations according to their water solubility and therefore conform to relevant environmental conditions as well as conditions given in the OECD TG 305. Sorption was determined following a modified method according to OECD TG 106.

Freely dissolved analyte concentrations in the aqueous phase were measured by SPME. The amount of sorption was determined indirectly, by a reduced recovery rate of analytes in the presence of matrix, compared to the recovery rate in the absence of matrix.

All water used in the laboratory experiments was in Milli-Q Quality (Milli-Q Advantage A10 System, Millipore). All materials used in experiments were of glass or polytetrafluoroethylene to minimize the risk of sorption to materials.

Matrices concentrations of 1, 2, 5, and 10 mg L-1 TOC were weighed in glass bottles (250 mL Schott), equilibrated with 250 mL water for at least two hours and spiked with the stock solution to an analyte concentration of 0.8 – 5 µg L-1 (the concentration was kept within the water solubility of the analytes). The content of methanol (0.003 %) was below 0.01 % which is allowed according to OECD TG 305 as solubilizing agent. Spiking of samples was done by eVol® dispensing system (Thermo scientific).

The spiked samples were equilibrated for 24 h on an overhead shaker to accelerate the distribution of analytes between aqueous phase and sorbent. The time was defined according to the results from kinetic experiments.

After equilibration, aliquots of 20 mL were transferred with volumetric glass pipettes in duplicates to brown glass vials (headspace vials 20 mL with magnetic caps, CS Chromatographie Service, Langerwehe, Germany) and extracted by SPME and measured by GC/MS. The volumetric pipettes were washed with the samples two times prior to use.

The remaining 170 mL were extracted by liquid-liquid extraction. Here, the aqueous phase was asymptotically extracted three times with n-hexane (50 mL, 20 mL, 10 mL). The extract was dried with sodium sulfate (Na2SO4) and reduced by rotary evaporator including 100 µL n-heptane as keeper. Extracts were adjusted to 200 µL with n-heptane and measured by GC/MS. Extracts included 1,2,3,4-tetrachloronaphthalene as internal standard for the extraction procedure.

Instrument settings SPME and GC/MS

Extraction of freely dissolved analyte concentrations was performed by using negligible depletion-SPME in the dynamic range and in immersed mode. The samples were thermally equilibrated for 5 min at 30 °C prior to extraction with 100 µm PDMS fibers (Supelco) for 30 min at 30 °C. Samples were shaken in the agitator during pre-equilibration and extraction with 250 rpm. Processing of the sample sequence was performed by automation with an autosampler (CTC-Analytics, Combi Pal PAL-System).

After extraction, the fiber was thermodesorbed in the injection system for 3 min in splitless mode at 280 °C. Before extraction and after thermodesorption, the fiber was additionally desorbed in a needle heater at 280 °C for 4 min, respectively, to avoid analyte residues on the fiber. This was assured by frequently measuring pure water samples (blanks).

To adjust measurements from variability of instrument and fiber, external standards/reference samples (spiked water without sorbents) were measured periodically before and after each duplicate of sorbent concentration.

Separation and detection of analytes was carried out by gas chromatography (GC) coupled to ion trap mass spectrometry (MS) on two instruments. The GCs (Thermo Finnigan and Thermo Trace GC Ultra) were equipped with 30 m × 0.25 mm fused silica capillary columns with 0.25 µm coating (TG-5HT, Thermo). Temperature program of the GC oven is given in Table 2. The transfer lines to the MS were heated to 290 °C. The MSs were used in ms/ms mode (GCQ, Thermo Finnigan) or rather SIM mode (ITQ, Thermo) with respective segments for each analyte (parameters see Table 3). Ionization of molecules was performed by electron impact ionization (70 eV). Helium 5.0 was used as carrier gas with a flow rate of 1.2 mL per minute. LLE extracts were measured with the same instrument settings (injection volume 1.0 µL).

Table 2: Temperature program in GC oven (total time 31 min).

Temperature [°C]	Rate [°C min^{-1}]	Hold [min]
60	-	3
200	20	0
240	3	0
290	40	5
305	40	1

Table 3: Parameters of the test substances for MS detection in ms/ms mode.

Analyte	Structure	CAS RN	Molar mass [g mol^{-1}]	Retention time [min]	Precursor Ion	Quantifying Ions
HCB	C_6Cl_6	118-74-1	284.78	8.86	284	284
NP [a]	$C_{15}H_{24}O$	104-40-5	220.35	10.20	107	107
OTP [a]	$C_{18}H_{14}$	84-15-1	230.30	10.33	230	230
BDE 15	$C_{12}H_8Br_2O$	2050-47-7	328.00	11.76	168	139
PCB 153	$C_{12}H_4Cl_6$	35065-27-1	360.88	15.75	360	290+360
PCB 138	$C_{12}H_4Cl_6$	35065-28-2	360.88	16.76	360	290+360
DEHA	$C_{22}H_{42}O_4$	103-23-1	370.57	17.45	111	111
PCB 156	$C_{12}H_4Cl_6$	38380-08-4	360.88	18.94	360	290+360
BDE 47 [a]	$C_{12}H_6Br_4O$	5436-43-1	485.79	20.03	326	326
DEHP D_4 [a]	$C_{24}H_{38}O_4$	93951-87-2	394.56	20.46	153	153
OCR	$C_{24}H_{27}NO_2$	6197-30-4	391.49	22.96	248	248

[a] two scans inside of one segment.

Kinetics

Determination of sorption kinetics was investigated with DOM from FF, FR, and AHA. Samples were measured in duplicates after 5 min, 1 h, 3 h, 6 h, 12 h, 24 h, 48 h, 72 h, and 96 h of equilibration according to quotient approach measurements (see “experimental design” and “Quotient approach”) at a concentration of 10 mg L-1 DOC.

Kinetic sorption studies were repeated with a biocide (sodium azide, NaN3) added to the samples for gaining knowledge of a possible impact of test substance or rather organic matter degradation throughout the experiments. NaN3 was added in a concentration of 100 mg L-1, which should be low enough to exclude molecule degradation according to Chefetz et al. (2006).

Quotient approach

Sorption was determined in a sequence of reference samples and samples with increasing sorbent concentrations (1, 2, 5, and 10 mg L-1 TOC or DOC). Spiked analyte concentrations were constant for each analyte in all samples (see “experimental design”). Each sequence N with four sorbent concentrations was repeated several times (1-7 times), with n=2 duplicates per sorbent concentration everytime.

Quantification of sorption

Sorption strength was measured indirectly from reduced recovery rates of analytes in the presence of organic matter (COM), organic matter quantified as TOC (CTOC), and DOM quantified as DOC (CDOC), compared to a reference sample without sorbents (C0). The slope of a linear regression as function of quotients made up of C0/COM and organic matter concentration leads to sorption coefficients Kd, whereas the regression of C0/CTOC and TOC concentration yields sorption coefficients KOC, and the regression of C0/CDOC and DOC concentration yields sorption coefficients KDOC. The slope was calculated with mean values of cumulated quotients obtained from all measured sequences. Outliers were excluded from calculation of sorption coefficients. Outliers were determined in box plots as values outside the 1.5fold or rather 3fold distance of the range between median and upper or lower quartile (Z-Q1) or (Q3-Z) when at least four quotients per sorbent concentration existed. For detailed

information see Müller-Wegener (1982), Yabuta et al. (2004), Böhm and Düring (2010). Values have not been corrected for dry matter.

5.3 Results and discussion

5.3.1 Characterizing parameters

Results from elemental analysis for further characterization of sorbents are given in Table 4. Because samples did not contain inorganic carbon, C total is identical with TOC. The content of organic carbon is regarded as main parameter to affect the amount of sorption, for that reason sorption coefficients Kd are normalized to the content of TOC resulting in sorption coefficients KOC.

Dry matter content was 96 % and 95 % for FF and FR, respectively.

Table 4: Elemental characterization (C, N, S) of sorbents [%].

Sorbent	FF	FR	AHA
C total	49.9	28.5	38.4
N total	9.39	4.11	0.57
S total	0.86	1.08	1.44

5.3.2 Sorption studies

Kinetic studies

Kinetic studies were performed in addition to the project specifications. The results show the time dependent characteristics of sorption of the test substances within aqueous phases containing DOM from AHA, FF, and FR for equilibrium times between 5 min and 4 days. In addition, experiments were repeated with sodium azide as biocide to reveal possible influences on sorption from molecule degradation over time.

According to the data on sorption kinetics, the sorption to AHA, FF, and FR did not increase very much over time. For most of the test substances, the amount of sorption within the first hour was similar to the amount of sorption after one or more days of equilibration time.

The results of the kinetic studies will be integrated in a forthcoming publication on the effect of organic matter on the bioavailability of HOCs.

Sorption studies with organic matter

Results of sorption studies confirm the hydrophobic characteristics of the investigated test substances as shown by their high affinity to bind to organic substances. Depending on sorbent quality, studies resulted in sorption coefficients Kd from approx. 29000 to 740000 (log Kd 4.46 – 5.87) and TOC normalized coefficients KOC from approx. 75000 to 2000000 (log KOC 4.87 – 6.30) (Table 5 to Table 7and Figure 4 to Figure 10). These values correspond to values from literature, if available (Table 1). Relative standard deviation (RSD) of results is between 0.1 and 0.2 for most values (min 0.02, max 0.3), resulting from the fact, that no internal standards can be used to correct variations in fiber sensitivity and instabilities of the instrument (MS). However, this drawback of the method was compensated by a high number of replicates N (up to seven reruns).

The content of organic carbon is regarded as the main parameter to affect the amount of sorption. However, the amount of sorption is not solely explainable by OC content, and may also depend on differences in organic matter quality (sorption increases with increasing complexity of organic matter, e.g. the complexity of and sorption to humic substances increases from fulvic acids to humic acids to humin).

Increasing Kd values from FR (28.5 % TOC) to AHA (38.4 % TOC) reflect the high impact of OC content on the sorption of the analytes to the investigated matrices.

However, when comparing AHA and FF (49.9 % TOC) it is noticeable, that Kd values for AHA are higher than values for FF. This might be due to the complex, macromolecular structure of humic acid, whereas the main sources of carbon in FF are lipids. This difference is adjusted by TOC normalization of Kd values for FF and FR. The largest KOC values were estimated for AHA; KOC values of FF and FR were similar.

Besides quality of the organic matter, the molecular characteristics of the test substances are very important. Most relevant for the amount of sorption is the hydrophobicity of the analytes. Three groups of analytes with a similar range of sorption coefficients for AHA were identified (Figure 6 to Figure 8). Group 1 contains the analytes HCB, NP, OTP, and BDE 15 with log KOW values from 5.5 to 5.8. Group 2 contains the unhalogenated substances DEHA, DEHP, and OCR with log KOW values from 6.1 to 7.6. Group 3 contains the halogenated compounds PCB 153, PCB 138, PCB 156, and BDE 47 with log KOW values from 6.8 to 7.8. As shown in Figure 8, there is an overlap of group 2 and 3 for FF and of group 1 and 2 for FR, which demonstrates the effect of the interaction of molecular structure and organic matter quality on the actual amount of sorption.

The relation between experimentally determined sorption coefficients Kd and KOC and KOW values from literature is shown in Figure 3.

Figure 3: log K_d and log K_{OC} values of the eleven test substances resulting from sorption studies with the sorbents Aldrich humic acid (AHA), fish feed (FF), filter residue (FR) plotted against their log K_{OW} values.

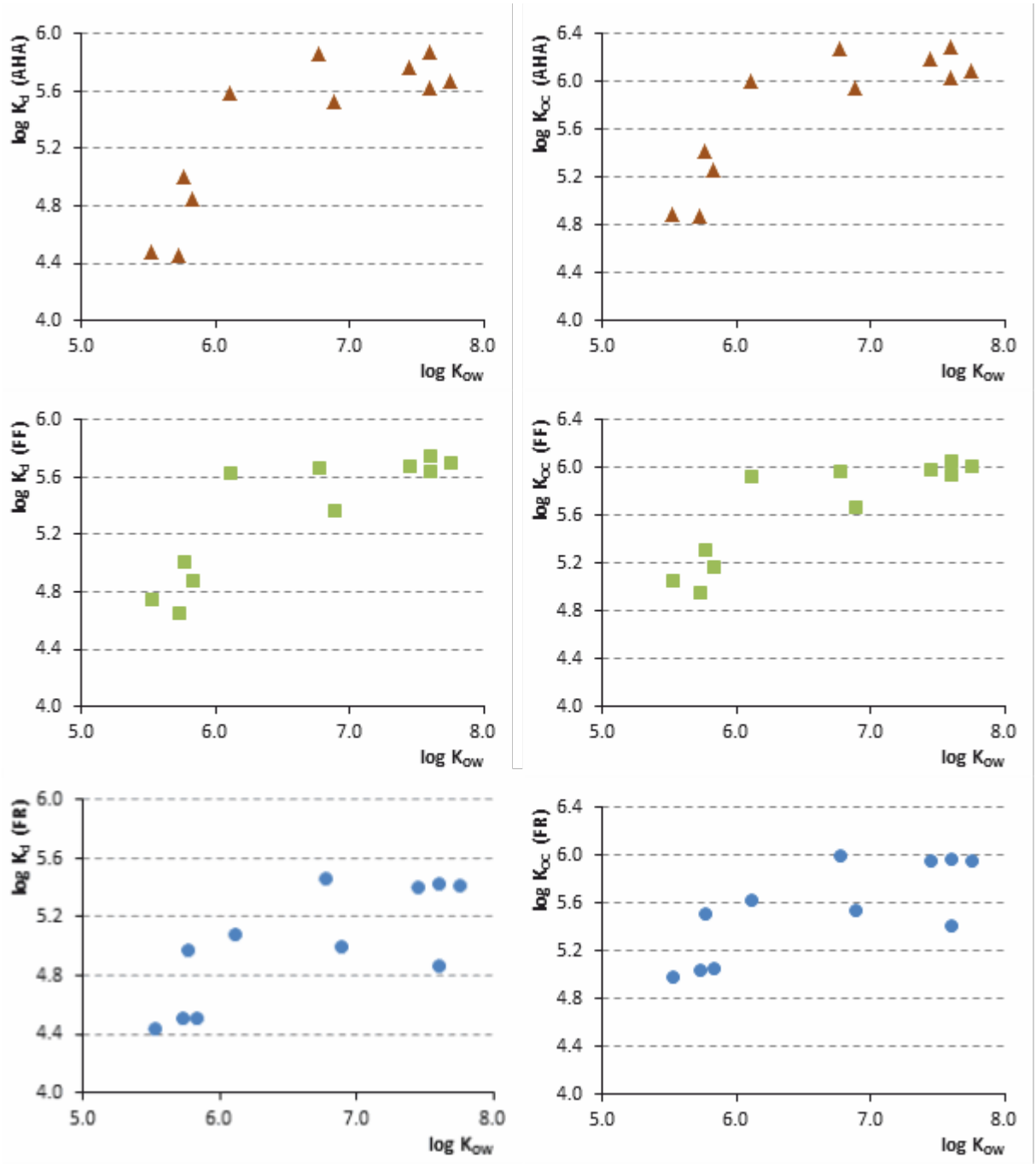
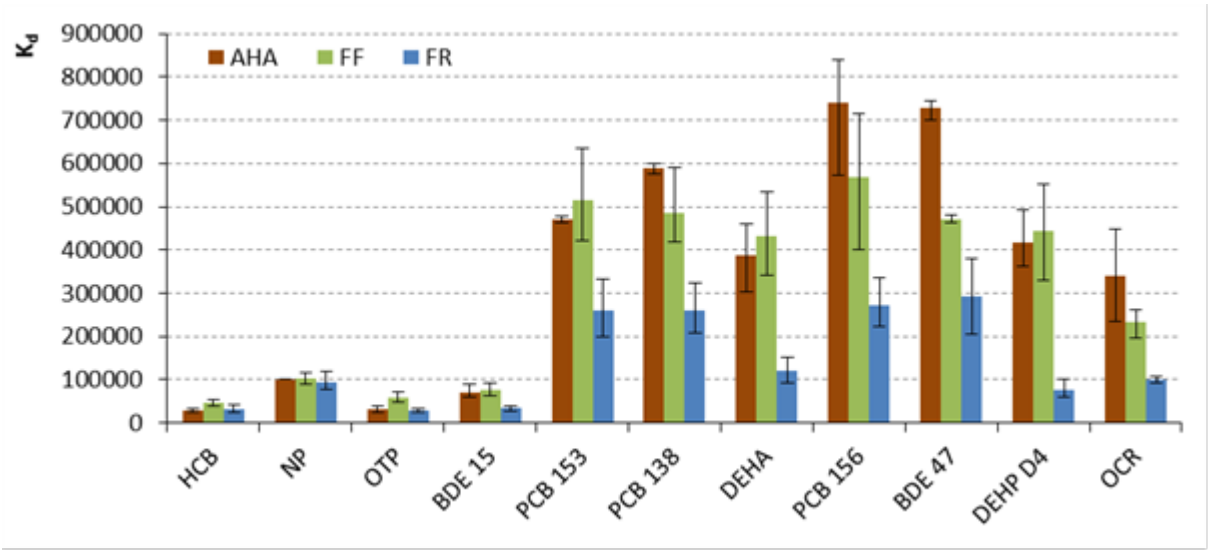
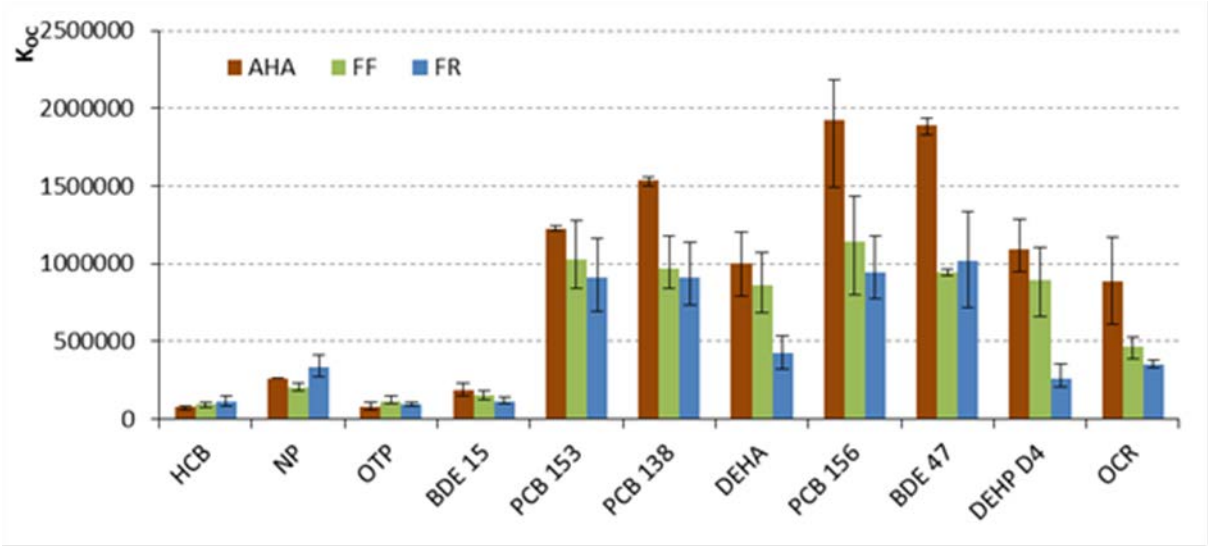


Figure 4: K_d values of test substances for investigated matrices. Extraction of freely dissolved test substances by SPME, order of test substances according to chromatographic separation.



AHA: Aldrich humic acid, FF: fish feed, FR: filter residue

Figure 5: K_{OC} values of test substances for investigated matrices. Extraction of freely dissolved test substances by SPME, order of test substances according to chromatographic separation.



AHA: Aldrich humic acid, FF: fish feed, FR: filter residue

Table 5: Partitioning coefficients K_d and K_{OC} for Aldrich humic acid as sorbent.

Analyte	K_d			log K_d			K_{OC}			log K_{OC}			r^2	RSD K	RSD log K	N
HCB	28729	±	3986	4.46	±	0.06	74854	±	10386	4.87	±	0.06	0.9927	0.10	0.01	6
NP	100826			5.00			262704			5.42			0.9969			1
OTP	30077	±	7952	4.48	±	0.11	78366	±	20719	4.89	±	0.11	0.9847	0.19	0.02	7
BDE 15	70343	±	16176	4.85	±	0.10	183281	±	42148	5.26	±	0.10	0.9968	0.19	0.02	7
PCB 153	470457	±	72163	5.67	±	0.06	1225788	±	188021	6.09	±	0.06	0.9953	0.11	0.01	6
PCB 138	588622	±	82132	5.77	±	0.06	1533670	±	213997	6.19	±	0.06	0.9985	0.10	0.01	6
DEHA	385979	±	48666	5.59	±	0.05	1005677	±	126800	6.00	±	0.05	0.9886	0.14	0.01	3
PCB 156	739613	±	274871	5.87	±	0.15	1927080	±	716182	6.28	±	0.15	0.9961	0.25	0.02	6
BDE 47	727279	±	169165	5.86	±	0.10	1894943	±	440763	6.28	±	0.10	0.9929	0.21	0.02	5
DEHP D4	417673	±	59775	5.62	±	0.06	1088258	±	155746	6.04	±	0.06	0.9550	0.16	0.01	3
OCR	338903	±	97215	5.53	±	0.13	883021	±	253296	5.95	±	0.13	0.9990	0.27	0.02	6

Table 6: Partitioning coefficients K_d and K_{OC} for fish feed as sorbent.

Analyte	K_d			log K_d			K_{OC}			log K_{OC}			r^2	RSD K	RSD log K	N
HCB	45486	±	5447	4.66	±	0.05	91069	±	10907	4.96	±	0.05	0.9928	0.09	0.01	6
NP	102665	±	26091	5.01	±	0.11	205547	±	52237	5.31	±	0.11	0.9857	0.22	0.02	4
OTP	57110	±	13785	4.76	±	0.11	114341	±	27599	5.06	±	0.11	0.9959	0.20	0.02	6
BDE 15	75543	±	13256	4.88	±	0.08	151246	±	26540	5.18	±	0.08	0.9987	0.17	0.02	5
PCB 153	514616	±	96191	5.71	±	0.08	1030323	±	192586	6.01	±	0.08	0.9809	0.17	0.01	5
PCB 138	485286	±	94885	5.69	±	0.08	971603	±	189971	5.99	±	0.08	0.9877	0.17	0.01	5
DEHA	430606	±	92231	5.63	±	0.09	862126	±	184657	5.94	±	0.09	0.9942	0.19	0.01	4
PCB 156	569067	±	154266	5.76	±	0.12	1139342	±	308859	6.06	±	0.12	0.9861	0.24	0.02	5
BDE 47	471738	±	148079	5.67	±	0.15	944477	±	296473	5.98	±	0.15	0.9926	0.26	0.02	4
DEHP D4	444925	±	110939	5.65	±	0.11	890794	±	222114	5.95	±	0.11	0.9899	0.25	0.02	3
OCR	234355	±	25329	5.37	±	0.05	469207	±	50712	5.67	±	0.05	0.9965	0.11	0.01	4

Table 7: Partitioning coefficients K_d and K_{OC} for filter residue as sorbent.

Analyte	K_d		log K_d		K_{OC}		log K_{OC}		r^2	RSD K	RSD log K	N
HCB	32232	± 8858	4.51	± 0.12	112977	± 31049	5.05	± 0.12	0.9840	0.23	0.02	4
NP	94842	± 20665	4.98	± 0.09	332429	± 72433	5.52	± 0.09	0.9674	0.21	0.02	3
OTP	28029	± 3920	4.45	± 0.06	98244	± 13739	4.99	± 0.06	0.9974	0.10	0.01	5
BDE 15	32821	± 4607	4.52	± 0.06	115040	± 16147	5.06	± 0.06	0.9958	0.13	0.01	4
PCB 153	260409	± 58950	5.42	± 0.10	912756	± 206626	5.96	± 0.10	0.9957	0.22	0.02	4
PCB 138	259746	± 49711	5.41	± 0.08	910431	± 174242	5.96	± 0.08	0.9978	0.21	0.02	4
DEHA	121033	± 25594	5.08	± 0.09	424232	± 89710	5.63	± 0.09	0.9932	0.21	0.02	4
PCB 156	270335	± 51307	5.43	± 0.08	947545	± 179834	5.98	± 0.08	0.9952	0.20	0.02	4
BDE 47	291558	± 86361	5.46	± 0.13	1021935	± 302702	6.01	± 0.13	0.9985	0.29	0.02	4
DEHP D4	74808	± 19979	4.87	± 0.11	262210	± 70030	5.42	± 0.11	0.9914	0.30	0.03	3
OCR	100429	± 2424	5.00	± 0.01	352013	± 8495	5.55	± 0.01	0.9929	0.02	0.00	3

Figure 6: Sorption of test substances in an aqueous phase containing Aldrich humic acid. Extraction of freely dissolved test substances by SPME, order of test substances according to chromatographic separation, error bars show min/max.

