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Triclosan toxicity in a model cyanobacterium (*Anabaena flos-aquae*): Growth, photosynthesis and transcriptomic response

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ABSTRACT

Exposure to triclosan (TCS) has been reported to reduce photosynthetic pigments, suppress photosynthesis, and inhibit growth in both prokaryotic and eukaryotic algae including *Anabaena flos-aquae* (a model cyanobacterium). In particular, cyanobacteria are more sensitive to TCS toxicity compared to eukaryotic algae possibly due to the structural similarity to bacteria (target organisms); however, whether TCS exerts its toxicity to cyanobacteria by targeting signaling pathways of fatty acid biosynthesis as in bacteria remains virtually unknown, particularly at environmental exposure levels. With the complete genome sequence of *A. flos-aquae* presented in this study, the transcriptomic alterations and potential toxic mechanisms in *A. flos-aquae* under TCS stress were revealed. The growth, pigments and photosynthetic activity of *A. flos-aquae* were markedly suppressed following a 7-day TCS exposure at 0.5 μg/L but not 0.1 μg/L (both concentrations applied are environmentally relevant). The transcriptomic sequencing analysis showed that signaling pathways, such as biofilm formation – *Pseudomonas aeruginosa*, two-component system, starch and sucrose metabolism, and photosynthesis were closely related to the TCS-induced growth inhibition in the 0.5 μg/L TCS treatment. Photosynthesis systems and potentially two-component system were identified to be sensitive targets of TCS toxicity in *A. flos-aquae*. The present study provides novel insights on TCS toxicity at the transcriptomic level in *A. flos-aquae*.

Introduction

Triclosan (TCS) has been synthesized and used for five decades as a broad-spectrum antimicrobial agent (Goodman et al., 2018; Milanović et al., 2021; Regös et al., 1979). It is widely used in hygiene products in clinical and medical settings (e.g., antiseptics, disinfectants, preservatives), personal-care items (e.g., toothpastes, hand sanitizers, soaps, facial cleanser, shampoos), household goods (e.g., cooking utensils, textile products, packaging materials), etc. (Goodman et al., 2018). Due to the extensive use of TCS and its incomplete removal (58%–98%) by wastewater treatment plants, TCS and its degradation products are ultimately discharged into the

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aquatic environment through effluents, application of reclaimed sludges in agricultural activities, and water runoff (Dar et al., 2022).

For the detected TCS concentrations on a global scale, up to 86.2 μg/L in influents, up to 5.37 μg/L in effluents, and up to 0.1 μg/L in seawater have been reported (Bedoux et al., 2012; Jagini et al., 2019; Milanović et al., 2021). The ubiquitous presence of TCS in the aquatic environment has contributed to the development and spread of antimicrobial resistance genes, posing ecological risks to the health of non-target species (Guo and Iwata, 2017; Xue et al., 2019). In particular, compared to organisms of higher trophic levels such as invertebrates (e.g., daphnids, shellfish) and vertebrates (e.g., fish, birds, mammals), algae at the bottom of food chains are more sensitive to TCS toxicity (Dar et al., 2022; Guo et al., 2018a, 2018b; Kumar et al., 2021). Notably, exposure to TCS has been reported to cause growth inhibition, photosynthesis suppression, reduction in pigment contents, induction of oxidative stress, as well as alterations in cell shape and size in several algal species, including *Chlamydomonas reinhardtii, Chlorella vulgaris, Raphidocelis subcapitata*, and *Microcystis aeruginosa* (Dai et al., 2021; Huang et al., 2016; Machado and Soares, 2021; Pan et al., 2018; Taştan et al., 2017). TCS has also been reported to alter the structures and function of phytoplankton community and biofilms that includes cyanobacteria (Clarke et al., 2019; Drury et al., 2013; Eriksson et al., 2015; Lawrence et al., 2015; Nietch et al., 2013; Pinckney et al., 2017). Furthermore, cyanobacteria are more sensitive to TCS toxicity compared to other algal species (EMA, 2016, 2018). For example, using growth inhibition as a measured endpoint, the 96-hr half maximal effective concentration (EC_{50}) value of TCS for *Anabaena flos-aquae* was 0.97 μg/L, which is much lower than that of diatoms (*Skeletonema costatum, Navicula pelliculosa*) and green algae (*Raphidocelis subcapitata, Scenedesmus subspicatus*) with a EC_{50} values of >66.0, 19.1, 4.46, and 2.8 μg/L, respectively (Orvos et al., 2002). As primary producers, cyanobacteria serve as suppliers of energy and materials in the food chains while playing fundamental roles in the cycle of carbon, nitrogen and oxygen in the ecosystem (Agnihotri, 2014). Due to the high ecological relevance, rapid growth, easy cultivation and great sensitivity, *A. flos-aquae* is a recommended species for chemical toxicity testing by the Organization for Economic Co-operation and Development (OECD, 2011). Nevertheless, the genome of *A. flos-aquae* has not been fully sequenced, and no attempt has been made to decipher the potential molecular mechanisms of environmental stressors on this species.

