The effect of temperature and salinity on growth rate and azaspiracid cell quotas in two strains of *Azadinium poporum* (Dinophyceae) from Puget Sound, Washington State

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ABSTRACT

Azaspiracids (AZA) are novel lipophilic polyether marine biotoxins associated with azaspiracid shellfish poisoning (AZP). Azaspiracid-59 (AZA-59) is a new AZA that was recently detected in strains of *Azadinium poporum* from Puget Sound, Washington State. In order to understand how environmental factors affect AZA abundances in Puget Sound, a laboratory experiment was conducted with two local strains of *A. poporum* to estimate the growth rate and AZA-59 (both intra- and extracellular) cell quotas along temperature and salinity gradients. Both strains of *A. poporum* grew across a wide range of temperatures (6.7 °C to 25.0 °C), and salinities (15 to 35). Growth rates increased with increasing temperature up to 20.0 °C, with a range from 0.10 d^{-1} to 0.42 d^{-1}. Both strains of *A. poporum* showed variable growth rates from 0.26 d^{-1} to 0.38 d^{-1} at salinities from 15 to 35. The percentage of intracellular AZA-59 in both strains was generally higher in exponential than in stationary phase along temperature and salinity gradients, indicating higher retention of toxin in actively growing cells. Cellular toxin quotas varied by strain in both the temperature and salinity treatments but were highest at the lowest growth rates, especially for the faster growing strain, NWFSC1011.

Consistent with laboratory experiments, field investigations in Sequim Bay, WA, during 2016–2018 showed that *A. poporum* was detected when salinity and temperature became favorable to higher growth rates in June and July. Although current field data of *A. poporum* in Puget Sound indicate a generally low abundance, the potential of local *A. poporum* to adapt to and grow in a wide range of temperature and salinity may open future windows for blooms. Although increased temperatures, anticipated for the Puget Sound region over the next decades, will enhance the growth of *A. poporum*, these higher temperatures will not necessarily support higher toxin cell quotas. Additional sampling and assessment of the total toxicity of AZA-59 will provide the basis for a more accurate estimation of risk for azaspiracid poisoning in Puget Sound shellfish.

1. Introduction

Azaspiracids (AZA) are novel lipophilic polyether marine biotoxins, with 62 isomers identified (Tillmann et al., 2018; Krock et al., 2019). The first azaspiracid was isolated and characterized from shellfish following a toxic incident which resulted in at least eight humans sickened from azaspiracid shellfish poisoning (AZP) in the Netherlands in 1995 after eating blue mussels (*Mytilus edulis*) harvested from Killary Harbour, Ireland (Satake et al., 1998; Ofuji et al., 1999). Since then, AZP incidents and/or AZA contained shellfish have been reported in various countries (Ofuji et al., 1999; Braña Magdalena et al., 2003; James et al., 2002; Taleb et al., 2006; Twiner et al., 2008). Azaspiracids have been isolated from different shellfish (mussels, oysters, scallops, cockles, clams; Salas et al., 2011) and crabs (Furey et al., 2003; Torgersen et al., 2008). The symptoms of AZP are very similar to diarrhetic shellfish poisoning (DSP) and include nausea, vomiting,

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diarrhea and stomach cramps (Satake et al., 1998; Twiner et al., 2008). Although the mechanism of AZA toxicity in humans has not been fully characterized, some analogues are proven to cause widespread organ damage and tumors in mice (Ito et al., 2002), teratogenic effects to finfish (Colman et al., 2005) and modulation of calcium concentrations in human lymphocytes (Alfonso et al., 2006).

The dinoflagellate genus *Azadinium* Elbrächter & Tillmann and the species *Azadinium spinosum* Elbrächter & Tillmann was identified in 2009 as the first AZA producer (Krock et al., 2009; Tillmann et al., 2009), over ten years after the first occurrence of AZP. Species of *Azadinium* are small dinoflagellates ($< 20\,\mu m$) and can be difficult to identify in routine phytoplankton surveys (Tillmann et al., 2018b). *Azadinium* can be fed upon by other plankton grazers, leading to accumulation of AZA in those organisms (Krock et al., 2009; Salas et al., 2011), and the heterotrophic dinoflagellate *Protoperidinium crassipes* (Kofoid) Balech was once considered as the causative species of AZP (James et al., 2003).

To date, 13 species in the genus *Azadinium* have been described (Tillmann and Akselman, 2016; Tillmann, 2018a), of which three species are known to produce AZA: *A. spinosum* (Tillmann et al., 2009; Salas et al., 2011), *A. poporum* Tillmann & Elbrächter (Krock et al., 2012; Gu et al., 2013) and *A. dexteroporum* Percopo & Zingone (Percopo et al., 2013). One other dinoflagellate, *Amphidoma languida* Tillmann, Salas & Elbrächter, within the same family (Amphidomataceae) as *Azadinium,* has been shown to produce AZA (Krock et al., 2012; Tillmann et al., 2018). *Azadinium* species are widely distributed around the world: the North Sea (Tillmann et al., 2009, 2011), the Irish Sea (Salas et al., 2011), the Mediterranean Sea (Percopo et al., 2013; Luo et al., 2017), the North Atlantic (Tillmann et al., 2014), the China Sea (Potvin et al., 2012; Luo et al., 2013; Gu et al., 2013), the Southeastern Atlantic in Argentina and Brasil (Tillmann and Akselman, 2016; Tillmann et al., 2016; Cavalcante et al., 2018), the northern Gulf of Mexico (Luo et al., 2016), the Southeast Pacific (Tillmann et al., 2017) and the Northeast Pacific (Kim et al., 2017). This wide distribution and toxin producing capability illustrates that AZP risk in many areas is likely to be underestimated. AZA production by Amphidomataceae is species specific but also varies among different strains of the same species (Luo et al., 2017; Rossi et al., 2017; Tillmann et al., 2019), and a very wide range of AZA cell quota has been reported for different strains of *A. poporum* (Kim et al., 2017; Luo et al., 2018). These characteristics attract attention to understanding which environmental factors may drive toxin production and growth rates in these dinoflagellates.

