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Pilot system for engineering sustainable aquatic food webs: Utilizing cyanobacteria for continuous secretion of ω-polyunsaturated fatty acids

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ABSTRACT

This study introduces a pilot system that uses genetically engineered and marker-less strains of *Synechocystis* sp. PCC 6803 for enhanced secretion of ω-polyunsaturated fatty acids (ω-PUFAs), specifically intended for aquaculture applications. By inactivating the native acyl-acyl carrier protein synthetase (Aas) and expressing the heterologous Fatty Acid Export 1 (FAX1) gene from *Arabidopsis thaliana*, transgenic cyanobacterial lines were obtained that continuously secrete PUFAs in the C_{16} to C_{18} range. Cellular integrity was maintained in these lines, as evidenced by the monitoring of growth rates and photosynthetic parameters. In a controlled aquaculture set-up with the planktonic crustacean *Daphnia magna*, using dialysis tubing for spatial separation, the engineered strains significantly enhanced the survival, the proportion of egg-bearing females, and the reproductive output of *D. magna*. These results underline the potential of genetically engineered algae to act as a sustainable feedstock in aquaculture, enhancing both nutritional quality and environmental safety.

1. Introduction

Aquaculture, defined as the controlled cultivation of freshwater and marine species of fish, invertebrates and algae, serves both food and technical industries. Unlike traditional fishing practices, aquacultures utilize specifically designated areas – including ponds, floating cages, and even isolated systems that are detached from natural environments, for farming [1]. This approach offers a more sustainable way to meet the increasing demand for fish by mitigating the severe ecological damage brought about by open-water overfishing.

Polyunsaturated ω-fatty acids (ω-PUFAs; 16–18 carbon atoms) and highly unsaturated ω-fatty acids (ω-HUFAs; 20–24 carbon atoms, >3 double bonds) are vital for the metabolic and developmental needs of healthy juvenile fish [2,3], and are therefore in great demand in aquatic environments [4]. For example, the consumption of ω-3 fatty acids has been found to be essential for the development of the nervous system and optimal brain function in all animals, including humans [5–8]. In addition, ω-fatty acids play a critical role in aquatic food webs, acting as essential dietary components for many species of zooplankton [9,10]. Currently, fish oil remains the only economically viable source of essential fats such as ω-3 FAs for aquafeed [11]. Access to these resources is therefore a critical limiting factor for growth in aquaculture.

The freshwater cladoceran *Daphnia magna* for example, is one of the best feedstocks for rearing young fish [12]. This small crustacean primarily feeds on bacteria, protozoa, detritus, as well as microalgae [13,14] by using filters on its trunk limbs [15]. As a secondary producer and a key component of zooplankton, fresh and dried *D. magna* has been a mainstay of aquaculture, providing a significant source of the nutrients in the microdiets of several fish species ([16]; MM [17]). *D. magna* likely lacks the ability to synthesize PUFAs de novo and must obtain them through its diet, typically by grazing on PUFA-rich microalgae such as *Scenedesmus obliquus*. While *D. magna* may convert dietary C_{18} PUFAs into HUFAs, the rate of biosynthesis appears insufficient to meet its physiological needs fully [18,19]. In fact, microalgal feed is effectively utilized in hatchery production of zooplankton, mollusks, crustaceans, shrimps and in fish farming [1].

The role of cyanobacteria, as part of the microalgal community, for sustainable free fatty acid (FFA) production has been well established

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[20]. As outlined by Wijffels et al. [21], relative to eukaryotic microalgae, cyanobacteria are the organism of choice in applications such as cell factories for the production of small molecules like FFAs, for which cellular storage capacity is not crucial. In cyanobacteria, the ω-PUFAs linoleic acid (LIN, $C_{18:2(\omega-6)}$) and α-linolenic acid (ALA, $C_{18:3(\omega-3)}$) are predominant in such settings [22,23]. Furthermore, many cyanobacteria are able to fix nitrogen and their light use is complementary to eukaryotic algal species. However, these organisms are of limited use as a food source for *D. magna* in aquatic environments, owing to their small size and robust cell wall, which hinder their efficient ingestion by *Daphnia* [24,25].

The freshwater cyanobacterium *Synechocystis* sp. PCC 6803 (hereafter *Synechocystis* 6803) is a model organism that is readily accessible to metabolic engineering [26,27]. Various approaches, such as the expression of desaturases and thioesterases sourced from various organisms, as well as FA exporters, have led to enhanced FFA production and secretion (for a comprehensive review, see [20]). Most of these studies have involved the inactivation of the acyl-acyl carrier protein synthetase gene *aas*, a key enzyme that is crucial for the esterification of intracellular FFAs to acyl-ACPs in the cytoplasm, and for the uptake and incorporation of exogenously added FFAs into membrane lipids [28]. Aas is localized at the plasma membrane (Fig. 1A; [107]) and is absent from thylakoids [29]. Disruption of the *aas* gene alone results in the accumulation and secretion of FFAs [28,30,31]. Furthermore, knockout of Aas prevents the natural uptake of fatty acids [28,32–35].

The introduction of heterologous solute transporters for substances like lactic acid [36], sucrose [37] and FFAs (Kojima et al. [33] has previously been shown to facilitate the secretion of compounds of low molecular weight into the medium. In this study, the gene for Fatty Acid Export 1 (FAX1; [38]) from *Arabidopsis thaliana* was inserted into the *Synechocystis* 6803 genome to boost the secretion of PUFAs into the surrounding medium. In a proof-of-concept approach for an engineered aquatic food web, transgenic *Synechocystis* 6803 cells were then co-cultivated with both the green alga *S. obliquus* – a conventional dietary component of *Daphnia* cultures – and *D. magna* to assess the impacts of secreted cyanobacterial FFAs on the life-history traits of *D. magna*, including survival rates and offspring production.

2. Materials and methods

2.1. Cultivation of Synechocystis 6803

The glucose-tolerant *Synechocystis* 6803 wild-type strain [108] was cultivated in solid or liquid BG_{11} medium [39] For mixotrophic

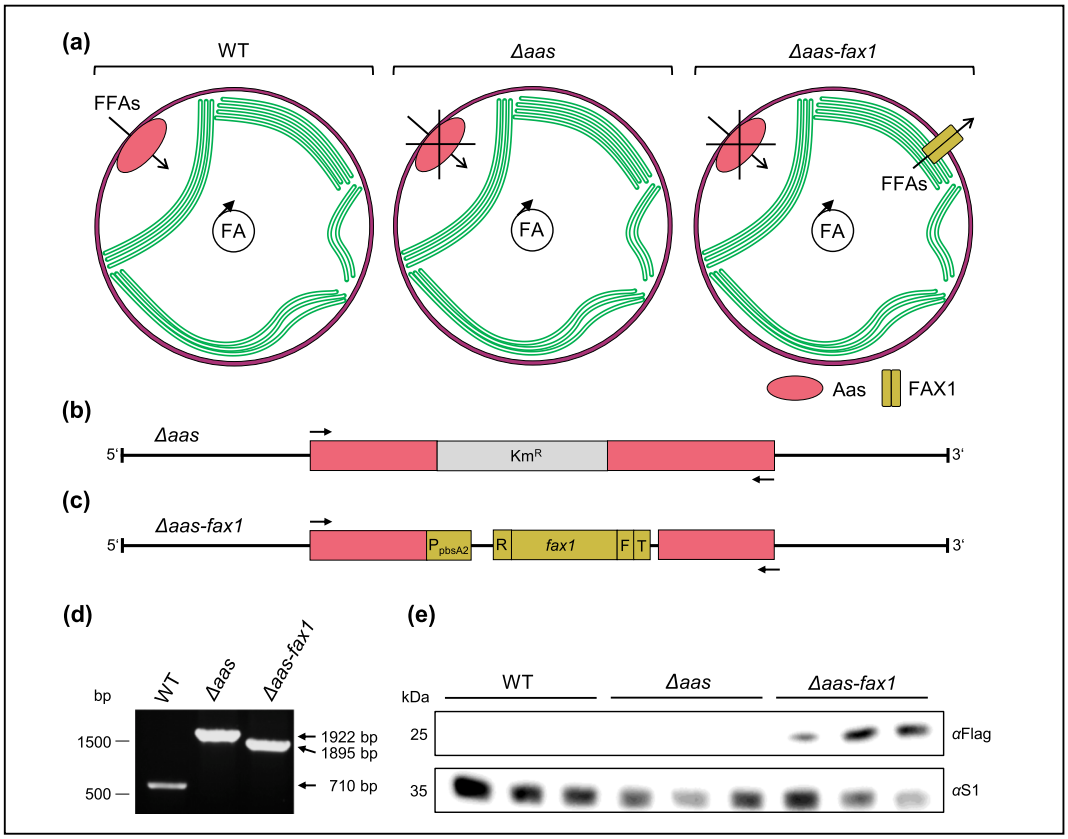

Fig. 1. Engineering of FFA-secreting *Synechocystis* 6803 strains.
(a) Schematic illustration of FFA-secreting strains. The wild-type (WT) cell architecture is depicted with the plasma membrane shown in purple, the thylakoid membranes in green, while fatty-acid synthesis (FA) takes place in the cytoplasm. The native Aas protein is indicated in pink, and the FAX1 exporter from *A. thaliana* is highlighted in yellow-brown. (b) Generation of the *Δaas* mutant. The aas gene was disrupted by the insertion of a kanamycin resistance cassette (Km^R), which effectively precludes the uptake of free fatty acids (FFA). (c) Generation of the *Δaas-fax1* mutant. The *fax1* gene was inserted via the marker-less gene replacement strategy described by Viola et al. [42], simultaneously inactivating the *aas* gene. See Supplemental Fig. S1 for further details. (d) Segregation analysis. Verification of the genetic modifications in *Δaas* (b) and *Δaas-fax1* (c) mutants was conducted via PCR using the primers P2071 and P2072 (Supplemental Table S2), as indicated by the arrows. (e) Validation of FAX1 expression in *Δaas-fax1*. Aliquots (30 μg) of whole-cell protein extracts from three wild-type, *Δaas* and *Δaas*-fax1 clones were fractionated by 12 % SDS-PAGE and subjected to immunoblotting using the specified antibodies directed against αFlag and αS1. Blots with αS1 served as loading controls. The detected signal at the expected size of 25 kDa verifies the presence of the FAX1 protein in the *Δaas-fax1* strain. Abbreviations: F, Flag-tag; FA, fatty-acid synthesis; FFA, free fatty acid; P, promotor; R, ribosome-binding site; T, terminator. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

cultivation, media were supplemented with 5 mM glucose as a carbon source.