TCS is known to target fatty acid biosynthesis and thereby effectively suppress bacterial growth (McMurry et al., 1998). Cyanobacteria are procaryotic organisms with a structural resemblance to bacteria; however, whether the target sites of TCS toxicity on cyanobacteria are similar to that of bacteria remains unclear. Importantly, toxicological studies of TCS on cyanobacteria are considerably lacking, especially their toxic mechanisms at environmental-related exposure levels. Transcriptomics, in the toolbox of omics technologies, can be used for global gene profiling and the identification of dysregulated molecular signaling pathways in algae including cyanobacteria in response to environmental toxicants (Mo et al., 2022; Yang et al., 2020). This mechanistic information generated may provide some insights into the algal species sensitivity in TCS toxicity.

In this study, potential effects of TCS at environmentally realistic exposure levels (0.1 and 0.5 μg/L corresponds to the commonly detected levels in surface water receiving treated effluents and municipal wastewater effluents, respectively) on the growth, pigment contents, and photosynthetic activity of *A. flos-aquae* were investigated. Meanwhile, the whole genome of *A. flos-aquae* was sequenced to facilitate the transcriptomic profiling of *A. flos-aquae* under TCS stress. Using *A. flos-aquae* as a model cyanobacterium, it was hypothesized that molecular signaling pathways related to biosynthesis of fatty acids were the most sensitive targets in the TCS-inhibiting cyanobacterium *A. flos-aquae*. This study showed that the growth inhibition of *A. flos-aquae* was related to reduced photosynthetic pigments, suppressed photosynthetic activities, and dysregulated signaling pathways including two-component system, RNA degradation, starch and sucrose metabolism, and photosynthesis.

1. Materials and methods

1.1. Chemicals and reagents

TCS (CAS# 3380-34-5, HPLC ≥ 98%) was provided by the Yuanye Bio-Technology Company (Shanghai, China). Chemicals of reagent grade were used for preparing the BG11 (Blue-Green) medium. Thereafter, 0.01 g TCS powder was weighted and added into a volume of 1 L sterilized BG11 medium, and the resulting TCS stock solution (10 mg/L) was subjected to ultrasonication and membrane filtration (Mo et al., 2022).

1.2. Cultivation of cyanobacteria

A cyanobacterium *A. flos-aquae* (No. FACHB-245) was purchased from the Institute of Hydrobiology, Chinese Academy of Sciences (Wuhan, China) and cultivated according to the OECD guideline No. 201 (OECD, 2011). In brief, *A. flos-aquae* (2×10^4 cells/mL) at the exponential phase were seeded in Blue-Green (BG11) medium (pH 7.1) with a total volume of 150 mL in each Erlenmeyer flask (250 mL). Erlenmeyer flasks with inoculated *A. flos-aquae* were incubated in an algal culture chamber with the illumination of cool-white lights (approximate 5500 lux light intensity) under a controlled temperature (22 ± 2)°C. The cyanobacteria cultures were shaken manually thrice every day. Sonification was applied to break up the cell chains of *A. flos-aquae* prior to the cell counting using a haemocytometer, and the absorbance of algal cultures at 680 nm were recorded as well (OECD, 2011).

Based on these data, the Eq. (1) was generated to calculate the cell density of *A. flos-aquae* (Y) in a specific algal culture with the measured absorbance (X):

$$Y = 7932.2 \times X - 310.56 \quad (1)$$

1.3. TCS exposure experiments

The exposure experiments of TCS to *A. flos-aquae* were performed following the OECD guideline No. 201 (OECD, 2011). No-

tably, before the execution of TCS exposure, a batch of algal samples from the control treatment group were collected for DNA extraction and whole-genome sequencing. The experimental details were shown in the Appendix A Texts S1–S3.

To prepare the TCS exposure medium, a calculated volume of the TCS stock solution was added into the BG11 medium within a flask. *A. flos-aquae* at the exponential phase were then seeded into the BG11 medium (the control treatment) and the TCS exposure medium (TCS exposure treatments), respectively. Exposure of *A. flos-aquae* (2×10^4 cells/mL) to TCS at environmental-related concentrations (0.1 and 0.5 μg/L) continued for 7 days, and their cell densities were determined. At the exposure day 7 upon the termination of the TCS exposure experiments, a proportion of *A. flos-aquae* were collected through membrane filtration for the measurement of photosynthetic pigments, and the remaining samples were centrifuged (10,000 r/min, 4°C, 10 min), transferred into new tubes, and kept at −80°C for the subsequent transcriptomic analysis.

1.4. Determination of photosystem II performances and pigment contents

The chlorophyll *a* fluorescence of *A. flos-aquae* were measured using a fluorometer (AquaPen-P AP-P 110-P, Photon Systems Instruments, Czech Republic) following the protocol provided by the manufacturer as described in Piovár et al. (2011). The dark-adapted maximum quantum yield of photosynthesis system (PS) II (F_v/F_m) of *A. flos-aquae* under the stress of TCS was determined.