A previous series of experiments have tested environmental and nutritional effects on growth and AZA production of *A. spinosum* (Jauffrais et al., 2013a), showing a general inverse trend between AZA cell quota and growth rate. Such trends have been also observed in other toxic dinoflagellates (Anderson et al., 1990; Morton et al., 1994; Navarro et al., 2006; Guerrini et al., 2007). The effects of different nutritional factors on the growth and AZA cell quota of *A. poporum* were shown for strains from the South China Sea (Li et al., 2016). However, little research has been done to determine how environmental drivers influence toxin production and growth rate of *Azadinium*, recently identified in North America.

In Puget Sound, Washington State, several lipophilic toxins including DTX-1, PTX-2, YTX and AZA-2 have been measured in phytoplankton samples collected by the SoundToxins program (Trainer et al., 2013). Recently, four species, *A. poporum*, *A. cuneatum* Tillmann & Nézan, *A. obesum* Tillmann & Elbrächter and *A. dalianense* Luo, Gu, & Tillmann were isolated from incubated sediment samples, and a new AZA (AZA-59) as the sole AZA was detected at a maximum cell quota of 106 fg cell^{-1} in Puget Sound *A. poporum* by liquid chromatography tandem mass spectrometry (LC–MS/MS) analysis (Kim et al., 2017). A small amount of AZA-59 phosphate was also measured. To understand how temperature and salinity gradients affect AZA cell quota and growth rate, a laboratory experiment was conducted with two strains of *A. poporum* isolated from Puget Sound sediment. Field observations of *A. poporum* and their relationship with actual temperature and salinities in Sequim Bay, WA were also examined. Together these data will allow scientists and managers to evaluate the windows of opportunity for the highest AZP risk and to estimate the potential for future AZA exposure in Puget Sound.

2. Materials and methods

2.1. Azadinium poporum isolation and culture

Two strains of *Azadinium poporum*, 967B8 (Kim et al., 2017; here named NWFSC1011) and NWFSC1018, were germinated and isolated from sediment collected on 16 Jan 2016 from Hood Canal, WA, USA (47°47.502'N, 122°51.540'W) (Kim et al., 2017). Both strains were maintained in an environmental incubator at $14.0 \pm 2.0\,°C$ on a 14:10 h light:dark cycle, with a photosynthetic photon flux density (PPFD) of 80–100 $\mu E\ m^{-2}\ s^{-1}$. The medium used for isolation and culture of *A. poporum* was ESNW-Si (Bill et al., 2016)

2.2. Azadinium poporum culture experiments

2.2.1. Temperature gradient bar

A temperature gradient bar (TGB) incubator (Bill et al., 2016) was used to test the effect of temperature on AZA-59 cell quota and growth rate of *A. poporum*. The TGB uses 50 mL culture tubes arranged in a six row × 19 column pattern and can be regulated to ensure temperature stability between rows and gradient uniformity between columns. The temperature gradient was set from 6.9 °C to 25.0 °C at $\sim 1.0\,°C$ increments, as a preliminary experiment showed that *A. poporum* (strain NWFSC1011) had a negligible growth rate at 6.9 °C and could not survive at 26.0 °C or higher. Light from below the culture tubes was set to a 12:12 h light:dark cycle and the average PPFD was $170 \pm 9\ \mu E$ $m^{-2}\ s^{-1}$ for the middle 4 rows and $122 \pm 7\ \mu E\ m^{-2}\ s^{-1}$ for the first and the 6th row. Since light intensity impacted the growth of *A. poporum* (data not shown), only the middle 4 rows were used for this experiment. In order to minimize temperature and light shock during the TGB experiments, cultures were adapted for 2–3 weeks in the TGB incubator at four temperatures (7.8 °C, 12.4 °C, 18.2 °C, 23.1 °C) for strain NWFSC1011 and three temperatures (10.0 °C, 17.2 °C, 22.3 °C) for strain NWFSC1018. The acclimated cultures were distributed into 30 mL (final volume) of fresh medium at 34 salinity (ESNW-Si; Bill et al., 2016) in 50 mL glass tubes at an initial abundance of ~ 200 cells mL^{-1}. The two strains were staggered between columns throughout the TGB, e.g. strain NWFSC1011 was incubated in columns 1, 3, 5, 7, 9, 11, 13, 15, 17 and 19, while strain NWFSC1018 was incubated in columns 2, 4, 6, 8, 10, 12, 14, 16 and 18, with four replicate tubes per column.

2.2.2. Salinity gradient experiment

The salinity gradient experiment was conducted in an environmental incubator at 16.0 °C on a 12:12 h light:dark cycle, with an average PPFD of 420 $\mu E\ m^{-2}\ s^{-1}$. Both strains of *A. poporum* were acclimated at salinities of 15, 20, 25, 30 and 35 for 2–3 weeks to minimize salinity shock. The lower salinity medium was made by diluting with MilliQ water before adding nutrients. The highest salinity of 35 was made by adding NaCl to the ESNW-Si medium. Salinity effects were tested using four replicate tubes per salinity. The acclimated cultures were distributed into 30 mL (final volume) fresh medium (ESNW-Si) in 50 mL glass tubes at initial abundance of ~ 200 cells mL^{-1}.

2.3. Growth rates, cell counts, toxin extraction and analysis

2.3.1. Growth rate calculations and growth curves

The growth of *A. poporum* was monitored daily in each tube by measuring the *in vivo* fluorescence of chlorophyll-*a* via fluorometer

(Model 10-AU, Turner Designs, Sunnyvale, CA). The *in vivo* fluorescence measurements, recorded as relative fluorescence units (RFU), were used to calculate average maximum specific growth rates (for the exponential phase of the growth curve) and to determine what two time points to use for toxin analysis (i.e. one at exponential and one at stationary growth). Specific growth rates (μ, d^{-1}) were calculated following Brand et al. (1981).