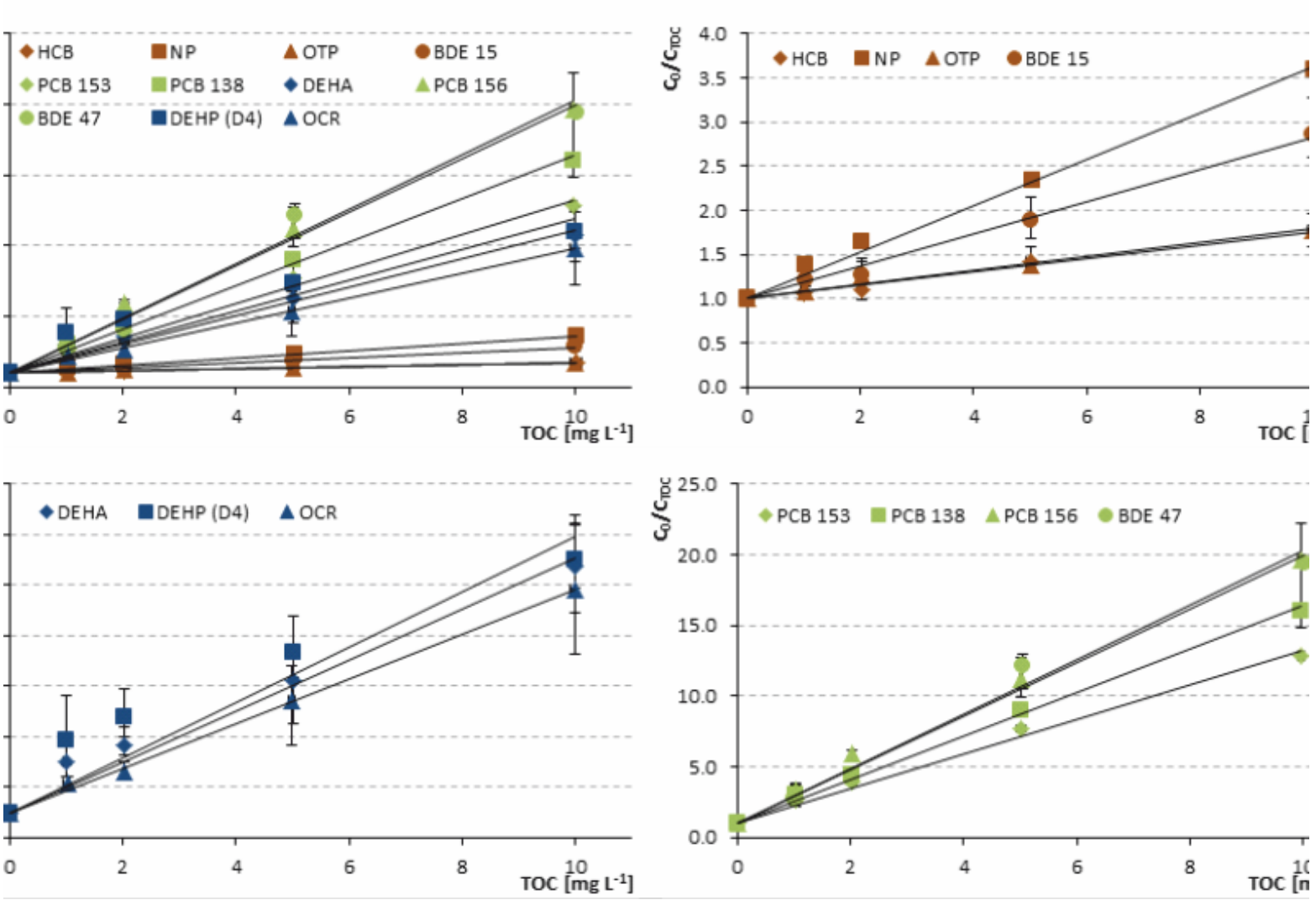


Figure 7: Sorption of test substances in an aqueous phase containing fish feed. Extraction of freely dissolved test substances by SPME, order of test substances according to chromatographic separation, error bars show min/max.

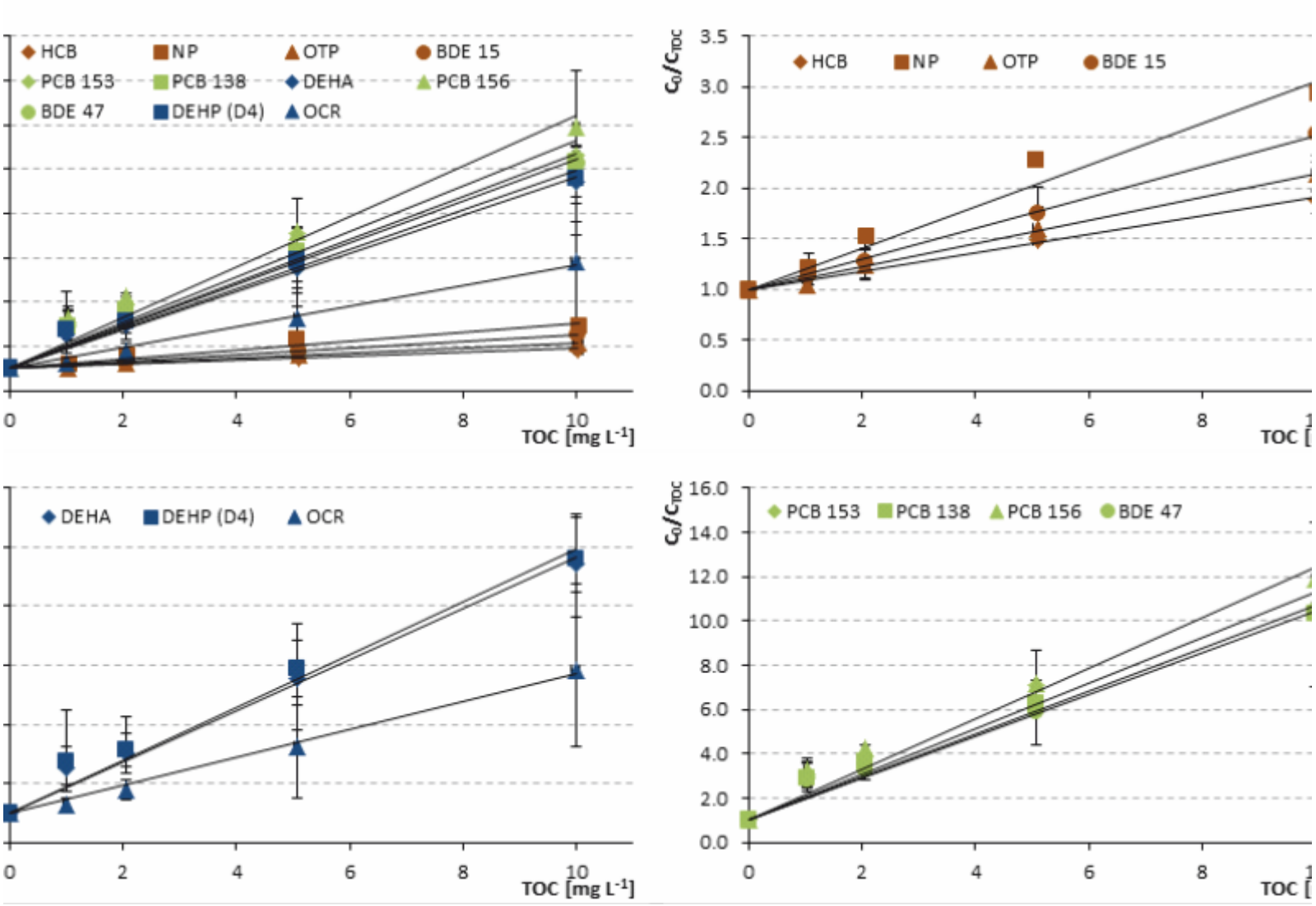
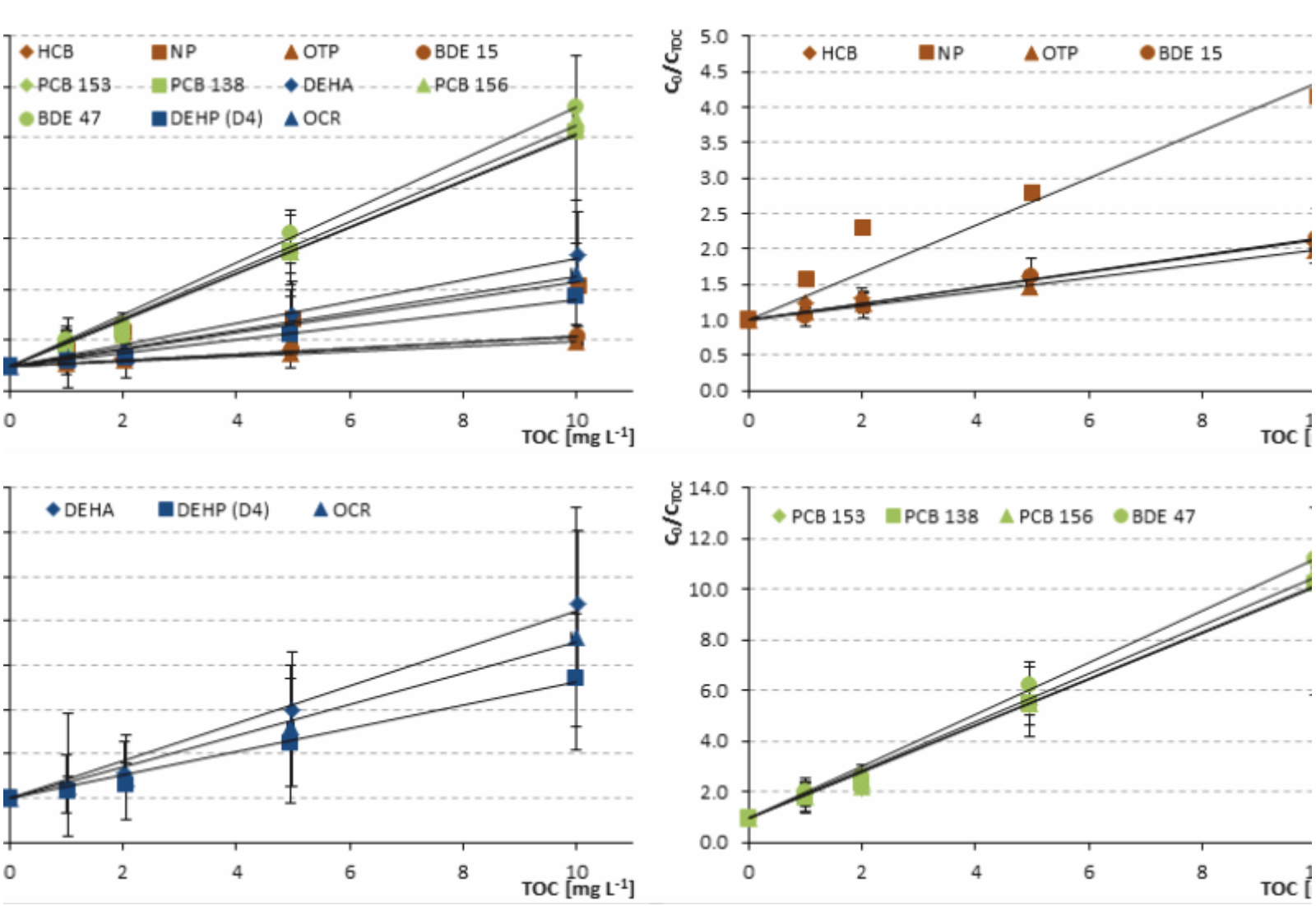


Figure 8: Sorption of test substances in an aqueous phase containing filter residue. Extraction of freely dissolved test substances by SPME, order of test substances according to chromatographic separation, error bars show min/max.



Sorption studies with dissolved organic matter

Higher K_{OC} values compared to K_{DOC} values were to be expected, because sorption increases with increasing molecular size, which is smaller for dissolved molecules (< 0.45 µm) compared to particles. This is supported by data evaluated for AHA, with significantly smaller K_{DOC} values than K_{OC} values (Table 8, Figure 9).

However, DOM from FF could be identified as very potent sorbent, since K_{DOC} values for FF are significantly higher than their K_{OC} values (Table 9, Figure 10) This might be explained by the structural composition of FF. Since the feed consists of fiber, ash, protein, and fat, it is assumed, that fiber and ash do not contribute to sorption but remain particulate, whereas the lipids from the fat fraction should contribute most to the sorption and are assumed to get dissolved within the process of DOM preparation.

According to the sorption data for FR, sorption coefficients K_{DOC} for FR as sorbent are estimated to be smaller than the K_{OC} values for FR.

Sorption coefficients K_{DOC} for FR as sorbent are smaller than the K_{OC} values for FR. However, in the low range of DOC (0 – 10 mg L^{-1}) used within this study, the determination method was not robust enough to sensitively measure the weaker sorption of FR_{DOC} compared to the other sorbents.

Table 8: Partitioning coefficients K_{DOC} for Aldrich humic acid as sorbent.

Analyte	K_{DOC}			log K_{DOC}			r^2	RSD K	RSD log K	N
HCB	40044	±	9129	4.60	±	0.10	0.9530	0.23	0.02	3
NP	51813			4.71			0.8152			1
OTP	32206	±	1963	4.51	±	0.03	0.9926	0.07	0.01	3
BDE 15	27772	±	11918	4.44	±	0.20	0.9657	0.61	0.06	2
PCB 153	254637	±	63914	5.41	±	0.11	0.9996	0.25	0.02	4
PCB 138	275677	±	88830	5.44	±	0.14	0.9967	0.33	0.03	3
DEHA	160200	±	63001	5.20	±	0.18	0.9903	0.39	0.04	3
PCB 156	353245	±	41008	5.55	±	0.05	0.9936	0.16	0.01	2
BDE 47	124326	±	46262	5.09	±	0.15	0.9936	0.42	0.03	3
DEHP D4	207039	±	618	5.32	±	0.00	0.9726	0.00	0.00	2
OCR	150028	±	9009	5.18	±	0.03	0.9712	0.08	0.01	2

Figure 9: Partitioning coefficients K_d, K_{OC}, and K_{DOC} by comparison for Aldrich humic acid as sorbent.

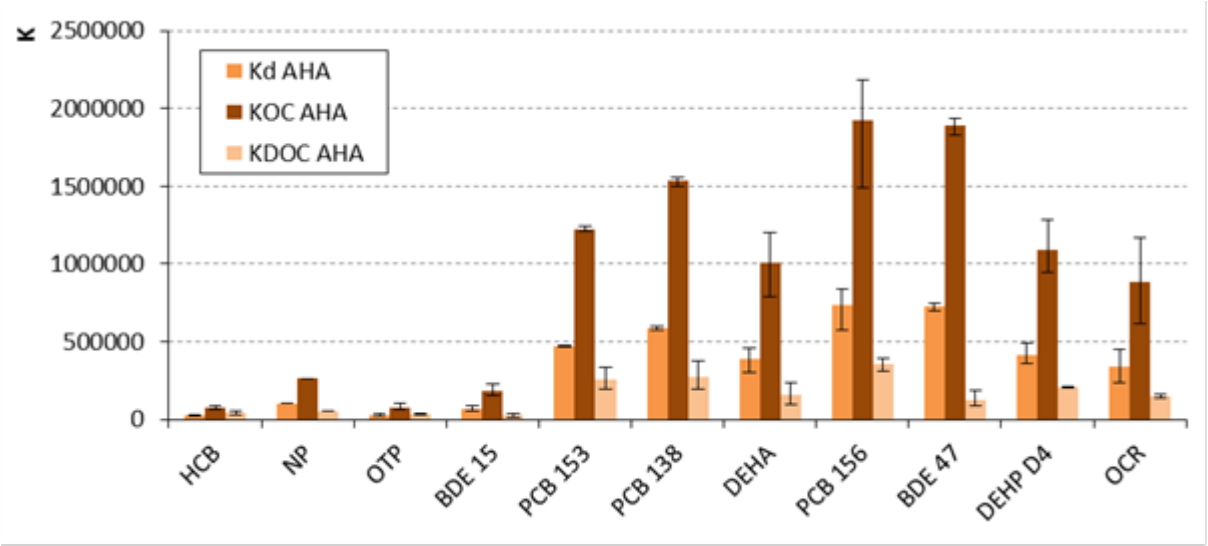
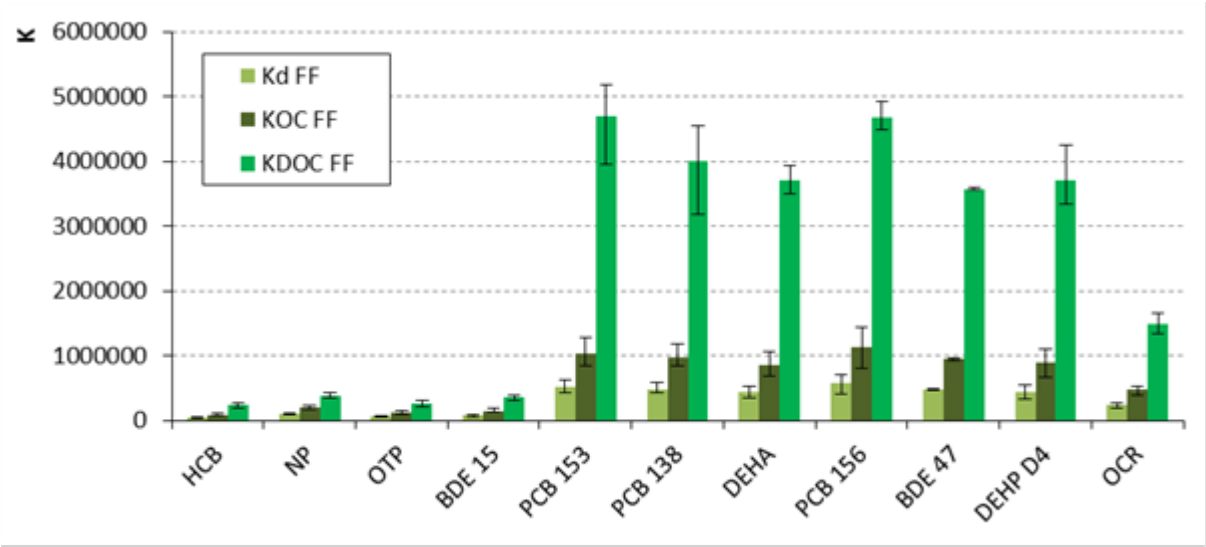


Table 9: Partitioning coefficients K_{DOC} for fish feed as sorbent.

Analyte	K_{DOC}	log K_{DOC}	r^2	RSD K	RSD log K	N
HCB	242640 ± 85408	5.38 ± 0.13	0.9904	0.31	0.02	3
NP	386109 ± 102862	5.59 ± 0.14	0.9803	0.32	0.03	3
OTP	260652 ± 74407	5.42 ± 0.14	0.9999	0.26	0.02	4
BDE 15	352523 ± 46840	5.55 ± 0.06	0.9992	0.12	0.01	4
PCB 153	4695087 ± 1500087	6.67 ± 0.19	0.9879	0.33	0.03	4
PCB 138	4006871 ± 1107512	6.60 ± 0.15	0.9878	0.27	0.02	4
DEHA	3712550 ± 1178634	6.57 ± 0.16	0.9995	0.35	0.03	3
PCB 156	4675245 ± 1552871	6.67 ± 0.19	0.9915	0.34	0.03	4
BDE 47	3574843 ± 397877	6.55 ± 0.04	0.9854	0.14	0.01	2
DEHP D4	3702391 ± 363946	6.57 ± 0.04	0.9951	0.10	0.01	3
OCR	1490957 ± 388088	6.17 ± 0.10	0.9935	0.21	0.01	4

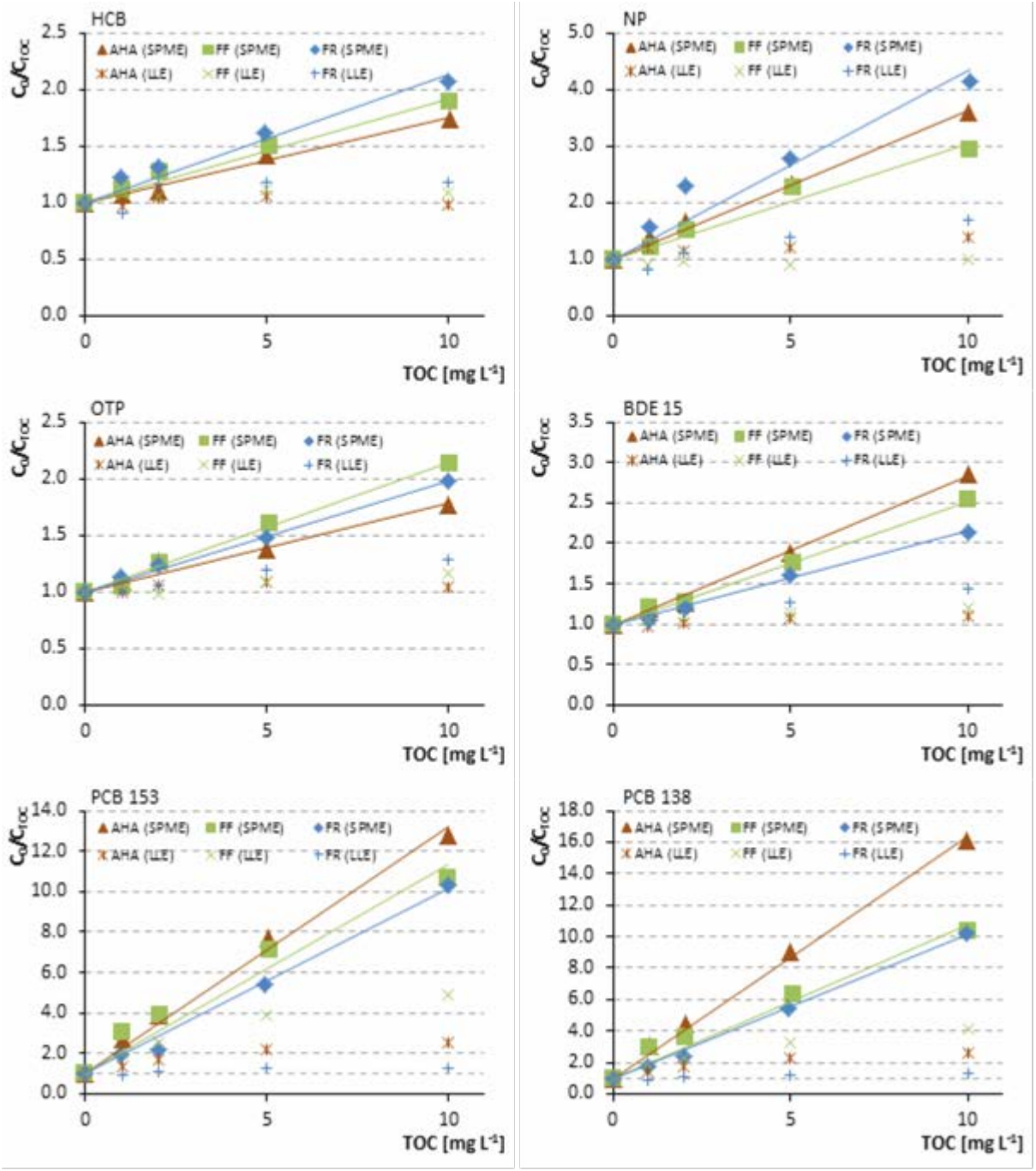
Figure 10: Partitioning coefficients K_d, K_{OC}, and K_{DOC} by comparison for fish feed as sorbent.



