



Front page for deliverables

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Authors and their organisation:
Thomas Junker, Thomas Knacker
ECT Oekotoxikologie GmbH, Boettgerstr. 2-14, D-65439 Floersheim Germany
(Partner 26)



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1 Introduction

The environmental fate of a compound is important for its risk assessment and is influenced by transformation, distribution and degradation processes. For the registration of compounds according to REACH (European Commission 2007a,b) the testing of their biodegradability is required. The biodegradability can be tested using standardized test methods according to OECD guideline 301. These screening tests are conducted in water systems. For the prediction of the metabolic fate of chemicals by mathematical models, data from different compartments (e.g. sediment, soil) are needed. Therefore, a new water-sediment-system was developed based on the OECD guideline 301 C within the framework of the EU-Project NoMiracle (Meller *et al.* 2005).

For a better understanding of the new screening test system the influence of different physical-chemical parameters, so-called “confounding factors”, has been analysed.

In the following, the experimental work between May 2007 and April 2008 is reported, including the following main aspects:

- Choice of confounding factors to be investigated
- Identification of a suitable test compound to investigate confounding factors
- Experimental examination of the influence of the chosen confounding factors in the water sediment system using a factorial design
- Statistical analysis of the test results
- Discussion of test results and comparison with literature data

Since search for additional degradation data for the aquatic compartment from literature and authorities has not been finished yet, these data will be presented within the deliverable report for the next project period together with literature data for the sediment compartment.

2 Material and Methods

Material and methods, including the test design, description of microbial inoculum, sediment, overlying water and the validation of the experimental procedures were described in Deliverables 2.3.1 (Meller *et al.* 2005) and 2.3.2 (Junker *et al.* 2006). For detailed information, please refer to these documents.

2.1 Choice of confounding factors and test substance

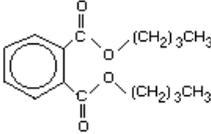
In Deliverable 2.3.7 (Junker *et al.* 2007), the confounding factors temperature, pH of the sediment and sediment composition were identified as most suitable for the first experiments. Furthermore, experimental biodegradation tests for a set of three test compounds (imidacloprid, glyphosate, cycloate) in water and sediment were performed. None of the tested compounds proved to be suitable for the investigation of factors which may influence biodegradation in the water-sediment system (confounding factors) because none of them was degraded within the test period of 28 days.

Eventually, the fourth potential test substance, dibutylphthalate (DBP), proved to be suitable for the experiments. Therefore, DBP was tested with regard to biodegradation in the water system and in the water-sediment system in parallel. Afterwards, the confounding factors "pH of the mineral medium" and "peat content of the sediment" were investigated in the water-sediment system using a factorial design.

2.2 Test substance

The physico-chemical parameters of the test substance DBP are shown in Tab. 1. At room temperature, DBP is a colourless liquid with an oily consistency, which is used as plasticizer in PVC, but also in paint, adhesives and cosmetics (Harris and Sumpter 2001). DBP is one of the most often detected phthalates in the environment (Xu *et al.* 2005). Its biodegradability is high (75 to 80% within 3 days) and DBP is classified as ready biodegradable (MITI-Test, European Commission 2000). However, DBP is known to be an endocrine disruptor (Harris and Sumpter 2001). DBP was chosen as test substance, since the compound is biodegradable, sorbs to sediment particles and is non-volatile (Streit 1991; European Commission 2000; Rippen 2000).

Tab. 1: Physicochemical parameters of the test substance DBP used in the studies with the water-only and the water-sediment system

Dibutylphthalate	
CAS-No.:	84-74-2 ^[1]
Empirical formula:	C ₁₆ H ₂₂ O ^[1]
Structural formula:	 [2]
Molecular weight [g/mol]:	278,34 g/mol ^[1]
Water solubility [mg/L]:	12 (10-30°C) ^[1]
Log P_{ow}:	4.61 ^[1]
Log K_{oc}:	3.4 ^[1]
Vapour pressure (20°C) [Pa]:	2.6*10 ⁻³ ^[1]
Henry constant [atm m³/mol]:	2.67*10 ⁻⁶ ^[3]
ThOD [mg/mg]:	2.242
Usage:	plasticizer

^[1] Rippen (2000); ^[2] www.inchem.org/documents/ehc/ehc/ehc189.htm; ^[3] Streit (1991)

2.3 Test design

2.3.1 Standard test design using the water-only and the water-sediment system

In order to investigate the influence of the sediment compartment, DBP was applied directly to the water phase and was mixed into the sediment of the water-sediment system. Due to its low water solubility of 12 mg/L (Rippen 2000) DBP was added to the mineral medium, which was dispersed for 30 minutes afterwards in order to achieve a homogeneous distribution of the test substance. The resulting dispersion with a concentration of 100 mg DBP/L was used as water phase for both the water-only and the water-sediment system. When applied to the sediment compartment, the sediment was mixed thoroughly due to the log K_{ow} of 4.61. The test design is summarized in Tab. 2. Aniline was tested in parallel in both test systems as reference substance at a concentration of 75 mg/L. In addition, aniline was tested together with DBP in two replicates of the water-sediment system in order to check if DBP inhibits the degrading micro-organisms. The test duration was 28 days.

