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Mass cultivation of the dinoflagellate *Alexandrium pacificum* for gonyautoxin-1,4 production

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Bioactive venoms and toxins are emerging as a promising source of drug leads. Optimized through evolution, these compounds display remarkable selectivity and ligand affinity toward a range of relevant pharmacological targets. The successful development of new drugs from toxins is hampered in some areas by the chemical complexity of the active compounds, which limits the possibility of using chemical synthesis or recombinant strategies for drug lead generation. Marine paralytic shellfish toxins produced by marine microalgae is one such family of compounds. These compounds are highly potent blockers of voltage-gated ion channels, involved in regulating a range of physiological processes and thus versatile targets for drug development. To overcome the supply issue, the current paper describes the development of a scalable production method to generate gram amounts of gonyautoxin-1,4 by mass cultivation of the dinoflagellate *Alexandrium pacificum* in artificial seawater. By selecting a high-producing strain and running a series of growth optimization experiments, we have scaled up production from 100 mL to 1150 L, with cellular yields of toxin 30 times higher than in a natural bloom. This allows commercial production of gram amounts of these promising compounds, thereby enabling their use in a range of applications beyond the analytical scale.

Keywords Gonyautoxin-1,4, *Alexandrium pacificum*, Toxin production, Artificial seawater, Dinoflagellate, Optimization

Natural toxins and venoms play an important and growing part in modern drug discovery and development[1–4]. It has been estimated that between 10 million and 50 million compounds exist in natural venoms and toxins, but of these, only 0.01% have been identified and characterized[5,6]. Most toxins are produced for defense, predation, or competitor deterrence[2,7]. These diverse compounds have been meticulously refined and optimized by evolution to be highly selective and potent ligands for a wide range of ion channels and receptors, of which the voltage-gated ion channels often represent a common molecular target group[1,2,7,8]. The number of approved drugs from toxins is still low, but those that have been developed are significantly aiding in the management of pain, cancer, and blood pressure, and in hemostasis control[7]. The majority of approved toxin and venom drugs originate from terrestrial sources, but there is growing interest in toxins derived from marine organisms[5,7,9]. The peptide toxin ziconotide, derived from a cone snail and used for the management of neuropathic pain, is a good example of a potent drug of marine origin[10]. In addition to ziconotide, several of the cytotoxic warheads used in approved antibody-drug conjugates, such as brentuximab vedotin and belantamab mafodotin, are of marine cyanobacterial origin[9,11,12].

Marine microalgae are also emerging as a promising source of bioactive compounds with interesting pharmacological properties[13,14]. For instance, dinoflagellates ubiquitously found in aquatic ecosystems can synthesize paralytic shellfish toxins (PSTs) and other neurotoxins that display an array of bioactivites[15–17]. Examples of these toxins include dinoflagellate-produced toxins such as the spirolides, saxitoxin and analogs, and portimine (Fig. 1), which display a high specificity and potency at sub- to low nanomolar concentrations, making them interesting as drug candidates for both pain management and cancer treatment[16,18,19]. Several of these, and other related toxins such as tetrodotoxin (TTX), have also been shown to display promising activities in pre-clinical and clinical trials[20]. Of particular interest are studies using both TTX and neosaxitoxin as site-I

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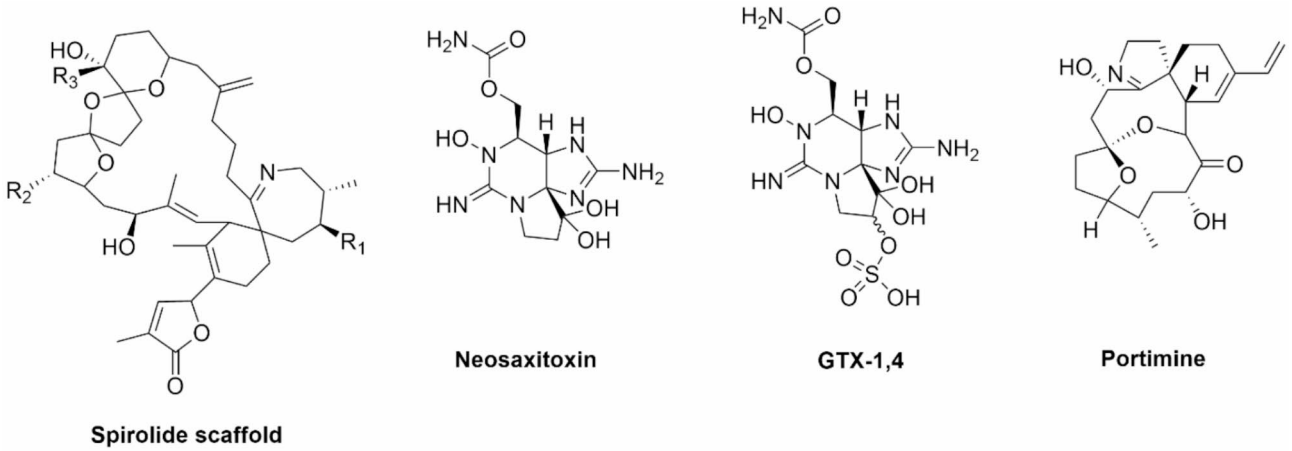

Fig. 1. Chemical structures of diverse bioactive toxins produced by dinoflagellates, illustrating their high complexity.

voltage-gated sodium channel blockers, together with bupivacaine and epinephrine, to obtain significant pain relief lasting up to 72 h in humans[21,22].

Despite their proven potential, purified PSTs are not widely available, and their complex non-peptidic structures often prevent rational chemical or recombinant production, which is a well-established challenge in marine drug development[11]. As a result of the intricate chemical structures, only a few PSTs are commercially produced, at a relatively small scale and high price, hampering further research and development[23]. More importantly, accessing sufficient quantities of compounds for clinical trials is often a long and windy bottleneck to production, even when only milligram to gram amounts of compounds are needed for commercial-scale applications[11]. While recent and impressive synthetic breakthroughs have enabled access to the portimine scaffold[24], saxitoxin and its analogs remain elusive at scale despite decades of work[25,26]. Where chemical, heterologous expression, or recombinant production are impossible or non-scalable, the remaining option is to use direct biosynthesis from the dinoflagellates[11]. To produce bioactive compounds from dinoflagellates in sufficient quantities and establish the production of novel voltage-gated sodium channel inhibitors at scale, mass culturing of these organisms is therefore critical. However, given the fragile and slow-growing nature of dinoflagellates, this task is challenging[27]. In addition, the yield of toxins ideally needs to be increased to allow for a feasible production at scale.

To date, only a handful of studies have reported scaled-up cultivation of dinoflagellates up to 315 L for metabolite production (e.g. lipids[28,29]) or up to 226 L for toxin production (Table 1).

Focusing on the latter, genetic background and biotic (e.g. bacteria[42]) and abiotic factors can influence toxin production in dinoflagellates[43]. Consequently, medium optimization and parameter control are key requirements for promoting growth and toxin production in these organisms[27].

