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Biofilm as a Food Resource for Shorebirds at the Salton Sea

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Cover Photo: Shorebirds feeding at the edge of the Salton Sea. Photo by Marlene Walters.

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Least Sandpipers foraging at the Seawall site, 25 July 2023. | Photo: Marlene Walters

Summary

The Salton Sea, the largest lake in California, is an essential habitat for birds that rely on wetlands and open water to breed and migrate long distances along the Pacific Flyway in a state where over 90% of historic wetland has been lost. As water levels decline and salinity rises in the Sea due to a reduction of water inflow, the resulting die-off of prey species has driven precipitous declines in fish-eating birds and has altered the bird communities that visit and breed at the Sea. Despite this, the Salton Sea remains a valuable resource for hundreds of thousands of shorebirds *(Charadriiformes)* that forage along the Sea's shoreline. The recent discovery of biofilm as a major component of the diets of migrating small-bodied shorebirds (e.g., *Calidris* spp.) on coastal mudflats elucidates these birds' complex ecological role as both primary and secondary consumers and emphasizes the need to conserve all foraging resources, including biofilm. This study investigated whether biofilm occurs at the Salton Sea, and if so, whether it was likely that shorebirds were feeding on it. Results showed a higher abundance of diatoms, a group of nutritious algal organisms that partially comprise biofilm, at the observed feeding locations of sandpipers during spring migration compared to summer feeding sites. The formation of biofilm at intertidal mudflats, where it has been more extensively studied, is driven by spring and fall mixing of salt and fresh water; this pattern appears at the Salton Sea as well, where freshwater inflows occur in spring and fall and dry up in summer. While further studies are needed to confirm the presence of biofilm in shorebird diets, this initial study suggests that it is important that freshwater inflow to the Salton Sea remain reliable during migration periods to ensure the availability of biofilm as an essential energy source for small-bodied shorebirds.

Snowy Plover (*Charadrius nivosus*) chicks at the Seawall site, 25 July 2023. | Photo: Marlene Walters

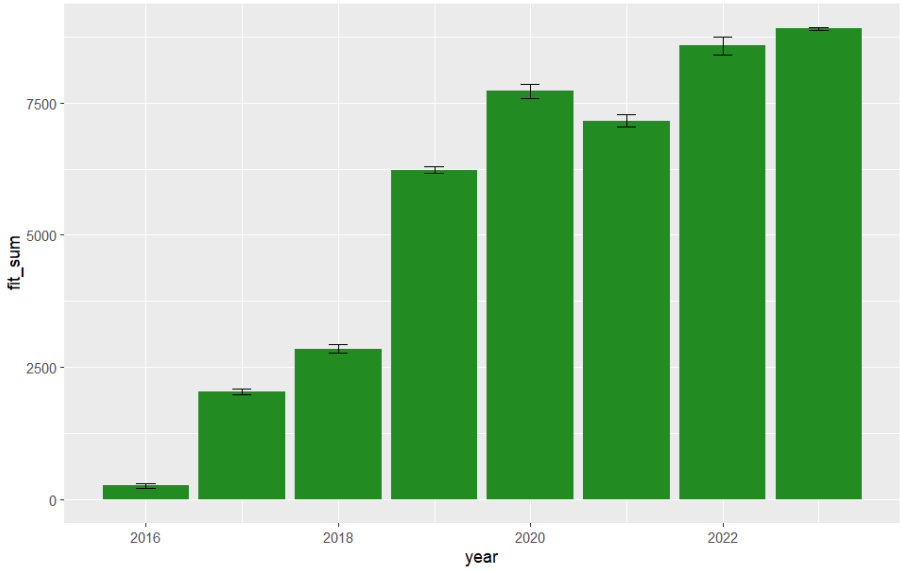
Introduction

Since 1970, 37% of shorebird abundance in North America has been lost (Rosenberg et al., 2019). Shorebird declines are steep and accelerating, highlighting the urgency to counteract threats to their survival (Smith et al., 2023). Most shorebird species undertake long-distance migrations each spring and fall and rely on a limited number of suitable stopover habitats to fuel their journeys (Myers et al., 1987). Thus, shorebirds are particularly vulnerable to habitat loss as the coastal mudflats, wetlands, and lakeshores they depend upon are lost to development or climate threats like drought and sea level rise. The State of California has already lost over 90% of its historic wetland habitat in the last 100 years (Dahl, 1990), which emphasizes the need to protect the State's remaining productive coastal and interior wetland habitats.