Pigment contents including chlorophyll *a* (Chl-*a*), Chl-*b* and total carotenoids were determined as described previously (Ma et al., 2021; Wellburn, 1994). At the end of the TCS treatment (day 7), samples (5 mL) from each replicate were filtered through membranes (0.22 μm) to collect the cyanobacteria. The collected algal samples were merged in methanol. The extraction of pigments was performed in dark under 4°C for 24 hr. Finally, after centrifugation (10,000 r/min, 15 min), absorbance of the supernatant were recorded at 470, 653 and 666 nm, respectively, using a microplate reader (TECAN, Switzerland).

The contents of Chl-*a* ($C_{Chl\text{-}a}$, mg/L), Chl-*b* ($C_{Chl\text{-}b}$, mg/L) and total carotenoids (C_{Total}, mg/L) were calculated as in Guo et al. (2016) according to the Eq. (2), Eq. (3) and Eq. (4), respectively:

$$C_{Chl-a} = 15.65 \times A_{666} - 7.34 \times A_{653} \quad (2)$$

$$C_{Chl-b} = 27.05 \times A_{653} - 11.21 \times A_{666} \quad (3)$$

$$C_{Total} = (1000 \times A_{470} - 44.76 \times A_{666})/221 \quad (4)$$

where, A_{666}, A_{653}, and A_{470} is the absorbance measured at 666, 653 and 470 nm, respectively.

1.5. Transcriptomic analysis

The whole genome of *A. flos-aquae* was sequenced in the present study (detailed in the Appendix A Texts S1-S5) to facilitate the transcriptomic profiling. Total RNA of *A. flos-aquae* in these samples were extracted and purified (Mo et al., 2022). The RNA quality was checked using a Bioanalyzer 2100 (Agilent Technologies, USA). RNA with an integrity number >9.0 were considered as qualified samples and used for the construction of cDNA library and sequencing (paired-end reads, 150 bp read-length) using an Illumina Novaseq platform (Illumina, USA).

In the bioinformatic analysis, the 3' adapter sequences of the sequencing raw data were trimmed, and the low-quality reads were filtered out using Cutadapt (Martin, 2011). The resulting clean reads (Phred score > 20) were mapped to the genome sequenced in the present study using HISAT2 (Pertea et al., 2016). The quantification of read counts were performed using HTSeq (Anders et al., 2015). The method of fragments per kilo base per million fragments (FPKM) was used for the normalization of gene expression. Finally, differential expression analysis was conducted using the DESeq2 package in R (Love et al., 2014).

Pearson's correlation analysis and principal component analysis were performed in R. The absolute $\log_2$(Fold change) > 1 and $p < 0.05$ were used as threshold for selecting differentially expressed genes (DEGs). These DEGs were used for the functional enrichment analyses of Gene Ontology

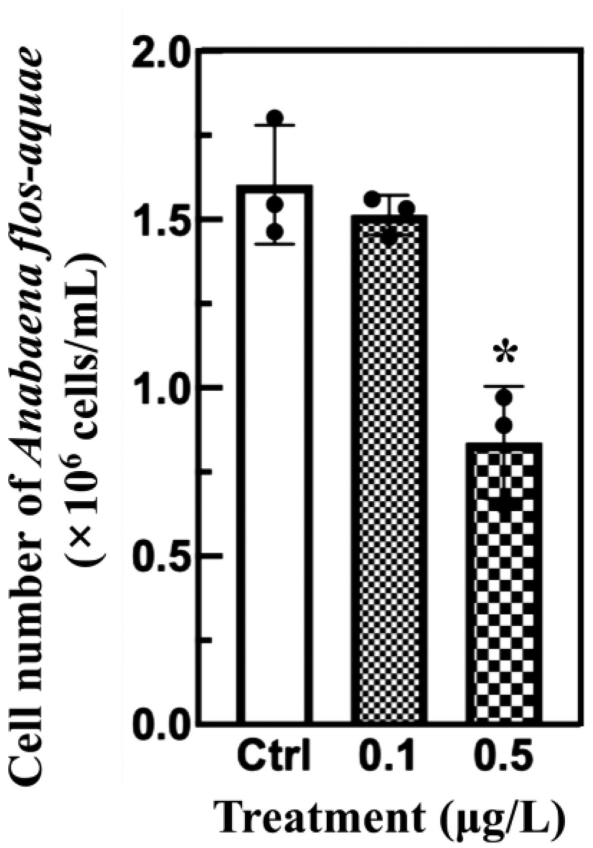

Fig. 1 – Growth performances of the cyanobacterium (*A. flos-aquae*) altered by a 7-day triclosan (TCS) exposure. Data are shown as mean ± standard deviation ($n = 3$). An asterisk (*) indicates a significant difference ($p < 0.05$) between the TCS treatment (0.1 or 0.5 μg/L) and the control (Ctrl), analyzed by one-way ANOVA with Tukey's post hoc test.

(GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway. GO terms and KEGG pathways with a $p < 0.05$ were deemed significant.

1.6. Statistical analysis

Data of algal growth, photosynthetic activities and pigment contents were statistically analyzed using GraphPad Prism 9.0 (San Diego, USA), in which a one-way analysis of variance (ANOVA) with Tukey's post hoc test was applied. In the bar charts, statistical significance ($p < 0.05$) between the TCS treatment groups and the control was indicated by an asterisk (*).

2. Results

2.1. Growth performance of A. flos-aquae altered by TCS exposure

The alterations in growth performance of *A. flos-aquae* following a 7-day TCS treatment (0.1 and 0.5 μg/L) are indicated in Fig. 1. While the cell density of the 0.1 μg/L TCS treatment was comparable to the control, it decreased significantly (one-way ANOVA, $p < 0.05$) in the 0.5 μg/L TCS treatment. Specifically, a growth inhibition by approximate 47.76% was identified in *A. flos-aquae* treated with 0.5 μg/L TCS in comparison to the control.

2.2. Photosynthetic pigments and activity of A. flos-aquae altered by TCS exposure

The potential changes in photosynthetic pigments and activity of *A. flos-aquae* following a 7-day TCS treatment (0.1 and 0.5 μg/L) were determined (Fig. 2). In agreement with the reduced performance, compared with the control, exposure of *A. flos-aquae* to 0.5 μg/L TCS caused a significant (one-way ANOVA, $p < 0.05$) reduction by approximate 63.63% in the dark-assimilated maximum quantum yield of PS II (Fig. 2a). Moreover, the contents of Chl-*a*, Chl-*b* and total carotenoids were significantly (one-way ANOVA, $p < 0.05$) reduced by approximate 55.37%, 58.87%, and 64.32%, respectively, in the 0.5 μg/L TCS treatment (Fig. 2b–d), compared with the control. These photosynthesis parameters remained unchanged in the 0.1 μg/L TCS treatment group (Fig. 2).

2.3. Transcriptomic alterations in A. flos-aquae exposed to TCS

The alterations in gene expression pattern and the dysregulation of molecular signaling pathways in *A. flos-aquae* following exposure to 0.5 μg/L TCS for 7 days was evaluated using transcriptomic sequencing. The raw data generated by the transcriptomic sequencing are high-quality (Q20 > 97.23%), and an average of 25.5 million clean reads per sample were obtained. Overall, the average mapping rate of clean reads were 91.10% to the sequenced genome of *A. flos-aquae*. Of which, there are approximate 22.80 million unique mapping reads in each sample. The similar mapping rate of replication samples in both the 0.5 μg/L TCS treatment and the control suggest that the uniformity and quality of the sequencing data are good. This is supported by the high correlation of samples (>0.91) within the same treatment group (Fig. 3a). In addition, replicates in TCS treatment or in the control were nicely clustered in the PCA analysis (PC1 and PC2 accounts for 44% and 35% of the variation, respectively) (Fig. 3b). In the heatmap, similar gene expression pattern was identified in the same treatment group but distinct from the other treatment group (Fig. 3c).

Using an absolute $\log_2$(Fold change) > 1 and a $p < 0.05$ as the selection threshold, a total of 221 DEGs including 168 down-regulated DEGs and 53 up-regulated DEGs (Fig. 3d) were found in *A. flos-aquae* treated with 0.5 μg/L TCS in comparison with the control.

The most significantly ($p < 0.05$) altered GO terms including BP (GO:0031412 gas vesicle organization, GO:0071840 cellular component organization or biogenesis, GO:0007154 cell communication, GO:0007229 integrin-mediated signaling pathway, GO:0065007 biological regulation, GO:0050896 response to stimulus, GO:0009635 response to herbicide), MF (GO:0033293 monocarboxylic acid binding, GO:0016899 oxidoreductase activity, acting on the CH-OH group of donors, oxygen as acceptor, GO:0005524 ATP binding, GO:0032559 adenyl ribonucleotide binding, GO:0060089 molecular transducer activity, GO:0035639 purine ribonucleoside triphosphate

