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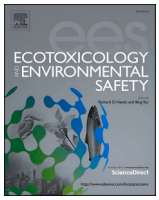

A novel extraction method of prymnesins from *Prymnesium parvum* whole culture samples and re-evaluation of existing protocols

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ABSTRACT

Harmful algal blooms caused by *Prymnesium parvum* can result in massive fish kills. The ichthyotoxins suspected to be responsible, known as prymnesins (PRMs), produced by *P. parvum* are classified into A-, B-, and C-types. In 2022, a severe HAB event within the Odra/Oder River impacted the environment via these toxins. The detection of PRMs is still a challenge due to a lack of analytical standards and stability issues along with losses during conventional extraction methods. In this study, PRM recovery was assessed for the three most common extraction methods for microalgae, solid-phase extraction (SPE), solid-liquid extraction (SLE), and liquid-liquid extraction (LLE). Furthermore, the impacts from sonication, freezing, and different solvents upon PRM stability were evaluated. Within methanol (MeOH), PRMs remained stable but within aqueous solvents, PRMs degraded rapidly. A novel 50 % MeOH SPE approach was developed, which outperformed LLE and SLE. This method offers advantages for salty whole culture samples through desalination and reduces PRM losses by the omission of reconstitution and evaporation steps. Ethanol as greener alternative to MeOH resulted in lower PRM yields compared to MeOH but showed similar recovery to LLE and still had advantages, besides the ecological ones, like faster and easier handling. The method's robustness was confirmed across all algal growth stages and for all PRM classes, including 6, 4 and 3 analogs for A-, B-, and C-types, respectively. The optimized SPE approach provides a faster, cleaner and more reliable extraction method for PRMs and improves analytical workflows for both natural and laboratory samples.

1. Introduction

Harmful algal blooms (HABs) are a global phenomenon which often lead to massive fish kills if toxic microalgae like *Prymnesium parvum* are involved (Svendsen et al., 2018; Hartman et al., 2021; Manning and La Claire, 2010). The toxins synthesized by this algal species are called prymnesins (PRM) and can be divided into three sub-groups namely A-, B-, and C-type PRMs, distinguished by the length of their carbon backbone with 91, 85, and 83 carbon atoms respectively (Rasmussen et al., 2016; Binzer et al., 2019). The species are therefore also categorized into A-, B-, and C-type producers. While A-type PRMs present the highest toxic properties (Varga and Prause et al., 2024), B- and C-types can also have a devastating impact on aquatic ecosystems. The most recent massive fish kill of a B-type *P. parvum* was in summer 2022 in the Odra/Oder River in Poland and Germany. According to estimates around 1000 tons of fish (Starck and Wolter, 2024), half of the whole population in the Odra/Oder River (Sobieraj and Metelski, 2023), were affected by this bloom. This species had not been reported in the river before. Presumably, high salinity and water temperatures in combination with low water levels led to a rapid increase of the algal population and associated PRMs (Free et al., 2023; Köhler et al., 2024). In contrast to other microalgal species like *Karlodinium veneficum* (Rasmussen et al., 2017; Binzer et al., 2020), PRMs seem to be mainly biomass bound. Therefore, the clear mechanism of how and under which circumstances PRMs reach and harm the affected fish is not fully understood. According to previous studies, around 25–46 % of the total PRM content is found in the supernatant after centrifugation, which is probably due to the loss of integrity of the algal cell membrane. Gentle filtration instead

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of centrifugation led to 4–18 % total PRM content in the filtrate (Svenssen et al., 2019). The impact of mechanical stress might also have played a role in the Oder scenario and could have contributed to the release of PRMs into the water, although this remains speculative at this stage.

An often used extraction method is solid-liquid extraction (SLE) of the algal cell pellet after centrifugation (Manning and La Claire, 2013; Rasmussen et al., 2016; Binzer et al., 2019; Taylor et al., 2020; Wagstaff et al., 2021; Anestis et al., 2021; Bannon et al., 2024), even though this method involves PRM losses during centrifugation and is limited to a certain cell number to create a cell pellet. Sonication is commonly used to support cell disruption in SLE protocols. In this study, it was evaluated whether using a sonication probe instead of the commonly applied ultrasonic bath could provide additional benefits. The use of a sonication probe is a common technique for biomass extraction, frequently applied in microalgae research, for instance, in the extraction of fatty acids (Castejón and Marko, 2022). Alternatively liquid-liquid extraction (LLE) of whole culture (containing biomass and culture medium) or cell-free supernatant without biomass was performed if SLE was not suitable (Medić et al., 2022). Despite higher workload, LLE, compared to SLE, has the advantage of a higher PRM yield, probably due to the use of whole culture instead of biomass only. However, both extraction methods still depend on a drying step which was found to be responsible for up to 75 % of the PRM losses (Svenssen et al., 2019). Solid-phase extraction (SPE) would address this problem, since the toxins are concentrated on the cartridge. Further, this would allow a complete desalination leading to cleaner samples (Dittmar et al., 2008; Brumovský et al., 2018). So far, the extraction yield from whole culture was not sufficient compared to the other two methods.

Within this project an optimization of the existing extraction methods and evaluation of various sample pretreatments was conducted to adapt the conditions and resulted in the development of a novel extraction method for PRMs in whole culture samples. The aim of this study was to further optimize this new extraction method, combining the advantages of all three mentioned extraction types to receive an easy, more efficient, and cleaner method for small scale natural or laboratory whole culture samples.

2. Material and methods

2.1. Preliminary note on method design and limitations of the study

Due to the lack of commercially available analytical standards for PRMs, quantification in this study is based on relative peak area comparisons. To minimize instrumental variability, all experiments involving direct method comparisons were conducted within a single whole culture batch on the same day. No comparisons were made across different LC-MS runs or over extended time periods.

The A-type *P. parvum* strain UTEX-2797 was chosen as model organism for most extraction experiments due to its fast growth and high PRM yield. This strain was not used as a quality control, but rather to standardize experimental conditions and reduce biological variability.

2.2. Reagents and chemicals

For extraction experiments and measurement, acetone (>99.8 %), acetic acid (≥99 %) and ethanol (EtOH) (analytical-grade) were sourced from Carl Roth GmbH & Co KG (Karlsruhe, Germany), while analytical-grade 2-butanol (2-BuOH) and LC-MS grade acetonitrile were obtained from Merck KGaA (Darmstadt, Germany). Formic acid (FA) (LC-MS grade) was obtained from Promochem/ScienTest-Biokemix GmbH (Wesel, Germany). LC-MS grade methanol (MeOH) and water were purchased from Honeywell-Riedel-de-Haën (Seelze, Germany) and VWR International GmbH (Radnor, USA) respectively. Ammonium formate (AF) was supplied by Fisher Scientific (Hampton, USA).

Reagents used for cell culture included Gibco™ Leibovitz (1x) L-15 medium without phenol red, Gibco™ Penicillin-Streptomycin (Pen-Strep) (10,000 U/mL penicillin, 10,000 µg/mL streptomycin), Gibco™ Trypsin, and Gibco™ Versene 1:5000, all purchased from Thermo Fisher Scientific Inc. (Waltham, USA). Fetal Bovine Serum (FBS) was purchased from Eurobio scientific (Les Ulis, France). Lugol's solution was prepared from 10 g potassium iodide and 5 g iodine in 100 mL deionized autoclaved water. Trypan Blue was sourced from Sigma Aldrich (St. Louis, USA). For the cytotoxicity assay a CellTiter-Blue® (CTB) reagent from Promega Corporation (Fitchburg, USA) was used. The ready-to-use L-15 medium containing 10 % FBS and 1 % penicillin-streptomycin is referred to as L-15 complete in the following.

2.3. Cultivation and harvest

Three different *P. parvum* strains were analyzed within this study, the A-type strain UTEX-2797 from the University of Texas at Austin Culture Collection of Algae (UTEX, Austin/TX, US), as well as the B-type ODER1 strain isolated from the Odra/Oder River during the bloom in 2022 (provided by Jan Köhler, Leibniz Institute of Freshwater Ecology and Inland Fisheries (IGB), Berlin, Germany) and the C-type strain RCC-7010 from the Roscoff Culture Collection of marine microalgae, macroalgae, protists, bacteria and viruses (Roscoff, France). The strains were cultivated under non-axenic conditions at 17 °C with a light:dark cycle of 16:8 h (30–40 µmol photons m^{-2} s^{-1}) and constant bubble aeration with sterile filtered air, except from the C-type strain. Filter-sterilized K-medium (Keller et al., 1987) with a salinity of 15 served as growth medium with the adjustment of artificial sea salt instead of actual sea water as described in the supplementary material (Table S1). Harvesting took place in the exponential growth phase and was performed for each experiment with the A-type strain at a similar timepoint around a cell density of 2.0×10^6 cells mL^{-1}. For the B-type 1.1×10^6 cells mL^{-1} were reached and only around 1.2×10^5 cells mL^{-1} for the C-type due to limited growth. Counting was performed with fixated cells (2 % Lugol's solution) on a Sedgwick-Rafter chamber. At least 400 cells per chamber were counted. A dilution with K-medium was conducted if cell density was too high for counting.

In this study, the term "whole culture" refers to unprocessed algal culture containing both cells and culture medium. The term "supernatant" refers to the cell-free culture medium obtained after centrifugation, while "biomass" indicates the pelleted cells.