Tab. 2: Number of replicates when testing DBP in the water-only and the water-sediment system

Treatment	Water-only system	Water-sediment system
Controls	2	2
Abiotic controls	2	-
DBP	4	4 (application via medium) 4 (application via sediment)
Reference substance	2	2
DBP + reference substance	-	2

2.3.2 Investigation of confounding factors in the water-sediment system using a factorial design

The factorial design allows for testing several parameters in parallel. All possible combinations of the investigated parameters are considered. Thus, it is possible to examine the effect of the parameters as well as their interaction. The test design chosen in the experiment with DBP was a so-called 2^3 -design, where three different parameters were investigated at two different levels (Morgan 1991). The first factor was the test substance (treatment vs. control), the second factor was the pH of the mineral medium (pH 6 vs. pH 8) and the third factor was the peat content of the artificial sediment (0% vs. 5%). The test design is summarized in Tab. 3. DBP was tested at a concentration of 100 mg/L. The test duration was 28 days.

Tab. 3: Investigation of confounding factors: Number of replicates when testing DBP in the water-sediment system

Test vessel No.	Application of DBP	pH of mineral medium	Peat content of sediment
1 - 3	no	6	0
4 - 6	yes	6	0
7 - 9	no	6	5
10 - 12	yes	6	5
13 - 15	no	8	0
16 - 18	yes	8	0
19 - 21	no	8	5
22 - 24	yes	8	5

2.4 Statistical evaluation

After determination of degradation kinetics, the test results were evaluated statistically using the program SPSS (Versions 7.5.2G and 11.5.1). Thereby, it is possible to check, e.g. whether the test substance has an inhibitory effect to the micro-organisms or if the composition of the artificial sediment affects the biodegradation kinetics.

3 Results and Discussion

3.1 Degradation test with DBP

The results of the degradation test with DBP in the water-only and in the water-sediment system are shown in Fig. 1.

