



Toxin production in bloom-forming, harmful alga *Alexandrium pacificum* (Group IV) is regulated by cyst formation-promoting bacteria *Jannaschia cystaogens* NBRC 100362^T

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ABSTRACT

Harmful algal blooms (HABs) caused by dinoflagellates like *Alexandrium pacificum* pose significant ecological and public health risks due to their production of paralytic shellfish toxins (PSTs). Bacterial populations, particularly *Alexandrium* cyst formation-promoting bacteria (Alex-CFPB), are known to significantly influence growth, encystment, toxin synthesis, the composition of toxic components, and bloom dynamics of these dinoflagellates. However, the role of Alex-CFPB in *Alexandrium* toxin synthesis and the mechanisms thereof are still unclear. Here, we show that co-culturing *A. pacificum* with cyst formation-promoting bacteria *Jannaschia cystaogens* significantly increases total intracellular PSTs content in the late stationary phase (including more cysts and less vegetative cells compared with axenic group). Our results demonstrate that the presence of *J. cystaogens* alters metabolic pathways in *A. pacificum* by upregulating key paralytic shellfish toxins synthesis genes and inducing downregulation of sulfotransferase *sxtN* (related to PSTs sulfation) which decreases sulfated PSTs components (low-toxicity), leading to an increase in high-toxicity PSTs content at a single-cell level. Furthermore, bacterial oxidative stress signals, nutrient competition, and quorum sensing contribute to increased toxin synthesis. These results provide insights into the major role of bacteria in modulating growth, physiology, and toxin production in bloom-forming algae, and the complex regulatory mechanisms therein. This study thus defines the critical function of microbial associations in bloom formation and toxin production with implications for managing HABs and mitigating their impacts.

1. Introduction

Harmful algal blooms (HABs) are increasing in range, frequency, and intensity throughout the world, primarily due to anthropogenic factors such as climate change, eutrophication, and anthropogenic transport (Anderson et al., 2008; Heisler et al., 2008; Anderson et al., 2012b; Gobler et al., 2017). *Alexandrium pacificum* is a HAB-forming dinoflagellate of particular interest due to its production of saxitoxin,

compounds that accumulate in shellfish and can result in paralytic shellfish poisoning (PSP) (Anderson, 1997; Kodama, 2010). In addition to presenting a human health hazard, blooms of *A. pacificum* can result in substantial economic losses due to the closure of shellfish beds and farms (Lewitus et al., 2012; Anderson et al., 2014; Vandersea et al., 2018). Incidents of PSP and human illness have been reported throughout the Globe, and Asia-Pacific in particular (Anderson, 1997; Anderson et al., 2012a; Hallegraeff et al., 2021). The first case of PSP

Abbreviations: Alex-CFPB, *Alexandrium* cyst formation-promoting bacteria; NO, nitric oxide; H₂O₂, hydrogen peroxide; T6SS, Type VI secretion system; CDI, Contact dependent inhibition; LuxL, LuxI-type acyl homoserine lactone synthase; LuxR, LuxR-type acyl homoserine lactone receptor.

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was recorded in the coastal area of Korea in 1986 when two people died after eating the mussel (Chang, 1987), and another such case was reported in 1996 on Geoje Island, which is adjacent to Busan and Jinhae-Masan Bay to the northeast (Lee et al., 1996).

Recently, the frequency of *A. pacificum* toxic blooms has increased worldwide, making it crucial to study the mechanisms of paralytic shellfish toxins (PSTs) synthesis during these blooms (Hadjadji et al., 2020; Hallegraeff et al., 2021; Shin et al., 2021). *Alexandrium* cysts are considered the seeds of HABs, and the seasonal outbreaks of *Alexandrium* are closely related to the germination characteristics of these dormant cysts (Shin et al., 2021). Multiple studies have indicated that bacteria influence the formation of HABs (Gallacher et al., 1997; Uribe and Espejo, 2003; Ramanan et al., 2016; Hattenrath-Lehmann and Gobler, 2017; Tanabe et al., 2023). In the case of *Alexandrium*, previous studies have used metagenomics to analyze free-living bacteria in seawater (Deng et al., 2023), and research conducted in regions experiencing annual *Alexandrium* blooms has identified the promotion of *Alexandrium* cyst formation by bacteria (Adachi et al., 1999; Adachi et al., 2004).

The existence of *Alexandrium* cyst formation-promoting bacteria (Alex-CFPB) during the annual blooms of toxic species *A. tamarensis* (species complex thence revised) was reported in Hiroshima Bay, Japan (Adachi et al., 1999; John et al., 2014). These studies identified a positive correlation between the abundance of Alex-CFPB and *A. tamarensis*, suggesting that Alex-CFPB may play a significant role in the process of encystment and bloom disintegration in the field (Adachi et al., 1999). This field-based correlation emphasizes the ecological significance of Alex-CFPB, but the mechanisms underlying these interactions remain largely unresolved. Notably, research has shown that *Jannaschia cystaurens*, a dominant member of Alex-CFPB, can actively induce cyst formation in *Alexandrium* through mechanisms such as oxidative stress signaling and quorum sensing (Adachi et al., 2004). Despite these findings, the relationship between bacterial density and *Alexandrium* density in controlled systems does not always mirror field observations. The current understanding of how Alex-CFPB influences toxin production is limited (Jauzein et al., 2015). Moreover, transcriptomic and molecular studies exploring these bacterial-algal interactions are sparse, creating a gap in knowledge regarding their regulatory mechanisms. Addressing these gaps is essential to elucidate the broader ecological and physiological implications of the role of Alex-CFPB in HAB dynamics.

In this study, we endeavor to establish a sterile cultivation environment by co-culturing *Alexandrium* cyst formation-promoting bacterium, *J. cystaurens*, and an axenic *A. pacificum* strain, with a focus on eliminating interference from other bacteria. This is followed by an exploration of the impact of *J. cystaurens* treatment on the toxin secretion levels of *A. pacificum*, with an aim to gain a deeper understanding of their mutual interaction. As the genome information of *J. cystaurens* is currently unknown, a comprehensive analysis of the entire genome was conducted. Recently, several studies have employed transcriptomic analysis aimed at unraveling the regulatory networks between genes and identifying crucial regulatory genes (Li et al., 2023; Song et al., 2023; Chen et al., 2024). Here, we use a combination of comparative transcriptomic analysis of toxin-related gene expression levels and the revelation of co-expression networks among toxin-related genes. With these tools, we envisage identifying genes playing key regulatory roles in this interaction, providing crucial insights for a deeper understanding of HAB formation and toxin production mechanisms. Further, the analysis of co-expressed genes related to toxin synthesis will contribute to the formation of a potential saxitoxin-related synthesis network.

2. Materials and methods

2.1. Culture of *A. pacificum* (Group IV)

A strain (KCTC-AG60910) of *A. pacificum* (Group IV) which was isolated from the Korean coastal area (34°59'36.71"N, 128°40'27.73"E), was obtained from the Korean Collection for Type Cultures (KCTC)

(henceforth *A. pacificum*). The strain was maintained in 2 L culture bottles (SPL, Korea) containing f/2 culture medium without silicate (Marine Water Enrichment Solution, Sigma Aldrich, USA), prepared with sterile seawater (salinity of 30) at 20 °C under a 12:12 h light: dark cycle with a photon flux density of approximately 65 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$. In this study, experimental cultures were established from exponentially growing cells. The original production of PSTs was shown in Table S1.

2.2. Axenic culture establishment and assessment of *A. pacificum*

The axenic cultures of *A. pacificum* strain were established using a protocol published previously with minor modifications (Shishlyannikov et al., 2011; Jiang et al., 2024). Approximately 25 mL of the mid-exponential phase xenic culture of *A. pacificum* was gravity-filtered through a 5 μm pluriStrainer membrane filter (pluri-Select). The cells were quickly rinsed with sterile f/2 medium and washed for approximately 1 minute in sterile medium containing 20 $\mu\text{g/mL}$ Triton X-100 detergent to remove surface-attached bacteria, then rinsed again with sterile f/2 medium. Subsequently, the cells on the filter were gently washed by pipetting into sterile medium containing an antibiotic mix (penicillin, 10,000 units/mL; streptomycin, 10 mg/mL; amphotericin B, 0.025 mg/mL). The cells were incubated in the antibiotic-containing medium for 24–48 hours under standard growth conditions (20 °C under a 12:12 h light: dark cycle with a photon flux density of approximately 65 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$). Finally, 0.5–1.0 mL of antibiotic-treated cells were transferred to antibiotic-free medium. Cultures were regularly monitored (every four or five transfers, ~1 month) for bacterial contamination by checking for bacterial growth in marine agar (MA) plates in addition to using DAPI staining and fluorescence microscopy (AxioCam 503 mono, Zeiss, Germany). Additionally, both axenic and xenic cultures of *A. pacificum* were examined using scanning electron microscopy (SEM) based on the method described by Shin et al. (2017).

2.3. Bacterial strain, genome sequencing and analysis

J. cystaurens NBRC 100362^T preserved at the Biological Resource Center (NBRC), National Institute of Technology and Evaluation (NITE) was used in this study (henceforth *J. cystaurens*). The whole-genome sequencing was carried out on the Illumina NovaSeq sequencing platform (Macrogen, Korea). Functional genes within each genome were also annotated using KEGG (Graham et al., 2018). Rapid annotation of microbial genomes using Subsystems Technology (RAST), Prokka, ROARY, and Clusters of Orthologous Genes (COGs) was also used to validate the annotations and particularly subsystems (Overbeek et al., 2014; Seemann, 2014; Page et al., 2015; Galperin et al., 2021).

2.4. Culture of axenic *A. pacificum* and co-culture with *J. cystaurens*

All experiments were conducted in f/2 medium. Co-culture experiments followed the procedures described by Amin et al. (2015). The bacterium *J. cystaurens* was grown, washed, and inoculated into axenic *A. pacificum* cultures at a final bacterial cell density of $\sim 1 \times 10^5$ – 2×10^5 cells mL^{-1} . The numbers of sexual cells, cysts and vegetative cells, and diameter of *A. pacificum* were determined periodically (every 5 days) over 30 days under the Benchtop FlowCam Cyano imaging flow cytometer (Yokogawa Fluid Imaging Technologies, USA). The system was equipped with a 532 nm excitation laser (or alternatively, a 488 nm excitation laser) and a color digital camera. A 1 mL syringe pump operated in laser-trigger mode was used to analyze 1 mL of each sample, capturing fluorescent particles. Quantification and identification of *A. pacificum* cells (10–60 μm diameter) were performed using a 10 \times objective and a 100 μm flow cell, ensuring high-quality imaging. All experiments were performed in triplicate.

For the transcriptome experiments, the axenic *A. pacificum* strain was

inoculated in sterile tissue culture flasks (Corning, USA). Treatments consisted of (1) *A. pacificum* and the bacterium co-culture, and (2) axenic *A. pacificum*. All treatments were in triplicate. Cells were harvested at final exponential growth (30 days after inoculation for all treatments) by filtering the culture through a 5 µm pore-size polycarbonate filter to capture the majority of dinoflagellate cells. Final cell densities at the time of harvesting were *A. pacificum* axenic $\sim 8.3 \times 10^4$ cells mL⁻¹ and *A. pacificum* co-culture $\sim 2.2 \times 10^4$ cells mL⁻¹. Filters were immediately flash-frozen in liquid nitrogen and later stored at -80 °C.

2.5. Saxitoxin measurements

2.5.1. Total toxin analysis

To determine the total intracellular toxin content, cells were cultured as mentioned above, subsequently 100 mL of each sample was harvested using 5µm filter after 30 day culture period. The filters were folded and frozen at -80 °C. Raw culture samples were preserved with Lugols (4 %) and stored to quantify cell densities. The frozen filters were thawed and homogenized with Mini-Bead Beater-16 (BioSpec Products Bartlesville, OK USA 74005). Then these samples were centrifuged at 10,000 rpm for 10 min and the supernatant was collected for further analysis.

Total saxitoxin was analyzed using Eurofins Abraxis saxitoxin-specific ELISA kits (Warminster, PA). The detection limit was 0.015 ng mL⁻¹ with minimal cross-reactivity to other PSP toxins. Results are reported as STX equivalents (eq), with concentrations interpolated using a 4-parameter logistic curve fit model based on standard absorbances and concentrations.

2.5.2. PSTs component analysis

In this study, toxin extraction was performed as described previously (Shin et al., 2020). Briefly, 2 L of each culture was filtered using a nylon net with a mesh size of 20 µm (CISA, Barcelona, Spain) and freeze-dried. The cell pellets were extracted with acetic acid 1 %, sonicated, and centrifuged at 10,000 g at 4 °C. The supernatant was purified by Solid Phase Extraction (SPE) with a C18 Plus Short Cartridge (Sep-PAK® Plus, 360mg C18, Waters WAT020515), based on established methods (Horwitz and Latimer, 2005; Van De Riet et al., 2011).

Analysis was performed by High-Performance Liquid Chromatography-Post-Column Oxidation-Fluorescence Detector (HPLC-PCOX-FLD), post-column reaction system and reagents, mobile phases, gradient conditions, and FLD wavelengths were employed as described in previous studies (Rourke et al., 2008; Van De Riet et al., 2011). Toxins (C1–2, GTX1–4, dcGTX2–3, dcNEO, dcSTX, neoSTX, STX) were quantified using certified calibration solutions (NRCC, Halifax, NS, Canada) and matrix-matched calibration curves. The final concentrations were obtained after correction by solid-phase extraction (SPE) recovery, with all PST components achieving recoveries exceeding 90 %. Limits of detection (LODs) for matrix-free toxins were determined from data for samples extrapolated to a signal-to-noise (S/N) ratio of 3.0. Limits of quantification (LOQs) were determined by multiplying the signal-to-noise ratio by 10.0 (Oshima, 1995; Magnusson, 2014). The results of the LOD and LOQ are shown in Table S2, based on the signal-to-noise ratio of toxin standard chromatographic peaks. The statistical significance of differences in each toxin expression was analyzed with a *t*-test.