The Salton Sea is an inland saline lake in southern California and the largest lake in the State. It is a critically important habitat for millions of birds that migrate along the Pacific Flyway of the western United States. The ecology of the Salton Sea has shifted dramatically over two decades of water loss and increasing salinity. Federal, state, and local water agencies implemented the Quantification Settlement Agreement (QSA) in 2003, which diverted some of the Colorado River water away from the Salton Sea to southern California municipalities. Mitigation inflows to the Salton Sea under the QSA ended in 2017, initiating rapid water loss and the exposure of thousands of acres of playa each year as the Sea's shoreline recedes. The Sea's current primary source of water inflow is from storms and agricultural runoff, which is further limited by improved water conservation practices on surrounding farmland. Numbers of fish-eating birds that historically visited and nested at the Sea have steeply declined, as the lake is now too saline to sustain adequate populations of tilapia, which served as a major food resource for pelicans, cormorants, and terns (Jones et al., 2019). However, numbers of shorebirds appear unaffected, and may be increasing as a result of exposed mudflats along the exposed shoreline as the Sea recedes (D. Orr, personal communication, 2023) (Fig. 1). Recent shorebird surveys at the Salton Sea by Point Blue Conservation Science and Audubon California further suggest historic numbers of shorebirds counted during an August 2023 survey (Audubon California, 2023).

FIGURE 1. Model predicted increase in counts of Western and Least Sandpipers from 2016–2023.

The y-axis is the summed count prediction from a mixed effect model of Western and Least Sandpiper abundance across sampled sites at the Salton Sea where site and season effects are controlled for. The x-axis is year. Error bars are 95% confidence intervals. (Audubon California, unpublished, 2024)



A key component of shorebird conservation is ensuring the reliable productivity of migration stopover habitats. Recent research has identified biofilm as a significant proportion of the diets of Calidris sandpipers (Elner et al., 2005; Kuwae et al., 2008). Biofilm, a nutritious substance rich in lipids and carbohydrates, forms when aggregations of bacteria and microalgae secrete mucus and attach to surfaces (Decho, 1990; Ollinik et al., 2021). Previously thought to consume mainly tiny invertebrates, morphological adaptations to the tongues of these birds show they have evolved to graze on biofilms that form on coastal mudflat and sandflat sediments (Elner et al., 2005). For the smallest shorebirds, biofilm may be a majority component of their diet during migration. For example, the diet of migrating Western Sandpipers (Calidris mauri) may comprise as much as 59% biofilm (Kuwae et al., 2008). This discovery identifies small-bodied shorebirds as holding a complex ecological role in food webs as both primary and secondary consumers (Kuwae et al., 2012). Thus, sandpipers and other small-bodied shorebirds are excellent ecological indicators of the health of shoreline habitats.

Previous published studies showing evidence for shorebird biofilm ingestion were conducted on intertidal mudflats or sandflats at the Fraser River estuary, British Columbia, Canada (Elner et al., 2005; Jardine et al., 2015; Jiménez et al., 2015; Kuwae et al., 2008, 2012); the Pacific Coast of Japan (Kuwae et al., 2012); the upper Bay of Fundy, New Brunswick and Nova Scotia, Canada (Quinn & Hamilton, 2012); Bourgneuf Bay, France (Drouet et al., 2015); and the Atlantic Coast of Portugal and East Africa (Lourenço et al., 2017). All of these sites represent coastal areas, and no published research investigates shorebird biofilm ingestion at inland migration stopover sites; however, biofilms are known to form at Great Salt Lake, Utah, another large saline lake and critical shorebird habitat in the western United States (Leffer, 2021).

American Avocet at Seawall site. | Photo: Marlene Walters

Biofilm is especially nutritious during shorebird migration periods because seasonal mixing of fresh and saltwater at coastal mudflats causes osmotic stress that triggers greater lipid production within diatoms (Schnurr et al., 2019). At the Salton Sea, freshwater agricultural runoff inputs create similar seasonal mixing conditions that may resemble those found on coastal mudflats. As strong evidence now shows that biofilm in an essential dietary component for several shorebird species during migration, it is imperative to complement coastal estuary research and investigate the occurrence of biofilm at inland stopover habitats as well. Recognizing where biofilm is present along migration paths and identifying the environmental conditions that promote production is vital for shorebird conservation planning.

The State of California is implementing the Salton Sea Management Program (SSMP) 10-year Plan to improve conditions at the Salton Sea through habitat restoration and dust suppression projects. As the State implements habitat projects as a part of this long-term plan, areas surrounding the Salton Sea that contain important food resources for shorebirds should be a conservation management priority. Investigating the presence of biofilm as a potential food source for shorebirds at the Salton Sea could inform the State of California's conservation and habitat strategies. To assess the availability of biofilm at the Salton Sea, shoreline sediments were sampled where either Western Sandpipers or Least Sandpipers *(Calidris minutilla)* were observed foraging in spring (late April), summer (late July), and fall (early October) and tested for the presence of organisms known to form the nutritious components of biofilm. This report will inform further exploration into the role of biofilm in shorebird diets at the Salton Sea and highlight the need to conserve this essential nutrition source.