2.3.2. Cell counts and toxin extraction

Direct cell counts, via inverted microscope (Zeiss Axiostar, Carl Zeiss, Oberkochen, Germany) were made at the beginning of the experiments and at each time point when toxin content was measured (i.e. exponential and stationary growth). Of the four replicate tubes for each temperature and salinity, two replicate tubes were harvested during both exponential and stationary phase (Supplementary Table 1, red circles indicate harvest dates for each treatment). For direct cell counts, 1 mL from each replicate tube was transferred to a 1.5 mL microcentrifuge tube and fixed using 40 μL of Lugol's iodine solution. Cell abundances were determined using a gridded Sedgewick-Rafter counting chamber. The remaining 29 mL culture from each replicate tube was vacuum filtered separately onto a 25 mm diameter, 3.0 μm pore size filter (Millipore Ultrafree, Eschborn, Germany). Vacuum pressure was kept to a minimum ($\leq$ 5 in. Hg) to avoid lysis of cells during filtration. The filter was transferred to a 15 mL centrifuge tube for extraction and quantification of intracellular toxin. The filtrate from each replicate tube was transferred to a 60 mL plastic bottle for extraction and quantification of extracellular toxin.

For extraction of intracellular toxin, 2 mL 100% methanol was added to the centrifuge tube containing the filter. The filter with methanol was probe sonicated for 30 s at half second interval bursts at the lowest power setting (Sonifier 450, Branson, USA). The methanol solution was then transferred to a 2 mL microcentrifuge tube and centrifuged for 10 min at 13,100 $\times$ g (Minispin plus, Eppendorf, USA) to pellet cell debris. The supernatant solution was collected into a 4 mL glass amber vial for LC–MS/MS analysis after a total extraction time of $\sim$ 20 min, including centrifugation.

For culture filtrate samples, 1 g (dry weight) of HP-20 resin (SUPELCO, Bellefonte, PA, USA) was activated by combining it with 5 mL of 100% methanol in a 10 mL plastic bottle for each replicate. After a 15 min incubation, a 20 μm sieve was used to separate the activated resin from the methanol. The resin was rinsed for 1–2 min with MilliQ water. Conditioned resin was transferred into each replicate bottle containing the culture filtrate. The bottles were then slowly mixed via rotation for a total of 72 h at 16 °C to allow dissolved toxin in the filtrate to adsorb to the resin. The resin was then separated from the culture medium, rinsed with MilliQ water and transferred to empty 12 mL solid phase extraction (SPE) reservoirs (Bond Elut, Agilent, Santa Clara, CA). SPE reservoirs were placed on a vacuum manifold and the resin was dried under low vacuum pressure for about 30 s. For toxin extraction from the resin, 10 mL of 100% methanol was added to the reservoir and incubated for 10 min before the methanol was slowly (over about 10 min) passed through the resin and collected into a clean glass culture tube. The solution was gently blown to dryness using filtered air from a vacuum pump. One mL of 100% methanol was added, tubes were vortexed and then reconstituted for 1 h before being transferred to 4 mL glass amber vials for LC–MS/MS analysis.

2.3.3. Toxin analysis

Samples and calibration standards were analyzed using a modification of a previously described LC–MS/MS method by UPLC (Acquity system, Waters Co., Milford, MA, USA) and a triple quadrupole tandem mass spectrometer (MS/MS, ABSciex 5500, Framingham, MA, USA; Trainer et al., 2013). Calibration and quality control standards in methanol were prepared from dilutions of previously analyzed toxic *A. poporum* cell extract. A quadratic natural-log normalized calibration curve was created by using 2 analyses of each of the 5 calibration

standards. Acquisition parameters of AZA-59 were as follows, precursor ion (m/z) = 860.6, product ion 1 (m/z) (used as quantitation transition) = 842.5, product ion 2 (m/z) (used as confirmation transition) = 362.3, dwell time (msec) (1/2) = 35/35, collision energy (V) (1/2) = 44/75. The limit of quantitation (LOQ) was 0.01 ng mL^{-1} and calculated based on instrument lowest level of calibration curve (detection limit) for AZA-59, as well as sample volume extracted and dilution factor of the extract. Ten microliters of each extract were injected into the LC–MS/MS.

2.4. Field sample collection and processing

Weekly seawater samples were collected using a Niskin bottle deployed to 2 m depth from a pier at a private residence adjacent to Sequim Bay State Park. Samples were transferred to containers for temperature and salinity measurement using a thermometer and refractometer, respectively (Fisher Scientific; Waltham, MA, USA). Whole water was stored at 4 °C until filtered in the laboratory within 24 h of collection for *A. poporum* DNA detection. Up to 1 L of surface seawater was filtered through 47 mm diameter, 3.0 μm pore size polycarbonate filter (MilliporeSigma, Burlington, MA, USA) under light vacuum (< 5 in. Hg). The filters were folded and placed into plastic 2.0 mL microcentrifuge tubes and frozen at $-$80 °C until DNA extraction.

2.5. Molecular detection of Azadinium poporum

2.5.1. DNA extraction

DNA was extracted from the material on the filters using DNeasy Plant mini kits (Qiagen, Hilden, Germany) with some modifications of the manufacturer's protocol. Briefly, 500 μL of buffer AP1 and 5 μL of RNase A were added to the tube containing the filter, tubes were vortexed for 30 s then incubated at 65 °C for 30 min. After addition of buffer P3 and a 5 min incubation on ice, the filters were removed from the tube, placed into the QiaShredder spin column and the lysate was then added to the QiaShredder spin column containing the filter. The manufacturer's protocol was then followed as written, except 2 eluates using 200 μL AE buffer were collected in separate tubes.

2.5.2. Quantitative polymerase chain reaction analyses

DNA was prepared for quantitative polymerase chain reaction (qPCR) analyses by mixing equal volumes of the 2 eluates from the DNA extraction process. Species specific analyses were conducted for *A. poporum*, in triplicate, following the procedure in Toebe et al. (2013). A standard curve for *A. poporum* was constructed using a cultured strain of *A. poporum* (NWFSC1012) that was collected from Puget Sound, WA. Known quantities *A. poporum* were filtered and extracted in the same manner as field samples. Ten-fold serial dilutions were analyzed to construct a standard curve.

Quantitative PCR analyses for *A. poporum* were performed on a Stratagene Mx3005 P (Stratagene, La Jolla, CA, USA) using the thermal profile in Toebe et al (2013). A standard curve, a positive control and a no template control were run with every set of environmental samples. Density of *A. poporum* in each reaction were automatically calculated using the MxPro (version 4.10) software. An average and standard deviation were calculated for the three technical replicates and used for further evaluation.

