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Hazard/Risk Assessment

Ecotoxicological Assessment of Immersion Samples from Facade Render Containing Free or Encapsulated Biocides

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Abstract: To protect house facades from fouling by microorganisms, biocides can be added to a render or paint before it is applied. During driving rain events, these biocides gradually leach out and have the potential to pollute soil or aquatic ecosystems. We studied the leaching behavior of biocides and toxicity of leachates from renders with either free or encapsulated biocides. Both render types contained equal amounts of terbutryn, 2-octyl-3(2H)-isothiazolinone (OIT), and 4,5-dichloro-2-n-octyl-4-isothiazolino-3-one (DCOIT). Nine leachate samples were generated over 9 immersion cycles according to a European standard, and biocides were quantified. The first and ninth leachate samples were tested using bioassays with algae, bacteria, and water fleas, the first sample was also tested with earthworms and springtails. Encapsulation reduced leaching of terbutryn, OIT, and DCOIT by 4-, 17-, and 27-fold. For aquatic organisms, the toxicity of water from render containing encapsulated biocides was always lower than that of render with free biocides. Furthermore, toxicity decreased by 4- to 5-fold over the 9 immersion cycles. Inhibition of photosynthesis was the most sensitive endpoint, followed by algal growth rate, bacterial bioluminescence, and water flea reproduction. Toxicity to algae was due to terbutryn and toxicity to bacteria was due to OIT. None of the samples affected soil organisms. Results demonstrate that combining standardized leaching tests with standardized bioassays is a promising approach to evaluate the ecotoxicity of biocides that leach from facade renders. *Environ Toxicol Chem* 2018;37:2246–2256. © 2018 SETAC

Keywords: Construction materials; Film preservatives; Leaching; Toxic effects

INTRODUCTION

Buildings and constructions that are exposed to environmental conditions are often protected against degradation by coatings like paints or renders. Fouling and microbial deterioration of such exterior coating products can be controlled by antimicrobial active substances (Paulus 2004; Sauer 2017). These biocides, also known as film preservatives, are added to water-based renders and paints that are sold 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, as dry-film preservation agents, exterior paints and renders may contain mere algaecides (e.g., terbutryn, diuron) as well as active ingredients with a primary fungicidal function (e.g., 2-octyl-3(2H)-isothiazolinone [OIT]; zinc pyrithione). Other biocides that are frequently used in products are 3-iodo-2-propynylbutylcarbamate, isoproturon, and 4,5-dichloro-2-n-octyl-4-isothiazolino-3-one (DCOIT). All these compounds are among the 25 substances currently authorized as film preservatives under European Union biocide regulations (European Chemicals Agency 2014a). In the present study, we focused on the 3 biocides with the greatest importance for exterior renders and paints: terbutryn, OIT, and DCOIT. These biocides are commonly applied in Swiss and German products (including facade renders) according to market research published in 2013 and 2015, respectively (Burkhardt and Dietschweiler 2013, 2015).

During wet conditions, such as rain events or directly following rain events, biocides migrate in the facade render and can leach out (Schoknecht et al. 2009; Burkhardt et al. 2011; Wangler et al. 2012; Bollmann et al. 2016). Leaching is particularly evident during the first rain events following product application (Burkhardt et al. 2012). In line with European legislation for the use of biocides in film preservatives (product type 7 in European Commission 1998), leaching tests have been suggested for the calculation of emission scenarios (van der Aa et al. 2004). Subsequently, international standards have been

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developed to support such evaluations and to determine the leaching behavior of biocides from film preservatives. For example, the technical specification CEN/TS 16637-2 of the European Committee for Standardization (2013) describes a laboratory method by which leaching from plate-like structures of construction products can be performed. Although recent progress has been made in understanding transport processes within different render matrices (e.g., Styszko et al. 2015) as well as material parameters and compound characteristics that control leaching (Bester et al. 2014), the picture is not complete.

Ultimately, when biocides wash out of a render, they enter the terrestrial and aquatic environmental compartments and have the potential to affect organisms in those compartments. To reduce risks to the environment—and at the same time extend the life-time of applied coatings—producers began to use encapsulated formulations of biocides in their products. When the biocides were encapsulated in polymers such as polyethyleneimines (Jämsä et al. 2013) or aminoplast resins (Breuer et al. 2012), it was shown that biocide leaching can be reduced significantly (Nordstierna et al. 2010; Breuer et al. 2012).

We investigated the leaching behavior of 3 biocides (terbutryn, OIT, and DCOIT) and studied the toxicity of leachates to the aquatic and terrestrial compartments using bioassays. Biocides were added to render in a free and encapsulated form provided by a producer of film preservatives (Thor GmbH, Speyer, Germany). Render immersion samples were generated using the European standard EN 16105:2011 intended for architectural coatings (European Committee for Standardization 2011). We compared the leaching of biocides between renders using chemical analyses and evaluated the toxicity of the leachates using a suite of 5 aquatic and terrestrial bioassays. The selection of bioassays was in accordance with a German scheme for testing leachates from construction materials (Deutsches Institut für Bautechnik 2011) as well as a recent technical report issued by the European Committee for Standardization (2017; see also Gartiser et al. 2017a). The main questions we aimed to address were: 1) How much does biocide leaching and toxicity of immersion samples differ between render with free and encapsulated biocides? 2) How well does the theoretical toxicity of individual biocides match the measured toxic effect of the biocide mixture present in the sample? and 3) Which sample dilutions are needed to avoid effects on aquatic and terrestrial test organisms?

MATERIALS AND METHODS

Preparation of facade renders on polystyrene panels

A base render with a frame formulation that is representative for market products was prepared according to Schoknecht et al. (2009) and split into 3 portions. One portion of the render without film-preserving biocides served as control. A second portion was spiked with 750 mg/kg of the biocidal active substance of each of the 3 biocides (terbutryn, OIT, and DCOIT) provided by Thor. The third render portion was spiked with 750 mg/kg of each of the 3 active ingredients in an encapsulated form, also provided by Thor (AMME^{TM} technology).

The 3 types of render were applied at 2.7 kg/m^2 onto extruded polystyrene panels (0.6 × 1.0 m; thickness, 30 mm; Supplemental Data, Figure S1) to produce a nominal biocide load of 2000 mg/m^2. Following 7 d of drying at room temperature, panels were cut into specimens of 100 cm^2 (125 × 80 mm) and subsequently used in the leaching experiment.