Liquid cultures were maintained at 30 °C under continuous illumination provided by cool white light (L 18 W/940 Lumilux De Luxe cool white, Osram, Munich, Germany) at an irradiance of 30 μmol photons m^{-2} s^{-1} and 120 rpm to ensure homogeneity and adequate gas exchange. The cell densities of cultures were monitored photometrically by measuring the optical density at 750 nm (OD_{750}) with a NanoPhotometer® P-Class 360 (Implen GmbH, Munich, Germany). Doubling times were determined in 24-h intervals.

For the selection of mutant strains, solid BG_{11} medium was supplemented with 100–500 μg ml^{-1} kanamycin for *Δaas* mutants and 5 % (w v^{-1}) sucrose for *Δaas-fax1* mutants.

2.2. Construction of Synechocystis 6803 mutants

Plasmids enriched in *Escherichia coli* DH5α cells were isolated using the NucleoSpin® Mini/ NucleoBond® Xtra Midi kit (Macherey-Nagel, Düren, Germany). DNA fragments for plasmid construction were amplified via PCR and purified from 1 % agarose gel using the NucleoSpin® Gel and PCR Clean-Up kit (Macherey-Nagel, Düren, Germany). Target sequences for *Synechocystis* 6803 were retrieved from Cyanobase (http://genome.kazusa.or.jp/cyanobase/Synechocystis). The plasmids, synthesized DNA fragments and primers used are listed in Supplemental Tables S1–3. Furthermore, a detailed scheme of mutant construction is illustrated in Supplemental Fig. S1.

The *aas* gene (ORF *slr1609,* BAA17024) was PCR-amplified from wild-type *Synechocystis* 6803 DNA utilizing the primers P2071/P2072. The resulting PCR fragment was cloned into the pJET1.2 vector via the CloneJET PCR kit (Thermo Fisher Scientific, Waltham, MA, USA), generating the *p994* construct. Subsequently, the kanamycin resistance cassette (Km^R) from *pBSL 15* [40] was inserted into *p994* at a single *Sma*I restriction site, using FastDigest SmaI (Thermo Fisher Scientific, Waltham, MA, USA). After clean-up, the Km^R fragments and the *p994* vector were ligated at a ratio of 3:1 to generate the plasmid *p995*, which was then used to transform *Synechocystis* 6803 (Eaton-Rye [41], thus giving rise to the desired *Δaas* mutant (Fig. 1c). After transformation, complete segregation was verified using the primer pair P2071/P2072 (Fig. 1b).

The *FAX1* gene (AT3G57280) from *A. thaliana* was codon-optimized for expression in *Synechocystis* 6803, and integrated using the single-vector strategy for markerless gene replacement described by Viola et al. [42]. This approach eliminates the need for a permanent antibiotic resistance marker. Simultaneously, the endogenous *aas* gene was inactivated by targeted insertion of *FAX1*.

The 582-bp *fax1* gene was split into two partially overlapping 5′ and 3′ segments, separated by a *nptl-sacB* double-selection cassette [43], thus introducing kanamycin resistance (*nptl*) and conferring lethal effects on Gram-negative cells grown on sucrose-supplemented BG_{11} media that trigger the expression of *sacB*. Each segment was 399 bp long and both shared a 216-bp overlap. A strong *Synechocystis* 6803 *psbA2* promoter (P_{psbA2}) [44] for constitutive *FAX1* expression and a FLAG-tag with a terminator sequence were added upstream and downstream, respectively. Synthetic DNA fragments *p1119* and *p1123* encoding 5′ and 3’ FAX1 segments, respectively, were synthesized by GenScript (New Jersey, USA) and inserted into pJET1.2 to create the constructs *p1121* and *p1130*. These were then combined using BamHI and *Eco*RI to form *p1133*.

The *nptl-sacB* cassette was amplified from plasmid *p1084* [42] with primers P2426/P2427 and ligated into pJET1.2, forming *p1117*. The cassette was then inserted into *p1133* via BamHI and *Sal*I. The resulting construct *p1136* was used to transform *Synechocystis* 6803, resulting in the *Δaas-fax1* strain. During the first recombination event, *aas* flanking sequences of the construct interacted with the corresponding sequences in the wild-type *Synechocystis* 6803 genome, thus disrupting the *aas* gene while *FAX1* remained inactive due to the *nptl-sacB* cassette. The functionality of the negative selection markers was assayed on BG_{11} supplemented with 5 % (w v^{-1}) glucose. Transformants were selected after plating on increasing kanamycin concentrations (5–100 μg ml^{-1}). Subsequently, a second recombination event led to the loss of the cassette and restoration of the functional *FAX1* gene. Selection on 5 % (w v^{-1}) sucrose media eliminated cells retaining the cassette, confirming successful markerless gene replacement (Fig. 1D, E, Supplemental Fig. S1).

2.3. Protein extraction and immunoblotting

To analyse whole-cell proteins [45], 50-ml culture samples were centrifuged at 3000 *g* for 5 min. The resulting pellets were resuspended in lysis buffer (50 mM Tris-HCl pH 7, 20 mM $MgCl_2$, 20 mM KCl, 0.5 % Triton X-100). Cell disruption was achieved using a BeadBug Homogenizer (Biozym, Hessisch Oldendorf, Germany) with 0.5-mm glass beads (Carl Roth GmbH & Co. KG, Karlsruhe, Germany). The process involved three 20-s agitation cycles, each followed by incubation on ice for 1 min, and after a final incubation on ice, the lysates we centrifuged for 1 min at 20,000 g and 4 °C. The resulting supernatants were then subjected to immunoblot analysis.

Protein concentrations were measured using RotiQuant (Carl Roth GmbH & Co. KG, Karlsruhe, Germany), in accordance with Bradford [46]. Equal amounts of proteins (30 μg per sample) were then subjected to 12 % SDS-PAGE, initially run at 80 V for 45 min and subsequently at 120 V for 135 min. Proteins were then transferred using transfer buffer (48 mM Tris; 39 mM Glycine; 0.037 % SDS; 20 % v v^{-1} MeOH) onto PVDF membranes via tank blotting at 100 V for 60 min, following the manufacturer’s instructions. Membranes were blocked with 5 % (w v^{-1}) milk in TBST (10 mM Tris-HCL pH of 7.5; 150 mM NaCl; 0.05 % v v^{-1} Tween 20) for 1 h before being cut and incubated overnight at 4 °C with the primary antibodies αFlag (1:1000; A00170–40, GenScript) and αS1 (1:2000; AS08 309, Agrisera).

Subsequently, membranes were washed three times with TBST for 10 min and incubated for 1 h with a secondary anti-rabbit antibody conjugated to HRP (1:10000; A-9687, Sigma). A final set of three 10-min washes in TBST was performed before capturing immunoblot images using an ImageQuant LAS 4000 system (GE Healthcare, Chicago, IL, USA). SuperSignal™ West Pico PLUS Chemiluminescent Substrate (Thermo Fisher Scientific, Waltham, MA, USA) was utilized for detection. Three separate biological replicates were analyzed for each strain. The molecular weight of the tagged FAX1 protein was predicted using the Protein Molecular Weight tool from BCCM (https://www.genecorner.ugent.be/protein_mw.html).

2.4. Physiological characterization of Synechocystis 6803 mutants

The cell number and volume were quantified using a flow cytometer (BD Accuri C6 Flow 4 Color Cytometer, Boston Industries Inc., Walpole, USA). For inoculation, pre-cultured *Synechocystis* 6803 cells were harvested in the mid-log phase. All experiments were prepared as biological triplicates.

2.4.1. PAM measurements

To assess the photosynthetic yield of Photosystem II (PSII), dilution series of the different *Synechocystis* 6803 strains were spotted onto solid agar media after adjusting the cultures to an OD_{750} of 1.0 and preparing a 10-fold serial dilution using fresh BG_{11} medium. Subsequently, 10-μl aliquots of each dilution were spotted onto the medium and incubated for 7 days. The maximum quantum yield of PSII was measured using pulse-amplitude modulation (PAM) fluorometry (FluorCam 800 MF, Photon Systems Instruments, Drásov, Czech Republic) by determining the F_v/F_m ratio [47] after a 10-min dark adaption. Measurement of the F_v/F_m ratio in cyanobacteria has been shown to accurately reflect the change in the rate of oxygen evolution from PSII [48], and can therefore be used as an indicator of photosynthetic efficiency and overall health.

