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A margin of safety approach for the assessment of environmentally realistic chemical mixtures in the marine environment based on combined passive sampling and ecotoxicity testing

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ABSTRACT

Organisms in the marine environment are being exposed to an increasing variety of chemicals. This research presents an effect-based monitoring method for the derivation of a margin of safety for environmentally realistic chemical mixtures. The method is based on a combination of passive sampling and ecotoxicity testing. First, passive sampling was performed using H_2O-philic divinylbenzene Speedisks during 3 sampling campaigns between 2016 and 2018 at 4 sampling locations in the Belgian part of the North Sea. Next we exposed the marine diatom *Phaeodactylum tricornutum* to Speedisk extracts that were reconstituted in HPLC-grade water and defined the MoS of each sample as the highest no-observed effect concentration, expressed as relative enrichment factor (REF). A REF was defined by comparing the concentrations of 89 personal care products, pesticides and pharmaceuticals in the biotest medium with those measured in water grab samples to relate exposure concentrations in the tests to environmental concentrations. Across eight marine samples, diatom growth inhibition was observed at REF ≥ 3.2 and margins of safety were found between REF 1.1 – 11.0. In addition, we found that reconstitution of extracts in HPLC-water was suitable to overcome the solvent-related challenges in biotesting that are usually associated with passive sampler extract spiking, whilst it still allowed REFs up to 44 in the biotest medium to be achieved. This method, however, likely covers mainly the polar fraction of environmentally realistic chemical mixtures and less the non-polar fraction. Nevertheless, for 5 out of 8 samples, the Margin of Safety (MoS) was found to be lower than 10, which represents the typically lowest possible assessment factor applied to no effects ecotoxicological data in conventional environmental risk assessments, suggesting ecological risks for these samples.

1 INTRODUCTION

Global chemicals sales have been predicted to grow about 3 % per year until 2050 (Massey et al. 2013). Along with an increasing production and consumption, more chemicals are expected to be released into the aquatic environment and ultimately into marine waters. Currently, a variety of substances can be found in a broad range of European marine waters (Ghekiere et al. 2013; Huysman et al. 2019; Moreno-Gonzalez et al. 2015; Noedler et al. 2014; Thomas and Hilton 2004; Vanryckeghem et al. 2019). Traditionally, environmental risk assessment couples monitoring of pollutant levels with ecotoxicity testing of individual chemicals (Shaw et al. 2009) on a substance-by-substance basis, but more recently the awareness for the necessity of characterizing the impact of complex mixtures to aquatic organisms is receiving increased recognition (Kim Tiam et al. 2016). The large variety of chemical substances and the even greater number of possible combinations of chemicals simultaneously present in aquatic environments requires methods for mixture toxicity assessment of environmentally realistic chemical mixtures (ERCMs) (Kim Tiam et al. 2016).

One promising approach is to transfer ERCMs from the field to the laboratory by using passive sampling devices for combined chemical and ecotoxicological monitoring. Passive sampling allows transfering ERCMs into biotest systems by either passive dosing (for equilibrium based samplers) or extract spiking (for integrative samplers). Combining passive sampling and ecotoxicity testing generally aims to realistically reconstitute the environmental chemical mixtures into the biotest medium. One of the major challenges to achieve this is the fact that different chemicals may experience different levels of enrichment on the sorbent of a passive sampler, often closely related to their physico-chemical properties (e.g. hydrophobicity) (Jahnke et al. 2016). Advantages and disadvantages of both methods have been discussed elsewhere

(Jahnke et al. 2017). Extract spiking is a well-established, simple, practical and efficient approach to transfer organic chemicals from the environment into biotest systems. However, quantitative, unskewed re-establishment of the original mixture as present in the sampled environment is very challenging. Additionally, reconstitution of passive sampler extracts is usually performed using the addition of solvents and, therefore, spiking extracts into biotest medium unavoidably also introduces these solvents into the biotest medium. This can be problematic (depending on the concentration of the solvent introduced) and may limit the maximum level of sample enrichment that can be investigated in the biotesting of passive sampler extracts (Moeris et al. 2019).

Applying passive sampler extract spiking in bioassays such as the 72 h growth inhibition test with the marine diatom *Phaeodactylum tricornutum* provides the opportunity of testing mixtures at a relatively high level of biological organization. The maximum test concentration that can be attained in a bioassay is highly dependent on the biotest volume, the available sample volume, and the presence of solvent in the PS extract (since too high solvent concentrations in a biotest may adversely affect the test organisms by themselves or may impact the partitioning of chemicals between all components). Nevertheless, one advantage of working with passive sampler extracts rather than passive dosing is the possibility to test ERCMs at a range of relative enrichment factors (REFs) (Shaw et al. 2009). Using this method to expose species of concern is a valuable tool to improve the relevance of test results for predicting real world effects (Shaw et al. 2009). Another challenge of combining passive sampling and biotesting is to obtain sufficient sensitivity in the biotest of choice. Sensitivity can be increased by selecting suitable endpoints or testing treatments with higher REF. The latter can be achieved by miniaturizing the test setup (Jahnke et al. 2016). Algae growth inhibition tests have shown to be especially sensitive

indicators of environmental stress and they have been standardized in a variety of international toxicity test guidelines for freshwater and marine species (Emelogu et al. 2014). Next to the high sensitivity, these tests can easily be adapted to testing in microplates instead of the more commonly used high-volume setups, which are usually performed in erlenmeyer flasks with volumes ≥ 50 mL. Microplate testing allows reduced extract consumption and can thus be used for high throughput screening (Brack et al. 2016).

Current environmental monitoring in the European Union is mostly focused on a limited set of priority pollutants as defined by the European Commission (European Commission 2013). Nevertheless, it is recognised that not all substances on the priority list are still representative of present day contamination (Busch et al. 2016). With regard to emerging chemicals, the Water Framework Directive Watch List suggests some additional chemicals for regular monitoring (European Commission 2018a) but inclusion of new chemicals for regular monitoring is generally a slow process. In addition, targeted chemical analyses on their own tend to underestimate mixture toxicity (Altenburger et al. 2015; Daughton 2005) and risk assessments based on chemical monitoring data provide only the lower boundary of the chemical risks at any given site or moment (Gustavsson et al. 2017). Indeed, low concentrations or below detection limit reports of priority pollutants are not sufficient to exclude any potential ecological risks (de Baat et al. 2018). Generally, awareness is increasing that targeted chemical monitoring and priority substance driven research alone cannot account for the complexity of chemical mixtures present in most aquatic environments (Escher et al. 2018). Due to our limited knowledge on the complex chemical mixtures that many aquatic organisms are exposed to and eventually affected by, there is an increasing need for effect-based rather than solely chemical analysis-driven monitoring and assessment tools.