Generating facade render leachates using a European standard

Test procedures followed a European standard (EN 16105; European Committee for Standardization 2011) with the following exceptions: 1) moderate horizontal shaking (30 rpm) was performed during immersion, to ensure more homogeneous leaching conditions; and 2) less water was used per immersion cycle (5 L/m^2 instead of 25 ± 5 L/m^2), to ensure less dilution of leached biocides so full concentration–response curves could be expected. Compared with the standard leaching conditions, shaking will likely increase leaching because equilibrium is probably not reached during a 1-h immersion cycle using stagnant conditions.

The leaching procedure lasted for 18 d and included 9 immersion cycles spaced out as shown in Table 1. An immersion cycle consisted of 2 1-h immersions with a drying phase of 4 h in between (humidity during drying was ~50%). Individual specimens were immersed face down into deionized water (50 mL) in glass boxes that were then sealed with plastic tops (Supplemental Data, Figure S2). Following the first 1-h immersion, water was collected and boxes refilled with fresh deionized water. Both 50-mL leachate samples generated in 1 d were pooled as a single 100-mL composite sample. Furthermore, to have sufficient sample volume for the various bioassays, we pooled the 100-mL aliquots of 12 replicate specimens from the control and encapsulated biocide renders into a 1.2-L pool. For render with free biocides, 8 replicates were pooled into 0.8 L; fewer replicates were needed because the sample was more toxic. All chemical and biological analyses were done with these pooled immersion samples. Between immersion cycles, render specimens were stored at room temperature in the dark at a humidity of approximately 50% for at least 42 h (see Supplemental Data, Figure S3 for a detailed scheme of European standard EN 16105).

Chemical analysis

Total organic carbon (TOC; an indicator for all leachable organic components), pH, and electrical conductivity were measured in every composite sample (Table 1). Samples were filtered (0.45 μm) and analyzed using high-performance liquid chromatography coupled with ultraviolet light detection and liquid chromatography–mass spectrometry (LC–MS) by the Federal Institute for Materials Research and Testing (Berlin, Germany) for terbutryn (and its degradation product M1 [N-tert-butyl-6-(methylsulfanyl)-1,3,5-triazine-2,4-diamine]), OIT, and DCOIT (see Schoknecht et al. 2009 and their associated

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TABLE 1: Overview of the leaching procedure of facade renders that were immersed intermittently on 9 d over a period of 18 d[a]

	Leaching experiment (days)																	
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
Sample tested in chemical analysis	1		2		3			4		5		6		7		8		9
Sample tested in bioassays[b]	1																	9

[a] For procedural details, also see European Committee for Standardization (2013).
[b] Samples were tested within 1 d in aquatic assays and within 2 d in terrestrial assays.

supporting information; for limits of quantification, see Table 2). LC–MS was performed on an Agilent 1100 series system with the following spray chamber parameters: nitrogen flow 5 L/min; gas temperature 350 °C; vaporizer temperature 200 °C; nebulizer pressure 40 psig; capillary voltage 2500 V; charging voltage 2000 V; and corona current 1.0 μA. Samples were injected directly or after dilution, and compounds were separated using LC on a Phenomenex Luna C18/2 100 A column (3 μm, 50 × 2.0 mm) using 2 mobile phases (A, 0.2% acetic acid in ultrapure water; and B, methanol) and the following gradient: 0 min (40% B); 0.5 min (40% B); 1 min (75% B); 4 min (75% B); 5 min (40% B); 7 min (40% B) at a flow of 0.5 mL/min. The following ions with their retention times were used for quantification: terbutryn, m/z 242.2 at 3.9 min; OIT, m/z 214.1 at 4.1 min; and DCOIT, m/z 282.1 at 5.5 min.

Aquatic bioassays

Inhibition of photosynthetic yield and algal growth rates. The assay with *Raphidocelis subcapitata* was performed as described in Escher et al. (2008). Briefly, a 2-fold dilution series of diuron served as a reference compound and 8 replicates of assay buffer as a negative control. Samples were first mixed 1:1 with double-concentrated culture medium. From this mix, 150 μL was added to the first row of the 96-well plate (CELLSTAR® Greiner Bio-One, HuberLab, Aesch, Switzerland), and a further 150 μL was 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 ($t = 0$).

Quantum yield (Y) of photosystem II (PSII) was measured using a Maxi-Imaging PAM (pulse amplitude modulation; Walz, Effeltrich, Germany) device after 2 h. Growth rate (μ) of the algae was measured by means of absorbance at 685 nm in a microtiter plate photometer (Synergy 4, BioTek) at 0, 2, 20, and 24 h. Inhibition of photosynthetic yield and algal growth rate were calculated using Equations 1 and 2.

$$\text{Inhibition } (\%) = \left(1 - \frac{Y_{\text{sample}}}{Y_{\text{control}}}\right) \times 100\% \quad (1)$$

$$\text{Growth } (\%) = \left(1 - \frac{\mu_{\text{sample}}}{\mu_{\text{control}}}\right) \times 100\% \quad (2)$$

Inhibition of bioluminescence in bacteria. Assays with *Aliivibrio fischeri* were performed as described in Escher et al. (2008). Briefly, a 2-fold dilution series of 3,5-dichlorophenol served as a positive control and 8 replicates of assay buffer as a negative control. Nine parts of a sample were mixed with 1 part 10-fold concentrated assay medium. Of this mix, 120 μL was added to a 96-well plate, and a 2-fold dilution series was made (in triplicate) using 2-fold concentrated assay medium. Finally, 100 μL was transferred from all wells to 100 μL of a bacteria solution.

Plates containing 100 μL of the bacteria culture/well were measured in a luminescence plate reader (Synergy 4) shortly before bacteria were exposed to the samples and 30 min after 100 μL of the samples had been added. Bioluminescence intensities of samples (I_{samples}) and controls (I_{controls}) were entered in Equation 3 to calculate the inhibition of bioluminescence.

$$\text{Inhibition } (\%) = \left(1 - \frac{I_{\text{sample}}}{I_{\text{control (corrected)}}}\right) \times 100\% \quad (3)$$

Inhibition of reproduction of water fleas. *Ceriodaphnia dubia* assays were conducted according to ISO/CD 20665 (International Organization for Standardization 2005) and Association Française de Normalisation T90-376 (2000) and performed

TABLE 2: Concentrations (μg/L) of terbutryn, 2-octyl-3(2H)-isothiazolinone (OIT), 4,5-dichloro-2-n-octyl-4-isothiazolino-3-one (DCOIT), and the metabolite desethyl-terbutryn (M1) in the first and ninth immersion samples

	Free biocides		Encapsulated biocides		Control	
	First immersion	Ninth immersion	First immersion	Ninth immersion	First immersion	Ninth immersion
Terbutryn	1640	460	400	120	<0.8	<0.8
M1	15	3	2	<0.4	<0.4	<0.4
OIT	7950	1200	410	100	<0.6	<0.6
DCOIT	120	10	4	<1	<1	<1

< indicates values below the respective limit of quantification.