2.4.2. Absorption spectra analysis

Absorption spectra were recorded, as changes in photosynthetic pigments are considered to serve as an indicator for stress levels in cyanobacteria [49]. For these measurements, *Synechocystis* 6803 cultures were adjusted to an OD_{750} of 1.0 using fresh BG_{11} medium. Subsequently, spectral data across the wavelength range from 350 to 750 nm were collected at room temperature (RT) using a GenesysTM 180 UV/VIS-Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). The data were normalized to the chlorophyll *a*bsorbance at 681 nm, and mean values were calculated from independent replicates. The difference in phycocyanin absorption was determined by dividing the mutant's absorption at 628 nm by the absorption of the wild type. Student's *t*-test was used for pairwise comparisons. $P < 0.05$ was considered statistically significant.

2.4.3. Determination of chlorophyll a content

Chlorophyll a (Chl a) contents of *Synechocystis* 6803 cells were determined according to Wellburn and Lichtenthaler [50]. Prior to extraction, *Synechocystis* 6803 cultures were adjusted to an OD_{750} of 1.0 using distilled water. Cell suspensions (1 ml each) were centrifuged at 10,000 *g* for 1 min at 4 °C. The pellet was resuspended in 1 ml of ice-cold 100 % MeOH, vortexed for 10 s, and incubated in the dark for 5 min at RT. After a second centrifugation at 10,000 *g* for 1 min, the OD_{666} and OD_{720} of the supernatant were measured to calculate the Chl a content.

2.5. Mass spectrometric analysis of FFA profiles

Cultures of *Synechocystis* 6803 strains were harvested during mid-log phase at an OD_{750} of 1. Subsequently, 2 ml of culture medium were transferred into 2-ml Safe Seal reaction tubes (Sarstedt AG & Co. KG, Nümbrecht, Germany). The cells were pelleted by centrifugation at 5000 *g* for 5 min at RT. Subsequently, 1 ml of supernatant was collected into a 1.5-ml Eppendorf Tubes® (Eppendorf AG, Hamburg, Germany), flash frozen in liquid nitrogen, and lyophilized using a rotational vacuum concentration (RVC) system to remove excess water. The system consists of a concentrator (Christ RVC 2–25 plus midi), a cold trap (Christ CT 02–50 SR) and a vacuum pump (Vacuubrand MD 4C NT), operated at 30 °C and 1550 rpm at a condenser temperature of −50 °C. The concentrated samples were overlaid with inert argon gas and stored at −18 °C until processed further.

The extraction procedure used for non-polar FFAs was adapted from Hummel et al. [51] and was performed for five independent biological replicates. The upper organic phase (for samples 550 µl, for standards 500 µl) containing the FFAs was transferred to fresh 1.5-ml Eppendorf tubes and concentrated using the RVC system, then stored at −80 °C.

FFA calibration standards for long-chain fatty acids ($n = 5$) were prepared using HPLC grade ethanol to calculate the fatty-acid secretion rates. The standards consisted of dilutions of stock solutions (each at a concentration of 1 g l^{-1}) of the following fatty acids: palmitic acid ($C_{16:0}$, 99 %), stearic acid ($C_{18:0}$, 98.5 %), oleic acid ($C_{18:1(\omega\text{-}9)}$, >99 %), linoleic acid ($C_{18:2(\omega\text{-}6)}$, 99 %) and stearidonic acid ($C_{18:4(\omega\text{-}3)}$, 98 %). In addition, palmitoleic acid ($C_{16:1(\omega\text{-}7)}$, 98.5 %) and α-linolenic acid ($C_{18:3(\omega\text{-}3)}$, 98 %) were included at a concentration of 500 mg l^{-1}. The fatty acids were sourced from Sigma-Aldrich (St. Louis, Missouri, USA) or Biomol GmbH (Hamburg, Germany). Preparation and extraction of these standards followed the protocol established for biological samples.

For HPLC-MS analysis, the dried extracts were resolved in 80 µl of isopropanol:acetonitrile (3:7) mixed with 20 µl of HPLC-grade water. After a final centrifugation step at 16,000 *g* for 5 min at 4 °C, 80 µl of the solution was transferred into glass vials equipped with conical glass inserts (µ-Insert 6 × 30 mm, 300 µl, tip 12–13 mm, Klaus Trott, Kriftel, Germany) and sealed with Snap-On LC caps. Samples (10 µl), and standards (3 µl each) were injected into a C8 reversed-phase column (Ultra C8 100 × 2.1 mm; Restek, Fuldabrück, Germany) at a flow rate of 300 µl per min at 60 °C. To prevent sample carry-over, a solvent blank was placed between every sample.

Analyses were performed using a Dionex Ultimate 3000 UHPLC system (Thermo Fisher Scientific) coupled with a timsTOF mass spectrometer (Bruker Daltonics, Billerica, USA) following the settings for both the solvent gradient and MS detection described by Espinoza-Corral et al. [52]. To detect FFAs, the electrospray ionization source was operated in negative ion mode. The spectra were acquired using otofControl 6.2. The compounds were annotated using a targeted approach based on the specific mass (m/z), retention time, and isotopic pattern. The data were analyzed using DataAnalysis 5.3 and MetaboScape 2021. Annotation % of FAs were calculated by considering only those peaks that matched the retention times and molecular weights of the respective standards, in our case C_{16} to C_{18} FAs. The FFA secretion rates were calculated in Excel (Microsoft Corporation, 2024) from standard curves based on the FFA calibration standards. Secretion rates are given in µg l^{-1} h^{-1}. Data are presented as means ± standard deviation. Student's *t*-test was used for pairwise comparisons. $P < 0.05$ was considered statistically significant.

2.6. Cultivation of Daphnia magna K217 and Scenedesmus obliquus

A single clone from the clonal line K217 of the cladoceran *D. magna* served as the consumer of the green alga *S. obliquus* in this study. This specific clone originated from Ismaninger Weiher, a pond located near Munich, Germany and has been maintained under controlled laboratory conditions for over two decades. To minimize maternal effects, *D. magna* were cultured under constant conditions for three generations in synthetic culture medium (SSS; [53]) before use in experiments. The medium was fully exchanged every second day to facilitate aeration and elevate oxygen levels. During the refreshment period, cultures were filtered through a 125-µm mesh sieve to remove excess algae, excrement and contaminants. Neonates released by females from the second clutch of the third generation were used as experimental individuals. They were kept in 250-ml jars ($n = 25$) as pure parthenogenic female lines in a climate-controlled chamber at 25 °C and 60 µmol photons m^{-2} s^{-1} of white light in a 16:8 h light/dark photoperiod (PWS-P530 plant growth cabinet, Rubarth Apparate GmbH, Laatzen). The cultures were fed daily ad libitum with *S. obliquus*.

S. obliquus was cultivated in *Z*-medium ([106], Supplemental Table S5) in a climate chamber at 20 °C and 60 µmol photons m^{-2} s^{-1}. The algae were then harvested at 1200 rpm for 8 min using an Eppendorf Centrifuge 5810R, and the resulting pellet was resuspended in pre-boiled tap water before being added to *D. magna* cultures.

2.7. Daphnia magna life history experiment

To investigate the impact of FFA-secreting *Synechocystis* 6803 mutants on *D. magna* life-history traits, i.e. survival and reproduction, *D. magna* were fed with *S. obliquus* (2.66×10^4 cells per ml/per day, i.e. beyond limiting levels), and exposed to treatments involving *Synechocystis* 6803 strains, which were spatially separated from the *D. magna* culture by a dialysis membrane (Membra-Cel™; MWCO: 14.0 kDa, Carl Roth GmbH, Karlsruhe, Germany), thus allowing only the diffusion of macromolecules. The membranes were sealed using 45-mm closure clips (Carl Roth GmbH, Karlsruhe, Germany). The abundance of the *S. obliquus* cell population was determined using a BD Accuri C6 Flow 4 Color flow cytometer (Boston Industries Inc., Walpole, USA). Throughout the experiment, cultures were incubated in the plant-growth cabinet, as previously outlined, with medium devoid of *Synechocystis* 6803 serving as the control. Each of the four treatments was replicated five times (with $n = 20$).

The experiment was conducted utilizing third-clutch 24-h age-synchronized *D. magna* neonates. These were prewashed with SSS medium to remove food residues on the carapace and ensure that old food had been discharged from the gastrointestinal tract [54,55]. Ten neonates per treatment were gently transferred using glass pipettes into 1-l WECK® jars filled with 900 ml of SSS to minimize handling stress. Fresh

Synechocystis 6803 cells were added daily at an OD_{750} of approximately 0.005 using fresh dialysis membranes.

The experimental design comprised two phases (hereafter Phase 1 and Phase 2, Figs. 4 and 5). During Phase 1 (P1; Days 0–16), *D. magna* were fed daily with freshly prepared *S. obliquus* and *Synechocystis* 6803 solutions. The medium was fully replaced every second day to mitigate bacterial contamination and remove residual algae. Phase 2 (P2; Days 14–20) was designed to encourage the accumulation of *S. obliquus* and secreted FFAs by refraining from changing the SSS medium. The feeding interval for *S. obliquus* was extended to every other day, while the *Synechocystis* 6803-containing dialysis membranes continued to be replaced daily until the end of the experiment. In addition, the cultures were monitored for the presence of male neonates, which were not observed at any point during the experiment.

