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Ecotoxicological Assessment of Immersion Samples from Façade Render

1st July 2013

On behalf of the Swiss Federal Office for the Environment (FOEN), Berne, Switzerland

Contracting Authority
Swiss Federal Office for the Environment FOEN
Division of Air Pollution Control and Chemicals
Biocides and Plant Protection Products Section
3003 Bern
Switzerland

Reference-Nr. REF-1011-06100
Credit number: A2111.0239 Umweltforschung + Bildung

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Citation: Burkhardt, M, Dietschweiler, C, Campiche, S, Junghans, M, Schifferli, A, Baumberger, D, Kienle, C, Vermeirssen, E, Werner, I (2013): Ecotoxicological Assessment of Immersion Samples from Façade Render. Report for the Swiss Federal Office for the Environment FOEN, Bern, p. 34.

Summary

To protect façade renders from growth of bacteria, fungi and algae, biocides can be added to a render before it is applied onto a façade. A comprehensive protection can be achieved by combining several biocides. During rain events and over time, biocides will gradually leach out and thus have the potential to affect soil or aquatic ecosystems.

In this project the leaching behaviour of biocides from three render formulations was evaluated: one render containing free, another render containing encapsulated biocides (Terbutryn, OIT, DCOIT) and a control render without biocides. The renders were applied onto extruded polystyrene panels and water samples were generated over nine immersion cycles of the panels in accordance with standard EN 16105. Concentrations of the biocides were measured using LC-MS. The toxicity of the first and ninth immersion samples was determined using bioassays. Toxicity to aquatic organisms was evaluated by assessing inhibition of photosynthesis and algal growth rate, inhibition of bacterial luminescence and inhibition of daphnid population growth. Toxicity to soil organism was assessed by determining avoidance behaviour of worms and reproductive output in springtails. For aquatic effects, the toxic potential of a sample was expressed as a 50% effect concentration (EC_{50}) based on sample dilution factors (DF; the sample volume and volume of culture medium used for dilution divided by the sample volume).

Encapsulation reduced the leaching of Terbutryn, OIT, and DCOIT 4-, 17-, and 25-fold compared to free biocides used in the same amounts in the render. Generally, the toxicity of water from render containing encapsulated biocides was always lower than that of render with free biocides and toxicity was considerably lower for the ninth immersion day compared to the first immersion day sample for both free and encapsulated samples. Thus, on the first immersion day, the free biocide sample had a DF EC_{50} of 630, and the encapsulated biocides sample a DF EC_{50} of 130. On the ninth immersion cycle, the free biocide sample had a DF EC_{50} of 120 and encapsulated biocide sample a DF EC_{50} of 30. Toxicity therefore decreased 4- to 5-fold over the nine immersion cycles for both free and encapsulated samples. For the aquatic organisms, inhibition of photosynthesis was the most sensitive endpoint, followed by algal growth rate, bacterial bioluminescence and daphnid reproduction. At all tested sample concentrations, none of the samples with biocides caused effects on soil organisms. No toxicity was observed in control immersion samples without biocides in render although TOC (total organic carbon) reached up 250 mg/L.

Results from bioassays matched quite well with expected bioassay responses based on chemical analysis and the toxicity of the individual biocides. It could be concluded, that the toxicity of given concentrations on algae is explained by Terbutryn whereas the toxicity on bacteria and daphnids is caused by DCOIT and OIT. The results thus indicated that other components in the render did not add to the toxicity of the individual biocides. Furthermore, the good agreement between the chemical analysis and the expected and observed biological effects indicates that the data are robust and that an assessment of the biological effect data using DF EC_{50} is a suitable evaluation tool for e.g. biocides released from treated articles or substances from construction products. Overall, the approach combining a standard leaching test with standard bioassays is very promising to evaluate the ecotoxicity of biocides leached out from façade renders.

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1 Introduction

Microbial deterioration of exterior coatings of buildings is controlled by antimicrobial active substances used in film preservatives (Paulus, 2004). Film preservatives are added to water based renders and paints available in cans for ready-to-use application. Although hundreds of end-products are offered on the market, the number of biocides for film preservation is rather limited. Typically, exterior paints and renders contain Terbutryn or Diuron (algicides), OIT, Carbendazim, and Zinc pyrithione (fungicides and bactericides). Less important are IPBC, Isoproturon, and DCOIT. These biocides are available as free substance or encapsulated in polymeric spheres (Burkhardt and Vonbank, 2011). During the wet state condition of the façade, biocides slowly migrate to the surface of the coating affecting organisms. Moreover, leached biocides might enter the environment.

The European biocide authorization has started with the environmental risk assessment of biocides. Based on the emission scenario documents (ESD) a leaching test for product type 7 "film preservatives" (PT 7) similar to the PT 8 "wood preservatives" test is suggested. A leaching test is recommended by the standard "Paints and varnishes - Laboratory method for determination of release of substances from coatings in intermittent contact with water" (EN 16105:2011). The EN 16105 has been proofed by a round-robin test published by Schoknecht *et al.* (2013).

The Swiss Federal Office for the Environment (FOEN) is responsible for the environmental risk assessment of formulated biocidal products placed on the Swiss market under the Swiss biocidal products regulation (Ordinance on Biocidal Products, OBP, SR number 813.12). Such products contain one or more active ingredients and are used directly (e.g. wood preservatives) or added to the final market product for end-users, like polymeric renders and paints. Ecotoxicological effects of released mixtures from end-products are normally not evaluated in the authorization. Further insights are lacking regarding the composition of the biocide mixture leached out over time and matrix substances released from the coating that may contribute to the toxicity.

In principle, the mixture toxicity can be derived by calculating the effects of individual substances in mixtures, e.g. using no observed effect concentrations (NOEC) or effect concentrations such as EC_{50}-values (concentration corresponding to 50% of the maximum effect), but possible lack of effect data would limit this approach.

In a laboratory study, water samples of the immersion test (EN 16105) are tested by a set of five bioassays to determine the toxicity with a focus on the following issues:

- How much does the ecotoxicity of immersion samples differ between render with free and encapsulated biocides as well as without biocides?
- How well does the theoretical ecotoxicity of individual biocides match the measured effect of the biocide mixture present in the sample?
- Which dilution is needed to avoid effects on aquatic and terrestrial test organisms?

The study is achieved in cooperation with UMTEC and Ecotox Centre of Eawag/EPFL.

2 Background Information

Exterior renders and paints are regularly divided into mineral and organic products, even though mineral coatings can contain polymeric binders. Polymeric paints and renders consist of up to 20 ingredients (Schoknecht *et al.*, 2009). According to EN 998-1 and EN 15824, mineral and organic modified renders are defined as coatings to protect the masonry physically against direct influence of weather. The thickness of render is usually 2-3 mm. Architectural paints and renders with polymeric binders have significant market share with a consumption quantity of about 26'000 t in Switzerland in 2011 (Burkhardt and Dietschweiler, 2013).

The emission of biocides occurs during wash-off events by driving rain. Highest emissions are observed in the early stage of exposure and early in each leaching event (Burkhardt *et al.*, 2009). In a later state of the coating lifetime, biocide concentrations tend to reach a fairly consistent range (Burkhardt *et al.*, 2012). Between wash-off events biocides are transported to the surface by diffusion (Wangler *et al.,* 2012).

The gradual release of biocides from façades to the environment depends on a number of factors such as properties of the active ingredient, product composition and environmental conditions. Factors specifically controlling the diffusion are for example water solubility and partitioning coefficient between octanol and water (Pow) (Table 1), total porosity of the matrix (pigment to volume concentration, PVC), or the connectivity of the porous network (tortuosity). Consequently, alone by product composition the emission from paints and renders can vary for the same initially added biocide at same concentration, e.g. up to a factor of 3 (Wangler *et al.*, 2012).

Table 1: Water solubility and logPow of the biocides used (Paulus, 2004; Schoknecht *et al.*, 2009).

Active ingredient	Water solubility (mg/L)	logPow (-)
Terbutryn	22	3.7
DCOIT	14	3.6
OIT	500	2.5

Microencapsulated biocides are on the market since 2001 (e.g. AMME™ products) and reach about 80% market share in Switzerland in 2011 (Burkhardt and Dietschweiler, 2013)[1,2]. In comparison with free biocides, microencapsulated biocides reduce leaching of biocides by a factor of 2 to 10 (Burkhardt and Vonbank, 2011; Breuer *et al.,* 2012). The results showed a decrease in leaching especially in the initial phase compared to free biocides.

[1] Mainly products based on „AMME®„-Technology are used (Advanced Micro Matrix Embedding). http://www.thor.com/biocideproducts.asp?AppID=2

[2] Sauer, F. (2013): Algen das Leben schwer machen. Farbe+Lack, 119:73-79.

3 Material and Methods - Leaching

In order to assess the ecotoxicity of free and encapsulated biocides used in organic render, two variants equipped with biocides and one variant without biocides as a control were prepared:

1. Render with free biocides (variant 1)
2. Render with encapsulated biocides (variant 2)
3. Render without biocide (control sample; variant 3)

3.1 Preparation of Specimen

In a frame formulation of a render, three pre-formulated biocides are used in free (variant 1) and encapsulated form (variant 2; AMME™ products):

- Terbutryn (CAS 886-50-0; N2-tert-butyl-N4-ethyl-6-methylthio-1,3,5-triazine-2,4-diamine)
- OIT (CAS 26530-20-1; 2-Octyl-3(2H)-isothiazolinone)
- DCOIT (CAS 64359-81-5; 4,5-dichloro-2-n-octyl-4-isothiazolino-3-one).

The biocides were obtained from Thor GmbH, 67346 Speyer, Germany. Variant 3 represents the reference material without film preservative enabling the assessment of ecotoxicological effects of leachable substances other than biocides. The composition of the frame formulation representative for market products has been successfully used in a round-robin test by Schoknecht *et al.* (2013).

The concentration of each biocide was set at 750 mg/kg and the amount of render applied was 2.7 kg/m^2 with 2000 mg biocides per m^2. In practice the concentrations of individual biocides are in the range of 100 to 1500 mg/kg. The exact amounts applied in the frame formulation, on the substrate, and their dry weights were controlled with a balance. As recommended in standard EN 16105, the render was applied on an inert material (1.0 x 0.6 m; extruded polystyrene panels, XPS) and dried at room temperature for 7 days. Finally, the XPS-plate was cut into 50 specimens, each 100 cm^2 sized (12.5 x 8.0 cm). The render formulation was prepared and applied on the substrate by a manufacturer of renders (Figure 1).

Figure 1: Preparation of XPS plate coated with the render.

3.2 Immersion Test and Chemical Analysis

The specimens were immersed with the coated surface top down in deionised water on 9 days over a period of 18 days (Table 2, Figure 2). A so-called "immersion day" consists of two immersion cycles of 1 hour each with a drying phase of 4 hours in between. The water was removed after every cycle and substituted by new deionised water. The two water samples per immersion day were pooled to a single composite sample for chemical analysis. During the immersion, the specimens were shaken moderately on a horizontal vibrating table by 30 rpm to ensure homogenous conditions. Glass boxes sealed with plastic tops were used to restrict water losses by evaporation (Figure 2). Between the immersion days specimens were stored at room temperature in the dark. The immersion test procedure was conducted according to EN 16105, except the moderate shaking of the immersed samples. Shaking enables more robust and reproducible conditions for diffusion controlled release of biocides.

Table 2: Overview of the testing procedure in accordance to EN 16105. Orange: immersion days on which water samples are taken for chemical analysis; white: days on which specimens are stored under dry conditions; blue: first and ninth water samples taken for biological tests.