Comparison of extractable amounts by SPME and LLE

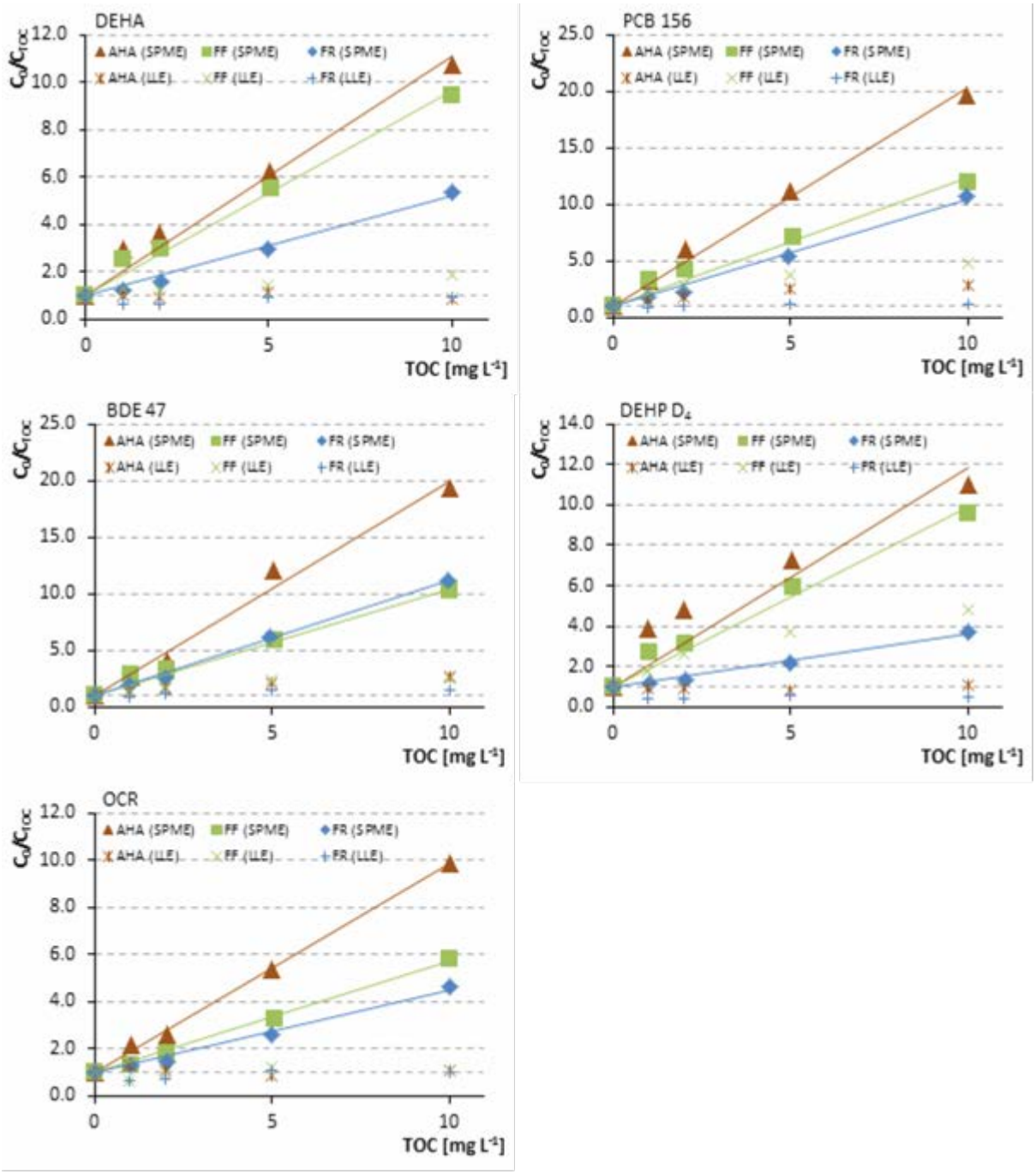
Extraction of the aqueous phases by LLE confirmed the method as tool for obtaining total analyte concentrations for most analytes and matrices. However, especially for aqueous phases containing FF and partially for AHA and FR, the amount of extractable analyte molecules slightly decreased with increasing TOC content for the highly hydrophobic substances. This is shown in Figure 11 in terms of the quotient C_0/C_{TOC}, which is the ratio of the recovery rate of a test substance in the absence of TOC (C_0) and the recovery rate of a test substance in the presence of TOC (C_{TOC}). In case of a total analyte extraction independent from TOC content, no differences should appear between recovery rates in the absence and presence of TOC, and the quotient should therefore be equal 1. Quotients > 1, especially systematically increasing quotients with increasing TOC content, indicate a reduced recovery rate due to the presence of organic matter. This is depending on the quality of organic matter, i.e. the high lipid content of fish feed can function as a competitor to n-hexane as solvent for the extraction. Therefore, the extracted amounts of test substance are reduced with increasing concentration of TOC. This is not the case for filter residue where for most test substances – independent of the TOC concentrations – equal amounts of the test substance are extracted. However, reduced extracted amounts by LLE do not correspond to the free concentrations of the test substance, which are bioavailable. Concentrations extracted by LLE are smaller than the total concentrations, but higher than the free concentrations (which can be extracted by SPME).

Figure 11: Extraction of test substances by SPME and LLE from aqueous phases containing different types of organic matter.



AHA: Aldrich humic acid, FR: filter residue, FF: fish feed. Sequence in order of chromatographic separation.

Figure 11 continued



5.4 Conclusions

Results show that SPME is capable of determining reduced bioavailability of hydrophobic test substances even in the presence of very low concentrations of OM as found in fish bioconcentration studies.

Results indicate that the influence of organic matter inside BCF test systems is highly dependent on the quality of organic matter and interacts with the molecular characteristics of the test substance. These interactions between structural quality of OM and analyte characteristics highly influence the amount of sorption.

LLE may capture significant amounts of molecules bound to OM which are not available to fish (Part III of this study). However, performance of LLE depends on the quality of OM. This method does not yield total analyte concentrations in every combination of organic matter quality and molecule characteristics. Especially for FF, extraction of highly hydrophobic analytes is often reduced. For FR, LLE yields total concentrations independent from TOC for most test substances. FF and FR are the most relevant sources of OM in BCF studies.

OM from BCF studies has a high sorption capacity for HOCs. However, resulting sorption coefficients represent a worst case scenario for BCF studies, as they are determined under equilibrium conditions in a closed system. Nevertheless, it is to be expected that the calculation of BCF values may lead to an underestimation of BCFs. This is supported by SPME analysis of flow-through fish test water concentrations (Part III of this study).

LLE results suggest, that the underestimation might be smaller than predicted from sorption coefficients, as extraction by LLE does not yield total concentrations as well for some combinations of matrix (OM quality) and analytes (analyte characteristics). Efforts should be done to choose an appropriate solvent, as extraction efficiency is also depending on the (quality of the) solvent used.

Nevertheless, even for the case of an insufficient extraction by LLE according to total analyte concentrations, LLE results will still contribute to an underestimation of BCF values, as the amount of extracted analyte concentrations is still higher than for free water concentrations extracted by SPME.

Sorption data for OM relevant to BCF studies (FF and FR) are missing in the literature, because subject of most studies is the interaction of test substances with humic substances as organic matter (natural and reference).

Different results are to be expected for feed and feces (or rather filter residue) obtained from studies on other test organisms. Relevance of SPME measurements might be further enhanced for other fish species such as carp, which shows a different feeding habit and consistency of fecal matter compared to rainbow trout, probably resulting in higher TOC contents in the water.

SPME should be used to measure bioavailable analyte concentrations in bioconcentration studies, to assess the presence of non- bioavailable molecules bound to OM in the test system.

6 Part II: Effect of different extraction procedures for water sample preparation on the result of BCF-studies

6.1 Background

The standard method for the extraction of organic compounds from aqueous samples collected during BCF-tests carried out according to OECD TG 305 is liquid-liquid extraction (LLE). The suitability of SPME to measure highly hydrophobic substances is described in the literature (Düring et al. 2012). The aim of this study was to investigate the effect of different extraction procedures on the result of BCF studies carried out under realistic experimental conditions. The bioconcentration potential of two highly lipophilic test substances - hexachlorbenzene (HCB, log K_{OW} = 5.7) and o-terphenyl (oTP, log K_{OW} = 5.5) – was investigated in a flow-through fish test on rainbow trout.

6.2 Objectives

- Performance of an aquatic bioconcentration study according to OECD TG 305 with two hydrophobic organic compounds (HOCs) characterized by log K_{OW} > 5 (hexachlorobenzene, HCB; o-terphenyl, oTP)
- Preparation of column generated concentrations of both HOCs over the maximum uptake period of 56 days.
- Measurement of total organic carbon concentration in the test water during the flow-through study
- Comparison of the results obtained by different extraction procedures (LLE vs. SPME) for water sample preparation

6.3 Material and methods

6.3.1 Preparation of the dosing system

Spiking of carrier matrix with the test items

A solution of HCB or oTP in methyl *tert*-butyl ether (MTBE) was mixed with Florisil 60-100 mesh in a round-bottomed flask to reach a loading of 1 mg g^{-1} or 5 mg g^{-1} for HCB and oTP, respectively.

Preparing the glass columns

On a rotary evaporator the solvent was subsequently evaporated to dryness and the material additionally dried in a drying cabinet for 3 h at 65°C. The dry carrier material of each test item was mixed with water and filled into glass columns (Dimension: diameter 55 mm, high 600 mm, end-capped with stainless-steel flanges). The top and the bottom of the fillings were covered with membrane filters to avoid the loss of matrix material. A constant flow of purified drinking water through each column from bottom to top was maintained at a level of 5–30 mL min^{-1}.

Supply of test items into the system

The outlet tubes of three columns (two with HCB, one with oTP) were connected and the resulting stock solution further diluted in a mixing chamber and continuously supplied to the single test chamber (Figure 12). The dosing system was tested for three weeks prior to the onset of the experiment to prove the continuous supply of constant test concentrations.

6.3.2 Holding and dilution water

Purified drinking water was used according to the OECD-Guideline (OECD, 2012). The purification included filtration with charcoal, aeration and passage through a lime stone column.

6.3.3 Test performance

The flow-through fish test was carried out with juvenile rainbow trout (*Oncorhynchus mykiss*) with an average size of 3.3 g ± 0.4g at the start of the experiment. Only healthy fish free of observable diseases and abnormalities were used in the study. The experimental animals were raised in the hatchery of Fraunhofer IME from fertilized eggs which were obtained from Fischzucht Rameil, Gleierbrück, Germany.

Two 100 L glass aquaria filled with 70 L of test solution (test group) or water (control group) were used as test vessels. In the flow-through tanks a continuous flow of approximately 22 L/h were maintained throughout the test using metering pump systems leading to 7 volume replacements through each test chamber per day.

Both tanks were stocked with 68 fish each. After the uptake phase scheduled for 56 d, the fish in the test group were transferred into a new aquarium with test substance-free dilution water for further 28 d (depuration period). According to OECD 305 the uptake phase should be run for 28 days unless it can be demonstrated that equilibrium has been reached earlier. The duration of the uptake period was extended to 56 days to prove that long-term exposure to constant column generated concentrations of HOCs is possible. Animals exposed to the two test substances were compared with unexposed control animals which were kept under comparable conditions. The water temperature in the experimental tanks was 15.1°C ± 0.2°C during the study period lasting 84 days.

Figure 12: Example of a test system with 4 loading columns.

The water in the test vessels was aerated via a stainless steel capillary. Observations were made throughout the test period on fish behavior and mortality. Temperature, pH and the oxygen concentrations in the test vessels were measured daily. The light/dark cycle was adjusted to 12/12 hours and light intensities were measured once during the test.

Rainbow trout in the control and test chamber obtained a daily ration equivalent to 1.5% of their body weight. A commercial feed for juvenile rainbow trout (Biomar, Inicio Plus 0.8mm) was used.

The total organic carbon concentration in the water (TOC) can be significantly affected by feed particles and feces. Therefore, the tanks were cleaned every day 30-60 minutes after feeding to avoid the accumulation of organic material. The TOC-concentration in the water was estimated on a weekly basis. Water samples were taken together with the samples for analysis of the test items: Water (1L) including solid particles were rinsed through a pre-weighed 0.4µm glass fiber filter. Water cleaned in this way was used to estimate the concentration of dissolved organic carbon (DOC). The glass fiber filters were dried and the amount of collected organic material estimated prior to combustion for analysis of total particulate organic carbon (POC). The calculated proportion of carbon in the particulate matter may also contain a small fraction of inorganic carbon. However, this fraction was ignored and the measure particulate carbon content defined as POC. Total organic carbon was then calculated as TOC = POC + DOC.

On a few sampling days further water samples were taken at different times during the day to monitor the trajectory of TOC in the water after feeding.

6.3.4 Sampling of fish and water samples

Samples of 4 fish were periodically removed from the test vessels (Table 10). The fish were immediately rinsed in dilution water, blotted dry and killed. The fish were immediately frozen in liquid nitrogen. Fish were analysed individually for the test item to generate four replicate data per sampling date. For the uptake and depuration phase, 8 and 6 sampling dates were scheduled, respectively. At the end of the uptake and elimination period four further fish were removed from both tanks for lipid analysis. At each sampling time during the uptake phase, adequate amounts of test water were analysed for the test item by LLE and SPME. An additional set of water samples was sent to Justus Liebig University Giessen (JLU) to carry out an independent SPME analysis.

Table 10: Sampling schedule.

	Day	Fish sampled
	0	4
Uptake	7	4
	14	4
	21	4
	28	4
	35	4
	42	4
	49	4
	56	4 (4)*
Depuration	1	4
	2	4
	4	4
	8	4
	16	4
	28	4 (4)*
Sum (per group)		68

*Fish used for lipid analysis

6.3.5 Chemical analysis (water samples)

Concentrations of HCB and oTP were measured in the water samples following SPME and LLE. SPME-GC/MS analysis and GC/MS analysis following LLE were carried out as described below:

SPME-GC/MS-analysis (JLU):

GC/MS-Unit:	GCQ (Fa. Thermo-Finnigan)
Column:	30m x 0.25mm TG-5HT (Fa. Thermo), 0.25µm film
Carrier gas:	Helium 1.2 mL/min, constant flow
Inlet:	Split/splitless-Injector at 280°C
Inlet liner:	SPME-Liner 1.0 mm ID, deactivated
Splitless time:	3 min
Oven program:	3 min 60°C -> 20°C/min to 200°C -> 3°C/min to 240°C
	-> 40°C/min to 290°C for 5 min -> 40°C/min to 305°C for 1 min
Run time:	31 min
Transfer line:	290°C
MS mode:	ms/ms
MS source:	200°C
Solvent delay:	5.5 min
Substances:	HCB-$^{13}C_6$ (IS to HCB), RT= 15.33 min, Target ion: 290 amu HCB, RT= 15.33 min, Target ion: 284 amu Terphenyl-d_{14} (IS to Terphenyl), RT=17.56 min, Target ion: 244 amu Terphenyl, RT= 17.62 min, Target ion: 230 amu
Autosampler:	CTC CombiPAL (Fa. CTC) with SPME-Unit
SPME-Fiber:	100 µm PDMS
Sample volume:	20 mL in SPME-Vial with SPME-Septum (Fa. CS-Chromatographie Service)
Sample shaker:	30°C
Incubation time:	5 min
Agitator speed:	250 rpm
Extraction time:	30 min
Vial penetration:	30 mm
Desorption time:	3 min
Bakeout unit:	280°C
Pre Bakeout:	4 min
Post Bakeout:	4 min

SPME-GC/MS-analysis (IME):

GC/MS-Unit:	GC 6890 N with MSD 5973 inert (Fa. Agilent)
Column:	30m x 0.25mm Rxi-5HT (Fa. Restek), 0.25µm film
Carrier gas:	Helium 1.2 mL/min, constant flow
Inlet:	Split/splitless-Injector at 280°C
Inlet liner:	SPME-Liner 0.75 mm ID, deactivated
Splitless time:	3 min
Oven program:	4 min 60°C -> 15°C/min to 135°C -> 8°C/min to 223°C
	-> 30°C/min to 320°C for 2.77 min,
Run time:	26 min
Transfer line:	280°C
MS mode:	Selected Ion Monitoring (SIM)
MS source:	250°C
MS Quadrupol:	180°C
Solvent delay:	16 min
Substances:	HCB-$^{13}C_6$ (IS to HCB), RT= 16.67 min, Target ion: 290 amu HCB, RT= 16.67 min, Target ion: 284 amu Terphenyl-d_{14} (IS to Terphenyl), RT=18.66 min, Target ion: 244 amu Terphenyl, RT= 18.73 min, Target ion: 230 amu
Autosampler:	MPS 2 (Fa. Gerstel) with SPME-Unit
SPME-Fiber:	100 µm PDMS
Sample volume:	20 mL in SPME-Vial with SPME-Septum (Fa. Gerstel)
Sample shaker:	35°C
Incubation time:	5 min
Agitator speed:	250 rpm
Extraction time:	30 min
Vial penetration:	30 mm
Desorption time:	3 min
Bakeout unit:	280°C
Pre Bakeout:	4 min
Post Bakeout:	4 min

GC/MS-analysis after liquid-liquid extraction (IME):

Extraction solvent:	tert.-Butylmethylether (changed to n-Hexane before Injection)
GC/MS-Unit:	450-GC with Ion-trap 240-MS (Fa. Varian)
Column:	30m x 0.25mm VF-5ms (Fa. Varian), 0.10µm film
Carrier gas:	Helium 1.0 mL/min, constant flow
Inlet:	Split/splitless-Injector at 280°C
Inlet liner:	4 mm-Gooseneck, deactivated
Splitless time:	1 min
Oven programme:	1 min 60°C ->20°C/min to 280°C for 5 min,
Run time:	17 min
Transfer line:	280°C
Solvent delay:	7 min
MS segment HCB:	7.00 – 8.30 min, mass range: 250 to 300
MS segment oTP:	8.30 – 9.50 min, mass range: 200 to 249
Substances:	HCB-$^{13}C_6$ (IS to HCB), RT= 7.78 min,Target ions: 290 / 292 amu HCB, RT= 7.78 min, Target ion: 282 / 284 amu Terphenyl-d_{14} (IS to Terphenyl), RT=8.70 min, Target ion: 244 amu Terphenyl, RT= 8.72 min, Target ion: 230 amu
Autosampler:	MPS 2 (Fa. Gerstel) Liquid-Injektor
Inj. volume:	1 µL

6.3.6 Fish Extraction and Analysis

Materials needed

- HCB-$^{13}C_6$ and o-Terphenyl-d_{14} as internal standards
- individual test compounds (HCB, oTP)
- extraction solvent (1:1 acetone / methylene chloride)
- Dionex Accelerated Solvent Extractor (ASE) 350 or equivalent pressurized fluid extractor
- 33 mL-stainless steel ASE extraction cells with cellulose filters
- Hydromatrix or equivalent drying reagent
- Metal spatulas
- analytical balance (0.0001 g)
- assorted volumetric flasks (100 mL) and pipettes
- Silica solid phase extraction (SPE) cartridges (Waters 2g/12mL) or equivalent
- SPE manifold

Fish Extraction

- The fish weights recorded on each sample vial for calculations were used representing the wet weight recorded at time of sampling.
- One whole fish was placed in a glass beaker along with approx. 5 g of pre-extracted Hydromatrix and homogenized mixture with metal spatula.
- The contents of the beakers were transferred to 33-mL ASE cells containing cellulose filters. Each beaker was rinsed with 2 mL of 1:1 acetone / methylene chloride and this was added to the respective ASE cells. Additional Hydromatrix was added as necessary to minimize cell dead volume.

The ASE cells were capped and extraction started using the following conditions:

- Heat Time: 5 min
- Solvent: 50% Acetone, 50% Methylenchloride
- Static Time: 5 min
- Percent Flush: 30
- Temperature: 120°C
- Purge Time: 60 sec
- Pressure: 1500 psi
- Cycles: 2

Each extract generated approximately 40 mL of solvent collected in 60 mL vials.

- The extracts were dried with Na_2SO_4. After centrifugation the supernatant was filled into 100 mL volumetric flasks. The Na_2SO_4 was rinsed two times with solvent. After re-centrifugation the extracts were combined.
- The raw fish extract was refrigerated (2-5°C)

Fish Extract Clean-Up

- Raw fish extracts were allowed to reach room temperature
- Silica SPE (2g/12mL) cartridges were conditioned with 8 mL of 1:1 acetone / methylene chloride followed by 8 mL hexane and dried under vacuum.
- Sampling glass tubes were placed in the manifold and the valve of each SPE cartridge closed
- An aliquote of each fish extract (0.25 – 1.5 mL) was reduced in a nitrogen stream to approx. 50 µL and resolved in 0.5 mL extraction mixture, internal standard was added.
- Each SPE cartridge was filled with 8 mL hexan and a previously prepared sample was added.
- After opening the SPE valves the cartridge eluent was collected together with additionally 2 x 2 mL of 1:1 methylene chloride / hexane mixture.
- The sampling tube was transferred to a heating block set at 40°C and the solvent was evaporated under a gentle stream of nitrogen to a final volume of 0.5mL.

- Finally, the samples were transferred to amber GC autosampler vials with insert, reduced to dryness, resolved in 250 µL hexane and measured by GC/MS.

Fish Extract Analysis

GC/MS-Unit:	450-GC with Ion-trap 240-MS (Fa. Varian)
Column:	30m x 0.25mm VF-5ms (Fa. Varian), 0.10µm film
Carrier gas:	Helium 1.0 mL/min, constant flow
Inlet:	Split/splitless-Injector at 280°C
Inlet liner:	4 mm-Gooseneck, deactivated
Splitless time:	1 min
Oven programme:	1 min 60°C ->20°C/min to 280°C for 5 min,
Run time:	17 min
Transfer line:	280°C
Solvent delay:	7 min
MS segment HCB:	7.00 – 8.30 min, mass range: 250 to 300
MS segment oTP:	8.30 – 9.50 min, mass range: 200 to 249
Substances:	HCB-$^{13}C_6$ (IS to HCB), RT= 7.78 min,Target ions: 290 / 292 amu HCB, RT= 7.78 min, Target ion: 282 / 284 amu Terphenyl-d_{14} (IS to Terphenyl), RT=8.70 min, Target ion: 244 amu Terphenyl, RT= 8.72 min, Target ion: 230 amu
Autosampler:	MPS 2 (Fa. Gerstel) Liquid-Injektor
Inj. volume:	1 µL

6.3.7 Gravimetric fish lipid determination using the Smedes extraction method

Smedes Method (based on de Boer et al. 1999):

- the 5-10g wet sample were weighed in a pre-weighed 100 mL-centrifugation glass beaker;
- 16 mL of propan-2-ol, and then 20 mL of cyclohexane were added to each centrifugation glass beaker using 20 mL pipettes;
- the sample was homogenized with the solvents for 2 minutes using e.g. UltraTurrax;
- **x** mL of deionized water were added to obtain a total of 22 g water including the water already in the sample,

x was calculated according to the following equation:

$$\mathbf{x}\ [\text{mL}] = 22 - \frac{\text{sample wet weight [g]} * \text{water content [\%]}}{100}$$

- the sample was again homogenized for 1 minute using an UltraTurrax;
- the phases were separated by centrifuging the mixture for 5 ± 1 minutes at 440 - 460 g;

- the upper cyclohexane phase was separated and volumetrically quantified, and transferred to a 10 mL glass centrifugation beaker;
- after addition of 20 mL of a mixture of cyclohexane/propan-2-ol (87:13, w/w) to the remaining lower watery phase, the phases were again homogenized for 1 minute and afterwards separated by centrifuging the mixture for 5 ± 1 minutes at 440 - 460 g;
- again, the upper cyclohexane phase was separated, volumetrically quantified, and transferred to the same 40 mL glass centrifugation beaker holding the first extract of the corresponding sample;
- the combined cyclohexane extracts were concentrated by gentle nitrogen stream to approx. 3 mL; the concentrated extract was quantitatively transferred into a pre-weighed 10 mL widemouth glass flask;
- the glass centrifugation beaker used for concentrating the extract was rinsed with 3 mL of the cyclohexane/propan-2-ol mixture using ultrasonication, and the solvent was added to the corresponding widemouth flask;
- the solvents in the flask was evaporated to dryness (e.g. with a rotavapor)
- the flask was further dried for 1 hour at 105;
- after cooling to room temperature in a desiccator, the dried flask was weighed, and the lipid content calculated according to:

$$\frac{(\text{weight of dry flask [g]-weight of empty flask [g]}) * 100}{\text{wet weight of sample [g]}} = \text{lipid content in \% of wet weight}$$

6.3.8 Estimation of time weighted average water concentrations (TWA)

Concentrations of HCB and oTP measured in the experimental tank during the uptake period were used to calculate a time weighted average (TWA) concentration for each test substance. First, weighted average concentrations were calculated by multiplying the average of two subsequently measured concentrations by the time period (h) between both measurements. All weighted average concentrations were then summed up and divided by the total time (h) of the uptake period resulting in the TWA concentration.

6.3.9 Calculation of bioconcentration factors (BCF)

The BCF was determined as steady state BCF (BCF_{ss}) and as kinetic BCF (BCF_k).

Steady state bioconcentration factor BCF_{SS}

The OECD Guideline defines the steady state as reached if “three successive analyses of concentration in fish (Cf) made on samples taken at intervals of at least two days are within ±20% of each other, and there are no significant differences among the three sampling periods.”

The BCF_{SS} was calculated as the quotient of the concentrations of HCB (and oTP) in the fish tissue (µg/kg fish) in steady state and the corresponding time weighted average exposure concentration (TWA) of HCB (and oTP) in the water (µg/L).

Kinetic bioconcentration factor BCF_k

The BCF_k was derived from the uptake and depuration rates k1 and k2.

The depuration rate constant (k2) was calculated by fitting a one-compartment model to the measured concentrations in fish during the depuration phase:

$Cf_{(t)} = Cf_{(ti)} * e^{(-k2*t)}$

$Cf_{(t)}$: concentration in fish at sampling time in µg/kg
$Cf_{(ti)}$: concentration in fish at start of depuration phase in µg/kg

For the fitting the concentrations were log_e-transformed to allow linear regression of log-concentrations versus time.

The uptake rate constant (k1) was calculated by non-linear regression analysis of the ratios Cf/Cw against time during the uptake phase and including the depuration rate k2 fitted before.

