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Algal toxins in Alaskan seabirds: Evaluating the role of saxitoxin and domoic acid in a large-scale die-off of Common Murres

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ABSTRACT

Elevated seawater temperatures are linked to the development of harmful algal blooms (HABs), which pose a growing threat to marine birds and other wildlife. During late 2015 and early 2016, a massive die-off of Common Murres (*Uria aalge*; hereafter, murres) was observed in the Gulf of Alaska coincident with a strong marine heat wave. Previous studies have documented illness and death among seabirds resulting from exposure to the HAB neurotoxins saxitoxin (STX) and domoic acid (DA). Given the unusual mortality event, corresponding warm water anomalies, and recent detection of STX and DA throughout coastal Alaskan waters, HABs were identified as a possible factor of concern. To evaluate whether algal toxins may have contributed to murre deaths, we tested for STX and DA in a suite of tissues obtained from beach-cast murre carcasses associated with the die-off as well as from apparently healthy murres and Black-legged Kittiwakes (*Rissa tridactyla*; hereafter, kittiwakes) sampled in the preceding and following summers. We also tested forage fish and marine invertebrates collected in the Gulf of Alaska in 2015–2017 to evaluate potential sources of HAB toxin exposure for seabirds. Saxitoxin was present in multiple tissue types of both die-off (36.4 %) and healthy (41.7 %) murres and healthy kittiwakes (54.2 %). Among birds, we detected the highest concentrations of STX in liver tissues (range 1.4–10.8 μg 100 g^{-1}) of die-off murres. Saxitoxin was relatively common in forage fish (20.3 %) and invertebrates (53.8 %). No established toxicity limits currently exist for seabirds, but concentrations of STX in birds and forage fish in our study were lower than values reported from most other bird die-offs in which STX intoxication was causally linked. We detected low concentrations of DA in a single bird sample and in 33.3 % of invertebrates and 4.0 % of forage fish samples. Although these results do not support the hypothesis that acute exposure to STX or DA was a primary factor in the 2015–2016 mortality event, additional information about the sensitivity of murres to these toxins is needed before we can discount their potential role in the die-off. The widespread occurrence of STX in seabirds, forage fish, and invertebrates in the Gulf of Alaska indicates that algal toxins should be considered in future assessments of seabird health, especially given the potential for greater occurrence of HABs in the future.

1. Introduction

Harmful algal blooms (HABs) in coastal environments are predicted to increase in intensity and frequency due to global warming and various anthropogenic influences (Glibert et al., 2014). Worldwide, many instances of morbidity and mortality among marine wildlife have been linked to ingestion of algal toxins (Burek et al., 2008; Lefebvre et al., 2016; Shumway et al., 2003), with HAB-related incidents becoming increasingly common (Landsberg et al., 2014). For example, strandings of California sea lions (*Zalophus californianus*) due to intoxication with the HAB toxin domoic acid (DA) were first documented in 1998 and now occur annually on the west coast of North America (Bargu et al., 2010; Gulland et al., 2002; Lefebvre et al., 1999). Numerous bird species have also experienced die-offs attributed to HABs (Shumway et al., 2003), including cormorants (Coulson et al., 1968; Fritz et al., 1992; O'Shea et al., 1991), terns (Nisbet, 1983), alcids (Peery et al., 2006; Shearn-Bochsler et al., 2014), pelicans (Work et al., 1993), and waterfowl (Forrester et al., 1977; Sasner et al., 1974). These mortality events, presumed to result from consumption of toxic prey, have occurred in various geographic locations and affected taxa with diverse

Abbreviations: DA, domoic acid; ELISA, enzyme-linked immunosorbent assay; GI, gastrointestinal; HAB, harmful algal bloom; HPLC, high-performance liquid chromatography; STX, saxitoxin

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foraging strategies and habitat preferences, suggesting that impacts of HABs occur broadly throughout the marine environment.

In late 2015 and early 2016, a large-scale die-off of Common Murres (*Uria aalge*; hereafter, murres) occurred in the Gulf of Alaska (Piatt et al., 2020). This mortality event coincided with a marine heat wave in the northeastern Pacific Ocean that resulted in sea surface anomalies 1–2 °C higher than normal (and exceeding 2 standard deviations above average) throughout the Gulf of Alaska and Bering Sea (Walsh et al., 2018). Murres also experienced widespread reproductive failures at major colonies in this region during summer (Dragoo et al., 2019; Zador and Yasumiishi, 2018; Piatt et al., 2020) and atypical foraging behaviors by seabirds were observed in Alaska in the fall (Robinson et al., 2018). Murre carcasses examined during the die-off were emaciated and starvation was identified as the proximate cause of death, presumably related to a decrease in forage fish availability (Zador and Yasumiishi, 2018; Piatt et al., 2020) and condition (von Biela et al., 2019). However, warm water anomalies have also been linked to increases in the diatom and dinoflagellate species that produce algal toxins, thus raising concern about potential impacts of HABs (Gobler et al., 2017; Ryan et al., 2017).

Indeed, during 2015–2016, elevated water temperatures in coastal regions of Alaska favored growth of the dinoflagellate *Alexandrium catenella*, which produces saxitoxin (STX), a potent neurotoxin that accumulates in the food web (Vandersea et al., 2018). Saxitoxin is responsible for paralytic shellfish poisoning, which causes respiratory paralysis and other deleterious impacts in humans, marine mammals, fish, and invertebrates (Landsberg, 2002; Landsberg et al., 2014). The widespread marine heat wave during this period was also associated with blooms of diatom species in the genus *Psuedo-nitzschia* along the west coast of the US and Canada (Ryan et al., 2017; Gibble et al., 2018). These blooms were characterized by record-breaking production of DA (McCabe et al., 2016), another neurotoxin responsible for amnesic shellfish poisoning, which results in seizures and impaired neurological function (Landsberg et al., 2014). Although HABs have been reported more commonly in temperate and tropical locations, STX and DA are known to occur in northern regions, including Alaska (Lewitus et al., 2012; RaLonde and Wright, 2011). Both of these toxins had recently been detected in marine mammals sampled over a broad area of coastal and shelf waters in Alaska (Lefebvre et al., 2016) where they overlap in distribution with many species of seabirds that consume similar prey (Piatt et al., 2018; Stephensen and Irons, 2003; Vermeer et al., 1987).