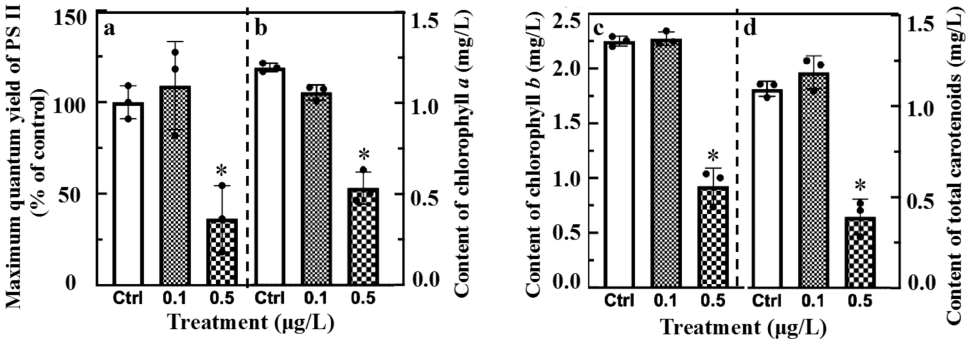

Fig. 2 – Photosynthetic activity and pigment contents of the cyanobacterium (*A. flos-aquae*) altered by a 7-day triclosan (TCS) exposure. The maximum quantum yield of PSII (a), contents of chlorophyll *a* (b), chlorophyll *b* (c), and total carotenoids (d) were determined. Data are shown as mean ± standard deviation ($n = 3$). An asterisk (*) indicates a significant difference ($p < 0.05$) between the TCS treatment (0.1 or 0.5 μg/L) and the control (Ctrl), analyzed by one-way ANOVA with Tukey's post hoc test.

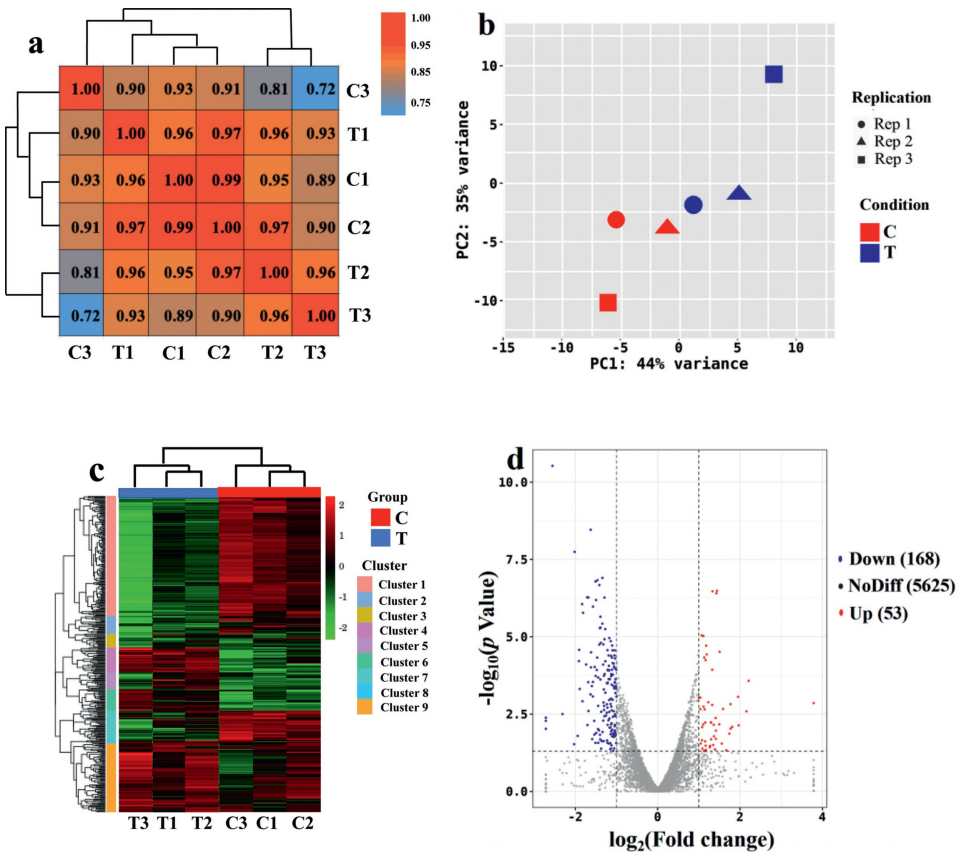

Fig. 3 – Transcriptomic response of the cyanobacterium (*A. flos-aquae*) following exposure to triclosan (TCS) for 7 days. (a) Correlation analysis of gene expression patterns; (b) Principal component analysis (PCA) of FPKM-normalized gene expression profiles in replication samples; (c) Clustering analysis showing the expression patterns of differentially expressed genes (DEGs) in each replication sample; (d) DEGs identified between the 0.5 µg/L TCS treatment group (T) and the control (C).

Table 1 – Key molecular signaling pathways altered in *Anabaena flos-aquae* following exposure to 0.5 µg/L triclosan for 7 days.

Pathway ID	Pathway	Up_DEGs	Down_DEGs	*p* Value
ko02025	Biofilm formation – *Pseudomonas aeruginosa*	–	*pilJ, pilI, pilG*	>0.01
ko03018	RNA degradation	–	*recQ, dnaK, hfq*	>0.01
ko02020	Two-component system	–	*pixH, pilJ, pilI, pilG, pixG*	0.01
ko00500	Starch and sucrose metabolism	*treA, treY*	–	0.02
ko00195	Photosynthesis	*psbA, atpf0c, psaJ*	–	0.04
ko04626	Plant-pathogen interaction	–	*Hsp90a*	0.04

DEGs: differentially expressed genes. A dash (–) indicates no up- or down-DEGs in a specific signaling pathway.

binding, GO:0016301 kinase activity, GO:0008144 drug binding), and CC (GO:0031411 gas vesicle, GO:0043227 membrane-bounded organelle, GO:0005773 vacuole, GO:0031090 organelle membrane, GO:0043231 intracellular membrane-bounded organelle, GO:0009521 photosystem), were identified in *A. flos-aquae* following the treatment of 0.5 µg/L TCS (Fig. 4a).