The fitted model assumes an attenuation of uptake by simultaneous depuration, increasing with increasing Cf up to equilibrium between uptake and depuration:

Cf/Cw = k1/k2 * (1-exp(-k2*t)

k1: uptake rate constant

k2: depuration rate constant
Cf: concentration in fish (µg/kg)
Cw: concentration in water (µg/L)

The kinetic bioconcentration factor (BCF_k) was calculated as the quotient k1/k2.

All calculations were done using Microsoft® Office Excel 2010 for calculation of means and SigmaStat 3.5 (Systat Software, 2006) for the regressions, using data for the parent compound in whole fish.

6.4 Results and Discussion

6.4.1 Study conditions and validity criteria

Concerning the validity criteria given in the OECD guideline 305 (OECD, 2012):

- No adverse effects or mortalities were observed during the study,
- Temperature during the test (daily measured during the evaluated periods) varied between 14.6 and 15.9°C (variation less than 2°C; Table 11),
- Oxygen saturation (daily measured during the evaluated periods) was between 70 and 98% (never below 60%;),
- pH (daily measured during the evaluated periods) was sufficiently constant at 7.81 to 8.62 with no considerable difference between treatments (Table 11),
- The concentration of the test item was stable during the uptake phase (deviations of TWA concentrations (Figure 16). □ 20%

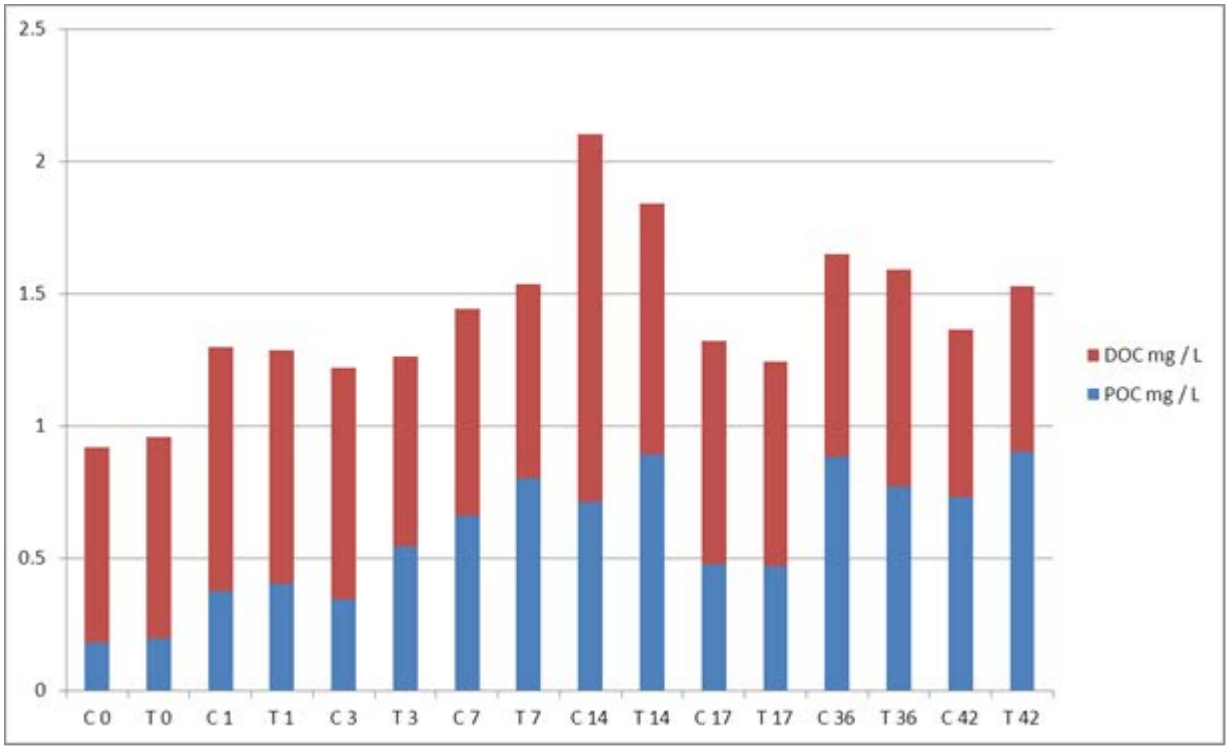
Table 11: Temperature, oxygen saturation and pH during the uptake phase.

	Temperature (°C)		pH		Oxygen (mg/L)		O_2 Saturation(%)	
	Control	Treatment	Control	Treatment	Control	Treatment	Control	Treatment
mean (oxygen, temp.) or median (pH)	15.1	15.1	8.2	8.2	7.7	7.7	86.4	85.4
Minimum value	14.6	14.6	7.81	7.92	6.3	5.8	71	70
Maximum value	15.9	15.8	8.61	8.62	8.8	8.9	98	95

6.4.2 Organic carbon analysis

The concentration of dissolved (DOC) and particulate (POC) organic carbon in the water of the control and experimental tank collected prior to feeding at different days during the experiment is presented in Figure 13. The results show that both chambers contained comparable concentrations of total organic carbon (TOC = DOC + POC). During the first week a slight increase of TOC was observed, however concentrations remained constantly below or close to a level of 2 mg / L and are thus even below the maximum acceptable value of TOC defined in the OECD TG 305 for dilution water. The TOC content of the dilution water used for the flow-through fish test was close to zero.

Figure 13: Dissolved (DOC) and particulate (POC) organic carbon measured in the water of the control and test chamber collected prior to feeding at different days during the experiment.

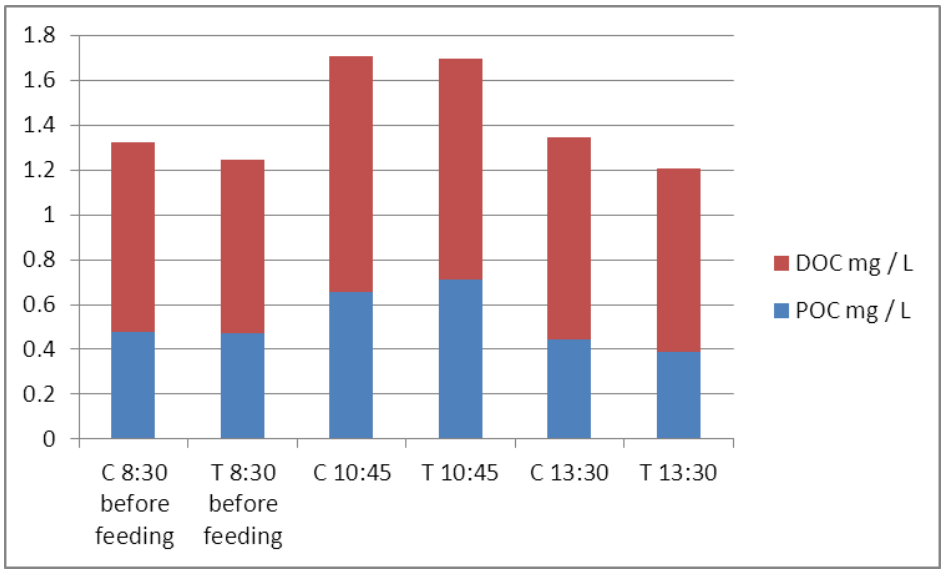


C0 = Control group on Day 0, T0 = Test group on Day 0

The trajectory of dissolved (DOC) and particulate (POC) organic carbon estimated during a single day of the study is described in Figure 14. Around one hour after removal of feces and uneaten feed particles the TOC concentration in the test chamber was clearly below 2 mg / L and further

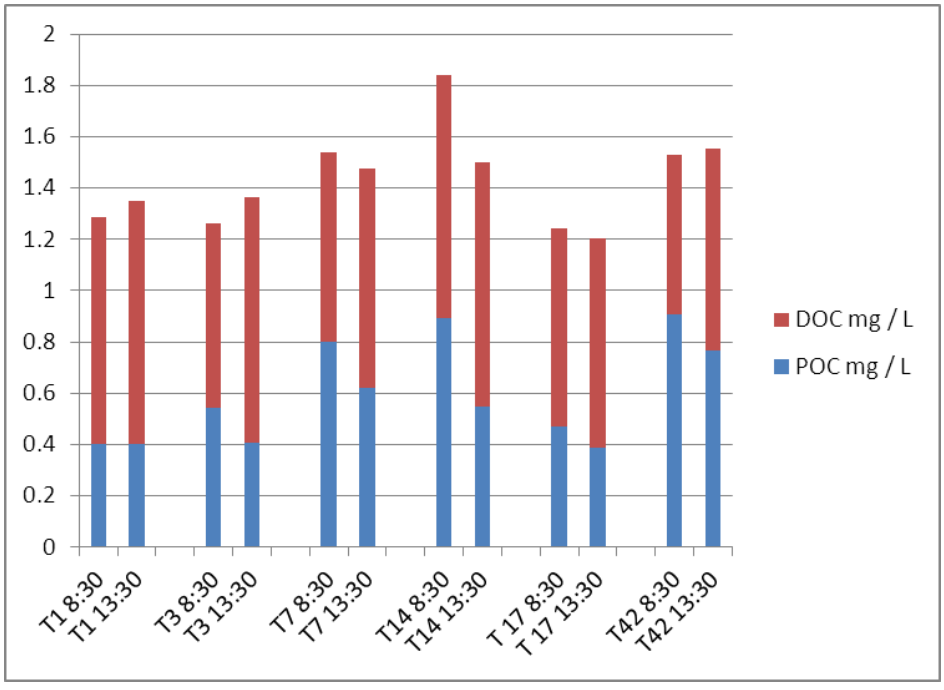
decreased to the level observed before feeding within three hours. Further measurements on different days confirmed this observation (Figure 15).

Figure 14: Trajectory of dissolved (DOC) and particulate (POC) organic carbon in the test chambers measured during a single day.



C = Control group, T = test group

Figure 15: Dissolved (DOC) and particulate (POC) organic carbon in the experimental tank measured before and five hours after feeding.



During feeding the TOC reaches a peak concentration which depends on the amount of feed supplied, however, due to the fast ingestion of the diet and the removal of feces after 30 to 60 minutes the effect of the temporarily increased organic carbon concentration on the bioavailability of the test items should be of minor importance. The revised guideline (OECD 305 § 28) defines that the concentration of organic carbon in the test vessels should not exceed the

concentration of organic carbon originating from the test substance by more than 2 mg / L (±10%). The results show that TOC concentrations below the threshold concentration can be maintained in the test chambers during the flow-through fish test.

6.4.3 Measurement of test concentrations with LLE and SPME

The test concentrations of HCB measured in the experimental tank during the uptake period are presented in Figure 16. The results obtained with LLE and SPME are compared and show that no major differences could be identified. Both extraction procedures were leading to a similar time weighted average concentration of about 0.390 and 0.396 µg/L, respectively (Table 12). The trajectory of the water concentrations was constantly within the range of mean ± 20% except of one value measured at the onset of the experiment by SPME. The test concentrations of oTP measured during the experiment are presented in Figure 17. Also in this case SPME and LLE were leading to comparable results with time weighted average concentrations of 4.30 and 4.53 µg / L, respectively (Table 12:). Under the given experimental conditions comparable BCF estimates can be therefore expected independent of the extraction procedure applied. The relatively constant water concentrations show that column generated concentrations are suitable for flow-through tests.

The GC/MS measurement of test substance concentrations following SPME was repeated by the partners from Justus Liebig University Giessen (JLU) (Düring-Lab). Concentrations comparable to the results obtained by the IME-Lab were estimated (Figure 18, Figure 19). However, mean measured concentrations were usually characterized by a higher standard deviation which might be explained by the transportation and short time storage of the water samples.

Figure 16: Concentration of HCB in the test chamber during the flow-through fish test measured by GC/MS after LLE and SPME (IME data).

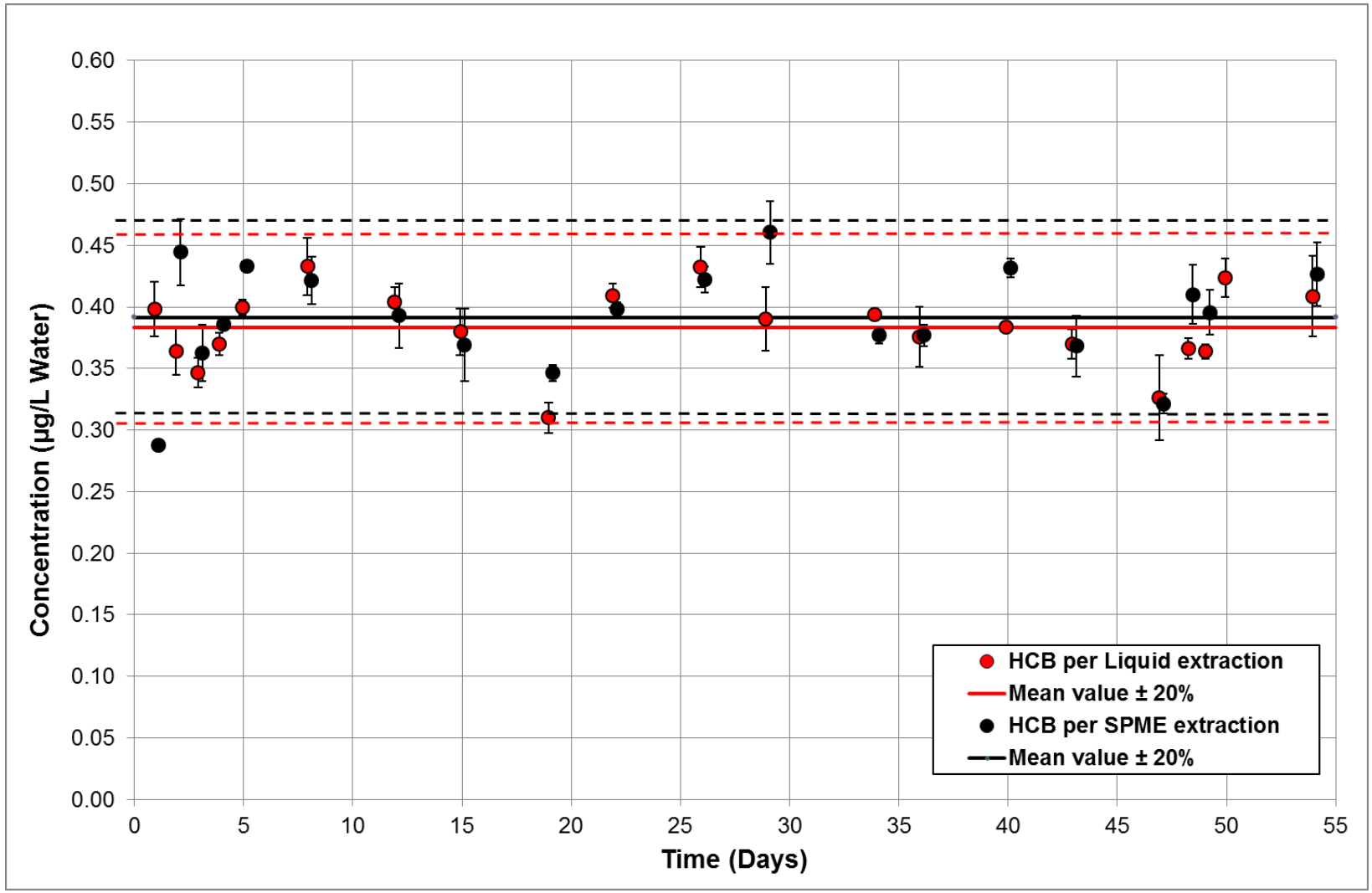


Figure 17: Concentration of oTP in the test chamber during the flow-through fish test measured by GC/MS after LLE and SPME (IME data).

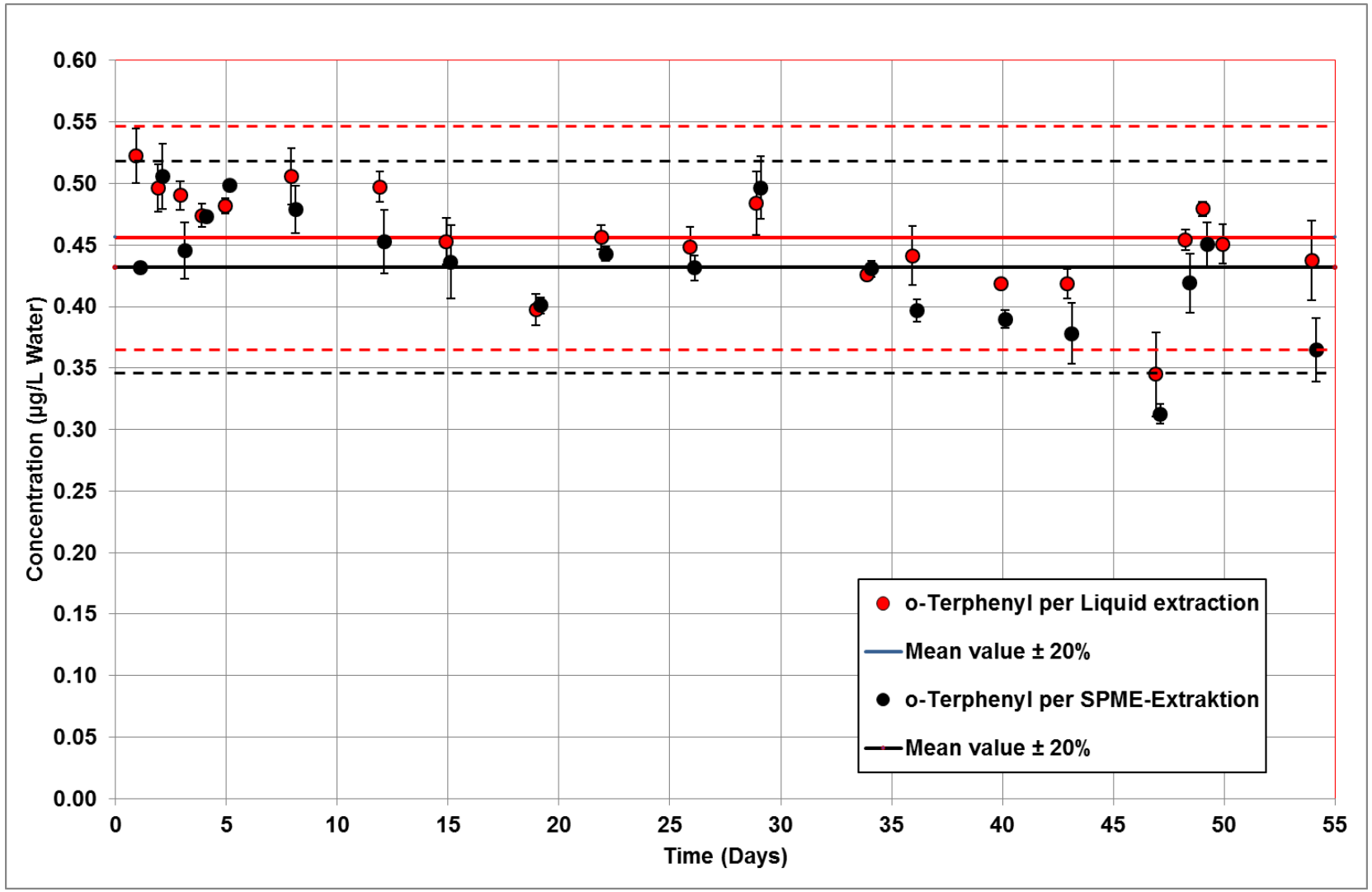


Figure 18: Concentration of HCB in the test chamber during the flow-through fish test measured by SPME (IME data vs. JLU Giessen data).

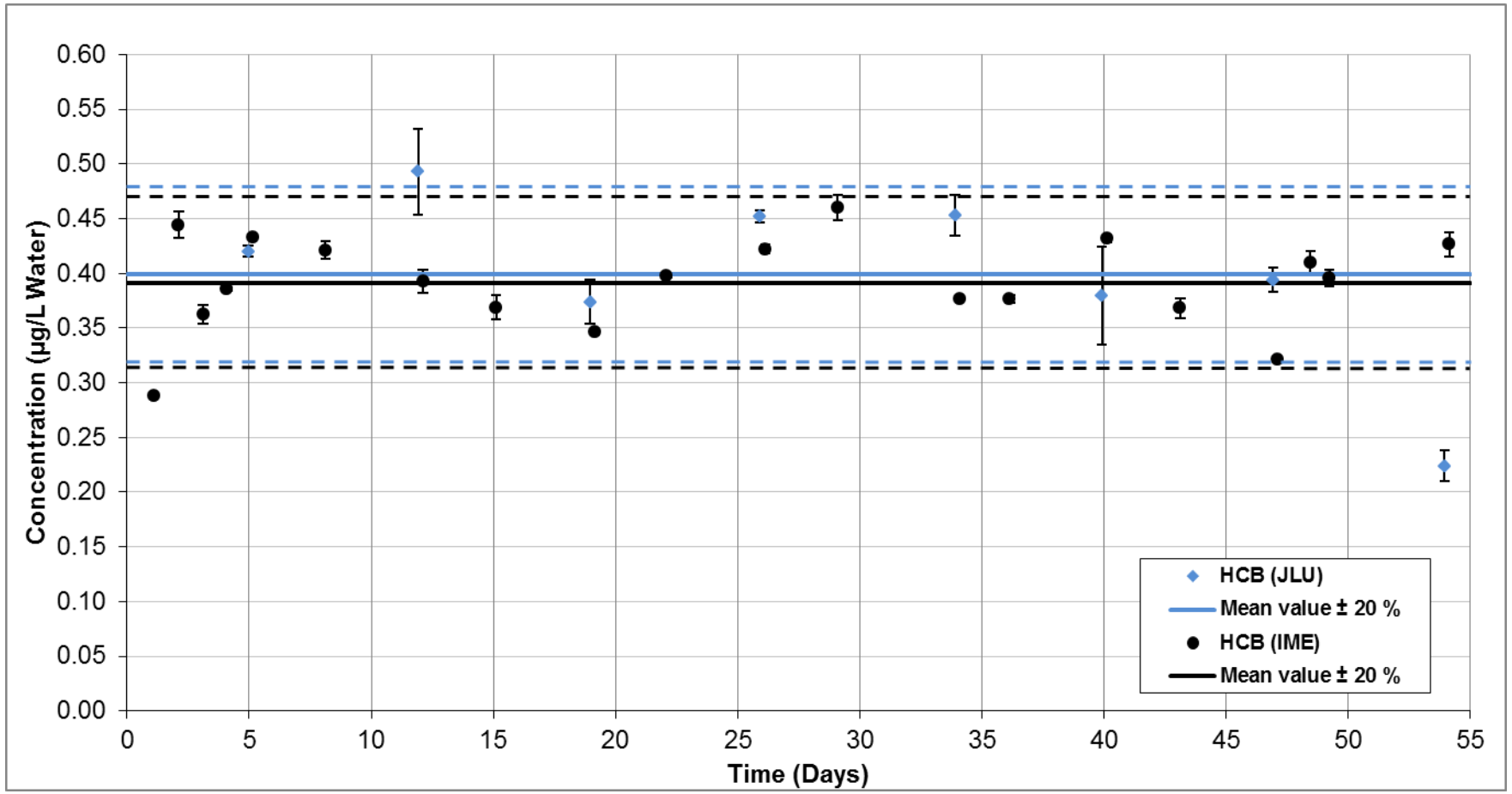
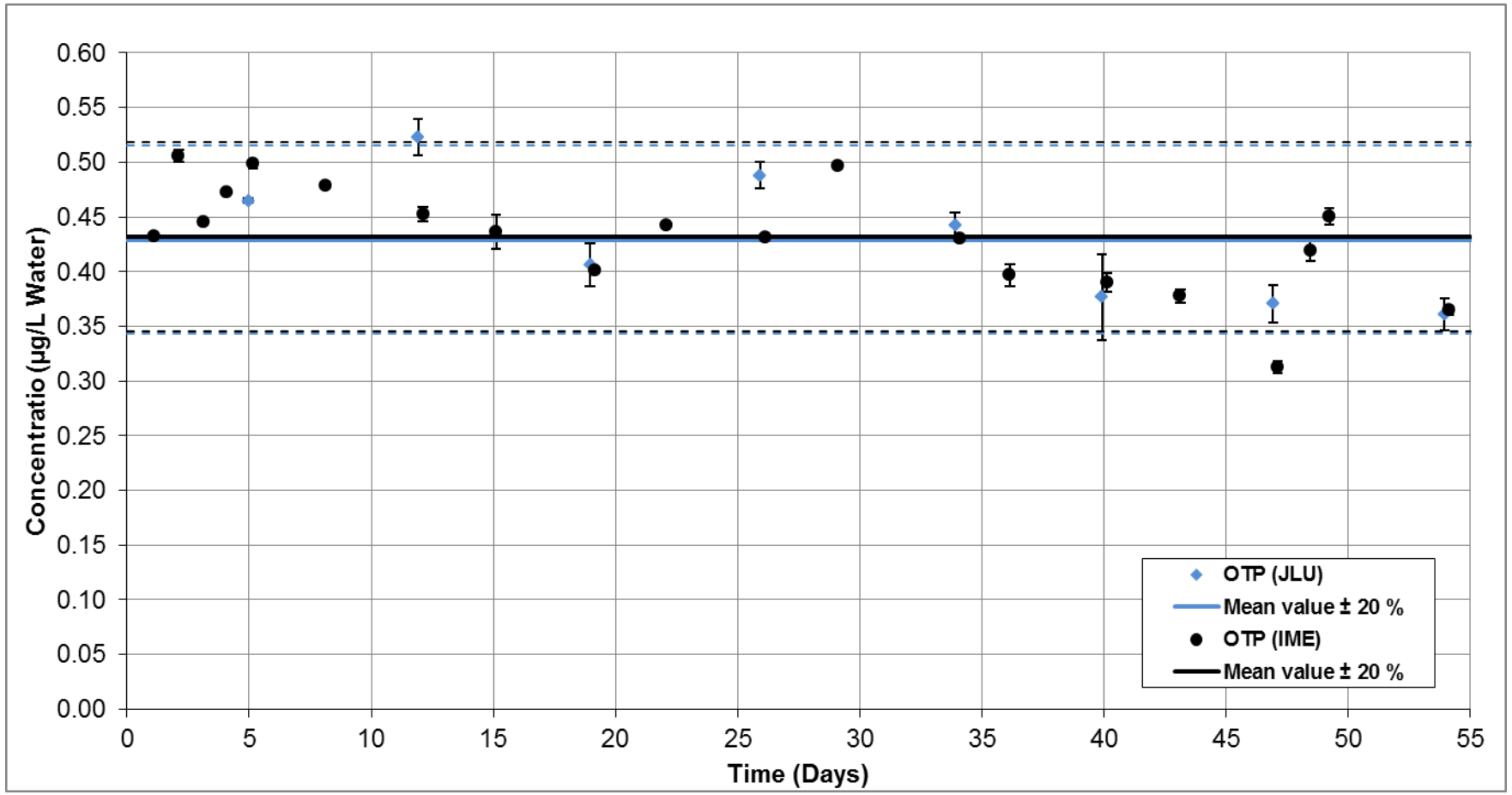


Figure 19: Concentration of oTP in the test chamber during the flow-through fish test measured by SPME (IME data vs. JLU Giessen data).