2.5.3. Saxitoxin synthesis core genes-sxtA, sxtG analyses

Total mRNA was isolated according to the manufacturer's instructions and further purified using the Mini Spin Columns of RNeasy Plant Mini Kit (Qiagen, Valencia, CA). RNA quality and quantity were assessed using a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Reverse transcription was performed to synthesize cDNA using a TOPscript™ cDNA Synthesis Kit (TOPscript™ RT DryMIX, Enzynomics, Daejeon, Korea). Specific primers targeting the *sxtA* and *sxtG* genes, previously developed by Murray et al. (2011) and Peerini et al. (2014), were employed. Thermal cycling conditions

included 30 cycles for genotyping or 40 cycles to test primer specificity, with the following steps: denaturation at 95 °C for 30 s, annealing at 58 °C for 20 s, and extension at 72 °C for 20 s. A T100 Thermal Cycler (Bio-Rad Laboratories, Hercules, CA, USA) was used for amplification. After PCR amplification, the samples were analyzed using a QX200 Droplet Digital PCR (ddPCR) system, which includes a droplet generator, droplet reader, and the T100 Thermal Cycler. The droplet reader (Bio-Rad Laboratories, Hercules, CA, USA) was employed to determine the average single-cell copy numbers for each *Alexandrium* species, following the protocol outlined by Lee et al. (2020).

2.6. Transcriptome sequencing, differential expression analysis, and functional annotation

Total RNA was extracted as mentioned above. The integrity and quality were determined using Qubit 4 (Thermo Fisher Scientific). The total RNA with high RNA integrity number (RIN) scores (criterion RIN > 8, range 9.1–9.8) from each sample were subjected to further sequencing with Illumina Nexa sequencers. Trimmomatic-0.36-Java-1.8.0_121 was used to remove adapters and filter those reads with low quality under criteria: mean Q < 30, a number (N) of over 5 % of the total nucleotide number, incomplete length < 50-bp, and repetitive reads with mutual information score > 0.5 (Bolger et al., 2014). Both reads in a pair were removed if either one of them failed in filtering. SOAPnuke was adopted for statistical analysis of the quality control data (Chen et al., 2018). Two methods, de novo assembly and reference genome-guided transcriptome assembly, were used in the present study. For de novo assembly, cleaned reads were subjected to Trinity (Grabherr et al., 2011), while for genome-guided assembly, clean reads were mapped onto the reference genome (*A. pacificum*) using Tophat and Cufflinks (Trapnell et al., 2012) and further assembled. RNA-Seq by Expectation Maximization (RSEM) was performed to calculate the abundance of reads mapped on genes or transcripts (Li & Dewey, 2011). Fragments per kilobase of exon per million fragments mapped (FPKM) was used to evaluate the expression abundance of genes or transcripts; and differential expression analysis was performed using the R statistical package, EdgeR (<http://www.biocductor.org/packages/release/bioc/html/edgeR.html>). Differentially expressed genes (DEGs) were defined as those with a fold-change (FC) and false discovery rate (FDR). Gene Ontology (GO) (<http://geneontology.org>) and Kyoto Encyclopedia of Genes and Genomes (KEGG) (<https://www.kegg.jp>) analysis were made via both databases and enriched pathways were further detected using cluster profile (Yu et al., 2012). Weighted Correlation Network Analysis (WGCNA) was used to do gene co-expression network analysis with dynamic tree cut method (GeneFrac threshold 0.5) (Langfelder & Horvath, 2008). The raw reads have been deposited in the NCBI database and are accessible under BioProject accession number PRJNA1187811. The associated Sequence Read Archive (SRA) accession numbers are SRR31414044, SRR31414043, SRR31414042, and SRR31414041.

2.7. Identification of STX genes and phylogenetic analyses of sxtA domains

TBLASTN analysis was performed with known *sxt* genes against the unigenes datasets in this study to identify genes potentially related to PSTs biosynthesis in *A. pacificum* (Table S3). The phylogenetic trees of *sxtA* domains were constructed separately within the *Alexandrium* species and compared with the *sxtA* of cyanobacteria and PKS-containing amino acid sequences of non-toxic bacteria and cyanobacteria (Table S4). Specifically, a dataset was constructed with 29 amino acid sequences, including the *Alexandrium* strain under study and 27 species of bacteria, cyanobacteria, and dinoflagellates. Phylogenetic trees were constructed using BI and ML methods (Ronquist et al., 2012; Zhang et al., 2020) with alignment done by MAFFT (Katoh & Standley, 2013). Bayesian posterior probabilities were calculated with MrBayes (Ronquist & Huelsenbeck, 2003), and phylogenetic trees were visualized

with iTOL (Letunic & Bork, 2016).

2.8. Statistical analysis

Statistical analyses were performed in R v4.1.1. Statistical significance for physiological measurements was calculated by Welch's *t*-test and one-way ANOVA with Tukey's HSD post-hoc analysis ($P < 0.05$).

3. Results

3.1. Development and characterization of axenic and co-cultured *A. pacificum*

The goal of an axenic dinoflagellate culture was pursued with a chemical treatment followed by antibiotics. The antibiotic mixture of penicillin, streptomycin, and amphotericin B was efficient in inhibiting bacterial growth and showed fewer effects on the dinoflagellate (volume: antibiotic mix=200:1). After repeated sub-culturing on f/2 medium for more than 2 months, a bacteria-free dinoflagellate culture was obtained. When dinoflagellate culture was plated on MA plate in the same condition for 5 days, no bacterial colonies were visible to the naked eye or under the fluorescence microscopy and SEM (Fig. 1A, B). The growth of *A. pacificum* was not significantly affected by the removal of its bacterial consortium in the short term. Over the longer term (>22 months), the majority of cultures experienced mortality, implying dependence on bacteria (data not shown).

The morphological characteristics between *J. cystaogens* NBRC 100362^T and *A. pacificum* (Group IV) were examined further under an SEM, as shown in Fig. 1B. Compared with *A. pacificum* cells in axenic single culture (Fig. 1B), *A. pacificum* cells co-cultured with *J. cystaogens* NBRC 100362^T, the bacterial flagella are inserted into the pore plates on the surface of the dinoflagellate. The insertion of bacterial flagella suggests that they may have established direct physical contact with the dinoflagellate, potentially influencing its metabolism or other physiological processes (Fig. 1B).

3.2. Growth of *A. pacificum* co-cultured with Alex-CFPB-*J. cystaogens*

As the bacterium established a close association with the dinoflagellate, the impact of the bacterium on the growth and physiology of the dinoflagellate was quantified. The maximum cell density of the dinoflagellate in the *A. pacificum*-*J. cystaogens* co-culture was low (2.2×10^4 cells mL⁻¹), whereas a relatively high maximum cell density (8.3×10^4 cells mL⁻¹) of *A. pacificum* was observed in the axenic condition ($P=0.04$) (Fig. 1C). During the initial 15 days, the maximum cell density of co-cultured group (2.0×10^4 cells mL⁻¹) was the higher than axenic group (1.4×10^4 cells mL⁻¹) (Fig. 1C). However, after 15 days and up to 30 days, the cell density of the axenic group surpassed that of the co-cultivated group.

In terms of cell size and morphology, the axenic group mainly maintained vegetative cells throughout the 30-day experiment, indicating that cells under axenic conditions remained in a stable asexual reproductive state (Cho et al., 2015). On the other hand, the co-cultivation group showed a noticeable growth slowdown after day 15, and signs of cyst formation were observed, indicating the initiation of the sexual reproduction phase (Fig. 1D). By day 30, the number of cysts in the co-cultivation group (1404 cells/1.5L) was significantly higher than in the axenic group (645 cells/1.5L) ($P < 0.001$), suggesting that the co-cultivation environment induced more cyst formation in the later phase (Fig. 1G).

3.3. Potential molecular indicators facilitating interaction between *A. pacificum* and *J. cystaogens*

Several studies have recorded *A. pacificum* stress responses in the

presence of algicidal bacteria such as activation of antioxidant enzymes, decreased protein content owing to the oxidation of certain amino acids including L-arginine to nitric oxide, and production of reactive oxygen species (ROS) (Li et al., 2015; Chen et al., 2022). *J. cystaogens* is an established Alex-CFPB and therefore, the whole-genome sequencing of *J. cystaogens* was performed to identify key genes that facilitate the interaction between *A. pacificum* and *J. cystaogens*. *J. cystaogens* utilizes direct contact mechanisms, such as the type VI secretion system (T6SS) and contact-dependent inhibition (CDI), to adjoin the cell structure of *A. pacificum* which is corroborated by the SEM images. Several copies of motility, flagellar/pili-related genes, and nitric oxide synthases (NOS) were present in the genome of the organism. *J. cystaogens* also hosts several genes that produce oxidative stress signals like hydrogen peroxide (H₂O₂) and nitric oxide (NO) through various metabolic processes. These genes which serve to attach themselves to *A. pacificum* and produce redox signals might trigger oxidative stress responses in *A. pacificum* (Fig. 2).

In addition, *J. cystaogens* competes with *A. pacificum* for essential nutrients like nitrogen and phosphorus by producing enzymes such as glutamine synthetase (GS), glutamate dehydrogenases (GDH), and polyphosphate kinase (PPK) (Table S5). The proximate physical interaction of *J. cystaogens* with *A. pacificum* demonstrated through SEM imaging and validated by the presence of essential genes in the genome of the bacterial strain led us to hypothesize that *J. cystaogens* may trigger stress responses in *A. pacificum*, leading to enhanced toxin synthesis as a defensive measure. Genome assembly results further suggested that autoinducer molecules, such as acyl-homoserine lactones (AHLs), produced by *J. cystaogens*, regulate various bacterial behaviors and interactions with the dinoflagellate. The QS system involves the *luxI/luxR* regulatory circuit, where *luxI* encodes the enzyme responsible for AHL synthesis, and *luxR* encodes the receptor that detects AHLs which may, in turn, activate gene expression related to toxin synthesis (Van Houdt et al., 2007; Berger et al., 2011; Ziesche et al., 2018; Wang et al., 2021) (Fig. 2).

3.4. RNA-seq analysis and identification of DEGs

Two samples were derived from two experimental groups, and each group was set with three replicates. All the retrieved data were assembled with Trinity. Finally, 451,847,813 reliable bases were identified from Trinity assembly, including 1,019,912 transcripts and 885,693 genes (Table S6). Analysis of 55,859 identified DEGs between axenic *A. pacificum* and co-culture samples included 6,567 upregulated and 49,292 downregulated genes.

3.4.1. Identification of *sxt* genes in the transcriptome of *A. pacificum*

The assembly of the *A. pacificum* transcriptomic library yielded 1,338 *sxt* genes in a range of 20–1,130 bp (Table S3). Both short and long isoforms *sxt* genes were identified, with high similarity to sequences of reference *A. pacificum* (Table S3). The results of the phylogenetic tree analysis revealed that the *sxtA* sequences identified in our study clustered within the clade containing *sxtA* sequences from toxic dinoflagellates (Fig. 3A). Reference and identified sequences are shown in Table S7. This suggests a close evolutionary relationship between the *sxtA* gene in our isolated strain and those found in other toxic dinoflagellates. Phylogeny analysis of the dinoflagellate *sxtA* gene formed a highly conserved and fully supported clade, separating the *Alexandrium* genus from other toxic, non-toxic cyanobacteria and bacteria with polyketide synthase. These findings provide additional evidence supporting the involvement of the identified *sxtA* gene in toxin production and its potential role in the toxicity of *A. pacificum*.

3.4.2. Comparison of the *sxt* gene cluster in *A. pacificum* with other bloom-forming species

RNAseq transcript analysis was performed to investigate the saxitoxin biosynthesis genes in *A. pacificum*. A comparative analysis was