KEGG pathways including biofilm formation – *Pseudomonas aeruginosa*, RNA degradation, two-component system, starch and sucrose metabolism, photosynthesis, and plant-pathogen interaction were significantly ($p < 0.05$) altered in *A. flos-aquae* following the treatment of 0.5 µg/L TCS (Table 1 and Fig. 4b).

3. Discussion

This study investigated TCS toxicity at environmentally relevant exposure levels in a model cyanobacterium (*A. flos-aquae*) and uncovered the potential molecular mechanisms. Contrary to our hypothesis, molecular signaling pathways related to biosynthesis of fatty acids (target of TCS toxicity in bacteria) were not within the most sensitive targets of the TCS toxicity in *A. flos-aquae*. Instead, key signaling pathways including biofilm formation – *Pseudomonas aeruginosa*, two-component

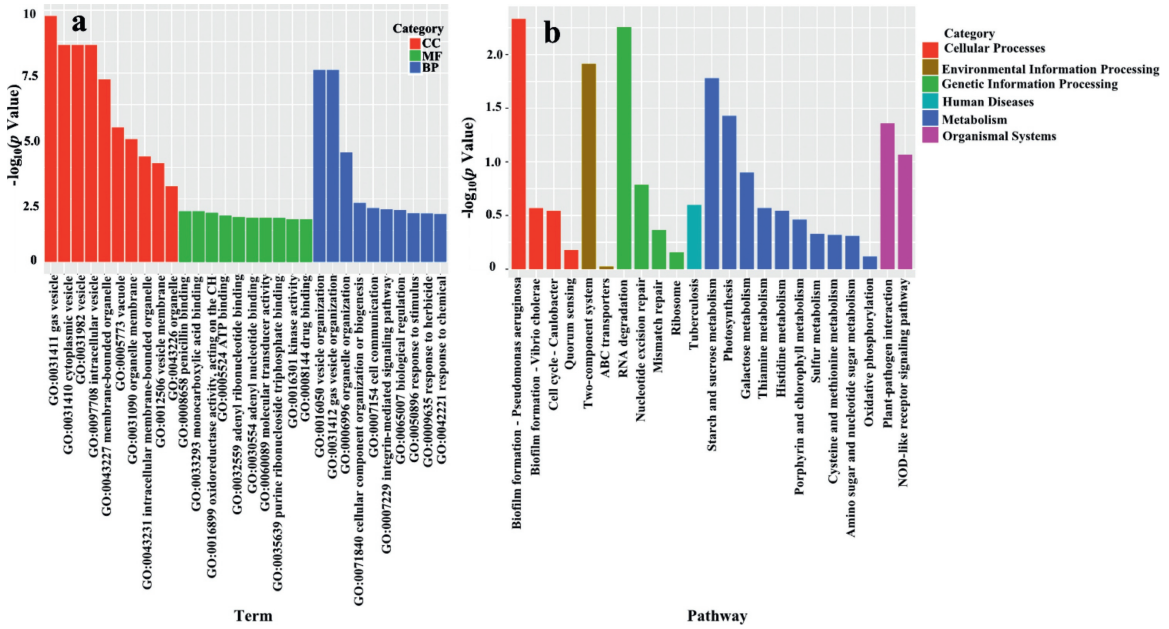

Fig. 4 – Analyses of Gene Ontology (GO) and KEGG pathways in *A. flos-aquae* following exposure to triclosan for 7 days. Go terms (a) includes cellular components (CC), molecular functions (MF) and biological processes (BP); KEGG pathways (b).

system, photosynthesis and starch and sucrose metabolism were altered in *A. flos-aquae* treated with 0.5 μg/L TCS (Table 1). Although the parent compound concentration was not measured in the present study at day 7 following TCS treatment, more than 80% of the nominal TCS in the algal exposure medium was detected at day 7 following TCS exposure, which suggests that TCS was rather resistant to degradation during the exposure (Xin et al., 2019). With the prolonged stress of TCS and its degradation products, the growth inhibition of *A. flos-aquae* was likely due to the reduction in photosynthetic pigments, suppression of photosynthetic activity, as well as potential dysfunction of two-component system and dysregulation of carbohydrate metabolism.