	Total Time (days)																
	1	2	3	4	5	6 7	8	9	10	11	12	13	14	15	16	17	18
Water Samples for chemical Analysis	1	-	2	-	3	___	4	-	5	-	6	-	7	-	8	-	9
Water Samples for Bioassay Tests	1	-	-	-	-	___	-	-	-	-	-	-	-	-	-	-	9

Initially, 250 ml water (25 L/m^2) were used for each 1-hour immersion cycle to produce a sample of 500 ml per immersion day (50 L/m^2 per day). This amount is typical for standard EN 16105. Aquatic toxicity was tested with the first and ninth immersion samples of all three variants; soil toxicity was tested with the first sample only. A preliminary "range finder" test was performed – using a broad range of sample dilutions and reduced replications – to determine the appropriate range for dilutions to be used in the main tests. Due to lacking effects in ecotoxicity tests to terrestrial organisms, more concentrated samples were produced for the soil test by reducing the amount of water per immersion cycle by a factor of 5 to 50 ml (5 L/m^2 per cycle and 10 L/m^2 per day). Briefly summarized, immersion time over nine immersion days was 18 h and cumulated water volumes were 4500 ml (450 L/m^2) for the first series and 900 ml (90 L/m^2) for the soil test.

The immersion samples of the three render variants are abbreviated as follows:

- Without biocides, immersion day 1: CTRL1
  Without biocides, immersion day 9: CTRL9
- Free biocides, immersion day 1: FREE1
  Free biocides, immersion day 9: FREE9
- Encapsulated biocides, immersion day 1: CAPS1
  Encapsulated biocides, immersion day 9: CAPS9

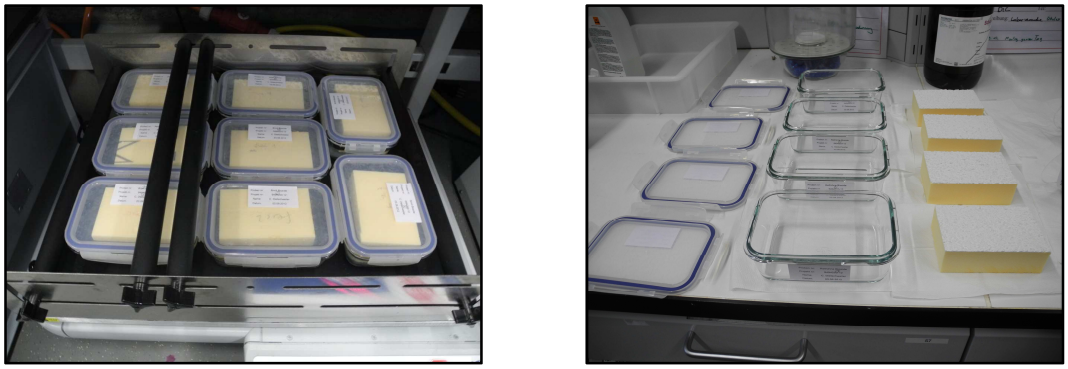

Figure 2: Immersion of specimens. Left: 1 h immersion in glass boxes, right: 4 h drying in the room.

Chemical analysis of Terbutryn and its degradation product M1 (N-tert-butyl-6-(methylsulfanyl)-1,3,5-triazine-2,4-diamine), OIT and DCOIT was performed by high pressure liquid chromatography coupled with UV-detection and mass spectrometry[3] (LC-MS) for all water samples of variant 1, 2, and 3 (nine samples each). Before analysis the samples were filtered (0.45 µm). Limits of detection and limits of quantification are listed in Table 3.

Additionally, total organic carbon (TOC) as an indicator for all leachable organic components, pH-value, and electrical conductivity were measured in every sample.

Table 3: Limits of detection and quantification of the biocides and the metabolite M1.

Substance	Limit of Detection (µg/L)	Limit of Quantification (µg/L)
Terbutryn	0.2	0.8
M1	0.1	0.4
OIT	0.1	0.6
DCOIT	1.6	5.2

4 Material and Methods – Ecotoxicity

The following five ecotoxicology tests were considered for aquatic and soil organisms:

- Combined Algae Test with *Pseudokirchneriella subcapitata* (aquatic)
- Bacterial Bioluminescence Inhibition Test with *Aliivibrio fischeri* (aquatic)
- Chronic Reproduction Test with *Ceriodaphnia dubia* (aquatic)
- Earthworm Avoidance Test with *Eisenia fetida* (soil)
- Collembolan Reproduction Test with *Folsomia fimetaria* (soil)

[3] Analysis performed by Federal Institute for Materials Research and Testing (BAM), Berlin, with the analytical method of by Schoknecht *et al.* 2009.

The algae serve as a substitute for primary producers, bacteria represent the decomposers and daphnia represent the primary consumers; earthworms and collembola are detritivores. These organisms are frequently used for ecotoxicity testing of environmental samples and for regulatory purposes. Furthermore, tests with these organisms can be conducted following internationally recognised standards (i.e. ISO or OECD).

In order to obtain full dose response curves in the bioassays, significant dilution of the samples was required. Dilution was expressed as "dilution factors" (DF; Equation 1). A certain volume of sample (V_{sample}) was diluted with a certain volume of culture medium ($V_{dilution\ medium}$). In this way an undiluted sample is represented by DF 1 and a DF of 2 indicates a 1:2 dilution. A 2-fold dilution results in a DF series of 2, 4, 8, 16, 32 and so on.

$$\mathrm{DF} = \frac{V_{sample} + V_{dilution\ medium}}{V_{sample}} \qquad \text{Equation 1}$$

The concept of DF for different species is also integrated in the German guidance document used for construction materials (DIBt, 2011).

4.1 Aquatic Bioassays

4.1.1 Combined Algae Test with *Pseudokirchneriella subcapitata*

The combined algae test (Escher *et al.*, 2008c) covers two endpoints:

- The inhibition of photosynthetic yield
- The inhibition of algal growth rate

Photosynthesis activity is determined by measuring the quantum yield and the growth rate was assessed by absorbance measurements. The species used in this test is the unicellular freshwater green alga *Pseudokirchneriella subcapitata*, a standard test organism for water quality evaluation (ISO, 2004). The assay is performed in 96-well microtiter plates and Diuron, a potent inhibitor of photosystem II (Schreiber *et al.* 2002), is used as a reference compound. Samples and the reference are typically prepared in ethanol, transferred to microtiter plates and diluted with ethanol in 2-fold dilution series. Solvents are then left to evaporate after which the samples and reference are redissolved in the assay medium. Subsequently, algae culture is added to the dissolved sample to start the assay (t=0).

In the current project, the above procedure was followed for the Diuron reference to produce a dilution series of 3×10^{-7} M to 2.3×10^{-9} M. In order to test the water samples at maximal concentrations, however, samples were first mixed 1:1 with double concentrated culture medium. From this mix 150 µl were added to the first row of the plate and a further 150 µl were added to the second row. From the second to the eighth row a 2-fold dilution series was produced using assay medium. Finally, 150 µl of algae culture was added to each well to start the assay (t=0). Samples from immersion days 1 and 9 were tested the day after they were produced.

The photosynthetic yield was measured using a maxi-Imaging PAM (pulse amplitude modulation, IPAM; Walz, Effeltrich, Germany) after 2 and 24 h (Escher *et al.*, 2008c; Schreiber *et al.*, 2007). The growth of the algae was measured by means of absorbance at 685 nm in a microtiter plate photometer (Synergy 4, Biotek, Winooski, United States) at 0, 2, ca. 20 and 24 h. Finally, the inhibition of both photosynthetic yield (Y) and the algal growth rate (μ) were calculated using Equation 2.

$$\text{Inhibition}\,[100\%] = \left(1 - \frac{Y_{sample}}{Y_{control}}\right) \cdot 100\% \quad \text{resp.} \quad \left(1 - \frac{\mu_{sample}}{\mu_{control}}\right) \cdot 100\% \qquad \text{Equation 2}$$

Dose response curves of both inhibition parameters (Y and μ) were fitted using the software GraphPad Prism 5 (GraphPad Prism version 5.02 for Windows, GraphPad Software, La Jolla California USA) and Equation 3. This fit provided EC_{50} and EC_{10} values: the concentrations causing 50 and 10% of the maximum effect.

$$\text{Inhibition}\,[\%] = \frac{100\%}{1 + 10^{\left(log(EC50) - log(concentration)\right) \cdot slope}} \qquad \text{Equation 3}$$

Statistical differences were assessed using ANOVA followed by Dunnett's multiple comparison test (GraphPad Prism 5).

4.1.2 Bioluminescence Inhibition Test with *Aliivibrio fischeri*

The bioluminescence inhibition test is performed with the gram-negative marine bacterium *Aliivibrio fischeri*. Luminescence in this bacterium is coupled directly to the metabolic condition of the cell. Substances that interfere with the cellular energy metabolism cause a decrease in light emission which is indicative of general toxicity. To evaluate this non-specific toxicity, the level of bioluminescence is measured before bacteria are exposed to a sample and after 30 min of exposure to a sample (Escher *et al.*, 2008a; see also ISO 2007).

We performed assays on 96-well plates with 3,5-Dichlorophenol as a positive control and assay buffer as a negative control (Escher *et al.*, 2008a). 3,5-Dichlorophenol was tested in triplicate in a seven-step 2-fold dilution series starting at 3 x 10^{-4} M in the first well; eight replicates were used for negative controls. Immersion samples were tested in triplicate and in 2-fold dilution series. The lowest dilution factor that could be tested was 2.2. This high sample load was possible by mixing nine parts of an aqueous sample with one part of 10 times concentrated assay buffer. Of this mix, 120 µL were added to a 96-well plate and a 2-fold dilution series was made using one time concentrated assay buffer. Finally, 100 µL were transferred from all wells to 100 µL of a bacteria solution. As for the combined algal test, samples were tested in the bioluminescence inhibition test the day after immersion had been performed.

To calculate the inhibition of bioluminescence, plates containing 100 µL of the bacteria culture per well were measured in a luminescence plate reader shortly before bacteria were exposed to the samples and 30 min after 100 µL of the samples had been added. Bioluminescence values of samples ($I_{samples}$) and controls ($I_{controls}$) were entered in Equation 4.

$$\text{Inhibition}\,[\%] = \left(1 - \frac{I_{sample}}{I_{control\,(corrected)}}\right) \cdot 100\% \qquad \text{Equation 4}$$

Bioluminescence inhibition data were then fitted with Equation 3 to determine EC_{50} and EC_{10} values.

Statistical differences were assessed using ANOVA followed by Dunnett's multiple comparison test (GraphPad Prism 5).

4.1.3 Chronic Reproduction Test with *Ceriodaphnia dubia*

In this test, daphnids are exposed to dilution series of a reference and samples. The effects on mortality and reproduction are assessed over 7 to 8 days to assess chronic toxicity. For this project, tests were performed by the private laboratory "Soluval Santiago" (2108 Couvet, Switzerland) according to draft ISO/CD 20665 from 2005 (see ISO, 2008) and AFNOR T90-376 (AFNOR, 2000).

Tests were carried out with a slightly modified version of the standards: the dilution medium corresponded to a moderately hard water prepared by mixing 25% of Evian mineral water, 25% of Elendt M4 medium (Elendt and Bias, 1990) and 50% of deionised water, supplemented with selenium and vitamin B_{12}. Food consisted of a mixture of yeast, digested fish flake suspension (TetraMin®) and green algae (*P. subcapitata* and *Chlorella sp.*).

Neonates that were less than 24 h old, and within 8 h of the same age, were exposed for up to 8 days to different dilutions of the façade samples in a static-renewal system (12 replicates per concentration). Control water (i.e. dilution medium) was tested using 20 replicates. All tests were carried out at 25 ± 1℃ in a temperature controlled chamber; illumination ranged from 300 to 500 lux, with a light-dark period of 16:8 h. Water was renewed every day, except for day 1. On day 1 and each following day at the time of water renewal, survival of mothers was determined and offspring were counted. Physicochemical characteristics of the sample solutions (pH, dissolved oxygen [mg/L] and conductivity [µS/cm]) were measured during the test in regular intervals (n=5-6).

Results of the controls fulfilled the validity criteria: on the seventh day, mortality of mothers ≤ 20%; proportion of males ≤ 20%; at least 60% of mothers alive have produced a minimum of three broods, and the average number of offspring born per live mother ≥ 15. The inhibition of population growth was calculated using Equation 5.

$$\text{Inhibition}\,[\%] = \left(1 - \frac{offspring_{sample}}{offspring_{control}}\right) \cdot 100\% \qquad \text{Equation 5}$$

EC_{50}- and EC_{10}-values of the inhibition of population growth were determined by fitting the inhibition data with Equation 3.