To address the challenges associated with producing algal toxins for clinical and commercial work, we have investigated the feasibility of scaling up a high PST–producing dinoflagellate strain in artificial and controlled conditions. In the current paper, we report on our initial strain selection, followed by the production of > 1000 L of *Alexandrium pacificum* (Figure S1) in optimized artificial seawater. The focus of the study is to maximize the yield of gonyautoxins-1,4 (GTX-1,4, a mix of both gonyautoxin-1 [GTX-1] and gonyautoxin-4 [GTX-4]), as the gonyautoxins (GTXs) have been shown to display a promising analgesic potential with wide applicability and a high degree of safety[16,44]. The gonyautoxins, which have the same mode of action as saxitoxin, have for example been evaluated as intramuscular treatment of anal fissures[44]. The desulphated analog neosaxitoxin is currently being developed as a long-acting local anesthetic for post-operative pain management[18,21] and GTX-2 and GTX-3 has been reported as highly efficient against chronic tension-type headaches[45]. The high potency of the compounds enable efficient treatment with a single injection of low µg amounts and several other medical applications, such as osteoarthritis, muscle spasms and gastrointestinal disorder are under current investigation, with applications for humans and animals[16,46,47].

Results and discussion

Natural toxins and venoms hold great potential for drug discovery and development, but their impact in the clinic is hampered by restricted access to these complex compounds[11,23]. With ready access to PSTs, this class of compounds will be available for several medical applications. The approach we have used to develop a scalable PST production methodology from dinoflagellates is schematically presented in Fig. 2.

Strain selection

As the focus of the current study was to identify a dinoflagellate strain suitable for large-scale production of commercially valuable toxins, it was initiated by screening *Alexandrium* strains for toxin production and their potential for further scale-up and optimization. Eighteen strains belonging to three different *Alexandrium* species from the Cawthron Institute Culture Collection of Microalgae (CICCM)[48] were selected

Species	System	Scale (L)	Cell density (10^3 cells/mL)	Toxin	Cell quota (pg/cell)	Reference
Alexandrium						
Alexandrium tamarense ATCI01	Tank	20	15.5	C2[a]	33	30
Alexandrium minutum AMAD06	E-flasks	2	94	Gonyautoxins	0.32	31
Alexandrium minutum AMAD16	E-flasks	2	99	Gonyautoxins	0.26	31
AMAD06 & AMAD16	Panel PBR[b]	4	33	Gonyautoxins	0.42	31
Alexandrium tamarense CI01	Tank	40	15	C3[c]	38	32
Alexandrium tamarense ATHK	E-flasks	2	17	Saxitoxin	1.26	33
Alexandrium ostenfeldii CCMP1773	Column PBR	100	70	Spirolides	0.7	34
Alexandrium ostenfeldii CAWD135	Tubular PBR	5.4	56	Spirolides	2.3	27
Alexandrium pacificum HYM9704	Chemostat	2	4	Neosaxitoxin	0.92	35
Other genera						
Protoceratium reticulatum CAWD129	Carboys	226	14	Yessotoxins	178–205	36
Azadinium spinosum 3D9	Chemostat	100	300	Azaspiracids	0.04	37
Azadinium spinosum 3D9	Tubular PBRs	100	200	Azaspiracids	0.02–0.1	38
Azadinium spinosum 3D10	Chemostat	100	59–346	Azaspiracids	0.01–0.07	39
Prorocentrum lima	Flat PBR	100	26	Okadaic acid Dinophysistoxins	1–2.25 8–11	40
Amphidoma languida	Flat PBR	180	75	Azaspiracids	0.2–0.3	41

Table 1. Maximum cell densities and toxin cell quota reported for *Alexandrium* cultivated at different scales and for other dinoflagellates at scales > 100 L. [a]N-sulfocarbamoyl gonyautoxin C2, [b]Photobioreactor, [c]N-sulfocarbamoyl gonyautoxin C3.

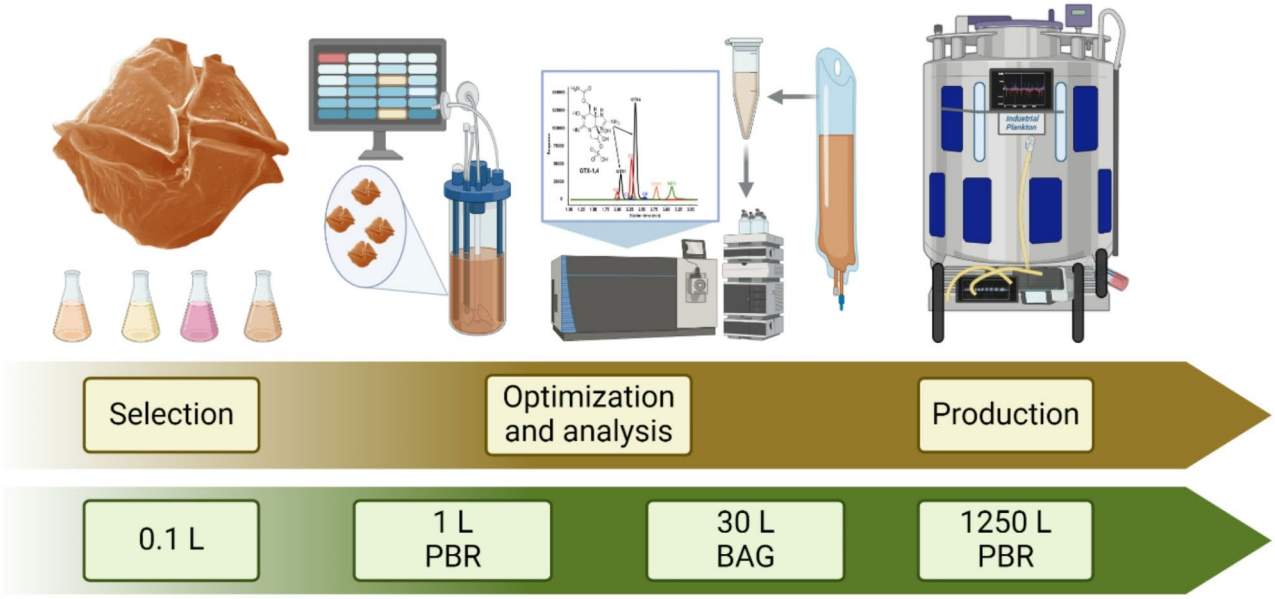

Fig. 2. Schematic presentation of the workflow employed toward a scaled-up production of paralytic shellfish toxins from marine dinoflagellates. Created in BioRender. Ingebrigtsen, R. (2025) https://BioRender.com/o94j330.

for initial strain evaluation (Table S1). Anecdotal observations from our earlier studies suggest that these strains range significantly in their propensity for toxin production when grown under identical conditions (unpublished data), and this guided our initial strain selection.