All measurements were taken prior to, or during each medium exchange. The contents of each glass jar were carefully filtered into a clean one prior to counting of *D. magna*. Each jar was screened daily for mortality and reproduction-related metrics. Key parameters recorded included the age of the *D. magna* individuals, the number of egg-bearing females with eggs present in the brood chamber, and the count of neonates. Offspring were removed from the jars during counting.

Data analysis was performed using the SPSS Statistical Software System 15.0 (SPSS, www.spss.com). Results are presented as mean values ± standard deviation. Each variable was tested for normality and homogeneity of variance employing Levene's tests. Statistical comparisons between groups were conducted using one-way ANOVA for normally distributed data, and the non-parametric Kruskal-Wallis test was used when variances were unequal. *P*-values were adjusted according to Bonferroni [56].

3. Results

3.1. Generation and characterization of Synechocystis 6803 strains for FFA secretion

Synechocystis 6803 naturally produces FAs for the synthesis of membrane lipids. Upon membrane degradation, most FAs are recycled via acyl-ACP synthetase (Aas) activity (Fig. 1A, [28]). Previous studies have shown that inactivation of the *aas* gene is essential for the prevention of both uptake and consumption of exogenously added FFAs in both *S. elongatus* 7942 [31] and *Synechocystis* 6803 [33,34].

Hence, as a first step towards the generation of cyanobacterial cell lines that efficiently secrete long-chain FFAs, the *aas* gene (ORF *slr1609*) of *Synechocystis* 6803 was disrupted by the insertion of a kanamycin resistance cassette into its single *Sma*I restriction site, thus inhibiting the re-uptake of secreted FFAs ([57]; Fig. 1A, B; Supplemental Fig. 1a). Complete segregation of the expected *Δaas* mutation was verified by PCR (Fig. 1d). Furthermore, the *FAX1* gene that codes for the FFA transporter in the plastid envelope (fatty acid export 1; [38]) was incorporated into the wild-type genome. FAX1 has been shown to predominantly export C_{16} and C_{18} FFAs from the chloroplast, and was therefore regarded as a suitable candidate for the secretion of long-chain FAs in the heterologous cyanobacterial system. The region coding for FAX1 in *A. thaliana* was fused to the P_{psbA2} promoter in *Synechocystis* 6803, an *α*Flag-tag epitope at its C-terminus and inserted into the *aas* site by using a markerless-gene replacement strategy (Fig. 1d, e; [42]). Integration at the *aas* site into the genome was verified by PCR (Fig. 1d). Moreover, immunoblotting using an αFlag antibody confirmed the heterologous expression of *FAX1* in the resulting mutant strain *Δaas-fax1* (Fig. 1e).

Both mutant strains exhibited growth patterns similar to that of the wild type during the exponential growth phase (0–72 h). The mutant strains also exhibited a prolonged late exponential phase extending for up to 96 h. Notably, during the stationary phase (96–216 h), the mutants grew significantly faster than the wild type (Fig. 2a, Supplemental Table S4). Hence, the genetic manipulations had no deleterious effects on the growth of *Synechocystis*. This finding also agrees with F_v/F_m ratios measured across cell lines over time (see section 2.4.1.), indicating that all strains maintained an equally healthy state, regardless of their genotype (Fig. 2b). Levels of photosynthetic pigments, a known indicator of stress in cyanobacteria, were also evaluated [49]. Although there were no notable variations in chlorophyll *a* content among the strains (~2.5 μg OD_{750}^{-1}), absorption spectra revealed a significant reduction in phycocyanin absorption in both the *Δaas* (15.5 ± 0.3 %) and *Δaas-fax1* mutants (21.0 ± 3.6 %), suggesting a slight decrease in phycobiliprotein content (Fig. 2c).

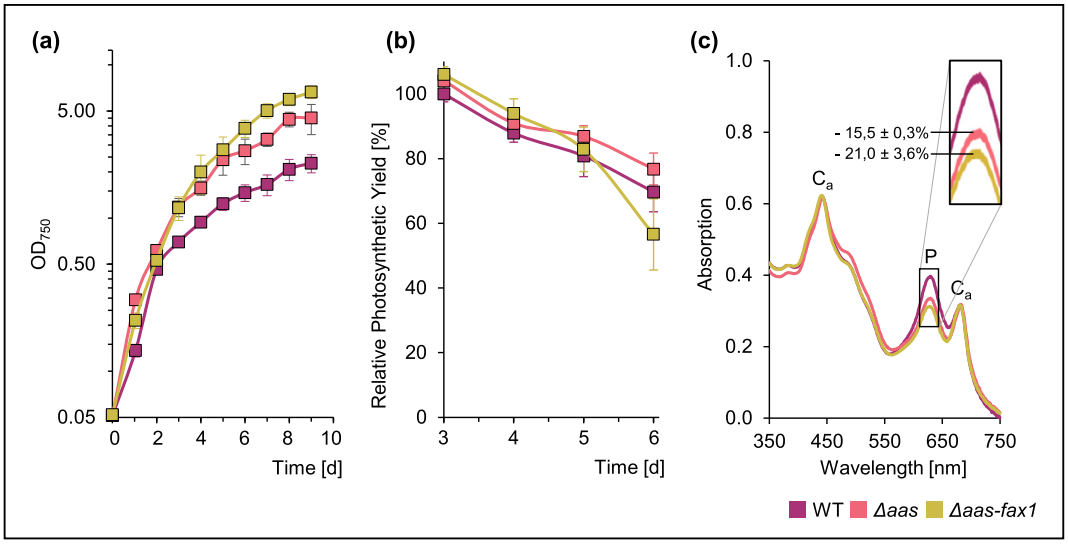

Fig. 2. Comprehensive analysis of growth and photosynthetic characteristics of *Synechocystis* 6803 strains.
(a) Growth curve analysis. Evaluation of liquid cultures growth under photoautotrophic conditions at 30 μmol photons m^{-2} s^{-1}, 30 °C and 120 rpm based on optical density measurements at 750 nm (OD_{750}). (b) Assessment of photosynthetic yields. Photosynthetic efficiency, i.e., the F_v/F_m ratio, was determined after 3–6 days of growth. Liquid cultures (10 μl) were spotted onto glucose-depleted solid BG_{11} agar plates and incubated at 30 μmol photons m^{-2} s^{-1} and 30 °C. (c) Whole-cell absorption spectroscopy. Absorption measurements were normalized to chlorophyll a (Chl a) at 681 nm. Peaks at 440 and 681 nm are indicative of Chl a (Ca), while the peak at 628 nm corresponds to phycocyanin (P). The phycocyanin absorption in *Δaas* and *Δaas-fax1* strains was significantly reduced by 15.5 ± 0.3 % and 21.0 ± 3.6 %, respectively, compared to the wild-type value ($P < 0.005$, Student's *t*-test).

Overall, verification of the construction of the *Δaas* and *Δaas-fax1* strains and their comprehensive physiological characterization confirms their functional integrity. Importantly, these mutants exhibited no significant defects or adverse alterations in growth relative to the wild type, thus establishing a solid foundation for subsequent FFA quantification.

3.2. Increased secretion of long-chain polyunsaturated FAs by Δaas and Δaas-fax1 strains

In the next step, the extracellular fatty-acid profiles of photoautotrophically grown wild-type, *Δaas* and *Δaas-fax1* strains were analyzed using LC-MS (Fig. 3, Table 1). Accurate quantification of FFA species was performed specifically for fatty acids in the C_{16} to C_{18} range, as these comprised the majority of FFAs found in the media in which the *Synechocystis* 6803 strains were grown (Supplemental Fig. S2). This analysis utilized the FFA calibration standards listed in section 2.5.

Overall, the composition of extracellular FFAs revealed that the saturated lipids palmitic acid (PA, $C_{16:0}$) and stearic acid (SA, $C_{18:0}$) predominated in all three strains (Table 1). However, in the media recovered from mutants, the C_{16}/C_{18} ratio was slightly shifted towards C_{16} FFAs. While $C_{18:0}$ remained the most prevalent extracellular FFA in all strains, in both *Δaas* and *Δaas-fax1* mutants, its relative level was reduced to 46.1 % and 46.2 %, respectively, compared to 59.4 % in the wild type (Table 1). Conversely, levels of the secreted polyunsaturated FFAs $C_{18:2}$ and $C_{18:3}$ increased in the media secreted by both strains, while the total amounts of $C_{16:0}$ and $C_{16:1}$ increased slightly in both mutants, which explains the shift in the C_{16}/C_{18} ratio. Other FFAs accumulated to only a minor extent (Supplemental Fig. S2).

In agreement with these results, both mutant strains secreted larger amounts of unsaturated C_{16}-C_{18} FAs relative to the wild type (Fig. 3, Table 2). In particular, the *Δaas-fax1* mutant secreted more than threefold higher levels of linoleic acid, α-linolenic acid and stearidonic acid (SDA, $C_{18:4}$, n-3) than either the wild type or the *Δaas* strain (Table 2). Palmitoleic acid (POA, $C_{16:1}$, n-7) levels were also significantly higher in both mutant strains, especially in the *FAX1*-expressing strain *Δaas-fax1* (Table 2). Following *aas* inactivation, the fraction of polyunsaturated C_{18} FFAs ($\sum C_{18:2}$-$C_{18:4}$) increased from 3.3 % in the wild type to 10.0 % and 12.4 % in *Δaas* and *Δaas-fax1*, respectively.