Materials and Methods

DATES AND LOCATIONS

Samples were collected at 3 areas on the eastern shore of the Salton Sea between 27–29 April 2023, 24–25 July 2023, and 1 October 2023. In April and October, these areas were near the Salton Sea Visitors Center (33.503368, -115.916198), near Salt Creek Beach Campground (33.442906, -115.846074), and at the Bombay Beach wetlands (33.352264, -115.696734). In July, the locations were North Shore (33.52076, -115.94876), Bombay Beach wetlands (33.34213, -115.67856), and the Seawall (33.16221, -115.64925) (Fig. 2). Western and/or Least Sandpipers were observed feeding at these areas, and all of these areas had shoreline sediment firm enough for researchers to walk safely to the water's edge. Sandpipers were not observed feeding at either the Visitor's Center or Salt Creek campground during the July visit. Migratory shorebirds do not return to the section of eastern shoreline fed by Salt Creek until inflow returns in late summer; in the summer of 2023, shorebirds returned to Salt Creek sites later than expected due to exceptional dryness (R. McKernan, personal communication, 2023).

FIGURE 2.

A map of the locations sampled in spring and fall (pink circles) and summer (yellow circles).

SEDIMENT SAMPLING

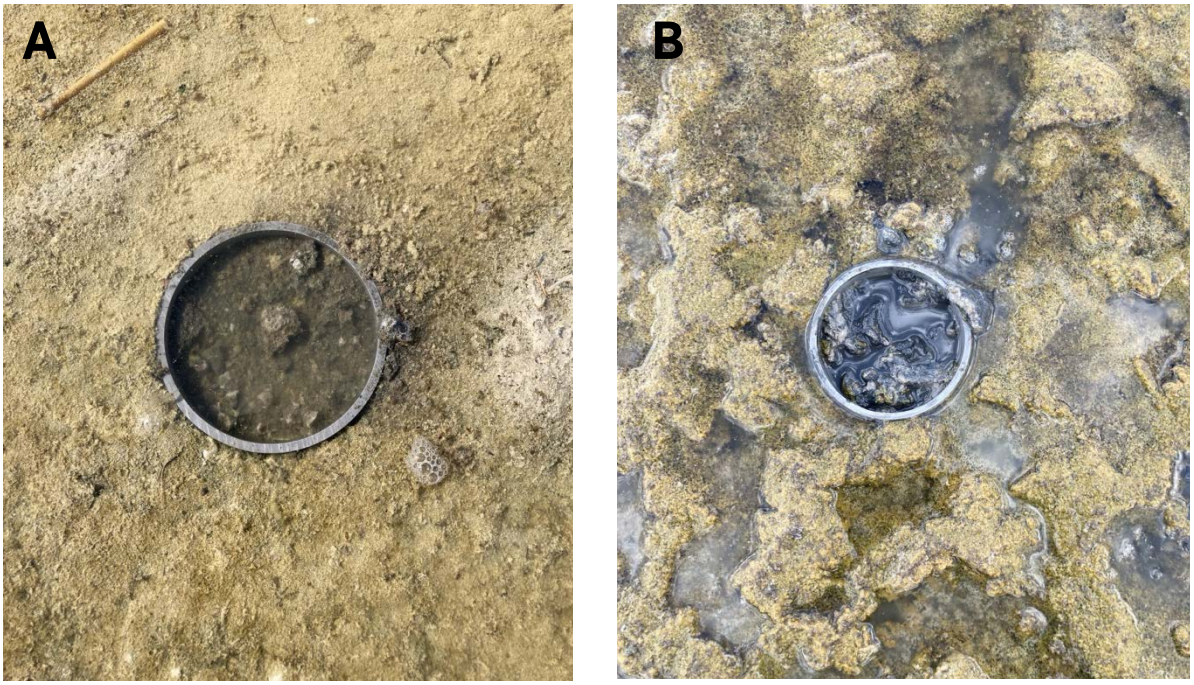
At each of the sampling areas, we collected sediment samples from 4 to 8 shoreline locations, depending on the amount of accessible foraging area to the researcher. We collected a surface sediment sample (~1–3 mm deep) and a below surface sample (~20–30 mm deep) at each of these locations. Western or

Least Sandpipers were observed feeding at each location immediately before sediment was taken. At least 30 m of distance separated each sampling location.

After approaching a space where sandpipers were observed feeding, a PVC ring with an inner diameter of 10 cm and depth of 3 cm was haphazardly tossed to select the sampling location. The ring was pushed into the sediment until flush with the surface (Fig. 3). A stainless-steel putty knife was used to scrape off the top layer of sediment within the ring. This layer was transferred to a 50 ml sampling tube. Next, remaining sediment within the ring was transferred with the putty knife to a 500 ml glass jar. In April, samples were combined with 99.5% ethanol for DNA preservation immediately after collection; this was not done in July or October under advisement of the lab performing analysis. The PVC ring and putty knife were wiped clean with ethanol between each sample.