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by Soluval Santiago (Couvet, Switzerland). Dilution medium (control water) was prepared by mixing 25% of Evian mineral water, 25% of Elendt M4 medium (Elendt and Bias 1990), and 50% of deionized water, supplemented with selenium and vitamin B12. Food consisted of a mixture of yeast, digested fish flake suspension (TetraMin®), and green algae (*R. subcapitata* and *Chlorella* sp.). Water fleas were exposed to dilution series of samples, with a maximum sample concentration of 90%. Each dilution was tested with 12 replicates, and each replicate held 1 water flea in 10 mL of test solution. Twenty replicates were used for controls. Inhibition of reproduction (Equation 4) was assessed over 7 to 8 d.

$$\text{Inhibition } (\%) = \left(1 - \frac{offspring_{\text{sample}}}{offspring_{\text{control}}}\right) \times 100\% \qquad (4)$$

Terrestrial bioassays

Earthworm avoidance. The assay was performed according to International Organization for Standardization standard 17512-1 (2008). Worms (*Eisenia andrei*) were obtained from Lombrico (Brandenburg an der Havel, Germany; www.natursache.de) and held under controlled conditions (temperature of 20 ± 2 °C with a 16:8-h light:dark cycle and a light intensity of 400–800 lux) in the laboratory in their original substrate (manure) for 12 d. They were acclimated for 24 h in a field-fresh standard loamy sand soil prior to the experiment. The standard soil according to the German soil classification (LUFA 2.2) was obtained from LUFA (Speyer, Germany; batch no. sp2.2-3012 4) with the following characteristics: maximum water holding capacity of 41.8%; pH 5.5; organic carbon 1.9% C; cation exchange capacity 10 meq/100 g. For the bioassay, we adjusted soil to 60% water holding capacity with either nanopure water (control soil), leachate sample, or leachate sample and nanopure water (diluted sample). Taking into account the 10% initial moisture content of the LUFA soil, the total amount of fluid that could be added was 134 mL/kg of soil. The tested sample doses/kg of soil were: 134 mL (undiluted or dilution factor = 1), 42 mL (mixed with 92 mL nanopure water, dilution factor = 3.2), and 13.4 mL (mixed with 120.6 mL nanopure water, dilution factor = 10).

Central dividers were placed in polystyrene plastic food containers (110 × 155 mm, 65 mm high) to create 2 sections. One section was filled with 450 g wet weight control soil. For negative controls ($n = 8$), the other section of a container was also filled with 450 g wet weight of control soil. For positive controls ($n = 5$), the other section of a container was filled with soil spiked with boric acid corresponding to the 75% effect concentration (708 mg of boric acid/kg dry wt; adjusted to 60% water holding capacity with nanopure water).

Samples were tested by pairing 450 g wet weight of control soil with soil wetted with sample at a sample dilution factor of 1, 3.2, or 10 and with 5 replicates/dilution factor. When all containers were prepared, dividers were removed, and 10 worms (adults, with individual masses of 300–600 mg) were placed on the line that divided the control and sample compartments. Subsequently, containers were closed with a fine mesh and a perforated lid and placed in a climate room with a 16:8-h light:dark cycle, a light intensity of 400 to 800 lux, and a temperature of 20 ± 2 °C. After 48 h, the divider was reinserted, and the number of worms in each compartment (n_{control} or $n_{\text{treatment}}$) was counted. The avoidance response of worms was calculated using Equation 5 (International Organization for Standardization 2008; Garcia et al. 2008).

$$\text{Avoidance } (\%) = \frac{n_{\text{control}} - n_{\text{treatment}}}{10} \times 100\% \qquad (5)$$

Inhibition of springtail reproduction. The 21-d assay was performed according to an Organisation for Economic Co-operation and Development (OECD) test guideline (Organisation for Economic Co-operation and Development 2008). The LUFA 2.2 soil was adjusted to 50% water holding capacity with either nanopure water (control soil), leachate sample, or leachate sample and nanopure water (diluted sample). Taking into account the 10% initial moisture content of the LUFA soil, the total amount of fluid that could be added was 97 mL solution/kg of soil. The tested sample doses/kg of soil were: 97 mL (undiluted or dilution factor = 1), 30 mL (mixed with 67 mL nanopure water, dilution factor = 3.2), and 9.7 mL (mixed with 87.3 mL nanopure water, dilution factor = 10). Boric acid at the 50% effect concentration given in the OECD test guideline (Organisation for Economic Co-operation and Development 2008) was used as positive control (100 mg of boric acid/kg dry wt; adjusted to 50% water holding capacity with nanopure water).

Glass test vessels were filled with 30 g wet weight of soil, and 10 female and 10 male springtails (*Folsomia fimetaria*; 23–26 d old) from a synchronous in-house culture were introduced into each test vessel. Control soil was tested with 8 replicates. Samples and the boric acid–positive control were tested with 5 replicates. After the vessels were stocked, they were closed with a black plastic lid and placed into a climate chamber (16:8-h light:dark cycle, 400–800 lux; 20 ± 2 °C). After 21 d, springtails were extracted from the soil using a controlled temperature gradient extraction technique (MacFadyen 1962) and counted to assess mortality and reproduction.

Data analysis of aquatic and terrestrial bioassays

Dilution factors were calculated to express the volume of sample in the assay relative to the volume of dilution medium in the assay (Equation 6). For the aquatic bioassays, effect data (e.g., inhibition of PSII) were fitted together with concentration (e.g., diuron) or dilution factor data using the software GraphPad Prism 5 (Ver 5.02 for Windows, GraphPad Software, La Jolla California, USA) and Equation 7 to determine 50% effect concentrations (EC50s) or 50% effect dilution factors (DF50s). Differences in terrestrial bioassay endpoints were assessed using analysis of variance followed by Dunnett's multiple comparison test (GraphPad Prism 5).

$$\text{Dilution factor} = \frac{V_{\text{sample}} + V_{dilutionmedium}}{V_{\text{sample}}} \qquad (6)$$

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$$\text{Effect } (\%) = \frac{100\%}{1 + 10^{(\log(\text{EC50}) - \log(\text{concentration or dilution factor})) \times \text{slope}}} \quad (7)$$

$$\text{TU}_i = \frac{\text{concentration of substance } i \text{ at sample dilution that causes 50\% effect}}{\text{EC50 of substance } i \text{ from single substance toxicity testing}} \quad (8)$$

Using Equation 8, toxic units (TUs; van der Ohe and de Zwart 2013) were calculated for the 3 biocides (*i*) and effects on algae and bacteria. For *R. supcapitatus* and *A. fischeri*, we determined EC50 values for the 3 compounds (Supplemental Data, Table S1).