The strains were cultured in small plastic containers and sampled for their ability to grow and produce toxins over 21 days. Briefly, a ~ 5 mL aliquot was used for both cell density measurements and PST quantification. Small-volume flask culturing was used at this initial screening stage as the goal was only to identify a suitable toxin producer. More comprehensive studies of the 18 strains was beyond the scope of the current study.

Data from growth and toxin production over 21 days can be found in the supporting information (Table S2). It revealed final cell concentrations ranging from 2.4×10^3 to 5.5×10^5 cells/mL under the conditions employed,

indicating different growth rates between strains (not measured). The cell densities were generally higher than those normally observed in a natural bloom (normally up to 4×10^3 cells/L[49]) and some strains clearly stand out as very efficient toxin producers under the conditions employed[50]. The PST profiles from each strain also ranged widely, illustrating that the *A. pacificum* strains were the best producers of PSTs compared to the *A. minutum* and *A. ostenfeldii* strains. Following on from this preliminary screening, *A. pacificum* CAWD234 was identified as one of the strains yielding the highest biomass and was a top producer of toxins, including high concentrations of C1&2, GTX-1,4, GTX-5, and neosaxitoxin (NEO), as shown in Fig. 3.

Consequently, CAWD234 was selected as a suitable candidate for further scale-up and optimization. No attempts were made at optimizing the toxin production at this initial stage and it cannot be ruled out that other strains could also be suited for scale-up.

Growth condition optimization

Investigations into growth cycles and cultivation conditions have shed light on how toxin production in dinoflagellates can be influenced or enhanced[51]. A robust understanding of the impact of environmental and nutritional factors affecting *A. pacificum* growth and toxin production is therefore critical to establish improved large-scale biomass and toxin production. Consequently, we designed and performed experiments to probe the influence of vitamin requirements, salinity, and light intensity on the growth and toxin cell quota of *A. pacificum* CAWD234. In addition, because trace elements may also impact growth and productivity, the use of artificial seawater from different manufacturers was also investigated and compared to natural seawater. Being able to use artificial seawater for large-scale production instead of natural seawater—with its fluctuating seasonal composition and range of possible contaminants, and hence significant batchwise variability—would also be crucial for a future process for toxins used for commercial scaled-up drug lead production that follows good manufacturing practice.

Artificial seawater

While the use of natural seawater is often a lower-cost option at large scales, it has drawbacks. These include inconsistency in composition based on environmental conditions at the time of collection, and the presence of unwanted organisms or contaminants, which need be removed prior to use. Beyond the potential effect on reproducible data, minimal batchwise variability is key from a pharmaceutical production perspective. From that aspect, working with artificial seawater (ASW) allows for batch reproducibility as ASWs are manufactured, with a well-defined composition and reduced risk of microbial contamination.

However, not all dinoflagellates grow well on artificial media and slight differences in media composition could influence growth rate and toxin production[52,53]. Consequently, after the initial strain selection, we

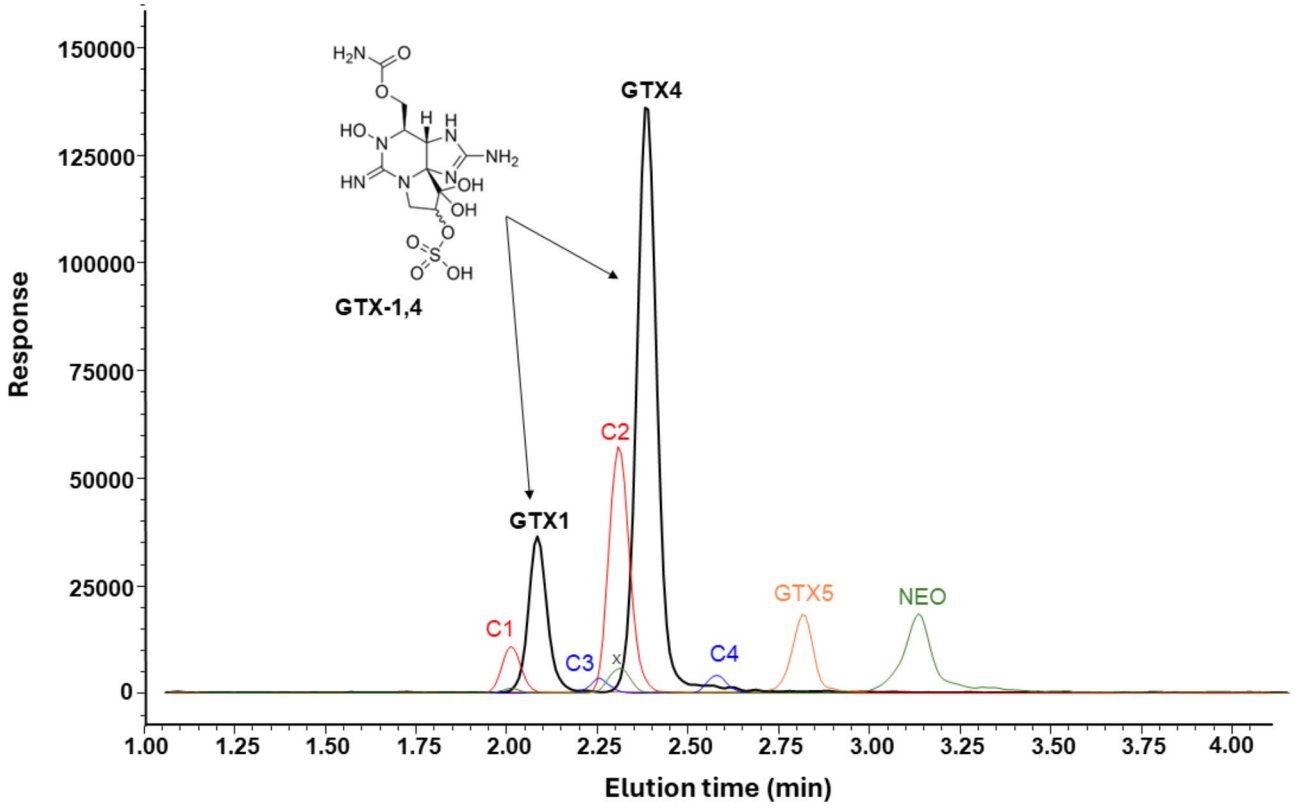

Fig. 3. Chromatogram illustrating the paralytic shellfish toxin profile of *A. pacificum* CAWD234. The chemical structure and peaks for GTX-1,4 are highlighted for clarity.

performed several growth cycles and the lead strain was grown in three different ASW formulations. The base recipe used (ASTM 2013) follows the American Society for Testing and Materials (ASTM) standard protocol for preparing ASW representative of ocean water ASTM D1141-98(2021)[54]. The two other formulations trialed were modified versions of the ASTM 2013 recipe, with natural seawater (SW0010) included as control. All three formulations were prepared from reagent-grade salts (Merck, USA). The ASW formulations were selected based on the content of reagent-grade salts rather than brands that use dried natural seawater, which is still prone to inconsistencies in composition. The growth rate and cell yield were compared against cells grown in natural seawater (Fig. 4 and Table S3.).