Thus, the introduction of the *Δaas* and *Δaas-fax1* mutations altered the overall extracellular FFA profile, shifting it towards a higher proportion of polyunsaturated fatty acids, particularly in the C_{18} range. These data suggest that both mutations have an impact on fatty-acid metabolism and export, and as such align well with the reported preference of FAX1 for C_{16} and C_{18} fatty acids in *Arabidopsis* [38]. Taken together, these results validate the functionality of the engineered system for the secretion of PUFAs from *Synechocystis* 6803.

Table 1
Relative distribution of C_{16} and C_{18} FFAs in the media of photoautotrophically-grown *Synechocystis* 6803 mutants.

	% of total C_{16} and C_{18} FFAs							
Strain	16:0	16:1	18:0	18:1	18:2	18:3	18:4	*PUFAs
WT	34.6	0.6	59.4	2.0	1.8	1.7	0.3	3.3
Δaas	39.6	1.7	46.1	2.6	3.1	6.4	0.5	10.0
Δaas-fax1	36.4	2.2	46.2	2.7	3.8	7.9	0.8	12.4

* Sum of the percentages of polyunsaturated FFAs secreted into the medium by the respective strains.

3.3. Impact of enhanced FFA secretion by Synechocystis 6803 mutant strains on the life history response of Daphnia magna

The impact of FFA-secreting *Synechocystis* 6803 mutant strains on the survival and reproductive performance of the water flea *D. magna* was assessed over a 20-day period. *D. magna* cultures fed with *S. obliquus* were exposed to the different *Synechocystis* 6803 strains, which were spatially separated from the crustaceans by dialysis tubing (2.7). During Phase 1 (P1), the synthetic SSS medium was refreshed on every second day in order to control bacterial growth and limit FFA accumulation (Fig. 4a). The sustainability of the growth of *Synechocystis* 6803 in synthetic SSS medium was verified, yet showing increased doubling times (13–48 %) compared to standard conditions with BG_{11} and reduced biomass (Fig. 4B; Supplemental Table S4). Notably, both the *Δaas* and *Δaas-fax1* strains exhibited significantly faster growth rates compared to the wild type.

Survival rates of *D. magna* fed *S. obliquus* alone declined over time, while those additionally exposed to *Synechocystis* 6803 strains demonstrated higher survival rates (Fig. 4a). The same is shown with *Synechocystis* 6803 but without *S. obliquus* as feed for *D. magna* (Fig. 4a). In Phase 1, *D. magna* exposed to *Synechocystis* 6803 showed a significant increase in average lifespan across genotypes relative to the control group, which had the lowest average lifespan of 10.78 ± 0.50 days. The lowest survival rate (12.18 ± 0.82 days) was observed upon exposure to the wild-type *Synechocystis* 6803 strain, closely followed by *Δaas* (12.20 ± 0.82 days) and *Δaas-fax1* (12.66 ± 0.75 days; Fig. 4c). In Phase 2, which allowed for FFA accumulation, these differences in lifespan were further accentuated. The *Δaas-fax1* mutant showed the greatest increase to 15.12 ± 0.98 days. The control group exhibited the lowest average age (11.42 ± 0.65 days) at the end of the experimental period, followed by the wild type (14.02 ± 1.02 days) and *Δaas* (14.10 ± 1.02 days; Fig. 4d). Thus, significantly increased survival in the presence of *Δaas* and *Δaas-fax1* indicated that provision of FFAs to growing cultures of *D. magna* has a positive influence on the survival of experimental females.

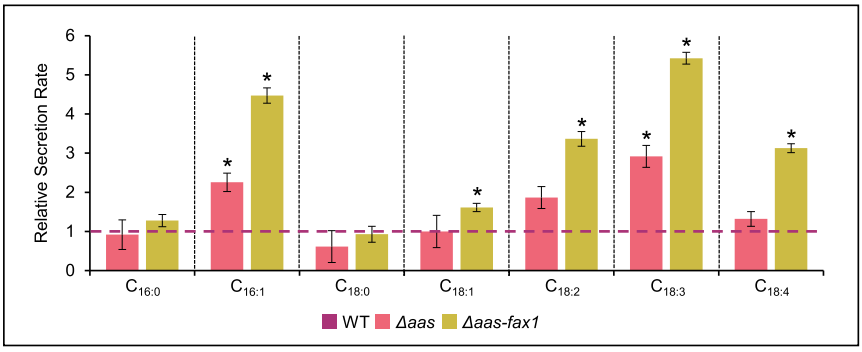

Fig. 3. Mass spectrometry-based quantification of secretion rates of C_{16} and C_{18} FFAs in *Synechocystis* 6803 strains.
Liquid cultures of wild-type Synechocystis 6803 (WT), *Δaas*, and *Δaas-fax1* strains were grown under standard conditions, and harvested at mid-log phase (OD_{750} = 1). WT levels were normalized to 1 (dashed purple line). Data are presented as means ± standard deviation ($n = 5$). Asterisks (*) indicate significant differences based on Student's t-test ($P < 0.005$). For total secretion rates, see Table 2. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 2
Quantification of secretion rates of C_{16} and C_{18} long chain fatty acids.

	FFA [µg l^{-1} h^{-1}]							
Strain	16:0	16:1	18:0	18:1	18:2	18:3	18:4	*SUM
WT	22.6 ± 3.8	0.6 ± 0.3	26.1 ± 5.1	1.0 ± 0.2	0.8 ± 0.2	1.0 ± 0.1	0.2 ± 0.0	52.4
Δaas	20.7 ± 7.9	1.5 ± 0.3	16.1 ± 6.6	1.0 ± 0.4	1.5 ± 0.4	2.9 ± 0.8	0.3 ± 0.1	44.0
Δaas-fax1	28.8 ± 4.5	**2.9 ± 0.6**	24.3 ± 4.9	1.7 ± 0.2	**2.8 ± 0.5**	**5.4 ± 0.8**	**0.7 ± 0.1**	66.6

* The total secretion rate was determined by summing the secretion rates of C_{16} to C_{18} fatty acids, as quantified by MS using standards. Secretion rates that differ significantly from both wild type and *Δaas* are shown in bold ($P < 0.005$, Student's *t*-test).

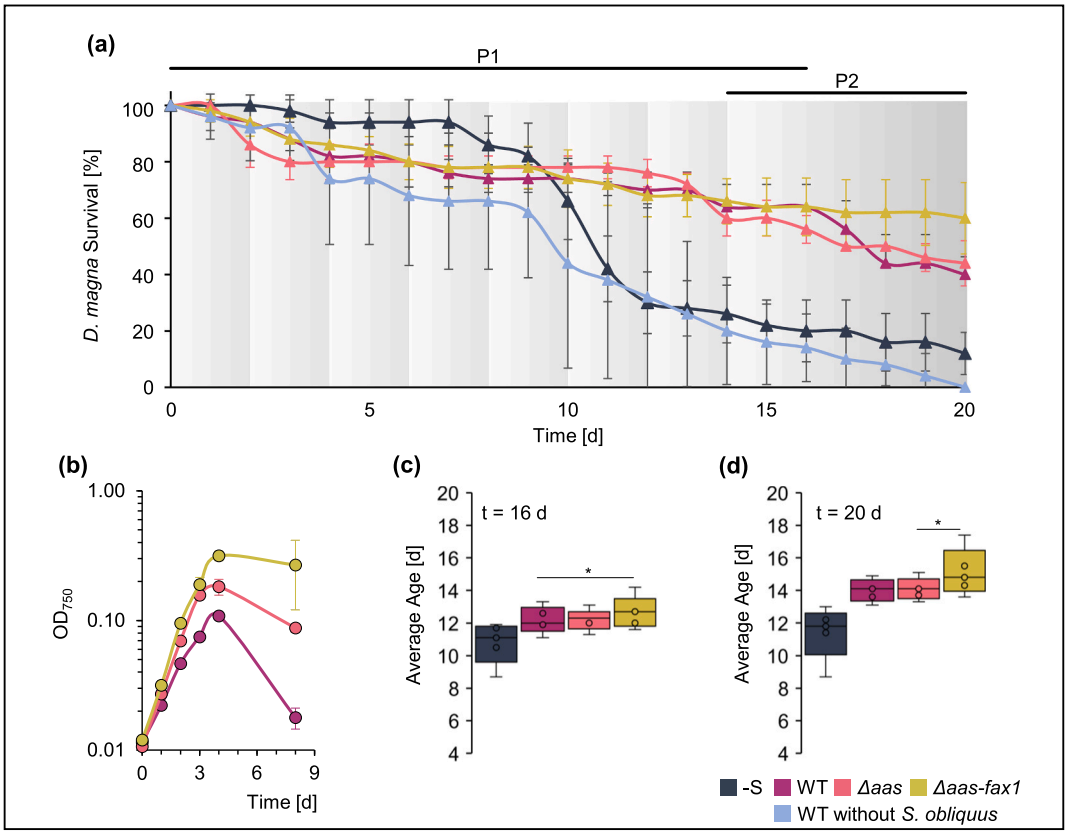

Fig. 4. Impact of FFA-secreting *Synechocystis* 6803 strains on the survival of *Daphnia magna* cultures.
(a) *D. magna* cultivation. Age-synchronized *D. magna* neonates from the third clutch were maintained in a climate chamber (25 °C, 60 µmol photons m^{-2} s^{-1}) and fed with *S. obliquus*. They were then exposed to wild-type (WT), *Δaas* and *Δaas-fax1 Synechocystis* 6803 strains, which were spatially separated from the *Daphnia* cultures by dialysis tubing that was replaced daily. Controls included *D. magna* that had not been exposed to *Synechocystis* 6803 (—S) and *D. magna* that had not been exposed to *S. obliquus* but WT *Synechocystis* 6803 (WT without *S. obliquus*). The synthetic SSS medium was refreshed every two days during the initial 16 days (Phase 1, P1). The medium was not exchanged from day 14-to day 20 (Phase 2, P2), in order to encourage FFA accumulation. During this period, *S. obliquus* was added on every second day. (b) Growth of *Synechocystis* 6803 strains in SSS medium. Liquid cultures were grown at 25 °C, 30 µmol photons m^{-2} s^{-1}, and 120 rpm to verify the viability of cells over the course of the experiment in synthetic medium. (c)-(d) *Daphnia* age metrics: The average age of *Daphnia* was recorded after 16 (c) and 20 (d) days of cultivation. Asterisks denote significant deviations from control conditions, as determined by the Kruskal-Wallis test with Bonferroni correction ($P < 0.05$, Kruskal-Wallis test). Data are presented as mean ± standard deviation (n = 5).