Statistical differences were assessed using ANOVA followed by Dunnett's multiple comparison test (GraphPad Prism 5).

4.2 Terrestrial Bioassays

4.2.1 Earthworm Avoidance Test with *Eisenia fetida*

In this test, earthworms are placed in a container that contains two soil compartments. One compartment holds control substrate and the other compartment substrate that is dosed with a reference chemical or a sample. After 48 h, worms in both compartments are counted. The ratio of the number of worms is indicative of the avoidance behavior towards the treated substrate. Tests were performed according to an ISO standard (ISO 17512-1; ISO, 2008).

Worms were obtained from Lombrico (wurmhandel.de) and held in the lab for 12 days prior to the experiment. A standard loamy sand soil was obtained from Lufa Speyer and used for all tests (Lufa 2.2 batch n° sp2.2-3012 [4], with a maximum water holding capacity (WHC) of 41.8%: pH 5.5, organic carbon 1.9% C, cation exchange capacity 10 meq/100 g). Nanopure water was added to the soil sample to reach 60% of the maximum WHC. As the soil was already moist to start with (i.e. at 24% of the maximum WHC), this meant that 134 ml of fluid had to be added per kg of soil[5].

Central dividers were placed in polystyrene plastic food containers (110 by 155 mm, 65 mm high) and one half was filled with 450 g of soil wetted with nanopure water (control soil). For negative controls (n=8), the other half of a container was also filled with 450 g of control soil. For positive controls (n=5), the other half of a container was filled with soil spiked with boric acid (708 mg of boric acid per kg dry weight; corresponding to the EC_{75}) before it was wetted with nanopure water to 60% WHC[5]. Samples were tested by pairing 450 g of control soil with soil wetted to 60% WHC with either full strength or diluted sample. Samples from the three treatments (i.e. CTRL, CAPS and FREE) from the immersion day 1 were tested with a DF of 1, 3.2 and 10; each treatment was tested with five replicates. When all containers were prepared – within 2 d following the generation of immersion samples – dividers were removed from the containers and 10 worms (adults, between 300-600 mg), were placed exactly on the line that divided the two compartments. Subsequently, the containers were closed with a fine mesh and a perforated lid and placed in a climate room with a light-dark cycle of 16:8 h, a light intensity of 400-800 lux and a temperature of 20 ± 2°C. After 48 h the divider was reinserted, the number of worms in each compartment ($n_{control}$ or $n_{treatment}$) was established and the avoidance response of worms was calculated using Equation 6 (ISO, 2008; Garcia *et al.*, 2008).

[4] http://www.lufa-speyer.de/images/stories/bodanalyse.pdf

[5] Bulk soil (60 kg) was mixed by hand in a 300 L tub, three ca. 10 g samples were taken, weighed and dried for 3 d at 105 ℃ to determine the water content. Information on the water content was used to calculate the amount of sample that could be added to reach 60% WHC (i.e. 134 ml/kg). The bulk soil was mixed again and ca. 2 kg aliquots were weighed out and stored in polystyrene plastic food containers, ca. 30 kg was weighed out to cover the controls in the experiment. The following day, soil aliquots were transferred to a steel bowl and samples (or dilutions of a 50 mg/ml boric acid stock solution) were added and mixed with the soil using a steel spoon; the ca. 30 kg of control soil was mixed by hand in a plastic tub. Three ca.10 g wetted soil samples were taken from each treatment to determine the water content and homogeneity of the mixing process, a further three samples (ca. 5 ml) were taken to determine the pH. At the end of the experiment (after 48 h) soil humidity and pH were assessed again in all treatments.

$$\text{avoidance } (\%) = \frac{n_{control} - n_{treatment}}{10} \times 100 \qquad \text{Equation 6}$$

Statistical differences were assessed using ANOVA followed by Dunnett's multiple comparison test (GraphPad Prism 5).

4.2.2 Collembolan Reproduction Test with *Folsomia fimetaria*

In the collembolan reproduction test, groups of 10 females and 10 males are placed on both control and treated soils. The mortality and reproductive output is recorded after 21 days. Tests were performed according to an OECD standard (OECD Test Guideline No. 232; (OECD, 2008)).

The springtails used for the test were originally provided by Dr Paul Henning Krogh from the Terrestrial Ecology Group, Aarhus University, Silkeborg, Denmark. These collembola have been successfully bred at the Ecotox Centre since 2009 according to OECD Test Guideline No. 232. Plastic test vessels were filled with 30 g of soil (Lufa 2.2) that had been wetted to 50% WHC (97 ml test solution per kg of soil) with either control (nanopure water; n=8) or sample (full strength or diluted samples from the first immersion day; n=5). Boric acid in soil (100 mg of boric acid per kg dry weight), wetted to 50% WHC with nanopure water (n=5), was used as a positive control[6]. Samples were tested with a DF of 1, 3.2 and 10. Ten female and 10 male *F. fimetaria*, 23-26 days old and from a synchronous culture, were introduced per test vessel. Subsequently, the vessels were covered with a black plastic lid and left for 21 d in a climate chamber (light-dark cycle 16:8 h, 400 to 800 lux; 20 ± 2℃). The test started within 2 d following the generation of the immersion samples. At the end of the test, all collembola were extracted from the soil using a controlled temperature gradient extraction technique (MacFadyen 1962) and counted to assess mortality and reproduction. Statistical differences were assessed using ANOVA followed by Dunnett's multiple comparison test.

[6] Samples and positive controls were mixed with soil as described for the earthworm avoidance test with the following changes: soil aliquots for collembola were only ca. 190 g, instead of ca. 2 kg; water content was not measured after sample and soil were mixed together, instead, vessels with 30 g soil and collembola were weighed on a weekly basis and any weight loss was topped up with nanopure water to maintain 50% WHC.

5 Results and Discussion

5.1 Immersion Test – Range Finder

Initially, samples were tested in all bioassays to determine the dilution range for the main tests. There was concern that concentrations in the CAPS9 sample would be too low to see effects in the aquatic bioassays and that more concentrated CTRL samples would be needed. For the aquatic bioassays, this test provided sufficient information to determine dilution factors that would produce appropriate results in the main study, but the terrestrial assays did not show effects in response to the range finder samples.

For this reason, a more concentrated sample was considered to be advantageous for terrestrial tests. To achieve this goal, the volume of water used for the render immersion in the main study was reduced to 50 ml compared to 250 ml in the range finder test. Using 5-fold less water, the concentration in the FREE1 sample rose by about factor 3 for Terbutryn and DCOIT and factor 4 for OIT. The measured concentrations and cumulated emissions are presented in Appendix A.

The concentration of M1 was too low compared to the parent substance Terbutryn. Therefore, further data analysis of M1 is not indicated.

None of the three biocides were detected in the control samples.

5.2 Immersion Test – Main Study

Results of the leaching pattern for free biocides are shown in Figure 3. The concentration patterns of Terbutryn and OIT followed an exponential decrease typical for coatings. Maximal concentrations in the first immersion sample of 7950 µg/L OIT, 1640 µg/L Terbutryn, and 120 µg/L DCOIT decreased to about 30% of the maximum in the last sample for Terbutryn and to about 15% for OIT. Therefore, the concentrations of each biocide and the ratios between them changed significantly. The concentration of DCOIT rose slightly over the first three immersion days followed by a sharp break down at the fourth immersion day. This is the case for substances with retarded diffusion.

The cumulative emissions compared with the initial amounts were as follows: 12.0% OIT, 3.4% Terbutryn, and 0.3% DCOIT (Table 4 and Appendix A). Comparison of the leaching of Terbutryn and OIT with their water solubility and logPow supports the existing ranking. Nevertheless, DCOIT was less mobile than expected, but behaved as published by Schoknecht *et al.* (2009) and Wangler *et al.* (2012).

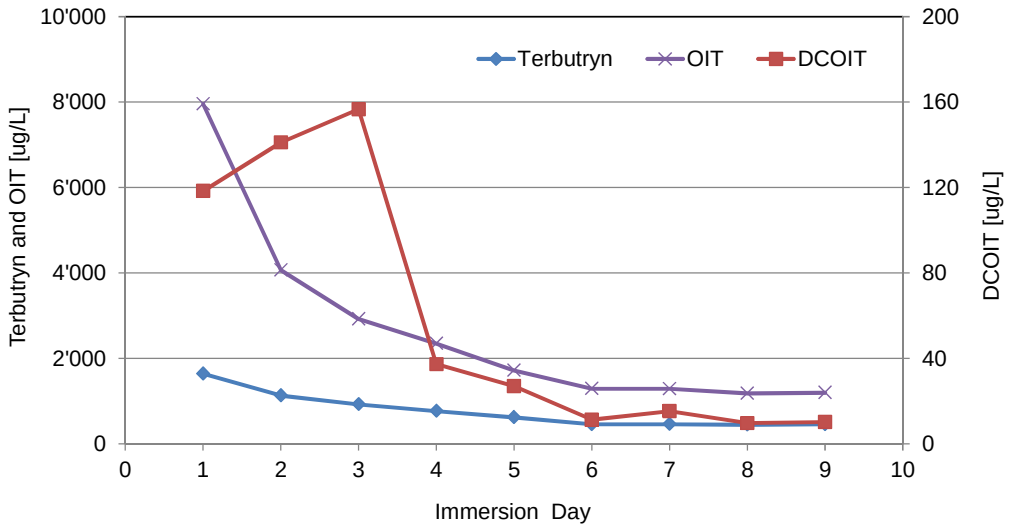

Figure 3: Leaching of biocides used in a free form in the render (FREE). Left axis: Concentrations of Terbutryn and OIT; Right axis: concentration of DCOIT.

The leaching patterns from render with encapsulated biocides were different to those with free biocide and the concentrations were significantly lower. Comparing the first immersion sample of free and encapsulated biocides, the initial concentrations of encapsulated form were for Terbutryn 4 times, for OIT 20 times, and DCOIT 30 times lower (Table 5). DCOIT varied in the range of the detection limit. Surprisingly, leaching of encapsulated Terbutryn and OIT behaved very similarly although their logPow and water solubility are very different (Figure 3). This indicates that on the one hand the rapid degradation of OIT might reduce the analysed amount in the immersion sample compared to Terbutryn. On the other hand, the capsule might play a role, e.g. in polymerisation, size, and thickness of the spheres. Consequently, cumulated losses resulted in 0.9% Terbutryn, 0.7% OIT and roughly 0.01% DCOIT.

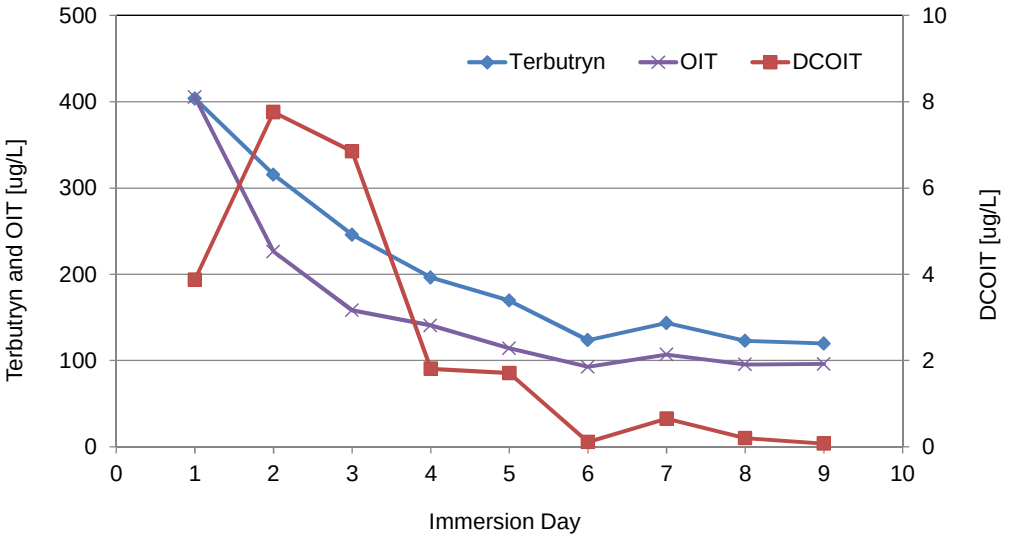

Figure 4: Leaching of biocides used in an encapsulated form in the render (CAPS). Left axis: concentrations of Terbutryn and OIT. Right axis: concentration of DCOIT.

