Prevalence of Dissolved Homarine in Puget Sound and North Pacific

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ABSTRACT

Due to the analytical challenges faced during metabolomic analyses of seawater, there has been very little research on the distribution and cycling of dissolved homarine in natural waters. Therefore, the role of homarine as a dissolved metabolite is unknown. This study uses water samples collected from the North Pacific Transition Zone, North Pacific Subtropical Gyre, and Puget Sound to evaluate, for the first time, the concentrations of homarine in different sea water samples. Metabolites were extracted using cation-exchange solid phase extraction (SPE) followed by high-performance liquid chromatography mass spectrometry (HPLC-MS). Homarine concentrations were compared to three other metabolites – dimethylsulfoniopropionate (DMSP), trimethylamine-n-oxide (TMAO), and proline – as also compared to physical, biological, and chemical factors. Homarine was most prevalent in Puget Sound with a concentration of 63.3 nM and least prevalent in the North Pacific Subtropical Gyre with a concentration of 0.8 nM. Homarine was negatively correlated with salinity and temperature but positively correlated with chlorophyll a and nitrate concentrations. This is similar to distributions with TMAO but opposite distribution with DMSP and proline. Between two different latitudes, North Pacific Subtropical Gyre versus the North Pacific Transition Zone, homarine made up 0.3% of the dissolved organic N pool in the lower latitude but 1.6% in the higher latitude. Homarine's dissolved to particulate ratio also increased from 0.49 to 0.91. This data leads to a broad prediction that there may be a greater source to sink ratio or lower affinity for homarine in the NPTZ.

PLAIN LANGUAGE SUMMARY

Metabolites are compounds produced by cells for metabolic processes and various functions such as signaling, growth, and development. Observing the distribution of different compounds in the ocean environment is crucial for understanding certain activities that marine microorganisms are undergoing with response to environmental stresses or molecular interactions. Homarine is an organic nitrogen compound produced by various marine phytoplankton but has never been studied in the natural environment due to analytical challenges. This study looks into how dissolved homarine from April 2016 in Puget Sound, North Pacific Transition Zone (NPTZ), and North Pacific Subtropical Gyre (NPSG) vary in concentration. This study looks into how homarine correlates with biological, physical, and chemical factors to understand what variables may be relevant to homarine's distribution. Comparisons include three other supplemental metabolites - dimethylsulfoniopropionate (DMSP), trimethylamine-n-oxide (TMAO), and proline. Results showed that dissolved homarine was most abundant in Puget Sound and least abundant in the Subtropical Gyre. Homarine showed a negative correlation with salinity and temperature but a positive correlation with chlorophyll a and nitrate concentrations. Between the NPTZ and NPSG stations, NPTZ being the higher latitude, homarine was found to make up a greater percentage of the dissolved organic nitrogen (DON) pool and had a greater dissolved to intracellular concentration ratio. Homarine's percentage of the DON pool was over three times greater than the other compounds in the NPTZ while it was average in the NPSG. This leads to a broad prediction that there may be a greater source to sink ratio or lower affinity for homarine in the NPTZ. Findings from this research show how homarine can vary with different environmental conditions, furthering the understanding of how homarine's role as a dissolved metabolite is essential for marine organisms.

INTRODUCTION

Metabolites are the products and intermediate compounds produced by cells during metabolic processes. Cells produce metabolites for various functions such as signaling, growth, and development. Investigations through metabolomic approaches can allow scientists to understand the molecular pathways cells undergo when conducting certain activities or are exposed to environmental or physiological stresses (Ragg et al., 2020; Dickson and Kirst, 1986; Lavoie et al., 2018). Exometabolomics involves the study of extracellular metabolites to understand intercellular interactions or how cells interact with their environment. Cells may release metabolites as dissolved organic matter (DOM) for various reasons such as communication or nutrient acquisition. They may also be released as a result of grazing by zooplankton (sloppy feeding), viral lysis, or deterioration by age (Nagata, 2008). Identifying and understanding the function and purpose of specific metabolites is important because they can be used as biomarkers to detect environmental exposures and behavioral responses to stresses such as infection, lower pH, and changing salinities (Santos et al., 2020; Orrego et al., 2005; Yin, et al., 2020).

Homarine is a metabolite commonly produced intracellularly in marine phytoplankton including *Tisochrysis lutea*, *Dunaliella salina.*, *Platymonas subcordiformis,* and various diatoms (Dickson and Kirst, 1986; Iglesias et al., 2019; Scholz, 2014). Homarine concentrations in *P. subcordiformis* increased with increasing salinity, indicating that homarine plays a physiological role in osmotic regulation (Dickson and Kirst, 1986). Scholz (2013) found that five species of marine microphytobenthic diatoms accumulated more homarine and DMSP in low-temperature exposures while proline accumulated more in temperatures deviating from 18° C. This data indicates that homarine can play a role in cryoprotection for cells. In nitrogen limited conditions, DMSP production was favored over homarine and other nitrogen containing osmolytes (Keller et al., 1999).