For the toxic evaluation of FA as additive in the SPE protocol, the model cell line RTgill-W1, obtained from Kristin Schirmer (Department of Environmental Toxicology, EAWAG, Dübendorf, Switzerland) was used. It is an adherent growing cell line, derived from rainbow trout (*Oncorhynchus mykiss*) gills (Bols et al., 1994). Cultivation was performed as described in Pöchhacker et al. (2025) with Gibco™ Leibovitz's L-15 complete under sterile and dark conditions, at a temperature of 19 °C. Cells were passaged weekly. First, the old culture medium was removed, and the cells were rinsed using Gibco™ Versene solution. This was followed by detachment using 0.25 % Trypsin / 0.02 % ethylenediaminetetraacetic acid (EDTA) in phosphate-buffered saline (PBS). After detachment, the cells were resuspended in fresh medium and centrifuged at 50 rcf for 3 min at 21 °C. The supernatant was discarded, and the cells were again resuspended in fresh culture medium. They were then seeded into T-75 flasks at a density of $2–2.5 \times 10^4$ cells cm^{-1}.

2.4. Targeted UHPLC-MS analysis

For the targeted analysis of the PRM content multiple reaction monitoring (MRM) methods were developed and optimized to achieve high signals for all PRM analogs present in the samples. Due to the lack of analytical standards, method optimization was performed using flow injection analysis, column-based separation, and manual adjustment of instrument parameters. Transitions were selected based on enhanced product ion scans of selected precursors, as well as the PRM profiles reported for various A-, B-, and C-type P. *parvum* strains by Binzer et al.

(2019). Key mass spectrometer (MS) parameters, including de-clustering potential, collision energy, collisionally activated dissociation (CAD), spray voltage, source- and curtain gas and source temperature, were optimized to enhance signal intensity and improve overall method performance. Samples were measured on an ultrahigh-performance liquid chromatographic (HPLC) 1290 Infinity System (Agilent, Santa Clara, CA, USA) coupled to a QTrap® 6500 + (Sciex, Framingham, MA, USA) MS via an electrospray ionization source (ESI). Measurements were conducted in positive mode and fragmentation was achieved via collision-induced dissociation (CID). Corresponding MS parameters and transition lists for all three types are provided in Table S2 and S3 in the supplementary material. Separation took place using a Kinetex™ 1.7 μm F5 100 Å LC column 100 × 2.1 mm (Phenomenex, Aschaffenburg, Germany) at 35 °C with a flow rate of 0.4 mL min^{-1} and 5 μL injection volume. A gradient elution was performed with water as solvent A and acetonitrile/water (90:10, *v/v*) as solvent B, both with 0.1 % FA and 1 mM AF. For B- and C-type PRMs the runtime was adjusted due to shorter retention times. The chromatographic gradient is shown in Table S4 in the supplementary material. For data acquisition the vendor's software Sciex OS (version 3.3.1.43) and for data evaluation the open-source software Skyline (version 22.2.0.351) was used. Peak integration was performed manually in Skyline using consistent retention time windows and identical transitions across all samples within an experiment. Since all directly compared samples were measured within the same LC-MS run, inter-batch variability was excluded. In the supplementary material, Fig. S1 shows representative chromatograms for the selected transitions that served as the basis for relative quantification.

2.5. Pretreatment and reconstitution

In the following different pretreatments are described which were evaluated due to their properties to enhance the PRM yield. For all spiking experiments a highly concentrated crude PRM extract was applied. The A-type extract was generated by SLE from a cell pellet containing approximately 1.2×10^{10} cells which was stored at −80 °C. For the stability experiment (in 2.5.3.) an A-type and a B-type extract were utilized sourcing from pellets containing 1.2×10^{10} and 4.0×10^{9} cells respectively.

2.5.1. Sonication

A digital sonifier S-250D (Branson Ultrasonics™, CT, USA) and an ultrasonic bath USC200T (VWR®, Pa, USA) were compared regarding their cell disruptive efficiency. The two main goals were, first, to determine whether PRM could be released from the biomass in whole culture samples, allowing for the extraction of the supernatant. Second, the study aimed to assess whether the sonication probe could improve the efficiency of biomass extraction with MeOH, similar to how it is done during SLE. Pulse frequency was set to 0.1/2 s pulse on/ pulse off respectively and amplitude to 20 %, since preliminary experiments presented these settings as most promising. Besides that, different application times with the sonication probe compared to the default protocol of 30 min with the sonication bath (as described in 2.6.1.) were tested. During each sonication step, the samples were stored on ice to prevent heat buildup. Six different protocols were tested: whole culture with sonication times of 5, 7, 10, and 20 min and subsequent centrifugation; whole culture without centrifugation with a sonication time of 7 min; and, lastly, whole culture without any sonication or centrifugation. All treatments were performed in triplicate and extracted using SPE (as described in 2.6.3).

Furthermore, the impact of sonication was evaluated in different solvents to understand if the stability of PRMs depends on the solvent or the sonication process itself. Therefore, a spiking experiment was performed where a highly concentrated crude A-type PRM extract (see 2.5.) was spiked into whole culture, K-medium (salinity of 15), NaCl in distilled water (salinity of 15), distilled water, and MeOH. These mixtures, with a final dilution of 10:90 (*v/v*) crude extract to solvent, were sonicated for 7 min with an amplitude of 20 % and centrifuged at 4000 rcf for 10 min. The same procedure was performed on the non-spiked whole culture. From the supernatant 1.5 mL were transferred to an HPLC vial and stored at −80 °C until LC-MS analysis.

2.5.2. Freeze-thaw cycle

To evaluate the possible advantage of a freeze-thaw cycle for the extraction efficiency and what impact freezing has on the stability of PRMs in whole culture, the samples were stored at −80 °C and thawed at room temperature. The first extraction was conducted immediately with fresh whole culture without a freezing step (day 0). To evaluate the stability at −80 °C over a longer period of time, further extractions of thawed whole culture samples after 1, 7, 14, 35, and 56 days of storage at −80 °C were performed.

2.5.3. Stability in water and methanol

PRMs are known to have low stability in aqueous solutions, which is presumably one reason for which they are found in such low quantities in the cell culture medium and are mainly biomass bound (Svenssen et al., 2019). For this experiment a 1:50 dilution (1 mL in total) of crude PRM-A and -B extracts (see 2.5) in both LC-MS grade water and MeOH were prepared in 1.5 mL screw cap vials (short thread vial, 32 × 11.6 mm, 1st hydrolytic class, wide opening, ChromaGlobe, Kreuzau, Germany) and measured with UHPLC-MS for seven days (168 h) in total. Additionally, to test the impact of a possible adhesion to surfaces the same experiment was conducted in cylindrical micro-inserts (31 × 6 mm, clear glass, 1st hydrolytic class, max volume 0.35 mL, ChromaGlobe, Kreuzau, Germany) with 200 μL in total over six days (144 h).

2.5.4. Evaporation and reconstitution

For most extraction methods it is crucial to perform a concentration step by evaporating the extract to dryness and subsequent reconstitution in an appropriate solvent. For this experiment evaporation was achieved using a CentriVap −84 °C cold trap (Labconco Corporation; Kansas City, MO, USA) with 4 °C and 1725 rpm in the centrifuge. Evaporation of 10 mL solvent was performed overnight, and the dry residue was reconstituted with 1.5 mL MeOH/H_2O (90:10, *v/v*) by thorough mixing. The extract was then transferred to a HPLC vial after being centrifuged at 2000 rcf and 21 °C for 2 min to prevent potential particles from getting into the LC-MS system.

To evaluate the direct impact of evaporation and reconstitution on the PRM yield, 1.5 mL of a highly concentrated crude A-type extract (see 2.5.) was added to 8.5 mL MeOH and 2-BuOH respectively. Afterwards it was evaporated and reconstituted as described above. This experiment was conducted in triplicate and compared to measurements of the untreated crude extract.

2.6. Fundamental extraction approaches

In the following the protocols for each extraction method in terms of the procedure and solvent quantities are described. For each extraction an aliquot of 10 mL of a previously harvested whole culture was used. All extractions and subsequent measurements of each experiment were performed within a single batch and on the same day to ensure comparability and minimize variability.

2.6.1. Solid-liquid extraction

For this extraction each aliquot of whole culture was centrifuged at 4000 rcf, 21 °C, for 10 min, with low acceleration/deceleration to prevent cell disruption as much as possible. The resulting cell pellet was extracted as follows. As a purification step, 5 mL ice-cold (-20 °C) acetone was added to the cell pellet, thoroughly vortexed, and afterwards centrifuged (4000 rcf, 5 acc./dec., 4 °C, 15 min). The supernatant was decanted and the same amount of LC-MS grade MeOH was added. The suspension was vortexed and afterwards sonicated for 30 min in the

ultrasonic bath USC200T (VWR®, PA, USA). After centrifugation (4000 rcf, 9 acc./dec., 21 °C, 15 min) the supernatant was collected. The MeOH extraction step was repeated, and the combined MeOH extracts were evaporated and reconstituted as described in chapter 2.5.4.