RESULTS AND DISCUSSION

Encapsulation reduces biocide leaching from renders

For renders with free and encapsulated biocides, a continuous reduction in terbutryn and OIT concentrations was observed over the 9 immersion cycles (Figure 1). Concentrations of DCOIT in the leachates, however, increased during the first few cycles before DCOIT release started to decline. In line with earlier observations (Nordstierna et al. 2010; Burkhardt and Vonbank 2011; Breuer et al. 2012), our data show that encapsulation of biocides reduces biocide emissions from renders. Concentrations of terbutryn, OIT, and DCOIT were 4-, 20-, and 30-fold lower in leachates from encapsulated biocide render (first immersion cycle) compared with the render containing free biocide (Figure 1 and Table 2). Over the entire experiment, encapsulation reduced leaching of terbutryn, OIT, and DCOIT by factors of 4, 17, and 27, respectively (Table 3 and Supplemental Data, Figure S4). Reduced emission through encapsulation has important consequences for possible risks caused by biocides to the aquatic and spoil compartments. For example, the European Union Water Framework Directive environmental quality standard for terbutryn is very low (65 ng/L for chronic effects and 340 ng/L for acute effects) and is much lower than the measured concentrations in the leachate samples (up to 1640 μg/L). The same is true for DCOIT, with measured concentrations up to 120 μg/L and a recently proposed environmental quality standard of 27 ng/L (Martins et al. 2018). Consequently, any reduction in leaching emissions will contribute markedly to reducing possible risks to the relevant environmental compartments. With respect to DCOIT and OIT, it has to be considered that they show excellent degradability (Sakkas et al. 2002; European Chemicals Agency 2014b; Bollmann et al. 2017), which also reduces possible environmental impacts.

TABLE 3: Cumulative emissions of biocides (mg/m^2) over 9 immersion cycles and from renders with free or encapsulated biocides[a]

	Free biocides		Encapsulated biocides	
Biocide	(mg/m^2)	(%)	(mg/m^2)	(%)
Terbutryn	69	3.4	18	0.9
OIT	240	12	14	0.7
DCOIT	5.3	0.3	0.2	0.01

[a]Relative emissions (%) indicate measured release relative to the nominally spiked amounts.
OIT = 2-octyl-3(2H)-isothiazolinone; DCOIT = 4,5-dichloro-2-n-octyl-4-isothiazolino-3-one.

Relative emissions of the 2 isothiazolinones were very different from each other. For the render with added free biocides, 12% of the OIT was released but only 0.3% of the DCOIT. A higher release of OIT relative to DCOIT has been previously reported (Schoknecht et al. 2009, 2013). There may

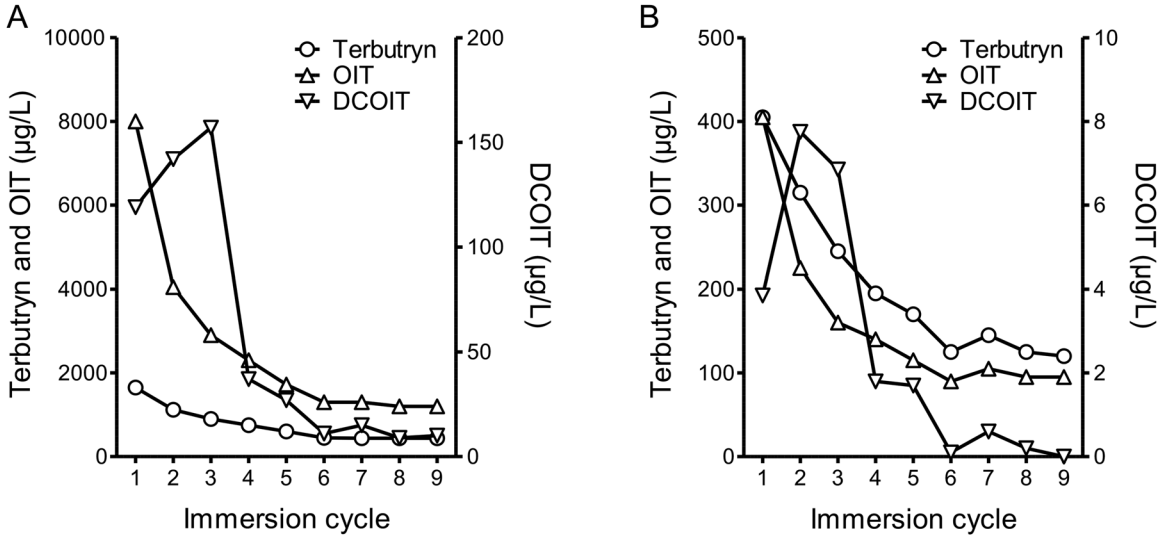

FIGURE 1: Concentrations of 3 biocides in immersion samples from renders with biocides in a free formulation (A) or an encapsulated formulation (B). Renders were immersed intermittently on 9 of 18 d (Table 1). OIT = 2-octyl-3(2H)-isothiazolinone; DCOIT = 4,5-dichloro-2-n-octyl-4-isothiazolino-3-one.

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be an association between release and the log octanol/water partition coefficent (DCOIT being more hydrophobic and less water soluble than OIT; Sauer 2017), but other factors, such as the pH in the porewater of the render, will also likely play a role with respect to substance mobility and release (Bester et al. 2014). In addition, for the render with encapsulated biocides, OIT emissions were much higher than DCOIT emissions (Table 3). Although we did not do a mass balance study (see, e.g., Wangler et al. 2012; Bollmann et al. 2017), it seems unlikely that rapid biocide degradation contributed to these differences. Although OIT can degrade with a half-life of 28 h under ultraviolet radiation (Bollmann et al. 2017), immersion samples were only exposed to light briefly (a few hours) before being frozen. Furthermore, even under natural solar irradiation, DCOIT had a half-life that is at maximum 18 d but is typically much shorter (Sakkas et al. 2002; European Chemicals Agency 2014b). This almost corresponds to the duration of the leaching experiment, most of which was conducted in the dark.

