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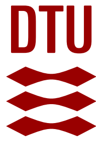

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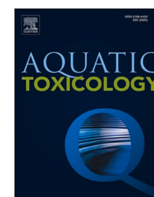

Toxicity of the antiparasitic lipopeptide biosurfactant SPH6 to green algae, cyanobacteria, crustaceans and zebrafish

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ABSTRACT

A lipopeptide with biosurfactant properties produced by the bacterium *Pseudomonas* H6 (SPH6) has antiparasitic effects and may serve as an alternative to chemotherapeutants against aquatic pathogens in aquaculture. We have elucidated its ecotoxicological potential by short-term standardized tests, including a growth rate inhibition test with algae (*Raphidocelis subcapitata*), a lethality test on the cyanobacteria *Phormidium autumnale*, a lethality test using crustaceans (*Daphnia magna*), a fish embryo acute toxicity test and a fish acute toxicity test using zebrafish (*Danio rerio*). The decrease of the biosurfactant concentration in zebrafish test water during 24 h was measured. The toxicity for crustaceans was highest ($LC_{50} = 20$ mg/L), followed by the test with the zebrafish embryo ($LC_{50} = 27$ mg/L). The juvenile zebrafish fish (complete mortality occurred between 40 and 80 mg/L), the cyanobacteria ($LC_{50} = 80$ mg/L) and the green algae ($EC_{50} = 170$ mg/L) showed higher tolerance. The determination of SPH6 concentrations in fish tank (up to 50% elimination over 24 h) suggested that the compound may become adsorbed to tank walls, absorbed by fish or degraded. Further studies should determine its impact under different environmental settings (*e.g.* temperature) relevant for different branches of the aquaculture sector.

1. Introduction

There is a demand in aquaculture for eco-effective solutions against ectoparasitic pathogens. Previously malachite green was applied worldwide but was banned decades ago. The usage of other chemotherapeutants such as formalin, hydrogen peroxide and peracetic acid has consequently increased but is also raising environmental concerns due to the toxicity of the products. In addition, concerns on fish welfare have been raised as the compounds may induce stress and injuries in fish (Buchmann et al., 2004; Pedersen et al., 2007; Jørgensen and Buchmann, 2007; Straus et al., 2018; Liu et al., 2017; Lieke et al., 2020). The search for renewable and sustainable control methods, including environment-friendly treatments, has pointed to the use of biosurfactants as a possible solution. These diverse amphiphilic molecules, produced by bacteria, fungi and yeast, effectively lower surface and interfacial tension, which may affect membranes of pathogens. As the compounds show lower toxicity and higher biodegradability they may have a potential application in various industrial fields (Shekhar et al., 2015) including aquaculture (Al-Jubury et al., 2018; Li et al., 2022). Lipopeptide biosurfactants (LPBSs) are complex and diverse structures consisting of a hydrophobic fatty acid part, linked with a hydrophobic peptide chain. LPBSs influence surface activity, anti-cellular and anti-enzymatic activities. The surfactants are also involved in biofilm formation and swarming motility. They represent a diverse group of secondary metabolites, synthesized through one or more non-ribosomal peptide synthesis pathways, and are produced mostly by bacteria such as *Bacillus* and *Pseudomonas* (Roongsawang et al., 2011). Members of the genus *Pseudomonas* are able to produce different biosurfactant molecules: glycolipids (rhamnolipids) and lipopeptides. The latter group comprises cyclic forms which have been sub-categorized into six groups: viscosin, amphisin, tolaasin, syringomycin, putisolvin and syringopeptin (Gross et al., 2009). The biological function may be to assist the colonization and survival of *Pseudomonas* in terrestrial, aquatic and marine habitats (Bollinger et al., 2020). The bacterium *Pseudomonas* H6

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produces a viscosin-like lipopeptide surfactant (SPH6) exhibiting lethal effects on some aquatic pathogens including the oomycete *Saprolegnia* (Liu et al., 2015), the parasitic ciliate *Ichthyophthirius multifiliis* (Al-Jubury et al., 2018; Li et al., 2022) and the amoeba *Vannella* sp. (Jensen et al., 2020). This suggests that the biosurfactant may have a potential to control some fish diseases but in this context a basic study on the toxicological impact of SPH6 on aquatic organisms is needed. The EU Water Framework Directive and national legislations request results from toxicological tests to determine environmental quality standards and consequently evaluate the chemical status of waters receiving various substances. The effects on the aquatic environment can be elucidated through tests on species belonging to different trophic levels. The aim of this study was to provide data for the toxicity of SPH6 by application of short-term standardized tests for environmental safety assessments using freshwater taxa related to three distinct trophic levels. These comprise green algae and cyanobacteria representing the primary producers (photosynthesizing organisms), the secondary producers (herbivores) represented by the crustaceans and finally tertiary producers (fish).

2. Materials and methods

2.1. Production of the lipopeptide surfactant SPH6

The surfactant was produced as described by Liu et al. (2015). In brief, bacteria grown on KMB agar plates were flooded by sterile demineralized water, the culture supernatant was sterile filtered and acidified (pH 2, HCl). The precipitate was dissolved in sterile distilled water and pH adjusted to pH 8 by use of NaOH. Following lyophilization and storage at −20 °C a stock solution of 2 mg/ml was prepared from which a dilution series of SPH6 was made in order to test extremes for the individual test organisms.