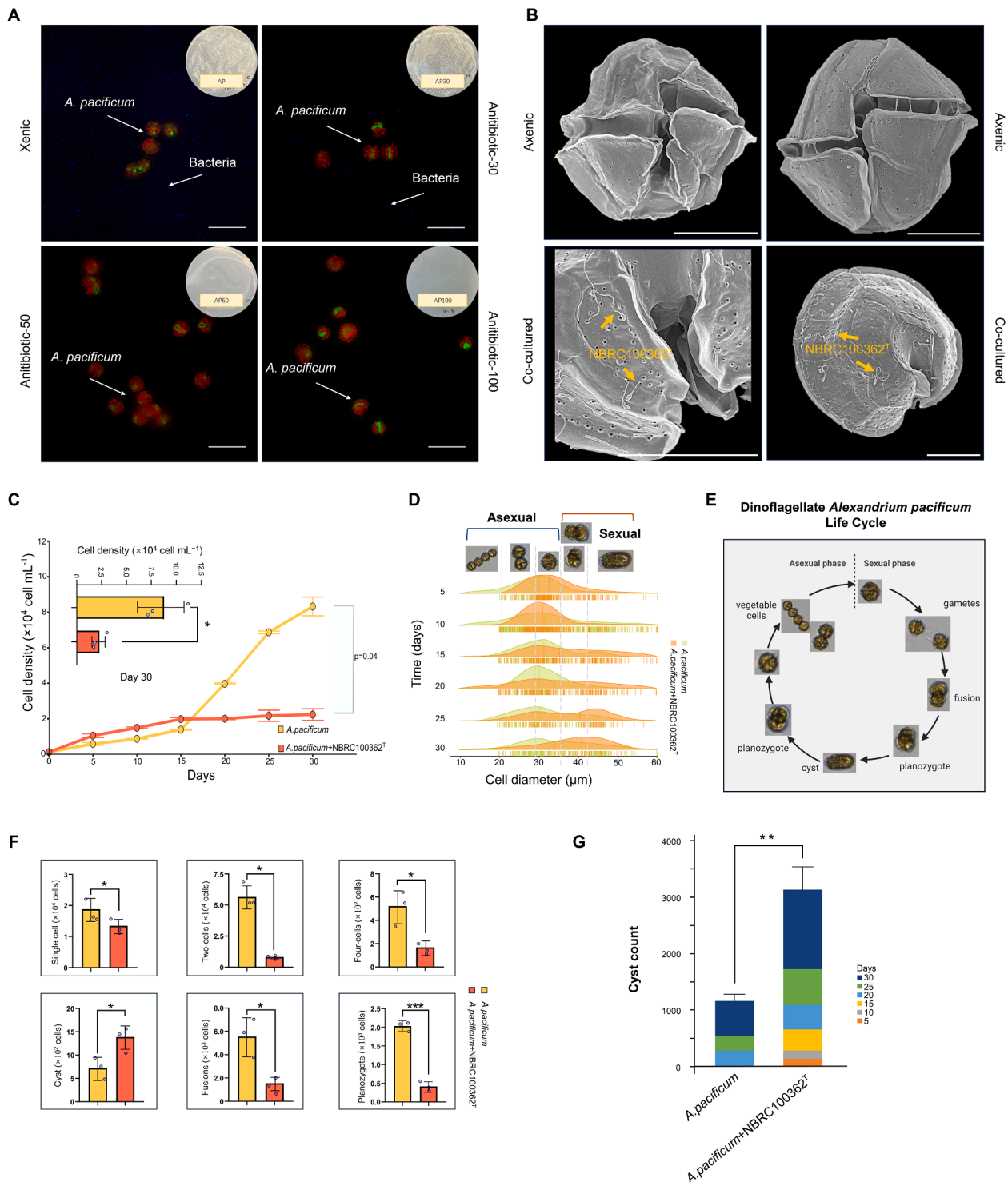


Fig. 1. (A) Fluorescence microscopy with MA agar plate images of axenic and xenic of *Alexandrium pacificum*. The xenic images of *A. pacificum* and phycosphere microbiology, AP: MA agar plate culture spread of xenic *A. pacificum* culture; AP30: MA agar plate spread with the *A. pacificum* culture incubated with 3 $\mu\text{L mL}^{-1}$ antibiotic mix. axenic images of *A. pacificum*. The nearly axenic images of *A. pacificum*, AP50: MA agar plate spread with the *A. pacificum* culture incubated with 5 $\mu\text{L mL}^{-1}$ antibiotic mix; AP100: MA agar plate spread with the *A. pacificum* culture incubated with 10 $\mu\text{L mL}^{-1}$ antibiotic mix. The scale bars correspond to 20 μm . DAPI-stained cell showing the position of the nucleus (green), and chloroplast (red) distributed around it. The xenic culture of *A. pacificum* showed bacteria (blue) appeared. (B) SEM images of an axenic and co-cultured group of *A. pacificum* at different magnifications. The scale bars correspond to 10 μm . (C) Growth curve showing the distribution of diameter with phenotypic dynamics every 5 days of the growth experiment over a period of 30 days. Combined with the differences in cell density at day 30 of the axenic and co-cultured group. Significant differences between the control and treatments at day 30 are indicated by * ($P < 0.05$). (D) The Gaussian Kernel Density curve shows the distribution of diameter with phenotypic dynamics every 5 days of the growth experiment over a period of 30 days. Combined with the corresponding growth morphology detected by FlowCam. (E) The life cycle images of dinoflagellate *A. pacificum* captured in this study. (F) The differences in life cycle stage cell density of axenic and co-cultured group. Significant differences between the control and treatments, as determined by a *t*-test, are indicated by * ($P < 0.05$) *** ($P < 0.01$). (G) The cyst density of axenic and co-cultured group every 5 days. Significant differences between control and treatments, as determined by one-way ANOVA, are indicated by ** ($P < 0.01$).

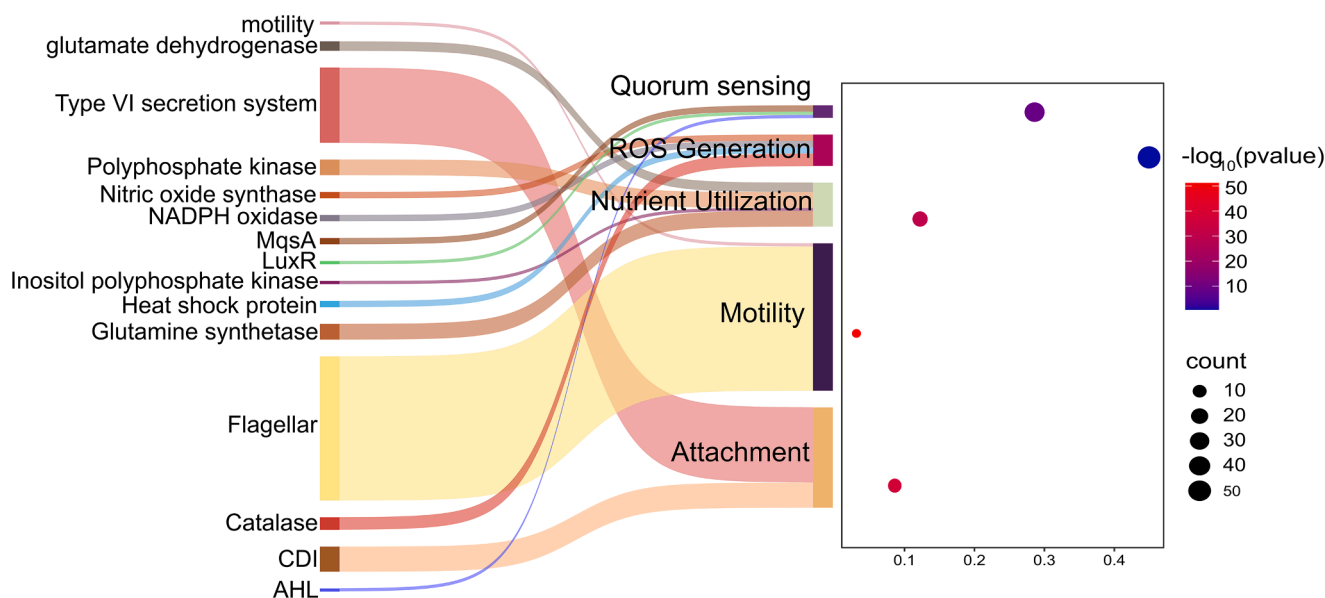


Fig. 2. The pathway enrichment analysis of metabolites in the *Jannaschia cystaogens* NBRC 100362^T genome based on the KEGG and Pfam database.

conducted with *A. tamarensis* GROUP IV and *Cylindrospermopsis raciborskii* T3. The analysis revealed that out of the 34 saxitoxin biosynthesis genes identified in *C. raciborskii*, 24 genes (*sxtD*, *sxtB*, *sxtA*, *sxtF*, *sxtG*, *sxtH*, *sxtI*, *sxtL*, *sxtM*, *sxtN*, *sxtX*, *sxtW*, *sxtV*, *sxtU*, *sxtT*, *sxtS*, *sxtR*, *sxtQ*, *sxtP*, *sxtO*, *sxtY*, *sxtZ*, *ompR*, *hisA*) were also found in *A. pacificum*, indicating that *A. pacificum* shares more than half of the biosynthetic pathway involved in saxitoxin production with *C. raciborskii* (Kellmann et al., 2008). Additionally, *A. pacificum* exhibited the presence of 12 similar *sxt* homologs as found in *A. tamarensis* Group IV (Hackett et al., 2013) (Fig. 3B). These findings suggest a significant overlap in the saxitoxin biosynthetic pathway between *A. pacificum*, *C. raciborskii*, and *A. tamarensis* Group IV. The presence of shared gene homologs indicates potential conservation of the saxitoxin biosynthetic pathway across these organisms.

3.4.3. DEGs in PSTs biosynthesis-related metabolic pathways in co-culture samples

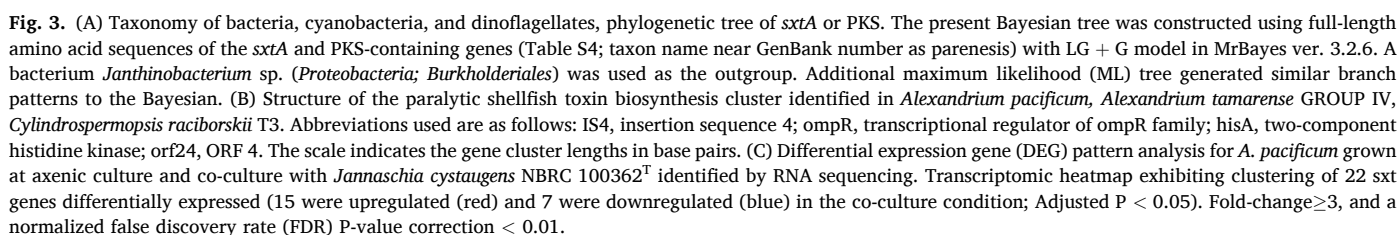
To investigate the reciprocal gene responses during co-culturing, we compared the expression differences of PSTs biosynthesis-related genes in *A. pacificum* transcriptome between the co-culture group and the control group. The co-cultivation of *A. pacificum* with *J. cystaogens* for 30 days resulted in significant changes in gene expression. A total of 15 genes were found to be upregulated, while 7 genes showed downregulation in the co-culture group compared to the control group (Fig. 3C; Table S8). Among the core PSTs biosynthesis-related genes (*sxtA*, *sxtG*, *sxtB*, *sxtD*, *sxtU*, *sxtH/T*, and *sxtI*) that are directly involved in the biosynthesis of STX, only *sxtD* (encoding sterol desaturase) was significantly downregulated when *A. pacificum* was co-cultivated with *J. cystaogens* for 30 days. Among tailoring genes (*sxtL*, *sxtN*, and *sxtX*) encoding proteins that modify the STX molecules to their analogs, *sxtN* (encoding N-sulfotransferase) which is involved in the biosynthesis of low-toxicity sulfated PSTs, and *sxtL* (encoding GDSL-lipase) were downregulated. In addition, *sxtSUL* (encoding O-sulfotransferase) was downregulated. Meanwhile, *sxtX* (encoding cephalosporin hydroxylase) which is involved in the biosynthesis of the highly toxic PSTs components GTX1&4 and neoSTX, was upregulated. Therefore, co-cultivation of *A. pacificum* with *J. cystaogens* for 30 days results in significant upregulation of PSTs biosynthesis-related genes in *A. pacificum* cells directly at the transcription level. On the contrary, genes playing a major role in lowering the toxicity of PSTs through sulfation of highly toxic analogs were downregulated in co-cultured samples. In addition, the highest kME values were that of quinone-related genes upregulated in

the co-culture samples adding credence to the slight increase in ROS-related genes of *A. pacificum* under co-cultivation (Table S9).

3.4.4. Gene co-expression networks and modules

All DEGs with expression levels below 1 were deleted, and the rest of 13,734 DEGs in the axenic and co-culture samples, were analyzed by WGCNA. Four modules were clustered in axenic *A. pacificum* and co-cultured *A. pacificum*, respectively, as shown in Fig. 4A. With a threshold line of kME value higher than 0.7, there were 687, 1102, 7820, and 154 DEGs collected in the blue, brown, turquoise, and yellow modules respectively (Fig. 4B). With the highest number of DEGs collected, the brown and blue modules showed no significant correlation with axenic and co-culture samples (Fig. 4A, B). Among these modules, the yellow and turquoise modules were significantly correlated with co-culture samples. The two showed the most significant differences in the genetic information processing both in total and turquoise module KEGG enrichment, and one that showed the most significant differences in the organismal system ($P < 0.05$) were observed for Ribosome biogenesis in eukaryotes (ko03008; ko03110), and Plant-pathogen interaction (ko04626) (Fig. 4C, D). For the turquoise module, KEGG and GO pathway analysis identified 100 DEGs enriched in various metabolic and organismal system pathways. The major pathways related to glutathione, quinone, photosynthesis, metal ion binding, and protein transport were related in the turquoise module. Nitrogen metabolism and phospholipid metabolism were related in the yellow module (Table S9). The hub genes of the yellow module were screened by the top five kME values ($kME > 0.9971$), whereas the hub genes of the turquoise module were screened by the top five kME values ($kME > 0.9999$). Further analysis of hub genes revealed that genes encoding Menaquinone biosynthesis protein MenD played the core functions in turquoise modules of *A. pacificum* when co-cultured with bacteria *J. cystaogens*. Ferredoxin-dependent glutamate synthase (Fd-GOGAT) and 1-AGP acyltransferase 8 (1-AGPAT 8) played the core functions in yellow modules of co-cultured *A. pacificum* (Table S9).

Among these modules, all upregulated genes involved in saxitoxin synthesis are contained within the turquoise module, while the downregulated gene falls within the yellow module (Fig. 4E). Additionally, gene expression related to oxidative stress (antioxidants, ascorbate peroxidase APX, glutathione peroxidases GPX, glutathione S-transferase GST) were upregulated in *A. pacificum* co-cultured with bacteria *J. cystaogens* (Table S10).