FIGURE 3.

Photos of the PVC ring used to collect samples pressed into the sediment, on (A) 29 April 2023 at Bombay Beach and (B) 1 October 2023 at Salt Creek Campground.



ANALYSIS

Samples were shipped to Jonah Ventures (Boulder, CO) for DNA barcoding analysis. Jonah Ventures is an environmental DNA laboratory that has assisted academic and agency scientists nationwide in quantifying and identifying DNA, leading to peer-reviewed, published research. The Appendix provides a complete description of test methods provided by Jonah Ventures.

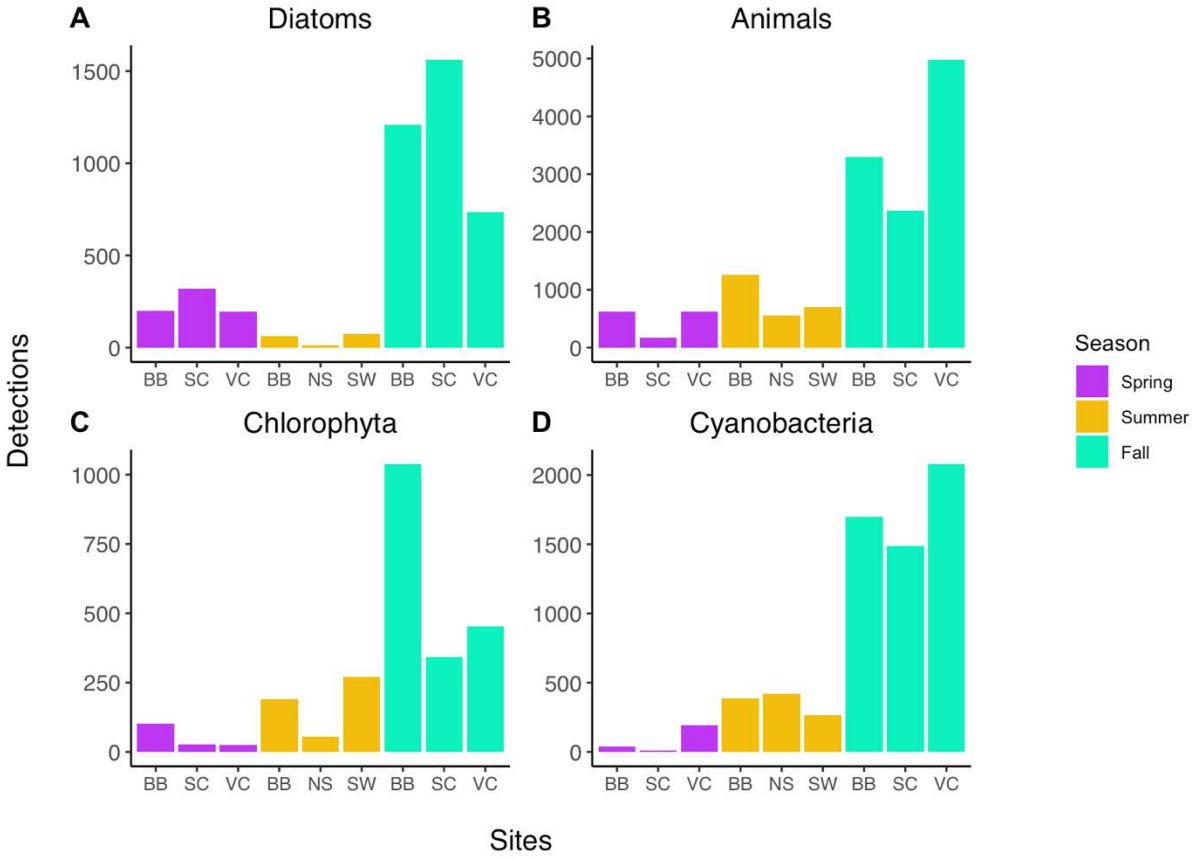
Statistical analyses were performed in R using the lme4 package. Using the glmer function, generalized linear models were constructed to evaluate the effect of season on total DNA detections for each taxon, including site as a random effect.

Results

The species composition across spring, summer, and fall foraging sites differed in both microorganisms (algae, bacteria) and meiofauna (invertebrates). Detections of diatoms in spring surface sediment samples were significantly more than detections in summer samples (Fig. 4a). Conversely, summer foraging sites had more animal detections (Fig. 3b) and significantly more detections of chlorophytes (Fig. 4c) and cyanobacteria (Fig. 3d) compared to spring sites. However, fall samples contained significantly more detections of all four taxon groups compared to both spring and summer (Fig. 4).

FIGURE 4.