2.2. Parasiticidal effect

Before the toxicity tests were performed, we confirmed, by conducting an *in vitro* test at 20 °C, the antiparasiticidal effect of the SPH6 on two stages (tomocysts and theronts) of the parasitic ciliate *Ichthyophthirius multifiliis*. Parasites were obtained from infected rainbow trout in the laboratory. In brief, infected fish were euthanized by immersion into 300 mg/L MS222 (Sigma-Aldrich, Denmark) and subsequently transferred to a plastic tray with tank water, in which trophonts escaped the fish surface as tomonts. These free-swimming stages were then collected by pipette and incubated in a 200 mL plastic tray containing 0.22 μm filtered tank water, whereafter they were transformed into sessile tomocysts. Some of these tomocysts were used for the test, whereas others were allowed to develop further and release theronts within 30 h at room temperature. Both parasite stages were exposed to different concentrations (6.25, 12.5, 25.0 mg/L) of SPH6 in concave wells on a glass plate (thickness 6 mm). The parasites in the concavities (diameter 25 mm, depth 3 mm, maximum water capacity 2000 μl) were studied under a dissection microscope (40 × magnification). The exposure of the specific parasite stage (theront or tomocyst) to each concentration was performed in triplicate and the number of theronts per well varied between 85 and 120, whereas the number of tomocysts (each carrying 500–1000 tomites) varied from 4 to 6 per well. Viability of the parasites was assessed by microscopy and total lack of motility (including beating by cilia and movements) of the parasites was recorded as mortality. The survival of parasites was recorded at time points 0, 15, 30, 45, 60 and 75 min post-exposure.

2.3. Algal growth rate inhibition test

The algal toxicity tests was conducted with the freshwater green algae *Raphidocelis subcapitata* (synonyms *Selenastrum capricornutum* and *Pseudokirchneriella subcapitata*) obtained from the Norwegian Institute of Water Research, Oslo, Norway (NIVA). It was grown continuously in ISO 8692 algal medium (ISO, 2012) in 20 mL glass vials fitted with a screw cap lid with a hole to allow for CO_2 exchange with the atmosphere. The vials were incubated in the LEVITATT (LED Vertical Illuminated Table for Algal Toxicity Test) described in detail by Skjolding et al. (2020). In short, nine wells each containing five scintillation vials fastened with metal clamps on the side of the wells were illuminated from below with an LED source sandwiched between two acrylic plates. The LED source delivers a “cool-white” light with an intensity of 108±10 $\mu E \bullet m^{-2} \bullet s^{-1}$ measured with a LI-189 Quantum/Radiometer/Photometer (LI-COR, Nebraska, USA). The LEVITATT was mounted on an orbital shaker (IKA® KS 260 basic) at 200 rpm and placed in a temperature controlled incubator at 23±2 °C. The pH value was 7.9. The SPH6 was dissolved in ISO 8692 media to a final concentration of 5, 10, 20, 40, 80, 160 and 320 mg/L and inoculated with 10^4 cells/mL. For each concentration, three replicates were prepared containing 4 mL of the respective inoculated concentrations. For the control, 6 replicates were prepared in ISO 8692 media with no addition of SPH6 inoculated with 10^4 cells/mL. The samples were incubated at identical conditions to the cultivation.

Samples of 0.4 mL were taken at times 0, 48 and 72 h. The algal growth rates were calculated based on the *in vitro* fluorescence of algal pigments as a surrogate for biomass as described by Mayer et al. (1997). The fluorescence was 430 nm and 670 nm for excitation and emission wavelengths. A blank sample, containing medium and acetone, served as background correction of the samples. The test was compliant with the ISO 8692:2012 algal growth inhibition test protocol (ISO, 2012), *i.e.* a control growth rate of minimum 1.4, no more than a maximum increase in pH of 1.5 relative to the pH of the growth medium and the variation coefficient of the growth rate in the control ≤ 5%.

2.4. Cyanobacteria toxicity test

The organism applied was collected (as an aggregate of filamentous *Phormidium autumnale* cyanobacteria) in the freshwater lake Bastemose on June 13, 2021 (Island of Bornholm, Baltic sea). The jellylike dark-green colony was incubated in sterile filtered tap water at 20 °C with weekly shifts of water for two months before experimentation. It was then subdivided and placed at 20 °C in 200 mL glass beakers with sterile filtered water from a recirculated zebrafish culture system (Aquaschwarz, Germany), pH of 7.4 and conductivity 550 μS. The identification of the organisms was performed by PCR and sequencing according to Nübel et al. (1997). In brief, genomic DNA from a subsample (approximate 3 mg) was purified by the means of QIAamp DNA Mini Kit (cat.no. 61306, Qiagen, Denmark) using a final elution volume of 50 μL. In order to obtain a partial sequence of the 16S gene, a 60 μL PCR was performed using the primers CYA106F (5′ CGGACGGGTGAGTAACGCGTGA 3′) and CYA7811R(a) (5′ GACTACTGGGGTATCTAATCCCATT 3′) (Nübel et al., 1997). The PCR mixture was composed of 1 μM of each primer (TAG Copenhagen, Denmark), 1 mM of dNTP-mix (cat.no. N8080260, Fisher Scientific, Denmark), 1.25 units of BIOTAQ DNA polymerase (cat.no. BIO-21060, Nordic BioSite, Denmark), 1.5 mM MgCl2, and 6 μL 10 × PCR buffer and RNase free H_2O (cat.no. 10977049, ThermoFisher Scientific, Denmark). The PCR protocol used comprised initial denaturation at 95 °C for 5 min; 40 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s, and elongation at 72 °C for 1 min; and finally an elongation step at 72 °C for 7 min. The PCR product was visualized by gel electrophoresis. The remaining PCR product was purified using the Illustra™ GFX™PCR and Gel band purification kit (cat.no. 28-9034-71, VWR, Denmark), and subsequently sequenced by Macrogen Inc. (Amsterdam, the Netherlands) using the same PCR primers. The PCR is a 663 bp long product and a Blast analysis of the sequence showed 100% identity towards 16S of the cyanobacterium *Phormidium autumnale*, GenBank accession number MT153248.