2.6.2. Liquid-liquid extraction

For better recovery whole culture samples were adjusted to a salinity of 30, according to the findings of Svenssen et al. (2019). The original protocol was translated to a small-scale extraction in 15- and 50-mL polypropylene tubes instead of using a separatory funnel. To allow proper mixing, 50-mL tubes were used until the final washing step, where 15-mL tubes offered a smaller surface and facilitated the transfer of the organic phase. This adjustment allowed the extraction of smaller volumes from whole culture and to perform the separation of the solvent phases in the centrifuge to speed up the process. The solvents and ratios used were adopted from Svenssen et al. (2019) but scaled down. Technically 5 mL 2-BuOH were added to 10 mL of whole culture and mixed for five minutes on an overhead shaker before centrifugation (2000 rcf, 21 °C, 5 min). After centrifugation the upper organic phase was transferred to another tube. This step was repeated twice with 2.5 mL 2-BuOH. After combining the organic phases three washing steps using LC-MS grade water were conducted. The same procedure as before was performed with respect to volume and centrifugation. The final extract was evaporated and reconstituted as described in 2.5.4.

2.6.3. Solid-phase extraction

For solid-phase extraction Isolute® C8 columns (100 mg/ 3 mL) (Biotage, Uppsala, Sweden) were used, based on the protocol of Svenssen et al. (2019). The protocol was performed with some adjustments to the original procedure as follows. SPE cartridges were conditioned with 3 mL MeOH and equilibrated with 3 mL H_2O, both containing 1 % FA. After sample loading, a washing step with 3 mL H_2O/MeOH (60:40, *v/v*) and 1 % FA was conducted, followed by an elution with 1.5 mL MeOH/H_2O (90:10, *v/v*) directly into a HPLC vial.

Small adjustments regarding the use of FA were implemented. Due to the toxic nature of FA in cell culture, which could interfere with the toxicity of PRMs in respective cell culture experiments, it was evaluated whether FA could be eliminated from the entire protocol or at least from the final elution step. Alternatively, the possibility of replacing FA with a potentially less toxic compound, such as acetic acid (HAc, 1 %), was investigated (see 2.9).

2.6.4. Combination of extraction methods

The three previously described extraction methods each have their own advantages and disadvantages. To maximize their benefits, different approaches were tested to develop a method that combines high extraction efficiency, low matrix effects, and ease of use.

The concept involved combining SLE with SPE by separately extracting the cell pellet (as described in 2.6.1) without the drying step, then loading both the supernatant and the crude extract onto the same SPE cartridge for concentration and purification. To enable loading the crude MeOH extract onto the cartridge, the appropriate dilution needed to be identified to prevent premature elution. Various protocols were tested, using eluents with increasing MeOH content, starting from 10 % MeOH to 50 % MeOH. Preliminary results suggested that adding NaCl (final salinity of 15) to the water enhanced interactions between PRMs and the solid phase of the cartridges.

Based on these findings, the protocol was adapted to simplify the process. Instead of extracting the cell pellets separately, the entire culture was directly mixed with MeOH before loading onto the SPE cartridge. This step was also tested with varying MeOH concentrations based on the results of earlier experiments. Technically, 10 mL whole culture was mixed with the respective amount of MeOH (30, 40 and 50 % MeOH in total) 20 min before loading. The loading process was performed with an approximately flow rate of 1 drop per second. It was crucial to keep the incubation time before loading and the loading time itself constant throughout the experiments to guarantee reproducibility. Fig. 1 shows a schematic of the final SPE method, which uses a 50:50 mixture of whole culture and MeOH, with FA omitted in the elution step.

2.7. Green extraction

With respect to greener extraction and the amount of solvent used, the extraction efficiency of EtOH compared to the classic MeOH SPE was evaluated. Since MeOH has higher polarity, the elution behavior of PRMs in EtOH was examined within a gradient elution experiment to find the right composition for each SPE step. Finally, a direct comparison between MeOH and EtOH in the SPE approach and LLE was conducted. Based on the first results, the washing step was adjusted to only 20 % EtOH.

2.8. Application for different PRM types

Due to the different lengths of the backbones, A-, B- and C-type PRMs show distinct elution behavior. With a total of 83 carbon atoms in the backbone, C-type PRMs elute first during a MeOH gradient elution on a reversed phase SPE cartridge. A-type PRMs with the longest backbone, containing 91 carbon atoms elute after B-type PRMs with 85 carbon atoms (Binzer et al., 2019). The number of sugar conjugates also has an impact on the polarity of the molecules and needs to be taken into consideration. To achieve the highest PRM yield possible, the ideal MeOH content for each type was evaluated by testing different concentrations, always in comparison to LLE.

2.9. The impact of formic acid during SPE and cell culture experiments

For the use of PRM extracts in cell culture experiments, such as toxicity tests, the impact of the solvents used on the model cell line RTgill-W1 was evaluated. First of all, the concentration of MeOH that can be applied to the cells without influencing viability and in a next step the use of additives during the SPE protocol was evaluated. To apply realistic conditions, the full SPE protocol (as described in 2.6.3) was performed but instead of whole culture, the respective blank K-medium was used. Four different conditions were tested, the a) standard protocol with 1 % FA as additive in all steps, b) HAc (1 %) as substitute for FA, c) omitting FA in the elution step and d) without the use of any additive. The cell viability assay CellTiter-Blue® (Promega Corporation, Fitchburg, MA, USA), was performed in 96-well cell culture plates, according to Pöchhacker et al. (2025). The respective extracts were diluted 1:10, 1:30, 1:50 and 1:100 in Leibovitz's L-15 complete medium (Gibco™) and incubated for three hours. Pure Leibovitz's L-15 complete medium was applied to cell containing wells and served as reference for 100 % viability.

Besides decreasing toxicity within cell culture experiments, PRM extraction efficiency still plays a major role. Therefore, each of these settings was also tested on whole culture to see whether FA could be left out or replaced at some point.

2.10. Extraction over the growth curve of an A-type producer

To test the extraction efficiency of this method in each point of the growth curve, hence different cell densities, extractions from fresh whole culture aliquots were conducted over 58 days with an initial cell density of 1×10^3 cells mL^{-1}. This time span includes the initial lag phase, as well as the final lethal phase. Additionally, LLE was performed regularly over that time to serve as reference, since it was the original way to extract whole culture. For cell counting two aliquots of the carefully but thoroughly mixed whole culture were withdrawn, fixated with 2 % Lugol solution and applied to one Sedgwick-Rafter chamber each. At least 400 cells per chamber were counted. A dilution with K-medium was conducted at later timepoints when cell density was too high for counting.

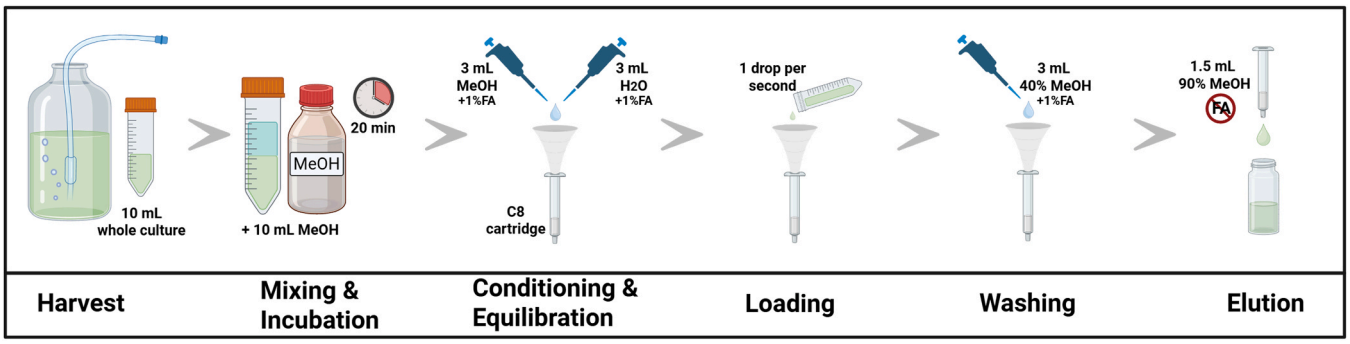

Fig. 1. Recommended solid-phase extraction (SPE) workflow used for prymnesin extraction from whole culture samples. A 50:50 (v/v) mixture of whole culture and methanol (MeOH) was prepared, briefly mixed by hand and incubated for 20 min at room temperature. The sample was then loaded onto a conditioned (MeOH with 1 % formic acid (FA)) and equilibrated (water with 1 % FA) C8 SPE cartridge. Following a washing step with 3 mL MeOH/water (40:60, v/v) was conducted. Elution was performed using 1.5 mL MeOH/water (90:10, v/v), without the addition of FA. The eluate was collected and stored at −80 °C for further analysis.

2.11. Statistical analysis

Statistics were performed in R version 4.4.0 and visualization of the results was conducted in OriginPro 2022 (9.9.0.225). Tukey HSD served as post-hoc test to assess significant differences between groups after one-way ANOVA.

3. Results

3.1. Pretreatment and reconstitution

3.1.1. Sonication probe

The results of the sonication experiments presented a negative impact of sonication in whole culture samples with regard to the total PRM yield (Fig. 2A). The lowest yield was obtained by extracting from the supernatant only, followed by sonicated whole culture. In comparison, loading whole culture directly on the SPE column had a significantly higher yield than any of the sonication treatments ($F(5, 12) = 47.86$, $p < 0.001$).