2.5.3. qPCR acceptance criteria

Tests were designated as below the limit of quantification for samples in which there was amplification in all three technical replicates, but the calculated cell density were less than the lowest point on the standard curve. The limit of quantification for *A. poporum* ranged from 4 to 108 cells L^{-1} depending on the amount of water filtered. Samples were labeled as negative tests if there was amplification in two or fewer of the technical replicates and the calculated cell density were below the lowest point on the standard curve. In tests where calculated
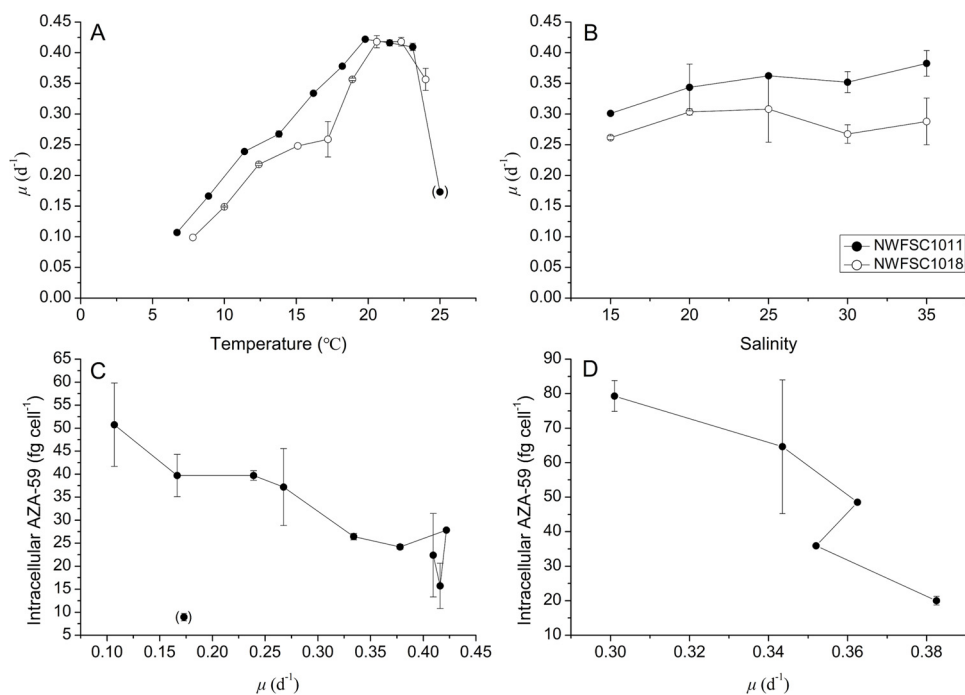
Fig. 1. Growth rate (μ) per day of *A. poporum* along a temperature (A) and salinity (B) gradient. The relationship of various growth rates in NWFSC1011 at different temperatures (C) and salinities (D) on intracellular AZA-59 content is shown. The dying culture at 25 °C is shown in parenthesis in panels A and C. Error bars indicate the range in replicate cultures. When no error bar is visible, it is contained within the symbol.

cell density were within the limits of the standard curve but with amplification in only one or two of the three technical replicates, the tests were repeated and re-evaluated. Samples with amplification in two or fewer of the technical replicates in repeat tests were considered to be negative.

3. Results

3.1. The effect of temperature and salinity on growth rate of A. poporum

The growth response of both strains of *A. poporum* (NWFSC1011 and NWFSC1018) with changing temperature was similar. Maximum growth (measured by fluorescence) occurred at temperatures ranging between about 20 °C and 23 °C (Supplementary Fig. 1 A–B; Fig. 1A). Specific growth rates of both strains increased from the lowest temperature tested (6.7and 7.8 °C, respectively, with growth rate ~ 0.10 d^{-1}) with increasing temperature up to 20 °C, with growth rate ~ 0.05 d^{-1} for each temperature step increase of approximately 2.0 °C, remained fairly stable from 20 °C to 23 °C (where maximum growth rates of ~ 0.42 d^{-1} were observed), and then declined at higher temperatures (Fig. 1A). Strain NWFSC1011 showed a steep decrease in growth rate above 23 °C (Fig. 1A) and did not survive at temperatures above 26 °C (data not shown). The growth rate of strain NWFSC1018 began to decrease above 22.3 °C (Fig. 1A). Strain NWFSC1011 reached a maximum abundance (100,670 cells L^{-1}) at 16.2 °C and strain NWFSC1018 (59,280 cells L^{-1}) at 20.6 °C (Supplementary Table 1). Maximum abundances were observed during stationary growth for strain NWFSC1011 (59,440 cells L^{-1}) at salinity of 20 and for NWFSC1018 (18,340 cells L^{-1}) at salinity of 15 (Supplementary Table 2).

Growth rates were not as sensitive to salinity as they were to temperature. The growth rate was 0.30 – 0.38 d^{-1} for strain NWFSC1011 and 0.26 – 0.31 d^{-1} for strain NWFSC1018 at salinity range of 15–35 (Fig. 1B). Overall, strain NWFSC1011 grew on average about 22% faster than NWFSC1018 across the salinity range (Fig. 1B). Both strains of *A. poporum* showed no growth at salinity of 10 and died within 12 days after inoculation.

3.2. The effect of temperature on the production of AZA-59 in A. poporum cultures

AZA-59 production and release in *A. poporum* was strain dependent and showed variable responses to temperature. In both strains, at most temperatures, AZA-59 cell quota (both intracellular and extracellular) was generally greater in stationary phase compared to exponential phase (Fig. 2A–D). Intracellular AZA-59 cell quota in strain NWFSC1011 generally decreased with increasing temperature, with cell quota ~ 18 times greater and 4 times greater at 8.9 °C than at 18.0–23.0 °C during stationary phase and exponential phase, respectively (Fig. 2A). Intracellular AZA-59 cell quota in strain NWFSC1018 was relatively constant with temperature during both exponential and stationary phases, ranging from 24 to 67 fg cell^{-1} (Fig. 2A–B).

Extracellular AZA-59 cell quota of both strains generally decreased with increasing temperature during both growth phases, except for at the highest temperature during stationary phase. Extracellular AZA-59 cell quota was higher in stationary phase compared to exponential phase for both strains. The cell quota of extracellular AZA-59 in strain NWFSC1018 was always higher than that measured in strain NWFSC1011 during stationary phase (Fig. 2C–D).