Previous research found homarine to be one of the most abundant metabolites in environmental samples (Heal, 2020). However, due to homarine being a zwitterion, a compound that has equal numbers of positively and negatively charged atoms, there are analytical challenges in measuring homarine in the dissolved phase with past techniques that utilize a styrene–divinylbenzene copolymer (PPL) resin. PPL is better at retaining large uncharged compounds but is inefficient in retaining zwitterions and positively charged cations. Thus, there are no publications on the role of free homarine in the open ocean and little literature on the importance of homarine in phytoplankton, making the cycling and distribution of this metabolite poorly understood and uncertain.

Nitrogen-containing organic molecules, such as homarine, contribute to a major fraction of nitrogen in the ocean and become a bioavailable source of nitrogen for most primary producers after bacterial mineralization. It has become increasingly acknowledged that, in addition to heterotrophic bacteria, autotrophic microbes are also utilizing similar inorganic and organic N substrates (Mulholland & Lomas, 2008). While autotrophs use CO_2 as a source of carbon, heterotrophs use DOM and release CO_2. Because of this, it is important to answer questions on the role of homarine as a dissolved metabolite in order to understand its contribution to the global C budget. By understanding specific compounds in terms of who they're released by, why they're released, and who utilizes them, we can better understand how the dissolved organic nitrogen (DON) pool and microbial communities can promote harmful algal blooms or enhance phytoplankton survival in oligotrophic environments. A potential way that homarine may be cycled is from heterotrophs assimilating the dissolved homarine for nitrogen and carbon sources or for osmotic regulation. Methylated amines, such as homarine, may be sources of nitrogen in nitrogen-depleted environments for heterotrophs. This can be compared with trimethylamine-n-oxide (TMAO), a zwitterion metabolite that can be used by marine heterotrophic bacteria as an exogenous source of nitrogen and promote adenosine triphosphate (ATP) production (Lidbury et al., 2015).

In this study, I ask the questions: Do physical (salinity and temperature), chemical (nitrogen availability) and biological (chlorophyll) factors correlate with the prevalence of dissolved homarine in natural water environments? How do homarine concentrations compare to concentrations of DMSP, proline, and TMAO? How do homarine concentrations vary between estuarine and ocean water samples in comparison to dissolved organic carbon (DOC) and dissolved organic nitrogen (DON)? And how does dissolved homarine compare to particulate homarine availability? These questions frame my hypothesis of **homarine distribution being affected by physical, chemical, and biological factors**. I expect homarine to be highest in locations with higher biomass and nitrogen availability. I also expect differences in homarine concentrations between different latitudes due to varying temperatures, nitrogen availability, and phytoplankton abundance. Lower temperatures, higher salinity, and higher nitrogen availability would lead to higher homarine concentrations. Because both homarine and TMAO are zwitterions and methylated amines, I predict for them similar affinity parameters, thus similar distributions. Lastly, I predict homarine and proline to have similar distribution patterns while DMSP would have the opposite due to their varying relationships with nitrogen availability (Keller et al., 1999).

METHODS

Data Acquisition

For this research, I used metabolomic analyses of samples taken April 2016 from the North Pacific Transition Zone (NPTZ), North Pacific Subtropical Gyre (NPSG) at Station ALOHA, and Puget Sound. Both North Pacific samples were taken at a depth of approximately 10 m using a CTD rosette and filtered through a 0.2 micron filter (Figure 1) (Table 1). The Puget Sound sample was taken off the coast of Golden Gardens at a depth of approximately 8 meters. In addition, I've included water samples from a *Phaeodactylum tricornutum* (PT) culture in University of Washington's Microbial Metabolomics Research Center in analyses.

The technique used for conducting this experiment was a cation-exchange solid phase extraction, derived from Glabonjat's (2017) methodology of extracting and determining cationic arsenicals in seawater. Each sample was acidified using nitric acid to a pH of 2 and loaded onto a 50 mL, 300 x 18 mm polystyrene pipette. The pipette was packed with 40 mL of DOWEX 50WX8, H+ form, resin beads. To remove any residual salts, the column was first rinsed with 50 mL of water before adding and eluting 1M of ammonia. Eluted solvents were collected in 10 mL fractions. The sample where the effluent becomes basic was then combined with fraction eluted before and the two fractions eluted after to be dried down and reconstituted. For each sample, there were groups were made with no spike (NS), a spike before extraction (SBe), and a spike after extraction (SAf). Each spike contained 25 nM of each specific compound. Extracted samples are analyzed with a Q-Exactive orbitrap mass spectrometer and incorporate a reverse phase high-performance liquid chromatography (HPLC) column. Resulting mass spectrometric data are collected in positive and negative ion modes with a mass range of 50-800 m/z.