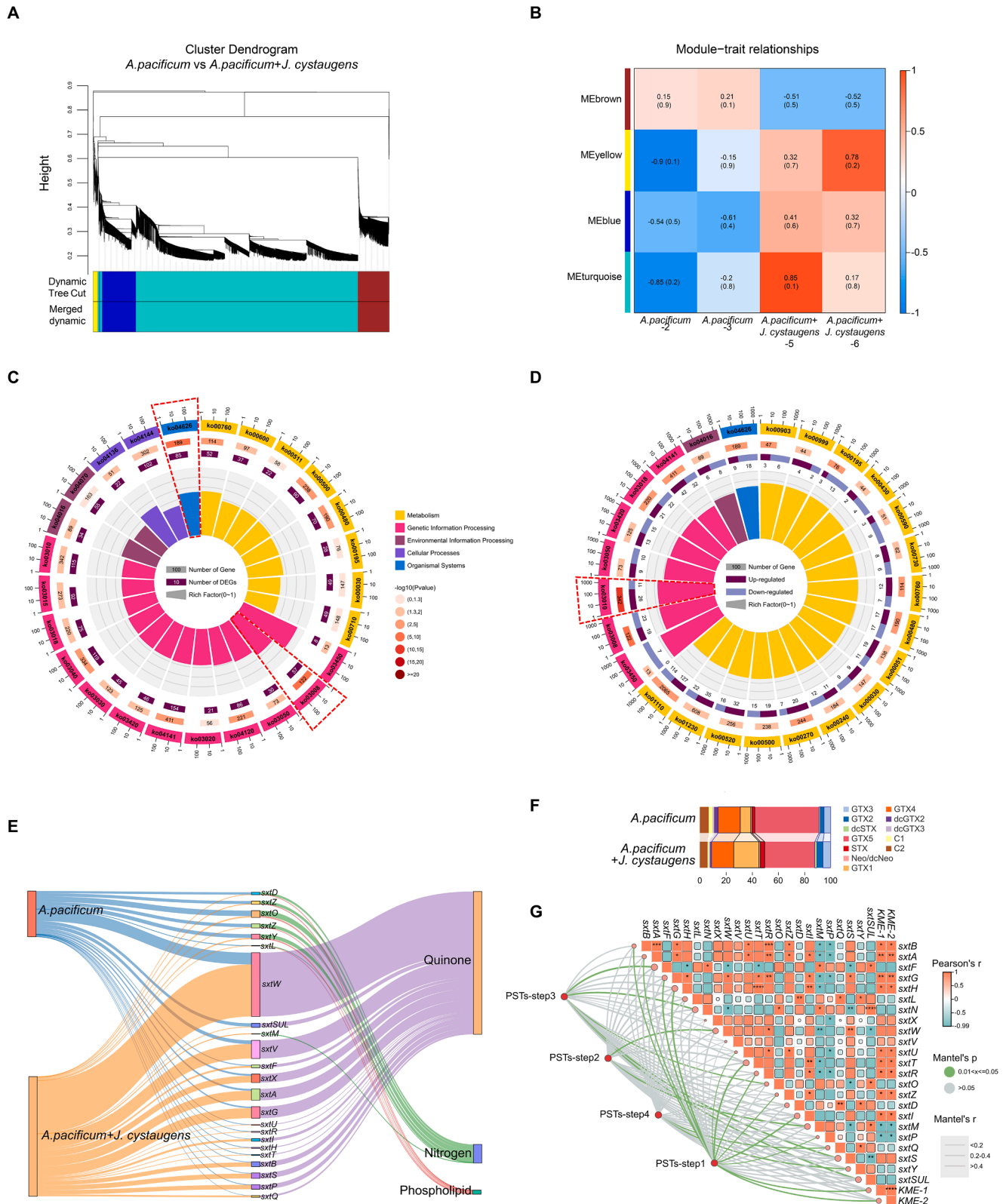


Fig. 4. WGCNA analysis. (A) Clustering dendrogram of genes, with gene dissimilarity based on topological overlap, together with assigned module colors. (B) Module-trait relationship. Replicated group axenic group -2, -3; co-cultured group -5, -6. (C) Top 25 turquoise and (D) yellow module KEGG pathway enrichment of DEGs in the transcriptome of two condition of *Alexandrium pacificum*. The first circle: top 25 KEGG pathways. The number outside of the circle is the coordinate ruler of the gene number. Different colors represent different KEGG classes; the second circle: the number and P value of the KEGG pathway in the background gene. The more genes, the longer the bars, and the smaller the P value, the redder the color. The third circle: a bar chart of the proportion of up and down-regulated genes, dark purple represents the proportion of up-regulated genes, and light purple represents the proportion of down-regulated genes; the specific values are shown below. Fourth circle: RichFactor value of each KEGG pathway. (E) The relationship of group-*sxt* genes-model key metabolism. (F) Comparison of the PSTs content of two groups. (G) The relationship between *sxt* genes and PSTs synthesis process.

3.5. Changes in the level of intracellular PSTs component, core gene expression, and toxicity of *A. pacificum* when co-cultured with *J. cystaogens*

Co-culture with *J. cystaogens* clearly affected the *A. pacificum* STXs profile as well as the total saxitoxins equivalents (STXs eq). The total saxitoxin analysis revealed a significant difference in saxitoxin levels between the bacterial-inoculated group and the control group (*, $P < 0.05$). The average STXs concentration in the bacteria-inoculated group was 0.0126 ± 0.0041 ng cell⁻¹, whereas the control group exhibited an average saxitoxin concentration of 0.0063 ± 0.0011 ng cell⁻¹. The intracellular saxitoxin level within the cell of *A. pacificum* in the co-culture group increased by two-fold compared to the axenic group (Fig. 5A).

Using digital PCR technology, we assessed the expression levels of the *sxtA* and *sxtG* genes in the bacterial-inoculated and control groups. The results revealed a significant upregulation of the *sxtA* gene in the bacterial-inoculated group compared to the control group (****, $P < 0.0001$). The bacterial-inoculated group exhibited an average expression level of 62.7000 ± 3.7211 copies μL^{-1} for the *sxtA* gene, while the control group had an average expression level of 9.0500 ± 0.6557 copies μL^{-1} for the *sxtA* gene, presenting a 7.0-fold increase in comparison to control axenic *A. pacificum* culture group (Fig. 5B). Similarly, the *sxtG* gene demonstrated a significant increase in expression in the bacterial-inoculated group (****, $P < 0.0001$). The bacterial-inoculated group showed an average expression level of 178.9750 ± 1.0210 copies μL^{-1} for the *sxtG* gene, whereas the control group had an average expression level of 62.2000 ± 2.8249 copies μL^{-1} (SD = X) for the *sxtG* gene (Fig. 5B). The *sxtG* gene showed higher transcript levels (about 3-fold) when axenic *A. pacificum* were exposed to *J. cystaogens*.

Toxin profiles of *A. pacificum* cultures in axenic and co-culture groups were dominated by 12 analogs (STX, neoSTX, GTX1, GTX2, GTX3, GTX4, GTX5, dcGTX2, dcGTX3, dcSTX, C1 and C2) (Fig. 5C, 5D; Table S11). Compared between axenic and co-cultured *A. pacificum* samples, dcGTX2, C2, C1, and dcGTX3 were the four major analogs (>70 % of the total toxin content), followed by GTX2, GTX5, GTX3, and NEO, of the axenic group. Four additional analogs (GTX1, GTX4, STX, and dcSTX) were also detected, albeit at very low levels (<1 fmol cell⁻¹). These analogs were all observed in axenic and co-culture groups, but the concentration and proportion in single-cell were different. The total toxin content of the co-cultured group (29.4980 ± 1.4111 fmol cell⁻¹) is higher than the axenic group (24.5204 ± 1.5941 fmol cell⁻¹). In the co-culture group, the cultures of *A. pacificum* produced more GTX3, GTX2, neoSTX, STX, GTX1, GTX4, and dcSTX (Fig. 4F, 5D; Table S11). On the contrary, the axenic *A. pacificum* produced more dcGTX3, GTX5, dcGTX2, C1, and C2 compared to co-cultured *A. pacificum*.

4. Discussion

4.1. The role of *J. cystaogens* in enhancing high-toxicity PSTs synthesis and altering toxin profiles in *A. pacificum*

Alexandrium species can produce resting cysts during their life cycle. Dinoflagellate cysts are the seeds formed by dinoflagellates, and they are crucial for the formation of PSTs-producing dinoflagellates bloom (Kim et al., 2002; Kim et al., 2020). Although it has been demonstrated that Alex-CFPB influences growth and cyst formation in dinoflagellates, the role of bacteria in enhancing the amount of toxin synthesis in *Alexandrium*, and the mechanisms and machinery involved are unclear (Hold et al., 2001; Adachi et al., 1999).

Studies conducted in regions with annual *Alexandrium* blooms have revealed that bacteria play a crucial role in promoting cyst formation in *Alexandrium* species like that of Hiroshima Bay, Japan (Adachi et al., 1999). To elucidate the mechanisms by which these bacteria influence *Alexandrium* blooms, we chose the previously described *Alexandrium* cyst formation promoting bacterium *J. cystaogens* to analyze its

interaction with *A. pacificum*.

Our measurements of transcriptomic and toxin profiles were taken at the late stationary phase of *A. pacificum* (30 days). This focus on the late stationary phase is significant because it is a critical period for toxin production and metabolic activity in dinoflagellates. Studies have shown that during the late stationary phase, toxin production often reaches its peak as the cells respond to nutrient limitations and other environmental stresses, also replicating the late bloom characteristics (Smith et al., 2012). For instance, research on *A. tamarense* demonstrated that the highest toxin content is observed during the late stationary phase when nutrient uptake and metabolic activities are heightened (Wang and Hsieh, 2005). Similarly, *Dinophysis acuminata* retains high levels of intracellular toxins during the late stationary phase, which are crucial for maintaining cellular toxicity and ecological competitiveness (Jia et al., 2019). Our focus on this growth phase allows us to capture the peak of these critical processes, providing insights into the metabolic strategies and toxin production mechanisms of *A. pacificum* under stress conditions.

4.1.1. Promotion of total toxin, *sxtA*, *sxtG* gene expression, and changes in PSTs component content

In this study, co-culturing *A. pacificum* with *J. cystaogens* significantly increased the STX content. The average intracellular saxitoxin level in the co-culture group demonstrated a two-fold increase compared to the axenic group (Fig. 5A). This enhancement in toxin production highlights the impact of *J. cystaogens* on *A. pacificum* metabolic processes.

To further understand the impact of *J. cystaogens* on STX synthesis in *A. pacificum*, we examined the expression levels of the core genes *sxtA* and *sxtG*, which are critical in the initial steps of STX synthesis in dinoflagellates. These genes play essential roles in the biosynthesis of saxitoxins, with *sxtA* initiating the toxin synthesis pathway and *sxtG* involved in subsequent steps (Stüken et al., 2011; Perini et al., 2014; Bui et al., 2022). In our experiments, the expression levels of *sxtA* and *sxtG* were significantly higher in the co-culture group compared to the control group (Fig. 5B). The *sxtA* gene showed a 7.0-fold increase, and the *sxtG* gene displayed a 3.0-fold increase in expression levels in the bacterial-inoculated group. These findings suggest a direct correlation between the enhanced expression of these key toxin synthesis genes and the observed increase in total toxin content. The upregulated expression of *sxtA* and *sxtG* implies that the presence of *J. cystaogens* not only boosts initial cell growth but also enhances the metabolic processes involved in toxin synthesis. This relationship underscores the role of Alex-CFPB in modulating both the growth dynamics and toxin production of *A. pacificum* (Adachi et al., 2003; Adachi et al., 2004).

4.1.2. Induction of high-toxicity PSTs component synthesis

The toxin profile of *A. pacificum* under co-culture conditions with *J. cystaogens* showed significant changes, particularly in the synthesis of high-toxicity PSTs components. The concentrations of GTX4, GTX1, STX, and neoSTX were markedly higher in the co-culture group. During the synthesis of high-toxicity PSTs, levels of neoSTX and GTX1&4 which are the highly toxic components and hydroxylation products formed under the action of hydroxylase (encoded by *sxtX*) from STX and GTX2&3, respectively, significantly increase (Fig. 6). According to the transcriptome results, the gene *sxtX* (encoding cephalosporin hydroxylase), which is related to GTX1&4 and neoSTX biosynthesis, was significantly upregulated in the co-culture group (Fig. 6). This upregulation indicates an enhancement in the biosynthesis pathway leading to more toxic PSTs components.

In contrast, the common feature of the less toxic PSTs components C1&2, GTX2&3, and GTX5 in algal cells is that they are sulfated products derived from precursor toxins. According to the putative PSTs biosynthesis pathway in *Alexandrium* species, GTX2&3 is synthesized through an oxygenation reaction under the action of phenylpropionate dioxygenase (encoded by *sxtH/T*) and a sulfonation reaction under the action of N-sulfotransferase (encoded by *sxtN*) successively from STX.