6.4.4 TWA concentrations of HCB and oTP in water during the uptake period

Time weighted average concentrations of HCB and oTP were determined for the entire uptake period. The results show that comparable results were obtained by LLE and SPME (Table 12).

Table 12: TWA concentrations of HCB and oTP during uptake period measured by LLE and SPME

Day	HCB (µg/L)	oTP (µg/L)
LLE	0.390	0.453
SPME	0.396	0.430

6.4.5 Results of the analyzed fish samples

At each sampling time, four separate samples (individual fishes) were taken out of the test and control tank. The results of the analyzed fish samples are listed in Table 13.

Table 13: Measured concentrations of HCB and oTP in fish (mg/kg) during uptake period

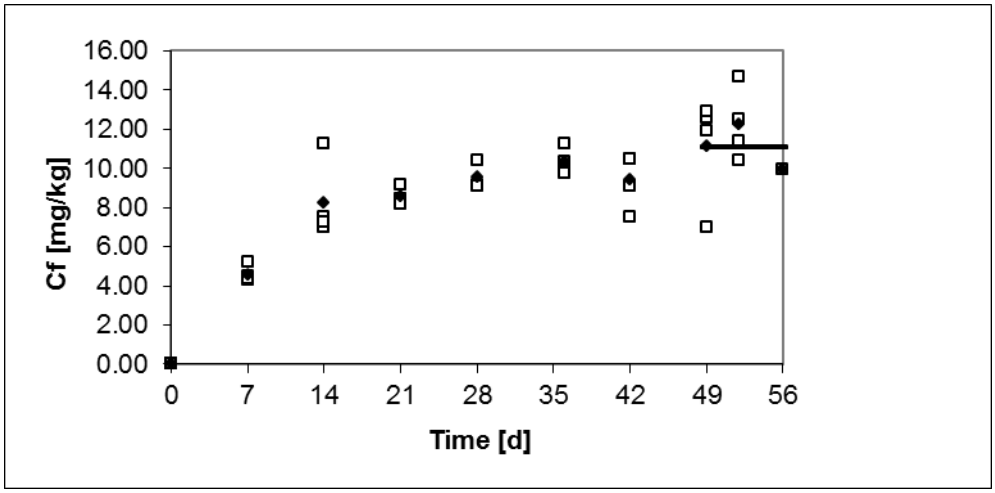
Day	Mean measured test concentration (mg/kg)	
	HCB	oTP
0	<LOQ	<LOQ
7	4.59	4.27
14	8.25	5.09
21	8.57	5.07
28	9.57	4.10
36	10.31	4.98
42	9.39	2.80
49	11.09	5.97
52	12.23	3.54
56	9.94*	2.71

*Single measurement; 3 samples excluded due to measurement artefact

6.4.6 Uptake of HCB in fish and BCF_{SS}

The concentrations of HCB in fish tissue (Cf) indicate a fast uptake, reaching a plateau after 49 days (Figure 20). Thus, the concentrations measured from day 49 to day 56 were used for calculation of the BCF_{SS} (Figure 20). Except for two samples collected on day 49 and 52 which were characterized by a very low and high lipid content in ASE fraction, respectively, Cf data were all within ± 20 % of the mean. Using the mean concentration measured in the water (Table 12; TWA based on LLE) and the mean concentration in fish from day 49 to 56 (11.1 µg/g), the BCF_{SS} was calculated to be approximately 28453.

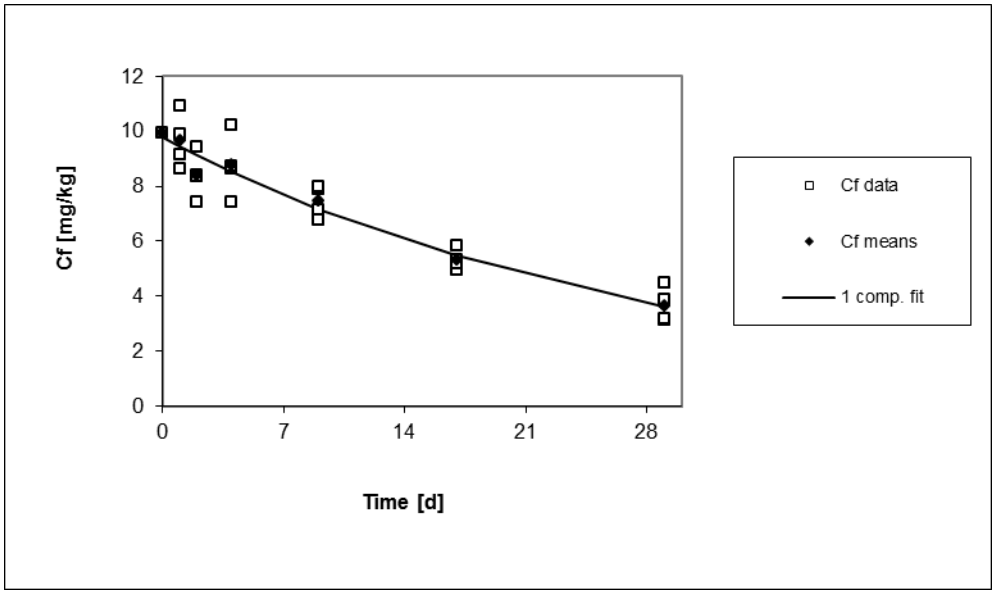
Figure 20: Concentration of HCB in fish (Cf) during the uptake phase.



6.4.7 Uptake and depuration rates and BCF_k

Within 29 days after start of the depuration phase, HCB concentrations did not fall below the limit of quantification in the test group (Figure 21). Log-linear fitting revealed a depuration rate of 0.0341/d. The quality of the fit was sufficiently high (r^2 = 0.987) and the one-compartment model was thus considered as sufficient to describe the elimination kinetics. More complex models were therefore not considered to be appropriate.

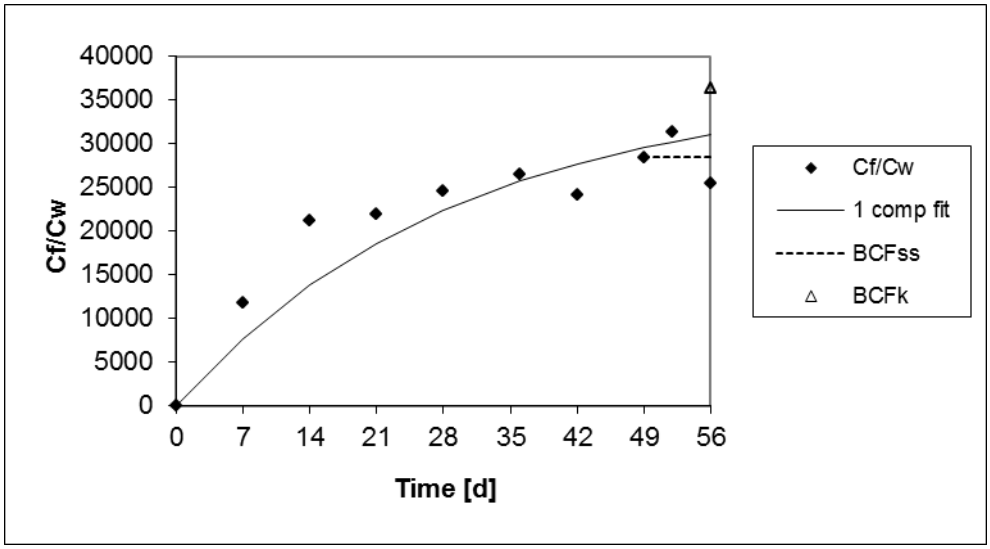
Figure 21: HCB elimination from fish during the depuration phase of 29 days.



Data are means of 4 fish, except of time zero where only one sample was available.

The uptake rate constant (k1) was calculated by fitting the Cf/Cw ratios calculated during the uptake phase (Figure 22) over time under consideration of the depuration rate (k2) fitted before. From the uptake and depuration rates the BCFk was calculated to be 24303 (Table 14).

Figure 22: Concentration factor of HCB (Cfish/Cwater) during the uptake phase.



The data are means of 4 individual fish, except of day 56 where only one sample was available.

Table 14: Summary of relevant study results ± standard errors.

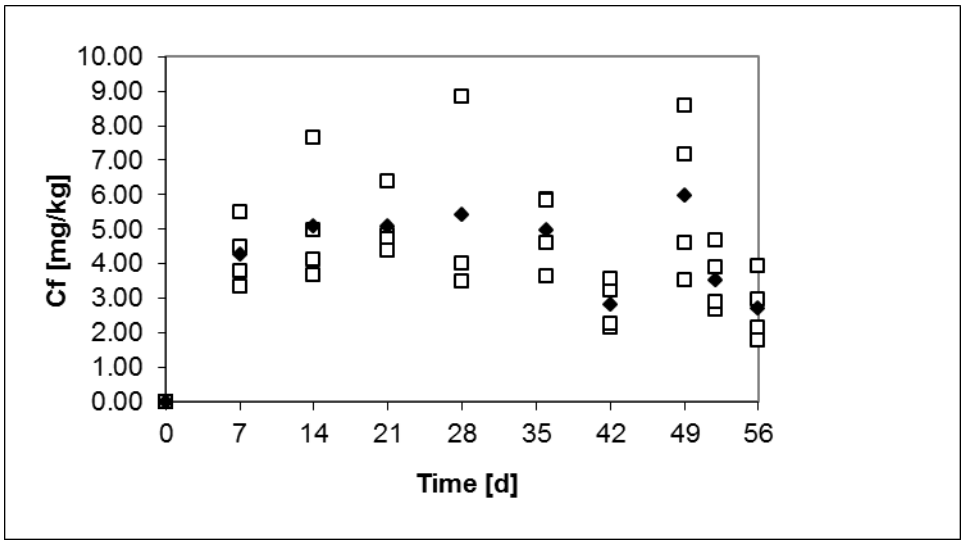
Parameter	estimation
BCFss (LLE)	28453
BCFss (SPME)	28022
BCFk	36363
K1 (uptake)	1240
K2 (depuration)	0.0341

Dimension of Rates k1, k2: 1/d.

6.4.8 Uptake of oTP in fish and BCF_{SS}

The concentrations of oTP in fish tissue (Cf) indicate a fast uptake, reaching a plateau after 14 days (Figure 23). However, concentrations measured from day 28 to day 56 showed a very high variability and even tended to decrease from day 35 towards the end of the study. Therefore, no BCF_{SS} could be determined. The data give clear indications for adaptive effects in rainbow trout after longterm exposure to oTP. Further studies have been initiated to prove this hypothesis.

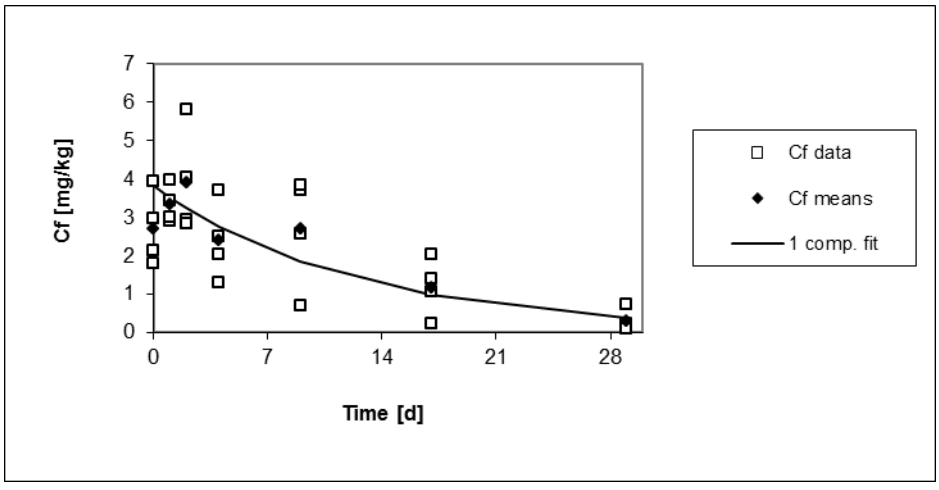
Figure 23: Concentration of oTP in fish (Cf) during the uptake phase (Fish exposed to 0.453 µg/L).



6.4.9 Uptake and depuration rates and BCF_k

Within 29 days after start of the depuration phase, oTP concentrations felt below the limit of quantification in the test group (Figure 24). Log-linear fitting revealed a depuration rate of 0.0808/d. Fish samples collected on day 2 of the depuration phase were characterized by comparatively high (3.89 µg/g) oTP concentrations in comparison to the concentration (2.71 µg/g)measured at the end of the uptake period. Samples collected during the depuration phase showed a high variability with respect to the oTP concentrations. However, the quality of the fit was sufficiently high (r^2 = 0.916) and the one-compartment model was thus considered as sufficient to describe the elimination kinetics. More complex models were therefore not considered to be appropriate.

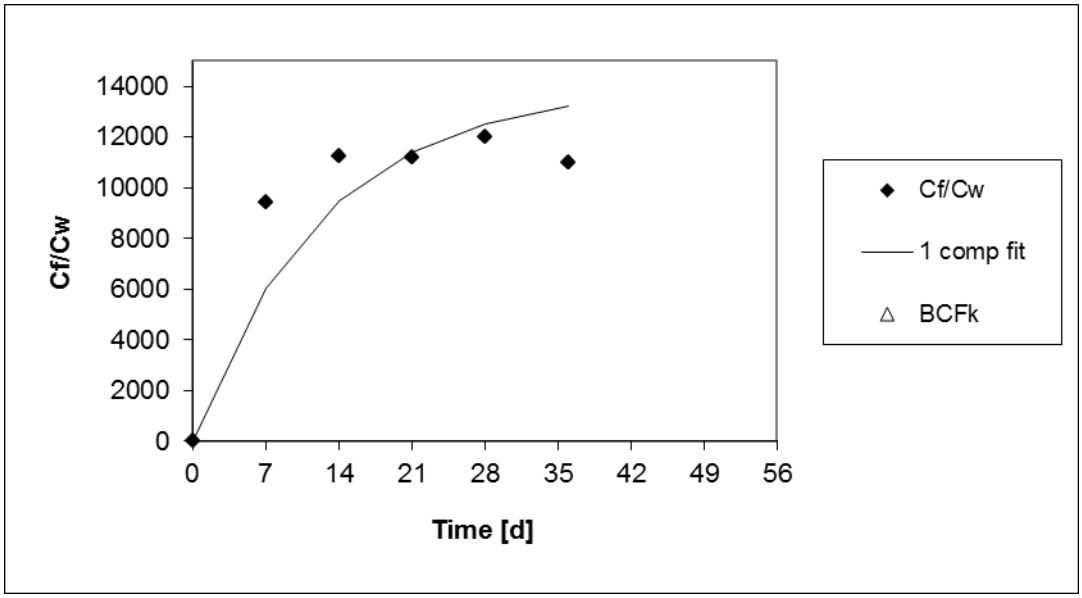
Figure 24: oTP elimination from fish during the depuration phase of 29 days.



Data are means of 4 fish.

The uptake rate constant (k_1) was calculated by fitting the Cf/Cw ratios calculated during the uptake phase (only day 0 to day 36) over time (Figure 25) under consideration of the depuration rate (k_2) fitted before. From the uptake and depuration rates the BCF_k was calculated to be 13960 (Table 15).

Figure 25: Concentration factor of oTP (Cfish/Cwater) during the uptake phase (day 0 to 35).



The data are means of 4 individual fish.

Table 15: Summary of relevant study results.

Parameter	estimation
BCFk	13960
K1 (uptake)	1128
K2 (depuration)	0.0808

Dimension of Rates k1, k2: 1/d.

6.4.10 Fish lipid content

The mean lipid content of the control fish sampled during the study period for lipid analysis ranged from 5.0% at the onset of the experiment, to 6.7% at the end of the depuration period. The mean lipid content of the test animals was comparable to the control animals until the end of the uptake period. However, a lower lipid content was observed at the end of the depuration period with only 5.8% compared to 6.7% in the control group (Table 16).

Table 16: Lipid content of fish sampled at the start, middle and end of the study.

Time	Control	Treatment
Start Uptake	5.0	5.0
End Uptake	5.9	5.8
End Depuration	6.7	5.8

6.5 Conclusions

The results of the first flow-through test show:

- that constant column generated concentrations of highly lipophilic substances can be continuously dispensed in a flow-through system over a 8 weeks period without using solvents.
- that the experimental conditions are characterized by low concentrations of TOC which are always below the threshold concentrations defined in the OECD TG 305.
- that the analysis of water samples is leading to comparable results by LLE and SPME under the experimental conditions applied in this study.
- that longterm exposure might lead to adaptive effects with respect to the metabolism of test compounds as observed for oTP.

7 Part III: Effect of different extraction procedures on the result of BCF-studies

7.1 Background

The standard method for water sample preparation in BCF tests is liquid-liquid extraction (LLE) providing total analyte concentrations. Solid-phase microextraction (SPME) enables the determination of total and freely dissolved aqueous concentrations. Highly hydrophobic organic chemicals (HOC, with log KOW > 5) readily bind to organic matter (OM) leading to a reduced bioavailability for fish which may lead to an underestimation of the true uptake when total aqueous concentrations are measured. However, clear estimates for the reduction of freely dissolved analytes by OM are missing.

7.2 Objectives

- Performance of an aquatic bioconcentration study according to OECD TG 305 with two hydrophobic organic compounds (HOCs) characterized by log KOW > 7 (PCB153; Dibenz[a,h]anthracene, DBA)
- Comparison of the results obtained by different extraction procedures (LLE vs. SPME) for water sample preparation
- Measurement of total organic carbon concentration in the test water during the flow-through study
- to estimate the freely dissolved analytes in the test medium by SPME
- to investigate the effect of total organic carbon (TOC) present in the test water on the result of BCF-studies

7.3 Material and methods

7.3.1 Preparation of the dosing system

Spiking of the carrier material with the test items was carried out analogous to 4.3.1 with solutions of PCB 153 in MTBE and DBA in dichloromethane leading to a loading of 0.2 mg/g for PCB 153 and DBA, respectively.

Three columns loaded with DBA and one column loaded with PCB 153 were used in the test. The water flow through the columns was in the range of 5 – 12 mL/min.

Column generate concentrations were further diluted in a mixing chamber as described in 4.3.1.

7.3.2 Holding and dilution water

Purified drinking water was used according to the OECD-Guideline (OECD, 2012). The purification included filtration with charcoal, aeration and passage through a lime stone column.

7.3.3 Test performance

The flow-through fish test was carried out with juvenile rainbow trout (*Oncorhynchus mykiss*) with an average size of 3.841 g ± 0.437g at the start of the experiment. Only healthy fish free of observable diseases and abnormalities were used in the study. The experimental animals were raised in the hatchery of Fraunhofer IME from fertilized eggs which were obtained from Fischzucht Rameil, Gleierbrück, Germany.

The study was principally carried out as the first flow-through test. Two 100 L glass aquaria filled with 70 L of test solution (test group) or water (control group) were used as test vessels. In the flow-through tanks a continuous flow of approximately 21 L/h were maintained throughout the test using metering pump systems leading to 5.8 volume replacements through each test chamber per day. A high water-to-fish ratio in order to minimise the reduction in test concentration caused by the addition of the fish at the start of the test was used. However, a slightly lower water replacement rate than during the first study was applied to avoid a significant dilution of the column generated test concentrations.

Both tanks were stocked with 68 fish each. After the uptake phase scheduled for 56 d, the remaining fish in the test group were transferred into a new aquarium with test substance-free dilution water for further 56 d (depuration period). An extended uptake period of 56 days was required in order to reach steady-state concentrations in fish and to enable the calculation of a steady state bioconcentration factor (BCFss) for each test item. The depuration period was terminated after 56 days, a sufficient period for determination of loss rate constants.

Animals exposed to the two test substances were compared with unexposed control animals which were kept under comparable conditions. Experimental conditions were comparable to the first flow-through test.

7.3.4 Sampling of fish and water samples

The TOC-concentration in the water was measured on a weekly basis. Water samples for TOC-analysis were taken together with the samples for analysis of the test items: Water (1L) including solid particles were rinsed through a pre-weighed 0.4µm glass fiber filter. Water cleaned in this way was used to estimate the concentration of dissolved organic carbon (DOC) as described above.

Samples of 4 fish were periodically removed from the test vessels (Table 17) and processed as described for the first study. At the end of the uptake and elimination period four further fish were removed from both tanks for lipid analysis. At each sampling time during the uptake phase, adequate amounts of test water were analysed for the test item by LLE and SPME. An additional set of water samples was sent to Justus Liebig University Giessen to carry out an independent SPME analysis.

Table 17: Sampling schedule (Fish sampled from treatment and control group).