To process the metabolomics data, raw data files were converted to .mzXML format through MSConvert (Chambers et al. 2012). I processed each compound using Skyline software for peak area integration (Adams et al., 2020). Quality control was checked using Best-Matched

Internal Standard Normalization technique to account for ion suppression and instrument drift (Boysen et al., 2018).

Quantifying Metabolite Concentrations

For each sample, NS groups are quantified as the background concentration times the extraction efficiency (EE), SBe groups are EE multiplied by the sum of 25 nM and the background concentration, and SAf groups are 25 nM plus the product of EE and the background concentration. EE varies for each sample's compound. NS has the smallest spectral signal, assuming that the background concentration is below 25 nM. SBf is going to be in the middle due to the fixed value, EE, reducing the amount of 25 nM spike retained. SAf should have the largest spectral signal in every case.

After exporting the spectral integrations for each sample's compound, I first calculated EE using the following equation:

$$\mathbf{EE} = \frac{SBe - NS}{SAf - NS} \tag{1}$$

After calculating EE, I calculated the response factor, a fixed ratio that tells us how much signal is made for an amount of compound. This value is fixed for each sample's compound and is calculated using the following fraction:

$$\textbf{Response Factor} = \frac{25\ nM\ of\ Compound}{SAf - NS} \tag{2}$$

Finally, to quantify the environmental concentration, I calculated:

$$\textbf{Concentration} = \frac{NS}{EE} \times \text{Response Factor} \tag{3}$$

Databases

North Pacific data, except for particulate organic carbon (POC), particulate organic nitrogen (PON), and particulate homarine, was exported from the Simons CMAP Database (Table 2). Each value was an average of all data taken from April 2016 at longitude (-158.25°)-(-157.75°) and a depth of 5-15 m. NPSG data took the average value from latitudes of (22.25°)-(22.75°) while NPTZ data took the average value from a latitude 37.25°. All particulate data is taken from Heal (2020). Puget Sound data was taken from the Salish Cruise of April 2017 at station P28, values averaged from depths of 5-10m. As an exception, DOC data was requested personally from the UW Marine Chemistry Laboratory through Aaron Morello.

RESULTS

The distribution of dissolved homarine between Puget Sound, NPTZ, and NPSG showed that homarine was most abundant in Puget Sound with a concentration of 63.3 nM and least abundant in NPSG with a concentration of 0.8 nM (Figure 2A). This distribution is also similar to TMAO's with 30.7 nM in Puget Sound and 0.5 nM in NPSG (Figure 2B). In contrast, while proline's distribution also showed highest abundance in Puget Sound with a concentration of 2.6 nM, proline was least abundant in NPTZ with a concentration of 0.8 nM. DMSP also had a different distribution where most dissolved DMSP was found in NPTZ with a concentration of 5.6 nM while Puget Sound and NPSG had similar abundances of 1.7 nM and 1.4 nM respectively. PT culture sample data is included to demonstrate that all the metabolites are released by *P. tricornutum.*

Of the three sample locations, metabolites are overall most abundant in Puget Sound while metabolites are least abundant in NPSG (Figure 3A). When looking at the relative percentages, homarine makes up about 64.3% of the metabolite pool in Puget Sound and decreases to 62.4% in NPTZ, though is roughly the same (Figure 3B). However, in NPSG, the relative percentage drops to 21.4%. Similarly, TMAO makes up 31.2% of the pool in Puget Sound, decreases to 14.4% in NPTZ, and decreases to 12.6 in NPSG. This is opposite of proline as proline makes up 2.7% and 2.9% of Puget Sound and NPTZ respectively but increases in relative percentage to 27.9% in NPSG. This pattern is also prevalent in DMSP as DMSP makes up 1.7% of the pool in Puget Sound, 20.3% in NPTZ, and 38.1% in NPSG.

Evident correlations between the metabolites and physical factors are that homarine and TMAO concentrations decreased with increasing temperature and salinity (Figure 4A & 4B). DMSP showed no correlation with temperature or salinity while proline showed a negative correlation with salinity but no correlation with temperature. For biological factors, homarine, TMAO, and proline concentrations increased with increasing biomass (Figure 4C). DMSP showed no correlation. When looking into chemical factors, only DMSP showed any correlation with ammonium, decreasing with increasing ammonium concentrations (Figure 4E) (Table 3). All metabolites except for DMSP showed a positive correlation with nitrate concentrations (Figure 4D).

When looking at the percentages each metabolite made up of the DON and DOC pool, each compound had a percent less than 0.5% except for homarine in NPTZ (Figure 5). Homarine was about 1.6% of the DON and DOC pool. In Puget Sound, all compounds were about 0.1% or less of the DOC pool except for homarine which was about 0.5% of the DOC pool.