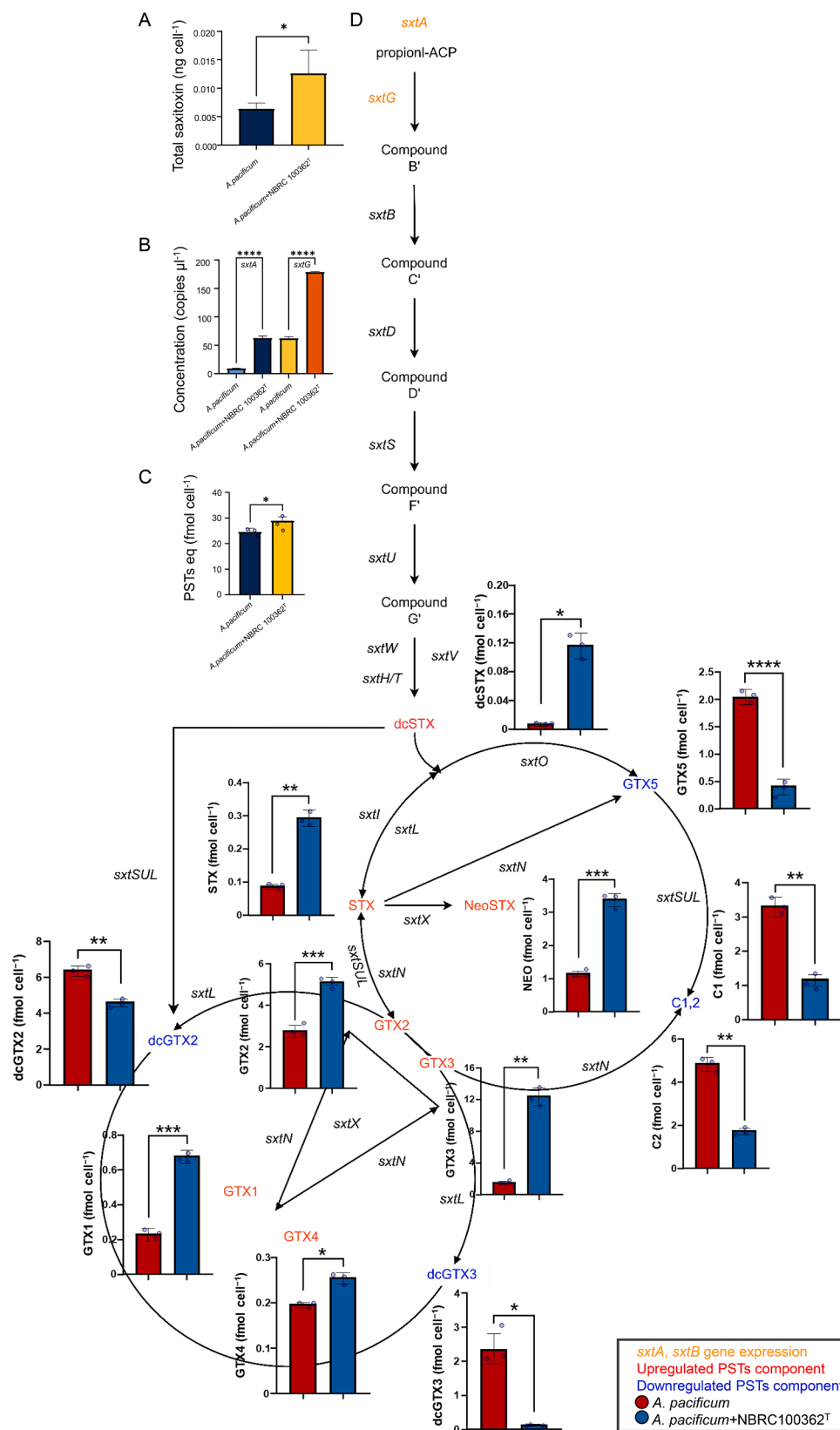
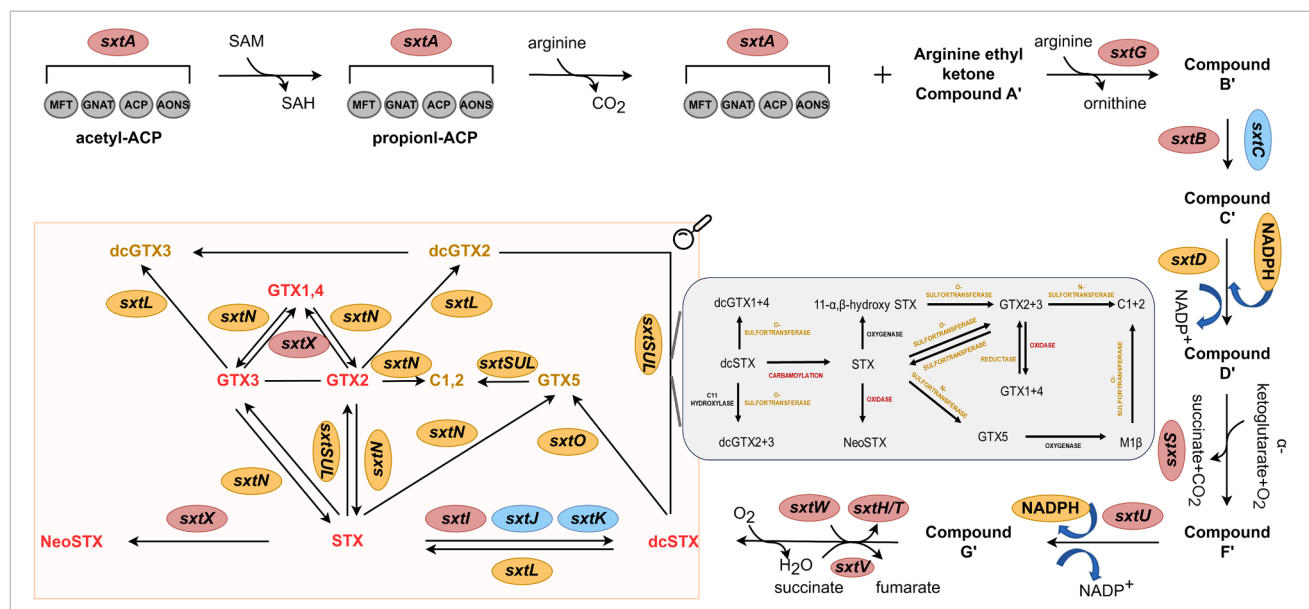


Fig. 5. Saxitoxin biosynthesis pathway combined with the changes in toxins profile of *Alexandrium pacificum* cultivated under axenic culture and co-culture with *Jannaschia cystaogens* NBRC 100362^T. (A) Total saxitoxin quantified by enzyme-linked immunosorbent assay (ELISA). Toxin content (ng cell⁻¹) of *A. pacificum* grown at axenic culture and co-culture with NBRC 100362^T. *, $P < 0.05$. (B) Gene expression levels of *sxtA* and *sxtG* in *Alexandrium pacificum* under axenic culture and co-culture with *Jannaschia cystaogens* NBRC 100362^T. Error bars represent standard deviations. Significant differences between the control (30 psu) and treatments, as determined by a one-way ANOVA, are indicated by ****, $P < 0.0001$. (C) Changes in total saxitoxins equivalent (STXs eq) of *A. pacificum* cultivated under axenic culture and co-culture with *J. cystaogens* NBRC 100362^T. (D) Changes in toxins profile of *Alexandrium pacificum* cultivated under axenic culture and co-culture with *J. cystaogens* NBRC 100362^T.



Similarly, GTX5 and C1&2 are sulfated products formed under the action of N-sulfotransferase (encoded by *sxtN*) from STX and GTX2&3, respectively (Fig. 3C). After 30 days of co-culturing, the genes *sxtN* (encoding N-sulfotransferase), *sxtSUL* (encoding O-sulfotransferase), and *sxtO* (encoding adenylylsulfate kinase) were all significantly downregulated. This downregulation suggests that the presence of cyst formation-promoting bacterium induces the PSTs biosynthesis pathway to favor the production of more toxic PSTs components, while the production of sulfated PSTs components, which facilitates a relatively easier detoxification of the PSTs in the marine environment, is diminished (Fig. 3C, 5D).

the toxin, a critical step in the conversion to C1/C2 forms (Soto-Liebe et al., 2010). Finally, the conversion to dcGTX2&3 shows significant correlations with *sxtQ* and *sxtY*, indicating these genes are central to the final stages of toxin synthesis. *sxtQ* and *sxtY* are thought to regulate toxin-specific tailoring enzymes that fine-tune the structure of the decarboxylated toxins.

These findings underscore the impact of Alex-CFPB on the toxin composition of *A. pacificum*, demonstrating a shift towards higher toxicity PSTs components. This shift is mediated by the upregulation of genes involved in hydroxylation processes and the downregulation of genes responsible for sulfation, leading to a more toxic profile under co-culture conditions. Such a shift can significantly impact the ecological dynamics and toxicity profiles of harmful algal blooms.

4.2. The multifaceted impact of *J. cystaugens* on toxin production and cyst formation in *A. pacificum*

The formation of cysts in *Alexandrium* species plays a crucial role in their life cycle and ecological impact, as these cysts act as a seed bank for HABs, facilitating the recurrence of blooms under favorable conditions. Generally, when *A. pacificum* cells are under external environmental stress, they enhance their toxin biosynthesis activity and defend against unfavorable environments with cyst formation (Ribeiro et al. 2011; Miyazono et al. 2012). Cyst formation-promoting bacteria may also affect the physiological state of cells and place external stress on algal cells (Anderson et al., 1984; Hwang and Lu, 2000; Hamasaki et al., 2001; Frangópulos et al., 2004; Ganini et al., 2013; Roy et al., 2014; Shin et al., 2018; Wang et al., 2022a). Numerous studies have linked cyst formation with environmental stressors such as nutrient depletion, oxidative stress, and bacterial interactions. These stressors drive the transformation of vegetative cells into cysts, a survival strategy which also facilitates their spread within marine ecosystems (Adachi et al., 1999). Furthermore, cyst formation in *Alexandrium* species is closely linked to toxin dynamics. Research suggests that resting cysts can contain significant amounts of paralytic shellfish toxins (PSTs), which may contribute to the toxicity of future blooms when these cysts germinate (Oshima et al., 1992).

4.2.1. *Alex-CPFB-J. cystaogens-induced stress promotes transition to sexual reproduction and cyst formation*

To investigate how *J. cystaogens* influences the life cycle of *A. pacificum*, we focused on understanding its effects on key transitions in the reproduction and development of the dinoflagellate. Our findings indicate that *J. cystaogens* significantly impacts the life cycle of *A. pacificum*, accelerating the transition from asexual to sexual reproduction, and leading to cyst formation. Using FlowCam technology, we measured cell size, density, and morphology every five days, allowing us to clearly capture the process of cyst formation. This approach provided detailed insights into the dinoflagellate life cycle, capturing changes in cell size and shape, especially the transition from vegetative cells to cysts. The morphological changes observed during the cyst formation process were consistent with those reported in field samples, demonstrating similar developmental stages under both laboratory and natural conditions (Ichimi et al., 2001; Adachi et al., 2003). During the 30-day experiment, the axenic group maintained mostly vegetative cells, while the co-cultured group underwent a clear shift towards sexual reproduction after day 15 (Fig. 1C, D). The co-cultured cells became larger (Fig. 1D), and through FlowCam imaging, we observed distinct stages of sexual reproduction, including gamete fusion and the planozygote stage, where two cells merged, with two flagella (Fig. 1D, E, F). This observation is consistent with the patterns reported for *Alexandrium* species earlier, where nutrient limitation or stress triggers the formation of planozygotes and subsequent cyst formation (Figueroa et al., 2007).

By the 30th day, the cyst count reached its peak in the co-cultured group, indicating the culmination of the cyst process. The strong cyst-promoting influence of *J. cystaogens* is consistent with other reports where bacteria from the *Roseobacter* group were found to induce cyst formation in *Alexandrium* species (Adachi et al., 2003; Adachi et al., 2004). To further explore the regulatory mechanisms behind this transition, we conducted transcriptome analysis on day 30, focusing on the genes involved in sexual reproduction and stress responses, as this is a critical period for cyst formation. This detailed analysis of cell size and morphological changes during the life cycle offers valuable insights into the stress-induced shift from asexual reproduction to sexual reproduction and highlights the importance of *J. cystaogens* in modulating the growth and reproduction of *A. pacificum* under co-cultivation conditions.

4.2.2. *Enhanced toxin production during encystment as a defense mechanism*

In our study, the co-cultivation of *A. pacificum* with *J. cystaogens* significantly altered the growth dynamics and reproductive cycle of the dinoflagellate. During the first 15 days of the experiment, the co-cultured group exhibited a similar or slightly higher growth rate compared to the axenic group. However, after day 15, the growth of the co-cultured group slowed considerably as sexual reproduction was initiated, leading to the formation of cysts. By day 30, the cell density in the co-cultured group was nearly one-quarter of the final cell density of the axenic group, highlighting the significant drop in cell density, and underscoring the shift from asexual proliferation to sexual reproduction and encystment.

The sexual reproduction process in *A. pacificum* resulted in notable changes in cell morphology and size, as cells transitioned into the planozygote stage, characterized by larger cell sizes and the fusion of two cells. This was followed by the formation of resting cysts, a critical phase in the life cycle that serves as a survival strategy under stressful conditions, such as those induced by bacterial interactions.

Interestingly, despite the reduction in overall cell numbers, the total toxin content per cell increased significantly in the co-cultured group. Specifically, the concentration of highly toxic saxitoxin analogs, such as GTX1&4, STX, and neoSTX, was elevated, indicating that *A. pacificum* enhances its toxicity during the encystment process. This is consistent with previous research on *A. tamarense*, where cysts were found to contain significantly higher toxin levels than vegetative cells. Cysts from

A. tamarense were reported to have six times more toxins than their vegetative counterparts, suggesting that toxin production may either continue or be conserved during cyst formation (Oshima et al., 1992).

These findings indicate that cysts not only serve as a dormant phase for survival but also as a reservoir for high toxin levels. Upon germination, these toxic cysts can release highly toxic vegetative cells back into the water, amplifying the impact on marine ecosystems and contributing to the recurrence of HABs. The increased toxicity per cell, combined with the formation of resistant cysts, highlights the dual role of cysts in both the survival and ecological impact of *A. pacificum*.

4.2.3. *Direct physical interaction and nutrient competition as key drivers of physiological change*

The results from our SEM analysis provide the evidence of the direct physical interactions between *J. cystaogens* and *A. pacificum*. The SEM images revealed that bacterial flagella were inserted into the pore plates on the surface of *A. pacificum* cells in the co-cultured group, which contrasts with the smooth surface of *A. pacificum* cells in the axenic culture. These pore plates, which are part of the dinoflagellate thecal structure, are typically involved in processes such as gas exchange, nutrient uptake, and waste expulsion.

The insertion of bacterial flagella into these pores suggests that *J. cystaogens* may have established direct physical contact with the dinoflagellate, potentially disrupting or altering its normal metabolic activities. This physical interaction could serve as a pathway for the bacterium to influence *A. pacificum* in multiple ways, including modulating its nutrient uptake or introducing stress signals, thereby triggering defensive responses such as increased toxin production and cyst formation. Previous studies have suggested that direct contact mechanisms, such as those mediated by bacterial flagella or type VI secretion systems, can profoundly influence dinoflagellate physiology by inducing stress or altering cellular function (Filloux, 2024).