The results presented in Fig. 4 and Table S3 illustrate that *A. pacificum* could be grown in all the evaluated ASWs in densities similar to samples grown in natural seawater[49,50]. Minor but not significant differences ($p = 0.22$, two-sample t-test, 95% confidence) in toxin yield (µg/L) were observed between 2013 ASTM and natural SW0010. The treatment with ASSW yielded lower cell growth and GTX-1,4 content. Once it was established that both 2013 ASTM and ASTM-rounded artificial seawater were viable alternatives to natural seawater, 2013 ASTM was selected for the remaining optimization and for scale-up as it is commercially available (Lakes Products Company LLC, Missouri, USA) and meets the ASTM International standard for the preparation of substitute ocean water[54].

Vitamin requirements

A. pacificum CAWD234 was grown in the absence or presence of different mixtures of the B vitamins thiamine (B_1), biotin (B_7) and cobalamin (B_{12}). The effect of these mixtures on CAWD234 growth and productivity is summarized in Table 2.

As can be seen in Table 2, CAWD234 did not grow in the absence of vitamin B_{12}. Furthermore, no notable effect on growth from the inclusion of vitamins B_1 or B_7 was observed. With B_{12} supplementation, the cultures were 22 times denser and subsequently produced 17 times more toxin than those that were not supplemented with B_{12}. Therefore, *A. pacificum* requires vitamin B_{12} to achieve high cell densities.

The toxin cell quota (of GTX-4) was similar with or without B_{12}. This aligns with data showing that eukaryotic microalgae require vitamin B_{12}[55], which is often obtained from symbiosis with bacteria[56,57]. Vitamin B_{12} is usually supplemented in artificial media when growing algae, and it is essential when growing axenic cultures[58]. Although strain CAWD234 is not axenic, this data shows that cultures should be supplemented with vitamin B_{12} during large-scale production of *A. pacificum* to achieve high cell densities, while vitamins B_1 and B_7 can be excluded to save on production costs.

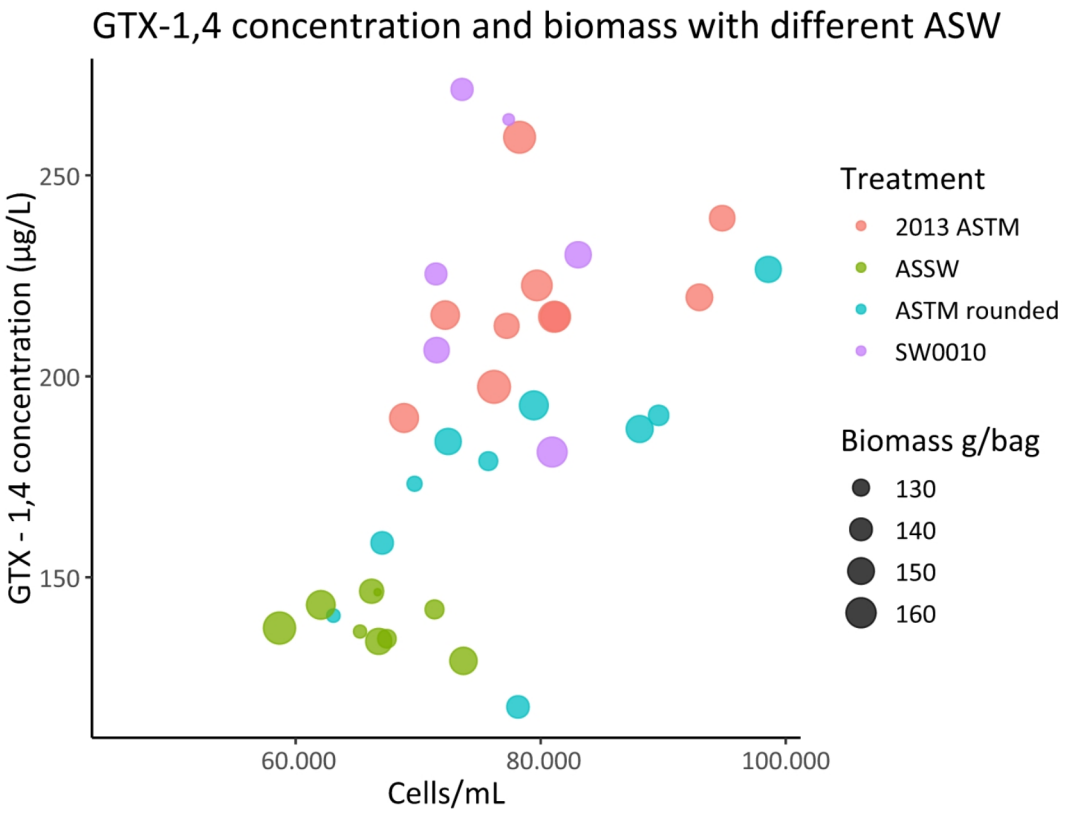

Fig. 4. Evaluation of different ASWs on the production of CAWD234. The plot visualizes values for cell density, biomass produced, and GTX-1,4 contents when cultivated in different types of ASW. Bubble size denotes biomass yield in grams per 28 L bag, and color indicates ASW type. The plot was made with ggplot2 in R version 4.3.2.

Vitamins	μ (d^{-1})[b]	Cmax (10^3 cells/mL)	GTX-1,4 (μg/L)	Cell quota (pg/cell)
–	–	3.38 ± 0.97	20.1 ± 5.54	5.99 ± 0.42
B_1	–	3.82 ± 1.00	23.1 ± 6.96	6.01 ± 1.02
B_7	–	3.78 ± 1.93	19.6 ± 8.96	5.36 ± 0.85
B_{12}*	0.22 ± 0.01	81.6 ± 4.03	360 ± 23.3	5.24 ± 0.35
B_1 and B_7	–	4.06 ± 0.93	22.1 ± 4.52	5.52 ± 0.71
B_1 and B_{12}*	0.22 ± 0.01	83.7 ± 4.30	350 ± 34.8	4.18 ± 0.45
B_7 and B_{12}*	0.22 ± 0.01	81.8 ± 2.86	360 ± 35.4	4.40 ± 0.49
B_1, B_7 and B_{12}*	0.23 ± 0.01	83.7 ± 1.71	352 ± 34.8	4.21 ± 0.45

Table 2. Effect of vitamin supplementation on microalgal growth and toxin production in CAWD234[a]. [a]Assessed after 28 days in batch culture in 28 L bags when grown with different vitamin mixtures. $n = 5$ biological replicates. [b]Growth calculated between day 14 and day 28, i.e. after the lag phase. *No statistical difference between the growth rates or maximum cell densities in the vitamin treatments involving B_{12} (p-value > 0.05, student t-test).