It was not possible to produce single cell cultures as recommended (OECD, 2011) due to the filamentous nature of the cyanobacterial colonies. They were therefore partly fragmented and separated by using a

strainer with a mesh size of 80 μm (Retsch GmbH & Co. Haan, Germany), which resulted in a suspension of cyanobacterial colony fragments (mean no. of cells per filament 20 (range 10–40)). Following determination of the fragment concentration by light microscopy (40–100 x magnification) the cyanobacteria were transferred to 24 TC well plates (Ref. 83.3922500, Sarstedt, Nümbrecht, Germany) (40 colony fragments per well). The test was run at 20 °C in triplicate and at pH 7.6. Each well contained a 500 μL solution of the SPH6 surfactant (concentrations 7.8, 15.62, 31.25, 62.5, 125, 250 mg/L). The number of aggregates in each well was then counted after 48 h incubation. In order to quantify dead cells by microscopy in the cyanobacterial colonies exposed to different concentrations of SPH6, we stained subsamples with the vital stain Trypan Blue (0.4%, Gibco, Life Technologies Corporation, NY, USA). In brief, the cyanobacteria were mounted in a drop of the vital stain on a microscope slide with cover slip and studied at 400 x magnification in the light microscopy (Leica DM5000B, Germany). Dead cells stained blue.

2.5. Acute toxicity test for crustaceans

The *D. magna* culture originates from Birkedammen, Denmark, collected in 1978 and since then continuously cultured at the Department of Environmental Engineering, Technical University of Denmark. For culturing, adult animals were kept in a 1 L glass beaker filled with 800 mL Elendt M7 medium (OECD, 2004). The culture medium was renewed twice a week, and the animals were fed green algae (*R. subcapitata*) daily ($2.5 \bullet 10^5$ cells/mL) and kept at a constant temperature of 20 °C and a light-dark cycle of 12:12 h. The acute toxicity tests with crustaceans were conducted according to the OECD TG 202 *Daphnia* sp. Acute immobilisation Test test protocol (OECD, 2004). The crustacean tests were carried out in 100 mL glass beakers in four replicates of 25 mL each, with approximately five neonates (< 24 h old) in each beaker. The following nominal concentrations were prepared for SPH6 in Elendt M7 media (OECD, 2004): 2.5, 5, 10, 20, 40 and 80 mg/L without the use of solvents or dispersants. The pH value was 7.4 and the oxygen content 8.6 mg/L (95% saturation) as measured initially and at the end of the test. The values were within the acceptable limits (OECD, 2004). The test was carried out in darkness at 20 °C for 48 h. The number of immobile and dead animals were counted after 24 h and 48 h. Immobile individuals were scored as dead for calculation of the lethal concentrations.

2.6. Fish embryo acute toxicity (FET) test

2.6.1. Fish and fish maintenance

A breeding stock of a wild-type (ABWT) zebrafish (*Danio rerio*), age (6–18) months, were used for egg production. The fish originated and were reared at the experimental zebrafish facility, University of Copenhagen, Frederiksberg, Denmark in a recirculated system (Aquaschwarz, Germany), with 10% daily water exchange, at 27°C, pH of 7.4 and conductivity 550 μS, under a light-dark cycle of 14 h L: 10 h D (stock density 5 adult fish/L and 1:1 sex ratio). Fish were fed with a pelleted dry feed (ZM Fish Food, UK) twice a day, supplemented once a day with live feed *Artemia* nauplii (Ocean Nutrition, Belgium).

2.6.2. Fish eggs and egg production

In order to stimulate breeding behavior, fish were placed, at 27°C, in tanks in the evening prior to the experiment. Empty pipette boxes (length, width, height: 12 × 8 × 5 cm, total volume 480 mL), were covered with mesh (3 × 3 mm) and submerged in the tanks. Next morning, around 2 h after start of fish spawning, eggs were collected from the boxes and rinsed in sterile filtered fish facility water. Fertilized eggs were distinguished from unfertilized under the dissection microscope (magnification x 6.3–40) (Leica S6E, Germany). For the fish embryo acute toxicity (FET) test fertilized eggs between 4 and 128 cell stage were used.

2.6.3. Test procedure

The test was carried out, at 27°C, according to the OECD test guideline 236 (OECD, 2013). Fertilized eggs were distributed in 24 well TC plates (Ref. 83.3922500, Sarstedt, Germany) containing 2.5 mL test solutions each. Six concentrations of SPH6 (2.5, 5, 10, 20, 40, and 80 mg/L) were prepared from a stock solution dissolved in sterile filtered facility system water (rearing medium). As a positive control, one plate with 4 mg/L 3.4-dichloroaniline was used. Each plate contained 20 eggs suspended in the individual test solution, and the remaining 4 eggs were placed in rearing medium only - internal plate control. An additional plate, containing 24 eggs in the rearing medium, served as negative control. Embryos were inspected under dissection microscope (Leica S6E, Germany) at 24, 48, 72 and 96 h of treatment. Embryo mortality observations were focused on four parameters: 1) coagulation of embryos, 2) lack of somite formation, 3) non-detachment of the tail and 4) lack of heart beats. Dead embryos were recorded after 48, 72 and 96 h. At 96 h hatching rates were observed. Initially and at the end of the experiment, pH and dissolved oxygen (pH 7.4, oxygen saturation >95%) were measured in both controls and for the highest tested concentration.

2.7. Fish acute toxicity test

Juvenile (two month old) wild-type (ABWT) zebrafish with an average weight 0.177 g and average length 1.72 cm, reared at the experimental zebrafish facility, University of Copenhagen, Frederiksberg, Denmark, were used. For housing conditions see 2.6.1 (fish maintenance). The fish acute toxicity test was performed, at 27°C, in accordance to the OECD test guideline (TG) 203 (OECD, 2019). Fish were exposed to five tested concentrations of SPH6 5, 10, 20, 40 and 80 mg/L for a period of 96 h (without feeding) under semi-static conditions (with a regular renewal of the test solutions every 24 h) according to the protocol. As a supplementary study additional observations were performed after 4 days, where feeding was resumed and test continued up to 10 days. Seven fish in each group were placed together in a tank (water volume 1.55 L) with a specific SPH6 concentration and the test were run in duplicate. Tested concentrations were prepared (see Section 2.6.3) and replaced daily. Standardized fish behavior observations were recorded 8 times on the first day of exposure and then twice a day and recorded in the standard forms (OECD, 2019) (Supplementary file S1). Regular measurements of water parameters (ammonia, nitrite, nitrate, pH) (Merck, Germany) were performed before and after daily renewal of tested solutions. At the end of the experiment all remaining fish were euthanized in 300 mg/L MS-222 (Sigma-Aldrich, Denmark).