	Day	Fish sampled
	0	4
Uptake	7	4
	14	4
	21	4
	28	4
	35	4
	42	4
	49	4
	52	4
	56	4 (4)*
Depuration	1	4
	2	4
	4	4
	8	4
	16	4
	32	4
	56	4 (4)*
Sum (per group)		72

*Fish used for lipid analysis

7.3.5 Chemical analysis (water samples)

Concentrations of PCB153 and DBA were measured in the water samples following SPME and LLE. SPME-GC/MS analysis and GC/MS analysis following LLE were carried out as described below:

SPME-GC/MS-analysis (JLU):

GC/MS-Unit:	Trace Ultra GC with ITQ MS (Fa. Thermo)
Column:	30m x 0.25mm TG-5HT (Fa. Thermo), 0.25µm film
Carrier gas:	Helium 2 mL/min, constant flow
Inlet:	Split/splitless-Injector at 320°C
Inlet liner:	SPME-Liner 1.0 mm ID.
Splitless-time:	3 min
Oven program:	3 min 90°C -> 15°C/min to 320°C for 2 min
Run time:	20 min
Transfer line:	325°C
MS mode:	Selected Ion Monitoring (SIM)
MS source:	250°C
Solvent delay:	5 min

Substances:	PCB 138 (IS to PCB 153), RT = 14.46 min, Target ion: 359.8 amu PCB 153, RT= 14.11 min, Target ion: 359.8 amu DBA-d_{14} (IS to DBA), RT = 18.57 min, Target ion: 292 amu DBA, RT= 18.62 min, Target ion: 278 amu
Autosampler:	CTC CombiPAL (Fa. CTC) with SPME-Unit
SPME-Fiber:	7 µm PDMS
Sample volume:	20 mL in SPME-Vial with SPME-Septum (Fa. CS Chromatographie Service)
Sample shaker:	30°C
Incubation time:	5 min
Agitator speed:	250 rpm
Extraction time:	60 min
Vial penetration:	30 mm
Desorption time:	3 min
Bakeout unit:	320°C
Pre Bakeout:	5 min
Post Bakeout:	5 min

SPME-GC/MS-analysis (IME):

GC/MS-Unit:	GC 6890 N with MSD 5973 inert (Fa. Agilent)
Column:	30m x 0.25mm Rxi-5HT (Fa. Restek), 0.25µm film
Carrier gas:	Helium 1.0 mL/min, constant flow
Inlet:	Split/splitless-Injector at 320°C
Inlet liner:	SPME-Liner 0.75mm ID.
Splitless-time:	2 min
Oven program:	2 min 60°C -> 30°C/min to 270°C for 2 min ->30°C/min to 360°C for 3 min
Run time:	17 min
Transfer line:	320°C
MS mode:	Selected Ion Monitoring (SIM)
MS source:	250°C
MS Quadrupol:	180°C
Solvent delay:	8 min
Substances:	PCB 138 (IS to PCB 153), RT= 11.03 min, Target ion: 359.8 amu PCB 153, RT= 10.70 min, Target ion: 359.8 amu DBA-d_{14} (IS to DBA), RT=14.32 min, Target ion: 292 amu DBA, RT= 14.35 min, Target ion: 278 amu
Autosampler:	MPS 2 (Fa. Gerstel) with SPME-Unit

SPME-Fiber:	7 µm PDMS
Sample volume:	20 mL in SPME-Vial with SPME-Septum (Fa. Gerstel)
Sample shaker:	35°C
Incubation time:	5 min
Agitator speed:	250 rpm
Extraction time:	60 min
Vial penetration:	30 mm
Desorption time:	2 min
Bakeout unit:	320°C
Pre Bakeout:	4 min
Post Bakeout:	5 min

GC/MS-analysis after liquid-liquid extraction (IME):

Extraction solvent:	Toluene
GC/MS-Unit:	GC 6890 N with MSD 5973 Network (Fa. Agilent)
Column:	30m x 0.25mm Rxi-5sil MS (Fa. Restek), 0.50µm film
Carrier gas:	Helium 1.0 mL/min, constant flow
Inlet:	Split/splitless-Injektor at 300°C
Inlet liner:	4 mm-Gooseneck-splitless-Liner deactivated
Splitless-time:	1 min
Oven program:	1 min 130°C ->20°C/min to 330°C for 5 min,
Run time:	16 min
Transfer line:	300°C
MS mode:	Selected Ion Monitoring (SIM)
MS source:	250°C
MS Quadrupol:	180°C
Solvent delay:	7 min
Substances:	PCB 138 (IS to PCB 153), RT= 10.35 min, Target ion: 359.8 amu PCB 153, RT= 10.07 min, Target ion: 359.8 amu DBA-d_{14} (IS to DBA), RT=15.00 min, Target ion: 292 amu DBA, RT= 15.08 min, Target ion: 278 amu
Autosampler:	MPS 2 (Fa. Gerstel) Liquid-Injektor
Inj. volume:	1 µL

7.3.6 Fish Extraction and Analysis

Fish extraction and clean-up of extracts were carried out as described above.

Fish Extract Analysis

Extracts were analyzed by GC-MS under the following conditions:

GC/MS-Unit:	GC 6890 N with MSD 5973 Network (Fa. Agilent)
Column:	30m x0.25 mm Rxi-5sil MS (Fa. Restek), 0.50µm film
Carrier gas:	Helium 1.0 mL/min, constant flow
Inlet:	Split/splitless-Injektor at 300°C
Inlet liner:	4 mm-Gooseneck-splitless-Liner deactivated
Splitless-time:	1 min
Oven program:	1 min 130°C ->20°C/min to 330°C for 15 min,
Run time:	26 min
Transfer line:	300°C
MS mode:	Selected Ion Monitoring (SIM)
MS source:	250°C
MS Quadrupol:	180°C
Solvent delay:	7 min
Substances:	PCB 138 (IS to PCB 153), RT= 10.35 min, Target ion: 359.8 amu PCB 153, RT= 10.07 min, Target ion: 359.8 amu DBA-d_{14} (IS to DBA), RT=15.00 min, Target ion: 292 amu DBA, RT= 15.08 min, Target ion: 278 amu
Autosampler:	MPS 2 (Fa. Gerstel) Liquid-Injektor
Inj. volume:	1 µL

7.3.7 Gravimetric fish lipid determination

Aliquots of the fish extracts obtained by ASE were used for lipid determination. The solvent extracts were evaporated to dryness under a gentle stream of nitrogen at 65°C and the lipid residue estimated. Based on the total lipid content of the original fish extract, the lipid content was calculated using the following formula:

Calculation:

$$\frac{\text{fish lipid weight (g)}}{\text{fish wet weight (g)}} \times 100 = \%\ \text{Lipid Content (wet-weight basis)}$$

The lipid content of all fish sampled during the experiment was compared with the lipid content of fish sampled at the end of the uptake and depuration period as well as the start of the study. These animals were extracted by the lipid extraction method originally described by Smedes (1999) and suggested by OECD 305 for fish lipid determination.

7.3.8 Estimation of time weighted average water concentrations (TWA) and bioconcentration factors (BCF)

Concentrations of PCB153 and DBA measured in the experimental tank during the uptake period were used to calculate a time weighted average (TWA) concentration for each test substance. The BCF was determined as steady state BCF (BCF_{ss}) and as kinetic BCF (BCF_k) as described above.

7.4 Results and Discussion

7.4.1 Study conditions and validity criteria

Concerning the validity criteria given in the OECD guideline 305 (OECD, 2012):

- No adverse effects or mortalities were observed during the study,
- Temperature during the test (daily measured during the evaluated periods) varied between 14.7 and 15.4°C (variation less than 2°C; Table 18),
- Oxygen saturation (daily measured during the evaluated periods) was between 85 and 100% (never below 60%; Table 18),
- pH (daily measured during the evaluated periods) was sufficiently constant at 7.76 to 8.21 with no considerable difference between treatments (Table 18),
- The concentration of the PCB 153 was stable during the uptake phase (deviations ≤ 20% of TWA concentrations; Figure 27).

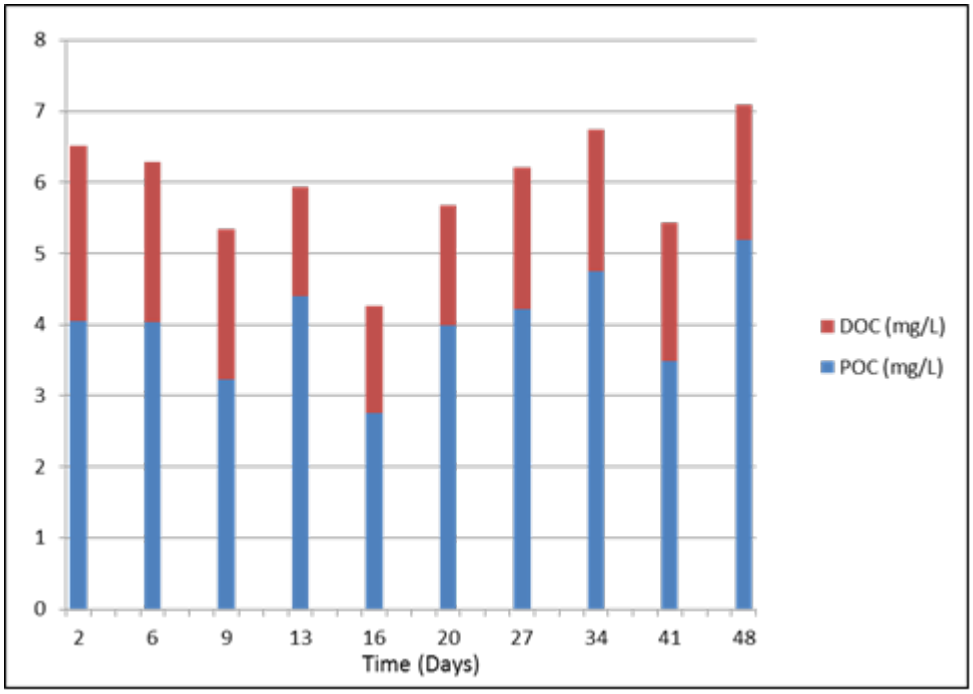
Table 18: Temperature, oxygen saturation and pH during the uptake phase.

	Temperature (°C)		pH		Oxygen (mg/L)		O_2 Saturation(%)	
	Control	Treatment	Control	Treatment	Control	Treatment	Control	Treatment
mean (oxygen, temp.) or median (pH)	14.98	14.98	7.97	8.02	8.36	8.64	90.93	93.78
Minimum value	14.7	14.7	7.77	7.76	7.6	7.8	85	86
Maximum value	15.4	15.4	8.18	8.21	9.5	9.7	98	100

7.4.2 Organic carbon analysis

The concentration of dissolved (DOC) and particulate (POC) organic carbon in the water of the experimental tank collected prior to feeding at different days during the experiment is presented in Figure 26. During the experiment a slight increase of POC was observed. Concentrations of DOC remained constantly below or close to a level of 2 mg / L and are thus even below the maximum acceptable value of TOC defined in the OECD TG 305 for dilution water. The TOC content of the dilution water used for the flow-through fish test was close to zero. Throughout the test, the concentration of TOC in the test vessels should not exceed the concentration of organic carbon originating from the test substance by more than 10 mg/L according to OECD TG 305. The results show that TOC (DOC + POC) concentrations below the threshold concentration can be maintained in the test chambers during the flow-through fish test.

Figure 26: Dissolved (DOC) and particulate (POC) organic carbon measured in the water of the test chamber collected prior to feeding at different days during the experiment. (Test group on Day 0-48)



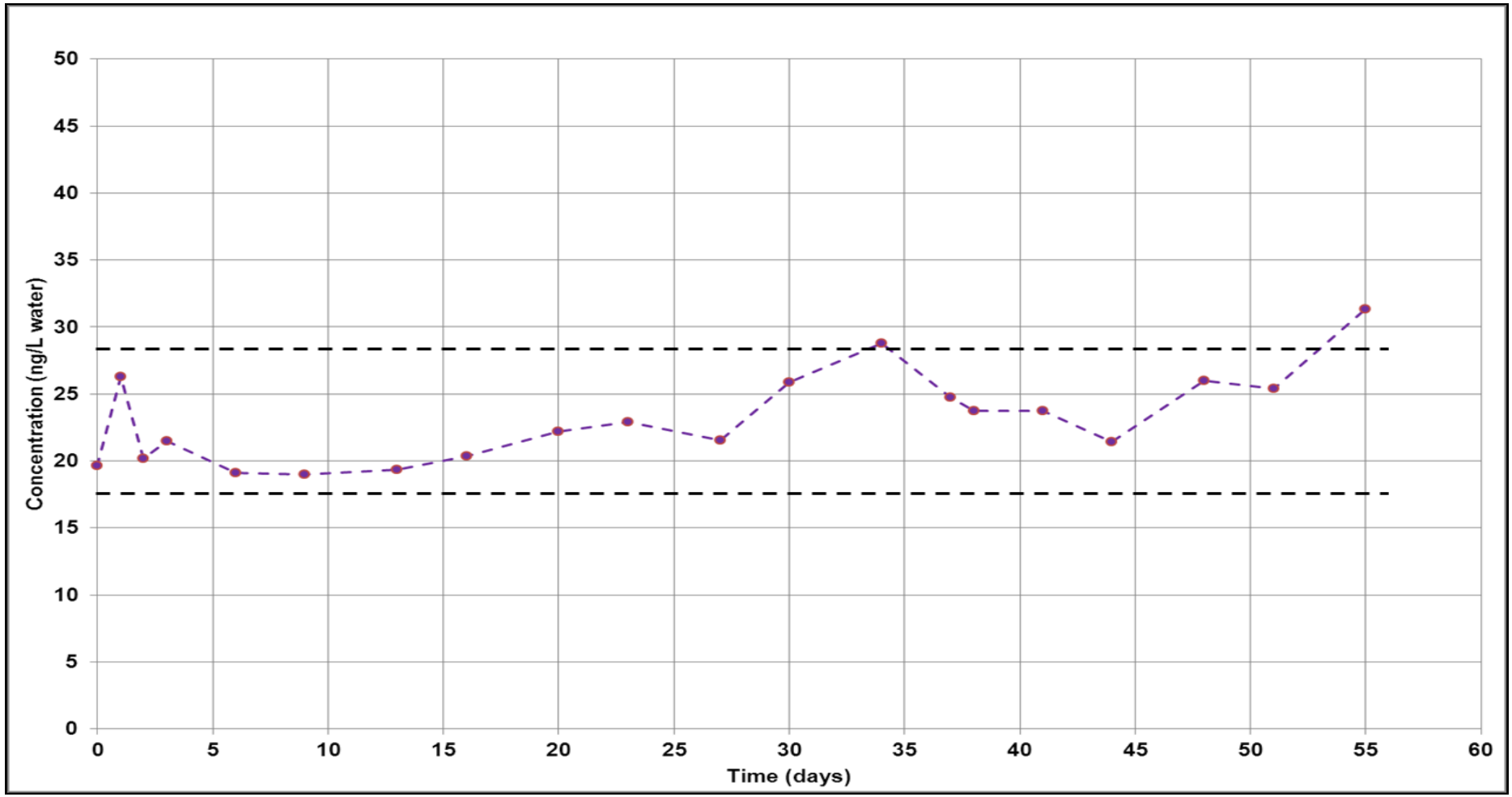
7.4.3 Measurement of test concentrations with LLE and SPME

The test concentrations of PCB 153 measured in the experimental tank during the uptake period after extraction with LLE are presented in Figure 27. The trajectory of the water concentrations was constantly within the range of mean ± 20% except of one value measured at the end of the experiment. The test concentrations of DBA measured during the experiment are presented in Figure 28. Constant water concentrations were monitored throughout the uptake period apart from two peak exposures on day 2 and 32 caused by a technical defect of the dosing system.

Freely dissolved concentrations, as well as total concentrations of test substances were determined by SPME ($SPME_{free}$ and $SPME_{total}$, respectively). Measurements were carried out by the partners from Justus Liebig University Giessen (JLU) (Düring-Lab).

$SPME_{total}$ concentrations are determined indirectly, from freely dissolved analyte concentrations ($SPME_{free}$): Within the extraction step, only the freely dissolved amounts of test substance and internal standard partition to the fiber. If an internal standard is added and equilibrated with the sample, the internal standard binds to the organic matter in an equal amount as the test substance, if an analogue of the analyte which is labeled with stable isotopes (D or ^{13}C) is used. Concentrations of the analyte and the internal standard are then compared to references of the internal standard in samples without organic matter (e. g. within the calibration). Free concentrations can then be corrected by the internal standard, resulting in total concentrations. Measurement of PCB 153 concentration in the test chamber during the flow-through fish test showed that the bioavailable fraction ($SPME_{free}$) was slightly decrease during the uptake period (Figure 29).

Figure 27: Concentration of PCB 153 in the test chamber during the flow-through fish test measured by GC/MS after LLE (IME data).



PCB 153 per LLE (Δ); Average concentration ± 20%.

Figure 28: Concentration of DBA in the test chamber during the flow-through fish test measured by GC/MS after LLE (IME data).

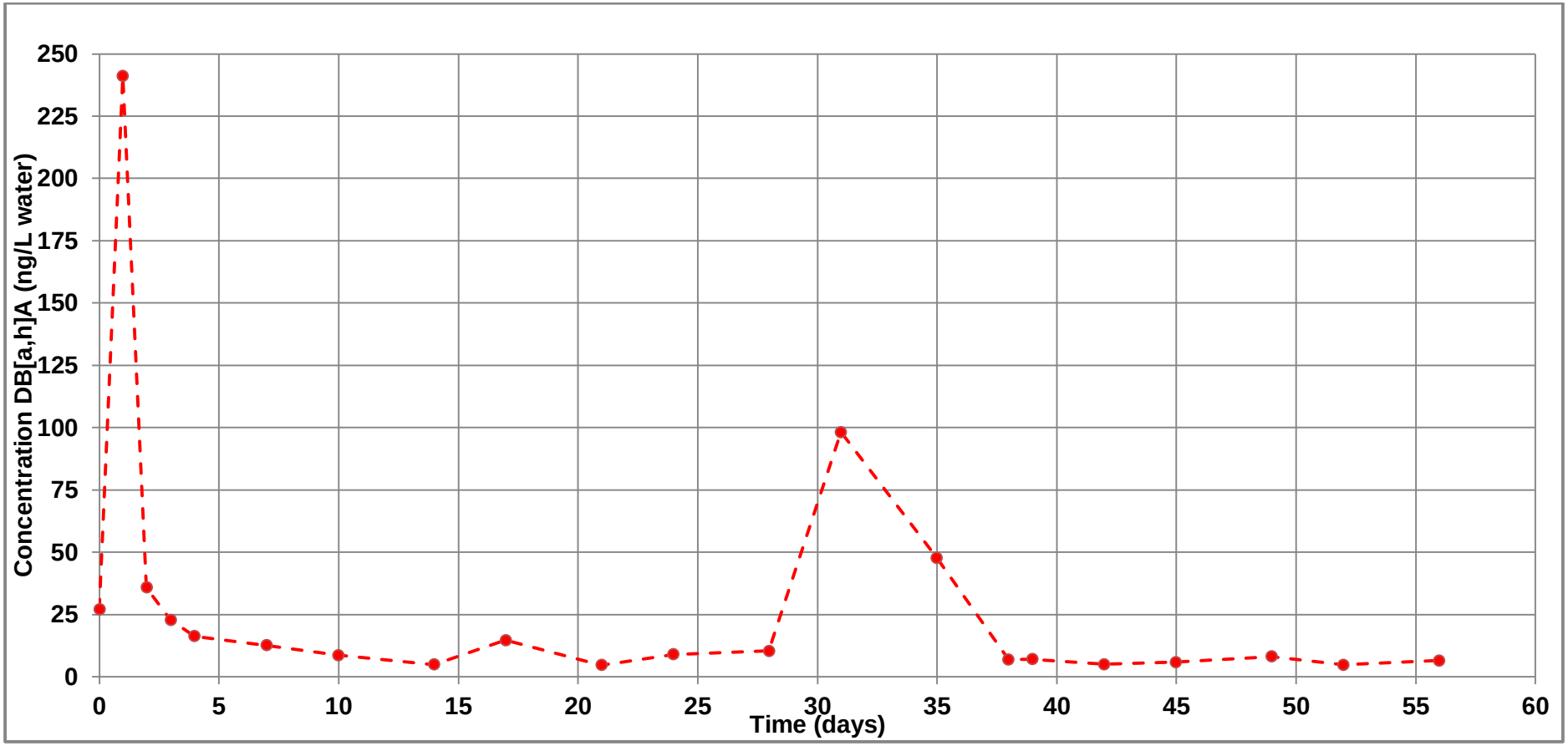
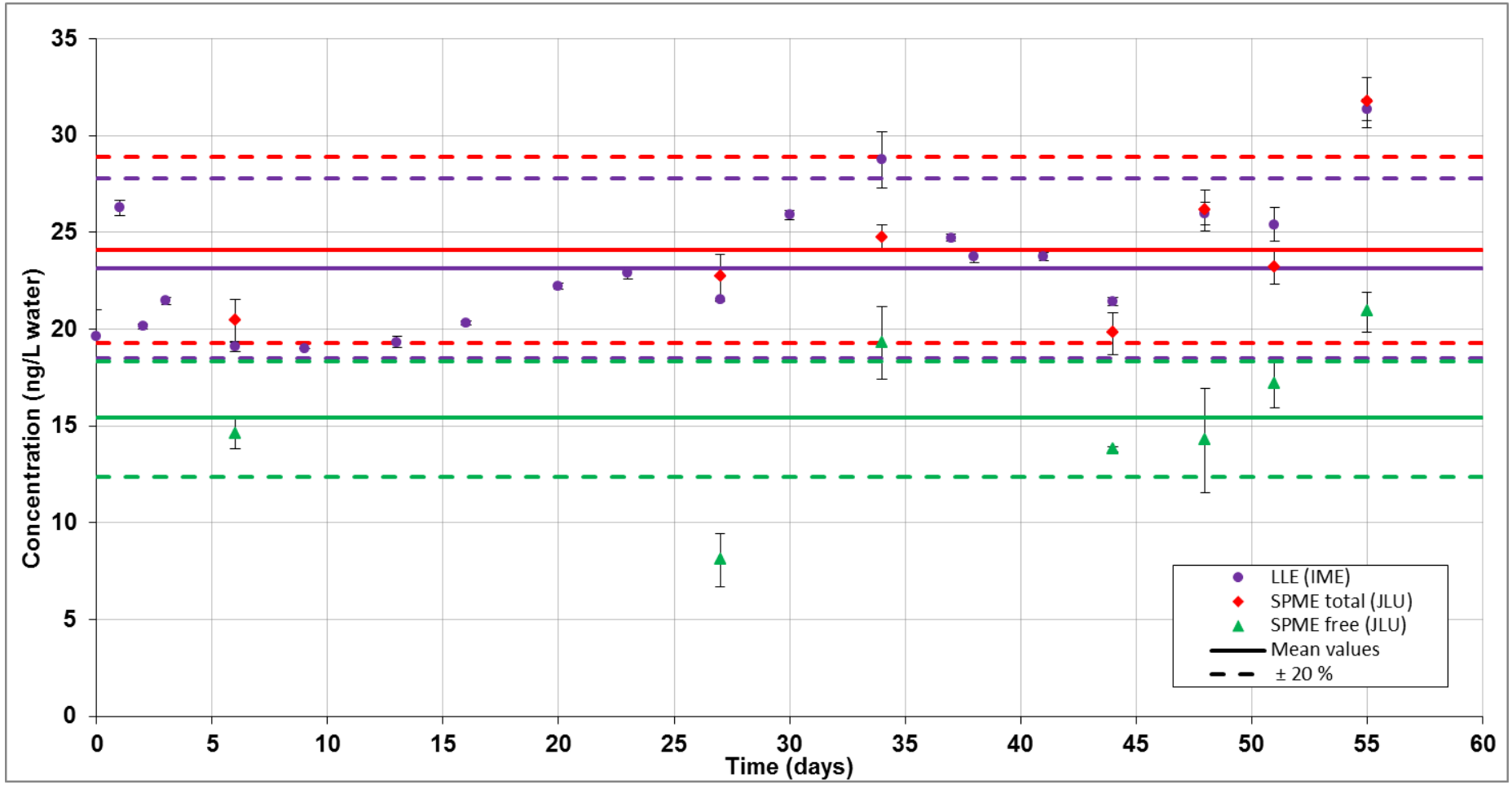


Figure 29: Concentration of PCB 153 in the test chamber during the flow-through fish test measured by IME (LLE) and JLU Giessen (SPME).



Means for LLE, $SPME_{total}$ and $SPME_{free}$ were 23, 24 and 15 ng/L, respectively.
$SPME_{free}$ = freely dissolved concentrations measured by SPME; $SPME_{total}$ = total concentrations measured by SPME.
n=4-8, outliers are excluded, error bars show RSD.

7.4.4 TWA concentrations of PCB 153 and DBA in water during the uptake period

Time weighted average concentrations of PCB 153 and DBA were determined for the entire uptake period Table 19.

Table 19: TWA concentrations of PCB 153 and DBA during uptake period measured by LLE and SPME (IME and JLU).

Day	PCB 153 (ng/L)	DBA (ng/L)
SPMEtotal	24	10.6
SPMEfree	15	
LLE	23	

7.4.5 Results of the analyzed fish samples

At each sampling time, four separate samples (individual fishes) were taken out of the test and control tank. The results of the analyzed fish samples are listed in Table 20.

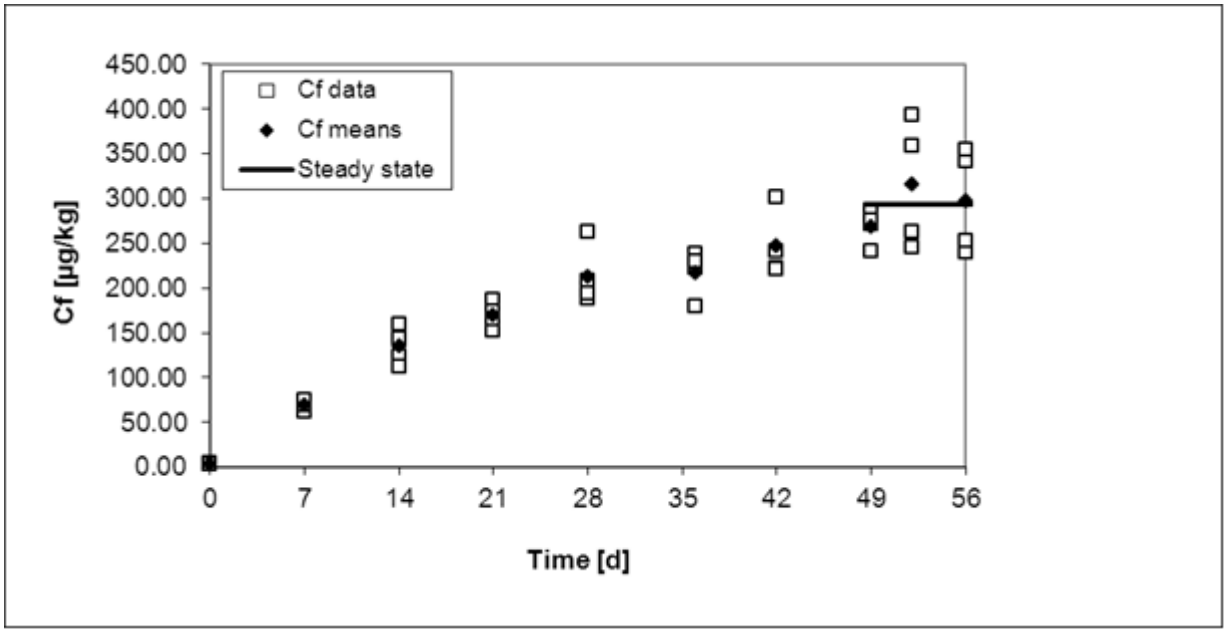
Table 20: Measured concentrations of PCB 153 and DBA in fish (µg/kg) during uptake period.