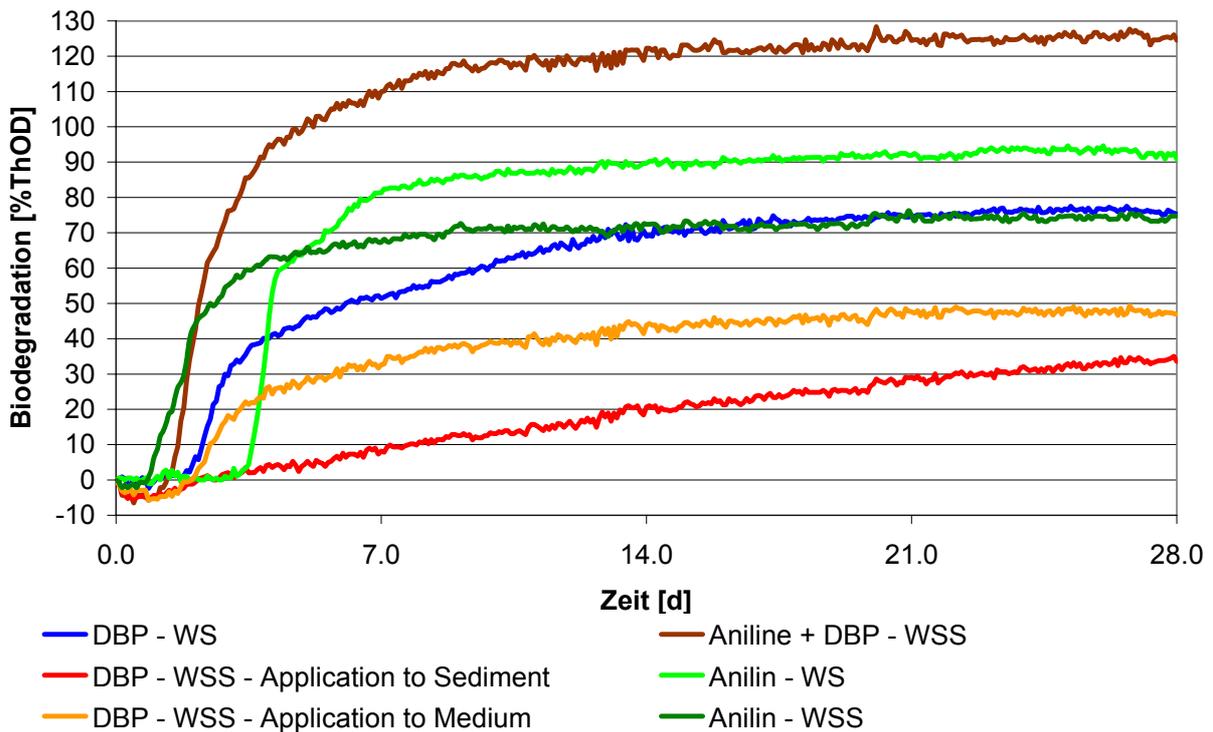


Fig. 1: Biodegradation of DBP and aniline (mean values) in the water-only system and in the new water-sediment system within 28 days (WS = water-only system; WSS = water-sediment system)

The reference substance aniline was degraded in both the water-only and the water-sediment system. As observed in previous tests, the lag-phase was shorter in the water-sediment system compared to the water-only system. However, the degradation at the end of the test period was lower in the water-sediment system (75% compared to 92.5% in the water-only system). With regard to statistical analysis significant differences (t-test, $p \leq 0.05$) between the test systems could be determined for the lag-phase as well as for the half-life.

The test substance DBP was also degraded in both, the water-only and the water-sediment system. For the water system, the exponential degradation phase ends at approx. 35% ThOD, followed by a slower, non-exponential degradation (for 10 days) and the plateau phase. The short exponential degradation phase (0-35% ThOD) could be explained by the limited availability of the test substance for the micro-organisms due to its low water-solubility. At first, the dissolved DBP is degraded by the bacteria. Afterwards, the remaining DBP dissolved little by little and is degraded non-exponential. It is stated in literature that water solubility plays a major role for the degradation of DBP (Mulligan *et al.* 2001; Xu *et al.* 2005). At the end of the test biodegradation was 75.5% ThOD. Since degradation was >60% within a 10-day window, DBP can be classified as readily biodegradable in water.

With regard to the water-sediment system, a clear effect of the sediment on the biodegradation of DBP could be observed and there are differences based on the application method (via the water phase or via the sediment). When applied to the water phase, the duration of the lag-period (2.3 d) is in the same range as for the water-only system (2.4 d). However, the biodegradation of 47% at the end of the test is clearly lower than for the water-only system. Furthermore, the exponential degradation phase is very short, followed by a non-exponential increase. With regard to the $\log K_{ow}$ of 4.61 and the $\log K_{oc}$ of 3.4, one might assume that firstly, DBP partly sorbed to the sediment, and became available for the degrading organisms by and by afterwards. This explanation is supported by the results after application of DBP to the sediment, where biodegradation does not follow a first-order kinetic. No exponential degradation phase but a more or less linear degradation could be observed throughout the test period, resulting in a degradation of approx. 35% ThOD at the end of the test. No degradation could be detected in the abiotic controls (not shown in Fig. 1). A summary of the degradation parameters is shown in Tab. 4.

Tab. 4: Summary of degradation kinetics for the test with DBP

	<i>lag-phase</i> (degradation <10% ThOD) [d]	Degradation after 28 days [% ThOD]	Degradation constant <i>k</i> [1/d]	<i>t</i> _{1/2} [d]
Aniline (water-sediment system)	1,1	75	0,3691	1,88
Aniline (water system)	3,6	92,5	1,1868	0,58
Aniline + DBP (water-sediment system)	1,6	124,5 (- DBP-Mean: 78)	1,0486	0,66
DBP (water system)	2,3	75,5	0,3526	1,97
DBP (application via water phase)	2,4	47	0,1784	3,89

For both, aniline and DBP, half-life is lower in the water system compared to the water-sediment system. The approach using aniline + DBP shows that the degradation of aniline is not affected by DBP, since the degradation at the end of the test (125% ThOD) is equivalent to the sum of the individual degradation of aniline (75% ThOD) and DBP (47% ThOD) in the water-sediment system. Consequently, DBP is not toxic to sewage sludge micro-organisms at 100 mg/L, which is in accordance with literature (ECB 2004). Furthermore, degradation constants for DBP are in the same range as described by Chang *et al.* (2007)