2.8. Ethics and legislation

Handling of the experimental fish followed the ethical guidelines of the University of Copenhagen. No license is required for work with embryos less than 120 h old. The experiment on the juvenile zebrafish was conducted under License 2021-15-0201-00951, and the work with rainbow trout was performed under License 2019-15-0201-00388 issued by the Experimental Animal Inspectorate, Veterinary and Food Administration, Denmark. When a fish displayed marked abnormal behavior (equilibrium disturbances) it was removed from the fish tank, euthanized in 300 mg/L MS222 and recorded as mortality.

2.9. Decrease of SPH6 content in test water during the fish acute toxicity test

During the Fish Acute Toxicity Test, 1 mL of water, at 27°C, from the tested solutions (with concentrations 5, 10, 20, 40 mg/L) were sampled 1 h and 24 h after renewal of rearing medium, for three consecutive days of the test. Water samples were subsequently stored at the temperature −20 °C for further analysis. Detection and quantification of SPH6 was

performed by High Performance Liquid Chromatography (HPLC) on the chromatography equipment from Agilent Technologies 1200 series equipped with Luna® 5 μm C18(2) 100 Å, LC Column 150 × 2 mm (Part No.#00F-4252-B0, Phenonemex). Mobile phases of water with 0.1% (v/v) trifluoroacetic acid (TFA) (Solution A) and acetonitrile (ACN) with 0.085% (v/v) TFA (Solution B) were used. The absorbance at 210 nm was measured to detect SPH6. A volume of 1–5 μL of a specific sample was injected onto the column. The flow rate was set at 0.9 mL/min. The HPLC running program was started with 90% of the solution A and 10% of the solution B, after running 2 min, the solution B was increased to 80% with gradient and the solution A reduced to 20%, after 8 min, the solution B was increased to 100% and kept running for 1 min. From between 9 to 10 min, the solution B was reduced back from 100% to 10% and the solution A was increased from 0% to 90%. SPH6 was purified by AnalytiCon Discovery GmbH (Germany) using preparative HPLC, and as standard a batch of SPH6 with > 99.5% purity for quantification was used. Chromatograms are presented in the Supplementary file S2.

2.10. Data analysis

2.10.1. Data analysis of the algal growth rate inhibition test

For the algal test, growth rates were calculated assuming exponential growth following Eq. (1)

$$\mu = \frac{\ln N_n - \ln N_0}{t_d} \tag{1}$$

where μ is the growth rate (d^{-1}), N_0 is the initial biomass, N_n is the final biomass and t_d is the length of the test period (d). Additionally, the inhibition is calculated as the growth rate of the control related to the growth in each individual exposure following equation 2.

$$I_i = \left(1 - \frac{\mu_i}{\mu_c}\right) * 100 \tag{2}$$

where I_i is the percentage inhibition of growth for concentration i, and μ_i is the mean growth rate for concentration I and μ_c is the mean growth rate for the control.

Growth inhibition based on growth rates (*R. subcapitata*) were plotted in the statistical software R loaded with the drc-package and used to estimate concentration-response curves. EC-values and their corresponding 95% confidence intervals was estimated using a Weibull function (Ritz, 2016).

2.10.2. Data analysis for the tests with cyanobacteria

The number of cyanobacterial aggregates were counted at each concentration after 48 h incubation and the numbers compared by the Kruskal-Wallis test. The survival of cyanobacterial cells were expressed as percentage of the total number of cells counted at each concentration following 48 h incubation and mean differences compared by the Kruskal-Wallis test. The probability level was set at 5%.

2.10.3. Data analysis of the acute toxicity test with crustaceans

For the *Daphnia* test, each beaker ($n = 4$) constitutes a replicate and the percent lethality is calculated as the number of dead organisms in each beaker compared to the total number of exposed organisms in the beaker. Lethality of the crustaceans (*D. magna*) were plotted in the statistical software R loaded with the drc-package and used to estimate concentration-response curves. LC-values and their corresponding 95% confidence intervals was estimated using a Weibull function (Ritz, 2016).

2.10.4. Data analysis of the fish embryo acute toxicity (FET) test

The FET test holds each embryo ($n = 20$) as a replicate. Lethality at each concentration was calculated as the ratio between the number of dead embryos to the total number of exposed embryos within each of the tested concentrations. Data were included in an Excel spreadsheet and further analyzed using Graph Pad Prism 9. Simple logistic regression with 95% confidence intervals was used to estimate concentration response curve and lethal concentration (LC_{50}) value.

2.10.5. Data analysis fish acute toxicity test

Data were analyzed using Microsoft Office Excel. The test considers each fish as a replicate ($n = 7$) and percentage of mortality was calculated as the number of dead fish compared to the total number of exposed fish for each concentration.

2.10.6. Data analysis of the degradation of SPH6

Data were analyzed using Microsoft Office Excel and GraphPad Prism 9. Differences were considered statistically significant at a probability level of 5% ($P<0.05$). Student's *t*-test was used to compare SPH6 concentrations in corresponding tanks at two different time points.

3. Results

3.1. Parasiticidal effect of SPH6

All tested concentration of 12.5 mg/L and higher killed all *I. multifiliis* theronts within 15 min. The lowest concentration tested (6.25 mg/L) was lethal within 45 min (Fig. 1A). Tomocysts were more resistant to the compound as it took 30 min to kill these parasites at a concentration of 25 mg/L. All tomocysts died within 75 min at 12.5 mg/L (Fig. 1B), whereas it took 24 h to kill all at a concentration of 6.25 mg/L (data not shown for 24 h).