Day	Mean measured test concentration (µg/kg)	
	PCB 153	DBA
0	3.25	<LOQ
7	69.50	<LOQ
14	134.5	<LOQ
21	169	17.5
28	213	<LOQ
36	217	1.75
42	246	0.33
49	268	7.00
52	315	169
56	297	<LOQ

7.4.6 Uptake of PCB 153 in fish and BCF_{SS}

The concentrations of PCB 153 in fish tissue (Cf) indicate a slow uptake, reaching a plateau after 49 days. Thus, the concentrations measured from day 49 to day 56 were used for calculation of the BCF_{SS} (Figure 30). Using the mean total concentration measured in the water (Table 19; TWA based on LLE) and the mean concentration in fish from day 49 to 56 (293.33 ng/g), the BCF_{SS} was calculated to be approximately 12736.

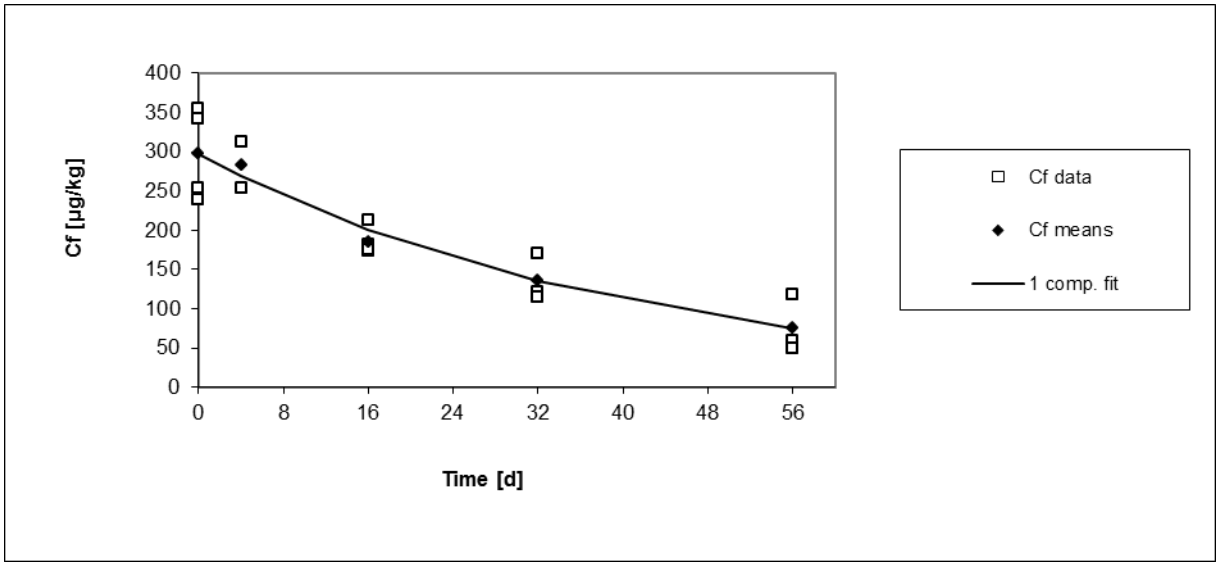
Figure 30: Concentration of PCB 153 in fish (Cf) during the uptake phase.



7.4.7 Uptake and depuration rates and BCF_k

Within 56 days after start of the depuration phase, PCB 153 concentrations did not fall below the limit of quantification in the test group (Figure 31). Log-linear fitting revealed a depuration rate of 0.0247/d. The quality of the fit was sufficiently high ($r^2 = 0.993$) and the one-compartment model was thus considered as sufficient to describe the elimination kinetics. More complex models were therefore not considered to be appropriate.

Figure 31: PCB 153 elimination from fish during the depuration phase of 56 days.



Data are means of 4 fish.

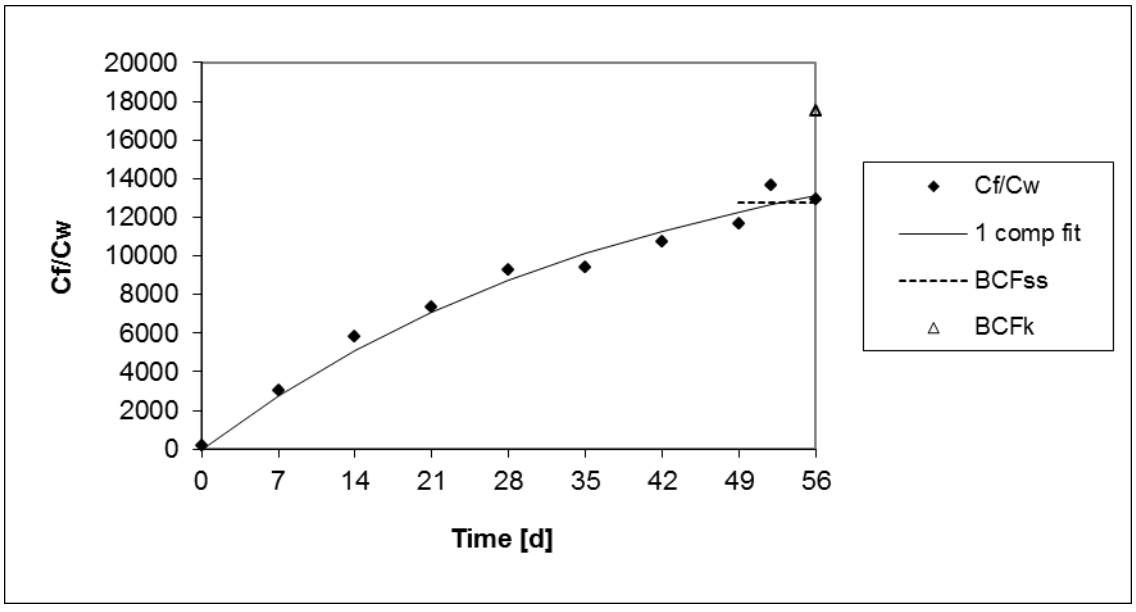
The uptake rate constant (k1) was calculated by fitting the Cf/Cw ratios calculated during the uptake phase (Figure 32) over time under consideration of the depuration rate (k2) fitted before. From the uptake and depuration rates the BCFk was calculated to be 17482 (Table 21).

Using the mean free concentration measured in the water ($SPME_{free}$) and the mean concentration in fish from day 49 to 56, the BCF value was calculated to be 19555. This results in an increase in BCF compared to the BCF calculated based on total water concentrations (LLE and SPME total). However, this increase is smaller than expected from sorption coefficients and the theory of an artificial phenomenon due to measuring artefacts.

Explaining factors might be:

- The relevance of biotic factors such as reduced membrane permeation for highly lipophilic compounds etc. which are debated in the context of hydrophobicity cutoff discussions.
- An insufficient extraction with LLE for highly lipophilic compounds in the presence of FF as organic matter (see “Comparison of extractable amounts by SPME and LLE” and “Conclusions” in Part I of this study).
- The study was carried out within a flow-through system: freely dissolved analytes are continuously supplied and weaken the effect of sorption to organic matter. (From the mean TOC concentration of 6.0 mg L^{-1} TOC in the test system and the freely dissolved PCB 153 concentration of 15 ng L^{-1} (compared to a total concentration of 24 ng L^{-1}) a sorption coefficient log K_{OC} of 5.0 could be determined. This value is significantly lower than the K_{OC} values determined in a closed equilibrium system of about log K_{OC} 6.0 (see Part I of this study)).

Figure 32: Concentration factor of PCB 153 (Cfish/Cwater) during the uptake phase.



The data are means of 4 individual fish, except of day 56 where only one sample was available.

Table 21: Summary of relevant study results.

Parameter	estimation
BCFss (LLE)	12737
BCFss (SPMEtotal)	13599
BCFss (SPMEfree)	19555
BCFk (LLE)	17482
K1 (uptake)	431.8
K2 (depuration)	0.0247

Dimension of Rates k1, k2: 1/d.

7.4.8 Uptake of DBA in fish and BCF_{SS}

The concentrations of DBA in fish tissue (Cf) indicate a fast metabolism, of the test compound (Table 20). Only negligible amounts were bioaccumulated during the uptake period of 56 d. Only after the peak exposure around day 32 a temporary increase of the tissue concentrations could be observed which completely disappeared (<LOQ) towards the end of the uptake period. Therefore, no BCF_{SS} or BCFk could be determined.

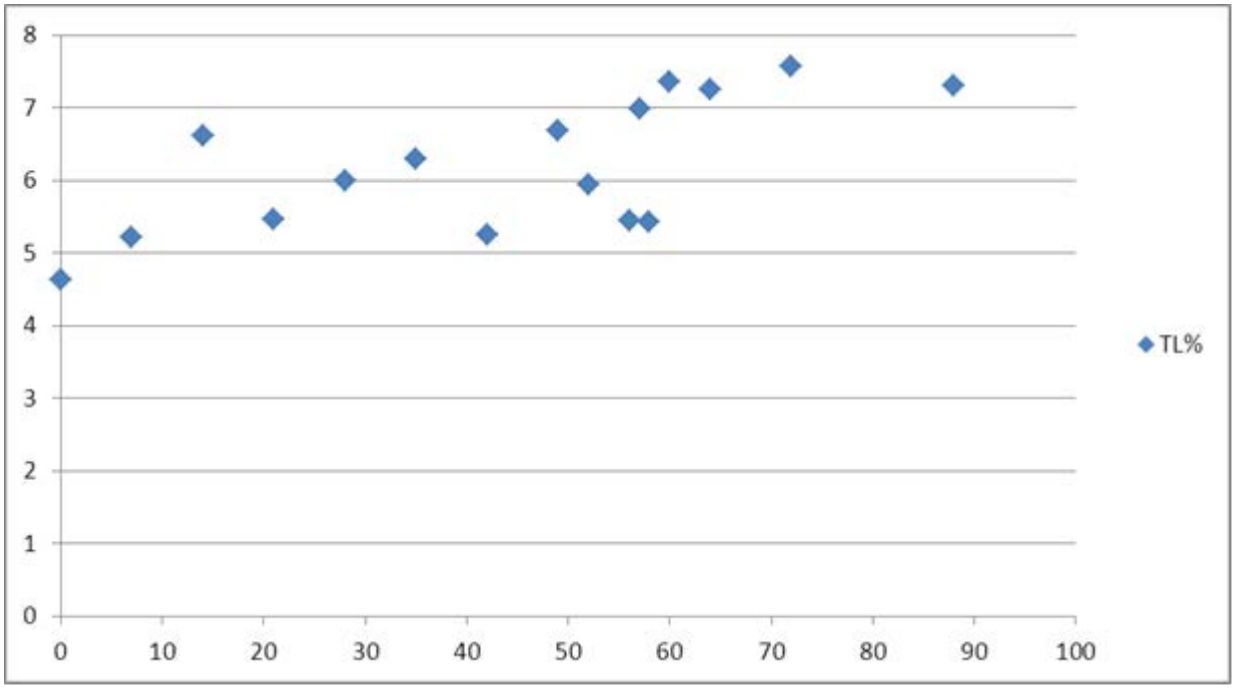
7.4.9 Fish lipid content

The mean lipid content of the control fish sampled during the study period for lipid analysis ranged from 6.1% at the onset of the experiment, to 8.9% at the end of the depuration period. The mean lipid content of the test animals was comparable to the control animals until the end of the depuration period. The observed differences were not significantly different (Table 22). The increase of lipid content was confirmed by lipid determination based on extracts from ASE of fish from test group (Figure 33).

Table 22: Lipid content of fish sampled at the start, middle and end of the study.

Time	Control	Treatment
Start Uptake	6.5	6.1
End Uptake	7.8	7.4
End Depuration	8.9	8.3

Figure 33: Lipid determination based on extracts from ASE of fish from test group.



7.5 Conclusions

The results of the second flow-through test show:

- That adsorption of the test substance to organic matter may reduce its bioavailability and therewith result in an underestimation of the BCF.
- That SPME can help to avoid possible issues with reduced bioavailability of poorly soluble and highly hydrophobic substances.
- that DBA is quickly metabolized by rainbow trout.

8 General Discussion and Conclusions

The performance of an aqueous exposure bioconcentration fish test according to OECD 305 requires the possibility of preparing stable, measurable dissolved aqueous concentrations of the test substance. For strongly hydrophobic substances (log KOW > 5 and a solubility below ~ 0.01-0.1 mg/L), testing via aqueous exposure may become increasingly difficult. For these highly hydrophobic substances the dietary test is recommended, provided that the test is consistent with the relevant regulatory framework and risk assessment needs (OECD 305). The dietary approach yields a biomagnification factor (BMF) rather than a BCF. Being able to estimate a BCF from the data generated in the dietary study would be a great advantage and would meet an accepted regulatory need. Therefore, the estimation of BCF values from data generated in the dietary studies is currently under investigation to keep the results of bioconcentration and biomagnification studies comparable [Crookes and Brooke, 2011]. However, this approach seems to be very complicated and might be even impossible due to the clear differences in the biomagnification and bioconcentration processes. In addition, defined regulatory cutoff criteria for the biomagnification potential of chemical compounds are still missing. Under such circumstances, the estimation of BCFs for highly HOCs in flow-through fish tests might remain essential in the future.

Measured bioconcentration factors (BCF) and n-octanol-water partition coefficients (KOW) of hydrophobic organic chemicals (HOC) show a curvilinear relationship up to log KOW 5-6. BCF values of more lipophilic substances tend to level of or decline (hydrophobicity cutoff). Debated reasons are steric effects, but also measurement artefacts. Jonker and van der Heijden (2007) suggested that the overestimation of bioavailable aqueous HOC by the presence of non-bioavailable HOC bound to dissolved organic matter, might lead to an underestimation of the true uptake. However, if bioconcentration factors for HOC are to be generated from the results of dietary studies, correct BCF estimates from aqueous exposure studies are required to verify the results of the suggested conversion procedure.

During the first part of this project the effect of organic matter on the bioavailability of HOCs was investigated in an analytical approach. Reduced bioavailability of HOCs could be shown by SPME/GC-MS analysis already in the presence of very low concentrations of OM as found in aqueous exposure bioconcentration fish tests. Results indicate that the influence of organic matter inside BCF test systems is highly dependent on the quality of organic matter and interacts with the molecular characteristics of the test substance.

To examine if there is an underestimation of fish BCFs caused by measuring artifacts, two flow-through fish tests with HOCs were carried out under realistic exposure conditions. Substances characterized by high (HCB, o-TP) and very high (PCB 153, DBA) lipophilicty were tested in the first and second study, respectively. The use of solvents and dispersants (solubilising agents) is not generally recommended for the preparation of stock solutions of test substances (OECD 305). A column elution method was therefore developed as part of this project to generate stable concentrations of the HOCs which can be maintained even throughout an extended uptake period. The analysis of the test media applied during the BCF studies showed that stable column generated concentrations of HOCs in the test system can be obtained over a period of 8 weeks without using solvents. Test concentrations were measured by SPME and LLE throughout the studies. Internal standard-corrected results obtained by SPME and LLE were equal; therefore, the results of BCF studies should be comparable independent of the method (LLE or SPME) used for the extraction of total water concentrations. Although already well established in environmental analyses to determine and quantify analytes mainly in aqueous matrices, SPME is still a rather unusual method in regulatory ecotoxicological research. Due to the potential

benefits (Duering et al, 2012), the use of SPME as an alternative analytical routine approach for aquatic bioconcentration studies should be considered.

The results of both BCF studies show that *Aqueous Exposure Bioconcentration Fish Tests* according to OECD 305 can be also carried out with HOCs up to a logKOW of 7.8. For three of the four test items it was possible to estimate kinetic and steady-state BCF values. However, as shown in the first part of this project natural organic matrices have a high sorption potential and can thus influence the bioavailability of HOC in BCF studies. DOC and POC concentrations in the test system were therefore monitored during the uptake periods. The results show that the experimental conditions are characterized by low concentrations of TOC. TOC levels can be kept below the threshold concentrations defined in the OECD TG 305 provided that the experimental tanks are kept as clean as possible. The results presented here were obtained from two flow-through bioconcentration studies on rainbow trout. Due to differences in feeding behavior and the stability of fecal material, flow-through tests on other fish species such as common carp may lead to different results regarding TOC concentrations.

SPME (without internal standard correction) can be used to measure bioavailable analyte concentrations in bioconcentration studies and help to assess the presence of non- bioavailable molecules bound to OM in the test system. SPME measurements carried out during the second BCF study showed that natural organic matrices present in the test system caused only a slightly reduced bioavailability of the HOCs. The recalculation of the estimated BCF values based on the bioavailable (freely dissolved) fraction showed that the presence of non-bioavailable molecules only caused a slight underestimation of the BCFs which cannot explain the hydrophobicity cutoff as suggested by Jonker and van der Heijden (2007). For the substance with the highest lipophilicity (PCB153, logKOW 7.8) still a lower BCF was estimated compared to the less lipophilic HOCs tested in this study, however, the decrease of the bioaccumulation potential must be caused by other factors, e.g. stearic effects, than measuring artifacts.

For the other test substance with a very high logKOW (DBA) no BCF could be determined. Obviously caused by the rapid metabolism of the test item no bioconcentration of the test substance could be observed. During the first BCF study animals were exposed for a longer uptake period (56 days) than required according to OECD 305 to reach the steady-state concentration. It was observed that tissue concentrations of oTP reached the steady state level already in the middle of the uptake period followed by a clear decrease of tissue concentrations toward the end of exposure. The results indicate that fish metabolism may adapt to a contaminant leading to a stronger elimination during the bioconcentration process. In contrast, tissue concentrations of HCB remained at a constant level after reaching the steady state. According to OECD 305 (§38) a steady-state is reached in the plot of test substance in fish (Cf) against time when the curve becomes parallel to the time axis and three successive analyses of Cf made on samples taken at intervals of at least two days are within ± 20% of each other, and there is no significant increase of Cf in time between the first and last successive analysis. Sampling should be continued (§59) during the uptake phase until a steady-state has been established. The steady-state BCF is then calculculated as Cf at steady-state (mean) / Cwater at steady-state (mean). An extension of the steady state period, as applied in this study, to take into account a potential decrease of the steady state concentrations is not in accordance with OECD 305.

The BCF estimates provided in this study are of high quality and can be used to verify the results of BMF to BCF conversion procedures. The project has shown that BCF studies can be carried out with HOCs reducing the need to run BMF studies which still do not fulfill the risk assessment requirements and thus challenge the use of experimental animals for such studies.

9 References

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10 Annex 1: Calibration functions

The following figures show for example calibration functions of the test items at the different analytical measurements. Plotted is the concentration of test item versus ratio Peak area test item * concentration IS / peak area IS.

Figure 34: Calibration graph of HCB in water per SPME-GC/MSD.

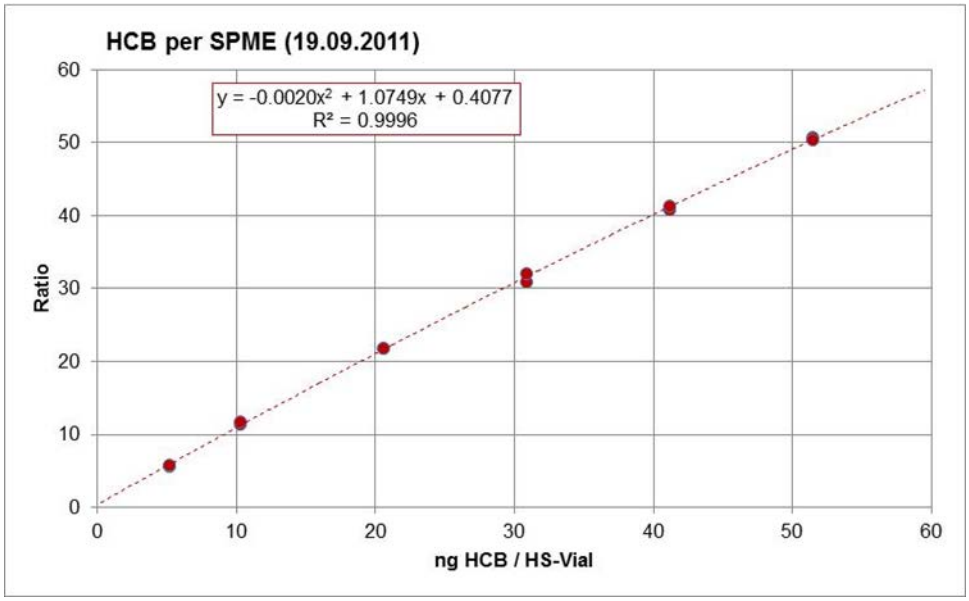


Figure 35: Calibration graph of Terphenyl in water per SPME-GC/MSD.

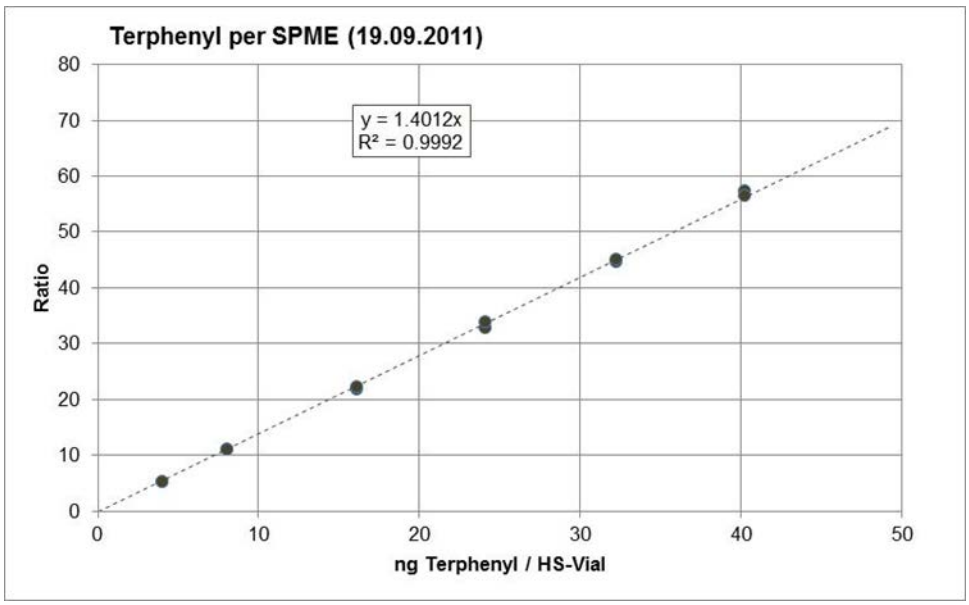


Figure 36: Calibration graph of HCB in water per LLE-GC/Ion Trap.

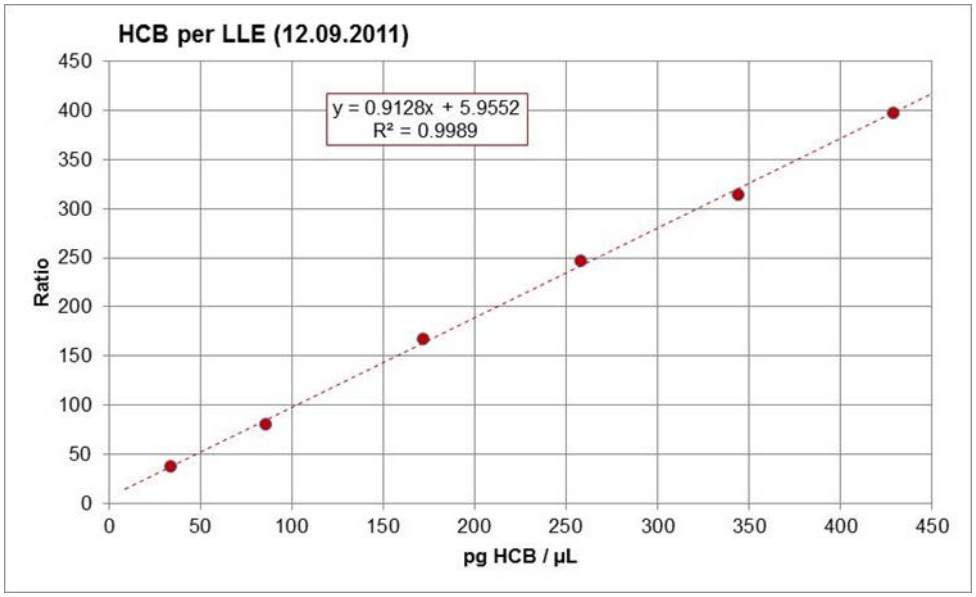


Figure 37: Calibration graph of Terphenyl in water per LLE-GC/Ion Trap.