To investigate the possibility that HABs contributed to murre deaths in the Gulf of Alaska, we analyzed carcasses collected during the 2015–2016 mortality event as well as healthy murres and Black-legged Kittiwakes (*Rissa tridactyla*; hereafter, kittiwakes) sampled from their breeding colonies in the preceding or subsequent summers for STX and DA. By comparing samples from die-off carcasses to those from healthy birds, we could determine whether the presence of algal toxins was correlated with the mortality event. Separate from bird collections, we also sampled forage fish and marine invertebrates in the food web during 2015–2017 to identify possible sources of STX and DA exposure for seabird consumers in the Gulf of Alaska.

2. Methods

2.1. Sample collection

2.1.1. Seabird samples

During the large-scale die-off of murres in 2015–2016, we collected beach-cast carcasses (n = 44) from multiple locations across the Gulf of Alaska (Fig. 1, Tables 1, S1). These carcasses were generally emaciated, with little to no fat, and no food in their stomachs. As much as possible, we selected intact, fresh carcasses for STX and DA testing; however, in some cases moderate decomposition was noted. Because die-off samples were collected opportunistically we could not control for potential effects of toxin degradation or microbial activity (see discussion below). As reference samples, we also collected tissues from apparently healthy (hereafter, "healthy") birds during the summers of 2015–2017. Healthy birds included murres and kittiwakes collected at or near breeding colonies in Cook Inlet (Chisik Island) and Kachemak Bay (Gull Island; Fig. 1, Tables 1, S1). We lethally collected murres (n = 5) and kittiwakes (n = 21) using a shotgun during July–August 2016 (ACUC permit #2016-11, USFWS permit #MB78758-4), live-captured murres (n = 4) and kittiwakes (n = 23) using telescoping fiberglass poles fitted with a monofilament noose during July–August 2016–2017 (ACUC permit #2016-11), and salvaged kittiwakes (n = 3) found recently dead near a feeding humpback whale in September 2016 (USFWS permit #MB78758-4). Additionally, we salvaged murres (n = 7) lethally caught in gillnets in Kodiak in May 2015 (USFWS permit #MB78758-4; Fig. 1, Tables 1, S1). Collection of reference samples coincident with the die-off event was not feasible because healthy birds could not be readily located and accessing remote offshore waters was logistically prohibitive. Basic necropsies (Work, 2000) revealed normal weights and no outward signs of disease in healthy birds.

For birds that were found dead or lethally collected, we analyzed samples of breast muscle, liver, upper gastrointestinal contents (stomach contents and/or entire stomach and gizzard), and cloaca (entire cloaca and/or cloacal contents) for STX and DA. From healthy, live-captured birds we analyzed feces (kittiwakes and murres) and regurgitant samples (kittiwakes only). Adult kittiwakes store partially digested food in their proventriculi for their chicks, and when disturbed (e.g., during noose pole capture) they readily regurgitate the contents (Barret, 2007). Not all tissue types were available from all individuals, and sample volume was often limited, thus precluding testing for both STX and DA in all tissues.

2.1.2. Forage fish and invertebrate samples

Forage fish (n = 60; Pacific capelin [*Mallotus catervarius*], Pacific herring [*Clupea pallasii*], Pacific sand lance [*Ammodytes personatus*], walleye pollock [*Gadus chalcogrammus*], longfin smelt [*Spirinchus thaleichthys*], juvenile lingcod [*Ophiodon elongatus*]; ADF&G permits #CF-15-106, CF-16-109, #CF-17-065) and invertebrates (n = 26; euphausiids, shrimp, zooplankton, mussels, mysids) were opportunistically collected during the summers of 2015–2017 in Cook Inlet and Prince William Sound to evaluate potential sources of STX and DA in the food web relevant to marine consumers (Fig. 1; Tables 2, S1).

2.2. Quantification of STX in samples

Commercially-available enzyme-linked immunosorbent assay (ELISA) kits were used to test for STX in seabird tissues, forage fish, and invertebrates. A subset of samples with elevated STX values were subsequently analyzed by high-performance liquid chromatography (HPLC) to determine congener profiles.

2.2.1. Saxitoxin extraction for ELISA and HPLC

Seabird tissues and whole forage fish and invertebrates were extracted for STX analysis using the procedure of Lawrence et al. (2005). This method is recognized by the Association of Analytical Communities (AOAC) as a standard method for extracting STX from shellfish tissues. Optimally, 5 g of sample is recommended for the assay, but it was not always possible to obtain this much material from seabird and forage samples in this study. In those instances, dilution volumes and calculations were adjusted accordingly; however, it is possible that very low concentrations of STX may have been less consistently detectable in samples < 5 g. One to 5 g of each tissue was homogenized in a 50 mL tube using an Omni Tissue Homogenizer (Omni International, Kennesaw, GA), and extracted in 3 mL of 1 % acetic acid. Samples were vortexed for 30 s, boiled for 5 min with loose caps, allowed to cool to room temperature, vortexed again for 30 s, and centrifuged at 4700 rpm for 10 min. The remaining supernatant was poured into a graduated 15 mL tube (BD Falcon, New York, NY). Next, 3 mL of 1 % acetic acid