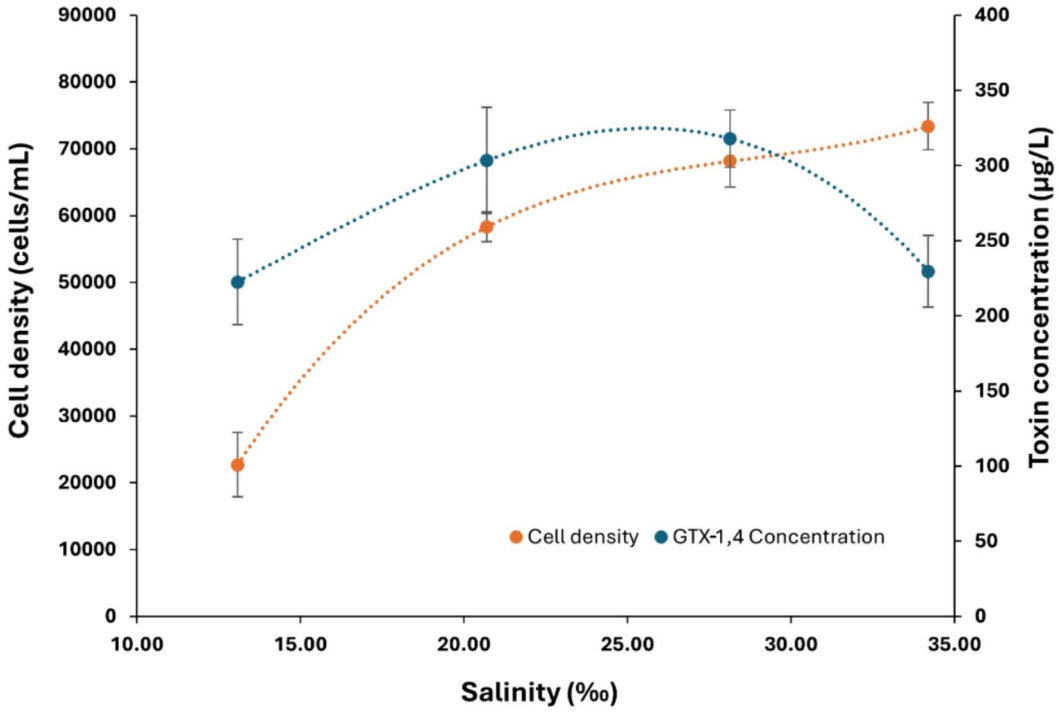

Fig. 5. Cell density and GTX-1,4 concentrations in CAWD234 at different salinity ranges after 12 days of growth. Day 12 sampling was specifically selected as the cells had reached stationary phase.

Salinity

The influence of salinity was investigated by diluting the seawater formulations in the bags to salinities of 13‰, 21‰, 28‰, and 34‰. The effect of this on growth and toxin production in CAWD234 is shown in Fig. 5 and Table S4.

The cell yield clearly positively correlated with salinity. While a similar trend was observed for the toxin cell quota at salinities between 13‰ and 28‰, cells at the highest salinities produced lower quantities of toxins. This observation corroborates with prior studies and illustrates that toxin production can be uncoupled from growth[39,59]. The low salinity could induce stress and a subsequent enhanced toxin production, but this needs to be investigated further. While the toxin cell quota was doubled at the low salinity, this overall toxin productivity gain was not significant enough to justify growing the cells at low salinities. Based on the results, a salinity of 28‰ was selected to balance efficient CAWD234 growth rates and toxin production with a subsequent reduced requirement for ASW salt use on a large scale.

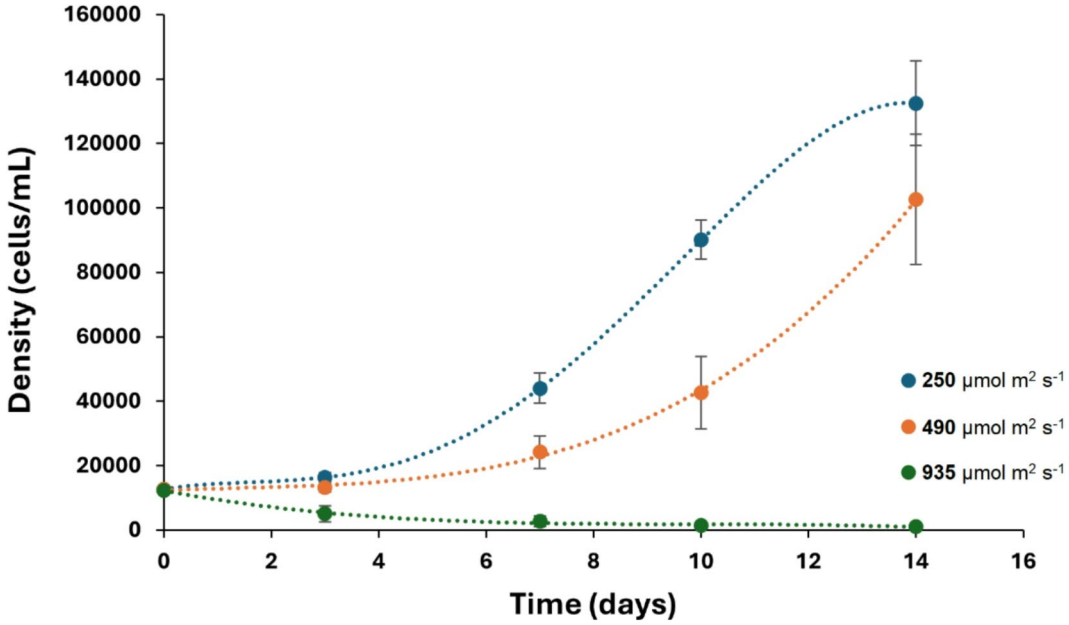

Fig. 6. The effect of light on CAWD234 growth over 14 days in 1 L PBR illuminated at three different light intensities (250 µmol m^2 s^{-1}(control), 490 µmol m^2 s^{-1}, and 935 µmol m^2 s^{-1}). Data represent average ± SD ($n > 3$).

Light intensity

While a 18 h:6 h light: dark photoperiod was kept constant during our experiments[27], the impact of different light intensities was evaluated. This was studied because, with increasing microalgal cell density comes increased cell shading and light limitation, leading to a reduction in growth rate and final cell densities[60]. Consequently, a stepwise increase in light intensity was investigated to determine if growth rates and final cell densities could be improved, without causing photoinhibition at lower initial densities. The algae grown in the control PBRs (initial light intensity of ~ 250 µmol m^2 s^{-1}, increasing to 490 µmol m^2 s^{-1} after 6 days) reached the highest cell density after 14 days (1.3×10^5 cells/mL; Fig. 6) and produced a cell quota of GTX-1,4 of 3.8 ± 0.62 pg/cell) as shown in Figure (Fig. 6).