In this research, we developed an effect-based monitoring method to derive a Margin of Safety (MoS) of ERCMs in the marine environment and applied it in a case study to the Belgian Part of the North Sea (BPNS). The method consists of passive sampling of an ERCM, followed by sampler extraction, and biotesting (here: the diatom *P. tricornutum*) of a range of dilutions of this (enriched) extract, with each dilution representing a REF in terms of chemical concentrations relative to the actual aquatic concentrations in the environment. The MoS is defined as the highest REF with no significant reduction of diatom growth rate compared to a control.

2 MATERIALS AND METHODS

2.1 *Active and passive sampling*

This research is part of the larger project called NEWSTHEPS (www.newstheps.be), which consisted of five field sampling campaigns. The methods presented in this manuscript, were only applied in three of those campaigns, i.e. sampling campaign 2, 3, and 5 (SC2, SC3, and SC5). For the sake of comparability between different publications (Huysman et al. 2019; Vanryckeghem et al. 2019), related PhD theses (Huysman 2019; Moeris 2020; Vanryckeghem 2020) and the final project report (www.newstheps.be/final report), we kept the project-related numbering of the sampling campaigns in this manuscript as well. A detailed overview of the different sampling locations and time of deployment is given in Table 1. For passive sampling, Bakerbond H_2O-philic divinylbenzene Speedisk (Filter Service S.A, Eupen, Belgium) were deployed inside the harbor of Zeebrugge (HZ, 51°20'25.68"N; 3°12'12.11"E), along the coast in front of Zeebrugge harbor (OZ_MOW1, 51°21'37.78"N; 3°6'49.01"E), and inside the harbor of Ostend (HO, 51°13'34.68"N; 2°56'8.00"E), along the coast in front of this harbor (OO_X, 51°15'33.00"N; 2°58'1.20"E), in the Belgian Part of the North Sea (BPNS), between November 2016 and May 2018 (Figure 1). Coastal sampling locations were located 3-5 km in front of the respective harbors. Samplers were deployed in triplicate at a depth of approximately 3m below the surface at the harbors, and above 2 m from the seabed at the coastal locations. Additional information about the passive sampler configuration and deployment is given in the supportive information. Before deployment, the samplers were pre-rinsed with 20 mL HPLC grade methanol : HPLC grade acetonitrile (1:1, v/v), followed with 20 mL HPLC water and finally stored in acid-washed glass bottles filled to the top with HPLC water until deployment. After retrieval, the samplers were immediately stored in empty glass bottles and kept in the dark at

4 °C until extraction. In addition, one Speedisk per sampling campaign was kept in a glass bottle filled to the top with HPLC-grade water during the complete sampler deployment time to serve as procedural blank. Procedural blanks were subsequently treated equally to the deployed passive samplers. Time between sampler recovery and extraction was 19 months (SC2), 15 months (SC3) and 4 months (SC5). It has been shown previously that chemical mixtures are very well preserved on passive sampling devices like speedisks and thus these different storage times are not expected to have had any impact on the mixture compositions (Challis et al. 2017). All samples were tested for toxicity within less than 2 weeks of extraction and chemical analysis occurred no later than 1 month after extraction.

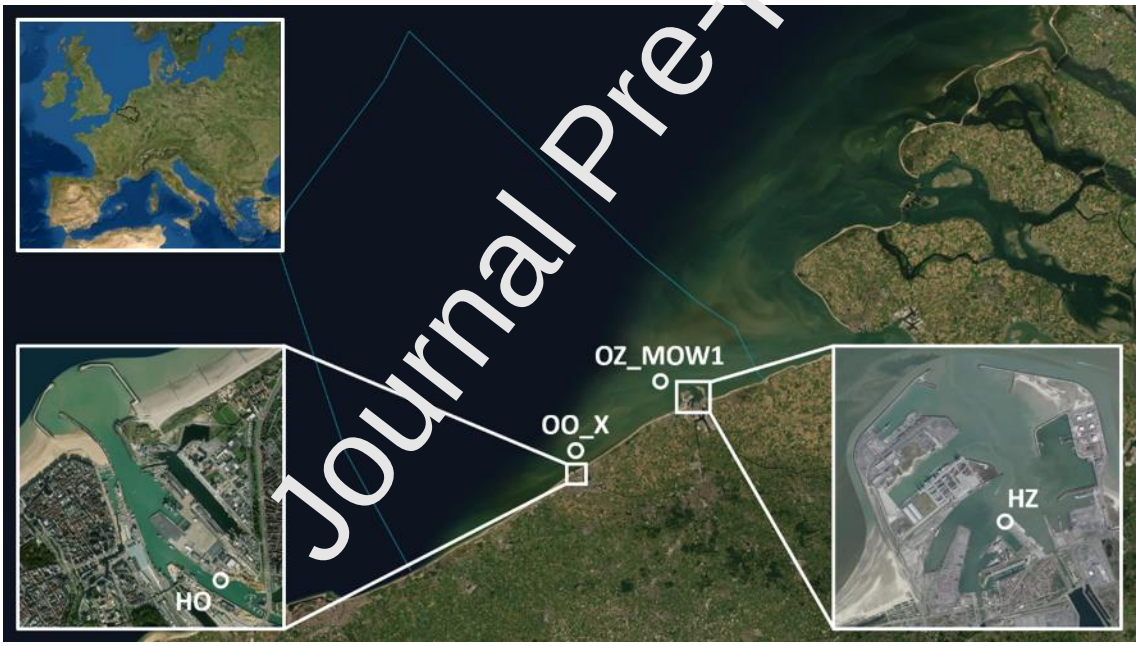


Figure 1 Map of the Belgian Part of the North Sea indicating the four sampling locations. HO and OO_X stand for "harbor Ostend" and "coast Ostend", respectively while HZ and OZ_MOW1 stand for "harbor Zeebrugge" and "coast Zeebrugge". The blue line shows the borders of the Belgian part of the North Sea.

In addition to passive sampling, active grab water samples were taken at the time of passive sampler deployment and retrieval to allow a comparison with passive sampler extract concentrations. For this purpose, 3000 mL water was collected and divided into 3 sub-samples of 1000 mL each. As such, these sub-samples cannot be considered as real sampling replicates but rather as analytical replicates (3 analytical measurements of one grab sample). This was mainly due to limited time at the respective sampling locations since our sampling campaigns were usually part of joint expeditions at the BPNS. Grab water samples were stored in amber glass bottles pre-cleaned with methanol and ultrapure water. Upon arrival in the laboratory, sub-samples were filtered using Whatman GF/D glass fiber filters (2.7 µm, 90 x 90 mm). $Na_2EDTA{\cdot}2H_2O$ at a concentration of 1 g L^{-1} was added to the grab water samples and the pH was adjusted to 7 by addition of formic acid. Then, samples were stored at 4 °C in the dark until extraction and analysis. Finally, chemicals were extracted from the samples by means of solid-phase extraction (SPE) using Oasis Hydrophilic-Lipophilic Balance (HLB) (6 mL, 200 mg sorbent, Waters, Belgium). Further details are described in Vanryckeghem et al. (2019).