Significant effects were observed in the fraction of egg-bearing females and the number of neonates produced at later stages of culture. A time-resolved plot reveals that egg production began essentially synchronously on day 4 (Fig. 5a), regardless of the treatment regime. The fraction of gravid females often increased as the medium approached exhaustion. Notably, the average fraction of gravid females was significantly higher in cultures that were exposed to the *Δaas-fax1* mutant compared to other groups, reaching a maximum of 42.9 ± 3.9 % in *Δaas-fax1* compared to 20.5 ± 1.4 % in the control group during P1 (Fig. 5b). The corresponding fractions upon exposure to the wild-type and *Δaas* strains were 34.0 ± 3.2 % and 35.4 ± 1.5 %, respectively. This trend was even more pronounced after phase P2, with the fraction of egg-bearing females in the presence of *Δaas-fax1* reaching 45,7 ± 4.6 %, relative to 7.1 ± 2.1 %, 20.3 ± 2.1 %, and 25.7 ± 2.0 % in the control, wild-type and *Δaas* treatments, respectively (Fig. 5c).

The average number of neonates produced throughout the experiment was negligible under control conditions (0.2 ± 0.2; Fig. 5d), implying that *D. magna* cultures are essentially unsustainable in the absence of added FFAs. It is also important to note that in the absence of *S. obliquus* as feed (Fig. 4a), *D. magna* failed to produce any neonates when cultivated solely with different *Synechocystis* 6803 strains. In contrast, the addition of *S. obliquus* and exposure to *Δaas-fax1* led to a fourfold increase in neonate production (160.6 ± 50.4) compared to treatment with wild-type *Synechocystis* 6803 (38.6 ± 4.6) and was significantly higher than that in the control treatment. Conversely, exposure to the *Δaas* mutant resulted in the lowest neonate production

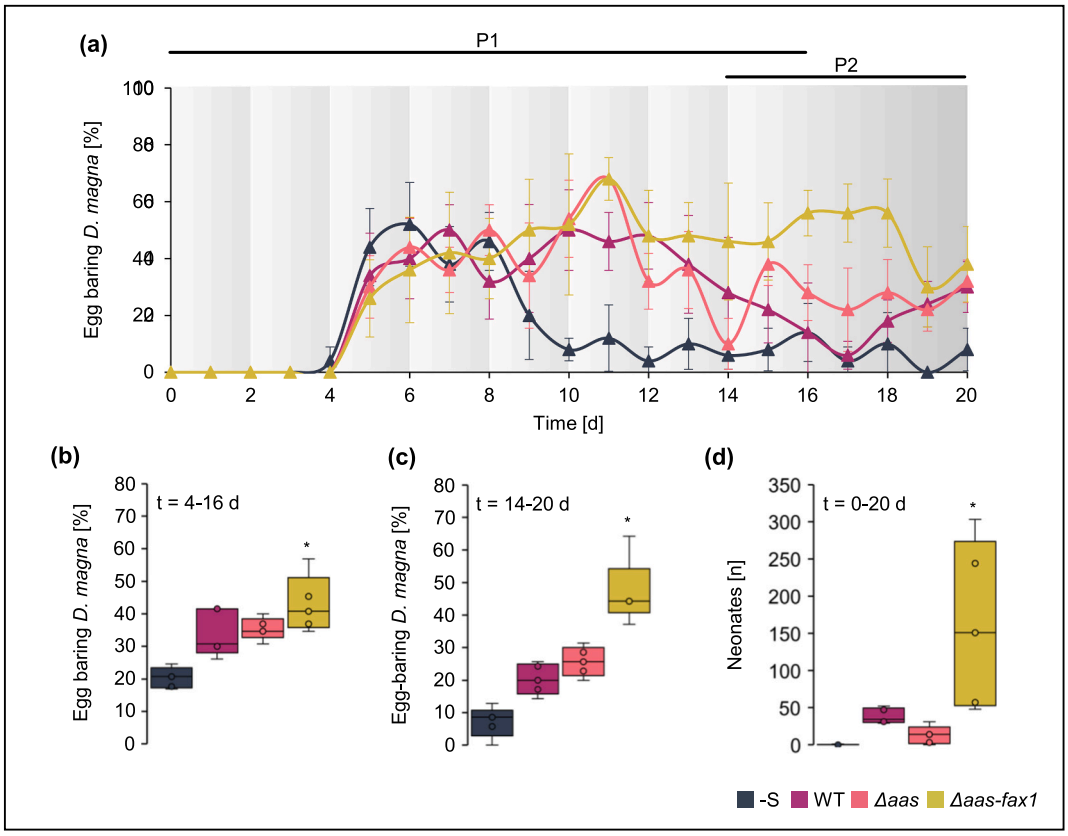

Fig. 5. Effects of FFA-secreting *Synechocystis* 6803 strains on the reproductive performance of *Daphnia magna* cultures.
Age-synchronized *D. magna* neonates from the third clutch were maintained in a climate chamber (25 °C, 60 μmol photons m^{-2} s^{-1}) and fed with *S. obliquus*. They were then exposed to wild-type (WT), *Δaas* and *Δaas-fax1* strains of *Synechocystis* 6803, which were spatially separated from the *Daphnia* cultures by dialysis tubing that was replaced daily. Controls included *Daphnia* that were not exposed to *Synechocystis* 6803 (—S). The synthetic SSS medium was refreshed every two days during the first 16 days (Phase 1, P1). On days 14–20 (Phase 2, P2), the medium was not exchanged, so as to encourage FFA accumulation. During that period, the intervals between feeding of *S. obliquus* were extended to every second day. (a) Daily fraction of egg-bearing daphnids. This parameter was monitored daily throughout the experimental period in which *Daphnia* cultures were exposed to either wild-type *Synechocystis* 6803, *Δaas* or *Δaas-fax1* strains. (b) Ratio of egg-bearing daphnids averaged over the period from day 4 to day 16, during the initial phase (P1). (c) Ratio of egg-bearing daphnids averaged over the second phase, during which the medium was not exchanged (P2). (d) Cumulative neonate production. Total number of neonates produced after 20 days. Asterisks denote significant deviations from control conditions, as determined by the Kruskal-Wallis Test with Bonferroni correction ($P < 0.05$, Kruskal-Wallis Test). Data are presented as mean ± standard deviation (n = 5).

(13.0 ± 5.5) among the *D. magna* cultures (Fig. 5d).

Our results thus demonstrate that exposure to engineered FFA-secreting *Synechocystis* 6803 mutant strains significantly enhanced both the survival and reproductive performance of *D. magna*. Thus, the FFAs secreted by the *Δaas* and *Δaas-fax1* mutants markedly improved the life-history traits of *D. magna* relative to wild-type *Synechocystis* 6803 that lacked these compounds. These positive effects, evident in an extended average lifespan and a higher fraction of egg-bearing females and neonate production demonstrate the beneficial impact of added FFAs on the vitality and fecundity of *D. magna*.

4. Discussion

4.1. Replacement of the aas gene by FAX1 enhances secretion of ω-3 PUFAs by Synechocystis 6803

Our study introduces a novel pilot system that utilizes genetically modified *Synechocystis* 6803 as a source of beneficial PUFAs for aquaculture. Direct export of these fatty acids by the cyanobacterial cells eliminates the need for resource-intensive filtration, cell lysis and product purification. Furthermore, the choice of marker-less gene replacement, as well as the spatial separation of the modified cyanobacteria from the consumers of the supplied FAs, mitigates environmental concerns linked to the use of GMOs [58]. Hence, this concept demonstrates how genetically altered cyanobacterial cells can be responsibly employed for biotechnological applications in aquatic environments.

The strategy for the generation of PUFA producer strains followed a two-step process involving (i) the inhibition of FFA uptake via inactivation of the endogenous *aas* gene and (ii) the parallel introduction of the FFA exporter *FAX1* transgene from *A. thaliana*. Inhibition of Aas activity was found to be crucial during mutant strain construction (Fig. 1). Aas has been identified as a key importer of FFAs [57], and its inhibition prevents the reabsorption of secreted FFAs and recycling of FFAs [28,35]. This not only mitigates the toxicity associated with the accumulation of extracellular FAs [28,59], but also facilitates the release of FFAs into the surrounding medium. However, the exact mechanism of the secretion pathway involved – whether active or passive – remains elusive [28].