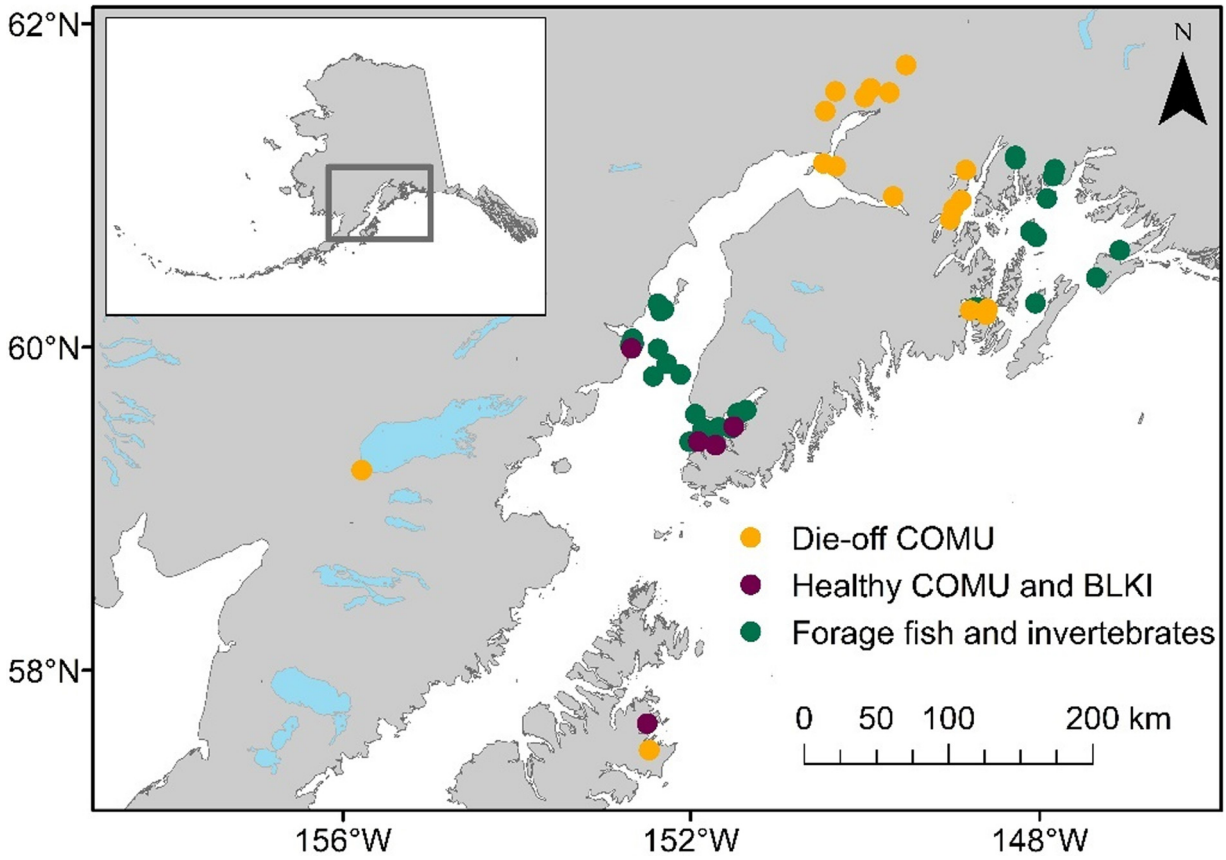

Fig. 1. Map of sampling locations for Common Murres (COMU), Black-legged Kittiwakes (BLKI), forage fish, and marine invertebrates collected in Alaska in 2015–2017 and tested for saxitoxin and/or domoic acid. Die-off COMU were associated with a large-scale mortality event during winter 2015–2016. Healthy COMU and BLKI and forage samples were collected during the preceding and following summers.

was added to the residue-containing 50 mL tube and vortexed for 30 s again. The 50 mL tube was again centrifuged, and the supernatant was added to the previous 3 mL in the 15 mL tube. When additional filtration was required, samples were cleared by passing through a 0.45 µm Millex HA syringe filter (Millipore, Billerica, MA). The combined extracted supernatant was then diluted to a final volume of 10 mL using Mill-Q water and stored at -20 °C until ready for analysis.

2.2.2. Validation of STX ELISA for use with seabird tissues

Little published information was available for the applied use of the STX ELISA, originally designed for shellfish, to test seabird tissues. Consequently, a validation of the assay performance was required. Potential matrix effects (interference with the assay based on characteristics of the sample type) was addressed by diluting representative extracts 1:25, 1:50, 1:100, and 1:250 with ELISA kit assay buffer and determining estimated STX concentrations. Initial dilutions yielding unexpectedly low STX values compared to higher dilutions that yielded the same corrected toxin concentrations were considered inhibited. To determine percent recovery we identified homogenized liver, muscle, upper GI, cloacal, and fecal samples that contained no detectable STX by ELISA. Five g aliquots from these samples were then spiked with purified STX to create a sample containing the equivalent of 20 µg 100 g^{-1} STX. The samples were subsequently extracted and analyzed by ELISA as described above. The recovery percentage was calculated as concentration estimated in the extracted sample divided by the calculated concentration from the spiked toxin amount into the sample times 100. Because the STX levels were low in the sample, this experiment also served to test whether 1:250 and 1:1000 dilutions gave

Table 1
Saxitoxin (STX) in seabird samples collected in the Gulf of Alaska during 2015–2017. Table shows number of samples (n), percent of samples with detectable concentration of STX (%), and maximum STX concentration (max STX conc.) for die-off Common Murres (COMU) that were associated with a large-scale mortality event, as well as healthy COMU and healthy Black-legged Kittiwakes (BLKI).