2.2 *Passive sampler treatment*

Prior to the extraction, Speedisk passive samplers were rinsed with 18 mL HPLC water and dried for 5 min under vacuum. Then, Speedisk extraction was performed with 10 mL HPLC grade methanol : acetonitrile (1:1, v/v). Three additional Speedisks deployed at SC3 HO were extracted and extracts split into two equal parts of 4.9 mL each, while all other extracts were kept undivided before full evaporation of the extraction solvent under a gentle nitrogen stream at 25 °C. The separation of SC3 HO extracts occurred in order to compare a previously applied extraction procedure where extracts were reconstituted in a water-solvent mixture (Moeris et al. 2019) with the newly developed one (reconstitution in pure HPLC water). Finally, the

concentrated liquid of half of the split SC3 HO extracts was reconstituted in 490 µL of HPLC methanol : HPLC water (1:9, v/v) acidified with 0.1 % formic acid and 0.01 % $Na_2EDTA.2H_2O$ (further called "MeOH extracts") while the other half was reconstituted in 490 µL HPLC water (further called "H_2O extracts"). For all other samples, the concentrated liquid was reconstituted in 1 mL HPLC water. Reconstituted extracts were ultra-sonicated for 1 min, vortexed for 20 s and centrifuged at 3,000 rpm for 5 min. Finally, the liquid phase was transferred to a HPLC vial and stored at – 20 °C until instrumental analysis and biotesting.

Prior to biotesting, concentration series of the passive sampler extracts were prepared. For this purpose, 500 µL original extract was diluted by adding 2.5 mL growth medium (International Organization for Standardization 2006). This growth medium was prepared in a way to account for dilution via spiking with Speedisk extracts. Consequently, salts and vitamins were added at 105 % of the concentrations described in the guideline. For the following treatment, 3.5 mL of this solution were diluted by adding 7 mL non-adjusted growth medium. This was repeated 6 more times to obtain a serial (1:3) dilution with a total number of 8 concentration treatments per Speedisk extract. The extract concentrations are expressed as sum-analyte concentrations, i.e. the sum of the concentrations of all quantified chemicals in a Speedisk extract, throughout this manuscript.

2.3 *Chemical analysis*

Based on previous experience (Moeris et al. 2019) Speedisk extracts were analyzed chemically and tested for toxicity within maximum four weeks of extraction. Targeted chemical analysis was performed using ultra-high performance liquid chromatography (UHPLC) and focused on the quantification of 89 personal care products (n = 9), pesticides (n = 28) and pharmaceuticals

(n = 52). Additional information about the analytical method can be found in Vanryckeghem et al. (2019) (Vanryckeghem et al. 2019) and in the supportive information.

Previous research has shown that it is better to predict biotest concentrations based on those chemical concentrations determined in Speedisk extracts in order to maintain a maximum of available information since concentrations in microplate wells conflict with analytical detection limits (Moeris et al. 2019).

2.4 *Algae growth inhibition testing*

The marine diatom *Phaeodactylum tricornutum* Bohlin was obtained from the Culture Collection of Algae and Protozoa (CCAP 1052/1A, Oban, United Kingdom). The organisms were cultured according to ISO 10253 (International Organization for Standardization 2016). Further details on growth medium and culturing conditions can be found in ISO 10253.

Four days prior to toxicity test start, a pre-culture was prepared by inoculating fresh growth medium with 10,000 cells mL^{-1}. At test start, microplate wells were inoculated with < 50 µL of the pre-culture to achieve an initial algae concentration of 10,000 cells mL^{-1}. In total, we performed three independent 72 h algae growth inhibition tests for samplers from the three different sampling campaigns in non-treated sterile 24-well tissue culture plates. All test plates were allowed to grow under continuous white light (100 – 120 µmol m^{-2} s^{-1}) at 22 ± 1 °C. In addition to the concentration treatments, 3 wells per plate were filled with 2 mL of pure growth medium serving as controls (CTL), and 1 well per CT was filled with extract-spiked growth medium but not inoculated to serve as particle blank. Further, a calibration series ranging from 10,000 to 400,000 cells mL^{-1} based on cell counts using an electronic particle counter (Coulter

Counter model DN, Harpenden, Herts, UK) was prepared and fluorescence measured at toxicity test start.

During the tests, well plates were constantly shaken at 150 rpm, and the algae fluorescence was measured after 0, 24, 48 and 72 h using a microplate reader (TECAN Infinite 200 PRO microplate reader). The pH of the test medium was measured in one replicate per concentration treatment at test start and end, and the temperature (in a reference beaker filled with biotest medium for the whole test setup) was measured constantly throughout the 72 h test period. After particle blank subtraction of the different treatments, the fluorescence was converted to cell counts by means of the calibration series. Next, the specific growth rate μ was calculated for the controls and each concentration treatment by applying the SLOPE function (Excel 2016) on the ln transformed cell counts of the measurements of day 0 to day 3. The percentage of growth inhibition in an individual test well ($I_{\mu w}$) was then calculated as shown in Equation 1:

$$I_{\mu w} = \frac{\bar{\mu}_c - \mu_w}{\bar{\mu}_c} * 100 \qquad \text{(Equation 1)}$$

with $\bar{\mu}_c$ as the average growth rate [d^{-1}] of the controls and μ_w as the growth rate [d^{-1}] in each individual test well.

Test concentrations were expressed as relative enrichment factors ($REF_{geomean}$) of all measured substances calculated as the geometric mean of individual REF per substance (REF_i). The REF_i was calculated from the analyte concentrations measured in both Speedisk extracts and grab samples (see supportive information Table SI2) taken at the respective sampling location during sampler deployment and retrieval (mean of sampler deployment and retrieval). A REF = 1 corresponds to environmental concentrations (C_w), as applied in previous research and further described by Equation 2 (Kim Tiam et al. 2014), where d indicates the applied dilution factor of

the treatment in the toxicity test. Substances with a measured concentration in procedural blank Speedisks were excluded from the dataset and not accounted for in all REF calculations. REFs of individual compounds were calculated as follows:

$$REF_i = \frac{\frac{C_{i,extract}}{\left(\frac{(C_{i,grab\ sample\ deployment} + C_{i,grab\ sample\ retrieval})}{2}\right)}}{d} \quad \text{(Equation 2)}$$

Then, the REF of the ERCM (as present in the Speedisk extract) was determined as the geometric mean ($REF_{geomean}$) of the individual REF_i across all measured substances:

$$REF_{geomean} = \sqrt[n]{\prod_{i=1}^{n} REF_i} \quad \text{(Equation 3)}$$

Substances with concentrations below the grab sample method detection limit (MDL) (Vanryckeghem et al. 2019) were not included in the calculation of the REF. An overview of the REF_i per sampling location and campaign is given in the supportive information Table SI3.