3.1. *Xenobiotic metabolism genes in TCS-inhibiting cyanobacteria*

The detoxification system of algae is composed of three phases of reaction, in which the cytochrome P450 system is responsible for the phase I and II reactions, while ABC transporter superfamily in the phase III export the metabolized xenobiotic generated from the phase I and II to the extracellular environment (Guo et al., 2021a; Wang et al., 2018). In a previous study conducted by Mo and colleagues, downregulation of CYP genes, *lut1* (encoding carotenoid epsilon hydroxylase) and *prxq* (encoding thioredoxin dependent peroxidase) were observed in *R. subcapitata* following exposure to TCS for 7 days at concentrations of 75–100 μg/L (Mo et al., 2022). However, in the present study, only one gene (*ftsE*) that encodes an essential component of the ABC transporter FtsEX and involves in cellular division, was found to be downregulated in *A. flos-aquae* treated with 0.5 μg/L TCS. The downregulation of *ftsE* may compromise the excretion of TCS in *A. flos-aquae* and cause growth inhibition. Nonetheless, the detoxification of TCS and toxicity of TCS degradation products in cyanobacteria including *A. flos-aquae* warrant further investigation.

3.2. *Photosynthesis and carbohydrate metabolism in TCS-inhibiting cyanobacteria*

Chlorophyll (e.g., Chl-*a*, Chl-*b*) is essential for light harvesting and transferring in oxygenic photosynthesis of eucaryotic algae and many prokaryotic algae including *A. flos-aquae* (Green and Durnford, 1996). Chls bind to antenna proteins to form the hardcore of the photosynthetic apparatus, including PSI, PSII, the light-harvesting complex I (LHCI) and LHCII. Carotenoids also have a key role in absorbing lights while providing photoprotection through non-photochemical quenching during the photosynthesis in organisms of oxygenic photosynthesis including *A. flos-aquae* (Brunet et al., 2011). The reduction in dark-assimilated maximum quantum yield of PS II and photosynthetic pigments including Chl-*a*, Chl-*b* and total carotenoids consistently suggests that structural and functional impairments of the PS were induced in *A. flos-aquae* following 0.5 μg/L TCS exposure.

Several studies have reported that organic contaminants, such as antimicrobial drugs, bisphenol A, nonylphenol, perfluorooctanoic acid etc., adversely affected photosynthesis in model algal species (e.g., *Raphidocelis subcapitata, Chlorella pyrenoidosa, Phaeodactylum tricornutum*) at the transcriptomic level (Feng et al., 2022; Guo et al., 2021a, 2021b; Li et al., 2021a, 2021b; Liu et al., 2020; Mo et al., 2022; Peng et al., 2021). To our knowledge, no study has been performed to reveal the transcriptomic alterations of *A. flos-aquae* potentially caused by environmental stressors. In the present study, to uncover the genome-wide gene expression patterns and the potential alterations in molecular pathways caused by TCS exposure, the genome of *A. flos-aquae* was sequenced (Appendix A Fig. S1

and Tables S1–S5) and used for the transcriptomic analysis. In agreement with the reduction in pigments and suppression of photosynthetic activity, the transcriptomic analysis revealed that signaling of photosynthesis was altered by TCS exposure. Intriguingly, genes including *psbA* (encoding photosystem II P680 reaction center D1 protein), *atpf0c* (encoding F-type H+-transporting ATPase subunit c) and *psaJ* (encoding photosystem I subunit IX) enriched in the signaling of photosynthesis were upregulated. This is in accordance with upregulation of photosynthesis system genes (e.g., *atpF, atpH, petC, petF, petH, psaE, psbR*) in tylosin-inhibiting *R. subcapitata* (Li et al., 2021b). The upregulation of *psbA, atpf0c* and *psaJ* may act as negative feedbacks to possibly restore the depletion of photosynthetic pigments, dysfunction of photosystems, and oxidative stress-induced damages in algae under TCS stress (González-Pleiter et al., 2017; Huang et al., 2016; Machado and Soares, 2021; Pan et al., 2018; Wang et al., 2020; Xin et al., 2019). In terms of TCS toxicity on algal photosynthesis at the transcriptomic level, however, it was distinctly different in a model green alga (*Raphidocelis subcapitata*) recently reported (Mo et al., 2022) where genes encoding antenna proteins and proteins of PSI, PSII and the electron transport chain were downregulated, and it aggravated with the TCS exposure concentration increased from 5 to 100 μg/L (Mo et al., 2022). In another study performed by Larras and colleagues, exposure to TCS at concentrations ranging from 0.1 to 70.7 μg/L for 14 hr reduced photosynthetic pigments and inhibited photosynthesis in *Scenedesmus vacuolatus*; however, the signaling pathway of photosynthesis, intriguingly, was not dysregulated in the transcriptomic analysis (Larras et al., 2020).