	Die-off COMU			Healthy COMU			Healthy BLKI		
	n	%	Max STX conc. (µg 100 g^{-1})	n	%	Max STX conc. (µg 100 g^{-1})	n	%	Max STX conc. (µg 100 g^{-1})
Collected birds	44	36	10.8	12	42	1.3	24	54	4.6
Liver	29	24	10.8	12	0	NA	24	21	2.7
Muscle	28	11	DBNQ	12	25	DBNQ	24	17	3.1
Upper GI	22	32	1.0	10	10	1.3	22	36	4.6
Cloaca	10	50	4.8	6	50	DBNQ	12	8	DBNQ
Live-sampled birds	NA	–	–	4	0	BD	23	17	DBNQ
Feces	NA	–	–	4	0	BD	5	0	BD
Regurgitants	NA	–	–	NA	–	–	23	17	DBNQ

BD = below detection; DBNQ = detectable but not quantifiable; NA, not applicable.

Table 2
Saxitoxin (STX) in forage fish and marine invertebrate samples collected in the Gulf of Alaska during summers 2015–2017. Table shows number of samples (n), percent of samples with detectable STX (%), and maximum STX concentration (max STX conc.) by sample type.

Group	n	%	Max STX conc. (μg 100 g^{-1})
Forage fish			
Capelin	10	20	DBNQ
Pacific herring	16	31	5.5
Pacific sand lance	17	6	DBNQ
Walleye pollock	13	31	1.4
Other	3	0	
Total	59	20	5.5
Invertebrates			
Crustacea	11	46	30.5
Zooplankton	6	17	DBNQ
Mussels	9	89	8.7
Total	26	54	30.5

DBNQ = detectable but not quantifiable.

equivalent STX estimates, indicating that 1:250 dilutions were not inhibited.

Initial method development for adapting the ELISA assay for use in bird tissues was conducted at the NOAA Laboratory in Beaufort, North Carolina. After the method was finalized, the protocol was duplicated at the USGS Alaska Science Center, with both laboratories subsequently analyzing tissues, and conducting cross-laboratory validations.

2.2.3. Saxitoxin analysis with ELISA

We used the Abraxis STX microtiter plate assay (Abraxis LLC, Warminster, PA) to test seabird, forage fish, and invertebrate samples. We followed the manufacturer's protocols with minor modifications. Extracts were diluted 1:250 instead of 1:1000 as recommended for shellfish samples. Prior to the two 30 min incubations specified in the kit instructions, the microtiter plate containing the samples was placed on a MSI S1 Minishaker (IKA Works, Inc., Wilmington, NC; NOAA) set at 600 rpm or on a Wellwash microplate washer (Thermo Scientific, Waltham, MA; USGS) set on high for 60 s to ensure the reagents in the microtiter wells were thoroughly mixed. Plates were washed with an ELx 50 plate washer (Biotek, Winooski, VT; NOAA) or Wellwash microplate washer (Thermo Scientific, Waltham, MA; USGS). Final absorbance of the standard curve and samples were measured at 450 nm on either a ClarioStar spectrophotometer (BMG Labtech, Cary, NC; NOAA) or an Emax Plus microplate reader (Molecular Devices, San Jose, CA; USGS). The standard curve for the ELISA was constructed as described in the manufacturer's protocol. The linear portion of the standard curve in which samples are quantifiable falls between B/B_0 values of ~20 % and 80 % as described in the Abraxis protocol. Above 80 %, the relationship between the B/B_0 and STX concentration becomes non-linear. Saxitoxin can be detected at these higher B/B_0 values, but not reliably quantified. To determine the point at which STX was no longer detectable, we examined the negative control (no STX) B/B_0 values and found they ranged from 97.1%–102.9 % (n = 34, mean = 100 ± 1.3 % SD). In theory, samples with values < 97.1 % could be considered STX positive. However, to reduce the chances of false positive readings we used a conservative B/B_0 range of 80–90 % for defining a sample as containing detectable but not quantifiable (DBNQ) levels of STX. B/B_0 values above 90 % were considered negative. The STX ELISA is sensitive (detection limit of 1–2 μg STX equivalents per 100 g tissue) and allows for rapid screening of a large number of samples.

2.2.4. Saxitoxin analysis by HPLC

Seabird tissues and forage samples estimated to contain > 7 μg 100 g^{-1} by ELISA were analyzed by HPLC using the standard method of Lawrence et al. (2005). The extraction procedure was the same as used for the ELISA kits with the addition of a further cleanup step. Once the STX extracts were prepared as described above, a 1 mL aliquot of extract was cleaned using solid phase extraction (SPE) cartridges (Sigma-Aldrich, St. Louis, MO) and a Phenomenex HyperSep 10 port Glass Vacuum Manifold (Phenomenex, Torrance, CA) to remove any interferences affecting HPLC analysis (Lawrence et al., 2005). Extraction efficiency was found to be 105 ± 5 %. Saxitoxin standards were purchased from the Certified Reference Materials Program of the Institute for Marine Biosciences, National Research Council (Halifax, Canada). The standards/toxins analyzed were as follows: dcGTX2,3; C1,2; dcSTX; GTX2,3; GTX5; STX; GTX1,4; and NEO.

2.3. Quantification of DA in samples

For DA extractions, 1–2 g of homogenized tissue was added to a tared 50 mL conical tube and the weight recorded to the nearest 0.01 g. Next, 18 mL of 50 % methanol: water mixture was added, and the samples vortexed at high speed for 2 min. Once the extraction was completed, the tubes were centrifuged for 20 min at 4700 rpm. As with the STX samples, if centrifugation did not clear the samples, they were transferred to a syringe, then passed through a 0.45 μm Millex HA syringe filter to remove the remaining particulates (Litaker et al., 2008).

Samples were screened for the presence of DA using the commercially available ELISA kit manufactured by Biosense Laboratories (Bergen, Norway) or the DAK-36 Domoic Acid test kit (Mercury Science, Inc., Durham, NC) following the manufacturers' protocols. Both kits are quantitative for DA and yield concentrations equivalent to those obtained using HPLC (Litaker et al., 2008). Previous studies have used ELISA assays to test for DA in seabird tissues (Gibble et al., 2018) and fish (Lefebvre et al., 2007).