The percentage of intracellular versus extracellular AZA-59 was generally the same for both strains in exponential phase (Fig. 3A–B). In exponential phase, the AZA-59 in strain NWFSC1011 was over 90% intracellular while in strain NWFSC1018 the intracellular toxin ranged from 60% to 95%. The percentage of intracellular AZA-59 during stationary phase in both strains was slightly lower than in exponential phase at all temperatures except at the highest temperature tested for each strain (Fig. 3C–D), where the percentage of extracellular toxin was higher than the percentage of intracellular.

3.3. The effect of salinity on the production of AZA-59 in A. poporum cultures

The two strains of *A. poporum* showed differences in AZA-59 cell quota over the salinity range tested. In exponential phase, intracellular cell quota of both strains decreased with increasing salinity (Fig. 4). In stationary phase, maximum intracellular cell quota in strain NWFSC1011 increased to a peak (58 fg cell^{-1}) at salinity 30 and then
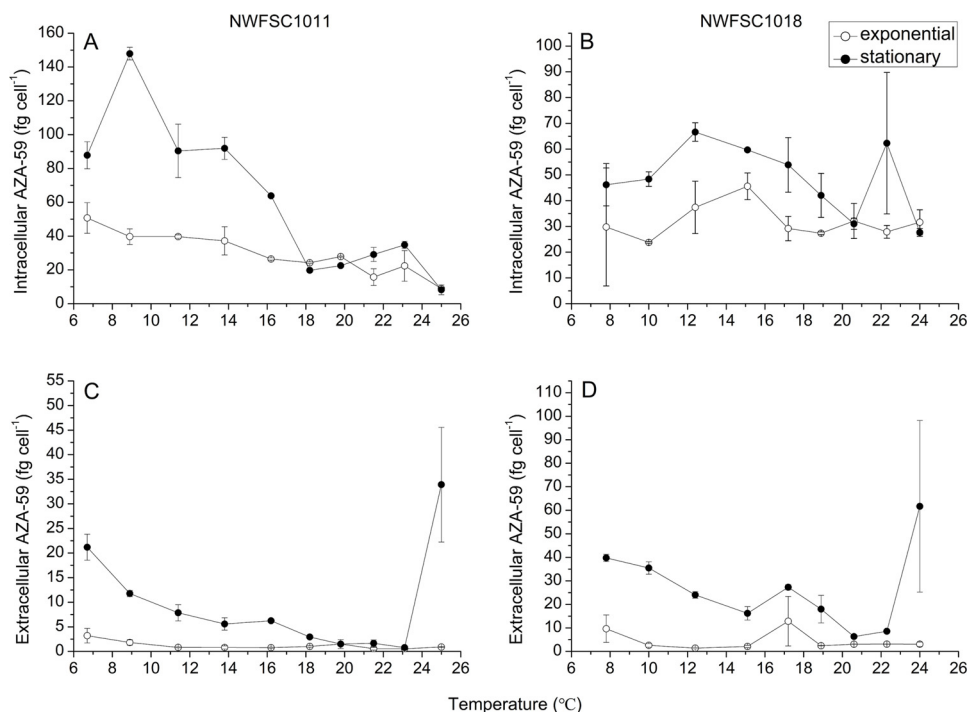
Fig. 2. Intracellular (A and B) and extracellular (C and D) AZA-59 cell quota in *A. poporum* strains NWFSC1011 (left panel) and NWFSC1018 (right panel) along a temperature gradient. Extracellular AZA-59 is expressed on a per cell basis for ease of comparison with intracellular AZA-59. Error bars indicate the range in replicate cultures. When no error bar is visible, it is contained within the symbol.

decreased with increasing salinity while intracellular cell quota in strain NWFSC1018 peaked at salinity 20 (147 fg cell^{-1}; Fig. 4A–B). The extracellular cell quota was higher in stationary phase compared to exponential phase over the entire range of salinities tested. The extracellular cell quota was variable in strain NWFSC1011, ranging from 2.0 to 5.3 fg cell^{-1} in exponential phase and 2.9–26.9 fg cell^{-1} in stationary phase (Fig. 4C). In NWFSC1018, the range in exponential phase was 3.1–12.3 fg cell^{-1} and in stationary phase from 46.9 to 84.3 fg cell^{-1} (Fig. 4C).

The relative percentages of intracellular compared to extracellular

AZA-59 for each salinity treatment is shown in Fig. 5. The percentage of extracellular toxin was higher in stationary phase versus exponential phase in nearly all salinity treatments for both strains. The only exception was the salinity 20 treatment in strain NWFSC1011. In both strains, the percentage of intracellular toxin in exponential phase showed a decreasing trend with increasing salinity (Fig. 5A–B). Stationary phase cultures showed little change in the proportion of intracellular versus extracellular toxin for strain NWFSC1011, however there was a slight increase in the percentage of extracellular toxin with increasing salinity for strain NWFSC1018 (Fig. 5C–D).
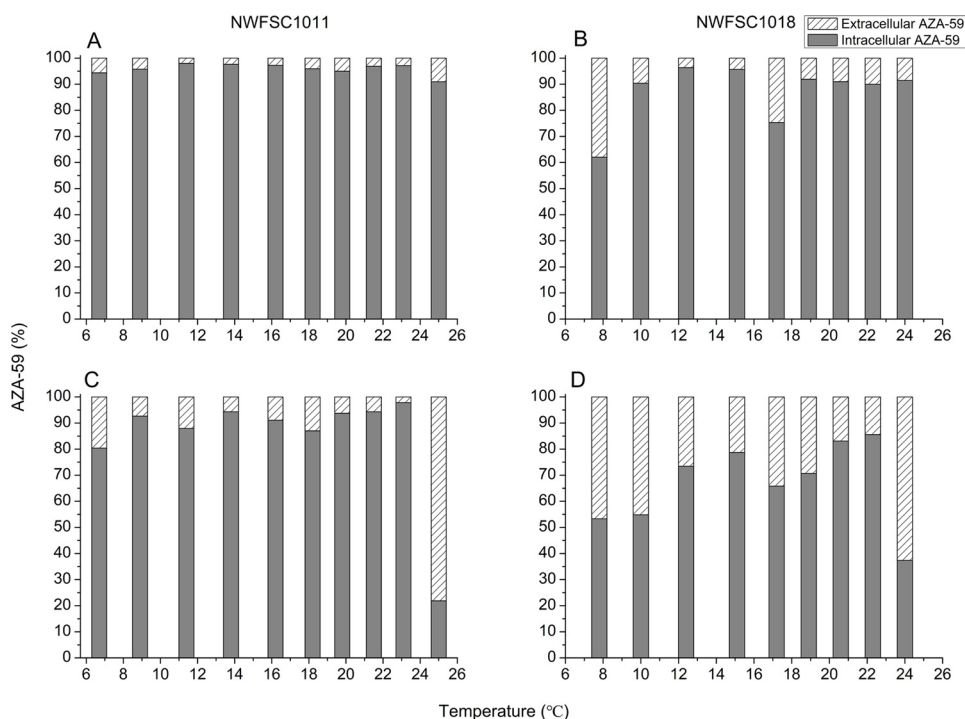