Comparative abundance of (A) diatoms, (B) animals, (C) Chlorophyta, and (D) cyanobacteria between spring (green) and summer (gold) foraging sites. Site names are abbreviated: BB = Bombay Beach, SC = Salt Creek Campground, VC = Visitor's Center, NS = North Shore, SW = Seawall. Bars represent the sum of individual detections across all DNA sequences of the indicated taxon at each site, after averaging total counts for each sequence across samples.



Three hundred nine (309) unique diatom DNA sequences were matched in surface samples. Of these, 82 were identified to at least the genus level. Forty-eight sequences matched the genus *Halamphora*. Other diatom genera matched were *Navicula* (14 matches), *Amphiprora* (7 matches), *Chaetoceros* (7 matches), *Nitzschia* (3 matches), *Pleurosigma* (1 match), and *Tryblionella* (1 match). Overall, spring sites had 5.90

(2.36 -14.98, 95% C.L.) times as many diatom detections as summer sites (p = 0.0000237). Fall sites had 28.37 (11.36- 72.02, 95% C.L.) times as many diatom detections as summer sites (p = 0.0000160), and 4.81 (1.92- 12.05, 95% C.L.) times as many diatom detections as spring sites (p = 0.0000237).

Most animals detected were arthropods, with order Lepidoptera (moths and butterflies), family Corixidae (water boatmen), *Tanytarsus hastatus* (a non-biting midge), and two unknown species representing the five most common matches. The most abundant insect detected was not identified beyond order Lepidoptera; detections were abundant in all three seasons. Corixidae detections were abundant in summer and fall, correlating with recent annual outbreaks. *Tanytarsus hastatus* were detected in high abundance at Salt Creek in fall. Other frequent detections included Scleractinia (stony corals), Urocoptidae (a family of land snails), *Proales similis* (a rotifer), *Culicoides* (a genus of biting midges), and *Diplolaimella ariakensis* (a nematode). All phyla detected were Arthropoda, Bryozoa, Chordata (*Cyprinodon macularius,* the endangered desert pupfish), Cnidaria, Gastrotricha, Mollusca, Nematoda, Platyhelminthes, Porifera, and Rotifera. Overall, summer sites had 1.94 (0.90-4.22, 95% C.L.) times as many animal detections as spring sites (p = 0.0602); this result is not significant. Fall sites had 4.28 (1.97- 9.28, 95% C.L.) times as many animal detections as summer sites (p = 0.0000395), and 8.32 (3.84- 18.05, 95% C.L.) times as many animal detections as spring sites (p = 0.00000000209).

Chlorophyta (green algae) of the genera *Picochlorum*, *Tetraselmis*, *Choricystis*, and the family Selenastraceae were detected across both surface and 3 cm-depth sediment samples. The majority of these detections represent the species *Picochlorum soloecismus* from below the sediment surface. Chlorophyta detections increased from spring to summer and were most abundant in fall. Summer sites had 3.43 (1.16-10.14, 95% C.L.) times as many Chlorophyta detections as spring sites (p = 0.0128). Fall sites had 3.84 (1.30-11.35, 95% C.L.) times as many Chlorophyta detections as summer sites (p = 0.00652), and 13.17 (4.46-38.93, 95% C.L.) times as many Chlorophyta detections as spring sites (p = 0.000000192).

The change in cyanobacteria detections between seasons was greater than any of the other taxa analyzed. Summer sites had 8.61 (2.22-33.59, 95% C.L.) times as many cyanobacteria detections as spring sites (p = 0.000502). Fall sites had 4.97 (1.28-19.25, 95% C.L.) times as many cyanobacteria detections as summer sites (p = 0.00936), and 42.83 (11.08-167.00, 95% C.L.) times as many cyanobacteria detections as spring sites (p = 0.000502).

Discussion

We observed that small-bodied sandpipers, particularly the Western Sandpiper, select different foraging sites depending on the season, as they were not present at the Salt Creek or Visitor's Center sites during the July visit, when inputs of fresh water were reduced. We also observed that prey availability at spring, summer, and fall foraging sites was different. This suggests that prey availability for sandpipers changes seasonally at each of the study locations. In fall, numbers of detections significantly increased across all taxa analyzed. This may be due to more frequent and intense precipitation events beginning in August 2023, following several years of persistent drought (National Drought Mitigation Center, 2024).

Diatoms were abundant at the feeding locations sandpipers selected in spring and fall compared to summer locations. Samples from spring and fall locations were rich in *Halamphora, Amphiprora, Navicula,* and *Chaetoceros* species. Many genera of diatoms are notable for accumulating high quantities of lipids, making them nutrient-rich food sources for migrating shorebirds, and are often researched for their potential as producers of biofuel because of their high lipid contents (Bhattacharjya et al., 2020; Jayakumar et al., 2021; Popovich et al., 2012; Stepanek et al., 2016). This finding indicates a likelihood

that biofilm is potentially a rich food source for the Western Sandpiper and other related species during migration stopover at the Salton Sea.