To test whether the solvent or the sonication process itself influences the PRM yield, spiking experiments with different solvents were conducted. Clear differences in PRM yield between the different solvents were observed (Fig. 2B) ($F(5, 12) = 33.02$, $p < 0.001$). The highest recovery was determined in spiked MeOH, followed by spiked water and spiked NaCl in water (salinity of 15). Even though whole culture itself contained PRMs, the spiked whole culture had a slightly lower, but non-significant, PRM yield than the MeOH sample. There was no difference in spiked MeOH observed whether it was sonicated or not ($t(4) = 1.09$, $p = 0.34$) (Fig. S2A). The extraction efficiency of the sonication probe in SLE instead of ultrasonic bath showed no difference with respect to the overall PRM yield between these two methods ($t(3) = 0.78$, $p = 0.49$) (Fig. S2B).

3.1.2. Stability of PRMs under different treatments

Due to significant changes in PRM yield in different solvents, the long-term stability of PRMs in water and MeOH was evaluated. In 1.5 mL vials there was a significant difference after 24 h between MeOH and water for both, the A-type and the B-type extract (Fig. 3A and B).

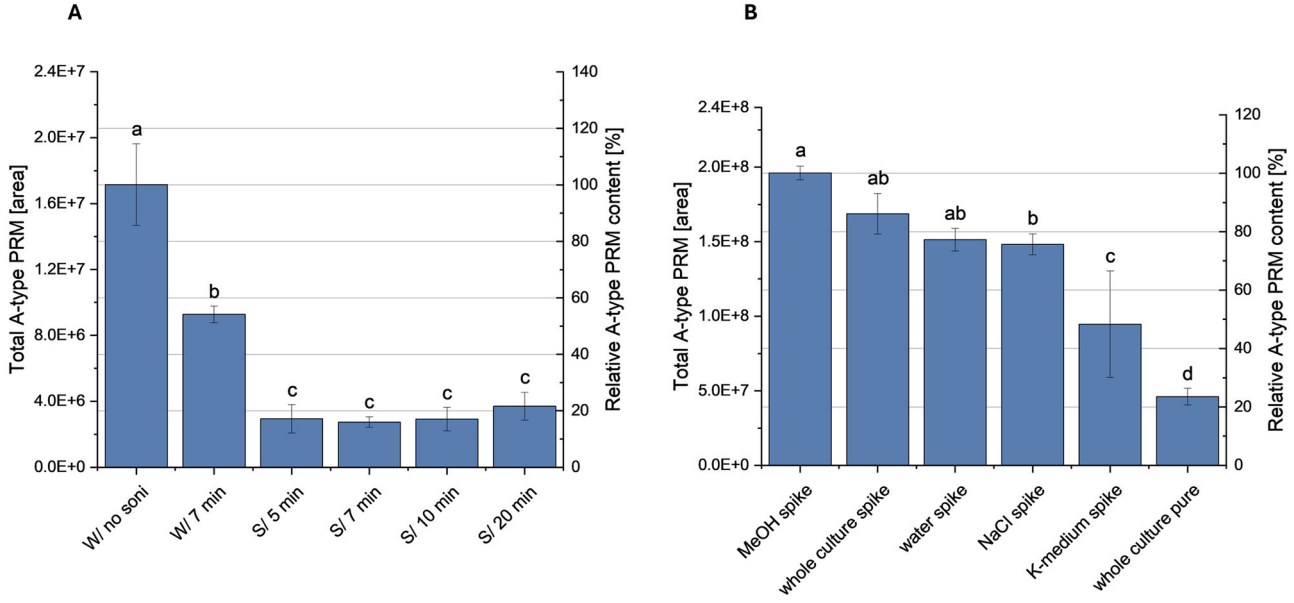

Fig. 2. A) Sonication probe treatment of A-type prymnesin (PRM) whole culture followed by solid-phase extraction (SPE). Sonication was performed with an amplitude of 20 % for the respective time period. Treatments marked with "S" indicate that after sonication centrifugation was conducted and only the supernatant was further extracted with SPE. Samples marked with "W" represent those which were extracted directly as whole culture. B) Sonication probe treatment (7 min and 20 %) on different solvents spiked with an A-type PRM extract (1:10, v/v). For comparison, SPE was performed on spiked and pure whole culture. Statistically significant differences between treatments ($n = 3$) were assessed by one-way ANOVA followed by Tukey's HSD test ($p < 0.05$). Different letters indicate significant differences.

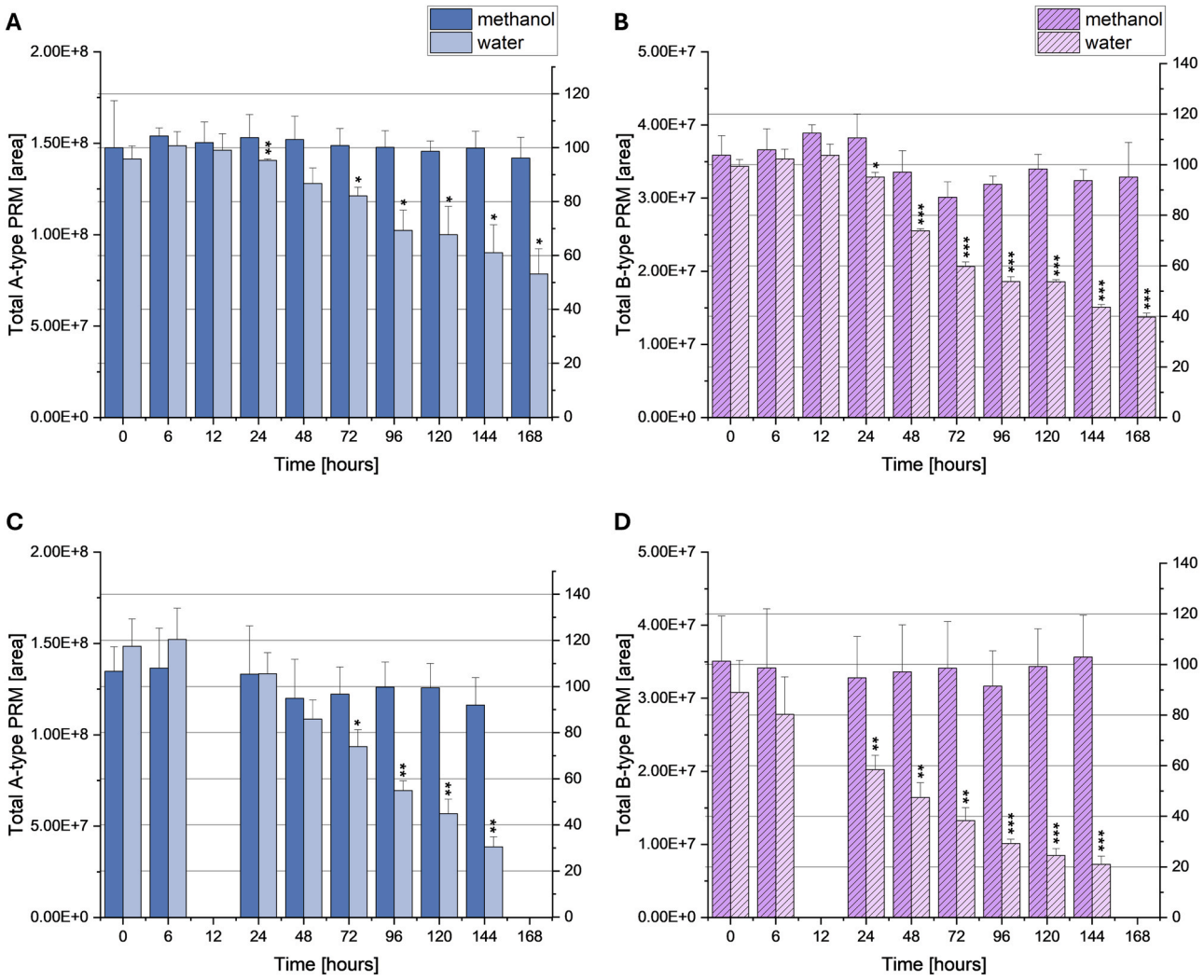

Fig. 3. A) and B) The stability of A- and B-type prymnesins (PRMs) spiked in 1 mL water and MeOH (1:50, v/v) was evaluated over seven days (168 h). C) and D) The stability of A- and B-type prymnesins (PRMs) spiked in 200 µL water and MeOH (1:50, v/v) in cylindrical micro-inserts was evaluated over six days (144 h). Statistically significant differences between treatments (n = 3) were assessed by one-sample *t*-test in comparison to the mean value of the MeOH measurements (set as 100 % in the graph). Statistical significance is indicated by *, ** or *** for p-values < 0.05, < 0.01 and < 0.001 respectively.

After seven days (168 h) only 53 % and 40 % of A- and B-type PRMs, respectively, were present in water compared to the MeOH mean value. For the samples measured in cylindrical micro-inserts only 31 % in the A-type and 21 % in the B-type sample were determined after six days (144 h), which is half the amount of the respective measurement in 1.5 mL vials (Fig. 3C and D).

The impact of storing whole culture samples at −80 °C on the PRM yield was evaluated. This experiment showed that already one freeze-thaw process had a negative impact on the PRM yield ($F(5, 12) = 25.17$, $p < 0.001$) but the duration of storage did not show a clear trend due to a high variation between measurements. Still, each of the thawed samples was significantly different to day 0 (Fig. S3).