The PBRs that started at a light intensity of 490 µmol m^2 s^{-1} had a longer lag period (~ 5 days vs. 3 days in the control) and reached a final cell density 23% lower than in the control. Furthermore, the final GTX-1,4 concentration was 29.8% lower than in the control, although the cell quotas were not significantly different (3.8 pg/cell in the control vs. 3.5 pg/cell in the treatment, $p = 0.59$, two samples, t-test at 95% confidence), indicating that the reduction in toxin yield was due to a lower cell density. The highest light intensity (935 µmol m^2 s^{-1}) led to cell death, and the final cell density at day 14 was more than 10-fold less than the starting cell density (Fig. 6). Cells exhibited signs of bleaching typical of a light intensity that cannot be mitigated by pigment production[61]. This also resulted in a GTX-1,4 concentration 150-fold lower than in the control, at 3.4 µg/L.

Although the difference between the final cell densities of the control vs. the 490 µmol m^2 s^{-1} group were not significant ($p = 0.15$, two samples, t-test at 95% confidence), the effect of starting at 490 µmol m^2 s^{-1} light increased the lag period before cells entered exponential growth, and at day 10, the cell density was 4.2×10^4 cells/mL vs. 9×10^4 cells/mL in the control. The results of this trial suggested that, at a starting density of approximately 1×10^4cells/mL, 450 µmol m^2 $s^{-1}$is at the upper tolerance limit for light intensity for the selected CAWD234 strain. This was further confirmed during an additional experiment during which five different light regimes between 250 µmol m^2 $s^{-1}$and 490 µmol m^2 s^{-1} were evaluated. From this experiment, no difference in growth rates (Cmax in the range 1.3–1.4 × 10^5 cells/mL) and cell toxin yield (7.6–8.8 pg/cell) were observed (Figure S2 and Table S5).

As only the inoculum differed between these experiments, the somewhat faster acclimatization of the cells at the 490 µmol m^2 s^{-1} light intensity during the second experiment was unexpected but could be due to the health of the inoculum. While it has previously been suggested that photosynthesis in dinoflagellates plays an important role in the synthesis of toxins[62], no significant difference in the toxin cell quota was measured between the different light treatments. While additional confirmation is needed, our results suggest that toxin production in *A. pacificum* is independent of photosynthetic activity under the conditions employed. Based on the data, starting with a more conservative light intensity (i.e. ~ 250 µmol m^2 s^{-1}) would be beneficial as it means that the initial cell density can be lowered without causing cell damage. Once the cells have had two doubling cycles (reaching ~ 4×10^4 cells/mL), the light intensity can be safely increased to enable a high productivity without it being a limiting factor (Table S5). Overall, the data provided important information about the most practical light regime and was used to set up the large-scale PBRs (Industrial Plankton, Victoria, BC, Canada). A light

regime starting at 250 µmol m^2 s^{-1} at inoculation, increasing to 490 µmol m^2 s^{-1} at day 6, was selected as standard for the growth of CAWD234.

Alexandrium pacificum mass cultivation

Dinoflagellates have been grown at different scales in batch cultures or chemostats (Table 1). Batch cultivation has advantages, including ease of sterility control and a lower risk of contamination, compared to semicontinuous or chemostat cultures, which require more complex setups[63]. However, batch cultures often operate at low volumes (1–10 L). Thus, there is a need for specific setups and technology to produce larger amounts of biomass, a critical step for structure elucidation, biodiscovery programs, feed experiments, nutraceuticals, and specific compound isolations[63,64].

During our optimization study, light intensity, salinity, and nutrient composition were selected to optimize growth and toxin yield following initial batch culture optimization in small-scale reactors. All the cultures of CAWD234 were grown in ASW because it has the advantage of a consistent and defined composition, making it the preferred choice for the production of pharmaceutical precursors. The cultures were not seemingly inhibited by the method of agitation (i.e. bubbling in bag vs. stirring and bubbling in PBRs), and CO_2 sparging for pH control did not seem to be an issue, suggesting that CAWD234 has a high shear tolerance. In general, it was observed that the growth rate in bioreactors (i.e. bags or PBRs) was higher than in flasks (Figure S3). The lack of agitation or pH control in the flasks could explain this observation. Once optimal conditions were selected, algal production was transferred to a 1250 L PBR to provide a meaningful and scalable industrial setting. Algal cell growth and production of GTX-1,4 at the 1250 L scale matched that observed in the previously trialed smaller PBRs. Several 14-day runs at the large scale were performed, with final cell densities ranging between 8×10^4 cells/mL and 1.3×10^5 cells/mL, and toxin production between 1000 µg/mL and 1500 µg/mL, illustrating that some variability is to be expected among runs.

As the runs were performed under similar conditions, this variability could be due to differences in the inoculum. This behavior was also observed in the optimization experiments. When comparing the different experiments and parameters, it became apparent that the inoculum may have an impact on the toxin production, with cell toxin production ranging from 2 pg/cell to 10 pg/cell between the experiments, and with good consistency within each experiment. Hence, no comparison was attempted between the absolute values recorded in the different studies. Beyond cell density, the inoculums were not analyzed or studied before use. Initial experiments looking at the impact of age of the inoculum on production suggest that there is room for further improvement and consistency in both cell density and cell quotas (Figure S4) in future studies. This was reflected in the numerous runs in the 1250 L reactor, which delivered ranging yields. While all the yields were high and satisfactory, optimization and standardization of the inoculum used could improve the process even further in the future.

In general, it is often costly to produce microalgal biomass in PBRs, and the species that require this culture method can be prone to culture crashes or yield variation[65,66]. Our work here on *A. pacificum* CAWD234 suggests that the strain is a robust producer with a consistent high yield under the conditions employed. In comparison to a reported intense bloom of *A. pacificum* in the wild (Bay of Plenty, New Zealand), the cell densities generated in the PBR in our study were about 100–500 times higher, and the concentration of GTX-1,4 was nearly 30 times higher[50], illustrating how efficient PBR production is under the selected conditions. The same study on the wild bloom also points out how natural isolates can have widely different toxin profiles and productivity, which was also supported by our initial strain screen and previous observations (unpublished data).

Total GTX-1,4 production over 14 days in the 1250 L PBR ranged between 1000 µg/L and 1500 µg/L, and while the method employed for PST analysis is rapid and quantitative, a robust simplified and scalable isolation method would be needed for industrial production given the other coeluting toxins (Fig. 3)[67]. Optimization of harvesting, extraction, and isolation of GTX-1,4 is under development. The toxin production in our developed system was in the high end of the range reported by others (Table 1). Nevertheless, based on published data on clinical trials using PSTs, it is estimated that 500 mg of toxin would be enough to generate $1–2.5 \times 10^4$ doses, depending on clinical application (normally low microgram doses) and toxin type[21,44]. Our study provides the methodology, and illustrates the possibility, for generating such amounts in 14 days via cultivation of *A. pacificum* in a single scalable automated PBR. As such, it represents the first feasible example of dinoflagellate mass production for subsequent potential pharmaceutical applications.