Conductivity, TOC, and pH of leachates were very similar across the 3 render formulations (Supplemental Data, Figure S5); any differences in biological effects between the control render and biocide-containing renders are thus considered to be due to the presence of free or encapsulated biocides. Conductivity values decreased from approximately 200 μS/cm in the first immersion sample to 25 μS/cm in the ninth immersion sample (this range is typical for soft surface waters). Total organic carbon declined in line with conductivity, from 200 to 250 mg/L to approximately 10 mg/L. The dominant part of the TOC (up to 250 mg/L) came from the organic render formulation containing several organic compounds, for example, polymeric binder, dispersing, antifoam, and hydrophobing agents (Schoknecht et al. 2009; extruded polystyrene releases only low amounts of TOC; e.g., see 2.4 mg/L in Gartiser et al. 2016), and the biocide fraction accounted for less than 5% (~10 mg/L; Table 2). Consequently, there were no major differences between TOC values from renders with or without biocides (Supplemental Data, Figure S5). The pH ranged between 6.8 and 8.0, which is a common pH range in surface waters. Thus, all measurements are well within the tolerance range of test organisms.

Terbutryn drives toxicity in the combined algae test

Concentration–response curves of the diuron reference and leachate samples from the first and ninth immersion cycles are shown in Figure 2. Samples from the free biocide and the encapsulated biocide renders induced nearly 100% inhibition of PSII yield and growth rates at the highest sample concentrations (i.e., the lowest dilution factor values). Effect levels of the control sample were low and showed no concentration–response

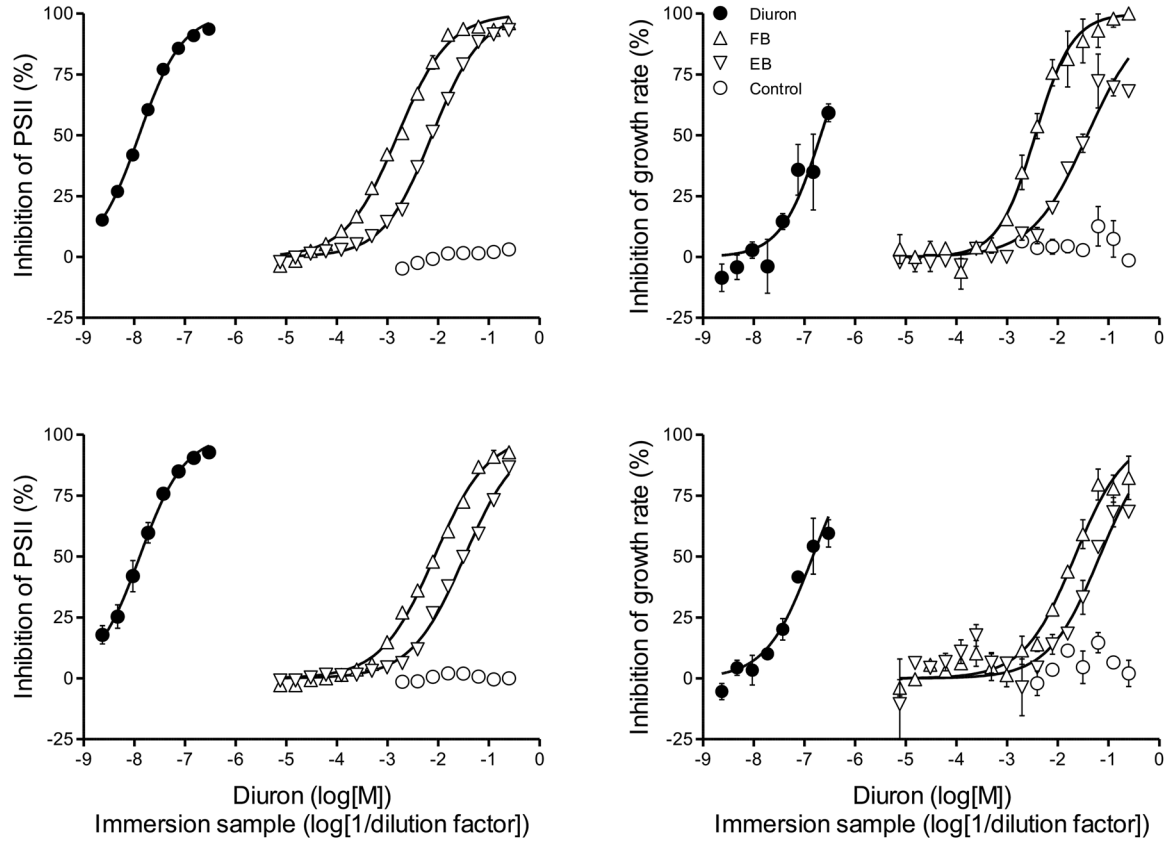

FIGURE 2: Effects of the diuron reference and immersion samples from 3 render types (control; encapsulated biocides [EB]; and free biocides [FB]) on photosystem II (PSII, left) and growth rates (right) in algae. Samples from the first immersion cycle are shown on top; samples from the ninth immersion cycle are shown below. Lines show the fit of Equation 7 to the data (average and standard deviation of 3 technical replicates).

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relationship; thus we conclude that no effects were caused by the render matrix itself (e.g., elevated TOC or conductivity). The maximum observed effect level in a control sample was 3.1% inhibition of PSII at a dilution factor of 4 (first immersion cycle sample) and 12.6% inhibition of growth at a dilution factor of 16 (ninth immersion cycle sample). As is typically observed for the combined algae test, the technical replicates of the PSII endpoint hardly scatter (i.e., have minimal standard deviations), whereas the growth rate endpoint often shows considerable variability (e.g., Vermeirssen et al. 2017).

Results on algae toxicity match those of chemical analyses in that the render with encapsulated biocides released less biocide and caused lower toxic effects than the render with free biocides. Data on sample toxicity and sample biocide concentrations (Table 2) were combined in a toxic unit analysis. Concentration–response curves (Figure 2) provided the DF50 values that were used to calculate the concentrations of the 3 biocides in the assay at DF50 (Table 4). Subsequently these biocide concentrations, as well as the EC50 values from single-substance testing (Supplemental Data, Table S1), were used to calculate the toxic units. In the combined algae test, OIT and DCOIT had a very low potency (Supplemental Data, Table S1), and thus they did not contribute to the effects on PSII and growth rates in algae (i.e., max $TU_{OIT} = 0.02$; Table 4). Terbutryn has a toxic unit that ranges between 0.4 and 0.9. This provides support for the hypothesis that most effects seen in the combined algae test are driven by terbutryn. A perfect toxic unit of 1 will always be difficult to attain due to the 3 main factors involved in toxic unit calculations that may each carry an error: 1) concentration measurements in the sample, 2) the fitted DF50, and 3) the EC50 of the single compound.