Since evaporation and reconstitution is a common procedure within an extraction protocol, the recovery of PRMs during these steps was tested. There was a significant decrease in A-type PRM yield after evaporation and reconstitution in MeOH and 2-BuOH with a recovery of 92 % and 86 % respectively ($F(2, 6) = 112.2$, $p < 0.001$) (Fig. S4).

3.2. Methanol content for the loading process during SPE

To find the right composition of MeOH and whole culture, experiments were conducted with three different *P. parvum* strains (Fig. 4). Due to structural differences between A-, B-, and C-type PRMs, the polarity and hence the elution behavior, was distinct. C-type PRMs showed the highest polarity, followed by B- and A-types. For all three types, a MeOH content of 50 % during the loading process showed the best extraction efficiency. Since C-types have the highest polarity, a concentration of 30 % was tested as well but showed a significantly lower PRM yield than the 50 %. For all three types, LLE performed worse or as good as the new SPE protocol.

3.3. Substitution of methanol with ethanol as greener alternative during SPE

The gradient elution of A-type PRMs with EtOH instead of MeOH showed that a relevant amount of PRMs eluted already at 50 %. Due to the higher polarity of MeOH the elution behavior was shifted to lower concentrations (Fig. 5A) (Snyder, 1978). To support this finding, three different sample loading settings with varying EtOH concentrations were performed (Fig. 5B). In contrast to MeOH, a concentration of 40 % EtOH offered better extraction efficiency than 50 % EtOH but still resulted in a lower PRM yield than the MeOH protocol. Compared to LLE it showed similar results.

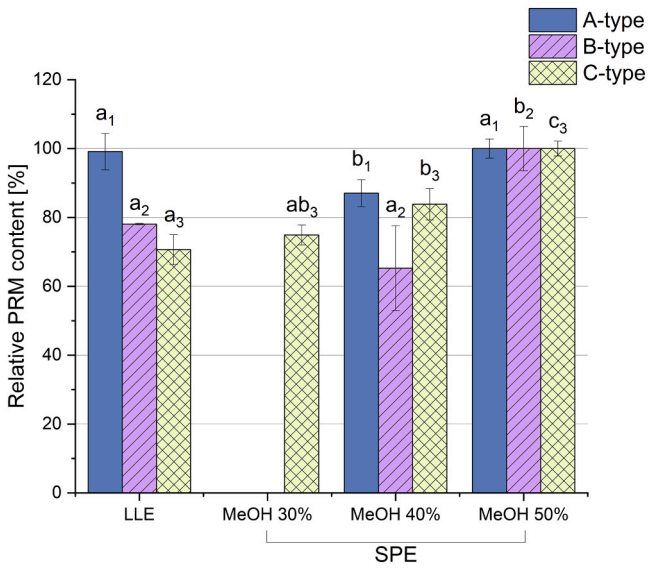

Fig. 4. For all three prymnesin (PRM) types the ideal amount of MeOH (30 %, 40 %, 50 %) during the loading process for solid-phase extraction (SPE) was assessed. This took place in comparison to liquid-liquid extraction (LLE). Statistically significant differences between treatments (n = 3) were assessed by one-way ANOVA followed by Tukey's HSD test ($p < 0.05$). Different letters indicate significant differences.

3.4. The role of formic acid in cell culture and PRM extraction

Cell culture experiments with the cell line RTgill-W1 showed that both additives, FA and HAc, had a significant impact on cell viability, with FA being more toxic than HAc (Fig. 6). No significant difference in cell viability was observed when omitting FA from the elution step only.

Regarding PRM extraction efficiency, no significant difference was present in SPE regardless of whether HAc or FA was used (Fig. 7A). The omission of additives from the whole protocol led to a significant decrease in PRM yield. A comparison between SLE, LLE and SPE with whole culture presented clear differences between the 50 % SPE protocol with additives and SLE and SPE with whole culture, while LLE showed no significant decrease in PRM yield. Similar to the results from the cell viability test, there was no significant difference in PRM yield when omitting FA from the elution step only (Fig. 7B).

3.5. Final comparison of the three most common extraction methods for microalgae

After the optimization of the SPE extraction protocol, the three extraction types, and pure whole culture on SPE were compared. The results showed that the 50 % MeOH SPE method offered the highest PRM yield followed by LLE (Fig. 8A). Additionally, the impact of these extraction methods on the ratio between PRMs with and without sugar conjugates was shown on the example of 3Cl-PRMs (Fig. 8B). The amount of sugar-conjugated analogs was significantly higher in LLE and 50 % SPE compared to SLE and SPE conducted with pure whole culture.

3.6. Performance of the extraction method over the growth curve of an A-type P. parvum strain

For 58 days, twice a week triplicates of whole culture, 10 mL each, were extracted by SPE with the newly developed 50 % method. At five timepoints also LLE was performed as reference method. SPE presented a similar or higher extraction yield compared to LLE. As presented in Fig. 9A, the algal growth showed a steep increase after a short lag phase, followed by a plateau and finally went through the lethal phase. The first extraction took place on day 7 with a cell density of around 2.2×10^4 cells mL^{-1}, at which PRMs were already detected (Fig. 9B). PRM concentration increased more slowly compared to cell number and was still increasing while the algal growth state was already in the plateau phase (Fig. 9A). After the onset of cell death, PRM concentrations also began to decrease after a short delay. On day 52 LLE presented an outlier (marked in red) with a drastic decrease in PRM yield, which can be explained by technical issues and longer processing times that day.

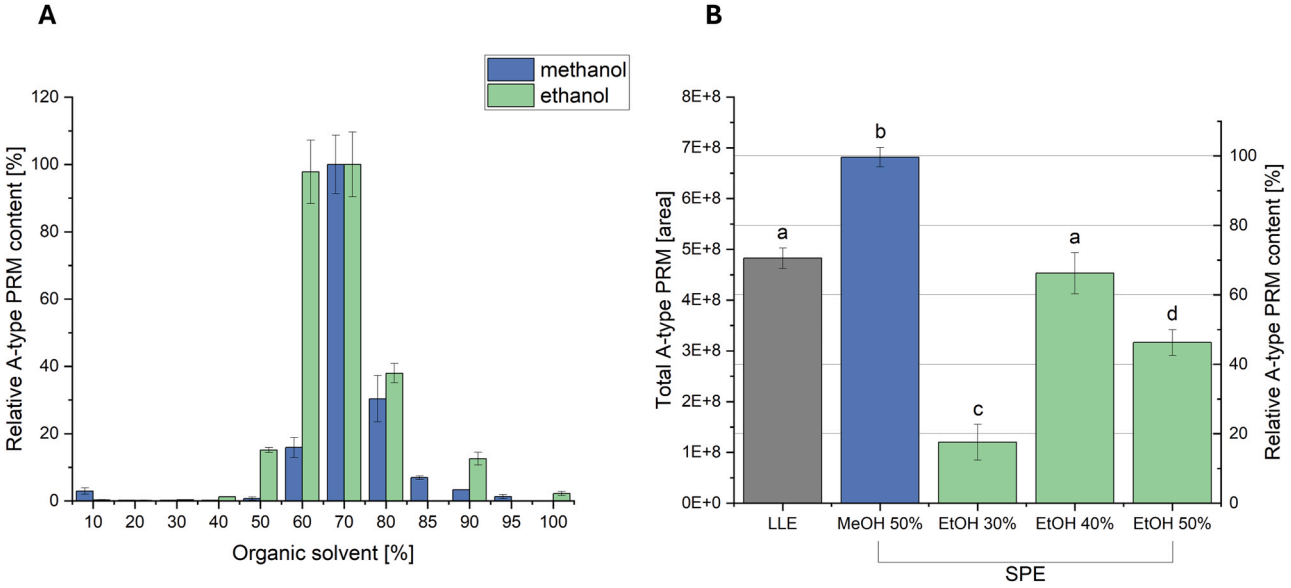

Fig. 5. A) Gradient elution of A-type prymnesins (PRMs) from solid-phase extraction (SPE) cartridges using increasing methanol (blue)/ethanol (green) concentrations. After loading the sample, the SPE cartridge was eluted stepwise with 1.5 mL aliquots at increasing concentrations (10–100 %). Each fraction was collected separately and analyzed for PRM content. B) Different ethanol concentrations (green) during the loading process of SPE were tested. It was compared with the results of liquid-liquid extraction (LLE) and SPE with 50 % MeOH loading. Statistically significant differences between treatments (n = 3) were assessed by one-way ANOVA followed by Tukey's HSD test ($p < 0.05$). Different letters indicate significant differences.

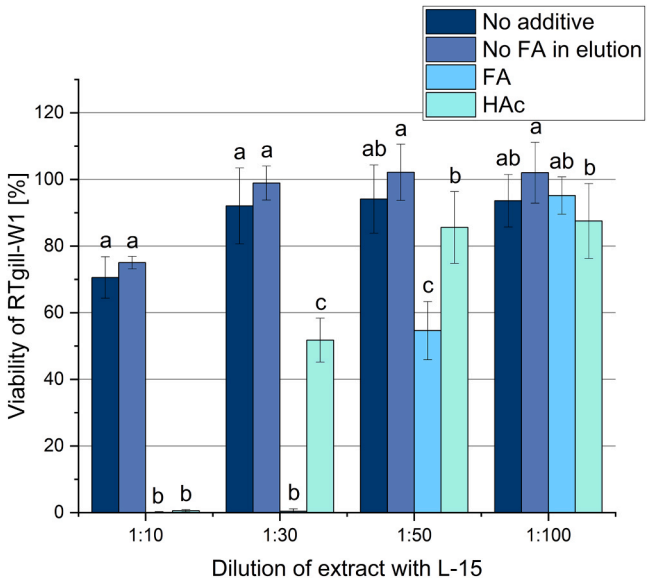

Fig. 6. CellTiter Blue® (CTB) assay: The impact of formic acid (FA) during cell culture experiments was evaluated as well as the substitution with acetic acid (HAc) and the omission of FA only in the elution step of solid-phase extraction (SPE). The respective extracts were diluted with L-15 complete medium as indicated on the X-axis. Statistically significant differences between treatments ($n = 3$) were assessed by one-way ANOVA followed by Tukey's HSD test ($p < 0.05$). Different letters indicate significant differences.