Briefly, 50 μL of DA antibody was added to each well, followed by 50 μL of control solution and sample, each added in the same sequence. Reagents and samples were brought to room temperature before use and the wells were shaken for 30 min (IKA Works mini-shaker or Wellwash microplate washer). Fifty μL of DA tracer was then added to each well and the plate was shaken again for 30 min. The strips were washed three times using the same plate washer described above, and 100 μL of substrate solution was added to each well and shaken for 5 min. Next, 100 μL of stop solution was added and shaken briefly and read at 450 nm on the spectrophotometer.

The Mercury Science ELISA reagents were standardized to such a degree that the slope and intercept of the B/B_0 standardized curves were highly reproducible (Litaker et al., 2008). The correlation between the concentrations of DA in shellfish and phytoplankton samples determined using the ELISA consistently had an $R^2 > 0.95$ to 0.99 and slope of between 0.97 and 1.06. In place of doing repetitive standard curves, a spreadsheet containing the standard curve parameters was developed by Mercury Science to analyze results and used to quantify the amount of DA in samples. For samples analyzed with the Biosense kits, endpoint absorbance was read using an Emax plus microplate reader and results were analyzed using Softmax Pro 7 software (Molecular Devices, San Jose, CA). Detection limits for the DA ELISA were 0.1 ppm using the Mercury Science kits and 0.02 ppm using the Biosense kits. Domoic acid has a single form that is recognized quantitatively by the ELISA and so measured concentrations represent actual, not minimal estimates, unlike STX.

2.4. Statistical analyses

We evaluated potential differences in the distribution of STX between the three groups of seabirds we sampled: beach-cast murres associated with the die-off event, healthy murres, and healthy kittiwakes. We had relatively small sample sizes and our data did not meet assumptions of normal distribution due to the preponderance of BD and DBNQ values in bird tissues; therefore, we applied the non-parametric

Kruskal-Wallis test (Program SAS, Version 9.4, SAS Institute, Cary, NC). Because this is a rank-based test, we assigned a value of 0.5 for DBNQ values, which was lower than the minimum quantifiable concentration but greater than 0 (BD). For this analysis, we used the maximum concentration measured in each bird and only included birds that were lethally collected or found dead because samples from live-captured birds were restricted to feces and/or regurgitants and thus were not directly comparable to those from die-off murres. We reasoned that due to the rapid depuration and unknown routing of STX into bird tissues this approach would provide insight into minimum exposure levels across groups. A bird need not have elevated values in all (or even multiple) tissues for saxitoxicosis to have occurred and there is a high likelihood of false negatives in field-collected samples (see discussion below). By using maximum values we assumed we would have the best chance of detecting potentially harmful exposure. However, we acknowledge that this analysis has necessarily limited inference and is intended only to provide a general comparison between groups. There were too few detections of DA in birds to conduct formal analyses but results for birds and forage samples are summarized below. All supporting data are available in Van Hemert et al. (2019).

3. Results

3.1. Saxitoxin

3.1.1. Saxitoxin ELISA validation

Preliminary STX dilution experiments showed that seabird tissue samples were inhibited at dilutions less than 1:100 (Table S2). Data from the spike recovery experiments, in which all the samples were run at both 1:250 and 1:1000 dilutions, yielded equivalent STX concentrations, indicating that dilutions of 1:250 or above were not inhibited (Table S3). The recovery rates for the liver, muscle, feces, and stomach contents samples spiked to a concentration of 20 μg 100 g^{-1} ranged from 92 % to 94 % (Table S3).

3.1.2. Saxitoxin concentrations in seabird tissues, forage fish, and invertebrates by ELISA

Saxitoxin was common in die-off murres (36.4 %, n = 44), healthy murres (41.7 %, n = 12), and healthy kittiwakes (54.2 %, n = 24; Table 1). There was no statistically significant difference between the three groups when the maximum concentration across all tissues by individual was considered (Kruskal-Wallis test; p = 0.37). However, the range of STX values varied among groups, with the highest concentration observed in die-off murres (10.8 μg 100 g^{-1}), followed by healthy kittiwakes (4.6 μg 100 g^{-1}), and healthy murres (1.3 μg 100 g^{-1}; Table 1; Fig. 2). The three highest STX concentrations were measured in liver samples of die-off murre carcasses collected in Prince William Sound (10.8 μg 100 g^{-1}) and Lake Iliamna (7.8 and 9.4 μg 100 g^{-}). Among die-off murres, STX was detected at the highest prevalence and concentration in liver tissue, whereas among healthy murres STX was most prevalent in cloaca but had the highest concentration in upper GI samples (Table 1; Fig. 2). Among healthy kittiwakes, STX was detected most commonly and at the highest concentration in upper GI samples (Table 1; Fig. 2). Other tissue-specific differences between the three groups included higher prevalence of STX in liver and GI tissues of die-off murres and healthy kittiwakes than healthy murres (Table 1). In contrast, STX prevalence was higher in muscle tissue of healthy murres than in die-off murres or healthy kittiwakes. Among healthy, live-captured birds, STX was detected in 17.4 % of regurgitant samples (n = 23) collected from kittiwakes but was not detected in any fecal samples from murres (n = 4) or kittiwakes (n = 5; Table 1). We detected STX in 20.3 % of forage fish (n = 59) and 53.8 % of invertebrates (n = 26; Table 2). Euphausiids collected in July 2016 in Prince William Sound had the highest STX concentration (30.5 μg 100 g^{-1}) among forage fish and invertebrate samples (Fig. 2).