The DNA analysis did not provide many detailed matches for the most often detected invertebrate species. The most abundant invertebrate identified to at least the family level was Corixidae, or water boatmen, which is consistent with a prior Audubon examination of infauna at the Salton Sea in 2020 (C. Bautista, personal communication, 2023). Summer outbreaks of water boatmen have inundated the Sea and surrounding communities, perhaps because their populations are no longer controlled by predatory fish. The most abundant invertebrate detected was not identified beyond order Lepidoptera. Observations of 51 Lepidoptera species occurring at the Salton Sea have been recorded by the Global Biodiversity Information Facility (GBIF.org, 2023). Notably, no DNA matches were returned for the family Ephydridae (brine flies), which occur at the Sea year-round and in summer densely coat some areas of shoreline, despite the application of several primers targeting this family.

Chlorophyta (green algae) of the genera Picochlorum, Tetraselmis, Choricystis, and the family Selenastraceae were detected across both surface and 2–3 cm-depth sediment samples taken in summer and fall. The majority of these detections represent the species Picochlorum soloecismus, a lipid-rich, halophilic green alga also researched for its potential as a biofuel (Gonzalez-Esquer et al., 2019). These species were more abundant in summer and fall and most detections were not on the surface, but in the sediment below and possibly not accessible to grazing sandpipers. Dierschke (1993) observed Purple Sandpipers (Calidris maritima) feeding on green algae attached to rocks and recovered it in droppings. Though diatoms have received more research focus recently as lipid-rich components of biofilm (Schnurr et al., 2019), green algae may provide similar nutrients and may be more prevalent than diatoms during summer months.

Cyanobacteria detections increased the most between spring, summer, and fall out of the four taxa analyzed. Summer heat gives cyanobacteria a competitive advantage against other types of phytoplankton. Cyanobacteria colonies form in floating mats; these are less likely to break up in summer because high heat stabilizes the water column (Jöhnk et al., 2008). As global temperatures increase, so does the potential for harmful cyanobacteria to outcompete diatoms during critical migration periods.

Future Directions

As part of the goal of the SSMP to identify and conserve areas around the Salton Sea with important food resources for migratory shorebirds, further progression of this research should include 1) confirmation of biofilm ingestion through biological sampling of shorebirds, and 2) nutritional analysis of biofilm and identification of diatom species. These investigations will determine the relative contribution of biofilm to shorebird diets at the Salton Sea. Following confirmation of biofilm ingestion by shorebirds, research can then identify shoreline areas with the highest potential for biofilm production based on inflow and sediment parameters.

SHOREBIRD SAMPLING

Given the presence of diatoms on sediment surfaces, as well as the selection of foraging sites by sandpipers where diatoms occur, it may be likely that avian migrants visiting the Salton Sea consume biofilm. However, the complete picture of shorebird diets cannot be ascertained without direct sampling of birds. Confirming that shorebirds feed on biofilm at the Salton Sea and determining what proportion of their diets comprise biofilm requires capturing birds to collect biological material, e.g., fecal samples.

Sandpiper along the Bombay Beach wetlands sampling site. | Photo: Camila Bautista

An analysis of shorebird diets will provide valuable information about the importance of each diet component and in turn the conservation efforts needed to sustain such diets. As sandpipers feed from both primary (e.g., biofilm) and secondary (e.g., invertebrates) trophic levels, they are both consuming and competing with invertebrates for food (Kuwae et al., 2012; Hall et al., 2021). Investigating the complex food web on the Salton Sea's mudflats through shorebird sampling will inform conservation decisions that promote the health of the full ecosystem.

Minimally invasive sampling techniques can confirm diets without harm to the captured animals (Jardine et al., 2015; Kuwae et al., 2008). Isotopic analysis of fecal samples is highly informative of diet and can provide accurate estimates of biofilm consumption similar to those derived from liver tissue (Hobson et al., 2022).

ANALYSIS OF BIOFILM

Biofilm can be analyzed for the presence of the most nutritious lipids. Nutritional breakdowns of biofilm can be compared across time to determine the optimal environmental conditions at the Sea to produce biofilm of the highest quality. They can also be compared to existing studies (Schnurr et al., 2020) to determine if biofilm at the Salton Sea is comparable in quality to those found at coastal mudflats, and if altering landscape conditions might improve nutritional quality.

Diatoms are more studied in marine environments compared to inland areas. The Salton Sea may be home to many undescribed diatom species (Stepanek & Kociolek, 2013). As the Salton Sea is twice as salty as the ocean, descriptions of diatoms adapted to hypersaline environments may assist researchers examining the quality of biofilm at similar inland stopover habitats. Studying these species in more detail may also determine if biofilm production at the Sea is at risk from increasing salinity, or if it can remain resilient if salinity continues to rise.