4. Discussion

The extraction of PRMs is essential for monitoring and research of *P. parvum* blooms but existing methods such as SLE, LLE, and SPE have significant limitations. To address these challenges, this study focused on optimizing PRM extraction by refining sample pretreatment and combining the advantages of all three methods.

Since PRMs are mainly biomass-bound (Svenssen et al., 2019), it was investigated whether a sonication probe would enhance extraction efficiency. The approach involved sonicating whole culture samples to release PRMs into the surrounding culture medium, followed by centrifugation and extraction of the supernatant. However, this method failed due to the low stability of free PRMs in the culture medium (Fig. 2A). Additionally, the observation that sonicated whole culture samples yielded higher PRM concentrations than their respective supernatants suggests that PRMs were not completely released by sonication and remained partially bound to the biomass. Stability issues alone cannot fully explain this result. Importantly, no difference was observed between sonicated and non-sonicated spiked MeOH samples (Fig. S2A), indicating that the sonication process itself does not degrade PRMs. The factors influencing PRM stability in the culture medium remain unclear, as water and saltwater did not produce comparable results. However, after 24 h, a significantly lower PRM content was observed in water compared to MeOH for both A- and B-type PRMs (Fig. 3). The experiment was conducted in 1.5 mL screw cap HPLC vials and 200 µL cylindrical micro-inserts and revealed a substantial deviation between these setups. After seven days (144 h) only half the relative amount of PRMs was detected in the water samples measured in micro-inserts compared to those in 1.5 mL HPLC vials. This was observed in A-type as well as in B-type samples. A-type PRMs seem more affected by micro-inserts, as their content dropped noticeably after just 24 h, while B-type PRMs only showed a significant change after 72 h. One hypothesis might be that A-type PRMs, due to their lower polarity present higher affinity to surfaces, such as in the HPLC vials. Due to the lower sample volume, the effect might be enhanced in micro-inserts. Based on this low stability of PRMs in water and K-medium (Fig. 2B, Fig. 3), and no advantage in extraction efficiency compared to the sonication bath (Fig. S2B), a sonication probe is not recommended for PRM extraction.

SPE proved to be the most suitable extraction method due to its efficiency in desalting, purification, and time savings. In contrast to SLE and LLE, SPE does not require additional evaporation and reconstitution steps. SLE and LLE are both time-consuming and a source of PRM losses, responsible for up to 75 % of the total PRM loss, as reported by Svenssen et al. (2019), and supported by the current results (Fig. S4). An initial experiment, in which SLE extracts were mixed with saltwater and pure water, respectively, prior to SPE, revealed improved PRM interaction with the sorbent material under saline conditions. Based on this finding, further experiments focused on optimizing the direct loading of whole

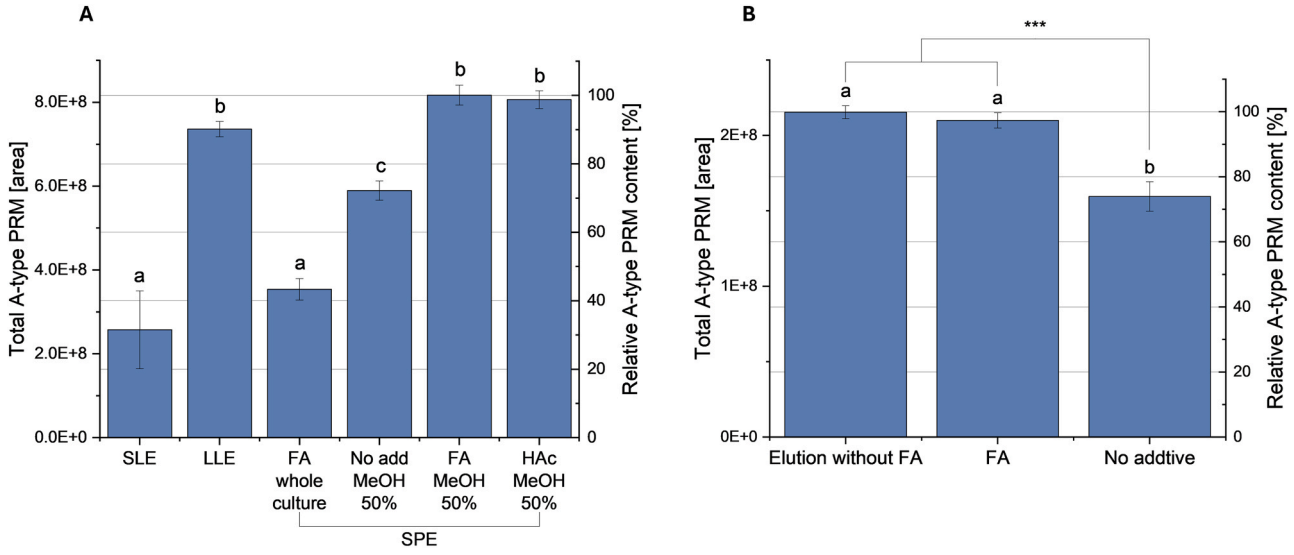

Fig. 7. Evaluation of the influence of additives on the prymnesin (PRM) yield during solid-phase extraction (SPE). A) displays a comparison between solid-liquid extraction (SLE), liquid-liquid extraction (LLE) and different SPE protocols. SPE was performed either with pure whole culture or 50 % methanol (MeOH) loading. The latter was conducted with standard formic acid (FA), acetic acid (HAc) or without any additives. B) Based on the findings shown in Fig. 6, the omission of FA in the elution step was tested. It was compared to an SPE protocol without additives and the standard SPE protocol with FA in all steps. Statistically significant differences between treatments ($n = 3$) were assessed by one-way ANOVA followed by Tukey's HSD test ($p < 0.05$). Different letters indicate significant differences.

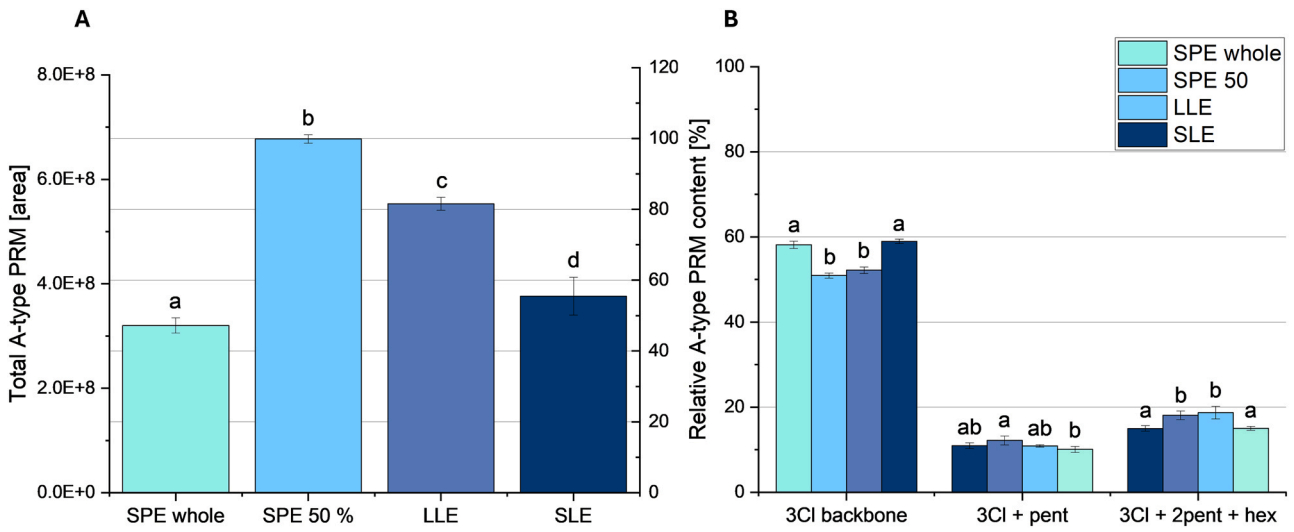

Fig. 8. A) Comparison of common extraction methods with respect to the total prymnesin (PRM) yield. B) The effect of different extraction methods on the ratio between sugar-conjugated analogs to only backbone on the example of A-type PRMs with a 3Cl backbone. Statistically significant differences between treatments ($n = 3$) were assessed by one-way ANOVA followed by Tukey's HSD test ($p < 0.05$). Different letters indicate significant differences.