In our study, this direct interaction may have contributed to the observed physiological changes in *A. pacificum* co-cultured with *J. cystaogens*, including the significant increase in cyst formation and toxin synthesis after day 15. Physical contact could intensify the effects of oxidative stress signals, nutrient competition, and quorum sensing signals, all of which were discussed earlier as key factors driving these changes. The insertion of bacterial flagella might also suggest a more intimate association between *J. cystaogens* and *A. pacificum*. Furthermore, the swarming motility increases their contact with dinoflagellates, amplifying the effects of oxidative stress signals, QS, nutrient competition, and direct contact mechanisms. This close association enhances the regulatory impact of bacteria on dinoflagellate toxin synthesis, leading to increased production of high-toxicity toxins and a decrease in low-toxicity sulfated toxins.

Thus, the SEM findings provide crucial insight into the potential mechanisms through which *J. cystaogens* directly interacts with *A. pacificum*, emphasizing the multifaceted nature of the bacterial influence, which includes both metabolic competition and physical interactions that together contribute to enhanced toxin production and cyst formation in the dinoflagellate.

In addition to the physical interactions discussed, our genomic analysis of *J. cystaogens* supports the hypothesis that the bacterium generates oxidative stress signals, such as H₂O₂ and NO, through various metabolic processes. NO is formed biologically through the oxidation of L-arginine by nitric oxide synthases (NO-synthesis), and H₂O₂ is produced as a byproduct of aerobic metabolism. These stress signals can further influence dinoflagellate responses. These reactive oxygen species act as redox signals, triggering oxidative stress responses and cyst formation in dinoflagellates (Ganini et al., 2013). This stress may induce increased ROS levels and increased antioxidant activity (Li et al., 2015). Under these stressful environmental conditions, dinoflagellates, in turn, may produce quinone-like molecules to mitigate oxidative damage (Fig. 4E). This stress-induced production of molecules aligns with bacterial quorum sensing (QS) systems.

Bacterial QS molecules, specifically acyl-homoserine lactones (AHLs), play a crucial role in regulating bacterial behavior and influencing dinoflagellate physiology. Through the *luxI/luxR* QS regulatory circuit, bacteria produce AHLs that, when detected by the *luxR* receptor, activate dinoflagellate genes involved in toxin synthesis pathways, promoting toxin production (Wang et al., 2022b). As bacterial populations reach a critical density, the QS mechanism is triggered, leading to coordinated bacterial activity that enhances stress responses in dinoflagellates. Furthermore, bacteria may compete with dinoflagellates for essential nutrients like nitrogen and phosphorus by producing enzymes such as Glutamine Synthetase (GS), Glutamate Dehydrogenases (GDH), and Polyphosphate Kinase (PPK) (Table S5, Fig. 2). These enzymes are central to nitrogen and phosphorus metabolism. The uptake and conversion processes mediated by GS and GDH are critical for managing nitrogen levels under competitive conditions. PPK regulates phosphorus storage and utilization, which is crucial under phosphorus-depleted conditions. This competition leads to the downregulation of nitrogen and phosphorus metabolism in dinoflagellates, resulting in increased stress responses. The increased stress due to nitrogen and phosphorus limitation promotes the synthesis of toxins. Specifically, phosphorus limitation can significantly increase total toxin content while altering toxin composition (Boyer et al., 1987).

This competition between bacteria and dinoflagellates for nutrients creates an additional layer of stress, leading to the downregulation of nitrogen and phosphorus metabolism in dinoflagellates. As a result, dinoflagellates may increase toxin production under nutrient-limiting conditions, particularly phosphorus limitation, which has been shown to significantly increase total toxin content and alter toxin composition (Boyer et al., 1987). The stress caused by nutrient competition and bacterial QS signaling highlights the complex microbial-dinoflagellate interactions that modulate the physiological responses of *A. pacificum* in co-cultured environments, where oxidative and nutrient stresses contribute to increased toxin synthesis.

This integration of microbial QS systems and nutrient competition emphasizes the multifaceted relationship between bacteria and dinoflagellates, with bacteria indirectly promoting toxin production in dinoflagellates through both oxidative and nutrient-related stress responses.

4.3. The role of environmental stress and bacterial interaction in enhancing PSTs synthesis in *Alexandrium pacificum*

4.3.1. Co-expression of high-toxicity PSTs synthesis genes and quinone metabolism

Transcriptomic analysis revealed a co-expression of high-toxicity paralytic shellfish toxins (PSTs) synthesis genes and quinone metabolism in *A. pacificum*. Higher quinone efficiency is typically associated with redox reactions and responses to oxidative stress. Environmental stress may lead to the production of more ROS in dinoflagellate cells, thereby triggering an oxidative stress response (Fig. 4E). In this context, the upregulation of quinone metabolism may serve as a mechanism for dinoflagellates to cope with oxidative stress. By increasing quinone metabolism, dinoflagellates can regulate the redox balance within the cell, reducing the damage caused by environmental stress. At the same time, the upregulation of quinone metabolism may also be related to increased toxin synthesis, as toxin production often involves the transfer of metabolic energy and the activation of cellular defense responses. Low-toxicity PSTs are sulfated toxins (sulfotransferase induced) (Fig. 6), the synthesis of which typically requires a large amount of ATP as an energy source. Therefore, in resource-limited conditions, dinoflagellates may reduce the production of these energy-demanding low-toxicity sulfated toxins, which can be redirected toward the synthesis of high-toxicity toxins. The relationship between quinone metabolism and toxin production is supported by the observation that redox quinone-related proteins, such as NAD(P)H, show increased expression during periods of high toxin synthesis (Wang et al., 2013).

The oxidative stress response, which mitigates the damaging effects of ROS, also promotes redox and toxin synthesis. Under oxidative stress conditions, genes involved in ROS detoxification and antioxidant enzyme expression are upregulated, facilitating both redox and the redirection of metabolic resources towards toxin production. This dual role of the oxidative stress response underscores its importance in the adaptive strategies of dinoflagellates (Ganini et al., 2013).

4.3.2. Co-expression of low-toxicity PSTs synthesis genes and nitrogen and phosphorus competition

Increased environmental stress from nitrogen and phosphorus competition is associated with the downregulation of low-toxicity sulfated toxin synthesis genes. This adaptive response helps dinoflagellates manage nutrient competition stress, influencing the overall toxin profile. For instance, under phosphorus-limited conditions, there is an enhancement of high-toxicity PSTs production, while nitrogen limitation leads to the suppression of overall toxin synthesis (Jiang et al., 2015).

The presence of Alex-CFPB can exacerbate these nutrient stresses, leading to a notable downregulation of genes encoding sulfotransferase enzymes responsible for the biosynthesis of low-toxicity sulfated PSTs. Sulfotransferases (ST) are crucial for transferring sulfate groups to various toxins, converting high-toxicity precursors into less toxic sulfated forms. Studies have shown that specific sulfotransferases are involved in this process, such as the enzyme transferring sulfate to gonyautoxin 2/3 to produce C1/C2 (Wang et al., 2007).

When nutrients are limited, particularly phosphorus, the downregulation of sulfotransferase genes such as *sxtN* and *sxtSUL* occurs, resulting in a decrease of low-toxicity sulfated PSTs and a shift towards more toxic forms. This shift is driven by the increased expression of high-toxicity toxin synthesis genes like *sxtA* and *sxtG* under nutrient stress, favoring the production of more toxic compounds (Perini et al., 2014) (Fig. 4E). Further studies may elucidate the overall regulatory and signaling mechanism influenced by the bacterium to facilitate a complete makeover of the toxin profile towards a more toxic dinoflagellate bloom through the formation of cysts.

5. Conclusion

Our study demonstrates the significant impact of *J. cystaogens* on the life cycle and toxin production of *A. pacificum*. Co-cultivation with *J. cystaogens* not only promotes the transition from asexual reproduction to sexual reproduction and cyst formation but also enhances the synthesis of high-toxicity PSTs. This bacterium-induced stress alters the growth dynamics and toxin profiles of *A. pacificum*, leading to a decrease in overall cell density and an increase in intracellular toxin content per cell, particularly the production of highly toxic saxitoxin analogs such as GTX1&4, STX, and neoSTX. The increased cyst formation and enhanced toxin synthesis suggest that cysts serve a dual role: as a survival strategy under stress and as a reservoir for toxic vegetative cells, contributing to the recurrence and persistence of HABs. These findings underscore the multifaceted influence of bacteria like *J. cystaogens* on dinoflagellate ecology, with implications for understanding bloom dynamics and managing marine ecosystems impacted by toxic blooms.

Ethics approval

The research does not report on or involve the use of any animal or human data or tissue.

CRediT authorship contribution statement

Yue Jiang: Writing – original draft, Conceptualization. **Rishiram Ramanan:** Writing – review & editing. **Sungae Yoon:** Writing – review & editing. **Bo-Mi Lee:** Writing – review & editing. **Yoon-Ho Kang:** Writing – review & editing. **Zhun Li:** Writing – original draft, Writing –

review & editing, Supervision, Funding acquisition.

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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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at [doi:10.1016/j.watres.2024.122930](https://doi.org/10.1016/j.watres.2024.122930).

Data availability

Data will be made available on request.