The ratio of dissolve to particulate homarine concentrations increased with increasing latitudes from NPSG to NPTZ with enrichment values of 0.49 to 0.91 (Table 4). In contrast, proline's dissolve to particulate ratio decreased from NPSG to NPTZ with enrichment values of 3.9 to 1.4.

DISCUSSION

Homarine was found most abundant in Puget Sound and least abundant in the Subtropical Gyre (Figure 3). Puget Sound is also the location that had the highest biomass and nitrate concentration which allows me to support my hypothesis that homarine would be highest in Puget Sound due to higher biomass and nitrogen availability. Varying concentrations between the transition zone and subtropical gyre also supports my hypothesis that homarine concentrations would be different due to varying temperatures, nitrogen availability, and diatom abundance at different latitudes. Diatom abundance is dependent on multiple factors including temperature and nutrient availability (Kingston & Birks, 1990 ; Korsman & Birks, 1996). Due to the downward Ekman pumping that occurs in the subtropical gyre, there is a limit of nutrient input to the mixed layer for diatom growth, thus a lower homarine concentration in the subtropical gyre. This distribution is also consistent with Scholz (2013) study showing that diatoms accumulate homarine in lower temperatures.

Because dissolved homarine was found at higher concentrations with lower temperatures and higher nitrogen availability, the data is able to support my hypothesis (Figure 4). However, my hypothesis that homarine would increase with higher salinities could not be supported. This is because, while homarine is found to be an osmotic regulator for cells, its prevalence is also reliant on other factors such as biomass and nitrogen availability (Dickson and Kirst, 1986). These factors become more available in environments closer to the coast. In this case, Puget

Sound is representative of coastal waters. Because there are only 3 sample location to extract data from, temperature and salinity are used as proxies for latitude and distance from the coast respectively. However, it is important to note that both Puget Sound and the North Pacific are both salty environments and are still exposing osmotic stress to microbial cells. Despite decreasing salinity, particulate homarine also increases when moving from the subtropical gyre to the transition zone, supporting the prediction that osmotic regulation isn’t a primary driving force of particulate homarine in the North Pacific (Table 4).

Throughout this study, homarine concentrations were much higher than TMAO concentrations, but their distribution patterns were very similar. This supports my hypothesis of there being a similar affinity for both homarine and TMAO as methylated amines due to their zwitterion characteristics, giving them both desirable characteristics as supplementary energy sources for heterotrophic bacteria. Catabolism of methylated amines, such as TMAO, can be used as sources of nitrogen or carbon and are efficient in producing biomass to give ecological advantage for heterotrophic bacteria (Moran and Miller, 2007). Because homarine and TMAO have very similar distributions, this strengthens the hypothesis that homarine may play a significant role in enhancing cell survival.

Homarine and proline had opposite distributions which goes against my hypothesis of homarine and proline having similar distributions due to having similar positive correlations with nitrogen availability. Despite proline being an organic nitrogen compound, it was found at higher concentrations in the subtropical gyre versus the transition zone. The transition zone has less biomass and less nitrogen availability so this indicates that there is a variation in source and sink variables between proline and homarine. As a proteinogenic amino acid, proline is required for biosynthesizing proteins. In contrast, homarine is a zwitterionic osmolyte of many that are available. With this in mind, nitrogen-limited environments can cause differing distributions between proline and homarine due to cells producing proline out of necessity but utilizing a compound other than homarine for osmotic regulation.

DMSP didn’t have an exact opposite distribution compared to homarine as I predicted but their distribution and correlation to other factors were very different. Homarine showed a correlation with every factor but ammonium concentration, DMSP only showed a correlation with ammonium concentration, decreasing with increasing concentrations. According to Keller (1999), DMSP production became favorable over other nitrogen-containing osmolytes when in nitrogen-limited conditions. In NPSG, the location with the lowest nitrogen availability, DMSP made up the highest relative percentage (Figure 3b). In Puget Sound, the location with the highest nitrogen availability, DMSP made up the lowest relative percentage. These findings support my hypothesis that DMSP would be found at higher concentrations than nitrogen-containing osmolytes in nitrogen-limited environments and show opposite distributions with homarine.

When comparing percent DON and DOC values, homarine greatly surpassed the other compounds in the transition zone (Figure 5). And despite homarine concentrations being highest in Puget Sound, homarine still had a higher percent of DOC value in NPTZ. This leads to the question: why does homarine make up such a big percentage of the DON and DOC pool in NPTZ compared to the other metabolites? A broad prediction would be that the source to sink ratios increase with increasing nutrient availability or that there is a lower affinity for homarine in the transition zone.