The dysfunction of photosynthesis systems and inhibition of photosynthesis, as discussed above, likely led to a reduction in energy production and dysregulation of carbohydrate metabolism (Atkin et al., 2000; Chen et al., 2006). Indeed, the signaling pathway of starch and sucrose metabolism was altered in *A. flos-aquae* treated with 0.5 μg/L TCS. Specifically, α,α-trehalose encoded by *treA* catalyzes the hydrolysis of α,α-trehalase into D-glucose, while *treY* encoding maltooligosyl trehalose synthase catalyzes the interconversion between maltodextrin and maltooligosyl-trehalose (De Smet et al., 2000; Vanaporn and Titball, 2020). The upregulation of *treA* and *treY* may facilitate the hydrolysis of α,α-trehalase to D-glucose for the purpose of energy supply (e.g., ATP production in glycolysis). These data seemingly suggest that catabolism was over assimilation in cyanobacteria under TCS stress. The potential dysregulation of carbohydrate metabolism may contribute to the TCS-induced growth inhibition of *A. flos-aquae,* and subsequent studies are required to test this hypothesis.

3.3. *The two-component system signaling in TCS-inhibiting cyanobacteria*

Molecular signaling pathways, such as biofilm formation – *Pseudomonas aeruginosa* and two-component system, were suggested to be inhibited and likely contributed to the grow inhibition of *A. flos-aquae* treated with 0.5 μg/L TCS. The two-component system, formed by a membrane-bound histidine kinase receptor and a response regulator, is a key signal transduction system that bacteria rely on to sense and respond to the changing environment (Ashby and Houmard, 2006). Microorganisms including cyanobacteria form biofilm in responds to the fluctuations of environmental factors and stressors through the two-component system (Liu et al., 2019). Downregulation of *pilJ* (encoding twitching motility protein PilJ), *pilI* (encoding twitching motility protein PilI), and *pilG* (encoding twitching motility two-component system response regulator PilG) in *A. flos-aquae* treated with 0.5 μg/L TCS were shared by these two dysregulated signaling pathways. Two additional downregulated genes, (*pixH* and *pixG* encoding two-component system, chemotaxis family, response regulator PixH and PixG, respectively) were enriched in dysregulated signaling of two-component system. The consistent downregulation of these genes enriched in the signaling pathway of two-component system suggests that the sense, signal transduction within the cells, and response to the environmental factors were likely disrupted, ultimately contributing to the growth inhibition of *A. flos-aquae* in the 0.5 μg/L TCS treatment (Fig. 1). Based on these findings, the aforementioned two signaling pathways, biofilm formation – *Pseudomonas aeruginosa* and two-component system, were identified as the potential primary targets of TCS toxicity in cyanobacteria.

3.4. *The RNA degradation signaling in TCS-inhibiting cyanobacteria*

In the present study, molecular signaling pathways including RNA degradation was also altered in *A. flos-aquae* treated with 0.5 μg/L TCS. The ATP-dependent DNA helicase RecQ encoded by *recQ* and *dnaK* encoded molecular chaperone DnaK are related to DNA replication, recombination and repair (Pathak et al., 2019; Rupprecht et al., 2007). Notably, the host factor-I protein encoded by *hfq* can bind to sRNA and interact with regulatory sRNA to promote the antisense interactions with their targets (Vasudevan et al., 2019). It can affect the protein synthesis by facilitating the degradation of mRNA or acting as a repressor of mRNA translation (Hajnsdorf and Boni, 2012). Downregulation of *recQ, dnaK* and *hfq* were enriched in the signaling of RNA degradation, which may serve as a negative feedback possibly to the energy crisis and structural deconstruction in *A. flos-aquae* under TCS stress. This is supported by experimental data from previous studies where exposure to TCS at higher concentrations caused membrane damages, altered the ratio of RNA/DNA, and induced DNA damages in eukaryotic algae (Ciniglia et al., 2005; Machado and Soares, 2021; Mo et al., 2022; Wang et al., 2013; Xin et al., 2019).

4. Conclusions

Exposure of *A. flos-aquae* to TCS at an environmentally relavent level (0.5 μg/L, within the detected levels of TCS in municipal wastewater effluents) reduced photosynthetic pigments, suppressed photosynthetic activities, and inhibited growth. Dysregulated signaling pathways including biofilm formation – *Pseudomonas aeruginosa*, two-component system, starch and sucrose metabolism, and photosynthesis were revealed to be associated with the changes in these apical endpoints. As cyanobacteria are significant contributors of the global primary production and cycle of carbon, nitrogen and oxygen, the inhibitory effects of TCS on cyanobacteria

may cause structural and functional disruptions on the food chains. Although exposure of *A. flos-aquae* to TCS at 0.1 μg/L for a 7-day period did not show significant alterations in the growth, pigment contents, and photosynthetic activity of *A. flos-aquae*, the long-term toxic effects of TCS at lower concentrations (e.g., 0.1 μg/L) on algae remain known, and further studies are required. The complete genome of *A. flos-aquae* sequenced in this study can be used for the molecular-based studies on the model cyanobacterium *A. flos-aquae* in response to environmental factors and stressors.

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 associated with this article can be found, in the online version at doi:10.1016/j.jes.2022.03.039.

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