3.2. Algal growth rate inhibition test

A reference test with 3,5-dichlorophenol was carried out using *R. subcapitata* yielding an EC_{50}-value of 3.8 mg/L (95% confidence interval [2.9–4.8]). The value is within the expected range for the substance (3.38 ± 1.3 mg/L) (ISO, 2012). The average control growth rate was 1.52 with a coefficient of variation of 2.5%. Consequently, the test fulfilled the validity criteria stated in the guideline (ISO, 2012). The concentration-response curve for the growth inhibition test with *R. supcapitata* is shown in Fig. 2. A concentration dependent increase in inhibition was observed in SPH6 concentrations from 20 to 320 mg/L with a maximum inhibition of 78%. The estimated effect concentrations resulting in 10, 20 and 50% inhibition was 56 mg/L $[50;61]_{95\%}$, 82 mg/L $[75;88]_{95\%}$ and 170 mg/L $[159;181]_{95\%}$ respectively.

3.3. Toxicity to cyanobacteria

The surfactant in concentrations 250, 125 and 62.5 mg/L showed a marked and significant effect on the cyanobacteria and their ability to aggregate (Fig. 3A). The low concentrations 7.8, 15.6 and 31.25 mg/L showed no effect on the cyanobacteria. The highest concentration (62.5 mg/L and above) showed a significant effect on *Phormidium autumnale* cell survival and the LC_{50}-value was measured to 80 mg/L (Fig. 3B).

3.4. Acute toxicity test for crustaceans

A reference test for the sensitivity of the organism was carried out with 3,5-dichlorophenol resulting in a LC_{50}-value of 2.7 mg/L with a 95% confidence interval of 2.4 to 3.0 mg/L. The pH (7.4) and dissolved oxygen values (8.6 mg/L) were measured initially and at the end of all experiments in the controls, and for the highest concentration tested, and were found to be within the limits of the respective testing guidelines (OECD, 2004). A SPH6 concentration dependent increase was observed from 2.5 to 40 mg/L and reached 100% lethality at concentrations higher than 40 mg/L, resulting in a full concentration-response curve (Fig. 4). The estimated LC-values corresponding to 10, 20 and 50% lethality were 7.5 $[4:10]_{95\%}$, 11 $[8:13]_{95\%}$ and 20 $[15:24]_{95\%}$ mg/L,

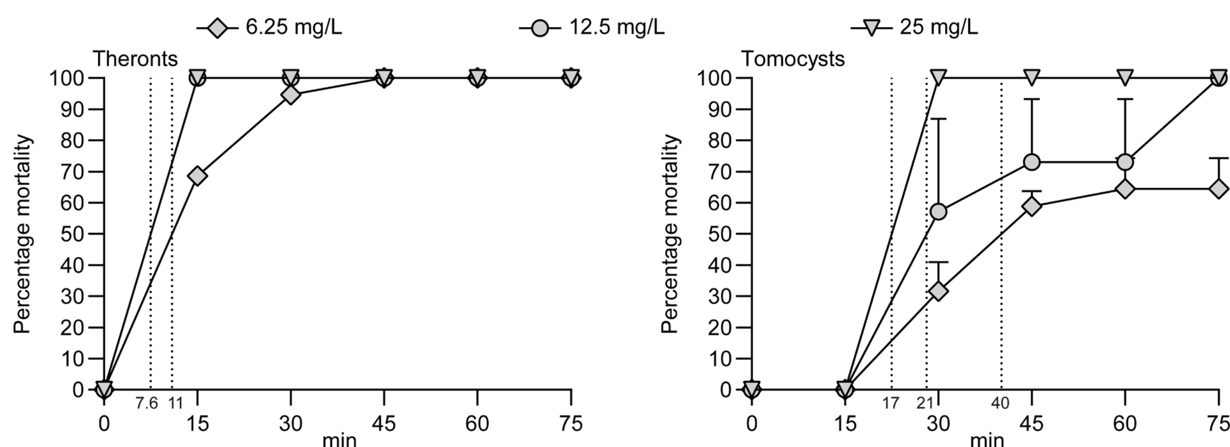


Fig. 1. Effects of SPH6 on the fish parasitic ciliate *Ichthyophthirius multifiliis* theronts (A) and tomocysts (B). The lines represent the mean of three trials ($n = 3$). Error bars included but are smaller than the size of the symbols and therefore not visible. The vertical dotted lines on the x-axis indicate time to 50% mortality.

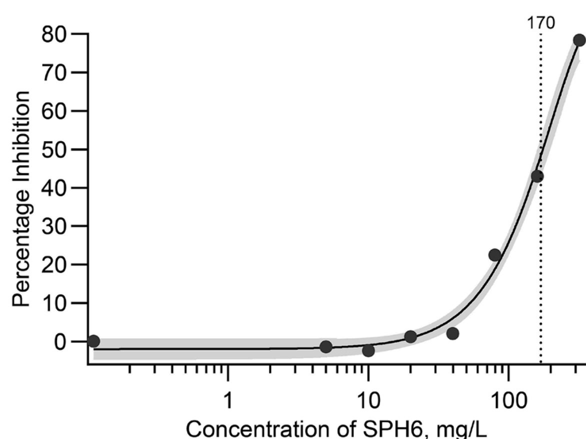


Fig. 2. Estimated concentration-response curve (black line) for a growth inhibition test with the green algae *Raphidocelis subcapitata* after 72 h exposure to SPH6 with a fitted 95% confidence interval (shaded area). The dots represent the average inhibition at the SPH6 concentration ($n_{exposure} = 3$, $n_{control} = 6$).

respectively.