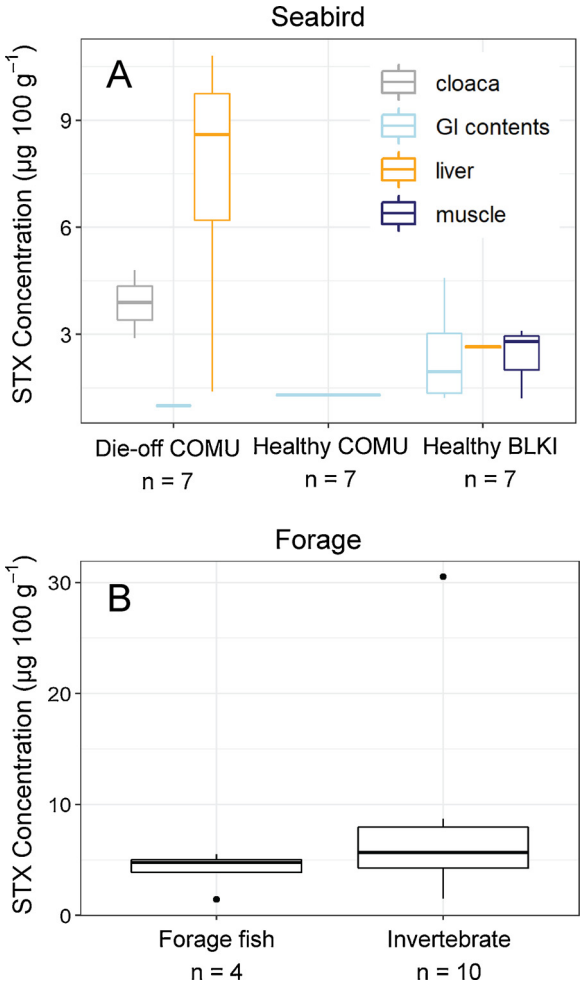

Fig. 2. Quantifiable concentrations of saxitoxin (STX) detected in (A) seabird tissues and (B) whole forage fish and marine invertebrates from the Gulf of Alaska during 2015–2017. (A) Samples were collected from die-off Common Murres (COMU) that were associated with a large-scale mortality event, healthy COMU, and healthy Black-legged Kittiwakes (BLKI). Tissues tested included breast muscle (muscle), liver, upper gastrointestinal contents (GI contents), and cloaca (entire cloaca and/or cloacal contents), although not all tissue types were available for every individual. (B) For forage fish and invertebrates, whole body samples were used. The boxplots show the median (horizontal line), 25th and 75th percentiles (lower and upper hinges of each box), range (whiskers), and outliers (points). Note that samples with detectable but not quantifiable concentrations of STX are not included here.

3.1.3. Saxitoxin concentrations in seabird tissues, forage fish, and invertebrates by HPLC

We tested forage (n = 4) and seabird (n = 3) samples with estimated STX ELISA values > 7 μg 100 g^{-1} by HPLC. Of these, only three invertebrate samples (two mussels and one euphausiid), had detectable STX concentrations (6.4, 6.9 and 7.0 μg 100 g^{-1} sample, respectively). All three of these samples contained predominantly STX, which is one of the congeners for which HPLC is maximally sensitive. Other samples likely contained congeners not easily detected by HPLC but detected by the ELISA.

3.2. Domoic acid measured using ELISA

Among birds, DA was detected in only one healthy kittiwake (9.1 %, n = 11, 0.1 ppm). Domoic acid was not detected in any tissues from healthy (n = 10) or die-off murres (n = 22), nor in feces or regurgitants from live-captured kittiwakes (n = 19). Among forage samples, DA was detected in 4.0 % of forage fish (n = 25) and 33.3 % of invertebrates

(n = 9). Concentrations in forage fish and invertebrates ranged from 0.03 to 0.30 ppm, with the highest concentration in a mysid (*Neomysis rayii*) from Cook Inlet.

4. Discussion

Our results demonstrated widespread exposure to STX among seabirds and forage taxa in the Gulf of Alaska, suggesting that marine consumers are routinely exposed to algal toxins in this region. Nonetheless, we did not find compelling evidence to support the hypothesis that acute exposure to algal toxins contributed directly to the 2015–2016 murre die-off event in Alaska. The occurrence of STX did not differ significantly among healthy seabirds and beach-cast carcasses when maximum concentrations across individuals were considered. Concentrations of STX in murres and kittiwakes were generally lower than those reported from most other studies that have established a clear link between STX ingestion and bird mortality, although sensitivity of seabirds to STX is unknown and interpretation of values from wild birds can be challenging. Of note, STX was present in 24% of livers from die-off murres, and these samples included the highest concentrations of STX of any seabird tissue we tested. The prevalence of STX was also relatively high in key forage taxa such as small pelagic fish and euphausiids, indicating a direct trophic pathway to seabirds and other marine predators. In comparison, DA was much less common in bird and forage samples.

4.1. Evaluating the role of algal toxins in the 2015–2016 murre die-off

Algal toxins can cause morbidity and mortality in a variety of wild avian species and have been implicated in numerous seabird die-off events (Shumway et al., 2003). Limited experimental studies have demonstrated acute toxicity of STX (Kvitek and Beitler, 1988) and DA (Silvagni, 2003) but harmful exposure levels have not yet been adequately established for birds or other wildlife, making direct assessments of ecological impacts from these toxins difficult. Additionally, reference samples are often lacking for mortality events among wild birds, and thus comparisons between affected and unaffected animals are not available. Consequently, evidence that HABs play a role in bird mortality is usually circumstantial.

A novel aspect of this study was the ability to collect samples from healthy seabirds (murres and kittiwakes) living near breeding colonies in summer as well as those from murres found dead during the mortality event (mostly in winter). Without this context, it would be tempting to conclude that because STX was present in a relatively high proportion (36.4 %) of die-off carcasses it may have contributed directly to bird mortality. However, since no difference was detected in STX distribution between die-off birds and healthy birds sampled within 6–9 months of the die-off, and because STX concentrations in the die-off samples were generally lower than those reported from most other HAB-induced mortality events, such a conclusion is not supported here. Nonetheless, it is important to note that healthy birds in our study were collected during summer, when warmer waters may induce bloom conditions (Vandersea et al., 2018), and we do not have reference samples from winter, when the die-off occurred and when birds may be more susceptible to compounding stressors. Additionally, most die-off birds were emaciated and did not have samples available from the GI tract, where the toxin is typically most concentrated. Even so, higher STX concentrations were detected in die-off murres than in live-sampled murres or kittiwakes, and more information is needed about toxicity levels and tissue routing in birds to assess the biological relevance of this difference.