OIT drives toxicity to bacteria

Samples from the first immersion cycle of render with free biocides showed the highest toxicity to bacteria. Leachate samples of render with encapsulated biocides and the control render were also toxic (Figure 3). In samples from the ninth immersion cycle, only the free biocide formulation caused toxicity. Over the 9 immersion cycles, the DF50 fell from 58 to 4.6, a 13-fold drop in toxicity.

It was unexpected that the control render leachates caused some toxicity in bacteria. The neutral pH and low conductivity of the sample were well within the working range of the assay (International Organization for Standardization 2009), and it thus remains unclear which compound(s) caused the inhibitory effect. Although the effect caused by the render itself—or perhaps caused by the extruded polystyrene panel—was fairly small (i.e., DF50 of 1.7; Table 4), it will have contributed to effects seen for renders with biocides. Because the render with encapsulated biocide had a DF50 of 5.0 for the first immersion cycle sample, approximately one-third of that effect (i.e., 1.7/5.0) could have been caused by the render itself and not by biocides.

Toxic unit analysis showed that the contribution of terbutryn and DCOIT to the effect on bacteria was negligible, but that OIT was the main contributor to the toxic unit. The OIT toxic units (TU_{OIT}) of the first immersion samples were fairly low (0.28 and 0.49), but, as highlighted just above, the control render itself

TABLE 4: Immersion sample dilution factors required to achieve 50% inhibition (DF50) of 4 endpoints in 3 bioassays[a]

		Biocide concentrations (μg/L) at DF50			Toxic units of biocides at DF50		
	DF50	Terbutryn	OIT	DCOIT	Terbutryn	OIT	DCOIT
Algae: inhibition of photosystem II							
EB first immersion	130	3.0	3.1	0.029	0.67	<0.01	<0.01
FB first immersion	630	2.6	13	0.19	0.58	0.01	<0.01
EB ninth immersion	30	3.9	3.2	—	0.87	<0.01	—
FB ninth immersion	120	3.8	10	0.086	0.84	0.01	<0.01
Algae: inhibition of growth rate							
EB first immersion	26	15	15	0.15	1.22	0.02	<0.01
FB first immersion	280	5.9	28	0.42	0.48	0.01	<0.01
EB ninth immersion	15	8.0	6.4	—	0.65	0.01	—
FB ninth immersion	46	9.9	26	0.22	0.80	0.01	<0.01
Bacteria: inhibition of bioluminescence							
C first immersion	1.7	—	—	—	—	—	—
EB first immersion	5.0	81	81	0.77	< 0.01	0.28	<0.01
FB first immersion	58	28	140	2.0	< 0.01	0.49	0.01
EB ninth immersion[b]	—	—	—	—	—	—	—
FB ninth immersion	4.6	98	260	2.2	< 0.01	0.90	0.01
Water flea: inhibition of population growth							
C first immersion[c]	1.7	—	—	—	—	—	—
EB first immersion	6.5	62	62	0.59	—	—	—
FB first immersion	21	78	380	5.6	—	—	—
EB ninth immersion[b]	—	—	—	—			
FB ninth immersion	7.0	65	170	1.5	—	—	—

[a] Samples came from a control render (C) and renders with added free (FB) or encapsulated biocides (EB). Concentrations (μg/L) of 3 biocides in the assay medium and toxic units were calculated at DF50; no toxic units could be calculated for water fleas as no EC50 data are available for the 3 biocides and *C. dubia*.
[b] No 50% effect level could be determined, and thus no biocide concentrations could be calculated.
[c] Concentrations were below the limit of quantification, and thus no biocide concentrations could be calculated.

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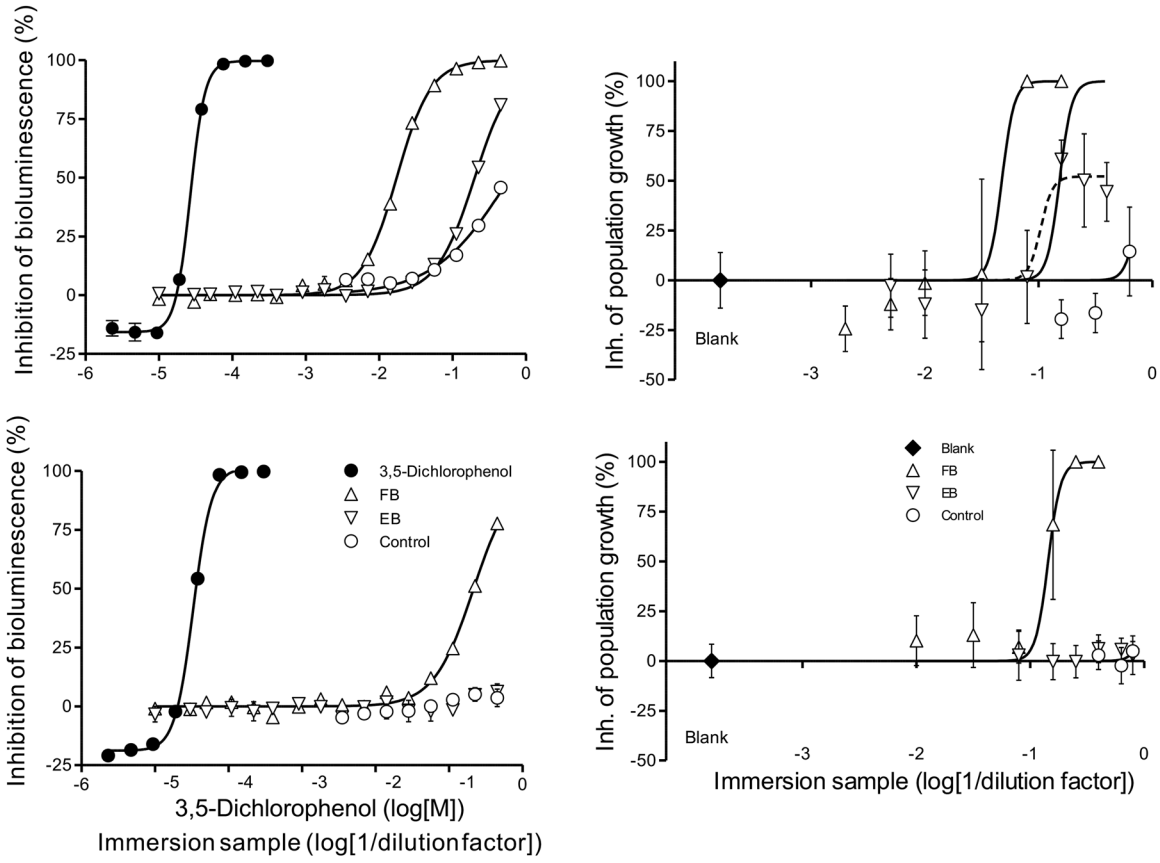