3.5. Fish embryo acute toxicity (FET) test

In the positive control with the 4 mg/L 3.4-dichloroaniline 100% lethality was observed within the first 48 h. This fulfills the OECD-protocol rule that exposure to this chemical in this concentration should result in a minimum mortality of 30% within 96 h. The two highest SPH6 concentrations of 40 and 80 mg/L elicited 100% mortality at 48 and 24 h, respectively. For the tested SPH6 solution containing 20 mg/L, a lethality of 10% of was recorded at 24 h. There was no embryo mortality detected at the concentrations 5 and 10 mg/L. In all the

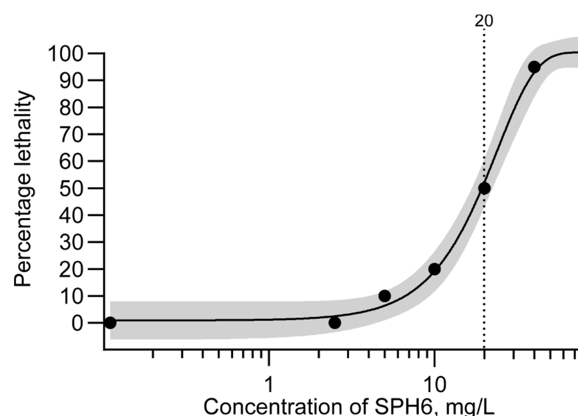
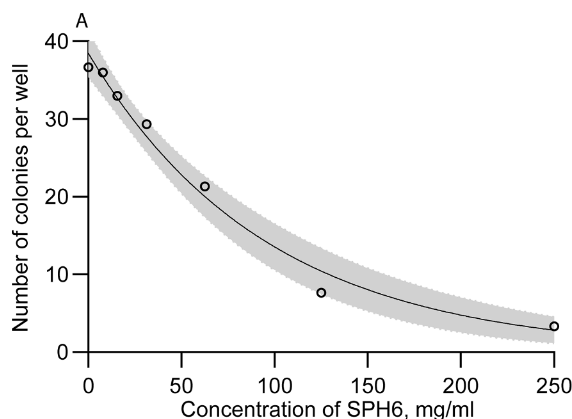


Fig. 4. Estimated concentration-response curve (black line) for a lethality test with *Daphnia magna* exposed 48 h to different SPH6 concentrations with a fitted 95% confidence interval (shaded area) ($n = 4$). Concentration for 50% inhibition indicated by the vertical line.



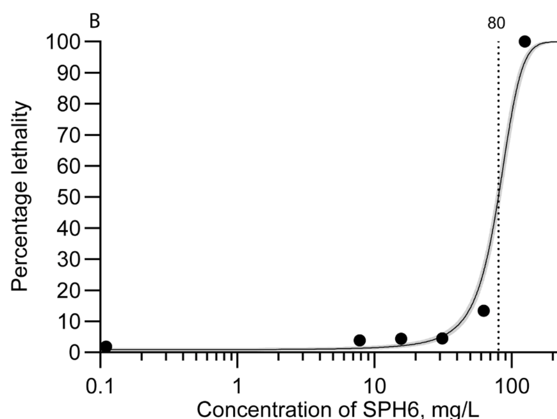


Fig. 3. Effects of different SPH6 concentrations on cyanobacteria *Phormidium autumnale.* Colony formation (aggregating of filaments) (A) and survival of cells (B) following 48 h incubation at room temperature. LC50 indicated by the vertical line.

internal controls 100% survival was recorded, and for the negative control 4% lethality was observed. The hatching percentage after 96 h in the negative control was 100% along with all SPH6 remaining concentrations. A full concentration-response curve was established (Fig. 5), and LC_{50} was calculated to be 27 mg/L. The FET test was performed with the compliance to the current accepted regulatory guidelines.

3.6. *Fish acute toxicity test with juvenile zebrafish (D. rerio)*

A fish survival of 100% was observed over all 10 days in the control tanks and in the 5, 10, 20, 40 mg/L SPH6 concentrations. At the concentration 80 mg/L 100% mortality was recorded within the first 24 h post exposure (Table 1). It was therefore not possible to calculate LC_{50}. At present we note that complete mortality occurs between SPH6 concentrations of 40 and 80 mg/L (Table 1). Observations of fish behavior during the exposure are listed in the supplementary file S1. After 96 h the feed-uptake was resumed by fish in all concentrations except in tanks with SPH6 concentrations of 40 mg/L, in which feed intake was lowered. Furthermore, irregular breathing, under-reaction to stimuli and hyperventilation were signs observed for all fish in the 40 mg/L. In the 20 mg/L, one single fish showed slightly irregular breathing after day 4 along with abnormal bottom-seeking behavior. The fish behavior in the control tanks and in the tanks with SPH6 concentrations 5 and 10 mg/L was normal. Daily measurements of the water parameters (pH, temperature, ammonium, nitrite, nitrate, dissolved oxygen) were performed and complied with the test regulatory guidelines (OECD, 2019).

3.7. *Decrease of the SPH6 content in test water during the fish acute toxicity test*

The decrease of the SPH6 concentration in water between 1 and 24 h was detected for all concentrations throughout the experimental course. There was a significant difference in concentration between the two measured time points for 20 and 40 mg/L ($p<0.05$) (Fig. 6).