References

- Adachi, M., Kanno, T., Matsubara, T., Nishijima, T., Itakura, S., Yamaguchi, M., 1999. Promotion of cyst formation in the toxic dinoflagellate *Alexandrium* (Dinophyceae) by natural bacterial assemblages from Hiroshima Bay, Japan. *Mar. Ecol. Prog. Ser.* 191, 175–185. <https://doi.org/10.3354/meps191175>.
- Adachi, M., Kanno, T., Okamoto, R., Itakura, S., Yamaguchi, M., Nishijima, T., 2003. Population structure of *Alexandrium* (Dinophyceae) cyst formation-promoting bacteria in Hiroshima Bay, Japan. *Appl. Environ. Microbiol.* 69 (11), 6560–6568. <https://doi.org/10.1128/AEM.69.11.6560-6568.2003>.
- Adachi, M., Kanno, T., Okamoto, R., Shinzaki, A., Fujikawa-Adachi, K., Nishijima, T., 2004. *Jannaschia cystaugens* sp. nov., an *Alexandrium* (Dinophyceae) cyst formation-promoting bacterium from Hiroshima Bay, Japan. *Int. J. Syst. Evol. Microbiol.* 54 (5), 1687–1692. <https://doi.org/10.1099/ijs.0.03029-0>.
- Amin, S.A., Hmelo, L., Van Tol, H., Durham, B., Carlson, L., Heal, K., Morales, R., Berthiaume, C., Parker, M., Djunaedi, B., 2015. Interaction and signalling between a cosmopolitan phytoplankton and associated bacteria. *Nature* 522, 98–101. <https://doi.org/10.1038/nature14488>.
- Anderson, D.M., 1997. Bloom dynamics of toxic *Alexandrium* species in the northeastern US. *Limnol. Oceanogr.* 42 (5part2), 1009–1022. <https://doi.org/10.1016/j.dsr.2.2013.05.034>.
- Anderson, D.M., Alpermann, T.J., Cembella, A.D., Collos, Y., Masseret, E., Montresor, M., 2012a. The globally distributed genus *Alexandrium*: Multifaceted roles in marine ecosystems and impacts on human health. *Harmful. Algae* 14, 10–35. <https://doi.org/10.1016/j.hal.2011.10.012>.
- Anderson, D.M., Burkholder, J.M., Cochlan, W.P., Glibert, P.M., Gobler, C.J., Heil, C.A., Kudela, R.M., Parsons, M.L., Rensel, J.J., Townsend, D.W., 2008. Harmful algal blooms and eutrophication: examining linkages from selected coastal regions of the United States. *Harmful. Algae* 8 (1), 39–53. <https://doi.org/10.1016/j.hal.2008.08.017>.
- Anderson, D.M., Cembella, A.D., Hallegraeff, G.M., 2012b. Progress in understanding harmful algal blooms: paradigm shifts and new technologies for research, monitoring, and management. *Annu. Rev. Mar. Sci.* 4 (1), 143–176. <https://doi.org/10.1146/annurev-marine-120308-081121>.
- Anderson, D.M., Couture, D.A., Kleindinst, J.L., Keafer, B.A., McGillicuddy Jr, D.J., Martin, J.L., Richlen, M.L., Hickey, J.M., Solow, A.R., 2014. Understanding interannual, decadal level variability in paralytic shellfish poisoning toxicity in the Gulf of Maine: The HAB Index. *Deep Sea Res. Part II Top. Stud. Oceanogr.* 103, 264–276. <https://doi.org/10.1111/j.0022-3646.1984.00418.x>.
- Anderson, D.M., Kulis, D.M., Binder, B.J., 1984. Sexuality and cyst formation in the dinoflagellate *Gonyaulax tamarensis*: cyst yield in batch cultures I. *J. Phycol.* 20 (3), 418–425. <https://doi.org/10.1111/j.0022-3646.1984.00418.x>.
- Berger, M., Neumann, A., Schulz, S., Simon, M., Brinkhoff, T., 2011. Tropodithetic acid production in *Phaeobacter gallaeciensis* is regulated by N-acyl homoserine lactone-mediated quorum sensing. *J. Bacteriol.* 193 (23), 6576–6585. <https://doi.org/10.1128/JB.05818-11>.
- Bolger, A.M., Lohse, M., Usadel, B., 2014. Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics* 30 (15), 2114–2120. <https://doi.org/10.1093/bioinformatics/btu170>.
- Boyer, G., Sullivan, J., Andersen, R., Harrison, P., Taylor, F., 1987. Effects of nutrient limitation on toxin production and composition in the marine dinoflagellate *Protogonyaulax tamarensis*. *Mar. Biol.* 96, 123–128. <https://doi.org/10.1007/BF00394845>.
- Bui, Q.T.N., Kim, H., Wang, H., Ki, J.-S., 2022. Unveiling the genomic structures and evolutionary events of the saxitoxin biosynthetic gene *sxtA* in the marine toxic dinoflagellate *Alexandrium*. *Mol. Phylogenet. Evol.* 168, 107417. <https://doi.org/10.1016/j.ympev.2022.107417>.
- Chang, D., 1987. A study on paralytic shellfish poison of sea mussel, *Mytilus edulis*, specimen caused food poisoning accident in Gamchun Bay, Pusan, Korea. *Bull. Korean Fish. Soc.* 20, 293–299. <https://doi.org/10.1248/jhs1956.30.19>.
- Chen, T., Chen, X., Sun, H., Zhang, H., Bai, J., 2024. Unveiling the responses of *Alexandrium pacificum* to phosphorus utilization by physiological and transcriptomic analysis. *Sci. Total Environ.* 911, 168759. <https://doi.org/10.1016/j.scitotenv.2023.168759>.
- Chen, X., Wang, D., Wang, Y., Sun, P., Ma, S., Chen, T., 2022. Algicidal effects of a high-efficiency algicidal bacterium *Shewanella* Y1 on the toxic bloom-causing dinoflagellate *Alexandrium pacificum*. *Mar. Drugs* 20 (4), 239. <https://doi.org/10.3390/md20040239>.
- Chen, Y., Chen, Y., Shi, C., Huang, Z., Zhang, Y., Li, S., Li, Y., Ye, J., Yu, C., Li, Z., 2018. SOAPnuke: a MapReduce acceleration-supported software for integrated quality control and preprocessing of high-throughput sequencing data. *Gigascience* 7 (1), gix120. <https://doi.org/10.1093/gigascience/gix120>.
- Cho, D.H., Ramanan, R., Heo, J., Lee, J., Kim, B.H., Oh, H.M., Kim, H.S., 2015. Enhancing microalgal biomass productivity by engineering a microalgal–bacterial community. *Bioresour. Technol.* 175, 578–585. <https://doi.org/10.1016/j.biortech.2014.10.159>.
- Cullen, A., D'Agostino, P.M., Mazmouz, R., Pickford, R., Wood, S., Neilan, B.A., 2018. Insertions within the saxitoxin biosynthetic gene cluster result in differential toxin profiles. *ACS Chem. Biol.* 13 (11), 3107–3114. <https://doi.org/10.1021/acschembio.8b00608>.
- Deng, Y., Wang, K., Hu, Z., Hu, Q., Tang, Y.Z., 2023. Toxic and non-toxic dinoflagellates host distinct bacterial communities in their phycospheres. *Commun. Earth Environ.* 4 (1), 263. <https://doi.org/10.1038/s43247-023-00925-z>.
- Figuerola, R.I., Garces, E., Bravo, I., 2007. Comparative study of the life cycles of *Alexandrium tamutum* and *Alexandrium minutum* (gonyaulacales, dinophyceae) in culture. *J. Phycol.* 43 (5), 1039–1053. <https://doi.org/10.1111/j.1529-8817.2007.00393.x>.
- Filloux, A., 2024. Bacterial type VI secretion system helps prevent cheating in microbial communities. *ISME J.* 18 (1). <https://doi.org/10.1093/ismej/wrae003>.
- Frangópoulos, M., Guisande, C., DeBlas, E., Maneiro, I., 2004. Toxin production and competitive abilities under phosphorus limitation of *Alexandrium* species. *Harmful. Algae* 3 (2), 131–139. [https://doi.org/10.1016/S1568-9883\(03\)00061-1](https://doi.org/10.1016/S1568-9883(03)00061-1).
- Gallacher, S., Flynn, K.J., Franco, J.M., Brueggemann, E., Hines, H., 1997. Evidence for production of paralytic shellfish toxins by bacteria associated with *Alexandrium* spp. (Dinophyta) in culture. *Appl. Environ. Microbiol.* 63 (1), 239–245. <https://doi.org/10.1128/aem.63.1.239-245.1997>.
- Galperin, M.Y., Wolf, Y.I., Garushyants, S.K., Vera Alvarez, R., Koonin, E.V., 2021. Nonessential ribosomal proteins in bacteria and archaea identified using clusters of orthologous genes. *J. Bacteriol.* 203 (11). <https://doi.org/10.1128/JB.00058-21>.
- Ganini, D., Hollnagel, H.C., Colepiccolo, P., Barros, M.P.d., 2013. Hydrogen peroxide and nitric oxide trigger redox-related cyst formation in cultures of the dinoflagellate *Lingulodinium polyedrum*. *Harmful. Algae* 27, 121–129. <https://doi.org/10.1016/j.hal.2013.05.002>.
- Gobler, C.J., Doherty, O.M., Hattenrath-Lehmann, T.K., Griffith, A.W., Kang, Y., Litaker, R.W., 2017. Ocean warming since 1982 has expanded the niche of toxic algal blooms in the North Atlantic and North Pacific oceans. *Proc. Natl. Acad. Sci. U. S. A.* 114 (19), 4975–4980. <https://doi.org/10.1073/pnas.1619575114>.
- Grabherr, M.G., Haas, B.J., Yassour, M., Levin, J.Z., Thompson, D.A., Amit, I., Adiconis, X., Fan, L., Raychowdhury, R., Zeng, Q., Chen, Z., Mauceli, E., Hacohen, N., Gnirke, A., Rhind, N., di Palma, F., Birren, B.W., Nusbaum, C., Lindblad-Toh, K., Friedman, N., Regev, A., 2011. Full-length transcriptome assembly from RNA-Seq data without a reference genome. *Nat. Biotechnol.* 29 (7), 644–652. <https://doi.org/10.1038/nbt.1883>.
- Graham, E., Heidelberg, J., Tully, B., 2018. Potential for primary productivity in a globally-distributed bacterial phototroph. *ISME J.* 12 (7), 1861–1866. <https://doi.org/10.1038/s41396-018-0091-3>.
- Hackett, J.D., Wisecaver, J.H., Brosnahan, M.L., Kulis, D.M., Anderson, D.M., Bhattacharya, D., Plumley, F.G., Erdner, D.L., 2013. Evolution of saxitoxin synthesis in cyanobacteria and dinoflagellates. *Mol. Biol. Evol.* 30 (1), 70–78. <https://doi.org/10.1093/molbev/mss142>.
- Hadjadj, I., Laabir, M., Frihi, H., Collos, Y., Shao, Z.J., Berrebi, P., Abadie, E., Amzil, Z., Chomérat, N., Rolland, J.L., Rieuvilleneuve, F., Masseret, E., 2020. Unsuspected intraspecific variability in the toxin production, growth and morphology of the dinoflagellate *Alexandrium pacificum* R.W. Litaker (Group IV) blooming in a South Western Mediterranean marine ecosystem, Annaba Bay (Algeria). *Toxicon* 180, 79–88. <https://doi.org/10.1016/j.toxicon.2020.04.005>.
- Hallegraeff, G.M., Schweibold, L., Jaffrezic, E., Rhodes, L., MacKenzie, L., Hay, B., Farrell, H., 2021. Overview of Australian and New Zealand harmful algal species occurrences and their societal impacts in the period 1985 to 2018, including a compilation of historic records. *Harmful. Algae* 102, 101848. <https://doi.org/10.1016/j.hal.2020.101848>.
- Hamasaki, K., Horie, M., Tokimitsu, S., Toda, T., Taguchi, S., 2001. Variability in toxicity of the dinoflagellate *Alexandrium tamarensis* isolated from Hiroshima Bay, western Japan, as a reflection of changing environmental conditions. *J. Plankton Res.* 23 (3), 271–278. <https://doi.org/10.1093/plankt/23.3.271>.