FIGURE 3: Effects of immersion samples from 3 render types (control; encapsulated biocides [EB]; and free biocides [FB]) on bacterial bioluminescence (left) and water flea reproduction (right). Samples from the first immersion cycle are shown on top, and samples from the ninth immersion cycle are shown below (average and standard deviations of 3 technical replicates for bacteria and 12 replicates for water flea). Bacteria data were fitted with Equation 7 without constraints (solid lines). Water flea data were fitted with Equation 7 using a fixed slope of 8 and a 0 to 100% effect range (solid lines); data from the sample with encapsulated biocides and the first immersion cycle were also fitted with an unconstrained maximal effect (dashed line).

explained approximately one-third of the toxicity seen in the leachate from the first immersion cycle of render with encapsulated biocides. When the effect caused by the render was assigned a TU_{render} of 0.33 and added to the toxic unit of OIT, then their combined toxic unit values began to approach 1 (i.e., encapsulated biocides first immersion: TU_{OIT} 0.28 + TU_{render} 0.33 = 0.61; free biocides first immersion: TU_{OIT} 0.49 + TU_{render} 0.33 = 0.82; assuming the modes of actions are the same and the toxic units can be summed).

Isothiazolinones—not terbutryn—are likely involved in water flea toxicity

The response pattern of *C. dubia* to the leachates of render with biocides was similar to that of *A. vibrio* but showed more variability. For both renders with biocides, the first immersion cycle samples were toxic. For samples from the ninth immersion cycle, toxicity was only observed for the render formulation containing free biocides. Fitting the data with Equation 7 was difficult, because concentration–response curves were steep and the first immersion sample from the render with encapsulated biocides had a submaximal response (~50%; Figure 3). For this reason, we fitted all data sets with Equation 4 using a fixed slope of 8 and a 0 to 100% effect range. Data for the render with encapsulated biocides were also fitted with an unconstrained maximal effect. These 2 fitting approaches only marginally shifted the DF50 of the sample from encapsulated biocides from 6.5 (Table 4) to 9.6.

For the *C. dubia* tests, a toxic unit approach could not be applied to establish the drivers of the toxicity, because no EC50 values are available for *C. dubia* and the 3 tested biocides. To further evaluate the toxicity seen in *C. dubia* tests, we explored available chronic effect data for *Daphnia magna* and the 3 biocides involved (Supplemental Data, Table S1). It has to be considered that, besides being a different water flea species, the *D. magna* test runs 3 times longer than the *C. dubia* test. Nevertheless, as is the case with the bacteria, terbutryn is much less toxic to *D. magna* than OIT (800-fold) and DCOIT (300-fold; Supplemental Data, Table S1). Given that the concentrations of terbutryn in immersion samples were only up to 50-fold higher than those of OIT and DCOIT (Figure 1), these higher terbutryn concentrations are not enough to overcome terbutryn's 300- to 800-fold lower toxicity compared with the isothiazolinones. Although this analysis extrapolates across water flea species and thus needs to be viewed with caution, it provides an indication that—as in bacteria—isothiazolinones (and not terbutryn) drive the toxicity to *C. dubia*.

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No effects on earthworm avoidance and springtail reproduction

None of the samples from the first immersion cycle caused effects in either of the terrestrial bioassays. Earthworms neither avoided nor were attracted to treated soil, whereas results from the positive control (boric acid) showed that the worms responded to chemical stimuli (Figure 4). No toxicity was observed in the bioassay with springtails (Figure 5) except for the positive control (boric acid).

There are 2 main difference between the terrestrial and the aquatic assays that may explain the absence of effects in terrestrial assays. First, whereas in aquatic assays fairly high sample concentrations could be applied (up to 90% sample in the water flea assay), the loadability of soil is fairly low due to limitations posed by the maximum water holding capacity. The maximum sample dose that could be added was 137 mL/kg soil in the earthworm assay (97 mL in the springtail assay). Second, whereas leached compounds in water samples are mostly available to the aquatic organisms, there is a very high sorption capacity for these compounds in soil. Thus, bioavailability of the biocides in soil is limited due to the adsorption to the solid matrix.

To assess whether effects from biocides should be expected, we calculated their concentrations in soil (Supplemental Data, Table S2) to compare them with known effect data. No literature data were found for the 3 biocides in relation to the avoidance behavior of worms or reproduction in springtails. However, the acute 14-d median lethal concentration of terbutryn for earthworms is reported to be 170 mg/kg (Tomlin 2009). Clearly, this value is far below the concentration reached in the present experiment (0.24 mg/kg; Supplemental Data, Table S2) and would explain why no acute (and probably also chronic) toxicity was observed in the earthworm avoidance test.

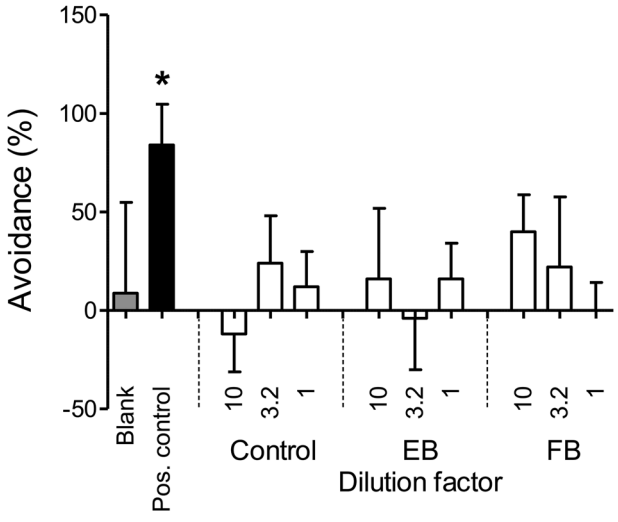

FIGURE 4: Effects of boric acid (positive control) and leachate from 3 types of render (control; encapsulated biocides [EB]; and free biocides [FB]) on avoidance behavior in earthworms. Open bars show the mean of 5 technical replicates with their standard deviation. Only the positive controls (black bar, $n = 5$) showed a significant difference from the assay blank (gray bar, $n = 8$).