To get further insight into the dissipation of OIT and efficiency of the microencapsulation technology, the concentration of Terbutryn per immersion day was normalized to the concentration of OIT. For the free application, the ratio between free Terbutryn and OIT varied from 0.2 to 0.4 whereas the ratio for the encapsulated application varied from 1.0 to 1.5. This indicates that leaching of encapsulated OIT is significantly reduced compared to the leaching of encapsulated Terbutryn (Figure 5). The reduction of OIT-leaching with concentrations even lower than Terbutryn is surprising and can only be explained by the known faster degradation compared to the stable Terbutryn.

Table 4: Cumulative emission (mg/m^2) and relative emission (%) of the biocides over the period of nine immersion days. The relative emission is based on the absolute emission normalized to the initial amount.

	Free biocides (FREE)		Encapsulated biocides (CAPS)	
	(mg/m^2)	(%)	(mg/m^2)	(%)
Terbutryn	69.0	3.4	18.4	0.9
OIT	239.7	12.0	14.4	0.7
DCOIT	5.3	0.3	0.2	0.01

The TOC, electrical conductivity and measured pH are listed in Appendix B. TOC and the conductivity showed a very similar pattern irrespective of the three variants (free, encapsulated biocides and references). The pH scattered between 7.5 and 8 in the first sample followed by a small drop below pH 7.5 for the last three immersion days.

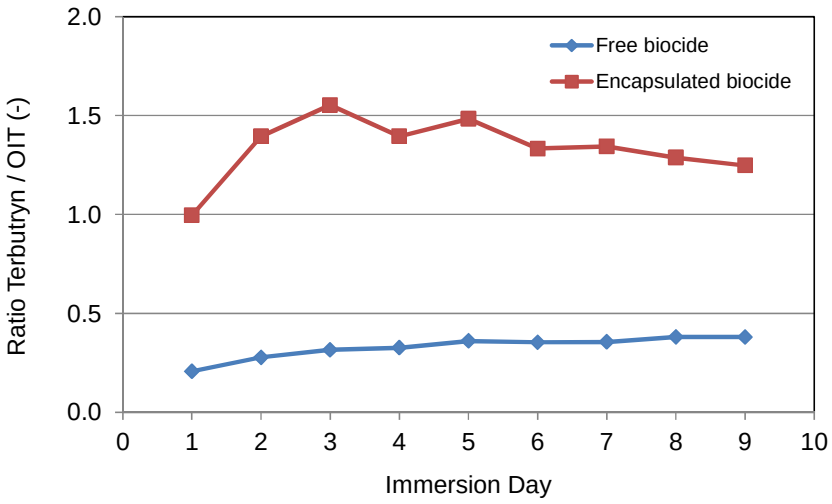

Figure 5: Ratio between concentration of Terbutryn and OIT for free and encapsulated application.

5.3 Aquatic Biotests

The measured concentrations of the biocides in the samples from immersion day 1 and 9 are listed in Table 5.

Table 5: Measured concentrations (µg/L) of the three biocides Terbutryn, OIT, and DCOIT and a metabolite (M1), in the original immersion samples from days 1 and 9. At given DF these concentrations were used to derive the calculated concentrations.

	Free biocides		Encapsulated biocides		Control	
	FREE1 (µg/L)	FREE9 (µg/L)	CAPS1 (µg/L)	CAPS9 (µg/L)	CTRL1 (µg/L)	CTRL9 (µg/L)
Terbutryn	1640	460	400	120	<5	<5
M1	15	3	2	<1	<1	<1
OIT	7950	1200	410	100	<3	<3
DCOIT	120	10	4	<1	<1	<1

M1 is a metabolite of Terbutryn and belongs to the same chemical class of compounds as Terbutryn (s-Triazines); the mode of action of M1 can be expected to be similar to Terbutryn. However, M1 is less potent than Terbutryn itself (ca. 10-fold (Okamura *et al.*, 2000)). In addition to a reduced potency, M1 concentrations were 100-fold lower than those of Terbutryn. For these reasons M1 was not further considered.

5.3.1 Combined Algae Test - Inhibition of PSII Quantum Yield

Dose-response curves of the Diuron reference and the samples from immersion day 1 and 9 are shown in Figure 6. Samples from the free biocide and the encapsulated biocide coatings induced up to 100% inhibition of PSII quantum yield at the lowest DF 4.

Water samples from the render that contain biocides displayed a near parallel dilution profile to the Diuron reference and could be fitted adequately with Equation 3. The EC_{50} and EC_{10}, derived from the algal assay using Equation 3, are listed in Table 6.

There was some indication that also the control sample affected PSII. Inhibition of PSII was significant up to a DF of 32 for immersion day 1 as well as at DF 32 and 63 for immersion day 9. Effect levels of negative control samples were very low, however, reaching a maximum value of 3.1% inhibition of PSII at a DF of 4 (immersion day 1).

The dose-response curves of immersion day 1 and 9 were very similar, showing that tests on both days were reproducible (Figure 6).

Photosynthetic yield was also assessed after 24 h. These 24 h data were almost identical to the 2 h data and were not considered further.

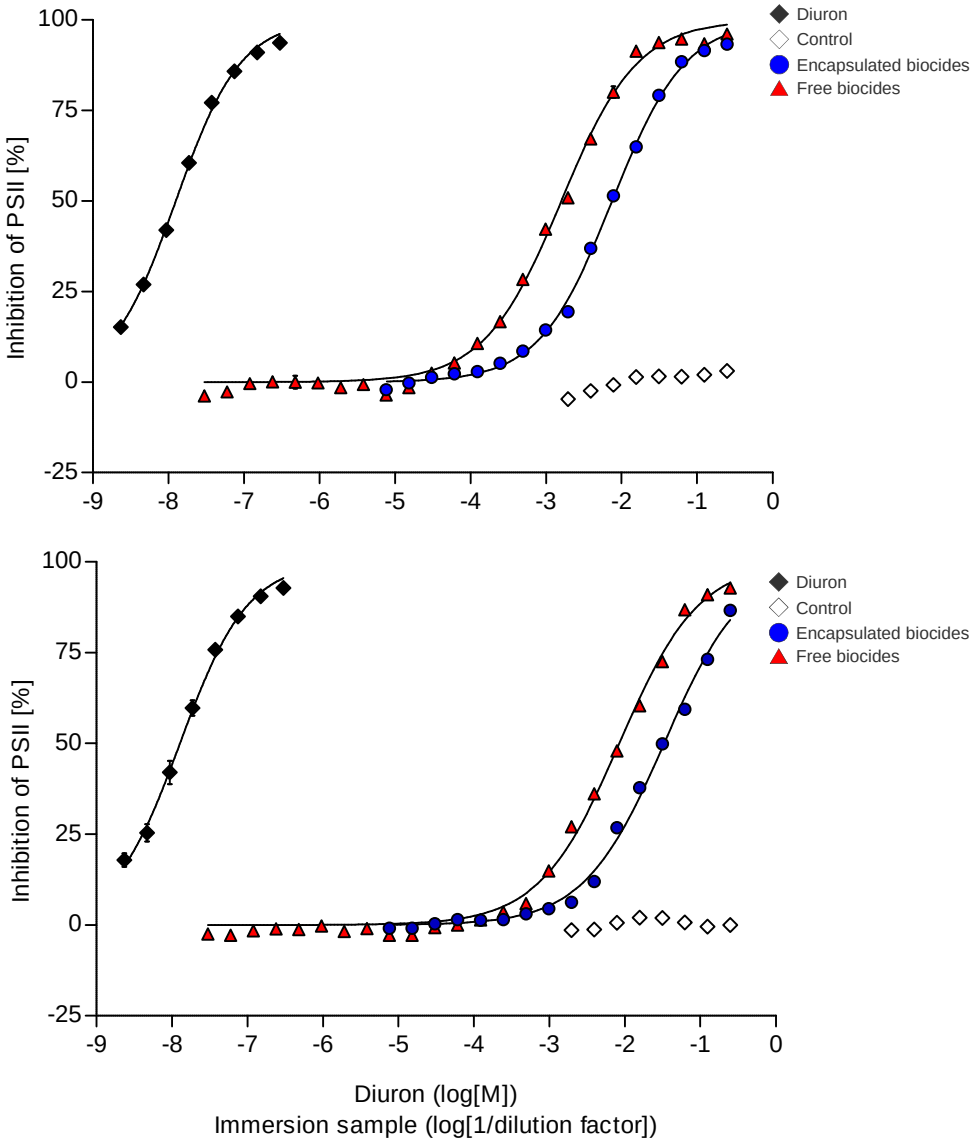

Figure 6: Inhibition of photosystem II (PSII) in algae in response to different Diuron concentrations (filled diamonds) and different sample dilution factors DF. Three kinds of samples were generated in the immersion test: control, open diamonds; encapsulated biocides, circles; free biocides, triangles. Samples from immersion day 1 are shown in the top panel, samples from immersion day 9 in the lower panel. Lines are fits of Equation 3 to the data (mean ± standard deviation), using a fixed effect range (0 to 100%) and a freely fitted slope.

According to the study of Vermeirssen *et al.* (2009), the EC_{50} of Terbutryn and for the 2 h endpoint in the combined algal test equaled $1.7 \cdot 10^{-8}$ M (Vermeirssen *et al.*, 2009). Taking into account the molecular weight of Terbutryn (241.4 g/M), this value corresponds to a concentration of 4.1 µg/L which is very close to the EC_{50} concentrations of Terbutryn of 3.0 µg/L at day 1 and 3.9 µg/L at day 9 (Table 6).

Table 6: Effect concentrations (EC_{50} and EC_{10}) of samples in the combined algal test and for the 2 h photosystem II inhibition endpoint expressed as a sample dilution factor (DF) and the corresponding concentrations of the three biocides Terbutryn, OIT, and DCOIT (µg/L) at given DF derived from the measured concentration in the original immersion sample by taking into account the DF.

		Immersion Day 1			Immersion Day 9		
		CTRL	CAPS	FREE	CTRL	CAPS	FREE
EC_{50}	**DF**	**-**	**130**	**630**	**-**	**30**	**120**
Terbutryn	(µg/L)	-	3.0	2.6	-	3.9	3.8
OIT	(µg/L)	-	3.1	13	-	3.2	10
DCOIT	(µg/L)	-	0.029	0.19	-	<LOQ	0.086
EC_{10}	**DF**	**-**	**1500**	**8200**	**-**	**440**	**1700**
Terbutryn	(µg/L)	-	0.26	0.20	-	0.27	0.27
OIT	(µg/L)	-	0.26	0.97	-	0.22	0.70
DCOIT	(µg/L)	-	0.0025	0.014	-	<LOQ	0.0060

Diuron Equivalents

EC_{50} values from Table 4 can be used to calculate a Diuron equivalent (DEQ) concentration for a sample by dividing the EC_{50} of Diuron by that of the sample (Equation 7).

$$\mathrm{DEQ} = \frac{\mathrm{EC_{50}(Diuron)}}{\mathrm{EC_{50}(sample)}} \qquad \text{Equation 7}$$

The DEQ for the free biocides sample from immersion day 1 was $7.9 \cdot 10^{-6}$ M. This DEQ can be transformed into an estimated Terbutryn concentration by multiplying the DEQ with the relative potency of a Terbutryn standard compared to the Diuron reference (0.76; i.e. Terbutryn and Diuron are almost equipotent; Vermeirssen *et al.*, 2009). Thus, the Terbutryn concentration in the sample is estimated to be $6.0 \cdot 10^{-6}$ M, which can also be expressed as 1.4 mg/L. This concentration is very close to the Terbutryn concentration determined by chemical analysis and calculated to be present at the DF of the EC_{50} (1.6 mg/L). DEQ values of the samples, as well as Terbutryn concentrations estimated in the samples using the above approach, are listed in Table 7. The relative good agreement between estimated and measured Terbutryn concentrations further supports the hypothesis that most of the observed effects on PSII are explained by Terbutryn.