4. Discussion

The lipopeptide surfactant isolated from *Pseudomonas* H6, termed SPH6 in this study, has been suggested as a potential biological control agent against pathogens in fish. *In vitro* studies performed in the laboratory have shown that the surfactant has a lethal effect on the oomycete *Saprolegnia* (Liu et al., 2015), the ciliate *Ichthyophthirius* (Al-Jubury et al., 2018) and the amoeba *Vannella* (Jensen et al., 2020). Further *in vivo* laboratory experiments showed that it can prevent ichthyophthiriasis in rainbow trout (Li et al., 2022). It is therefore possible that the surfactant may find application in aquaculture settings (for both cold and warm water fish) and replace the use of chemotherapeutants currently used. The biocide malachite green was used extensively against various diseases in the 20th century but was removed from the list of approved chemotherapeutants in 1990 (Alderman, 2008; Kanhere et al., 2014). In addition, concerns were also raised on the problematic effects of other chemotherapeutants, such as formalin (Buchmann et al., 2004; Meinelt et al., 2005; Jørgensen and Buchmann, 2007), hydrogen peroxide (Rach et al., 1997) and peracetic acid (Strauss et al., 2018). As the surfactant SPH6 could serve as an alternative and sustainable antiparasitic agent in aquaculture further ecotoxicological analyses are needed. In the present study we produced the surfactant SPH6, based on well described methods (Liu et al., 2015), and we confirmed the antiparasitic activity towards *I. multifiliis* corresponding to previous tests *in vitro* (Al-Jubury et al., 2018) and *in vivo* (Li et al., 2022). We then established new data on the effects of the surfactant on test organisms at different trophic levels (algae, crustaceans and fish), as such information may be used for an ecotoxicological evaluation of the new compound before field use is considered. Crustaceans, represented by *D. magna*, were in this study found to be the most sensitive organism followed by zebrafish (*D. rerio*) embryos, juvenile zebrafish, cyanobacteria (*M. autumnale*), and the least sensitive organism was green algae represented by *R. subcapitata*. In general, the toxicity of SPH6 is lower compared to effects of other chemotherapeutants presented in the literature (Table 2). The EC_{50} value of SPH6 for algal growth was estimated to be 170 mg/L. The test with algae is a growth inhibition test, thus there is no "LC" value but an "EC" value, as it is not recorded whether the algae is dead or alive, but only how growth is inhibited, when compared to the control. The SPH6 showed toxicity towards *D. magna* with LC_{50} value of 20 mg/L. In corresponding tests the LD_{50} values of other chemotherapeutants used in aquaculture clearly reflect a higher toxicity (Table 2). When applying the acute toxicity test on zebrafish embryos the lipopeptide biosurfactant SPH6 reached a LC_{50} value of 27 mg/L, which is clearly higher compared to literature data for other chemicals (Table 2). It was not possible to calculate the precise LC_{50} value for SPH6 and zebrafish juveniles, but we noted full mortality of juvenile zebrafish at concentrations between 40 and 80 mg/L. During the test juvenile zebrafish were two months old and had an average weight of 0.177 g and an average length of 1.72 cm. These fish were smaller when compared to fish used in other toxicity tests, in which chemicals used in aquaculture (formalin, hydrogen peroxide, peracetic acid) have been investigated (Reardon and Harrel, 1990, Rach et al., al., 1997, Resendes et al., 2018, Straus et al., 2018). However, even with these small fish the SPH6 toxicity was at level with or significantly lower compared to other chemicals tested (Table 2). Toxicity studies have been performed with several other fish species, which are not directly comparable to the present study. However, also in those cases the currently applied chemicals have shown a higher degree of toxicity. An acute formalin toxicity test using striped bass fingerlings at different salinities showed that the $LC_{50-96\ h}$ values ranged from 4.96 to 10.84 mg/L at 0 to 15 ppt salinity, respectively (Reardon and Harrel, 1990). The LC_{50} values of malachite green vary among species, from 0.0453 mg/L for smallmouth bass, 0.0728 mg/L for coho salmon, 0.283 mg/L for Atlantic salmon and 1.0 mg /L for freshwater catfish (Srivastava et al., 2004). A study on twelve fish species (fingerling size) exposed to peracetic acid

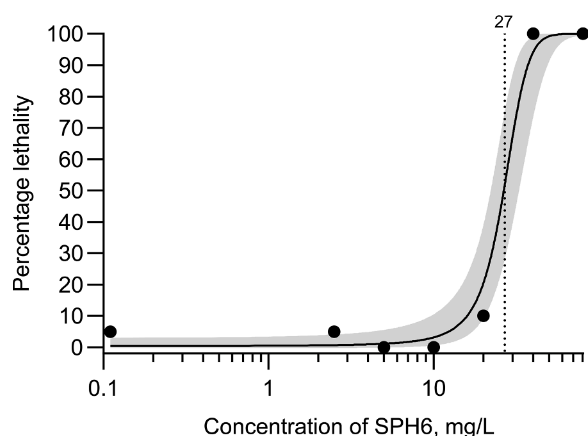


Fig. 5. SPH6 concentration-response curve for the Fish Embryo Acute Toxicity (FET) test using zebrafish embryo (*Danio rerio)* exposed over 96 h. The 95% confidence interval is indicated by the shaded area ($n = 20$). LC50 indicated by the vertical line.

Table 1
Mortality results for the Fish Acute Toxicity Test.

SPH6 concentrations	80 mg/L	40 mg/L	20 mg/L	10 mg/L	5 mg/L	Control
Number of fish used	7	7	7	7	7	7
Lethality 24 h	100%	0%	0%	0%	0%	0%
Lethality 96 h	–	0%	0%	0%	0%	0%
Lethality 10 days	–	0%	0%	0%	0%	0%

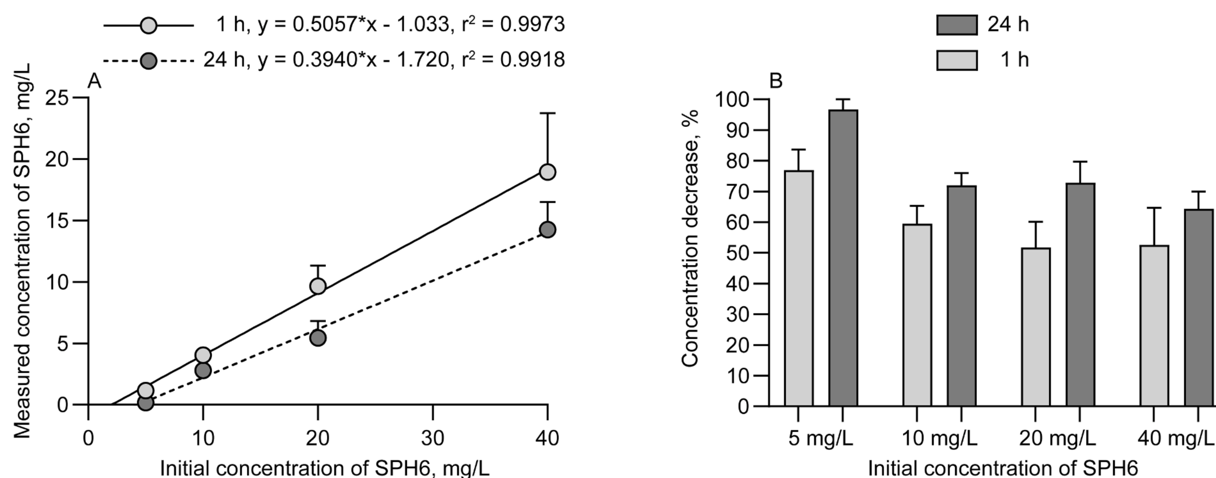


Fig. 6. Decrease of the different SPH6 concentrations in water after 1 h and 24 h. A. The x-axis represents different concentrations of SPH6 added to the fish tank water. The y-axis represents the mean SPH6 concentration (with SEM indicated, $n = 3$) measured after 1 and 24 h. B. The percentage decrease of SPH6 (with SD indicated, $n = 3$) at 1 and 24 h after adding the surfactant (at different concentrations indicated by the x-axis) to the fish tank. Error bars smaller than the size of the symbols are not visible.