Between the subtropical gyre to the transition zone, not only is homarine becoming more concentrated at the transition zone, but homarine’s dissolved to particulate phase ratios are also increasing (Table 4). Interestingly, proline has an opposite shift where proline's enrichment values decreased at the transition zone. Differences in enrichment between the two locations can imply that there may be differing significant factors that cause homarine to be released. NPSG has lower nutrient availability compared to the transition zone which can lead to more exudation due to photosynthate accumulation. With lower nitrogen availability, there may also be a greater affinity for homarine to undergo bacterial mineralization and support heterotrophic growth, leading to the lower observed enrichment value. On the other hand, NPTZ has more nutrient availability, thus higher productivity and biomass. With a higher biomass there can be more homarine release through sloppy feeding or viral lysis, leading to a greater accumulation in the transition zone versus the subtropical gyre. To evaluate this, we would need to further consider bacteria and phytoplankton community composition differences between these two locations.

CONCLUSION

The data from this study supported the hypothesis that homarine would vary between Puget Sound and the North Pacific due to various physical, biological, and chemical factors. Homarine was found to be more prevalent at higher latitudes and closer to the coast while also making up a greater percent of the DON pool in higher latitudes. Because only 3 locations at a single depth were analyzed, it is difficult to say that dissolved homarine is truly affected by these factors. Therefore, this study can be improved through metabolomic analyses for homarine in a wider transect with multiple depths. Research of homarine can also be enhanced through the annotation of genes involved in homarine catabolism, thereby enabling the identification of organisms that utilize homarine. Findings from my research can enhance the importance of studying homarine by leading us to further question why homarine prevalence is so variable and what organisms cause or are affected by homarine concentrations.

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TABLES

TABLE 1. Coordinates of water samples collected for metabolomic analysis.

SAMPLE NAME	LATITUDE	LONGITUDE
North Pacific	37.2994	-158.0007
ALOHA	22.45	-158
Puget Sound	47.69049	-122.4135

TABLE 2. Specific database sources for each sample location's data.

Sample Location	**Data**	**Database**	**Title**
NPSG and NPTZ	DOC, DON, Nitrate, Ammonium	Simons CMAP	Darwin Biogeochemistry Climatology Model
NPSG and NPTZ	Diatom Abundance	Simons CMAP	Darwin Biogeochemistry 3 Day Averaged Model of Phytoplankton
NPSG and NPTZ	Salinity, Temperature, and Chlorophyll a	Simons CMAP	2016 SCOPE Gradients, Downcast CTD Data
Puget Sound	Temperature, Salinity, Nitrate, Ammonium, Chlorophyll a	NANOOS	Salish Cruise April 2017
Puget Sound	DOC	UW Marine Chemistry Laboratory	*Data requested by email*

Table 3. Coefficients (R) for each variable and compound's correlation from figure 4. Compound vs variable relationships that showed relevant correlations are highlighted in orange.

VARIABLE	COMPOUND	COEFFICIENT (R)
Temperature	Homarine	0.829
	Proline	0.575
	TMAO	0.735
	DMSP	0.390
Salinity	Homarine	0.993
	Proline	0.969
	TMAO	0.999
	DMSP	0.301
Nitrate	Homarine	0.990
	Proline	0.975
	TMAO	0.999
	DMSP	0.325
Ammonium	Homarine	0.485
	Proline	0.141
	TMAO	0.347
	DMSP	0.766
Chlorophyll	Homarine	0.977
	Proline	0.989
	TMAO	0.998
	DMSP	0.397

Table 4. Summary of dissolved and particulate metabolite concentrations, measured in nM, and enrichment to quantify ratio of dissolved to particulate concentrations in NPSG vs NPTZ.

	NPSG			NPTZ		
Metabolite	Dissolved	Particulate	Enrichment	Dissolved	Particulate	Enrichment
Homarine	0.77	1.6	0.49	17	19	0.91
Proline	1	0.26	3.9	0.8	0.57	1.4