Table 7: Diuron equivalent (DEQ) concentrations (M) and measured and estimated Terbutryn concentrations (mg/L) in samples from the immersion test.

	Immersion Day 1		Immersion Day 9	
	CAPS	FREE	CAPS	FREE
DEQ (M)	$1.7 \cdot 10^{-6}$	$7.9 \cdot 10^{-6}$	$2.9 \cdot 10^{-7}$	$1.2 \cdot 10^{-6}$
Estimated Terbutryn (mg/L)	0.30	1.4	0.07	0.28
Measured Terbutryn (mg/L)	0.40	1.6	0.12	0.46

5.3.2 Combined Algae Test - Inhibition of Algal Growth Rate

For sample from immersion day 1, the free biocide sample induced 100% inhibition of algal growth at a DF of 4. The encapsulated biocide samples did not produce a full dose-response curve and reached a plateau at a maximum effect of around 70% for the three lowest DFs tested (16, 8, and 4). On immersion day 9, toxicity was generally reduced, as can be seen in Figure 7 by a shift of the curves to the right when comparing the upper and lower panels. The highest effect induced by the render control coating CTRL (14.6%, DF 16) occurred for the sample from immersion day 9.

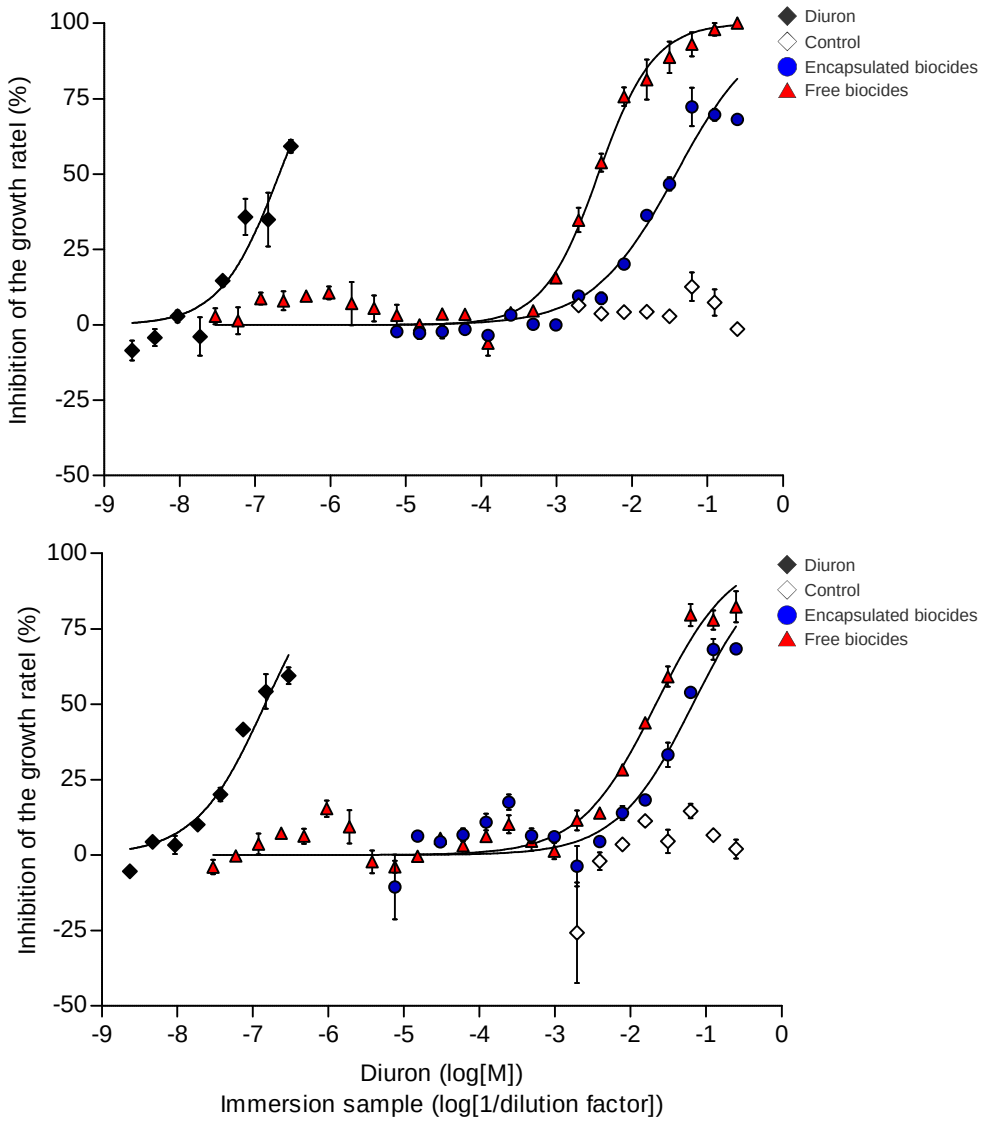

Figure 7: Effects of different Diuron concentrations (filled diamonds) and different sample dilution factors on the inhibition of the algal growth rate. Three kinds of samples were tested: control, open diamonds; encapsulated biocides, circles; free biocides, triangles. Samples from immersion day 1 are shown in the upper panel, samples from immersion day 9 in the lower panel. Lines are fits of Equation 3 to the data (mean ± standard deviation), using a fixed effect range (0 to 100%) and a freely fitted slope.

For this kind of assay, it is common for the data to scatter (Escher *et al.*, 2008b). Nonetheless, satisfactory dose response curves were obtained for the Diuron reference and the free biocide and encapsulated biocide samples. Although some dilutions of the control samples significantly affected the growth rate, it is difficult to evaluate these effects. Effects were not increasing with increasing sample concentration and no dose response curves could be fitted (i.e. immersion day 1: DF 8 and 16 reduced the growth rate; immersion day 9: at DF 512 the growth rate was enhanced and the standard deviation was large). EC_{50} and EC_{10} values derived from Equation 3 are listed in Table 8.

The endpoint "inhibition of algal growth rate" can respond to non-specific toxicants, as for the bioluminescence endpoint in *Aliivibrio fischeri* (Escher *et al.*, 2008b). However, in the presence of specifically acting toxicants, such as the PSII inhibitor Terbutryn, the specific effects mask the non-specific effects (Vermeirssen *et al.*, 2010). OIT and DCOIT do not specifically affect PSII at this concentration and thus do not contribute to the effect.

Table 8: Effect concentrations (EC_{50} and EC_{10}) of samples in the combined algal test and for the 24 h growth rate inhibition endpoint expressed as a sample dilution factor (DF) and the corresponding concentrations of the three biocides Terbutryn, OIT, and DCOIT (µg/L) at given DF derived from the measured concentration in the original immersion sample by taking into account the DF.

		Immersion Day 1			Immersion Day 9		
		CTRL	CAPS	FREE	CTRL	CAPS	FREE
EC_{50}	**DF**	**-**	**26**	**280**	**-**	**15**	**46**
Terbutryn	(µg/L)	-	15	5.9	-	8.0	9.9
OIT	(µg/L)	-	15	28	-	6.4	26
DCOIT	(µg/L)	-	0.15	0.42	-	-	0.22
EC_{10}	**DF**	**-**	**420**	**1800**	**-**	**190**	**590**
Terbutryn	(µg/L)	-	0.95	0.92	-	0.63	0.78
OIT	(µg/L)	-	0.96	4.5	-	0.50	2.0
DCOIT	(µg/L)	-	0.009	0.067	-	-	0.017

5.3.3 Bioluminescence Inhibition Assay with *Aliivibrio fischeri*

A 100% inhibitory effect was observed for the free biocide samples, whereas the encapsulated biocide samples were about 10-fold less toxic (i.e. one log unit shifted to the right, Figure 8). The control sample induced a maximal effect of 46% at a DF of 2.2. Samples from immersion day 9 were much less toxic. Incomplete dose-response curves were observed for the encapsulated biocides and control samples. The dose-response curve of the free biocide samples was shifted to the right when comparing the upper and lower panels in Figure 9. The EC_{50} and EC_{10} values are listed in Table 9.

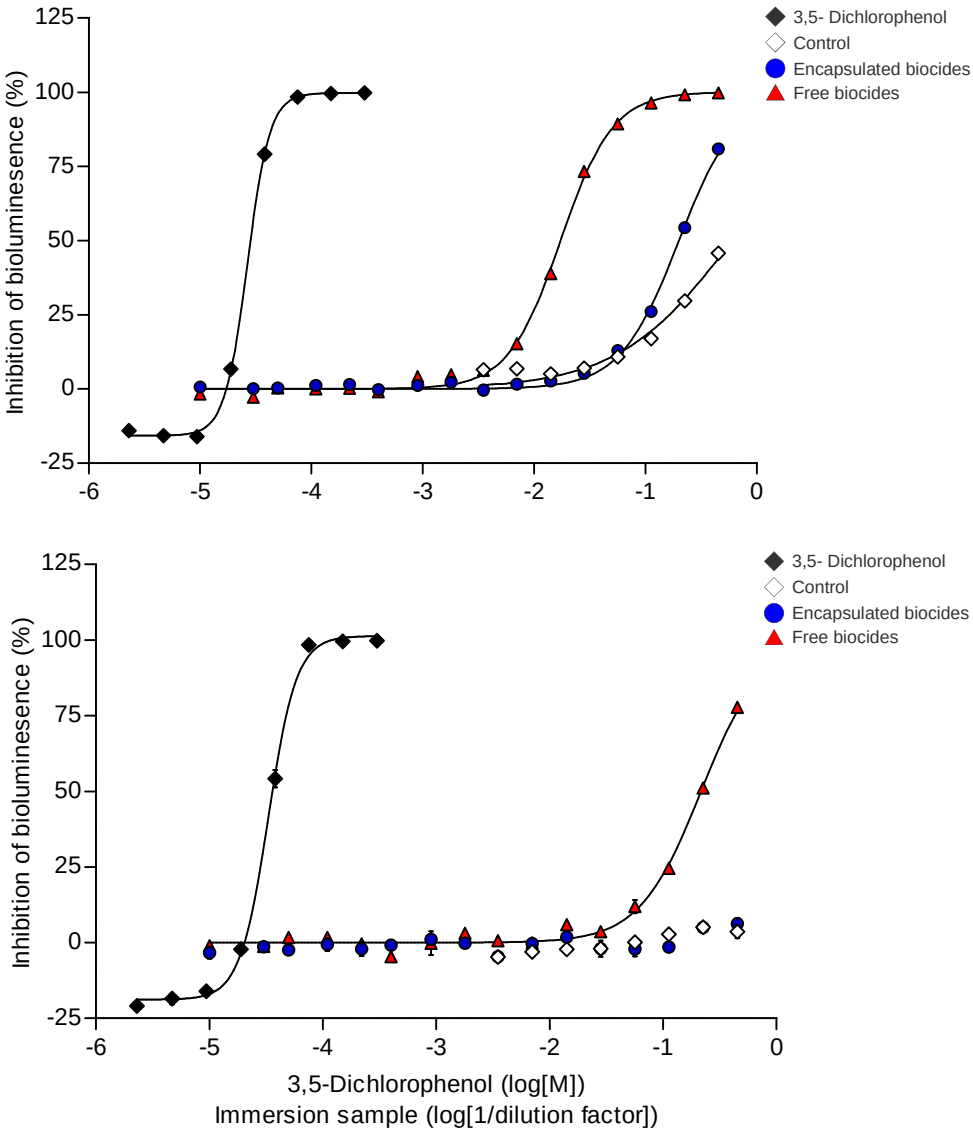

Figure 8: Effects of different sample dilution factors on bacterial bioluminescence; 3,5-Dichlorophenol serves as a positive control. Three kinds of samples were tested: control, open diamonds; encapsulated biocides, circles; free biocides, triangles. Samples from immersion day 1 are shown in the upper panel, samples from immersion day 9 in the lower panel. Lines are fits of Equation 3 to the data (mean ± standard deviation), using a fixed effect range (0 to 100%*) and a freely fitted slope. * At low concentrations, 3,5-Dichlorophenol always enhances bioluminescence. A reason for the effect is not known. Thus, the minimum effect is not constrained to 0% when fitting 3,5-Dichlorophenol to Equation 3.