Cultivation of the mutant strains described here did not result in growth deficits or reduced photosynthetic activity during the exponential phase of growth under standard conditions (Fig. 2a, b). The growth behavior of our *Δaas* mutant agrees with those reported in previous studies on Aas-deficient mutants in *Synechococcus* sp. PCC 7002 [30], *S. elongatus* PCC 7942, and *Synechocystis* 6803 [28], in which similar conditions did not significantly impair growth. Other studies that investigated high-light stress in Aas-deficient strains of *S. elongatus* PCC 7942 ([35], Kato et al.2015, 16) and *Synechocystis* 6803 [33] have

demonstrated PSII destabilization, owing to enhanced lipid diacylation and intracellular FFA accumulation at higher light intensities (180–400 μmol photons m^{-2} s^{-1}). However, such effects are irrelevant under the experimental conditions applied here.

Selective degradation of chlorophyll *a* pigment in response to FFA production and secretion, as described for *S. elongatus* PCC 7942 [31], was not observed. The decrease in phycocyanin absorption in the mutants involved here is a common stress response in cyanobacteria (Fig. 2C; [60]), but did not negatively impact growth under the conditions employed in this study.

In agreement with prior research, both mutant strains engineered for elevated production of ω-PUFA secretion showed enhanced growth during the approach to the stationary phase (Fig. 2A; [61,62]). It has been proposed that the synthesis of reduced carbons in triacylglycerols and fatty-acid synthesis pathways might act as an electron sink under photo-oxidative stress by utilizing excess electrons that accumulate in the photosynthetic electron transport chain, which might otherwise induce over-production of reactive oxygen species, thus causing damage to membrane lipids, proteins and other macromolecules [63]. Similar alleviating effects have been observed in sucrose-secreting *S. elongatus* PCC 7942 strains [37]. Furthermore, it has been shown that FFA-secreting strains are markedly more robust than wild-type cultures during the stationary phase [34], and generate lower levels of ROS formation [64]. Taken together, these factors probably explain how FFA secretion contributes to the prolongation of the exponential growth phase and enhanced growth during the stationary phase in the *Δaas* and *Δaas-fax1* strains (Fig. 2a). The introduction of FAX1-mediated FFA secretion appears to further enhance this positive effect, alleviating detrimental effects commonly associated with high intracellular FFA levels.

Various FFA-producing cyanobacterial mutants have already been engineered with the intention of maximizing FA productivity and enhancing secretion rates (Supplemental Table S6). Among the most prolific strains developed, derivatives of *Synechocystis* 6803 have demonstrated secretion rates as high as 438 μg l^{-1} h^{-1} [34] and even 1023 μg l^{-1} h^{-1} [64]. Although engineered mutants of *S. elongatus* PCC 7942 have generally shown lower productivity, studies such as that done by Kato et al. [65] have shown that extraction of product from the medium can substantially elevate total FFA secretion, achieving rates up to 1500 μg l^{-1} h^{-1}. In this study, our calculated total FFA secretion rates of 44 μg l^{-1} h^{-1} for *Δaas* and 67 μg l^{-1} h^{-1} for *Δaas-fax1* are notably lower than those reached in these earlier reports. The major factor that contributes to these reduced rates is the early harvest of samples during the exponential growth phase for HPLC-MS analysis. It has been well-documented that the majority of FFA secretion occurs during the stationary phase [30,31,34], and thus studies that aim to maximize FFA productivity typically allow cultures to mature into late stationary phase, often extending cultivation to between 168 and 480 h (Supplemental Table S6). Moreover, in our experiments, cells in exponential phase were promptly utilized for the feeding of *D. magna* cultures, owing to growth limitations in SSS medium (Fig. 4b), which prevent cultures from reaching high optical densities. Consequently, fatty-acid profiling was conducted during this earlier growth phase to assess the range of FA species secreted under these specific physiological conditions. Furthermore, the secretion rates reported in other studies were often obtained under conditions of high-light intensity, aeration with CO_2-enriched air or the addition of nitrate – conditions that were deliberately avoided in our experimental set-up.

The Fatty Acid Export 1 protein FAX1, isolated from *A. thaliana*, has been identified as a mediator of fatty-acid export from plastids to the cytosol [38]. Following its successful expression in the *Synechocystis* 6803 mutant strain *Δaas-fax1* (Fig. 1e), FAX1 significantly enhanced the secretion of unsaturated fatty acids, notably elevating levels of POA, LIN, ALA and SDA (Fig. 3, Table 1). In this strain, PUFAs accounted for 12.4 % of the total FAs, compared to 10.0 % in the *Δaas* mutant, and only 3.4 % in wild-type strains (Table 1), demonstrating the substrate specificity of FAX1 for PUFAs in *Synechocystis* 6803.This is supported by FAX1's preference for C_{16} and C_{18} fatty acids *in A. thaliana* [38]. It has been proposed that FAX1 selects substrates primarily based on chain length rather than the degree of unsaturation, with its transport activity being driven by the concentration gradients of FFAs. Consequently, the enhanced FFA secretion can be attributed to the increased intracellular concentrations of these fatty acids in *Δaas-fax1* cells, potentially/probably driven by the inactivation of Aas. This selective FAX1-mediated FFA export mechanism ensures the transport of nutritionally valuable PUFAs, thereby enhancing the nutritional profile of cyanobacterial excretions.

FFAs can cross the cell membrane either by passive diffusion [66] or via specific transporters. While passive diffusion effectively secretes middle-chain FFAs [34], it generally fails to efficiently export long-chain FFAs. This limitation highlights the importance of employing an exporter like FAX1 in our *Synechocystis* 6803 strain to target and enhance the export of nutritionally valuable long-chain PUFAs. Moreover, this targeted export might have enhanced intracellular FFA production by shifting the reaction equilibrium and possibly reducing feedback inhibition of acyl-ACP, as suggested by Afrin et al. [64].

In an attempt to facilitate secretion of FFA in the medium, earlier studies explored the effectiveness of various FFA efflux systems to boost cyanobacterial FFA production and secretion, such as overexpressing endogenous RND-type efflux pumps in *S. elongatus* PCC7942 [62] or introducing heterologous transporters like the FarB transporter [33] from *Neisseria lactamia* [67]. Although these systems enhanced secretion rates under specific conditions (e.g., high-light intensity and CO_2 supplementation), they predominantly secreted saturated and mono-unsaturated FFAs. For instance, secretion rates of up to 230 μg l^{-1} h^{-1} were achieved in *S. elongatus* PCC7942, yet the spectrum was limited to shorter and saturated fatty acids [62]. FarB-expressing *Synechocystis* 6803 *dAS11_farB* cells were able to secrete 60 % of FFAs as LIN and ALA under comparable levels of illumination (50 μmol photons m^2 s^{-1}). However, secretion rates were not significantly increased in wild-type cultures and FFA productivity was notably lower with 24 μg l^{-1} h^{-1} [33] as compared to 67 μg l^{-1} h^{-1} for *Δaas-fax1*. Conversely, *FAX1* expression in *Synechocystis 6803* significantly increased the secretion of PUFAs, including LIN, ALA, and notably SDA, which constituted 7.6 % of the secreted fatty acids – a unique feature among published FFA efflux systems to date (Fig. 3, Supplemental Table S6). Actually, *Synechocystis* 6803 is one of only four cyanobacteria that are known to produce SDA [68], unlike e.g. the *Synechococcus* species used in most other studies.

As the heterologous expression of *FAX1* enhances the secretion of ALA and SDA under conditions that do not require intense light or high CO_2 levels, such a system may be particularly suited for applications in aquatic environments, since it directly enhances the dietary availability of essential ω-3 fatty acids. ALA and SDA serve as precursors for the biosynthesis of long-chain ω-3 fatty acids, such as eicosapenta-enoic acid (EPA, $C_{20:5(\omega-3)}$) and docosahexa-enoic acid (DHA, $C_{22:6(\omega-3)}$). Upon consumption, ALA and SDA are readily converted into EPA and DHA [69,70], offering a direct route for the enrichment of dietary sources of ω-3 fatty acids in aquaculture settings.

While FAX1 has shown promise in enhancing the secretion of PUFAs, the substrate specificities of transporters can vary significantly between different host organisms. To further refine and enhance the efficiency of FFA secretion, it may be beneficial to explore the incorporation of additional or alternative transporters known for their efficacy in other systems [67,71,72] or to utilize completely different hosts. In this way, total FFA secretion could help to customize the fatty-acid profile according to specific needs, thus enhancing the commercial and ecological value of the production system.

4.2. Enhancing Daphnia magna lifespan and reproductive output with FFA-secreting Synechocystis 6803

Daphnia species play a pivotal role in aquatic ecosystems. They are

effective filter-feeders, significantly influencing phytoplankton populations and serving as a crucial link between primary production and the cultivation of numerous fish species [73]. The efficacy of microalgae as a dietary component for *Daphnia* is influenced by a number of factors, including size [74], shape, potential toxicity, stoichiometry [10], and biochemical composition [75,76]. Importantly, the availability of ω-HUFAs in their diet was found to be important for reproduction, egg development, and food quality in *Daphnia* [77–79].