Fig. 3. Percentage of intracellular (solid bar) and extracellular (hatched bar) AZA-59 cell quota cell^{-1} in *A. poporum* strains NWFSC1011 (left panel) and NWFSC1018 (right panel) in exponential (A and B) and stationary (C and D) phase along a temperature gradient.
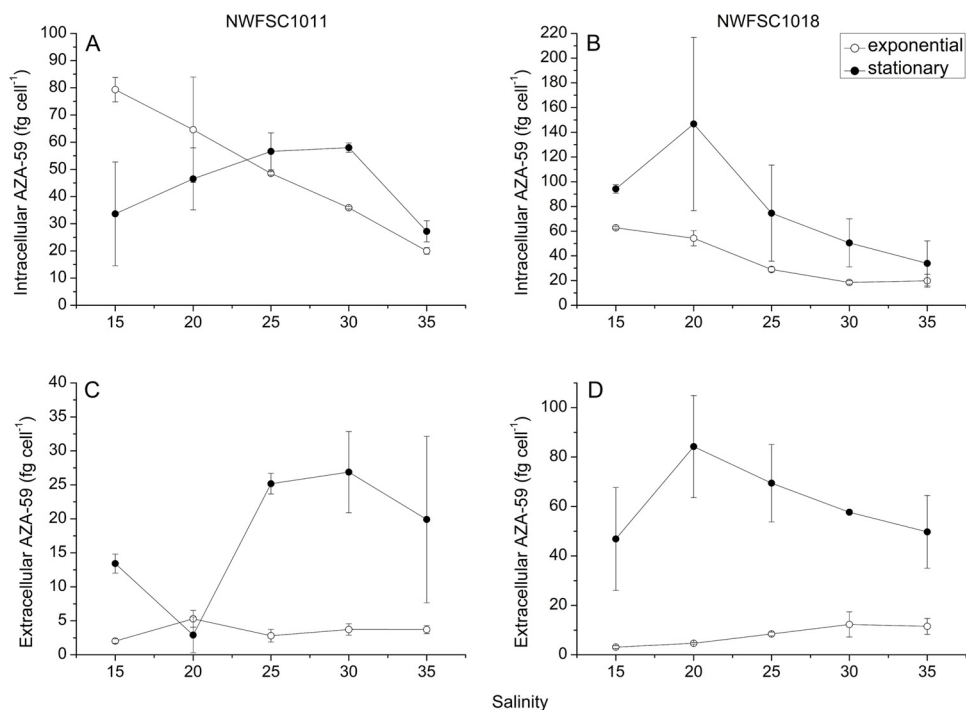
Fig. 4. Intracellular (A and B) and extracellular (C and D) AZA-59 cell quota in strain NWFSC1011 (left panel) and strain NWFSC1018 (right panel) in exponential (open circle) and stationary (filled circle) phase along a salinity gradient. Extracellular AZA-59 is expressed on a per cell basis for ease of comparison with intracellular AZA-59. Error bars indicate the range in replicate samples. When no error bar is visible, it is contained within the symbol.
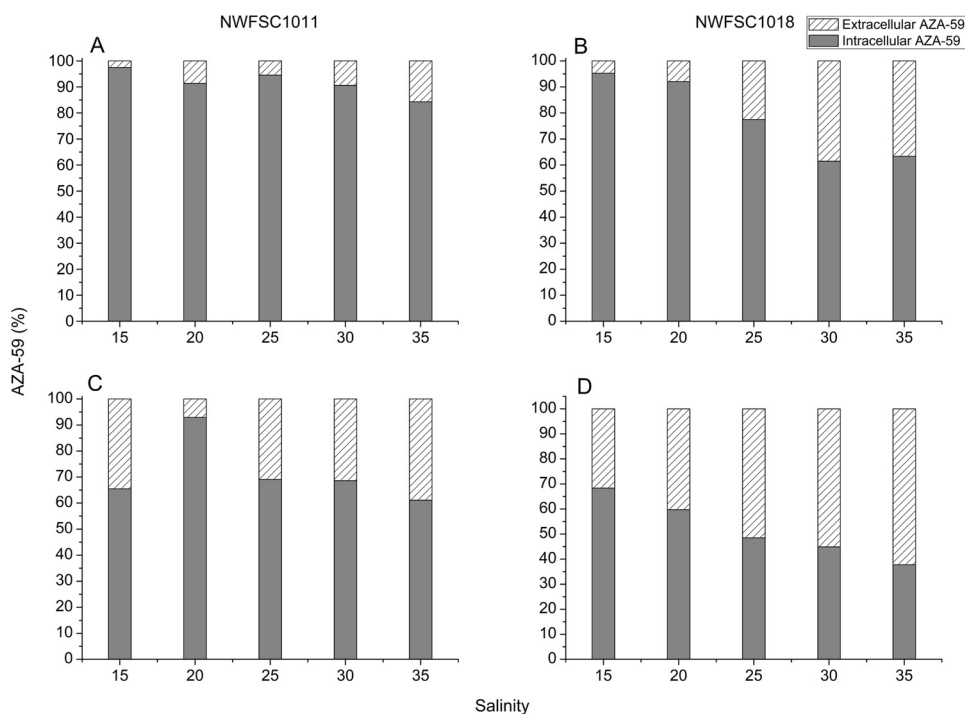

Fig. 5. Percentage of intracellular (grey bar) and extracellular (hatched bar) AZA-59 cell quota in *A. poporum* strains NWFSC1011 (left panel) and NWFSC1018 (right panel), in exponential (A and B) and stationary (C and D) phase along a salinity gradient.