MUDFLAT HABITAT CONSERVATION

Biofilm quality is dependent on environmental characteristics that may vary on small spatial scales, including sediment grain size, hydrodynamics, and salinity (Ollinik et al., 2021). Additionally, as the critical driver of lipid production in diatoms is osmotic stress (Schnurr et al., 2020), reliable freshwater inflow to the Sea is essential to produce high-quality food resources for shorebirds. Analysis of these abiotic conditions at locations around the Sea, in addition to biofilm sampling for nutritional quality, can identify shoreline areas with conditions best suited to biofilm production. These areas can then be prioritized for optimizing this resource.

Conclusion

The role of inland saline lakes like the Salton Sea in providing biofilm to migrating birds is a new and intriguing line of inquiry and emphasizes the already critical need to conserve the limited number of stopover habitats suitable for shorebirds. Saline lakes across the interior western U.S. are at risk of ecological collapse as fresh water is diverted away and salinity rises to unhealthy levels. This puts millions of birds already devastated by habitat loss at further risk and exposes human residents to toxic sediments as shorelines recede and form large dust clouds. Maintaining the ecological function of these lakes is essential to both public health and the recovery of migratory bird populations in the western United States.

The confirmation of biofilm production on the Salton Sea's mudflats, and the high likelihood that shorebirds ingest it, provides a new path forward under the SSMP to support shorebirds migrating along the Pacific Flyway. The conditions required to promote biofilm production include mudflat habitats where freshwater inflow can reach the Sea in large enough quantities to trigger osmotic stress in diatoms. Most importantly, these freshwater inflows must occur reliably throughout shorebird migration periods; further reduction of runoff during the spring and fall due to drought or agricultural practices may lead to lower-quality biofilm resources for shorebirds.

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Appendix

The following are the DNA barcoding test methods provided by Jonah Ventures.

SAMPLE PROCESS
1.7.1

Frozen samples were thawed for 1-2 hours before processing. Sample barcodes were recorded and assigned a well within the 96 well plate or numbered extraction tube. Under a laminar flow hood, sterile cotton swabs (Fisher, cat# 22-363-173) were coated with sediment matter, and the swabs were placed in the corresponding extraction plate or tube. Sterile tweezers and pliers are used to handle cotton swabs and remove the wooden ends of the cotton swab before extraction. Plates or tubes were immediately processed or stored in -20C until the extraction process could be performed.

EXTRACTION
2.1.3

Genomic DNA from samples was extracted using the DNeasy 96 PowerSoil Pro Kit (384) (Cat # 47017) according to the manufacturer's protocol. Genomic DNA was eluted into 100µl and frozen at -20°C.

PCR (ALGAE)
3.10.1

Forward primer: GGACAGAAAGACCCTATGAA

Reverse primer: TGAGTGACGGCCTTTCCACT

Primer notes: Algal

Primer reference: Sherwood & Presting 2007; Hamsher et al. 2011; Cannon et al. 2016

A portion of the chloroplast trnL intron was PCR amplified from each genomic DNA sample using the p23SrV_f1 and Diam23Sr1 23S primers. Both forward and reverse primers also contained a 5' adaptor sequence to allow for subsequent indexing and Illumina sequencing. Each 40 µL PCR reaction was mixed according to the Promega PCR Master Mix specifications (Promega catalog # M5133, Madison, WI) which included 12.5ul Master Mix, 0.5 µM of each primer, 1.0 µl of gDNA, and 10.5 µl DNase/RNase-free H2O. DNA was PCR amplified using the following conditions: initial denaturation at 94 °C for 3 minutes, followed by 40 cycles of 30 seconds at 94 °C, 45 seconds at 55 °C, and 1 minute at 72 °C, and a final elongation at 72 °C for 10 minutes.

PCR (BACTERIA)
3.4.1

Forward primer: GTGYCAGCMGCCGCGGTAA

Reverse primer: GGACTACNVGGGTWTCTAAT

Primer notes: 515F and 806R

Primer reference: Caporaso, J. G., C. L. Lauber, W. A. Walters, D. Berg-Lyons, C. A. Lozupone, P. J. Turnbaugh, N. Fierer, and R. Knight. 2011. Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample. Proceedings of the National Academy of Sciences 108:4516-4522.

A portion of mitochondrial 16S rDNA gene was PCR amplified from each genomic DNA sample. Both forward and reverse primers contained a 5' adaptor sequence to allow for subsequent indexing and Illumina sequencing. Each 25 µL PCR reaction was mixed according to the Promega PCR Master Mix specifications (Promega catalog # M5133, Madison, WI) which included 12.5ul Master Mix, 0.5 µl of each primer, 1.0 µl of gDNA, and 10.5 µl DNase/RNase-free H2O. DNA was PCR amplified using the following conditions: initial denaturation at 95 ˚C for 5 minutes, followed by 35 cycles of 45 seconds at 95 ˚C, 1 minute at 50 ˚C, and 90 seconds at 72 ˚C, and a final elongation at 72 ˚C for 10 minutes.