FIGURES

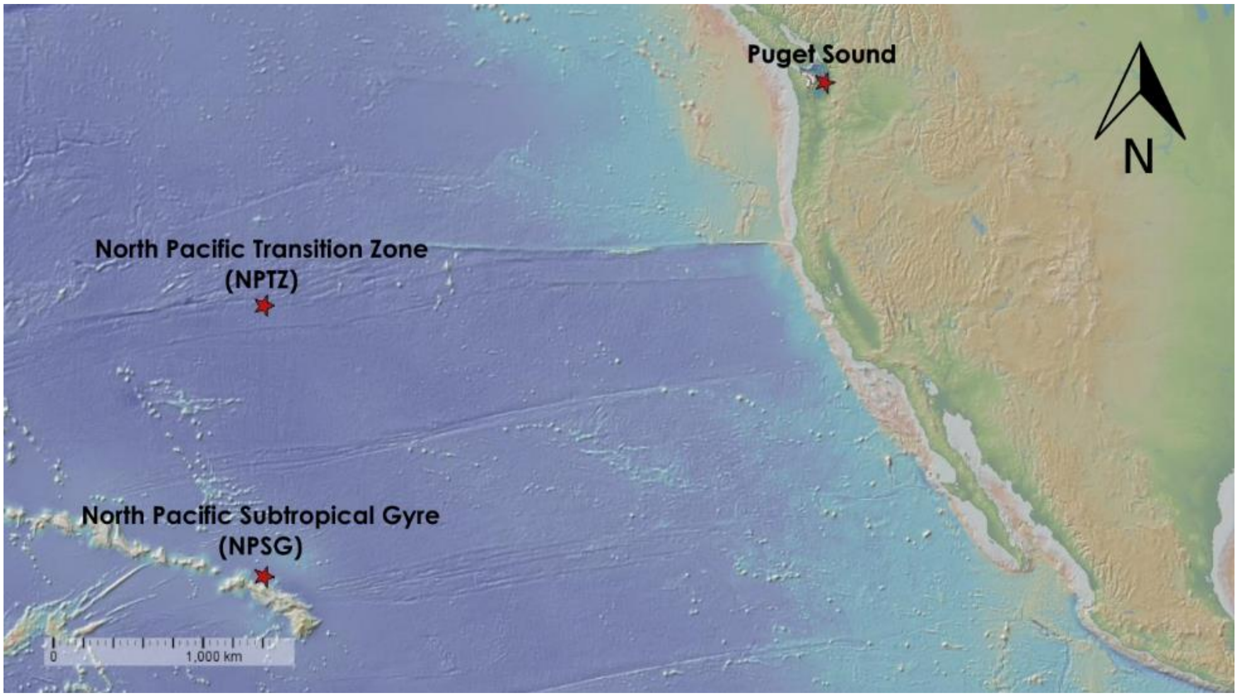

Figure 1. A map representing stations of data collection for metabolomic analyses. North Pacific, ALOHA station, and Puget Sound are labelled.

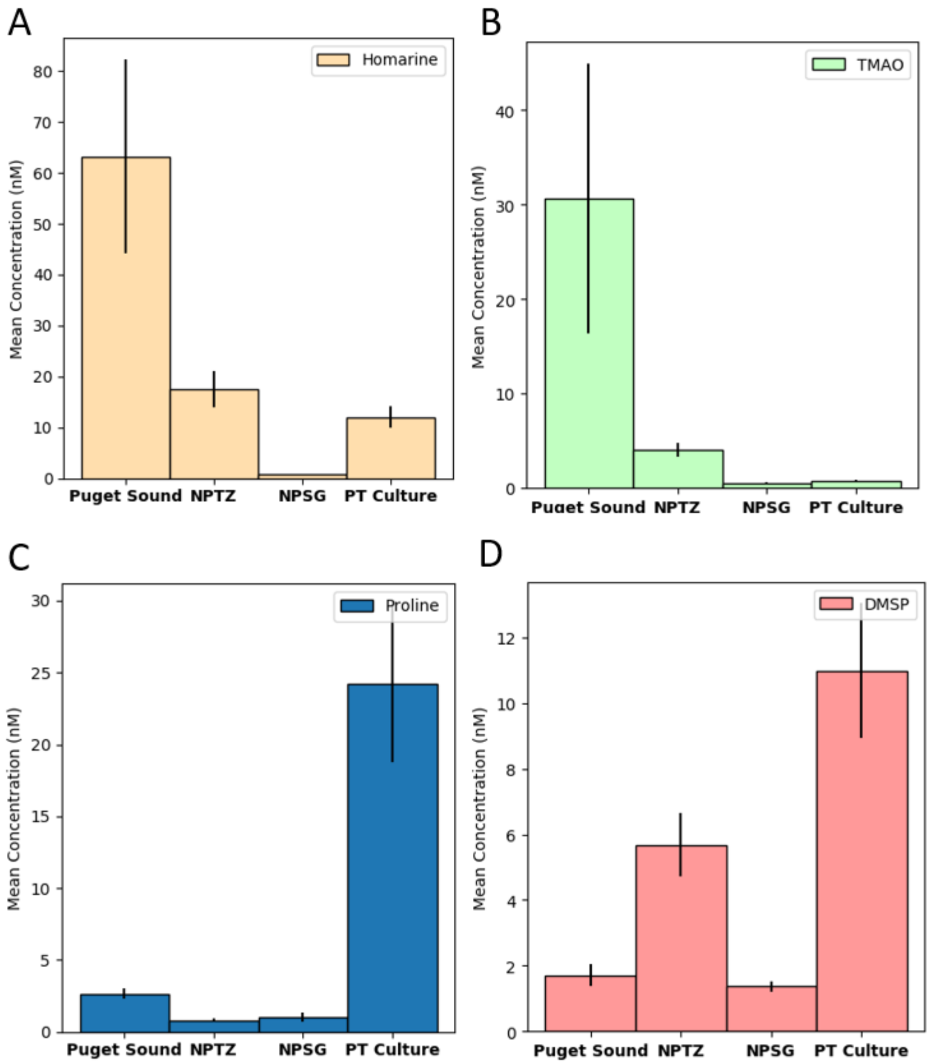

Figure 2. Bar plots graphing mean concentrations for a) homarine, b) TMAO, c) proline, and d) DMSP for all four samples. Error bars are shown in vertical black lines.