2.5 *Data analysis*

Finally, fluorescence measurements were converted to cell counts using a calibration series and diatom growth rates in all dilutions where calculated (equation 1) and statistically compared with those in the control using one-way ANOVA, followed by Dunnett's multiple comparisons test ($\alpha = 0.05$). The $NOEC_{REF}$ (NOEC expressed as $REF_{geomean}$) was defined as the highest $REF_{geomean}$ with no significant reduction of diatom growth rate compared to a control. The $NOEC_{REF}$ is also equal to the MoS for this diatom species. In the course of data analysis, additional efforts were undertaken to derive EC_{10} values by fitting log-logistic concentration-response models to the biotest data. This was only succesfully for 3 out of 8 samples (SC3 OO, SC5 HZ and SC5 HO).

Thus, we decided to use the NOEC despite being somewhat less informative than the EC_{10}. The MoS was determined for each sampling location based on growth inhibition of *P. tricornutum* exposed to various dilutions of Speedisk extracts.

Correlation analysis comparing the contaminant concentrations in MeOH extracts and H_2O extracts was performed using Pearson correlation ($\alpha = 0.05$) including the triplicate data for both extraction methods.

All statistical analyses of the toxicity test results and the chemical analysis were conducted using GraphPad Prism version 5.01 for Windows (Muzyka et al. 2007).

3 RESULTS

3.1 *Comparison of extraction methods*

Figure 2 shows the results of the chemical analysis of 89 target substances as a correlation plot of the contaminant concentrations in the MeOH extracts (x-axis) vs. the contaminant concentrations in the H_2O extracts. Correlation analysis of the averaged concentration for 39 substance pairs resulted in a significantly positive correlation ($p < 0.0001$, $r^2 = 0.9691$) of the contaminant concentrations in the different extracts. Details about the identity of the 39 compounds found in both types of extracts are shown in the supportive information Table SI6, samples HO.4 - HO.6). Besides the 39 substances detected in both extracts, four were detected in only one: propylparaben was detected in all three replicates and ketoprofen in one replicate of the MeOH extracts, while alprazolam was detected in two and lamivudine in one replicate of the H_2O extracts. The remaining 46 compounds were not detected in any of the extracts. For those substances detected in both extracts, 83 % had a concentration within a factor 2 and 96 % within a factor 3.

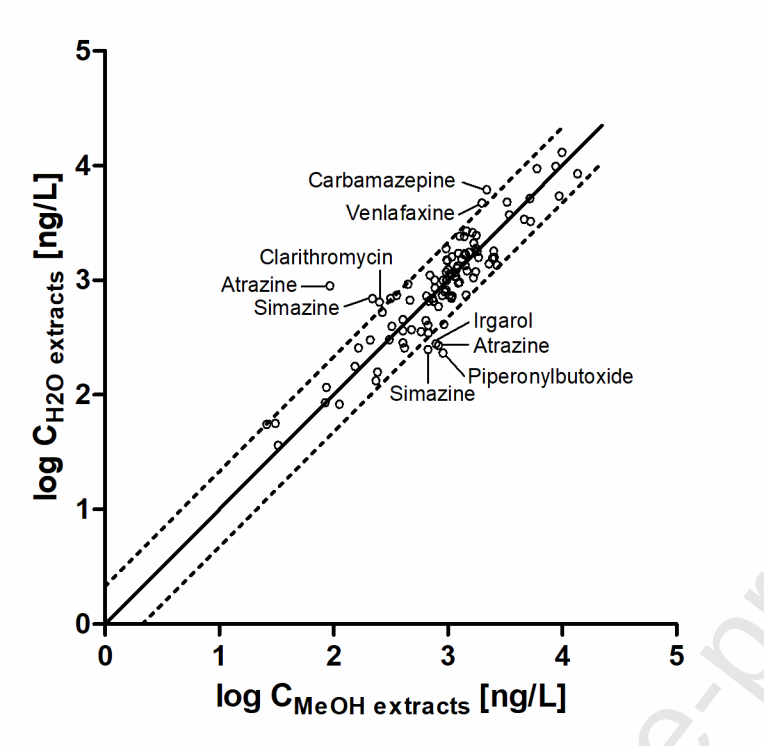
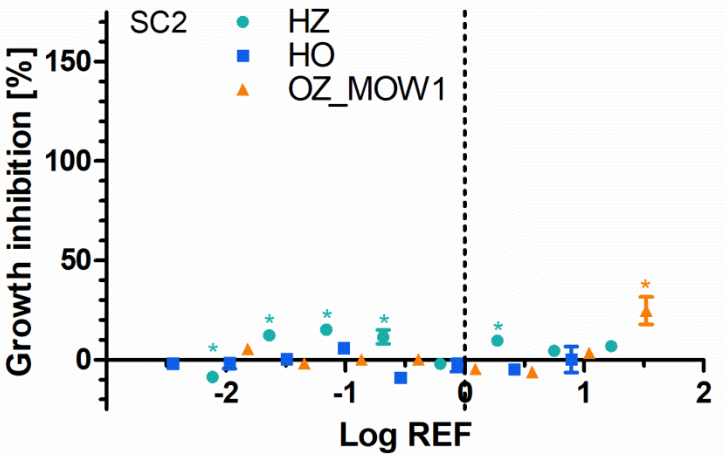


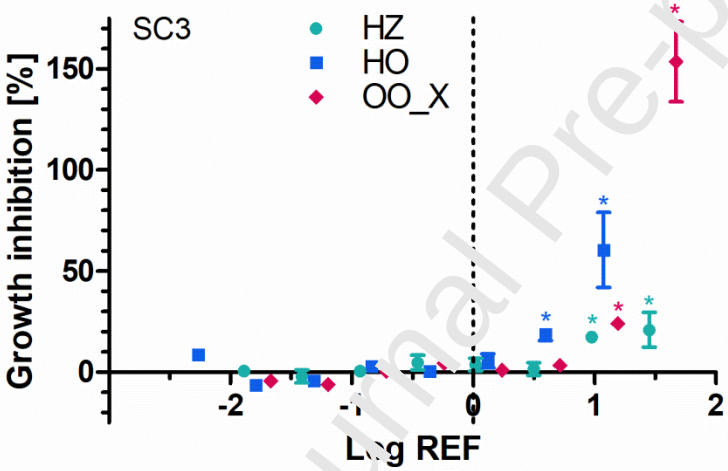
Figure 2 Correlation plot comparing the contaminant concentrations measured in the MeOH-extracts (x-axis) and the H_2O-extracts (y-axis) expressed as log_{10} concentration. The solid line shows the 1:1 trendline and dashed lines indicate the 2-fold line.