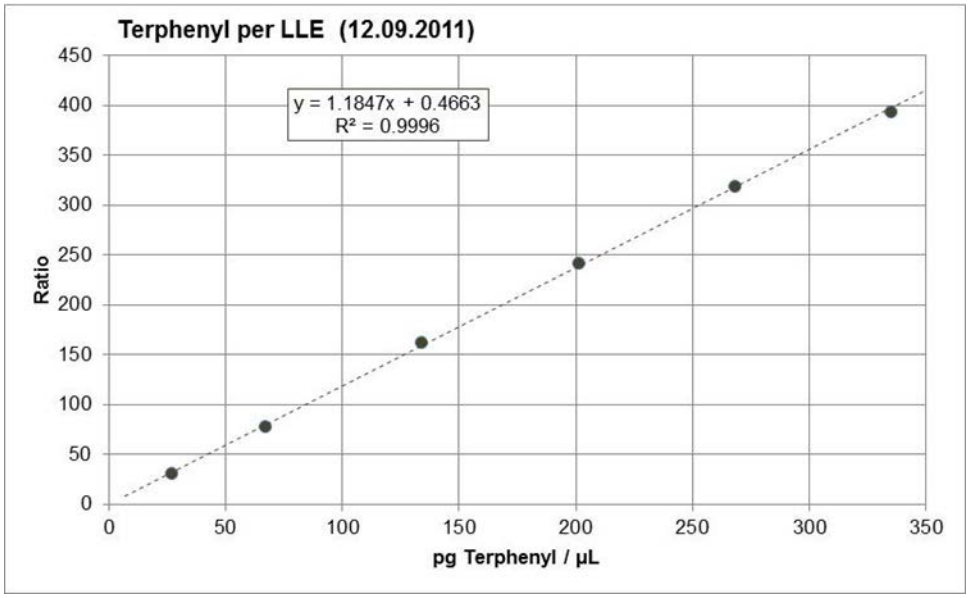


Figure 38: Calibration graph of PCB 153 in water per SPME-GC/MSD.

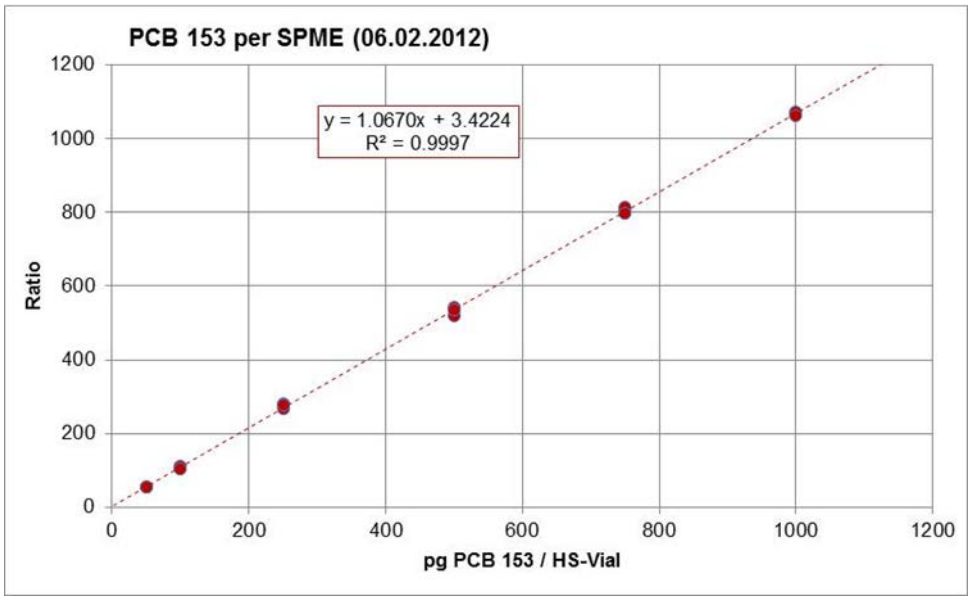


Figure 39: Calibration graph of DBA in water per SPME-GC/MSD.

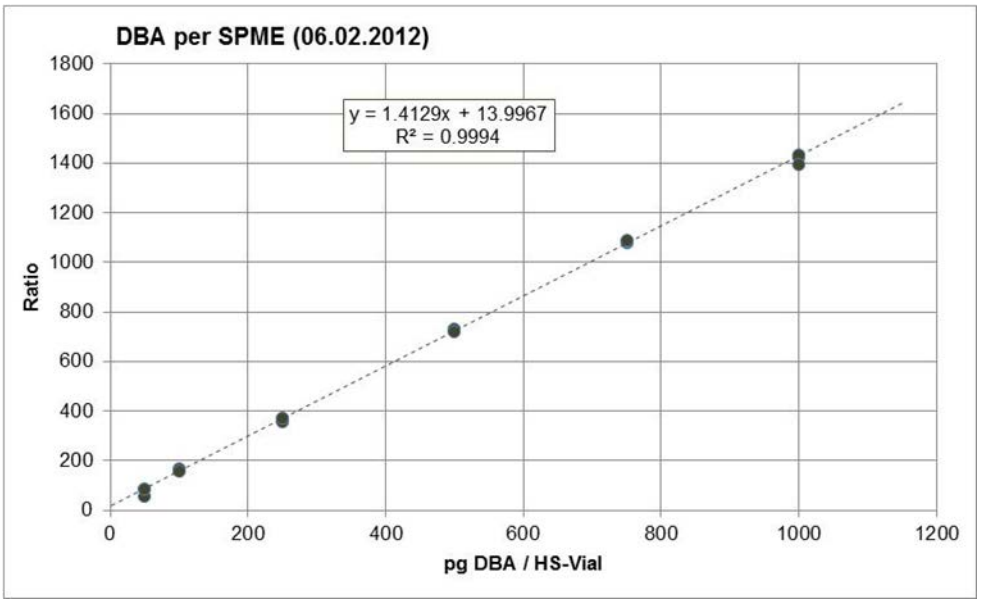


Figure 40: Calibration graph of PCB 153 in water per LLE-GC/MSD

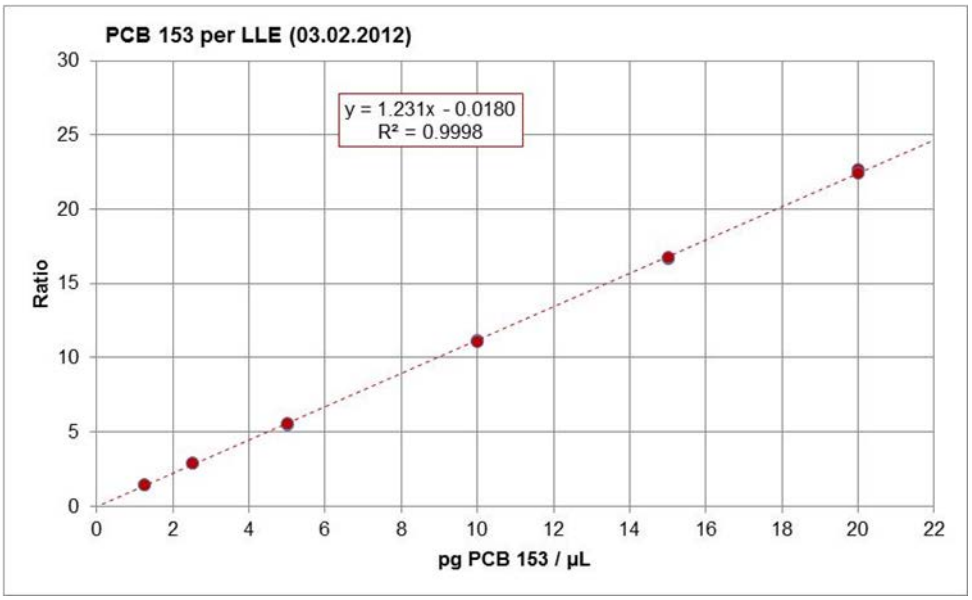


Figure 41: Calibration graph of DBA in water per LLE-GC/MSD.

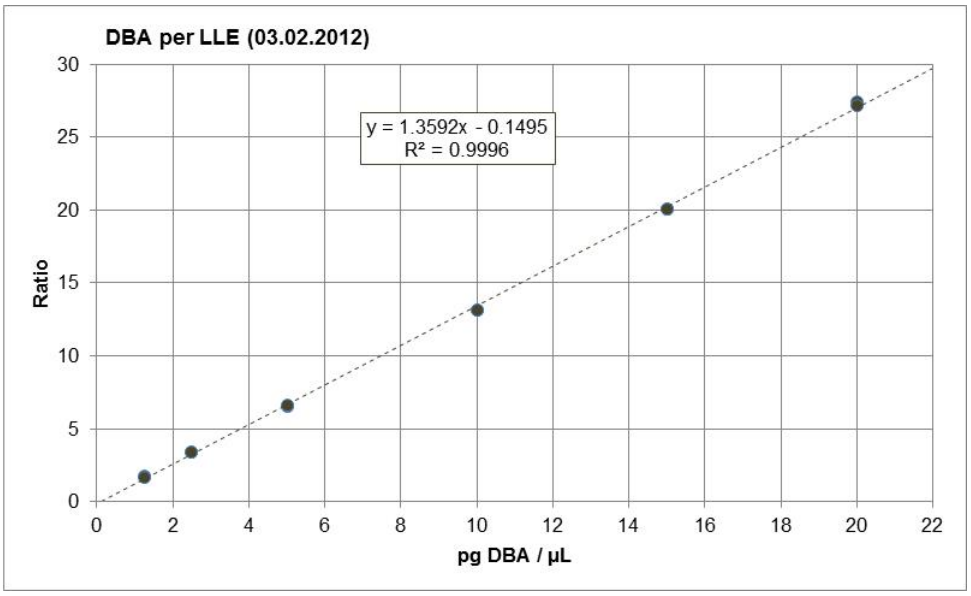


Figure 42: Calibration graph of PCB 153 in fish matrix with ASE and GC/MSD.

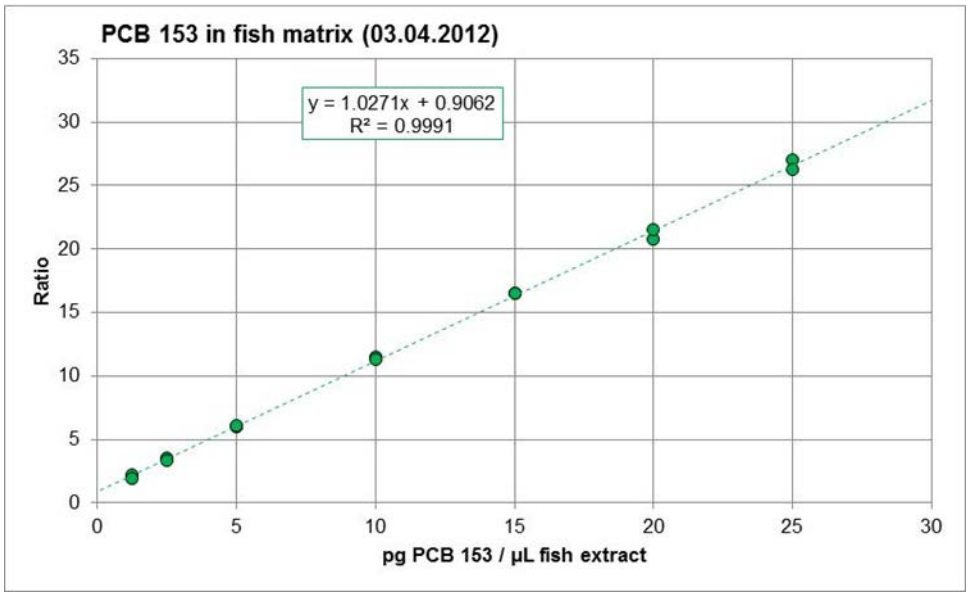
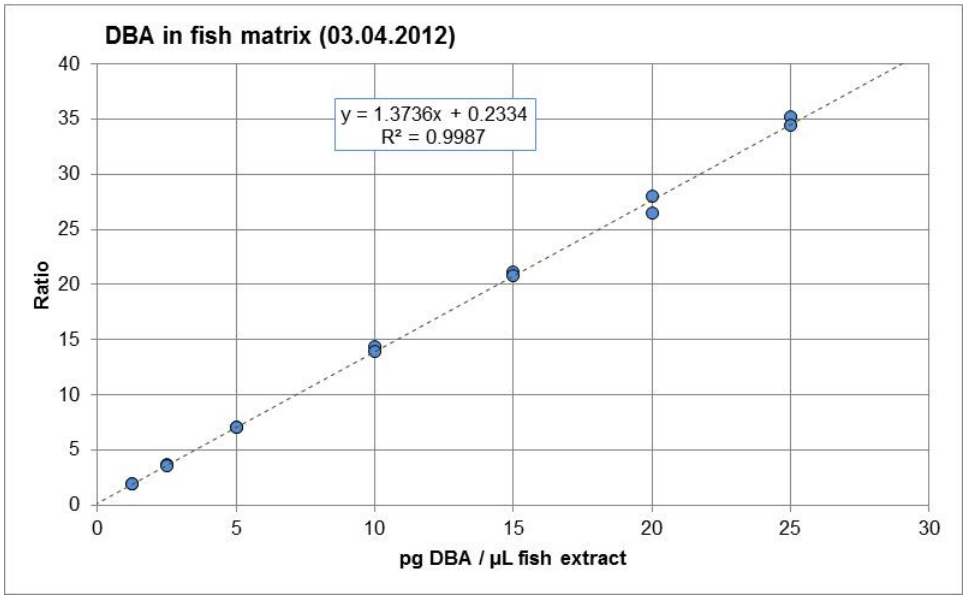


Figure 43: Calibration graph of DBA in fish matrix with ASE and GC/MSD.



11 Annex 2: Examples of GC/MS- Reports

Figure 44: GC-MS analysis report of HCB/Terphenyl in water per SPME.

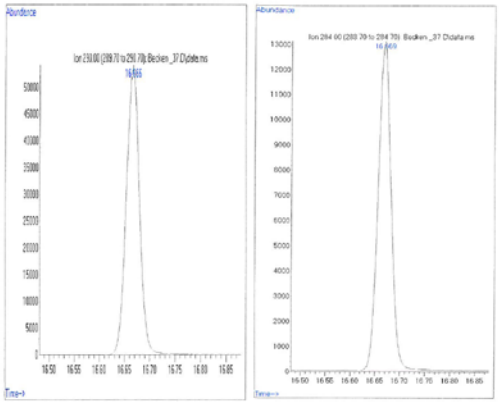
D:\Data\HCB_Terphenyl_Fischtest_SPME\Becken _37.D

Bestimmung von HCB und Terphenyl per SPME-Extraktion

Auswertung mit zwei markierten Standards

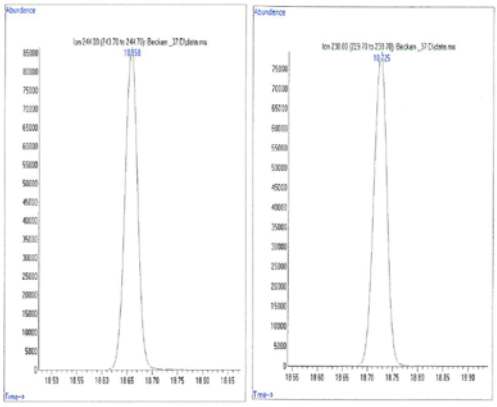
Data File Path	D:\Data\HCB_Terphenyl_Fischtest_SPME\
Data File Name	Becken _37.D
Operator	bru
Date Acquired	8/23/2011 11:46
Acq. Method File	HCB_Terph_SPME_SIM.M
Sample Name	Becken, 23.08.2011, 07:30, Probe 37
Vial Number	15

Name	**HCB-13C6**
Ret Time	16.67
Target Signal (amu)	290
Target Response	974525
Amount	26.80
Units	ng/Vial



Name	**HCB**
Ret Time	16.67
Target Signal (amu)	284
Target Response	234651
Amount	5.99
Units	ng/Vial

Name	**Terphenyl-d14**
Ret Time	18.66
Target Signal (amu)	244
Target Response	1517036
Amount	22.00
Units	ng/Vial



Name	**Terphenyl**
Ret Time	18.73
Target Signal (amu)	230
Target Response	1424102
Amount	13.84
Units	ng/Vial

Page 1 of 1 C:\msdchem\custrpt\HCB_TERPH_ SPME_2 IS.CRT 7/11/2013 10:46 AM

Figure 45: GC-MS analysis report of PCB 153/DBA in water per SPME.

D:\Data\PCB_PAK Fischtest\PCB_PAK_Becken 82.D

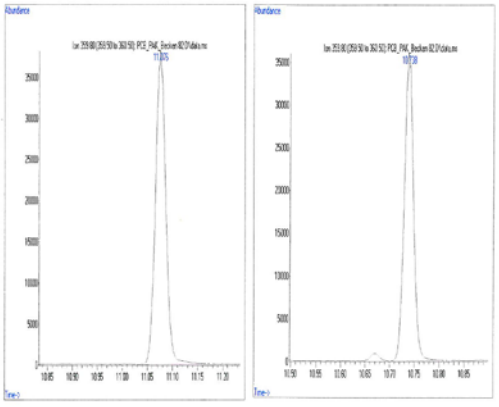
Bestimmung von PCB 153 und DB[a,h]A per SPME-Extraktion

Auswertung mit zwei Standards

Data File Path	D:\Data\PCB_PAK Fischtest\
Data File Name	PCB_PAK_Becken 82.D
Operator	bru
Date Acquired	3/9/2012 14:38
Acq. Method File	PCB_PAK_SPME_SIM3.M
Sample Name	Becken 09.03.2012, 08:30, Probe 82
Vial Number	5

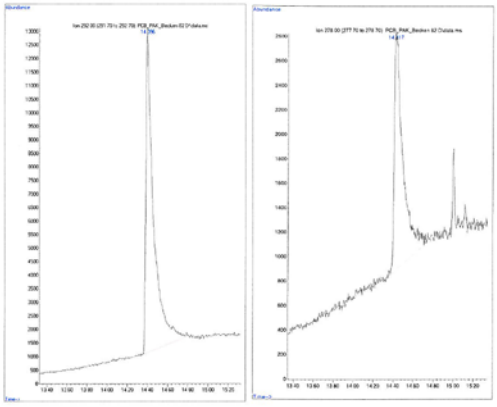
Name	**PCB 138 (IS)**
Ret Time	11.08
Target Signal (amu)	359.8
Target Response	535317
Amount	**500.00**
Units	pg/Vial

Name	**PCB 153**
Ret Time	10.74
Target Signal (amu)	359.8
Target Response	468048
Amount	**429.32**
Units	pg/Vial



Name	**DB[a,h]A-d14**
Ret Time	14.40
Target Signal (amu)	292
Target Response	638574
Amount	**500.00**
Units	pg/Vial

Name	**DB[a,h]A**
Ret Time	14.43
Target Signal (amu)	278
Target Response	118861
Amount	**66.29**
Units	pg/Vial



Page 1 of 1 C:\msdchem\custrpt\PCB_PAK_ SPME_2 IS.CRT 7/11/2013 10:52 AM

Figure 46: GC-MS analysis report of PCB 153/DBA in water per LLE.

D:\Data\PCB_PAK Fischtest\Becken 45.D

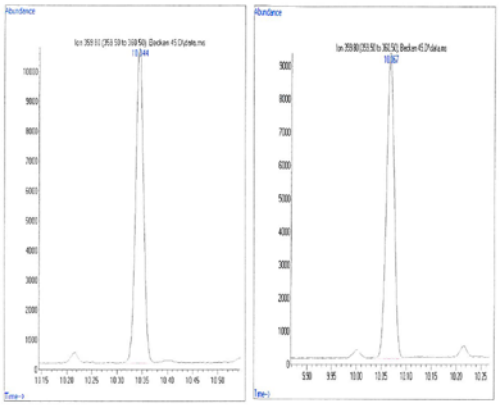
Bestimmung von PCB 153 und DB[a,h]A per Liquidextraktion

Auswertung mit zwei markierten Standards

Data File Path	D:\Data\PCB_PAK Fischtest\
Data File Name	Becken 45.D
Operator	bru
Date Acquired	2/3/2012 15:32
Acq. Method File	PCB_PAK_LLE_SIM.M
Sample Name	Becken 03.02.2012, 08:30 Uhr, Probe 45
Vial Number	65

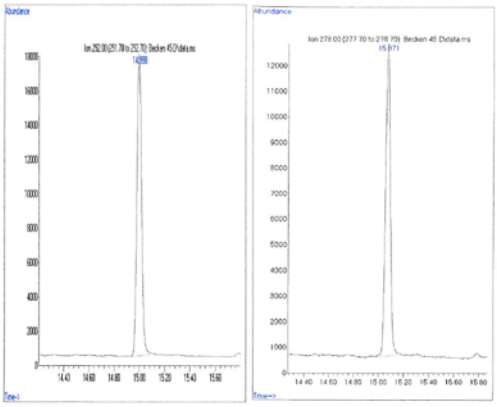
Name	**PCB 138 (IS)**
Ret Time	10.35
Target Signal (amu)	359.8
Target Response	114691
Amount	**10.00**
Units	pg/µL

Name	**PCB 153**
Ret Time	10.07
Target Signal (amu)	359.8
Target Response	100079
Amount	**7.59**
Units	pg/µL



Name	**DB[a,h]A-d14**
Ret Time	15.00
Target Signal (amu)	292
Target Response	443951
Amount	**10.00**
Units	pg/µL

Name	**DB[a,h]A**
Ret Time	15.07
Target Signal (amu)	278
Target Response	321138
Amount	**5.35**
Units	pg/µL



Page 1 of 1 C:\msdchem\custrpt\PCB_PAK_ SIM_2 IS.CRT 7/11/2013 10:41 AM

Figure 47: GC-MS analysis report of PCB 153/DBA in fish extracts per ASE.

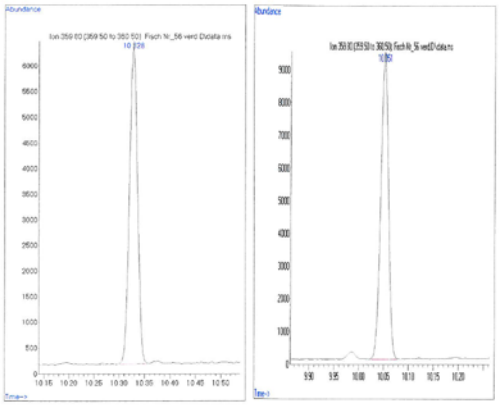
D:\Data\PCB_PAK Fischanalytik\Hauptversuch\Fisch Nr_56 verd.D

Bestimmung von PCB 153 und DB[a,h]A per Liquidextraktion

Auswertung mit zwei markierten Standards

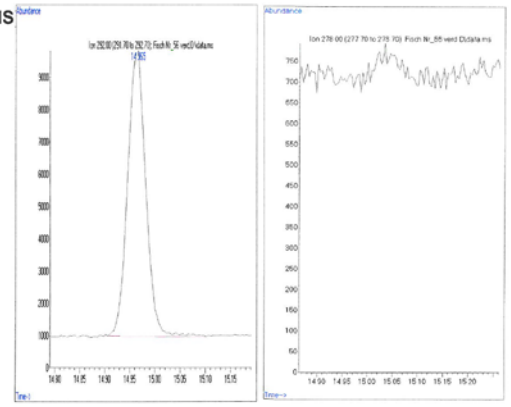
Data File Path	D:\Data\PCB_PAK Fischanalytik\Hauptversuch\
Data File Name	Fisch Nr_56 verd.D
Operator	bru
Date Acquired	5/9/2012 23:54
Acq. Method File	PCB_PAK_Fischanalytik 2.M
Sample Name	Fisch Nr. 56, 1:5 verd., 200 mL Extrakt
Vial Number	26

Name	**PCB 138 (IS)**
Ret Time	10.33
Target Signal (amu)	359.8
Target Response	68718
Amount	**10.00**
Units	pg/µL



Name	**PCB 153**
Ret Time	10.05
Target Signal (amu)	359.8
Target Response	100277
Amount	**13.46**
Units	pg/µL

Name	**DB[a,h]A-d14 (IS**
Ret Time	14.97
Target Signal (amu)	292
Target Response	221876
Amount	**10.00**
Units	pg/µL



Name	**DB[a,h]A**
Ret Time	0.00
Target Signal (amu)	278
Target Response	0
Amount	**0.00**
Units	pg/µL

Page 1 of 1 C:\msdchem\custrpt\PCB_PAK_ SIM_2 IS.CRT 7/11/2013 10:39 AM