In this study, we explored the impact of ω-PUFA supplementation by FFA-secreting *Synechocystis* 6803 on the effects of these FFAs on the survival and reproduction of *D. magna*. Our results reveal a notable improvement in the survival rates of *D. magna* cultures fed with *S. obliquus* and exposed to *Synechocystis* 6803 (Fig. 4a). Cultures exposed to the *Δaas-fax1* strain, which secretes higher levels of ω-PUFAs (i.e. LIN, ALA, and SDA) boosted the average lifespan of *D. magna* individuals by 17 % after 16 days (Fig. 4c), reaching 19 % after 20 days of cultivation with accumulation of FFAs (Fig. 4d). These results suggest that the survival enhancements observed in the experiment were primarily attributable to the differential secretion of ω-PUFAs by *Synechocystis* 6803, and not simply an effect of the presence of the microalga *S. obliquus*, which was provided at well above limiting levels and in equal concentrations across all treatments.

With regard to reproductive output, our study observed a substantially higher proportion of egg-bearing females (Fig. 5b, c) and an increase in neonate production (Fig. 5d). Specifically, the presence of *Δaas-fax1* led to a fourfold increase in neonate numbers (Fig. 5d), signifying an enhancement of FFA-secreting *Synechocystis* 6803 mutants as food additive for *D. magna*.

As the beneficial effects on survival and reproduction were more pronounced when medium exchange was omitted, the improvement can most probably be attributed to the enhanced availability of LIN, ALA and SDA in the medium, as these compounds are known precursors for ω-HUFAs, such as arachidonic acid (ARA, $C_{20:4(\omega-6)}$), EPA and DHA [80]. *Daphnia* can use ALA and LIN as precursors for the synthesis of EPA and ARA, respectively [79,81,82]. However, contrasting results have been reported for *Daphnia* species in which the conversion of ALA to EPA was limited [83–85]. In the metabolism of *D. galeata*, conversion was evident [79,86]; however, it has not been proved for certain clones of *D. magna* [87]. Therefore, uptake and conversion of secreted ω-PUFAs in our set-up is possible, but might be of secondary importance due to the energy-consuming nature of the conversion, as proposed by Sundbom and Vrede [88].

Alternatively, uptake, assimilation and conversion of FFAs by *S. obliquus* appear more plausible, leveraging FFA precursors to synthesize ω-HUFAs such as EPA and DHA. It has also been shown that de novo synthesis of fatty acids in *D. magna* accounts for only ~2 % of the total, whereas most accumulated fatty acids originate from their diet [89]. *Scenedesmus* sp. has been reported to synthesize EPA under specific conditions [90]. *S. obliquus* itself can contain between 0.97 % and 5.43 % EPA in total fatty acids [91]. A high dietary ω-HUFA content has been shown to increase food quality [92]. Previous studies with *Scenedesmus quadricauda* have indicated that the addition of encapsulated ALA positively influences *Daphnia* maturation and reproduction [88]. As reported for *S. obliquus* [9,93,94], *S. quadricauda* is rich in C_{18} ω-PUFAs, with ALA as the major ω-FA, and only trace amounts of EPA and DHA [95].

Finally, FFAs, including PUFAs, may provide several indirect benefits for *D. magna* cultures, since they have antioxidative properties [96] and anti-inflammatory effects [97]. Moreover, as reviewed by Desbois and Smith [98], PUFAs have shown antibiotic activity against industry-relevant pathogens, and pose virtually no environmental harm when leached into the water. These properties make FFA-secreting strains like *Δaas-fax1* especially valuable, as they contribute to healthier aquacultural environments. Future research should focus on elucidating how

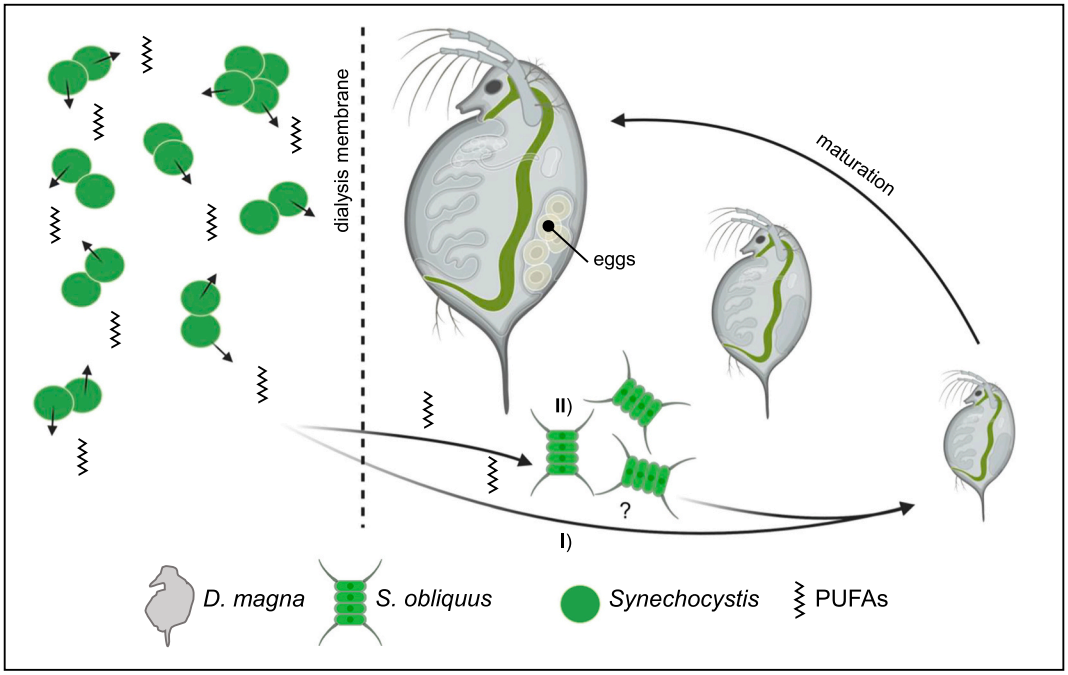

Fig. 6. Model depicting the benefits of the provision of free fatty acids to *Daphnia magna*.
This model illustrates the basic interactions in a simplified aquatic ecosystem comprising *D. magna*, *S. obliquus* and *Synechocystis* 6803. It explores potential cascade effects of fatty acids secreted by engineered *Synechocystis* 6803 strains on trophic interactions. In the direct pathway (I), *D. magna* may assimilate free fatty acids from the environment, enhancing maturation and reproductive output. Conversely, in the indirect pathway (II), *S. obliquus* absorbs FFAs, also using them as precursors for the synthesis of highly unsaturated fatty acids, such as eicosapentaenoic acid (EPA, C20:5ω3) and docosahexaenoic acid (DHA, C22:6ω3), which are subsequently ingested by *D. magna*. This could enhance the nutritional quality of crustaceans' diet, potentially promoting faster growth and increased reproductive rates. In additionally, the engineered production of FFAs could sustain a healthier *D. magna* population, impacting their lifecycle from neonates to egg-bearing adults. (Created with BioRender.com).

secreted ω-PUFAs confer these benefits on *Daphnia* populations in the context of this engineered simple food web (Fig. 6).

While we have demonstrated that the deployment of genetically engineered strains holds promise for enhancing aquaculture productivity, it also introduces potential risks that must be meticulously managed, particularly in open aquatic systems. These risks include, among others, potential competition with native phytoplankton species [58,99,100], and horizontal gene transfer [101,102]. In particular, the transfer of antibiotic resistances to human pathogens is a major concern [103]. In the light of these concerns, our study incorporates spatial separation via dialysis tubing, which simultaneously acts as a selective filter for macromolecules, allowing secreted FFAs to pass through.

We have also adopted a marker-less gene replacement technique similar to those previously reported in cyanobacterial research. Markerless mutations, including those using a *nptl-sacB* cassette [43,104] or selective agents such as FFAs [105], have demonstrated versatility in the construction of cyanobacterial mutants. By utilizing a similar markerless strategy, first described by Viola et al. [42], our modified strain is devoid of antibiotic resistance genes, thus enhancing its biosafety. This approach could reduce risks to the environment, and also has the potential to facilitate further modifications, enhancing the adaptability and utility of engineered strains.

The strategy of engineering *Synechocystis* 6803 to enhance FFA secretion, coupled with our method of *D. magna* cultivation, has obvious applications to other microalgae and zooplankton species. Utilizing genetically modified organisms in this manner also promises to reduce the risks associated with their release into natural environments, while providing important biochemical compounds, such as fatty acids. This approach not only enhances the efficiency of production, it contributes to the responsible use of biotechnological advances to the sustainability of aquaculture and its ecological management.

5. Conclusion

In this study, we successfully engineered a markerless *Synechocystis 6803* strain for direct ω-PUFA secretion, thus eliminating the need for resource-intensive extraction processes. This modified strain heterologously expresses *FAX1*, and significantly enhances the rate of secretion of nutritionally valuable ω-PUFAs, particularly LIN, ALA, and SDA. Utilized in a novel pilot system, the strain has demonstrated its potential as a sustainable aquacultural feedstock, owing to its positive impact on *D. magna*'s survival and reproduction.

CRediT authorship contribution statement

Maximilian K. Lübben: Writing – original draft, Visualization, Methodology, Data curation, Conceptualization. **Steffen Heinz:** Methodology, Conceptualization. **Sabine Gießler:** Resources, Methodology. **Martin Lehmann:** Writing – review & editing, Methodology. **Laura Kleinknecht:** Methodology. **Maria Stockenreiter:** Writing – review & editing, Methodology. **Matthias Ostermeier:** Writing – review & editing, Writing – original draft, Visualization, Supervision. **Herwig Stibor:** Writing – review & editing, Supervision, Methodology, Funding acquisition. **Jörg Nickelsen:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

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

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.algal.2025.103910.

Data availability

Data will be made available on request.

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