3.2 Investigation of confounding factors using DBP in the water-sediment system

The degradation of DBP in the water-sediment system depending on the pH in the water phase (mineral medium) and the peat content of the sediment is illustrated in Fig. 2. There are only small differences between the degradation curves. DBP is degraded in all four combinations of parameters, resulting in a degradation of 63-69% ThOD at the end of the test. Thus, the degradation rate is higher than in the first test with DBP (chapter 3.1), where a degradation of 47% could be observed after application to the water phase with a pH of 7. This is contrary to Chang *et al.* 2007, where the highest degradation rates for DBP could be observed at pH 7. However, the calculated degradation parameters (e.g. degradation constant and half-life) are in good accordance with literature data (Chang *et al.* 2007). A summary of the degradation parameters is shown in Tab. 5.

Tab. 5: Summary of degradation kinetics for the test with DBP (investigation of confounding factors)

	<i>lag-phase</i> (degradation <10% ThOD) [d]	Degradation after 28 days [% ThOD]	Degradation constant <i>k</i> [1/d]	<i>t</i> _{1/2} [d]
pH 6; 0% peat	1,3	69	0,2559	2,71
pH 6; 5% peat	1,2	68	0,2626	2,64
pH 8; 0% peat	1,9	65	0,2325	2,98
pH 8; 5% peat	1,4	63	0,2372	2,92

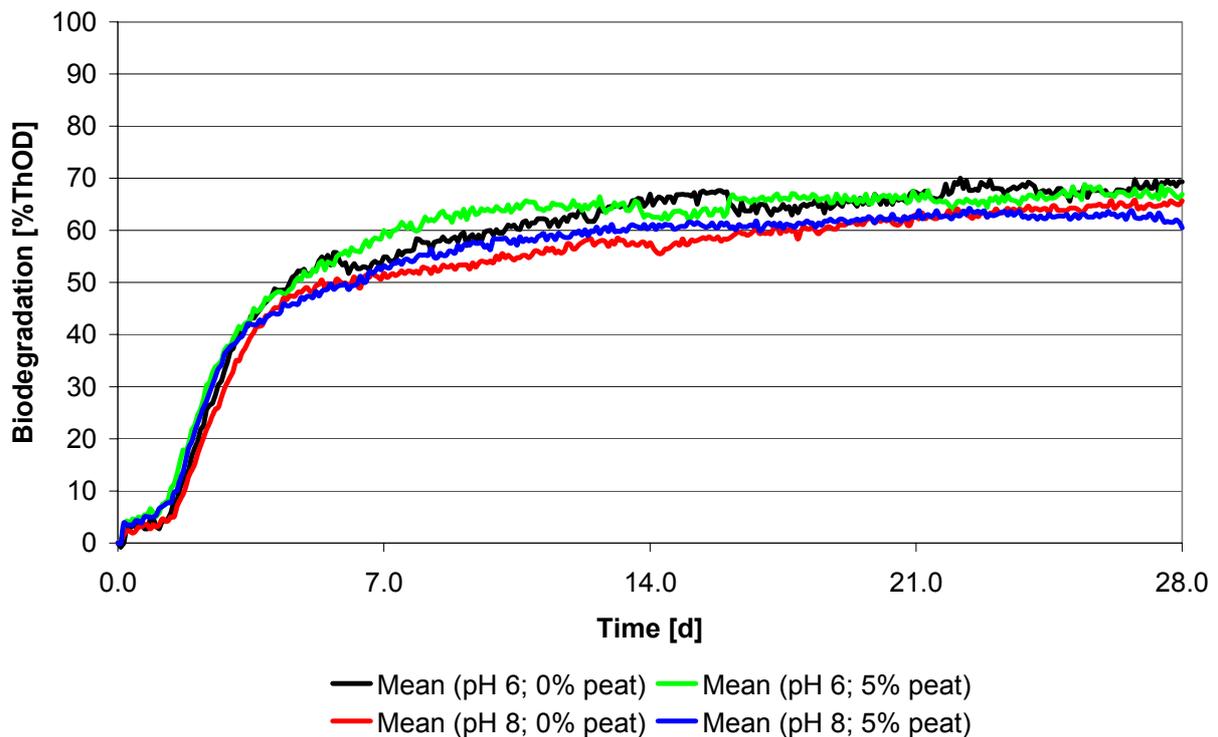


Fig. 2: Biodegradation of DBP (mean values; $n = 3$; 100 mg/L) in the water-sediment system within 28 days using a factorial design

The statistical evaluation of the test results using a multifactorial ANOVA resulted in an inhomogeneity of variances (Levene-test, $p \leq 0.05$) for all parameters (duration of lag-period, degradation constant, half-life, degradation at the end of the test). Furthermore, all values are normally distributed (Kolmogorov-Smirnov-test, $p \leq 0.05$).