When comparing the EC_{50} from the first and ninth immersion day samples, the toxicity of the free biocide samples reduced by 12.5-fold. For the encapsulated biocides sample, an EC_{50} could be established for the first immersion day and no effect was determined for immersion day 9. The response to the encapsulated biocide samples from day 9 was not different from the control, i.e. no toxicity was observed after 9 immersions. This means that Terbutryn was not toxic against *Aliivibrio fischeri* on day 9.

Table 9: Effect concentrations (EC_{50} and EC_{10}) of samples in the bacterial bioluminescence inhibition assay expressed as a sample dilution factor (DF) and the corresponding concentrations of the three biocides Terbutryn, OIT, and DCOIT (µg/L) listed for the respective DF and derived from the measured concentration in the original immersion sample by taking into account the DF.

		Immersion Day 1			Immersion Day 9		
		CTRL	CAPS	FREE	CTRL	CAPS	FREE
EC_{50}	**DF**	**1.7**	**5.0**	**58**	**-**	**-**	**4.6**
Terbutryn	(µg/L)	-	81	28	-	-	98
OIT	(µg/L)	-	81	140		-	260
DCOIT	(µg/L)	-	0.77	2.0		-	2.2
EC_{10}	**DF**	**22**	**19**	**190**	**-**	**-**	**17**
Terbutryn	(µg/L)	-	21	8.7	-	-	26
OIT	(µg/L)	-	21	42	-	-	69
DCOIT	(µg/L)	-	0.20	0.63	-	-	0.59

Toxic Unit Evaluation

EC_{50} of *Aliivibrio fischeri* are available for Terbutryn (33.2 mg/L, geometric mean of Gaggi *et al.*, 1995 and Menge 2005), DCOIT (3 ± 0.3 µg/L, Hernando *et al.*, 2003), and OIT (230 µg/L, Menge 2005). Using these measured EC_{50} values their respective contribution to the observed toxicity can be evaluated and expressed as toxic units (TU) as shown in Equation 8.

$$TU_i = \frac{concentration\ of\ substance\ i\ at\ sample\ dilution\ that\ causes\ 50\%\ effect}{EC_{50}\text{of substance i from single substance toxicity testing}} \qquad \text{Equation 8}$$

In general, substances with higher TUs have a higher influence on the observed toxicity. For example, OIT has an EC_{50} of 230 µg/L, this means that a 50% inhibition of bioluminescence is expected to occur at this concentration (230 µg/L). In the FREE sample from day 9, the OIT concentration in the dilution that caused 50% effect was 260 µg/L (see Table 9). From this follows that for the FREE sample from day 9, the TU_{OIT} = 260 µg/L / 230 µg/L = 1.130 (see Table 10). The sum of such individual compound TUs (i.e. TU_{OIT}, TU_{DCOIT}, $TU_{Terbutryn}$) can be added together to produce a ΣTU which is an estimate of the mixture toxicity. If the ΣTU = 1, then the mixture is expected to produce a 50% effect in the bioassay. If ΣTU is lower than 1, a smaller (than 50%) effect is expected. If ΣTU is higher than 1 a larger (than 50%) effect is expected.

In all tested samples DCOIT and OIT have almost the same TUs, whereas Terbutryn has distinctly lower TUs (Table 10). The sum of the derived TUs of DCOIT and OIT explains the measured toxicity quite well. This is the case irrespective of the immersion day and sample type (encapsulated or free). Hence DCOIT and OIT dominate the observed toxicity under these test conditions.

Table 10: Toxic unit (TU) analysis at the experimentally determined EC_{50} values. The concentrations given in table 9 were divided by the literature EC_{50} values for the three substances. If the ΣTU at experimental EC_{50} equals 1, the concentrations of the three substances fully explain the observed toxicity. The substance with the highest TU has the highest influence on the mixture toxicity and is marked in bold.

	Immersion Day 1		Immersion Day 9	
	CAPS	FREE	CAPS	FREE
ΣTU at experimental EC_{50}	0.611	1.276	-	1.867
TU Terbutryn	0.0024	0.00084	-	0.0029
TU OIT	**0.352**	0.609	-	**1.130**
TU DCOIT	0.257	**0.667**	-	0.733

5.3.4 Reproduction Test with *Ceriodaphnia dubia*

Samples coming from the free biocide coating showed the highest toxicity. The sample from the first immersion day, tested at DF 6.7 and 15, lead to complete mortality of the mothers and consequently 100% inhibition of population growth (Figure 9). The same result was obtained with the sample from the ninth immersion day, tested at DF 2.5 and 4. The sample from the encapsulated biocides coating from the first immersion day was also toxic at DF between 2.5 and 6.7; interestingly only a partial inhibition of population growth (sub-maximal) was observed (dashed line). The mechanism producing such a result is not known.

The highest dose of the control sample (DF 1.7) from the first immersion day also caused a slight inhibition of population growth (-14.5%). As expected, the toxicity decreases going from the first to the ninth immersion day. This can be seen in Figure 9 (curves shift to the right) and in the table containing effect concentrations (Table 11). The response to the sample is very steep, i.e. there are few or no data between 0 and 100% effect. This makes it difficult to fit the data in a robust way (with Prism software). For this reason, the slope of the curve was fixed for all dose-response curves to 8.

In the daphnid trial conducted with samples from the first immersion day, the population growth of the controls varied which is typical for daphnid population growth in test systems. Thus, some sample dilutions of the control, encapsulated biocides and free biocides samples had a population growth that was significantly higher compared to control values (i.e. negative inhibition of population growth, upper panel of Figure 9). Results from the second daphnid test appear more robust in this respect, the variability is lower and the population growth of CTRL is in line with growth observed for low concentrations.

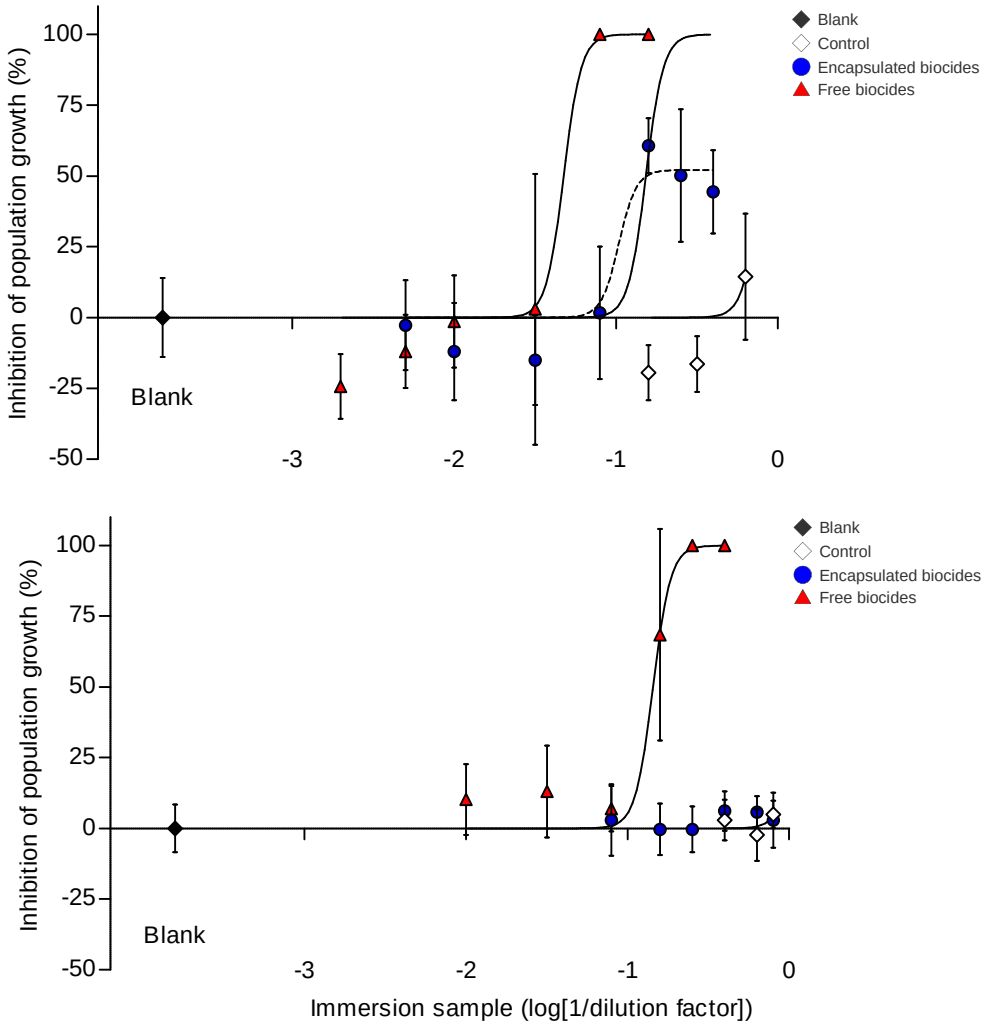

Figure 9: Inhibition of population growth of daphnids in relation to chronic exposure to façade immersion samples (upper panel, immersion day 1; lower panel, immersion day 9). Three kinds of samples were tested: control, open diamonds; encapsulated biocides, circles; free biocides, triangles. Data (mean ± standard deviation) were fitted with Equation 3 using a fixed slope of 8 and a 0 to 100% effect range (solid lines); data from the encapsulated sample from immersion day 1 were also fitted with an unconstrained maximal effect (dashed line).

Toxic Unit Evaluation

As for the bioluminescence assay, the contribution of the various biocides to the toxicity observed in daphnid reproduction tests can be estimated from published toxicity values. However, these values are only available for *Daphnia magna*, not for *Ceriodaphnia dubia*. Since this test is a chronic test, one has to compare the EC_{10} values with published NOEC (no observed effect concentration) or EC_{10} values. For Terbutryn a NOEC of 1300 µg/L (Le Blanc 1982a, cited in EU 2011a), for DCOIT a NOEC of 0.63 µg/L (EU 2011b) and for OIT a NOEC of 1.6 µg/L were retrieved in a literature search.

The TU evaluation shows a clear dominance of the two isothiazolinones. The TU of Terbutryn is in all cases more than 800 times lower than the sum of the TUs of DCOIT and OIT. The really low toxic units of Terbutryn indicate that the contribution of Terbutryn to the observed toxicity is almost negligible, as long as there is no significant transformation of the isothiazolinones over 7 day exposure period. However, this analysis has to be regarded with care, since it is based on toxicity data for different species (*Daphnia magna* instead of *Ceriodaphnia dubia*). Thus, it cannot be concluded with highest certainty that DCOIT or OIT contributed more to the toxicity.

Table 11: Effect concentrations (EC_{50} and EC_{10}) of samples in the daphnid reproduction tests expressed as a sample dilution factor (DF) and the corresponding concentrations of the three biocides Terbutryn, OIT, and DCOIT (µg/L) derived from the measured concentration in the original immersion sample by taking into account the DF.

		Immersion Day 1			Immersion Day 9		
		CTRL	CAPS	FREE	CTRL	CAPS	FREE
EC_{50}	**DF**	**-**	**6.5**	**21**	**-**	**-**	**7.0**
Terbutryn	(µg/L)		62	78			65
OIT	(µg/L)		62	380			170
DCOIT	(µg/L)		0.59	5.6			1.5
EC_{10}	**DF**	**1.7**	**8.6**	**28**	**-**	**-**	**9.2**
Terbutryn	(µg/L)	-	47	59			50
OIT	(µg/L)	-	47	290			130
DCOIT	(µg/L)	-	0.45	4.3			1.1

5.4 Terrestrial Bioassays

5.4.1 Earthworm Avoidance Test with *Eisenia fetida*

None of the immersion samples induced avoidance (or attraction) behavior in earthworms. Results from the positive control (boric acid) indicated that the worms responded to chemical stimuli (Figure 10). This means that there was no difference between the leachates and the control samples. This leads to the conclusion that the immersion samples were not toxic to earthworms at the tested sample doses.