3.2 *Marine Speedisk extract testing*

A detailed overview of the measured pH in the three biotests is given in Table SI4. Overall, the pH varied by 0.2 (7.9 – 8.1, SC2), 0.7 (7.6 - 8.3, SC3) and 0.6 (7.6 – 8.2, SC5) units during the tests. The temperature varied by 1.8 °C (21.0 °C – 22.8 °C, SC2), 2.0 °C (21.1 °C – 23.1 °C, SC3) and 2.0 °C (20.8 °C – 22.8 °C, SC5) throughout the test period. Both pH and temperature variation thus fulfilled the recommended validity criteria for 72 h growth inhibition testing with *P. tricornutum* (International Organization for Standardization 2016). A summary of the results of all algal growth inhibition tests with Speedisk extracts is shown in Figure 3. The NOEC could

be defined for 6 out of 8 samples as the highest test concentration showing no significant effect on algae growth. Unfortunately, the data generated in this study only allowed a reliable derivation of EC_{10} values for 3 out of 8 samples. Using log-logistic dose-response models, we derived EC_{10} values of REF = 15 (confidence interval: 11 - 20) for SC3 OO_X, REF = 9.3 (6.5 - 12) for SC5 HZ and REF = 6.2 (4.0 – 8.5) for SC5 HO. This corresponds approximately to the lowest effect REF for SC3 OO_X and falls in between the lowest effect REF and the MoS for SC5 HZ and SC5 HO.





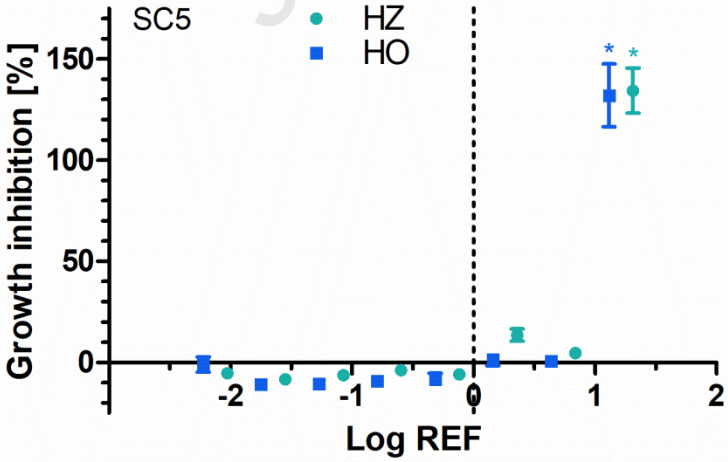


Figure 3 Summary of Speedisk extract testing for sampling campaigns 2, 3 and 5. Shown are the percentage growth inhibition vs. the log relative enrichment factor for Speedisk extracts from the different sampling locations HZ (Harbor Zeebrugge), HO (Harbor Ostend), OZ_MOW1 (Coast Zeebrugge) and OO_X (Coast Ostend). The vertical dashed line indicates the summed contaminant concentrations in corresponding grab samples representing realistic environmental concentration levels (REF = 1). Data shows the average growth inhibition of triplicate Speedisks for each location. Error bars represent the standard error of the mean and data marked with an asterisk showed significant growth inhibition as compared to the control treatments.

3.3 *Link to environmental concentrations and margin of safety determination*

Based on grab water samples taken at the start and end of the passive sampler deployment period, the average contaminant concentrations at each sampling location were determined. A comparison with the sum analyte concentrations in the Speedisk extracts allowed defining the REFs (Equation 2). The measured contaminant concentrations per extract and in the grab samples can be consulted in the supportive information (Tables SI4 – SI6). REFs tested ranged from 0.0035 (lowest test concentration) to 33 (highest test concentration) for SC2, from 0.0043 to 44 for SC3 and from 0.0058 to 19 for SC5. In general, the two to three highest concentration treatments represented enriched environmental mixtures while the lower five to six treatments were dilutions of realistic environmental mixtures. The third highest concentration treatment approximately represented realistic seawater levels as indicated by the vertical dashed line in Figure 3.

Margins of safety could be determined for all four sampling locations as shown in Table 2. Yet, analysis of SC2 HZ resulted in no concentration-dependent effects and no MoS could be determined. Speedisk extracts for SC2 HO did not exert any effects on algal growth resulting in

the highest REF to define the MoS. For SC3 and SC5 the MoS ranged from 1.1 to 6.5, and statistically significant effects (one-way ANOVA followed by Dunnett's multiple comparisons test, $\alpha = 0.05$) on the growth of *P. tricornutum* were measured as of REF = 3.2.

Table 2 Summary of algae growth inhibition testing of Speedisk extracts and link to environmental concentrations. Shown are the highest relative enrichment factors (REF) resulting in no statistically significant effect (= MoS) and the lowest REF resulting in a statistically significant effect on the growth of *Phaeodactylum tricornutum*. The MoS is given as the highest REF resulting in no-observed effect on algal growth. The number in parentheses indicates the measured % growth inhibition observed at the respective MoS or lowest effect REF. No results were determined (ND) for SC2 HZ because effects were not concentration-dependent. For SC2 HO no effects were observed and thus defining a lowest effect REF was not applicable (NA).

Sampling campaign (SC) & location	Margin of safety = Highest no-effect REF (% growth inhibition)	Sum analyte C (µg L^{-1}) MoS	Lowest effect REF (% growth inhibition)	Sum analyte C (µg L^{-1}) lowest effect REF
SC2 HZ	ND	ND	ND	ND
SC2 HO	>8.1 (0.18)	4.7	>8.1	NA
SC2 OZ_MOW1	11 (8.7)	1.5	33 (25)	4.4
SC3 HZ	2.8 (1.5)	0.22	8.3 (17)	0.67
SC3 HO	1.1 (5.6)	0.21	3.2 (19)	0.63
SC3 OO_X	4.9 (3.3)	0.26	15 (24)	0.78
SC5 HZ	6.5 (4.7)	0.60	19 (134)	1.8
SC5 HO	4.3 (0.51)	2.2	13 (132)	6.7