Most concentrations of STX in seabird tissues from this study were relatively low compared to those reported from other studies of marine birds and mammals known to have died from STX intoxication (Shumway et al., 2003). In the published literature, concentrations of STX in bird tissues range from < 1–110 μg 100 g^{-1} (ICES, 1998; Jones et al., 2019; Levasseur et al., 1996; Shearn-Bochsler et al., 2014). The lowest value reported for STX-induced mortality among adult birds was 36 μg 100 g^{-1} in the intestinal contents of a Herring Gull (*Larus argentatus*) during a HAB event in the St. Lawrence Estuary (ICES, 1998), which is more than three times higher than the maximum value we detected in murres. Few data exist for Alaskan seabirds, but an incident among nestling Kittlitz's Murrelets (*Brachyramphus brevirostris*) on Kodiak Island may provide insight into levels of STX that are harmful to seabirds in this region. Acute mortality in otherwise healthy nestlings was observed following consumption of forage fish (Lawonn et al., 2018); STX was subsequently detected in the tissues of most of these birds (Shearn-Bochsler et al., 2014). The method of tissue preservation (alcohol) was suspected to have resulted in falsely low values for samples collected in the first year, but data from three individuals collected in the second year under optimal conditions had STX concentrations in their tissues between 5.2–21.6 μg 100 g^{-1} (Shearn-Bochsler et al., 2014), which overlap with the range of values we detected in bird tissues from our study. Although sample sizes were small and toxicity levels of STX likely differ between nestlings and adult birds, these results suggest that deleterious impacts are possible at concentrations we observed in murres and kittiwakes. The only other published values for STX in Alaskan seabirds are from a die-off of Tufted Puffins (*Fratercula cirrhata*) in 2017 in the eastern Bering Sea. Trace levels of STX (< 0.01 μg 100 g^{-1}) were reported from stomach or cloacal contents of all four individuals tested but acute toxicosis was not suspected to have contributed to death in this case (Jones et al., 2019). In each of the studies mentioned above, only birds associated with a mortality event were tested and reference values were not obtained (ICES, 1998; Levasseur et al., 1996; Shearn-Bochsler et al., 2014; Jones et al., 2019).

Among birds, DA was detected in only a single healthy kittiwake and our results did not suggest a role for DA in the murre die-off. An experimental study of Common Murres intracoelomically injected with DA estimated the LD_{50} (lethal dose for 50 % of animals) at 4.14 ppm and the ED_{50} (effective dose for observational endpoint for 50 % of animals) at 0.96 ppm (Silvagni, 2003), both of which exceed ecologically relevant exposure based on our findings from forage samples collected in the Gulf of Alaska during 2015–2017. Additionally, concentrations of DA in the tissues of these experimentally-dosed murres (both those that died from acute intoxication and those that were exposed and survived) were much higher than any observed in bird samples from this study (Silvagni, 2003). During a concurrent mortality event of murres in California in 2015–2016, DA was detected in > 80 % of the birds tested, most at very low concentrations (Gibble et al., 2018). Although starvation was identified as the ultimate cause of death, the authors reported that DA exposure may have contributed to the die-off as a secondary factor. Our detection limits were higher than those reported in this study, so trace levels of DA may have been present but not detected among some of our samples.

It is also possible that sublethal effects of STX and DA on birds could influence behavior or compromise health without causing acute mortality (Gibble and Hoover, 2018; Shumway et al., 2003). Among mammals, repeated low-level DA exposure resulted in cognitive deficits, including impaired spatial memory (Lefebvre et al., 2017). In a captive study of murres experimentally exposed to sublethal doses of DA, thermoregulatory problems, depression, and lack of responsiveness were observed, all of which could contribute to decreased survival (Silvagni, 2003). Unusual distributional patterns were recorded among murres during the die-off period, including gathering in nearshore waters and traveling inland where these birds do not typically occur, but the underlying reasons for such behaviors are unknown (Piatt et al., 2020). Chronic exposure to STX might also lead to difficulties in foraging and negatively impact seabirds and other marine wildlife over a longer time scale (O'Neill et al., 2016). However, such impacts would be difficult to identify in a field setting, particularly in remote, offshore waters where murres and kittiwakes occur. Given the wide foraging

ranges of seabirds and lack of consistent algal toxin monitoring programs in Alaska, identifying the timing and location of HABs relevant to marine wildlife has proven to be challenging.

4.2. Algal toxins in forage fish and invertebrates

Detection of STX in key forage taxa collected in the Gulf of Alaska provided evidence for the occurrence of this toxin throughout lower trophic levels, over multiple seasons, and across a relatively broad area. Saxitoxin was common among the forage fish (20.3 %) and invertebrate samples (53.8 %) we tested (Table 2). These included sand lance, capelin, herring, juvenile pollock, and euphausiids, which comprise the majority of prey consumed by murres, kittiwakes, and other seabirds in the Gulf of Alaska (Piatt et al., 2018; Vermeer et al., 1987). Higher concentrations of STX in important forage taxa corroborate other studies that report the ability of these organisms to concentrate algal toxins (Deeds et al., 2008; Oyaneder Terrazas et al., 2017). Samples of prey items tested during previous STX-implicated seabird die-offs were as high as 4000 μg 100 g^{-1} (Shumway et al., 2003), and more than two orders of magnitude higher than maximum values reported in forage samples from this study (Table 2).