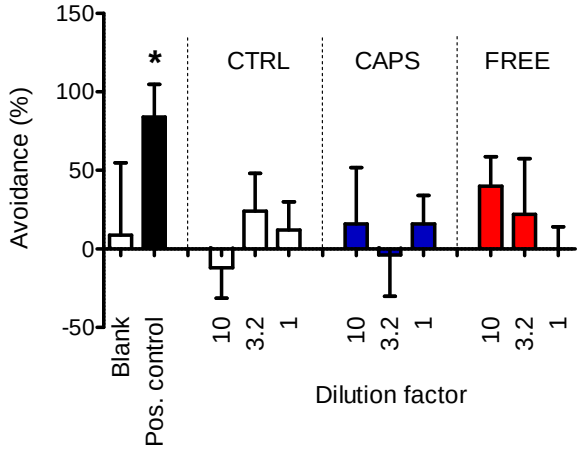

Figure 10: Avoidance response of earthworms (mean ± standard deviation) in relation to different dilutions of façade immersion samples (dilution factor, DF). Three kinds of samples were tested: control, CTRL; encapsulated biocides, CAPS; free biocides, FREE. Boric acid served as a positive control and nanopure water as blank (* significantly different from control; Student's t-test, $p<0.05$).

5.4.2 Collembolan Reproduction Test with *Folsomia fimetaria*

As for the earthworm assay, no toxicity was observed in the bioassay with collembola (Figure 11). Also in this case the positive control indicated that the test was working properly. This means that there was no difference between the leachates and the control samples. This leads to the conclusion that the immersion samples are not toxic to collembola at the tested sample doses.

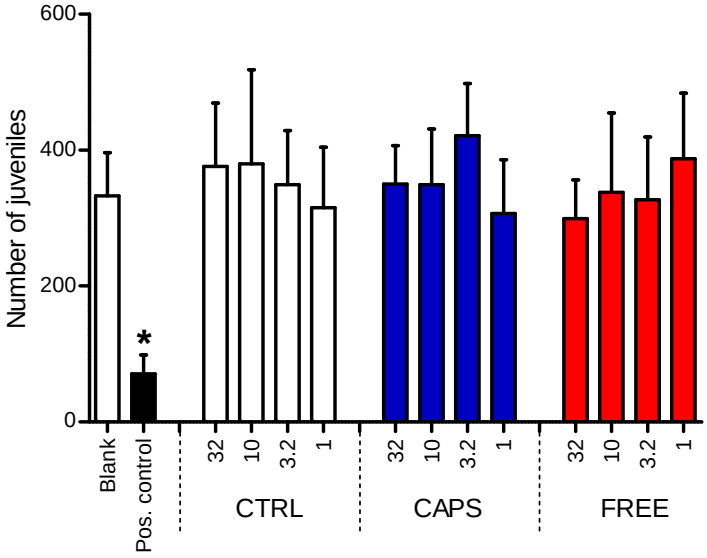

Figure 11: Collembola juvenile production (mean ± standard deviation) in relation to different dilutions of façade immersion samples (dilution factor, DF) of three kinds of façade immersion samples: control, CTRL; encapsulated biocides, CAPS; free biocides, FREE. Boric acid served as a positive control and nanopure water as blank (* significantly different from control; Student's t-test, $p<0.05$).

The loading of biocides into the soil was calculated using the data from chemical analyses and the amount of sample added to the soil (Table 12).

Table 12: Concentrations of biocides in soil (mg/kg) used in the earthworm and collembola bioassays. Concentrations were calculated for the highest sample dose (undiluted sample, DF 1) of the coating containing free biocides.

	Terbutryn (mg/kg)	OIT (mg/kg)	DCOIT (mg/kg)
Collembola	0.18	0.85	0.013
Earthworm	0.24	1.2	0.018

No literature data were found for the above three compounds in relation to the avoidance behavior of worms or reproduction in collembola. However, the acute LC_{50} of Terbutryn for earthworms at day 14 is 170 mg/kg[7]. Clearly, this value is far away from the concentration reached in the present experiment (Table 12) and would explain why no toxicity was observed, at least in the earthworm avoidance test and at the tested sample concentrations. Therefore, Terbutryn is not toxic in the analyzed concentrations.

[7] http://sitem.herts.ac.uk/aeru/iupac/Reports/624.htm

6 Conclusion and Outlook

Based on the introductory questions and elaborated insights, the following conclusions and outlook can be summarized:

- *How much does the ecotoxicity of immersion samples differ between render with free and encapsulated biocides as well as without biocides?*
  - Immersion samples from the render coating with free biocides always showed the highest toxicity compared to samples from façades with encapsulated biocides. The control façade samples only showed occasional and low levels of toxicity under enforced leaching conditions with 5-fold less water than proposed by the immersion standard EN 16105. The results thus indicate that additional constituents of the render formulation did not add to the toxicity of the individual biocides.
  - Toxicity decreased noticeably from the first to the ninth immersion day by up to a factor of 5 to 10 according the measured biocide concentrations.

- *How well does the theoretical ecotoxicity of individual biocides match the measured effect of the biocide mixture present in the sample?*
  - The Diuron equivalent concentrations DEQ of the 2 h endpoint in the PSII inhibition algae test can be explained very well by measured Terbutryn concentrations. Thus, under these test conditions Terbutryn is the compound responsible for inhibition of photosynthesis and not DCOIT or OIT. It is very likely that the inhibition of algal growth rate was also a consequence of Terbutryn and not DCOIT or OIT confirming already existing data that OIT and DCOIT do not affect PSII.
  - Inhibition of bioluminescence in bacteria was not caused by Terbutryn; toxic units of Terbutryn were 800-fold lower than those of DCOIT and OIT. OIT and DCOIT seemed to contribute equally to the toxicity for the FREE1 sample as their toxic units were identical and close to 1.
  - Toxicity on daphnid reproduction is likely dominated by isothiazolinones. It has to be considered though, that OIT and DCOIT degrade rapidly, limiting their presence in natural water and soil systems. As no measurements were made of the biocides during the aquatic or soil bioassays, we do not know how exposure developed over time in the various tests.
  - No effects in soil bioassays were observed. Although a concentrated sample was provided (immersion tests were performed with a reduced water volume), dosing of the sample to the soil is constrained by the WHC. No EC_{50} data are available for any of the compounds in relation to the endpoints that were tested using collembola or earthworms. However, such EC_{50}-values would be helpful to allow for a comparison between the achieved soil concentrations and the toxic potential of the tested compounds.

- *Which dilution is needed for no effects to aquatic and terrestrial test organisms?*
    - No effects were seen in terrestrial tests. Of the aquatic species tested, algae were most sensitive. The first immersion sample of the render with free biocides (FREE1) has to be diluted more than 630-fold to maintain the inhibition of photosynthesis below 50%. The sample from render with encapsulated biocides (CAPS1) has to be diluted 130-fold to reduce the effect on photo-synthesis below 50%. In the bacterial assay, the required dilutions to maintain inhibition of bioluminescence below 50% are 58-fold for FREE1 and 5-fold for CAPS1. In the daphnid test, FREE1 and CAPS1 require 21-fold and 6.5-fold dilutions respectively, to maintain inhibition of population growth below 50%.

Outlook

- Encapsulation (AMME™ products) reduces leaching of a biocide significantly compared to the use of the same substance in the same amount in the free form, even for substances with high mobility. The handling and application of encapsulated biocides is similar to free biocides. Therefore, free biocides should be substituted by encapsulated products as a source control measure to reduce leaching of biocides from façades and help prevent potential effects on aquatic and terrestrial organisms.

- It is expected that toxicity of runoff from real facades decreases over time with a similar trend as observed in the experimental set-up, according to the similar decrease of the leached concentration.

- An assessment of biological effects using DF EC_{50} is a suitable evaluation tool for e.g. biocides released from treated articles or substances from construction products. Combining the standard leaching test with standard bioassays is a useful approach to evaluate the toxic potential of leachates.

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8 Appendix A

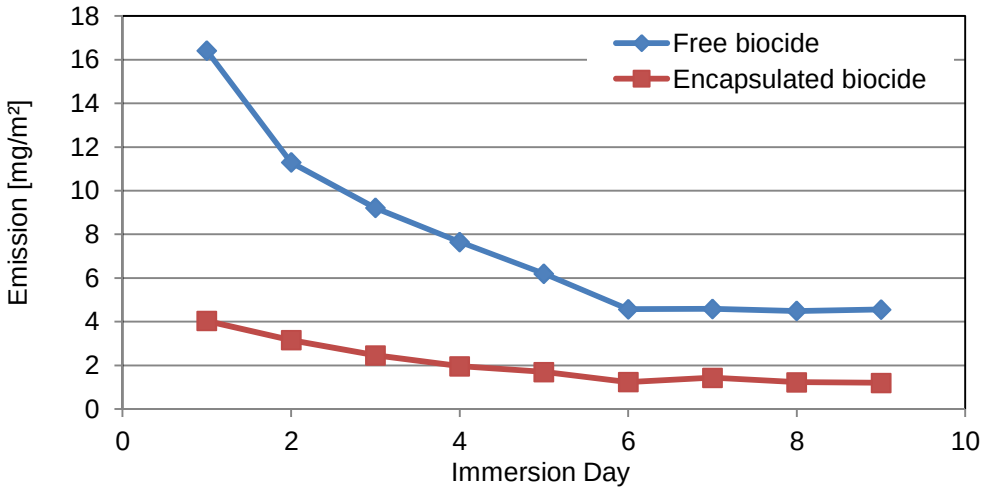

Figure 12: Emission of Terbutryn [mg/m^2].

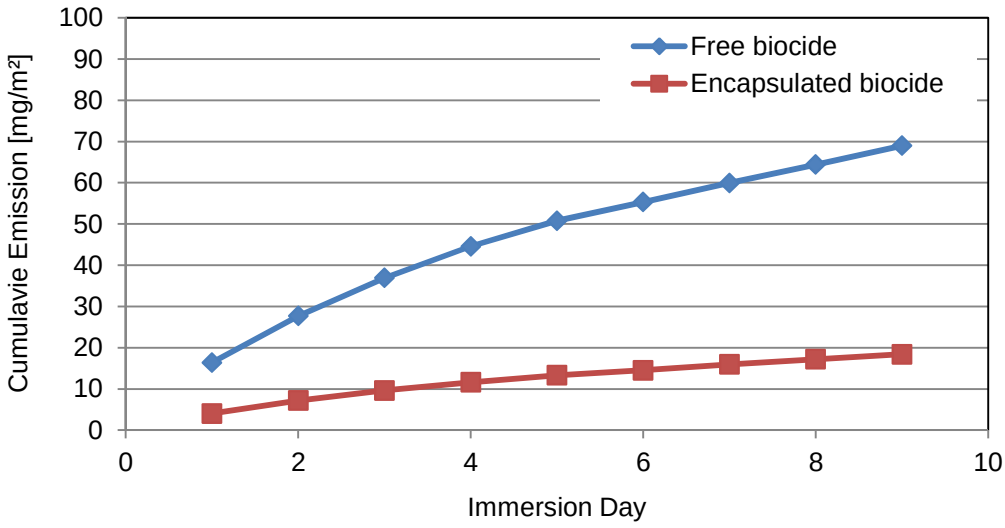

Figure 13: Cumulative losses of Terbutryn [mg/m^2].