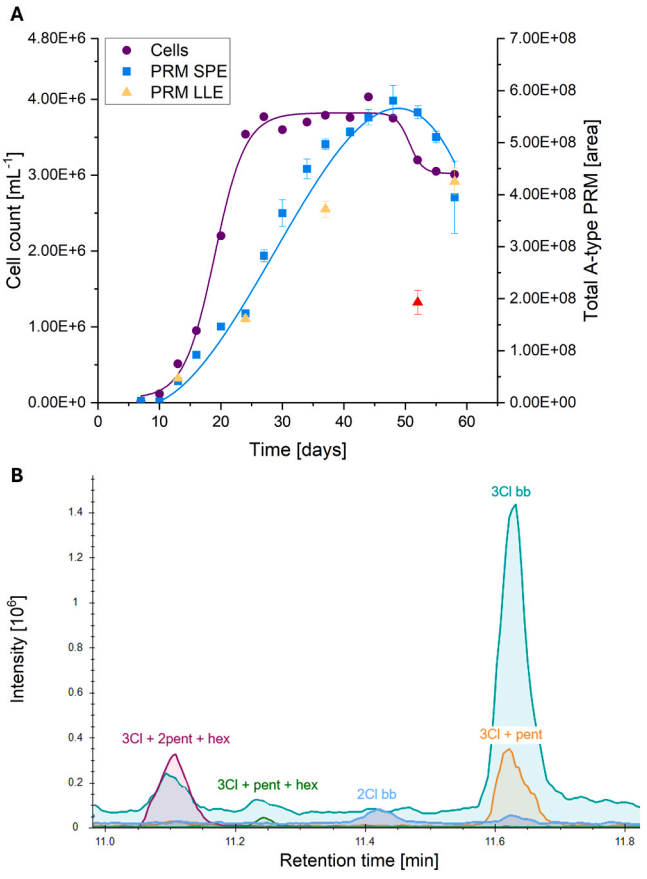

Fig. 9. A) Growth curve of the A-type strain UTEX-2797 ranging from day 7–58. Cell counting and extractions of 3 technical replicates were performed twice a week. The purple dots represent the cell count with the initial lag phase, exponential growth phase, plateau- and lethal phase. In blue squares the prymnesin (PRM) content as area is shown. Liquid-liquid extraction (LLE) (yellow triangles) was performed over the growth curve as a reference. Due to prolonged processing times during LLE an outlier occurred which is marked in red. B) Excerpt of an extracted-ion chromatogram of one replicate from day 7. Transitions of 2Cl- and 3Cl-backbone analogs with different sugar moieties are presented in color.

culture samples onto the SPE cartridge without prior SLE extraction. To determine the ideal MeOH concentration for the loading process, different MeOH-to-whole-culture ratios were tested and compared to LLE. The optimized SPE protocol, using 50 % MeOH for sample loading, provided the best results across all PRM types (Fig. 4). The improved performance of a MeOH-whole-culture mix compared to pure whole culture could be attributed to several factors. Immediately after mixing, a color change from brownish-green to light green was observed, indicating chlorophyll release due to cell lysis. The 20-min incubation prior to loading probably promoted extraction and facilitated PRM accessibility to the SPE stationary phase, while also preventing cartridge clogging. Clogging issues were particularly problematic with pure whole culture and lower MeOH concentrations.

Previous studies have shown that SPE cartridges can be reused, and the method requires less solvent than LLE, making it more cost-efficient and environmentally friendly (Dittmar et al., 2008; Robles-Molina et al., 2013). To find an even greener variant of the extraction method, the replacement of MeOH with EtOH was tested. A gradient elution experiment comparing MeOH and EtOH on A-type PRMs, revealed a shift to lower organic solvent concentrations (Fig. 5A). Comparisons between different EtOH concentrations and 50 % MeOH during SPE and LLE indicated that the 50 % MeOH protocol remains the method of choice (Fig. 5B). However, 40 % EtOH performed comparably to LLE while still offering advantages such as ease of handling, reduced time consumption, and high extract purity.

To validate the new extraction method across different growth stages and cell densities, an A-type *P. parvum* strain was cultured for 58 days, with extractions performed twice a week starting on day 7 after inoculation (Fig. 9A). Extractions were carried out immediately after sample collection, as freezing whole culture samples at −80 °C led to significant PRM losses after a single freeze-thaw process (Fig. S3). At extended intervals, LLE was conducted for comparison. On day 52 of the growth curve experiment, LLE produced an extreme outlier likely caused by technical issues that resulted in prolonged standing times (15 min instead of the standard 5 min). These findings highlight the importance of standardized extraction protocols and demonstrate the low robustness of LLE for this sample material. On the final day of extractions, high standard deviations were observed, likely due to poor culture conditions with many dead cells forming aggregates that interfered with SPE loading. These aggregates led to cartridge clogging and inconsistent loading times. Under real life conditions cell numbers between 3 and 4×10^6 cells mL^{-1} as present from day 24–58 are quite unlikely. During

the *P. parvum* bloom in 2022 in the Odra/Oder River a maximum of 1×10^5 cells mL^{-1} was recorded (UBA-Umweltbundesamt, 2022). Usually, ichthyotoxic *P. parvum* blooms are observed at cell densities around $\geq 10^4$ (Roelke et al., 2011; VanLandeghem et al., 2015) but exceptionally high cell numbers between 1 and 10×10^2 cells mL^{-1} have been reported (Caron et al., 2023). This study offered a reliable detection of PRMs already at the first time point with a cell number of 2.2×10^4 cells mL^{-1} (Fig. 9B). Since the amount of toxins produced and overall toxicity do not necessarily correlate with cell number, but are instead influenced by growth stage, nutrient availability, environmental stressors, and the considerable diversity between and within microalgal strains, placing too much emphasis on cell counts can be unreliable (Ross et al., 2006; Qin et al., 2020; Li et al., 2023; Linares-Maurizi et al., 2024; Pöchhacker et al., 2025). Recent findings also highlight genetic and metabolic variability among *P. parvum* strains, even within a single bloom. It was suggested that toxicity also depends on the analog of PRM produced, since strong cytotoxic effects have been present even at moderate cell densities (Mazur-Marzec et al., 2025). Therefore, efforts should focus on developing detection methods that are as sensitive as possible. This is particularly important in the context of early warning and monitoring systems.

In addition to generating clean extracts, it might be necessary to apply these extracts in cell culture experiments for toxicological testing. Tests on acidic additives showed that both FA and HAc negatively affected cell viability (Fig. 6) but could not be omitted due to their critical role in PRM extraction (Fig. 7A). A solution for this problem was found by omitting FA in the final elution step only, which minimized toxicity in RTgill-W1 cells without reducing PRM yield (Fig. 7B). Furthermore, the study demonstrates that FA can be effectively replaced by HAc, which is not only less toxic in cell culture experiments but offers clear advantages in terms of safety and regulatory requirements.

In conclusion, the newly developed SPE protocol utilizing a 50 % MeOH-whole culture mix offers cleaner, desalinated extracts with increased PRM yield, and reduced workload compared to other common extraction methods such as SLE and LLE. Since the method does not require a drying step, water samples can be collected and extracted directly on site. This enables real-time assessment without the risk of PRM degradation or continued algal growth and toxin production.

CRediT authorship contribution statement

Magdalena Pöchhacker: Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Alexander Conrad:** Methodology, Investigation, Formal analysis, Data curation. **Doris Marko:** Writing – review & editing, Supervision, Resources, Funding acquisition. **Elisabeth Varga:** Writing – review & editing, Supervision, Resources, Project administration, Methodology, Funding acquisition, Conceptualization.

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Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Elisabeth Varga reports financial support was provided by Austrian Science Fund. Elisabeth Varga reports financial support was provided by Federal Ministry of Education and Research Berlin Office. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2025.118745.

Data availability

Data will be made available on request.

References

Anestis, K., Kohli, G.S., Wohlrab, S., Varga, E., Larsen, T.O., Hansen, P.J., et al., 2021. Polyketide synthase genes and molecular trade-offs in the ichthyotoxic species *Prymnesium parvum*. Sci. Total Environ. 795, 148878. https://doi.org/10.1016/j.scitotenv.2021.148878.

Bannon, C.C., Wang, X., Uhlig, S., Samdal, I.A., McCarron, P., Larsen, T.O., et al., 2024. Influence of biotic and abiotic factors on prymnesin profiles in three strains of *Prymnesium parvum*. Algal Res. 78, 103390. https://doi.org/10.1016/j.algal.2024.103390.

Binzer, S.B., Svenssen, D.K., Daugbjerg, N., Alves-de-Souza, C., Pinto, E., Hansen, P.J., et al., 2019. A-, B- and C-type prymnesins are clade specific compounds and chemotaxonomic markers in *Prymnesium parvum*. Harmful Algae 81, 10–17. https://doi.org/10.1016/j.hal.2018.11.010.

Binzer, S.B., Varga, E., Andersen, A.J.C., Svenssen, D.K., Medeiros, L.S. de, Rasmussen, S. A., et al., 2020. Karmitoxin production by *Karlodinium armiger* and the effects of *K. armiger* and karmitoxin towards fish. Harmful Algae 99, 101905. https://doi.org/10.1016/j.hal.2020.101905.

Bols, N.C., Barlian, A., Chirino-Trejo, M., Caldwell, S.J., Goegan, P., Lee, L.E.J., 1994. Development of a cell line from primary cultures of rainbow trout, Oncorhynchus mykiss (Walbaum), gills. J. Fish. Dis. 17 (6), 601–611. https://doi.org/10.1111/j.1365-2761.1994.tb00258.x.