Conclusions and future outlook

Alexandrium spp., a species of bloom-forming dinoflagellates, is globally ubiquitous and, consequently, one of the most studied dinoflagellates[68]. This study of *A. pacificum* grown under different conditions provided insights into the physiology of this species. Importantly, while the toxin cell quota ranged from 2 pg/cell to 10 pg/cells between experiments, it did not vary significantly within those experiments, meaning that toxin production in *A. pacificum* is constitutive. The feasibility and efficacy of using ASW to reliably produce *A. pacificum* biomass at scale for PST extraction was investigated. We showed that *A. pacificum* growth and toxin production was not influenced by the system used (i.e. bags vs. photobioreactors), and consequently *A. pacificum* was successfully batch-produced in 1250 L PBRs. To the best of our knowledge, large-scale mass production of *A. pacificum* for toxin production has never been reported at that scale.

Earlier studies have shown that ASW can be used for producing and studying dinoflagellates in the laboratory[58]. In our current report, we have shown that it can also be efficiently employed at scale and tailored to yield bioactive compounds in commercially relevant amounts. Using bespoke ASW that is consistent in composition in large, closed PBR systems allows for large-scale precision cultivation of microalgae for clinical trials or other applications where highly consistent outcomes and standardization are necessary.

The implications of our findings are significant as they show that it is possible to optimize and produce reliable quantities of highly valuable and complex bioactive compounds from marine microalgae. The method developed is scalable, and large amounts can be rapidly generated in several parallel PBRs as needed. The scaled-up methods enable ready access to gram amounts of PSTs for a range of potential commercial applications that to date have been hampered by synthetic and recombinant hurdles.

Methods and Cultures maintenance

Strain selection

The Cawthron Institute Culture Collection of Microalgae (CICCM) maintains more than 700 strains of microalgae and cyanobacteria for scientific purposes[48]. Several of these strains produce PSTs, and 18 strains belonging to three different *Alexandrium* species (*A. minutum*, *A. pacificum*, and *Alexandrium ostenfeldii*) collected around the New Zealand coastline were selected for initial biomass and toxin production assessment. The selection of strains was based on earlier observations of toxicity (unpublished data) in the Cawthron Institute laboratory. The selected algae were all maintained in natural seawater/L1 media[69]in 70 mL containers at 17 °C under cool white fluorescent lighting at an intensity of 30 µmol m^2s^{-1} [48]. Information about the selected strains can be found in Table S1.

Artificial seawater

For drug development it is paramount to reduce the batchwise variability, and for that reason, different artificial seawaters (ASWs) were tested. A total of three ASW recipes were tested, with the formulations based on the American Society for Testing and Materials (ASTM) 2013 standard recipe (ASTM D1141-98)[54]for preparation of ASW representative of ocean seawater. Recipes for each are outlined in Table S6. Briefly, 2013 ASTM followed the standard recipe while 2013 ASTM-rounded contained a similar composition as 2013 ASTM, but with each salt concentration rounded to the nearest whole number and sodium fluoride removed. ASSW was a modified recipe where sodium sulphate and magnesium chloride were replaced by magnesium sulphate. All three formulations were initially prepared from reagent grade salts (Merck, USA) in-house. Once it was established that both 2013 ASTM and 2013 ASTM rounded were viable alternatives, 2013 ASTM was selected for the remaining optimization and for scale-up as it is commercially available (Lakes Products Company LLC, Missouri, USA) and meets the ASTM standard for preparation of substitute ocean water[54]. The suitability of the prepared ASWs for algae and toxin production was compared to natural seawater (SW0010). The final analyzed compositions of each recipe, including the SW0010 can be found in Table S7. ASWs were prepared on site as follows: Reverse osmosis (RO) water was generated using a Milli-Q HR 7000 Series system (Merck, USA) in a 4000 L food-grade medium-density polyethylene plastic tank (Advantage Plastics, New Zealand). ASW salts were mixed with the RO water in a secondary tank using an electric diaphragm pump (Husky 1050e, Graco, USA) until a stable conductivity reading was achieved.

Production systems

During the study, dinoflagellates were produced in different cultivation systems, with the overall goal of being able to scale up to pilot production (1250 L). Initial parameter optimization studies and selection were performed at small scale, while different larger systems were subsequently used for specific optimization experiments and scale-up trials. The culturing systems used are described below.

Culture flasks

Tissue culture flasks (175 cm^2, Nunc, ThermoFisher Scientific, USA) were used for strain selection and for cultivating *A. pacificum* (strain CAWD234, hereafter referred to as CAWD234) on a small scale (100 mL). Flasks were maintained in a growth cabinet (Climatron, Thermoline Scientific, Australia) at 17 °C, with a light cycle (4000 K, fluorescent bulbs at an intensity of approximately 240 µmol m^2 s^{-1}) of 18 h light:6 h dark.

Bbi photobioreactors

1 L PBRs (xCUBIO, Bbi Biotech, Germany) mounted with four types of light-emitting diodes, including white light at 445 nm, 625 nm and 645 nm wavelengths, were used to test the influence of light intensity on the growth of CAWD234.

Hanging bags

200 × 0.23 m rolls of polyethylene plastic (Amcor, New Zealand) were used to manually prepare hanging bags for CAWD234 culturing. Bags were prepared with a hanging loop at the top and a cone at the bottom where the aeration line was inserted. The largest volume of individual bags was 28 L, but volumes could be adjusted. Hanging bags were housed in a temperature-controlled room, and a single vertical cool white fluorescent light (4000 K, TLD 58 W/840, Philips, Netherlands) with adjustable intensity outputs was positioned behind each bag. Each bag was supplied with a pH probe connected to a controller (Apex, Neptune Systems, California, USA) and a solenoid valve to enable dosing of CO_2 when the pH exceeded the programmed set-point.

Photobioreactors

Scaled-up cultivations were performed in 1250 L Industrial Plankton PBRs (Victoria, BC, Canada). These are fully contained and sealed systems with temperature, pH, and light intensity (of a fixed spectrum) controls[70]. These reactors can be cleaned and sanitized in place (CIP and SIP), and represent a realistic commercial production system for PSTs.

Growth parameter optimization

Vitamins

Microalgae require vitamin for growth, and consequently vitamins are generally provided in artificial media[55]. The vitamin B_1, B_7, and B_{12} requirements of CAWD234 were tested in bag systems. Briefly, 28 L bags (Ancor, New Zealand) were filled with seawater and inoculated with CAWD234. 280 mL of inoculum/bag, at a density of 5×10^4 cells/mL, was used to prevent the introduction of appreciable levels of residual B vitamins. The bags were illuminated with fluorescent lights (cool white [Philips]) at a mean irradiance of 176 µmol m^2 s^{-1}). In sets of five, bags were randomly selected and supplied with the standard concentration of L1 medium (7 mL/L), with the B-vitamins omitted. Solutions of either one B-vitamin, or a mixture of B-vitamins (prepared from bioreagent-grade powders, Merck) were then added at the same concentration as is standard for L1 medium (B_{12} = 207 µg/L of seawater, B_1 and B_7 = 1.03 µg/L of seawater). As a negative control, five bags were not supplied with any vitamins. Samples were taken at days 0, 14, and 28 to measure cell numbers and for PST extraction and analysis.