Table 2
Comparison of SPH6 effects on cyanobacteria, green algae, crustaceans and fish with literature data on toxicity of previously and/or currently used chemotherapeutants. NA: not applicable.

Compound	Effect measured on cyanobacteria	Effect measured on green algae	Effect measured on crustaceans	Effect measured on fish embryos	Effect measured on fish	References
SPH6	LC_{50} for *M. autumnale* 80 mg/L	EC_{50} for *R. subcapitata* 170 mg/L	LC_{50} for *D. magna* 20 mg/L	LC_{50} for *D. rerio* 27 mg/L	Mortality *D. rerio* between 40 and 80 mg/L	Present study
Formaldehyde	NA	NA	LC_{50}: 3.26 mg/L	NA	NA	Ton et al., 2012
Formaldehyde	NA	NA	NA	LC_{50}: 5.71–6.33 mg/L	NA	Meinelt et al., 2005
Formaldehyde	NA	NA	NA	NA	LC_{50}: 45.73 mg/L	Resendes et al., 2018
Hydrogen peroxide	LC_{50}: 4.15–21.26 mg/L	LC_{50}: 4.15–21.26 mg/L	NA	NA	NA	Drabkova et al., 2007
Hydrogen peroxide	NA	NA	LC_{50}: 3.1 mg/L	NA	NA	Meinertz et al., 2008
Peracetic acid	NA	LC_{50}: 2.46 mg/L	LC_{50}: 0.74 mg/L	NA	NA	Chhetri et al., 2020
Peracetic acid	NA	NA	NA	LC_{50}: 2.24–7.14 mg/L	NA	Marchand et al., 2013
Malachite green	NA	NA	NA	LC_{50}: 0.044–0.047 mg/L	NA	White et al., 2012

recorded LC_{50}-values ranging from 2.8 to 9.3 mg/L (Straus et al., 2018). The SPH6 toxicity should therefore be further investigated for these fish species as well. Bacterial surfactants are generally milder to fish, and another cyclic lipopeptide isolated from *Bacillus amyloliquefaciens*, HAB-2, showed a LC_{50} of 22.20 mg/L to zebrafish (Jin et al., 2018), which indicates a similar or merely slightly higher toxicity compared SPH6 recorded in the present study.

When evaluating a possible use of a biosurfactant in aquaculture enterprises the elimination of the compound in water should be determined. In the present study, using water from a zebrafish production system, the SPH6 concentration showed a significant decrease over 24 h in water containing zebrafish. However, as the elimination may be caused by various factors (bacterial degradation, absorption to fish tank walls and absorption in fish) this parameter may vary significantly between production systems. Further studies on concentration decreases in various types of fish tank water (and at different temperatures and pH) are therefore recommended. As these physico-chemical parameters may influence toxicity further testing of other relevant fish species should be performed.

It should be noted that even though zebrafish did not show any mortality at a SPH6 concentration at 40 mg/L the compound may have affected the respiratory function of the fish. The exposed fish showed an increased ventilation rate suggesting that SPH6 affects oxygen uptake from water, directly or indirectly. Previous studies on rainbow trout exposed to SPH6 demonstrated an effect on mucus release from mucous cell in the skin and a slight inflammation in gills (Mathiessen et al., 2021), which indicates that the compound may interfere with functions in the surface epithelia of fish. Further, if the surfactant is considered for an application for parasite control in aquaculture, further toxicology studies should be performed. Thus, any effect on mammalian system including humans, should be studied in further depth.

5. Conclusions

The aim of the present study was to examine selected ecotoxicological effects of the SPH6 lipopeptide biosurfactant by the use of standardized tests using algae (*R. subcapitata*), cyanobacteria (*P. autumnale*), crustaceans (*D. magna*), zebrafish juveniles (*D. rerio*) and zebrafish embryos. The acute toxicity test for crustaceans showed that *D. magna* was the most sensitive of the tested organisms. Less sensitive were zebrafish embryos, and the fish acute toxicity test showed that juvenile zebrafish were more resistant. Cyanobacteria were tolerant to the compound at low to moderate concentrations, and the least sensitive of the organisms were green algae. This indicates a lower toxicity of the

tested SPH6 substance when compared to corresponding effects of chemicals used regularly in aquaculture. When searching for alternative and sustainable parasiticidals the SPH6 may be a sustainable candidate. The ecotoxicological profile indicated by the present study shows a high effect on a parasite but a low to moderate toxicity towards free-living organisms at different trophic levels. Future work should include an environmental safety assessment addressing effects on human cells, and specific elimination (degradation/adsorption/absorption) studies on the compound under practical fish farm conditions.

CRediT authorship contribution statement

Rozalia Korbut: Writing – original draft. **Lars M. Skjolding:** Data curation, Formal analysis, Writing – original draft. **Heidi Mathiessen:** Data curation, Formal analysis, Writing – original draft. **Rzgar Jaafar:** Data curation. **Xiaoyan Li:** Data curation. **Louise von Gersdorff Jørgensen:** Data curation, Formal analysis, Writing – original draft. **Per Walter Kania:** Formal analysis, Software. **Boqian Wu:** Resources, Formal analysis. **Kurt Buchmann:** Conceptualization, Data curation, Methodology, Funding acquisition, Writing – review & editing.

Declaration of Competing Interest

The company Sundew, employing co-author Boqian Wu, has established a pilot production of the surfactant investigated in the study. A patent application PCT/EP2018/081923 was filed with the European Patent Office November 20, 2018.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.aquatox.2021.106072.

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