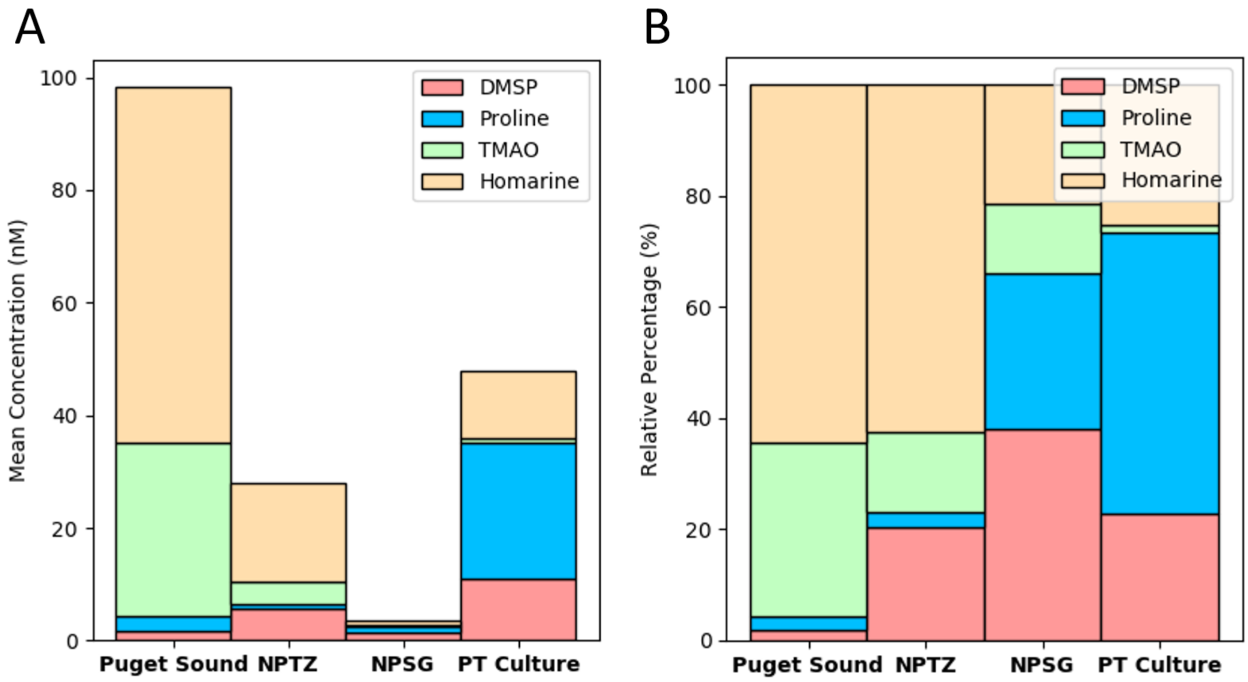

Figure 3. Stacked bar charts compare metabolite concentrations using a) mean concentrations and b) relative percentages of the four compounds.