4 DISCUSSION

In a preliminary test, we compared the contaminant mixtures in Speedisk extracts reconstituted in two different solvents (MeOH and H_2O extracts). Our results showed that there was a positive correlation between chemical concentrations in the extracts obtained from the two reconstitution methods. The concentrations for 88 % of the detected substance pairs was found to be within a factor 2. When exposing the marine diatom *P. tricornutum* to Speedisk extracts, we observed significant effects on its growth in treatments with REF ≥ 3.2. Based on these results, we defined MoS for all four sampling locations in the BPNS ranging from 1.1 to 11. Indeed, a REF > 13 always resulted in growth inhibition, while a REF ≤ 2.8 never resulted in growth inhibition, but REF values in-between were associated with cases of both growth inhibition ($LOEC_{REF}$) and no growth inhibition ($NOEC_{REF}$). From a conventional risk assessment point of view, there would typically be a need for applying an assessment factor of minimum 10 to the lowest of available species no-observed effect concentrations (NOECs) to account e.g. for laboratory to field extrapolations (European Commission 2018b). Thus, 5 of the 8 samples studied here would, according to conventional risk assessment, be considered at risk, even if chronic NOECs for fish and invertebrates would be higher than that of the diatom (i.e. even if diatoms were the most sensitive species).

4.1 *Seasonal trends and seasonality effect(s)*

There was no obvious difference of the MoS in offshore locations (4.9 - 11) compared with harbor locations (1.1 – ≥ 8.1), but sample size was too small for a definitive conclusion. On the other hand, there appeared to be some differences between SCs, with MoS between ≥ 8.1 – 11 (SC2), between 1.1 – 4.9 (SC3), and between 4.3 – 6.5 (SC5). From a use perspective, especially the use of pesticides and personal care products like e.g. UV blockers is subject to high

fluctuations. As an example, O'Brien et al. found a strong correlation of pesticide concentrations in an Australian estuary with the harvesting season during a 2-year monitoring campaign with monthly sampling (O'Brien et al. 2016). Our sampling was limited to three sampling campaigns over 2 consecutive years and detection of such trends would require a much higher sampling frequency. Nevertheless, the higher effects observed in tests with samples from SC3 and SC5 than from SC2 may be associated with an increased pesticide use in spring than in winter.

4.2 *Combining passive sampling and biotesting*

Combining passive sampling and biotesting has been applied and confirmed to be highly suitable to identify the ecotoxicological relevance of complex contaminant mixtures (Shaw et al. 2009). Similar to lowering the detection limit in analytical chemistry, there is a need for sample enrichment for biological effects detection. Indeed, the fact that all the lowest effect REF values were > 1 (Table 2), indicates that detecting significant effects in marine samples without enrichment is unlikely, at least for the diatom tested here. In the context of combining passive sampling and biotesting this is achieved by increasing the maximum REF that can be tested. Increasing the REF can be challenging depending on factors such as e.g. sample volume, biotest volume, deployment time, and solvent used for the SPE elution step. For toxicity testing, usually passive sampler extracts are dosed using solvents. This requires a REF of 10,000 – 100,000 in the extracts due to the maximum acceptable solvent concentrations (usually 0.1 – 1 %, v/v) in biotests to reach a REF of 100 depending on the solvent acceptability in the biotest system (Brack et al. 2016). Such high REF are easily reached with large volume solid-phase extraction (SPE) (Tousova et al. 2017) but unrealistic for passive sampling. Therefore we investigated the possibility to reconstitute passive sampler extracts in HPLC-water instead of in an organic

solvent (mixture). Chemical analysis showed a statistically significant positive correlation of the mixture compositions in MeOH and H_2O extracts, with 88 % of the substance's concentrations within less than a factor 2. Reconstitution in HPLC-water was thus suitable to overcome solvent-related challenges while allowing REFs up to 44 in the biotest medium. Therefore, and due to the fact that chemical analysis in this research was targeted at rather polar to mildly apolar compounds (log $K_{OW} \leq 4.9$), the reconstitution in HPLC water was used as the standard method for all biotests performed in this research. Reconstitution in water would probably be rather limited for substances with higher log K_{OW}. If present in the extract, more hydrophobic substances could theoretically be dissolved up to their respective solubility limits in the biotest medium at test initiation, butin contrast with more hydrophilic substances, the dissolved concentration of hydrophobic chemicals would likely decrease during the test (Smith et al. 2010).

While being a useful screening tool for realistic mixtures, testing passive sampler extracts is associated with one major restriction: each passive sampler has a specific binding capacity related to substance polarities. Kim Tiam et al (2016) stated that passive sampler extracts do not reveal the entire complexity of the studied water body, since each sampler has a defined selectivity in terms of polarity or charge. Most of the commonly used passive samplers have affinity ranges spanning about 3 - 4 log K_{OW} units (Vrana et al. 2005). For the "pharmaceutical" configuration of the Polar Organic Chemical Integrative Sampler (POCIS) (Mazzella et al. 2007) this affinity is in the range of log K_{OW} 1 – 4, while styrenedivinylbenzene (SDB) Empore® disks embedded in Chemcatcher have been shown to sample substances with a log K_{OW} 0 – 4 (Vermeirssen et al. 2009). Equilibrium passive samplers based on e.g. polydimethylsiloxane (PDMS) silicone rubber (SR) as the receiving phase or semi-permeable membrane devices

(SPMD) can be used to sample compounds in the hydrophobicity range of log K_{OW} 3 – 10 (Vrana et al. 2005). Data from Huysman et al. (2019) has shown that H_2O-philic divinylbenzene Speedisks are able to sample a broad polarity range spanning from log K_{OW} -0.13 – 9.85 and thus including both very polar and non-polar compounds. Consequently, Speedisks containing this sorbent considerably broaden the sampled polarity range as compared to commonly used passive sampling devices and their extracts are very likely to represent a relevant mixture of contaminants. When reconstituting these mixtures in water, one might expect that only the polar fraction of the mixture would be maintained in the sample. Nevertheless, a correlation analysis between log K_{OW} and REF_i did not support this expectation, at least for the polarity range from log K_{OW} -0.13 (thiamethoxam) to 4.9 (amitriptyline) as shown in the supportive information (Figure SI3). For substances with a log K_{OW} above this range it will become increasingly unlikely that they can be dissolved in HPLC water. This has previously been confirmed by Smith et al. (2015) (Smith et al. 2015).