- Hattenrath-Lehmann, T.K., Gobler, C.J., 2017. Identification of unique microbiomes associated with harmful algal blooms caused by *Alexandrium fundyense* and *Dinophysis acuminata*. *Harmful. Algae* 68, 17–30. <https://doi.org/10.1016/j.hal.2017.07.003>.
- Heisler, J., Glibert, P.M., Burkholder, J.M., Anderson, D.M., Cochlan, W., Dennison, W. C., Dortch, Q., Gobler, C.J., Heil, C.A., Humphries, E., 2008. Eutrophication and harmful algal blooms: a scientific consensus. *Harmful. Algae* 8 (1), 3–13. <https://doi.org/10.1016/j.hal.2008.08.006>.
- Horwitz, W., Latimer, G., 2005. AOAC International: Gaithersburg. MD, USA 18. 1 093/jaoac/81.1.240.
- Hwang, D.F., Lu, Y.H., 2000. Influence of environmental and nutritional factors on growth, toxicity, and toxin profile of dinoflagellate *Alexandrium minutum*. *Toxicon* 38 (11), 1491–1503. [https://doi.org/10.1016/S0041-0101\(00\)00080-5](https://doi.org/10.1016/S0041-0101(00)00080-5).
- Ichimi, K., Yamasaki, M., Okumura, Y., Suzuki, T., 2001. The growth and cyst formation of a toxic dinoflagellate, *Alexandrium tamarense*, at low water temperatures in northeastern Japan. *J. Exp. Mar. Biol. Ecol* 261 (1), 17–29. [https://doi.org/10.1016/S0022-0981\(01\)00256-8](https://doi.org/10.1016/S0022-0981(01)00256-8).
- Jauzein, C., Evans, A.N., Erdner, D.L., 2015. The impact of associated bacteria on morphology and physiology of the dinoflagellate *Alexandrium tamarense*. *Harmful. Algae* 50, 65–75. <https://doi.org/10.1016/j.hal.2015.10.006>.
- Jia, Y., Gao, H., Tong, M., Anderson, D.M., 2019. Cell cycle regulation of the mixotrophic dinoflagellate *Dinophysis acuminata*: Growth, photosynthetic efficiency and toxin production. *Harmful. Algae* 89, 101672. <https://doi.org/10.1016/j.hal.2019.101672>.
- Jiang, X.-W., Wang, J., Gao, Y., Chan, L.L., Lam, P.K.S., Gu, J.-D., 2015. Relationship of proteomic variation and toxin synthesis in the dinoflagellate *Alexandrium tamarense* C101 under phosphorus and inorganic nitrogen limitation. *Ecotoxicology* 24, 1744–1753. <https://doi.org/10.1007/s10646-015-1513-x>.
- Jiang, Y., Shin, H.H., Park, B.S., Li, Z., 2024. Potential siderophore-dependent mutualism in the harmful dinoflagellate *Alexandrium pacificum* (Group IV) and bacterium *Photobacterium* sp. TY1-4 under iron-limited conditions. *Harmful. Algae* 102726. <https://doi.org/10.1016/j.hal.2024.102726>.
- John, U., Litaker, R.W., Montresor, M., Murray, S., Brosnahan, M.L., Anderson, D.M., 2014. Formal Revision of the *Alexandrium tamarense* Species Complex (Dinophyceae) Taxonomy: The Introduction of Five Species with Emphasis on Molecular-based (rDNA) Classification. *Protist* 165 (6), 779–804. <https://doi.org/10.1016/j.protis.2014.10.001>.
- Katoh, K., Standley, D.M., 2013. MAFFT multiple sequence alignment software version 7: improvements in performance and usability. *Mol. Biol. Evol* 30 (4), 772–780. <https://doi.org/10.1093/molbev/mst010>.
- Kellmann, R., Mihali, T.K., Jeon, Y.J., Pickford, R., Pomati, F., Neilan, B.A., 2008. Biosynthetic intermediate analysis and functional homology reveal a saxitoxin gene cluster in cyanobacteria. *Appl. Environ. Microbiol* 74 (13), 4044–4053. <https://doi.org/10.1128/AEM.00353-08>.
- Kim, Y.-O., Park, M.-H., Han, M.-S., 2002. Role of cyst germination in the bloom initiation of *Alexandrium tamarense* (Dinophyceae) in Masan Bay, Korea. *Aquat. Microb. Ecol* 29 (3), 279–286. <https://doi.org/10.3354/ame029279>.
- Kim, Y.O., Choi, J., Baek, S.H., Lee, M., Oh, H.-M., 2020. Tracking *Alexandrium catenella* from seed-bed to bloom on the southern coast of Korea. *Harmful. Algae* 99, 101922. <https://doi.org/10.1016/j.hal.2020.101922>.
- Kodama, M., 2010. Paralytic Shellfish Poisoning Toxins: Biochemistry and Origin. Terrapub, Orono, ME, USA. <https://doi.org/10.5047/abms.2010.00301.0001>.
- Lee, H.-G., Kim, H.M., Min, J., Park, C., Jeong, H.J., Lee, K., Kim, K.Y., 2020. Quantification of the paralytic shellfish poisoning dinoflagellate *Alexandrium* species using a digital PCR. *Harmful. Algae* 92, 101726. <https://doi.org/10.1016/j.hal.2019.101726>.
- Leticia, A., Bork, P., 2016. Interactive tree of life (iTOL) v3: an online tool for the display and annotation of phylogenetic and other trees. *Nucleic. Acids. Res.* 44 (W1), W242–W245. <https://doi.org/10.1093/nar/gkw290>.
- Lewitus, A.J., Horner, R.A., Caron, D.A., Garcia-Mendoza, E., Hickey, B.M., Hunter, M., Huppert, D.D., Kudela, R.M., Langlois, G.W., Largier, J.L., 2012. Harmful algal blooms along the North American west coast region: History, trends, causes, and impacts. *Harmful. Algae* 19, 133–159. <https://doi.org/10.1016/j.hal.2012.06.009>.
- Li, B., Dewey, C.N., 2011. RSEM: accurate transcript quantification from RNA-Seq data with or without a reference genome. *BMC Bioinform.* 12, 1–16. <https://doi.org/10.1201/b16589-9>.
- Li, C., Li, Y., GUO, J., WANG, Y., SHI, X., ZHANG, Y., LIANG, N., YUAN, J., XU, J., Chen, H., 2023. The highly abundant mRNA m1A modification: a new layer of gene regulation in dinoflagellates. *bioRxiv*, 2023.2011.2004.565600. <https://doi.org/10.1007/s12374-02-009239-5>.
- Li, Y., Zhu, H., Lei, X., Zhang, H., Cai, G., Chen, Z., Fu, L., Xu, H., Zheng, T., 2015. The death mechanism of the harmful algal bloom species *Alexandrium tamarense* induced by algicidal bacterium *Deinococcus* sp. Y35. *Front. Microbiol.* 6, 992. <https://doi.org/10.3389/fmicb.2015.00992>.
- Miyazono, A., Nagai, S., Kudo, I., Tanizawa, K., 2012. Viability of *Alexandrium tamarense* cysts in the sediment of Funka Bay, Hokkaido, Japan: Over a hundred year survival times for cysts. *Harmful. Algae* 16, 81–88. <https://doi.org/10.1016/j.hal.2012.02.001>.
- Murray, S.A., Wiese, M., Stüken, A., Brett, S., Kellmann, R., Hallegraeff, G., Neilan, B.A., 2011. *sxtA*-based quantitative molecular assay to identify saxitoxin-producing harmful algal blooms in marine waters. *Appl. Environ. Microbiol.* 77 (19), 7050–7057. <https://doi.org/10.1128/AEM.05308-11>.
- Oshima, Y., Bolch, C.J., Hallegraeff, G.M., 1992. Toxin composition of resting cysts of *Alexandrium tamarense* (Dinophyceae). *Toxicon* 30 (12), 1539–1544. [https://doi.org/10.1016/0041-0101\(92\)90025-Z](https://doi.org/10.1016/0041-0101(92)90025-Z).
- Overbeek, R., Olson, R., Pusch, G.D., Olsen, G.J., Davis, J.J., Disz, T., Edwards, R.A., Gerdes, S., Parrello, B., Shukla, M., 2014. The SEED and the Rapid Annotation of microbial genomes using Subsystems Technology (RAST). *Nucleic. Acids. Res.* 42 (D1), D206–D214. <https://doi.org/10.1093/nar/gkt1226>.
- Page, A.J., Cummins, C.A., Hunt, M., Wong, V.K., Reuter, S., Holden, M.T., Fookes, M., Falush, D., Keane, J.A., Parkhill, J., 2015. Roary: rapid large-scale prokaryote pan genome analysis. *Bioinformatics* 31 (22), 3691–3693. <https://doi.org/10.1093/bioinformatics/btv421>.
- Perini, F., Galluzzi, L., Dell'Aversano, C., Dello Iacovo, E., Tartaglione, L., Ricci, F., Forino, M., Ciminiello, P., Penna, A., 2014. *SxtA* and *sxtG* gene expression and toxin production in the Mediterranean *Alexandrium minutum* (Dinophyceae). *Mar. Drugs* 12 (10), 5258–5276. <https://doi.org/10.3390/md12105258>.
- Ramanan, R., Kim, B.-H., Cho, D.-H., Oh, H.-M., Kim, H.-S., 2016. Algae–bacteria interactions: evolution, ecology and emerging applications. *Biotechnol. Adv.* 34 (1), 14–29. <https://doi.org/10.1016/j.biotechadv.2015.12.003>.
- Ribeiro, S., Berge, T., Lundholm, N., Andersen, T.J., Abrantes, F., Ellegaard, M., 2011. Phytoplankton growth after a century of dormancy illuminates past resilience to catastrophic darkness. *Nat. Commun.* 2 (1), 311. <https://doi.org/10.1038/ncomms1314>.
- Ronquist, F., Huelsenbeck, J.P., 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* 19 (12), 1572–1574. <https://doi.org/10.1093/bioinformatics/btg180>.
- Ronquist, F., Teslenko, M., Van Der Mark, P., Ayres, D.L., Darling, A., Höhna, S., Larget, B., Liu, L., Suchard, M.A., Huelsenbeck, J.P., 2012. MrBayes 3.2: efficient Bayesian phylogenetic inference and model choice across a large model space. *Syst. Biol.* 61 (3), 539–542. <https://doi.org/10.1093/sysbio/sys029>.
- Rourke, W.A., Murphy, C.J., Pitcher, G., van de Riet, J.M., Burns, B.G., Thomas, K.M., Quilliam, M.A., 2008. Rapid postcolumn methodology for determination of paralytic shellfish toxins in shellfish tissue. *J. AOAC Int.* 91 (3), 589–597. <https://doi.org/10.1093/jaoac/91.3.589>.
- Roy, S., Letourneau, L., Morse, D., 2014. Cold-induced cysts of the photosynthetic dinoflagellate *Lingulodinium polyedrum* have an arrested circadian bioluminescence rhythm and lower levels of protein phosphorylation. *Plant Physiol.* 164 (2), 966–977. <https://doi.org/10.1104/pp.113.2.9856>.
- Sako, Y., Yoshida, T., Uchida, A., Arakawa, O., Noguchi, T., Ishida, Y., 2001. Purification and characterization of a sulfotransferase specific to N-21 of saxitoxin and gonyautoxin 2+ 3 from the toxic dinoflagellate *Gymnodinium catenatum* (Dinophyceae). *J. Phycol.* 37 (6), 1044–1051. <https://doi.org/10.1046/j.1529-8817.2001.00119.x>.
- Seemann, T., 2014. Prokka: rapid prokaryotic genome annotation. *Bioinformatics* 30 (14), 2068–2069. <https://doi.org/10.1093/bioinformatics/btu153>.
- Shin, H.H., Li, Z., Kim, E.S., Park, J.-W., Lim, W.A., 2017. Which species, *Alexandrium catenella* (Group I) or *A. pacificum* (Group IV), is really responsible for past paralytic shellfish poisoning outbreaks in Jinhae-Masan Bay, Korea? *Harmful. Algae* 68, 31–39. <https://doi.org/10.1016/j.hal.2017.07.006>.
- Shin, H.H., Li, Z., Kim, H.J., Park, B.S., Lee, J., Shin, A.-Y., Park, T.-G., Lee, K.-W., Han, K. H., Youn, J.Y., 2021. *Alexandrium catenella* (Group I) and *A. pacificum* (Group IV) cyst germination, distribution, and toxicity in Jinhae-Masan Bay, Korea. *Harmful. Algae* 110, 102122. <https://doi.org/10.1016/j.hal.2021.102122>.
- Shin, H.H., Li, Z., Lim, D., Lee, K.-W., Seo, M.H., Lim, W.A., 2018. Seasonal production of dinoflagellate cysts in relation to environmental characteristics in Jinhae-Masan Bay, Korea: One-year sediment trap observation. *Estuarine, Coast. Shelf Sci.* 215, 83–93. <https://doi.org/10.1016/j.ecss.2018.09.031>.
- Shin, H.H., Li, Z., Réveillon, D., Rovillon, G.-A., Mertens, K.N., Hess, P., Kim, H.J., Lee, J., Lee, K.-W., Kim, D., 2020. *Centrodinium punctatum* (Dinophyceae) produces significant levels of saxitoxin and related analogs. *Harmful. Algae* 100, 101923. <https://doi.org/10.1016/j.hal.2020.101923>.
- Shishlyannikov, S.M., Zakharova, Y.R., Volokitina, N.A., Mikhailov, I.S., Petrova, D.P., Likhoshway, Y.V., 2011. A procedure for establishing an axenic culture of the diatom *Synedra acus* subsp. *radians* (Kütz.) Skabbitsch. from Lake Baikal. *Limnol. Oceanogr.* Methods 9(10), 478–484. <https://doi.org/10.4319/lom.2011.9.478>.
- Smith, J.L., Tong, M., Fux, E., Anderson, D.M., 2012. Toxin production, retention, and extracellular release by *Dinophysis acuminata* during extended stationary phase and culture decline. *Harmful. Algae* 19, 125–132. <https://doi.org/10.1016/j.hal.2012.06.008>.
- Song, W., Song, X., Cheng, R., Chi, L., Zhu, J., Yu, Z., 2023. Uncovering the regulation effect of modified clay on toxin production in *Alexandrium pacificum*: From physiological insights. *J. Hazard. Mater.* 454, 131516. <https://doi.org/10.1016/j.jhazmat.2023.131516>.
- Soto-Liebe, K., Murillo, A.A., Krock, B., Stucken, K., Fuentes-Valdés, J.J., Trefault, N., Cembella, A., Vásquez, M., 2010. Reassessment of the toxin profile of *Cylindrodorsomopsis raciborskii* T3 and function of putative sulfotransferases in synthesis of sulfated and sulfonated PSP toxins. *Toxicon* 56 (8), 1350–1361. <https://doi.org/10.1016/j.toxicon.2010.07.022>.
- Stüken, A., Orr, R.J., Kellmann, R., Murray, S.A., Neilan, B.A., Jakobsen, K.S., 2011. Discovery of nuclear-encoded genes for the neurotoxin saxitoxin in dinoflagellates. *PLoS. One* 6 (5), e20096. <https://doi.org/10.1371/journal.pone.0020096>.
- Tanabe, Y., Yamaguchi, H., Yoshida, M., Kai, A., Okazaki, Y., 2023. Characterization of a bloom-associated alphaproteobacterial lineage, 'Candidatus Phycosocius': insights into freshwater algal-bacterial interactions. *ISMe Commun.* 3 (1), 20. <https://doi.org/10.1038/s43705-023-00228-6>.
- Trapnell, C., Roberts, A., Goff, L., Pertea, G., Kim, D., Kelley, D.R., Pimentel, H., Salzberg, S.L., Rinn, J.L., Pachter, L., 2012. Differential gene and transcript expression analysis of RNA-seq experiments with TopHat and Cufflinks. *Nat. Protoc.* 7 (3), 562–578. <https://doi.org/10.1038/nprot.2012.016>.

- Uribe, P., Espejo, R.T., 2003. Effect of associated bacteria on the growth and toxicity of *Alexandrium catenella*. *Appl. Environ. Microbiol.* 69 (1), 659–662. <https://doi.org/10.1128/AEM.69.1.659-662.2003>.
- Van De Riet, J., Gibbs, R.S., Muggah, P.M., Rourke, W.A., MacNeil, J.D., Quilliam, M.A., 2011. Liquid chromatography post-column oxidation (PCOX) method for the determination of paralytic shellfish toxins in mussels, clams, oysters, and scallops: Collaborative study. *J. AOAC Int.* 94 (4), 1154–1176. <https://doi.org/10.1093/jaoac/94.4.1154>.
- Van Houdt, R., Moons, P., Aertsen, A., Jansen, A., Vanoirbeek, K., Daykin, M., Williams, P., Michiels, C.W., 2007. Characterization of a *luxI/luxR*-type quorum sensing system and N-acyl-homoserine lactone-dependent regulation of exo-enzyme and antibacterial component production in *Serratia plymuthica* RVH1. *Res. Microbiol.* 158 (2), 150–158. <https://doi.org/10.1016/j.resmic.2006.11.012>.
- Vandersea, M.W., Kibler, S.R., Tester, P.A., Holderied, K., Hondolero, D.E., Powell, K., Baird, S., Doroff, A., Dugan, D., Litaker, R.W., 2018. Environmental factors influencing the distribution and abundance of *Alexandrium catenella* in Kachemak bay and lower cook inlet, Alaska. *Harmful. Algae* 77, 81–92. <https://doi.org/10.1016/j.hal.2018.06.008>.
- Wang, D.-Z., Gao, Y., Lin, L., Hong, H.-S., 2013. Comparative proteomic analysis reveals proteins putatively involved in toxin biosynthesis in the marine dinoflagellate *Alexandrium catenella*. *Mar. Drugs* 11 (1), 213–232. <https://doi.org/10.3390/md11010213>.
- Wang, D.-Z., Hsieh, D.P., 2005. Growth and toxin production in batch cultures of a marine dinoflagellate *Alexandrium tamarense* HK9301 isolated from the South China Sea. *Harmful. Algae* 4 (2), 401–410. <https://doi.org/10.1016/j.hal.2004.07.002>.
- Wang, D., Zhang, S., Hong, H., 2007. A sulfotransferase specific to N-21 of gonyautoxin 2/3 from crude enzyme extraction of toxic dinoflagellate *Alexandrium tamarense* Cl01. *Chin. J. Oceanol. Limnol.* 25 (2), 227–234. <https://doi.org/10.1007/s00343-007-0227-1>.
- Wang, H., Kim, H., Ki, J.-S., 2021. Transcriptome survey, molecular identification, and expression analysis of stress-responsive genes in the toxic dinoflagellate *Alexandrium pacificum* under algicidal agents and metal stresses. *J. Appl. Phycol.* 33 (5), 3139–3151. <https://doi.org/10.1007/s10811-021-02509-w>.
- Wang, H., Kim, H., Park, H., Ki, J.-S., 2022a. Temperature influences the content and biosynthesis gene expression of saxitoxins (STXs) in the toxigenic dinoflagellate *Alexandrium pacificum*. *Sci. Total Environ.* 802, 149801. <https://doi.org/10.1016/j.scitotenv.2021.149801>.
- Wang, H., Wu, P., Zheng, D., Deng, L., Wang, W., 2022b. N-Acyl-homoserine lactone (AHL)-mediated microalgal–bacterial communication driving *Chlorella*-activated sludge bacterial biofloc formation. *Environ. Sci. Technol.* 56 (17), 12645–12655. <https://doi.org/10.1021/acs.est.2c00905>.
- Yu, G., Wang, L.G., Han, Y., He, Q.Y., 2012. clusterProfiler: an R package for comparing biological themes among gene clusters. *OMICS*. 16 (5), 284–287. <https://doi.org/10.1089/omi.2011.0118>.
- Zhang, D., Gao, F., Jakovlić, I., Zou, H., Zhang, J., Li, W.X., Wang, G.T., 2020. PhyloSuite: An integrated and scalable desktop platform for streamlined molecular sequence data management and evolutionary phylogenetics studies. *Mol. Ecol. Resour.* 20 (1), 348–355. <https://doi.org/10.1111/1755-0998.13096>.
- Ziesche, L., Rinkel, J., Dickschat, J.S., Schulz, S., 2018. Acyl-group specificity of AHL synthases involved in quorum-sensing in *Roseobacter* group bacteria. *Beilstein J. Org. Chem.* 14 (1), 1309–1316. <https://doi.org/10.3762/bjoc.14.112>.