3.4. Azadinium poporum in situ

Water samples collected in Sequim Bay from 2016 to 2018 had quantifiable levels of *A. poporum* during all three years of sampling (Fig. 6). Quantifiable densities of *A. poporum* were observed typically in June when water temperature ranged between 12–18 °C and salinity between 27–31 (Fig. 6). The highest densities of *A. poporum* were observed in late June 2016 (417 cells L^{-1}) with an associated temperature of 17.1 °C and salinity of 30, and in early June 2018 (562 cells L^{-1}) with an associate temperature of 13.2 °C and salinity of 29. *Azadinium*

poporum were below the limit of quantification in July and August of 2016 and August and September of 2017, but in 2018, *A. poporum* was only observed in May and June.

4. Discussion

The first description of *A. poporum* was based on samples from the southern North Sea off the Danish coast in 2011 (Tillmann et al., 2011). Since that time, this species has been detected around the world (Tillmann, 2018b). In a recent study by Kim et al. (2017), several
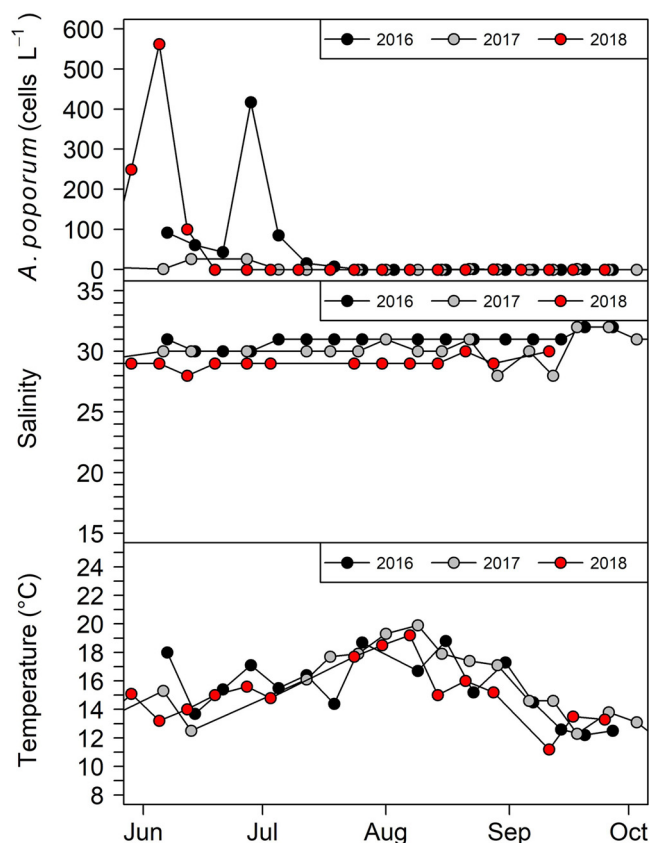
Fig. 6. *Azadinium poporum* abundance in Sequim Bay, Puget Sound, from late May-Oct, in 2016, 2017, and 2018 using quantitative real-time PCR. Temperature and salinity measurements were made at the same time that water samples were collected for *A. poporum* analysis. The range of temperature and salinity used in the y-axis legends are the same as those used in the Temperature Gradient Bar experiments.

Azadinium species were isolated from Puget Sound and a new toxin, AZA-59, was identified in cultured strains of *A. poporum*. Understanding the factors affecting the growth and toxicity of *A. poporum* relative to changing salinity and temperature will allow coastal shellfish managers to anticipate future windows of opportunity for the success of this species and also the risk of azaspiracids to human health.

4.1. Effect of temperature on AZA cell quota and toxin release

The proportion of toxin maintained within the cell compared to that which is released, or passively diffused across cell membranes, is an important consideration when estimating the risk of human exposure. In the present study, the AZA-59 cell quota of strain NWFSC1011 decreased 18-fold from 8.9 °C to 25 °C during stationary phase growth (Fig. 2A). This inverse relationship between toxin cell quota and temperature also has been found in other toxic dinoflagellates (Etheridge and Roesler, 2005; Navarro et al., 2006; Guerrini et al., 2007; Bill et al., 2016). In contrast, the AZA-59 cell quota for NWFSC1018 remained relatively constant during stationary growth, ranging from 31 to 67 fg cell^{-1} (Fig. 2A). This illustrates that strain differences need to be considered, including testing multiple strains for their potential toxin quotas which has been done for strains of several *Azadinium* species (Kim et al., 2017; Luo et al., 2017).

Extracellular AZA-59 cell quota in both strains during stationary phase were the highest not only at the lowest but also at the highest temperatures (Fig. 2C–D, solid circles), suggesting that release or diffusion of toxin across the cell membrane is higher at temperature extremes. At high temperatures where cell densities declined in stationary

phase, increases in extracellular AZA were due primarily to dying cells having poor membrane integrity. Both strains of *A. poporum* showed a greater proportion of AZA-59 in the extracellular fraction in stationary phase compared to exponential phase (Fig. 3A–D). A study by Jauffrais et al. (2013b) found that both live cells of *A. spinosum* and dissolved AZAs *in situ* are important sources of shellfish contamination and thus relevant to human health. This work, together with other studies confirming the accumulation of dissolved OA and DTX1 by shellfish (e.g., Li et al., 2018), demonstrates that dissolved toxin must be considered when estimating the impacts of azaspiracids to human health.

The balance between the temperature for maximum growth versus the temperature associated with the highest toxin quota must be considered when estimating risk. Both strains of *A. poporum* grew in a broad range of temperatures (Supplementary Fig. 1). However, maximum growth ($\mu > 0.35$ d^{-1}) occurred between 16.2–23.1 °C for NWFSC1011 and 18.9–24.0 °C for NWFSC1018 and growth was limiting by temperatures less than 16.2 and 18.9 °C for the respective strains (Fig. 1A, C). These differences in optimal temperature for growth observed between the two strains are notable as they were germinated and isolated from the same sediment sample.