Brumovský, M., Bečanová, J., Karásková, P., Nizzetto, L., 2018. Retention performance of three widely used SPE sorbents for the extraction of perfluoroalkyl substances from seawater. Chemosphere 193, 259–269. https://doi.org/10.1016/j.chemosphere.2017.10.174.

Caron, D.A., Lie, A.A.Y., Buckowski, T., Turner, J., Frabotta, K., 2023. The effect of pH and salinity on the toxicity and growth of the Golden Alga, *Prymnesium parvum*. Protist 174 (1), 125927. https://doi.org/10.1016/j.protis.2022.125927.

Castejón, N., Marko, D., 2022. Fatty acid composition and cytotoxic activity of lipid extracts from nannochloropsis gaditana produced by green technologies. Molecules 27 (12). https://doi.org/10.3390/molecules27123710.

Dittmar, T., Koch, B., Hertkorn, N., Kattner, G., 2008. A simple and efficient method for the solid-phase extraction of dissolved organic matter (SPE-DOM) from seawater. Limnol. Ocean Methods 6 (6), 230–235. https://doi.org/10.4319/lom.2008.6.230.

Free G., van de Bund W., Gawlik B., van Wijk L., Wood M., Guagnini E., *et al.* An EU analysis of the ecological disaster in the Oder River of 2022. Luxembourg, 2023. https://doi.org/10.2760/067386.

Hartman, K.J., Wellman, D.I., Kingsbury, J.W., Cincotta, D.A., Clayton, J.L., Eliason, K. M., et al., 2021. A case study of a *Prymnesium parvum* Harmful Algae Bloom in the Ohio river drainage: impact, recovery and potential for future invasions/range expansion. Water 13 (22), 3233. https://doi.org/10.3390/w13223233.

Keller, M.D., Selvin, R.C., Claus, W., Guillard, R.R.L., 1987. Media for the culture of oceanic ultraphytoplankton. J. Phycol. 23, 633–638. https://doi.org/10.1111/j.1529-8817.1987.tb04217.x.

Köhler, J., Varga, E., Spahr, S., Gessner, J., Stelzer, K., Brandt, G., et al., 2024. Unpredicted ecosystem response to compound human impacts in a European river. Sci. Rep. 14 (1), 16445. https://doi.org/10.1038/s41598-024-66943-9.

Li, D., Liu, Q., Zhao, Y., Lv, M., Tang, X., Zhao, Y., 2023. ROS meditated paralytic shellfish toxins production changes of *Alexandrium tamarense* caused by microplastic particles. Environ. Pollut. 338, 122702. https://doi.org/10.1016/j.envpol.2023.122702.

Linares-Maurizi, A., Awad, R., Durbec, A., Reversat, G., Gros, V., Galano, J.-M., et al., 2024. Stress-induced production of bioactive oxylipins in marine microalgae. Mar. Drugs 22 (9). https://doi.org/10.3390/md22090406.

Manning, S.R., La Claire, J.W., 2010. Prymnesins: toxic metabolites of the golden alga, *Prymnesium parvum* Carter (Haptophyta). Mar. Drugs 8 (3), 678–704. https://doi.org/10.3390/md8030678.

Manning, S.R., La Claire, J.W., 2013. Isolation of polyketides from *Prymnesium parvum* (Haptophyta) and their detection by liquid chromatography/mass spectrometry metabolic fingerprint analysis. Anal. Biochem. 442 (2), 189–195. https://doi.org/10.1016/j.ab.2013.07.034.

Mazur-Marzec, H., Grabski, M., Konkel, R., Cegłowska, M., et al., 2025. Genetic, metabolic and toxicological diversity within *Prymnesium parvum* (Haptophyte) from Polish waterbodies. Water Res. 282, 123744. https://doi.org/10.1016/j.watres.2025.123744.

Medić, N., Varga, E., van, Waal, D.B. de, Larsen, T.O., Hansen, P.J., 2022. The coupling between irradiance, growth, photosynthesis and prymnesin cell quota and production in two strains of the bloom-forming haptophyte, *Prymnesium parvum*. Harmful Algae 112, 102173. https://doi.org/10.1016/j.hal.2022.102173.

Pöchhacker, M., Tillmann, U., Marko, D., Varga, E., 2025. Intraspecific variability within *Karlodinium armiger* (Dinophyceae) on a toxicological and metabolomic level. Harmful Algae 143, 102808. https://doi.org/10.1016/j.hal.2025.102808.

Qin, J., Hu, Z., Zhang, Q., Xu, N., Yang, Y., 2020. Toxic effects and mechanisms of *Prymnesium parvum* (Haptophyta) isolated from the Pearl River Estuary, China. Harmful Algae 96, 101844. https://doi.org/10.1016/j.hal.2020.101844.

Rasmussen, S.A., Binzer, S.B., Hoeck, C., Meier, S., Medeiros, L.S. de, Andersen, N.G., et al., 2017. Karmitoxin: an amine-containing polyhydroxy-polyene toxin from the marine dinoflagellate *Karlodinium armiger*. J. Nat. Prod. 80 (5), 1287–1293. https://doi.org/10.1021/acs.jnatprod.6b00860.

Rasmussen, S.A., Meier, S., Andersen, N.G., Blossom, H.E., Duus, J.Ø., Nielsen, K.F., et al., 2016. Chemodiversity of ladder-frame Prymnesin polyethers in *Prymnesium parvum*. J. Nat. Prod. 79 (9), 2250–2256. https://doi.org/10.1021/acs.jnatprod.6b00345.

Robles-Molina, J., Gilbert-López, B., García-Reyes, J.F., Molina-Díaz, A., 2013. Comparative evaluation of liquid-liquid extraction, solid-phase extraction and solid-phase microextraction for the gas chromatography-mass spectrometry determination of multiclass priority organic contaminants in wastewater. Talanta 117, 382–391. https://doi.org/10.1016/j.talanta.2013.09.040.

Roelke, D.L., Grover, J.P., Brooks, B.W., Glass, J., Buzan, D., Southard, G.M., et al., 2011. A decade of fish-killing *Prymnesium parvum* blooms in Texas: roles of inflow and salinity. J. Plankton Res. 33 (2), 243–253. https://doi.org/10.1093/plankt/fbq079.

Ross, C., Santiago-Vázquez, L., Paul, V., 2006. Toxin release in response to oxidative stress and programmed cell death in the cyanobacterium Microcystis aeruginosa. Aquat. Toxicol. 78 (1), 66–73. https://doi.org/10.1016/j.aquatox.2006.02.007.

Snyder, L.R., 1978. Classification off the solvent properties of common liquids. J. Chromatogr. Sci. 16 (6), 223–234. https://doi.org/10.1093/chromsci/16.6.223.

Sobieraj, J., Metelski, D., 2023. Insights into toxic *Prymnesium parvum* blooms as a cause of the ecological disaster on the Odra river. Toxins 15 (6). https://doi.org/10.3390/toxins15060403.

Starck, S., Wolter, C., 2024. Resilience approach for assessing fish recovery after compound climate change effects on algal blooms. Sustainability 16 (14), 5932. https://doi.org/10.3390/su16145932.

Svendsen, M.B.S., Andersen, N.R., Hansen, P.J., Steffensen, J.F., 2018. Effects of harmful algal blooms on fish: insights from *Prymnesium parvum*. Fishes 3 (1), 11. https://doi.org/10.3390/fishes3010011.

Svenssen, D.K., Binzer, S.B., Medić, N., Hansen, P.J., Larsen, T.O., Varga, E., 2019. Development of an indirect quantitation method to assess Ichthyotoxic B-type Prymnesins from *Prymnesium parvum*. Toxins 11 (5). https://doi.org/10.3390/toxins11050251.

Taylor, R.B., Hill, B.N., Bobbitt, J.M., Hering, A.S., Brooks, B.W., Chambliss, C.K., 2020. Suspect and non-target screening of acutely toxic *Prymnesium parvum*. Sci. Total Environ. 715, 136835. https://doi.org/10.1016/j.scitotenv.2020.136835.

UBA-Umweltbundesamt (2022) Fischsterben in der Oder, August 2022. Available at: ⟨https://www.umweltbundesamt.de/sites/default/files/medien/2546/dokumente/statusbericht_fischsterben_in_der_oder_220930.pdf⟩ (Accessed: 15 February 2025).

VanLandeghem, M.M., Denny, S., Patiño, R., 2015. Predicting the risk of toxic blooms of golden alga from cell abundance and environmental covariates. Limnol. Ocean Methods 13 (10), 568–586. https://doi.org/10.1002/lom3.10048.

Varga, E., Prause, H.-C., Riepl, M., Hochmayr, N., Berk, D., Attakpah, E., et al., 2024. Cytotoxicity of *Prymnesium parvum* extracts and prymnesin analogs on epithelial fish gill cells RTgill-W1 and the human colon cell line HCEC-1CT. Arch. Toxicol. 98 (3), 999–1014. https://doi.org/10.1007/s00204-023-03663-5.

Wagstaff, B.A., Pratscher, J., Rivera, P.P.L., Hems, E.S., Brooks, E., Rejzek, M., et al., 2021. Assessing the toxicity and mitigating the impact of harmful *Prymnesium* blooms in eutrophic waters of the Norfolk broads. Environ. Sci. Technol. 55 (24), 16538–16551. https://doi.org/10.1021/acs.est.1c04742.