4.3 *Environmental realism in algae growth inhibition testing with passive sampler extracts*

Diatoms account for ~20 % of the photosynthesis occurring in global oceans (Falkowski and Woodhead 1992) and play a crucial role at the basis of the oceanic food web (Echeveste et al. 2010). In this study, exposure of *P. tricornutum* to enriched passive sampler extracts resulted in significant growth inhibition (17 – 134 %) as of 3.2-fold enrichment of realistic environmental mixtures. Similar observations have been reported before in other studies. Shaw et al. (2009) observed a significant yield inhibition in *P. tricornutum* exposed to SDB-reverse phase sulfonate (RPS) Empore disk extracts deployed at a river mouth in the Great Barrier Reef, Queensland, Australia. Similar to this study, they observed about 25 – 50 % inhibition at REF 5 – 10 (Shaw et

al. 2009). In an effect-based and chemical identification monitoring program of organic pollutants in European surface waters, Tousova et al. (2017) reported algae growth inhibition EC_{50} values for the green algae *Raphidocelis subcapitata* to occur at REF ≥ 17 and lowest observed effect concentration (LOEC) values at REF ≥ 11 (Tousova et al. 2017). Their LOEC values are in good agreement with our findings, except for two of our samples (SC3 HZ and SC3 HO), where REF at LOEC = 8.3 and 3.2, respectively. Due to the limited number of tested dilutions that actually exerted significant effects (maximum 2 per test), we could not reliably determine EC_{50} values. For those tests that showed growth inhibition ≥ 132 %, the EC_{50} values would likely be in the range 4.3 < REF < 44. This would situate some of our EC_{50} values in a range that is lower than the lowest reported by Tousova et al. (2017). In this specific case, growth inhibition values above 100 % indicate cell death. In this study growth inhibition was calculated based on fluorescence. As opposed to basing measurements on particle counting, fluorescence measurements integrate chlorophyll a content and viability of algae. Thus, in the case where algae growth is fully inhibited and cell walls of the diatoms disintegrated or their chlorophyll content decreases during the test, one might measure decreasing fluorescence over time and when converting these measurements to growth inhibition, this then leads to calculated growth inhibition values exceeding 100 %.

In another study investigating effects of trace levels of complex mixtures on oceanic phytoplankton, Echeveste et al. (2010) observed lethal effects on wild marine phytoplankton communities (Echeveste et al. 2010). These observations suggest an effect that goes beyond growth inhibition being an irreversible reduction of phytoplankton biomass and production. Similar effects were observed in our study for SC3 OO_X, SC5 HZ and SC5 HO extracts where exposure to REF ≥ 13 led to a decrease of the initial cell number, suggesting cell death (as

indicated in Figure 3 by growth inhibition values exceeding 100 %). Echeveste et al. (2010) concluded that levels of pollution reaching oceanic waters are approaching concentrations that significantly affect oceanic phytoplankton due to the complex cocktail formed by a mixture of a huge variety of chemicals (Echeveste et al. 2010).

4.4 *Ecotoxicity studies in the Belgian Part of the North Sea*

In previous studies with SR sheet passive samplers deployed in the same harbors as sampled in this study, Claessens et al. (2015) and Everaert et al. (2016) investigated effects of realistic mixtures of mainly non-polar contaminants on the growth of *P. tricornutum* (Claessens et al. 2015; Everaert et al. 2016). In these studies, the authors applied equilibrium-based partitioning (passive dosing) to spike algae test medium resulting in REFs of 1 under equilibrium conditions. When exposed to passive samplers deployed in three sampling campaigns between April 2008 and October 2009 at HZ and HO, *P. tricornutum* growth was inhibited up to 100 % in one fourth of the samples (Claessens et al. 2015). This suggests that the non-polar fraction of chemicals could significantly contribute to the toxicity of complex ERCMs on marine diatoms rather than the polar fraction of chemicals.

4.5 *Margin of safety approach – Possibilities & Limitations*

Overall the MoS approach allowed the definition of a "safety range" for the polar fraction of ERCMs in the BPNS. It provides a methodological concept for an effect-based monitoring approach that, with a few adaptations, could be transformed into a regulatorily useful effects-based method for mixture-based risk assessments. Below, we list possibilities and limitations.

4.5.1 Requirements for risk assessment

While our method was developed for testing with the marine diatom *P. tricornutum*, the basis for environmental risk assessment typically requires endpoints for at least 3 species representing three trophic levels (European Commission 2018b). Thus, in order to fulfill the requirements for the basic set of endpoints, our method would need to be adapted for the use with e.g. the 7-day larval development test with *Nitocra spinipes* and the fish early life-stage toxicity test. Of course, an extended test duration would be associated with additional challenges especially regarding the maintenance of constant exposure concentrations (Ribbenstedt et al. 2016).

4.5.2 Limited enrichment of extracts

In most cases, enriching ERCMs is needed in order to reach effect thresholds in various bioassays (Shaw et al. 2009). Most passive sampler extracts are spiked using a carrier solvent, hereby by default limiting the maximum extract concentration to a maximum of 0.1 – 1 % in biotests (Brack et al. 2016). The method applied here is not limited by any solvent since extracts are reconstituted in HPLC water. Nevertheless, limits are defined by the extract volume (1 mL) and the biotest volume (2 mL). For regulatory purposes testing a range of REF 1 – 10 would be ideal since i) testing of this small REF range reduces the spacing factor between each REF, whilst the number of tested REFs can remain the same, ii) when considering the typical assessment factor of 10 for e.g. laboratory to field extrapolation, observing no effects up to REF 10 can be considered equivalent to "no risks", and iii) testing this reduced concentration range is associated with reduced work load and costs.

4.5.3 Calculation of the REF

The calculation of the $REF_{geomean}$ is based on the individual REF_i of the measured contaminants. This is associated with one important limitation: targeted chemical analysis takes into account

only a pre-defined set of compounds and does not give any information about other substances present in the sampled mixtures. On the other hand, adsorption based samplers such as the Speedisks provide excellent enrichment of specific analytes (Jahnke et al. 2016). Further, chemical analysis and dosing of toxicity tests can easily be performed with the same set of samples (Moeris et al. 2019).

Further, we recognize that basing the MoS on the NOEC is less favorable than using the EC_{10} to derive the MoS. Yet, since a reliable EC_{10} derivation in this study was only possible for 3 out of 8 samples, we based our MoS definition on the NOEC here. Future studies might benefit from deriving the MoS from EC_{10} values rather than NOECs since the former are less dependent on the choice of test concentrations, spacing factors between consecutive concentrations in the test design, and power of the statistical test used. On the other hand, an accurate EC_{10} derivation requires the availability of sufficient test concentrations exerting actual effects in the biotest. A reliable and accurate EC_{10} will not always be guaranteed when working with passive sampler extracts and the final decision of whether to base the MoS on the EC_{10} or NOEC should then be made on a case-by-case evaluation.