PCR (ANIMAL)
3.9.1

Forward primer: GGWACWGGWTGAACWGTWTAYCCYCC

Reverse primer: TAIACYTCIGGRTGICCRAARAAYCA

Primer reference: Leray, M., J. Y. Yang, C. P. Meyer, S. C. Mills, N. Agudelo, V. Ranwez, J. T. Boehm, and R. J. Machida. 2013. A new versatile primer set targeting a short fragment of the mitochondrial COI region for metabarcoding metazoan diversity: application for characterizing coral reef fish gut contents. Front Zool 10:34.

A portion of the universal mitochondrial cytochrome c oxidase subunit I (COI) gene was PCR amplified from each genomic DNA sample using the UniCO1F and UniCO1R primers. Both forward and reverse primers also contained a 5' adaptor sequence to allow for subsequent indexing and Illumina sequencing. Each 25 µL PCR reaction was mixed according to the Promega PCR Master Mix specifications (Promega catalog # M5133, Madison, WI) which included 12.5ul Master Mix, 0.5 µM of each primer, 1.0 µl of gDNA, and 10.5 µl DNase/RNase-free H2O. DNA was PCR amplified using the following conditions: initial denaturation at 94 ˚C for 2 minutes, followed by 45 cycles of 15 seconds at 94 ˚C, 30 seconds at 50 ˚C, and 1 minute at 72 ˚C, and a final elongation at 72 ˚C for 10 minutes.

GEL
4.1.1

To determine amplicon size and PCR efficiency, each reaction was visually inspected using a 2% agarose gel with 5µl of each sample as input.

PCR AMPLICON CLEANUP
5.1.1

Amplicons were then cleaned by incubating amplicons with Exo1/SAP for 30 minutes at 37C following by inactivation at 95C for 5 minutes and stored at -20C.

BARCODING PCR
6.1.1

A second round of PCR was performed to complete the sequencing library construct, appending with the final Illumina sequencing adapters and integrating a sample-specific,12-nucleotide index sequence. The indexing PCR included Promega Master mix, 0.5 µM of each primer and 2 µl of template DNA (cleaned amplicon from the first PCR reaction) and consisted of an initial denaturation of 95 ˚C for 3 minutes followed by 8 cycles of 95 ˚C for 30 sec, 55 ˚C for 30 seconds and 72 ˚C for 30 seconds.

PCR NORMAL POOL
8.1.1

Final indexed amplicons from each sample were cleaned and normalized using SequalPrep Normalization Plates (Life Technologies, Carlsbad, CA). 25µl of PCR amplicon is purified and normalize using the Life Technologies SequalPrep Normalization kit (cat#A10510-01) according to the manufacturer's protocol. Samples are then pooled together by adding 5µl of each normalized sample to the pool.

SEQUENCING
9.7.1

Sample library pools were sent for sequencing on an Illumina MiSeq (San Diego, CA) at the Texas A&M Agrilife Genomics and Bioinformatics Sequencing Core facility using the v2 500-cycle kit (cat# MS-102-2003). Necessary quality control measures were performed at the sequencing center prior to sequencing.

BIOINFORMATICS
10.2.2

Raw sequence data were demultiplexed using pheniqs v2.1.0 [1], enforcing strict matching of sample barcode indices (i.e, no errors). Cutadapt v3.4 [2] was then used remove gene primers from the forward and reverse reads, discarding any read pairs where one or both primers were not found at the expected location (5') with an error rate < 0.15. Read pairs were then merged using vsearch v2.15.2 [3], discarding resulting sequences with a length of < 300 bp, > 380 bp, or with a maximum expected error rate [4] > 0.5 bp. For each sample, reads were then clustered using the unoise3 denoising algorithm [5] as implemented in vsearch, using an alpha value of 5 and discarding unique raw sequences observed less than 8 times. Counts of the resulting exact sequence variants (ESVs) were then compiled and putative chimeras were removed using the uchime3 algorithm, as implemented in vsearch. For each final ESV, a consensus taxonomy was assigned using a custom best-hits algorithm and a reference database consisting of publicly available sequences (GenBank [6]) as well as Jonah Ventures voucher sequences records. Reference database searching used an exhaustive semi-global pairwise alignment with vsearch, and match quality was quantified using a custom, query-centric approach, where the % match ignores terminal gaps in the target sequence, but not the query sequence. The consensus taxonomy was then generated using either all 100% matching reference sequences or all reference sequences within 1% of the top match, accepting the reference taxonomy for any taxonomic level with > 90% agreement across the top hits.

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