Salinity

The impact of media salinity was assessed using the hanging bags method of culturing with 23 L bags. For this experiment, 20 bags were randomly assigned a seawater dilution of either 40%, 60%, 80%, or undiluted, corresponding to salinities of 13‰, 21‰, 28‰, and 34‰, respectively. Natural seawater (at 34‰) was diluted to each of these concentrations using RO water and the bags were then inoculated with a 2.3 L inoculum (1.1×10^5 cells/mL) of CAWD234, with five replicates per salinity level. L1 nutrient mix was added at a flow of 7 mL/L to each bag, all of which were grown under cool white fluorescent light (176 µmol m^2 s^{-1}) on a 18 h light:6 h dark light cycle at 18 °C and a pH set-point of 8.60 (regulated via injection of food-grade CO_2). Samples were taken for salinity and cell density measurements at the beginning of the experiment and again after 12-days when the cells had reached stationary phase.

Light intensity

Small 1 L parallel PBRs (xCUBIO, Bbi Biotech, Germany) were used to determine the optimum light intensity for growing CAWD234. Eleven PBRs were used, with three selected as controls and the remaining eight as two sets of treatment groups with four replicates each. Experiments were performed in 28‰ ASW (including L1 media [7 mL/L] and 1 mL/L vitamin B_{12}) at pH 8.55 and 18 °C, and with a 18 h light:6 h dark cycle that was applied to all PBRs. The Industrial Plankton light spectrum was used (at a ratio 5:1:4:3 645 nm:625 nm:445 nm: white light) (Table S8) and the ratio of the wavelengths remained constant during the experiment. The control group received a stepwise increase in light intensity, starting at 250 µmol m^2 s^{-1}until day six, when it was increased to 491 µmol m^2 s^{-1}. The two treatment groups were grown under either 490 µmol m^2 s^{-1}or 935 µmol m^2 s^{-1} light intensity for the duration of the experiment. Each PBR was inoculated with CAWD234 at a cell density of 1.2×10^4 cells/mL in ASW. Samples of each PBR were taken every 3 days for cell density measurement, and a sample was taken at day 14 for PST analysis.

A second trial was carried out to determine a more refined light intensity regime for growing CAWD234 in the same PBRs. Fifteen PBRs were inoculated with CAWD234 at a cell density of 8.3×10^3 cells/mL. Standard growth conditions were used, i.e. 17 °C ± 1 °C, 14 mL/L L1 nutrient mix, 1 mL/L vitamin B_{12}in 28‰ ASW. In triplicate, each set of PBRs was given a different light regime, either starting at 250 µmol m^2 s^{-1} (ratios outlined in Table S8), 360 µmol m^2 s^{-1}or 490 µmol m^2 s^{-1} (Figure S5). Cell density measurements were made every 3 days to assess growth, and a sample of each PBR was taken on day 15 for PST analysis.

Selected optimized growing conditions

Following on from the growth optimization experiments, the selected growth conditions of 28‰ ASW (which includes 14 mL/L L1 media and 1 mL/L vitamin B_{12}), pH set-point 8.55, 17 °C ± 1 °C, and 18 h light:6 h dark cycle were applied to all systems. Some of the experimental parameters, such as photoperiod, temperature, and pH, were selected based on our previous work and knowledge of dinoflagellate growth[27]. In addition, several experiments highlighted that the cells were limited by nutrient concentration at maximum cell densities. In response, nutrients were therefore supplied in excess, i.e. 4 times more L1 media[69] was added.

Analyzes

Bag and reactor sampling

Hanging bags, xCUBIO PBRs, and the Industrial Plankton PBRs were aerated to keep the *A. pacificum* cells in suspension and homogenous. For the hanging bags and xCUBIO PBRs, a sampling line toward the middle of the water column was used to collect 30 mL samples, which were analyzed for both cell density and PSTs. One sample per bag or PBR replicate was taken for all the described experiments. Although the sampling port in the Industrial Plankton PBRs is located at the bottom of the reactor, once ~ 500 mL of culture has been drained to flush the port, representative samples can be collected (30 mL).

Microalgal cell density measurements

Cultures were regularly analyzed under a microscope and growth was reported as cell numbers per mL. *A. pacificum* culture concentrations (cells/mL) were determined using a Nikon Eclipse Ts2 inverted microscope equipped with a Nikon DS-Fi2-U3 camera. Samples fixed with Lugol's iodine were pipetted (5 mL) into individual wells of a six-well plate (Nunc) and left to settle for 1 h. The wells were photographed, and the images were processed using ImageJ 1.54 g (U.S. National Institute of Health, Bethesda, MD, USA) to enumerate cells.

PST analyzes

The toxin profile and GTX-1,4 production were regularly evaluated during the different experiments. Only the whole cultures were analyzed for toxins. Sample extraction for PST analysis was based on a method developed in the Cawthron Institute laboratory[67]. In brief, homogenous 10 mL samples of each culture were poured into 15 mL falcon tubes (Nunc) supplemented with 50 µL of glacial acetic acid (HOAc). The sample was heated at 85 °C for 10 min, cooled on ice, and then centrifuged at 4000 × g for 2 min. Solid-phase extraction (SPE) clean-up was carried out on each extract by conditioning a 6 mL/250 mg Supelclean™ ENVI-Carb™ SPE Tube (Merck) with 3 mL 20% acetonitrile (ACN) containing 1% HOAc, followed by 3 mL of 0.025% ammonia. One mL of each extract was then loaded onto the SPE column before washing with 3 mL of Milli-Q water. The samples were eluted with 20% ACN containing 1% HOAc, and each elution was diluted in ACN for analysis. Samples were analyzed by a Triple Quadrupole liquid chromatography–tandem mass spectrometer (Waters, USA) using certified reference standards from the National Research Council of Canada (Halifax, Canada) and Sigma-Aldrich (Merck Life Science Ltd, Buchs, Switzerland). Solvents and chemicals used were of analytical grade and supplied by Merck.

Statistical analysis and software

All conditions were tested using at least three biological replicates. Two sample t-tests were carried out to compare the means between two groups using the data analysis function from Excel. Means between groups were considered significantly different at a p-value lower than 0.05 (95% confidence). Figures were made using Microsoft Excel and ggplot2 in R v. 4.4.0. Chemical structures were drawn using ChemBioDraw Ultra 14.0 and Fig. 3 illustration was created using biorender.com.

Data availability

Data supporting the findings of this study is provided within the manuscript or supplementary information files.

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Author contributions

H.G., C.W., R.V.G., J.B., G.K., J.O., and A.I.S. performed the experiments. H.G., C.W., R.V.G., J.B., and A.I.S conceived and designed the study. H.G., C.W., J.B., M.P., R.I., J.S., and A.I.S. analyzed and interpreted data. H.G., M.P., R.I., J.S., and A.I.S. wrote the manuscript. All authors contributed to the final version of the manuscript and approved it.

Declarations

Competing interests

The authors declare no competing interests.

Supporting information

Algal strain information, artificial seawater composition data, light regimes information, and additional toxin production and growth data.

Additional information

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