5 CONCLUSION AND RECOMMENDATIONS

This research presents a novel effect-based method to derive the MoS for ERCMs in the marine environment based on passive sampling. H_2O-philic divinylbenzene, the sorbent included in the Speedisk passive sampler, has been shown to sample a broad polarity range of chemicals and reconstitution of the Speedisk extracts in HPLC water was a promising approach when combined with biotesting as it allows higher REFs to be tested. Samples with a REF > 13 have always been

shown to significantly affect the growth of *P. tricornutum*, and MoS were found to be in the range of 1.1 to 11 across four sampling locations (and three sampling campaigns) in the BPNS. For 5 out of 8 samples, the MoS was found to be lower than 10 which represents the typically lowest possible assessment factor applied to no effects ecotoxicological data in conventional environmental risk assessment, suggesting ecological risks exists for these sampling locations. In conclusion, we have taken a first important step in the development of a ready-to-use method for effect-based monitoring and risk assessment of ERCMs, which we here explored with the marine diatom *P. tricornutum* as test species. However, in order to make this methodology fully compliant with conventional risk assessment it would be required to extend the ecotoxicity test battery. We would recommend the adaptation of the method to at least one chronic test with a marine crustacean such as e.g. the larval development test with *N. spinipes* and one (sub)chronic test with fish such as e.g. the fish early life stage toxicity test with *Cyprinodon variegatus*. Strictly spoken, to be more applicable for the marine environment, two marine taxonomic groups should additionally be tested. In that case, and if it can be argued that (sub)chronic testing with 3 sensitive species from three trophic levels (plus 2 additional marine taxonomic groups) is sufficient to allow for an AF = 10, the MoS values could be translated into a conventional RCR of the ERCM, as follows.

$$RCR = \frac{10}{Minimum\ (MoS_{algae}, MoS_{crustacean}, MoS_{fish}, MoS_{marine1}, MoS_{marine2})} \quad \textbf{(Eq. 5.1)}$$

Optionally, the use of in-vitro assays such as e.g. the CALUX (Chemical Activated LUciferase gene eXpression) assay could help classifying the mode of action of the sampled ERCM to

identify the chemicals or groups of chemicals driving the toxicity within the ERCM. Alternatively, the combination of non-targeted chemical analysis and multivariate statistics is a promising approach for virtual EDA and may also help identifying substances driving toxicity or predicting toxic samples based on compositional similarity (Hug et al. 2015). Overall, our findings are in good agreement with other studies investigating potential effects of ERCMs to marine phytoplankton and confirmed that concentrations of ERCMs in marine waters are close to or even at levels that might be of concern for the Belgian marine environment.

Supplemental Data – The Supplemental Data are available on the Wiley Online Library at DOI:

CONFLICT OF INTEREST

The authors declare no conflict of interests.

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CONFLICT OF INTEREST STATEMENT

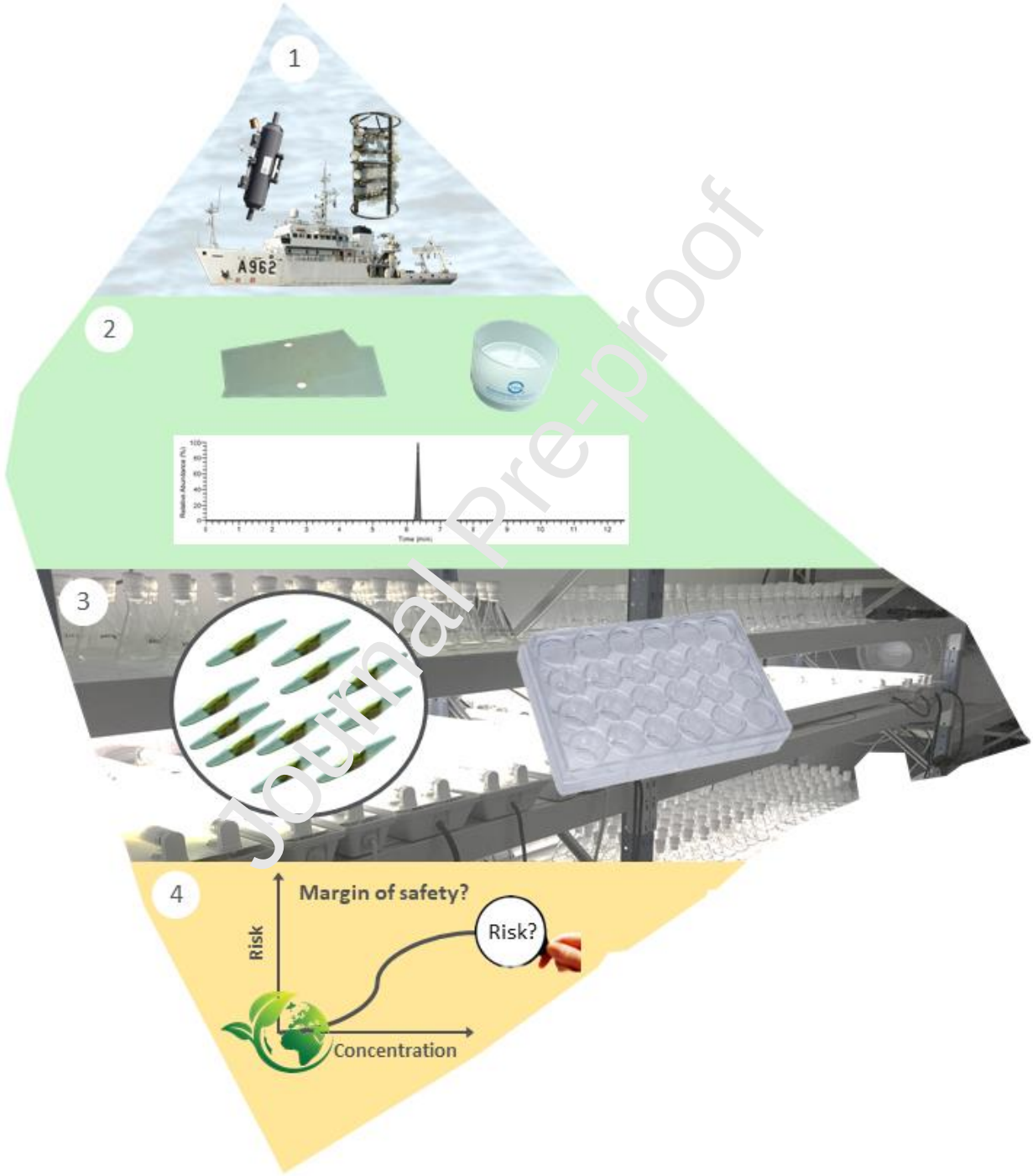
The authors declare no conflict of interests.

Declaration of interests

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

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Graphical abstract



HIGHLIGHTS

- Development of a novel effect-based method to derive a margin of safety for environmentally realistic chemical mixtures in the marine environment.
- Samples with a relative enrichment factor ≥ 13 always affected the growth of *P. tricornutum*.
- For 5 out of 8 samples the margin of safety was < 10, suggesting ecological risks for these samples