Because our collection of forage taxa occurred during discrete time periods that did not overlap with the murre die-off event, these samples were not intended to inform conclusions about causes of murre mortality but rather to determine potential sources of exposure. There is relatively little other information on algal toxins in forage taxa available for coastal and shelf waters of Alaska, where 95 % of North American seabird populations are found (Hatch and Piatt, 1995). Thus, detecting algal toxins commonly in forage fish and invertebrates in this region has important implications for murres, kittiwakes, and other marine consumers. Although variability in sampling timeframe and location and small sample sizes preclude direct comparisons between bird and forage samples, the highest STX values in known prey items (euphausiids and forage fish) roughly coincided with the highest STX values for healthy birds in summer 2016. During this period, notably elevated water temperatures and STX-induced shellfish closures were also reported in some parts of Alaska (Vandersea et al., 2018; Walsh et al., 2018). Domoic acid, while less prevalent than STX, was detected at low concentrations in 33.3 % of invertebrates and 4.0 % of forage fish, demonstrating that this toxin also occurs in the marine food web and provides a potential source of exposure to seabirds in the Gulf of Alaska.

4.3. Data gaps

Both STX and DA undergo rapid depuration upon ingestion (Andrinolo et al., 1999; Lagos and Andrinolo, 2000), presenting challenges for interpretation of data from wild birds. It is possible that higher concentrations of STX or DA were present in murre tissues, potentially causing harm but not immediate death, but had been metabolized or excreted prior to sample collection. The length of time that STX remains detectable in bird tissues after exposure is unknown, although studies from other taxa suggest that this period is relatively short, perhaps as little as several hours (Andrinolo et al., 1999; Lagos and Andrinolo, 2000). A single observation from a captive trial of European Starlings (*Sturnus vulgaris*) fed toxic clams indicated that STX was not detectable in intestinal contents after 24 h (Kvitek and Beitler, 1988). Residence time of DA is also typically brief; in an experimental study of DA in birds, dosed birds had no detectable DA in tissues 8–72 h post exposure (Silvagni, 2003). The duration of time that algal toxins persist in tissues of shellfish and other marine invertebrates varies; in the case of butter clams, STX is detectable for months after the initial bloom event, whereas in mussels and oysters elimination occurs much more rapidly (Bricelj and Shumway, 1998). Thus, depending on the prey species consumed, a HAB event need not occur contemporaneously for a seabird to be exposed to toxins.

The highest concentrations of STX were detected in liver samples from die-off murres. However, the pharmokinetics of HAB toxins in birds are poorly understood, making it difficult to interpret the biological relevance of this result. Analysis of tissues from Kittlitz's Murrelet chicks presumed dead from STX ingestion found relatively high concentrations of the toxin in both upper gastrointestinal and liver samples (Shearn-Bochsler et al., 2014). In contrast, the single published study of experimental dosing of STX in birds did not detect the toxin in liver, kidney, or heart tissues that were collected after birds died acutely (Kvitek and Beitler, 1988). Results are not directly comparable across studies due to methodological differences, but the observed variation highlights major gaps in our understanding of how STX is routed to various avian tissues upon ingestion.

It should also be noted that the length of time between death and collection of samples from beach-cast murres associated with the 2015–2016 die-off was unknown. Although most carcasses used for testing were intact and showed no or limited gross signs of decomposition, a subset of individuals from select locations (including Lake Iliamna, where the highest STX concentrations were recorded) were notably decomposed. Measured concentrations of STX (Donovan et al., 2008; Indrasena and Gill, 2000) and DA (Zabaglo et al., 2016) can change in response to microbial activity and environmental conditions, including temperature, UV exposure, and pH. Thus, it is plausible that toxin degradation may have occurred in some samples prior to our testing, but we could not control for any such bias due to the opportunistic nature of carcass collection.

4.4. Methodological considerations

Although ELISA assays have been applied to testing of marine mammal tissues and a variety of other taxa and are verified as a sensitive method for detecting STX, the Abraxis STX ELISA used in this study was originally designed for use with shellfish. As part of our quality control and quality assurance process, we evaluated matrix effects and percent recovery of STX in bird tissues. Even with these measures, however, sensitivity and specificity of the test has not been confirmed with experimental trials in which birds were exposed to known quantities of STX congeners. One important caveat of the STX ELISA used is that it is much less effective at detecting toxins other than STX. This means we may have failed to detect some related congeners, leading to underestimates of STX-equivalents present. Most of the samples tested by ELISA contained < 10 μg STX 100 g^{-1}, the lower detection limit of HPLC. Thus, we were unable to determine the complete suite of congeners to which birds may have been exposed. Also, the lower cutoff levels for both the DA and STX ELISA analyses were set conservatively to avoid reporting false positives. Without knowledge regarding the sensitivity of seabirds to STX or DA, we cannot be certain about the range of concentrations that may be biologically meaningful and whether the ELISA test effectively captured the lower extent of such values.

5. Conclusions

The widespread occurrence of STX among seabirds and their potential prey in the Gulf of Alaska suggests that marine consumers are regularly exposed to this toxin via the food web. Domoic acid, while less common than STX, was also present in forage taxa. Our results do not implicate STX or DA as a primary cause of the 2015–2016 murre mortality event, but additional research is needed to establish biologically meaningful toxicity levels for birds before the potential role of HABs can be fully discounted. Additionally, knowledge of pharmokinetics and sublethal effects of these toxins in birds would help with interpretation of field-based values. The likelihood of more frequent and severe HAB events in northern waters and the positive correlation between water temperatures and blooms of STX-producing algae suggests that algal toxins pose a growing problem that warrants additional

research (Glibert et al., 2014; Moore et al., 2008). Increased occurrence, geographic range, or distribution of STX or DA throughout the food web may present new challenges to seabirds and other marine wildlife and compound existing stressors related to rapid environmental changes in Alaska.

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.2019.101730.

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