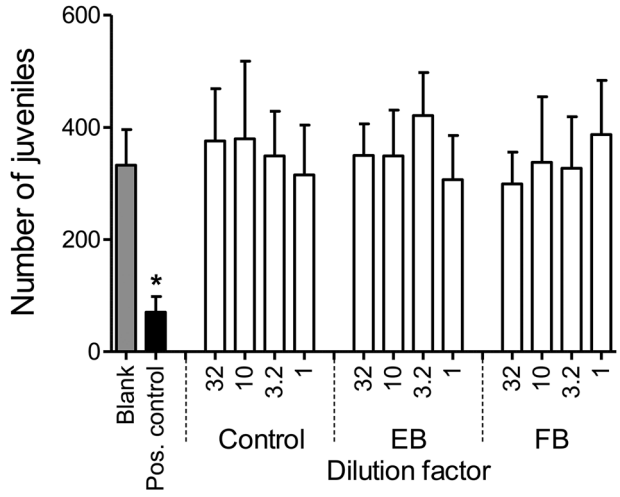

FIGURE 5: Effects of boric acid (positive control) and leachate from 3 types of render (control; encapsulated biocides [EB]; and free biocides [FB]) on reproduction in springtails. Open bars show the mean of 5 technical replicates with their standard deviation. Only the positive controls (black bar, $n = 5$) showed a significant difference from the assay blank (gray bar, $n = 8$).

Future developments concerning an ecotoxicological evaluation of construction materials: Combining standard leaching protocols with standardized bioassays

We combined a standard leaching protocol with both chemical as well as biological analyses of immersion samples from facade renders. Measured biocide concentrations explained the results of bioassays rather well, with toxic units ranging between 0.3 and 1.2 for endpoints measured in algae and bacteria (average toxic unit = 0.7, $n = 11$). These results illustrate the robustness of the approach of combining (partially) standardized bioassays with a standard leaching method on a real construction product containing biocides (termed the "treated article").

In our study, we mainly used acute aquatic bioassays, which are both relevant and cost effective. Also, the Deutsches Institut für Bautechnik scheme (2011) for testing eluates from construction products involves similar assays (see also European Committee for Standardization 2017). To improve risk assessment still further, additional bioassays—particularly chronic assays—could be added to construction material leachate sample testing schemes.

No effects were observed in bioassays conducted in soil. As discussed above (in the *No effects on earthworm avoidance and springtail reproduction* section), the possibility of adding sample to soil is limited by the soil's water holding capacity, and also the availability of biocides in soil is limited due to sorption processes. Under real-world exposure scenarios, however, there will be on the one hand continuous input of biocides into the soil compartment, with adsorbed biocide concentrations possibly increasing over time. On the other hand, various soil parameters will affect biocide removal and bioavailability. For example, a sandy soil adsorbs organic substances to a lesser degree compared with the tested loamy sand LUFA 2.2. Future

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experiments could be designed to investigate the effects of these factors on the outcome of terrestrial bioassays.

The product type that we tested—facade render—typically contains a combination of 2 to 3 biocides for dry-film preservation, and thus the compound mixture in our leachate samples was fairly simple (i.e., terbutryn, OIT, and DCOIT), which facilitated our evaluation and understanding. This situation is likely atypical when other product types containing biocides are considered (European Chemicals Agency 2014a), as well as construction materials without biocides. For example, in a parallel study on corrosion protection coatings, which are complex epoxy resin-based products (Vermeirssen et al. 2017), we were able to: 1) match bisphenol A concentrations in leachates very well with several endpoints monitored in algae and human cell lines, but 2) also detect very high toxicity to bacteria caused by (an) unknown compound(s). When the results from both studies are taken together, they illustrate that bioassays provide added value in terms of 2 aspects: 1) they can confirm that the chemical analysis was appropriate and covered relevant substances and 2) they can indicate the presence of toxic compounds that were missed by chemical analyses. The second aspect can be developed further, and at a relatively low cost, by using bioassays with an effect-directed analysis approach to identify unknown toxicants (Brack 2003). For the development of environmentally friendly products and performance characterization of leachates, bioassays deliver a rapid and relevant overview of toxicity and may help to proof product labels addressing leaching.

The added value of the implementation of bioassays in the testing of construction materials has been recognized earlier and builds on experience gained in the area of testing toxicity of waste materials (for a recent overview, see Pandard and Römbke 2013). For example, in Germany a scheme was used to assess possible effects of construction products on soil or groundwater that also included evaluations using bioassays (Deutsches Institut für Bautechnik 2011). Also, in 2017, the European Committee for Standardization adopted a technical report that provides guidance on the use of ecotoxicity tests applied to construction products. Other recent studies have emerged in this area (Gartiser et al. 2017a, 2017b; Vermeirssen et al. 2017; see also Menge 2005), showing that the topic is gaining momentum. This is important, given the extensive use of a diversity of construction products (with or without biocides) in the environment and the open questions concerning their ecotoxicological properties (see, for example, a review on geotextiles in Vé Wiewel and Lamoree 2016).

CONCLUSIONS

The toxicity of leachate from render containing encapsulated biocides (terbutryn, OIT, and DCOIT) was consistently lower than that of leachate from render containing free biocides. Toxicity decreased noticeably from the first to the ninth immersion cycle by up to a factor of 5 to 10; this was in line with measured biocide concentrations. Toxicity to algae was dominated by terbutryn, whereas toxicity to bacteria was dominated by OIT. No effects were observed in terrestrial assays, but the loadability of sample into soil is limited due to constraints caused by the soil's water holding capacity. Finally, combining standard leaching protocols with both chemical and biological analyses can provide added value for ecotoxicological evaluation of construction materials.

Supplemental Data—The Supplemental Data are available on the Wiley Online Library at DOI: 10.1002/etc.4176.

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Data availability—All bioassay concentration–response data are given in the Supplemental Data. Other raw data and data evaluation methods are available on request (etienne.vermeirssen@oekotoxzentrum.ch).

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