Temperature is an important and close regulator of phytoplankton growth (Sherman et al., 2016). When cell growth is regulated differently than the production rate of a secondary metabolite, higher or lower cell quota of these compounds may occur. This becomes obvious when cell growth is limited, e.g. by temperature, or when cells are in stationary phase but toxin production continues. The resulting elevated toxin cell quota then is caused by accumulation and not necessarily by changes of the actual rate of production (e.g. Jauffrais et al., 2013a). This is the case in the present study. The AZA-59 cell quota was quite variable at different temperatures (Fig. 2A, B) although strain NWFSC1011 showed higher AZA-59 per cell at lower temperatures (Fig. 2A), corresponding to lower growth rates and higher toxin quotas (Fig. 1C) at those lower temperatures (Fig. 2C). Observation of an 18-fold difference in AZA-59 cell quota for *A. poporum* strain NWFSC1011 between 8.9 °C and 25 °C is similar to what has been observed in *A. spinosum*, where the AZA cell quota was 20-fold elevated at 10 °C compared to 18–26 °C (Jauffrais et al., 2013a).

4.2. Effect of salinity on AZA cell quota

The euryhaline growth potential may allow *A. poporum* to expand its habitat to inshore waters, including estuaries and bays, and to take the advantage of, or not be affected by, temporary freshwater inputs, such as strong rainfall derived runoff (Lim and Ogata, 2005). This is consistent with the fact that *A. spinosum* was present in Irish bays (Salas et al., 2011) or Norwegian fjords (Tillmann et al., 2018) where salinity is highly variable due to freshwater inflow or heavy rainfall. However, both Puget Sound strains did not survive at salinity of 10. This salinity will be most common near the mouths of estuaries, and not typical in shellfish growing regions such as the Sequim Bay location shown in Fig. 6. Thus, this low salinity is not expected to be a major factor in the restriction of *Azadinium* growth near shellfish growing regions in Puget Sound. Similar salinity-dependent survival was observed in *A. spinosum* (Jauffrais et al., 2013a). However, overall growth rates for both strains used in the present study were not as dependent on changing salinity as they were to temperature (Fig. 2B). This suggests that temperature plays a more important role in seasonal change of *A. poporum* and azaspiracid production.

4.3. Azaspiracid risk in Puget sound

All of the global cases of azaspiracid poisoning have been caused by shellfish from the western coast of Ireland, including mussels from Killary Harbor (1995), mussels from Arranmore Island (1997), and mussels and scallops from Clew Bay and Bantry Bay in 1998 and 2000, respectively (Twiner et al., 2008). The primary producer of AZA-1 and

-2 was identified as *A. spinosum* (Tillmann et al., 2009). A strain (SM2) of *A. spinosum* from Bantry Bay, Ireland, showed a production of AZA (1 + 2) from ~16 to 30 fg cell^{-1} (Salas et al., 2011). A strain of *A. spinosum* from the Scottish east coast (3D9), grown in bioreactors set to variable growth rates, was found to produce a maximum cell quota of 98 fg AZA(1 + 2) cell^{-1} at a growth rate of 0.15 day^{-1}. At a similar growth rate observed at a temperature of 8.9 °C, the Puget Sound strain NWFSC1011 of *A. poporum*, achieved a maximum of 148 fg cell^{-1} of AZA-59 at 8.9 °C once the cells reached stationary growth (Fig. 2A). These specific toxin quotas are among the highest reported in all *Azadinium* strains to date (Krock et al., 2009; Jauffrais et al., 2012, 2013; Li et al., 2016; Kim et al., 2017), however because AZA cell quotas are highly variable among strains, during different growth phases, and under different environmental conditions, comparisons are fraught with problems. Assuming that results from culture experiments reflect the potential cellular AZA quotas *in situ*, the remarkably high AZA cell quota of local *A. poporum* strains increase the risk of AZA poisoning in Puget Sound. The balance of cellular toxin quotas with the ability of cells to reach a critical density and the absolute toxicity of the azaspiracid congener in a specific region all contribute to the consideration of risk. A separate study on mouse exposure to purified AZA-59 is in progress to assess its toxicity. However, to date there is no evidence of AZA-59 resulting in poisoning in the region. In fact, the maximum density of *A. poporum* observed in Sequim Bay, WA, using qPCR, was 562 cells L^{-1} (Fig. 6), several orders of magnitude less than the maximum cell quota achieved in culture (Supplementary Table 1). Although the density of *A. poporum* at this site were most often low or undetectable, the rare higher abundance of cells was foreshadowed by levels above background during the prior weeks (Fig. 6 data from 2016 and 2018), illustrating that regular monitoring will confer some predictability.

Based on deployment of passive solid-phase resin samples at 29 sites throughout Puget Sound during 2014–2015 and detection of measurable but low amounts of AZA-59 in shellfish in 2016 and 2017 (unpublished data), the current risk of human exposure to AZA is regarded as low (Kim et al., 2017). However, given that aquatic ecosystems are changing due to climatic factors, and temperature and stratification will increase due to warming of the planet (e.g. Hallegraeff, 2010; Paerl and Sott, 2010), estimated to increase by 2.3–3 °C by 2050 in one climate model (Mauger et al., 2015), the present study provides baseline data for a better understanding of the environmental controls of toxin production needed to evaluate the future risk of AZP in the region.

In summary, *A. poporum* grew across a temperature range from 6.7 °C to 25.0 °C and salinities ranging from 15 to 35. Temperature played a larger role than salinity in determining *A. poporum* growth rates in isolates from Puget Sound. Higher intracellular AZA-59 cell quota of *A. poporum* always occurred at lower temperature in stationary phase. Cellular toxin quotas were strain dependent in both the temperature and salinity treatments. It is clearly seen that both growth rates and toxin cell quota are critical for the estimation of exposure risk. However, the present study only touches the surface to complete our understanding of the multitude of factors that will cause human exposure to AZA in the region. Future work must include continued consistent monitoring of known "hotspot" sites together with multifactorial studies to test the suite of factors contributing to a risk of *Azadinium* bloom formation, such as grazing potential, nutrient inputs, stratification intensity, and competition among phytoplankton species.

Declaration of Competing Interest

None.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.hal.2019.101665.

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