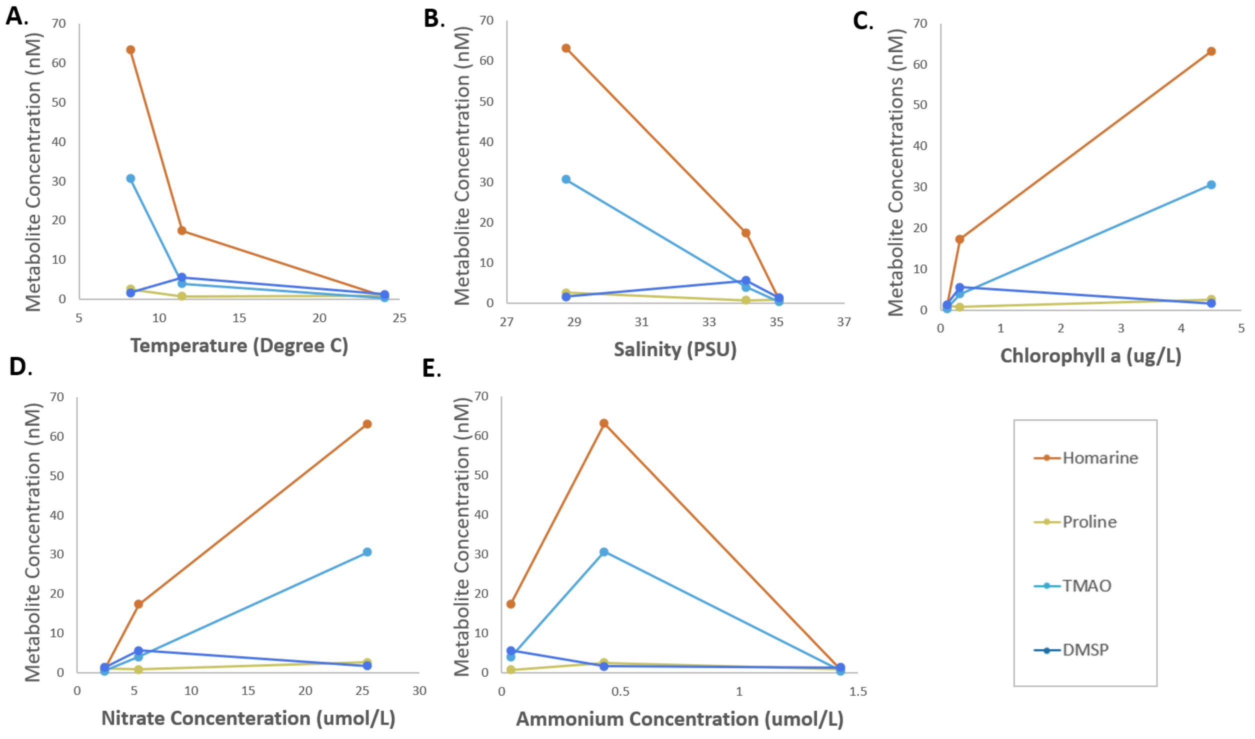

Figure 4. Correlation plots for homarine (yellow), TMAO (green), proline (blue), and DMSP (pink) concentrations with respect to a) temperature, b) salinity, c) chlorophyll, d) nitrate concentration, and e) ammonium concentration.

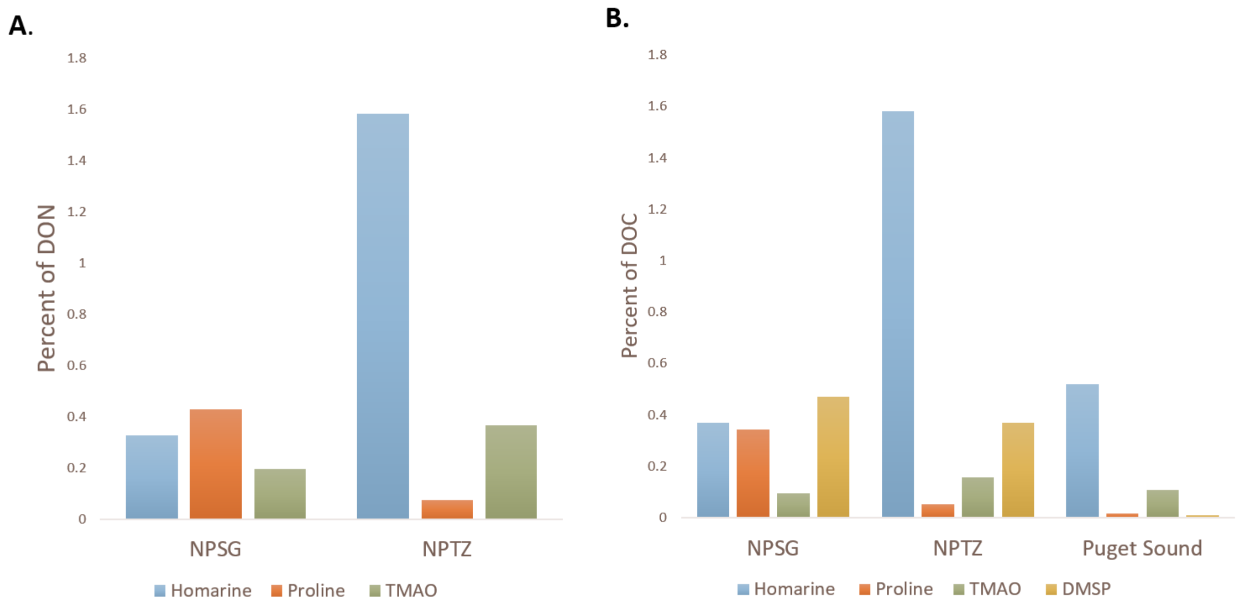

Figure 5. Bar charts presenting the percentages of the a) dissolved organic nitrogen pool (DON) and b) dissolved organic carbon pool that each metabolite makes up in each sample location.