The pH of the mineral medium has a statistical significant effect (ANOVA; $p \leq 0.05$) on all degradation parameters except the duration of the lag-phase, whereas the peat content of the

sediment has a statistical significant effect (ANOVA; $p \leq 0.05$) on all degradation parameters except the degradation constant. There is no interaction between pH and peat content with regard to duration of the lag-phase and with regard to degradation at the end of the test. However, an interaction between pH and peat content could be detected in terms of degradation constant and half-life. Table shows a summary of results of the statistical evaluation.

Tab. 6: Summary of results of the statistical evaluation of the test for investigation of confounding factors using DBP in the water-sediment system (+: significant; -: not significant)

	pH of mineral medium	peat content of sediment	interaction pH ↔ peat content
Degradation constant k	+	-	+
Half-life $t_{1/2}$	+	+	+
lag-phase	-	+	-
Degradation (28 d)	+	+	-

Moreover, a brown colouration of the water phase could be observed in the test vessels with a peat content in the sediment of 5%, indicating that dissolved constituents of the peat moved to the water phase Fig. 3. The pH values in the water phase at the end of the test are summarised in Tab. 7. The results show that the pH in the test vessels with pH 6 at the beginning of the test increased up to 6.8 in the vessels without peat and up to 7.7 in the vessels with 5% peat. In the test vessels with pH 8 at the beginning of the test, the pH remains constant (8.1) throughout the test in the vessels with a peat content of 5% in the sediment, whereas the pH decreased to 6.8 in the replicates without peat.

Tab. 7: pH values in the water phase at the end of the test for investigation of confounding factors using DBP in the water-sediment system

Approach	pH in the water phase at the end of the test
pH 6 - 0% peat - without DBP	6.7
pH 6 - 0% peat - with DBP	6.8
pH 6 - 5% peat - without DBP	7.4
pH 6 - 5% peat - with DBP	7.7
pH 8 - 0% peat - without DBP	6.8
pH 8 - 0% peat - with DBP	6.8
pH 8 - 5% peat - without DBP	8.1
pH 8 - 5% peat - with DBP	8.1



Fig. 3: Water-sediment test vessels with 5% (left) and 0% (right) peat content in the sediment at the end of the test (day 28)

4 Conclusion and Outlook

Indeed, statistically significant effects on the degradation of DBP in the water-sediment system could be detected with regard to the pH of the mineral medium and with regard to the peat content of the artificial sediment due to small variations in the results between the replicates of each approach. However, the differences between the degradation curves of the approaches and the related kinetic parameters are minimal. Thus, it is questionable if the statistically significant effects will also result in relevant biological and ecological effects in terms of the degradation of DBP in the environment.

Within the next project period biodegradation experiments will be continued for further selected substances. To ensure an optimal comparability of the results, each compound is tested using the water-only system and the water-sediment test system in parallel. In doing so, existing differences between the test systems will be identified in order to continually improve the comparability of experimental biodegradation data obtained for the different compartments.

With regard to confounding factors, it would be informative to test DBP at pH values of 6, 7 and 8 in parallel, in order to check the thesis by Xu *et al.* (2005) that the highest degradation rates for DBP are obtained at pH 7. This could not be evaluated in this work, since the first degradation experiment with DBP at pH 7 was performed using a peat content of 2% in the sediment. Furthermore, different batches of microbial inoculum were used for the experiments. In general, the composition of the microbial inoculum might affect the results of degradation tests. Further confounding factors, which might be investigated are the temperature and the particle size distribution of the sediment.

Moreover, the test with DBP showed that the addition of peat to the artificial sediment is not necessary to result in high degradation rates, since degradation was in the same range for both peat contents tested (0% and 5%). Thus, one might conclude that experiments should be performed using sediment without peat, because the preparation of the peat suspension for the artificial sediment is time consuming and different sources of peat might result in high variability. However, sediments with peat are closer to the real environment, where sediments without organic matter are unlikely.

Besides the experimental part, a systematic search for additional degradation data from literature and competent authorities will be performed for the aquatic compartment. The results of both, biodegradation experiments and literature search, will provide data which are needed by other partners in WP 2.3 to build a database for biodegradation parameters of organic compounds in water and sediment. Thus, an analysis of degradation parameters across chemicals and media will be possible and improved quantitative structure-property relationships (QSPRs) can be developed.

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