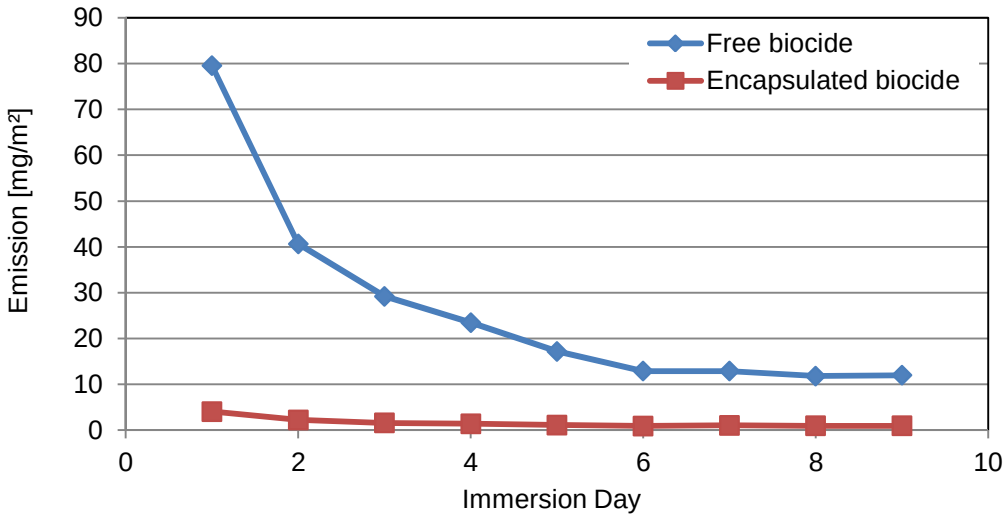

Figure 14: Emissions of OIT [mg/m^2].

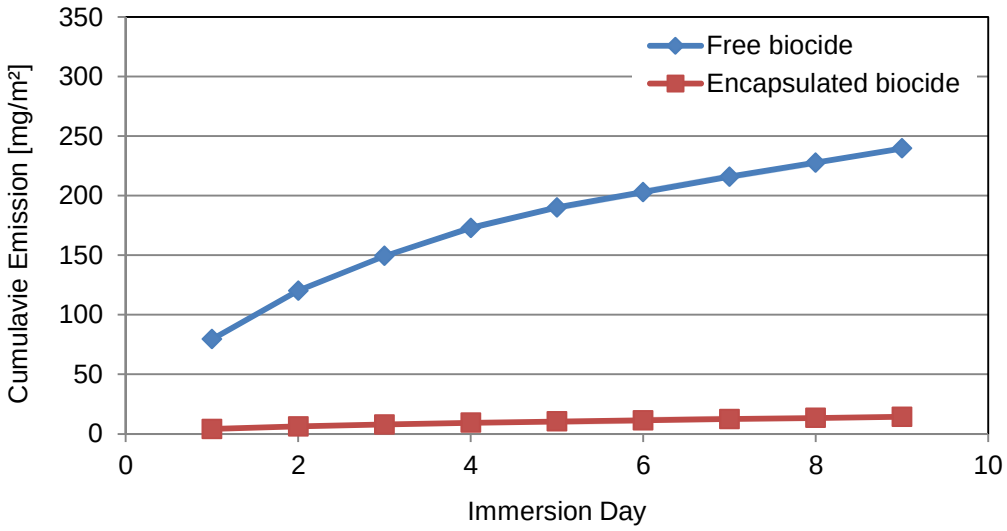

Figure 15: Cumulative losses of OIT [mg/m^2].

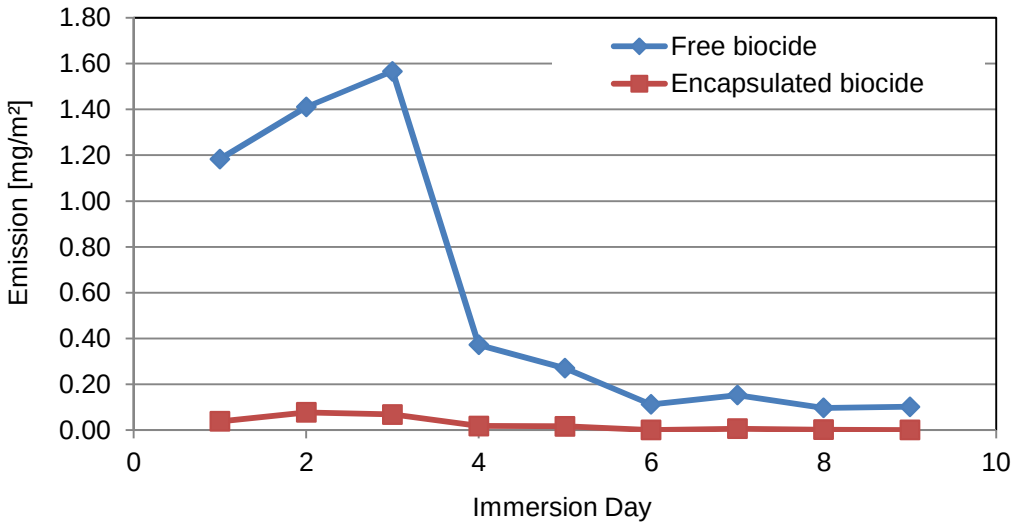

Figure 16: Emissions of DCOIT [mg/m^2].

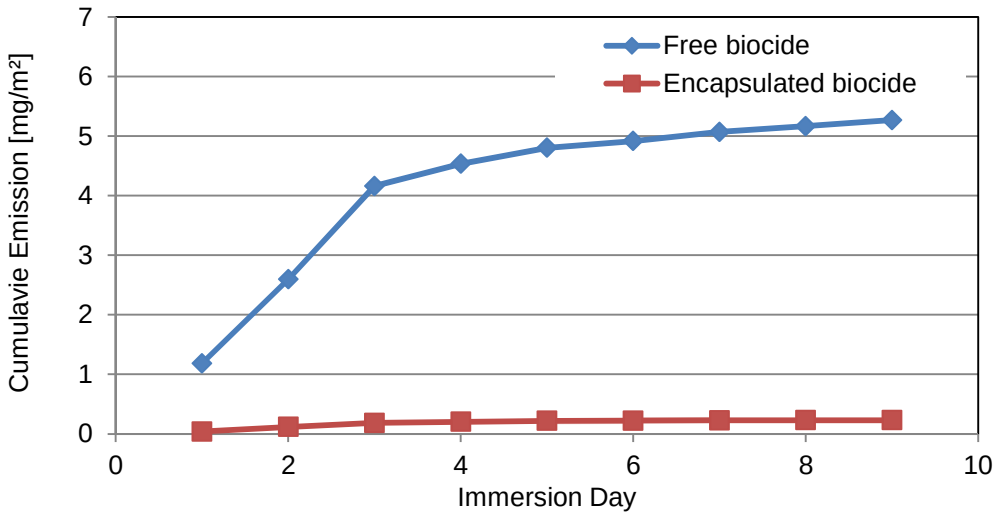

Figure 17: Cumulative losses of DCOIT [mg/m^2].

9 Appendix B

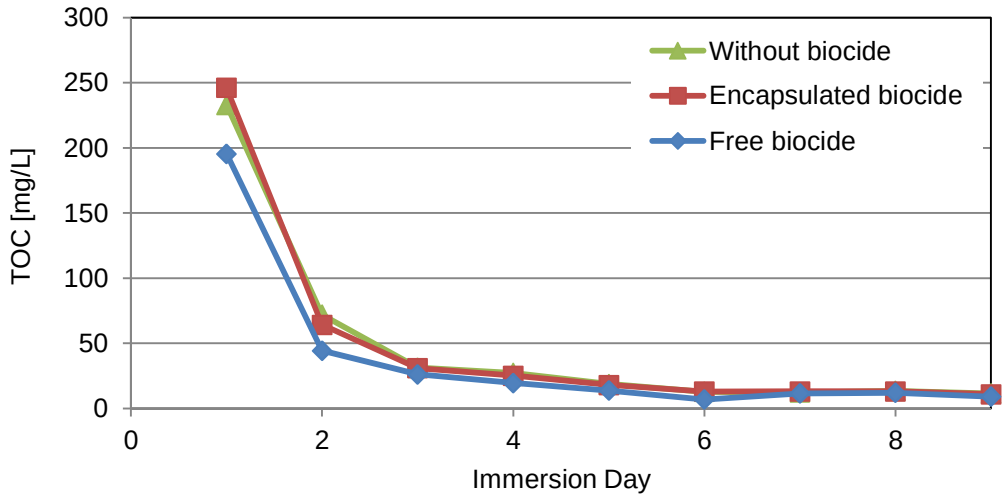

Figure 18: Total organic carbon (TOC) in the immersion samples.

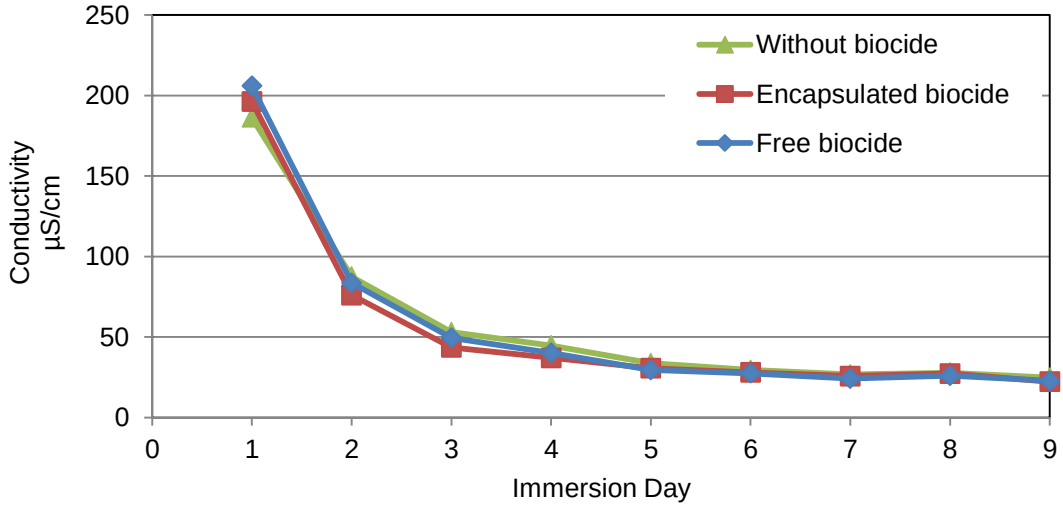

Figure 19: Electrical conductivity (µS/cm) in the immersion samples.

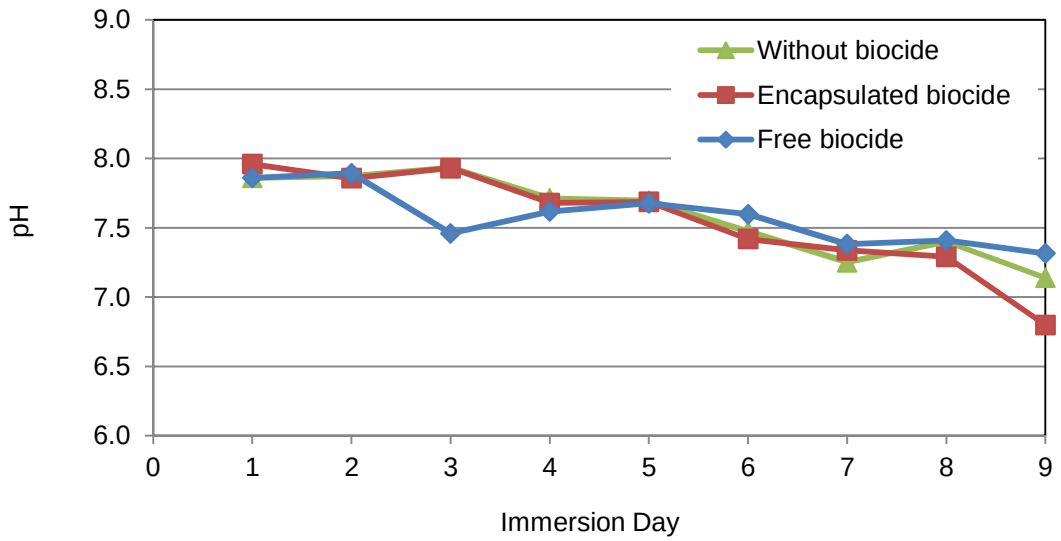

Figure 20: pH-values in the immersion samples.