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FEATURE ARTICLE

If You Build It, Will They Come? An Environmental DNA Assessment of Fish Assemblages on Artificial Reefs in the Northern Gulf of Mexico

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Abstract

Globally, estuarine and marine fisheries have declined over the past century, and a variety of approaches have been employed in an attempt to improve fisheries, including the development of protected areas, implementation of catch regulations, hatchery stocking, and habitat augmentation. The focus of this study was to assess the impact of the introduction of artificial reefs on the fish assemblages in the northern Gulf of Mexico (nGOM). Unfortunately, assessing the success of artificial reefs has been problematic due to the high turbidity of the region and the difficulty of using traditional sampling gears to assess species diversity at reefs. To accomplish this, we gathered environmental DNA metabarcode data (12S) to assess the impact of reef age (1–19 years), construction material (limestone, concrete, shell, and oil and gas), and season on the fish assemblages at nine artificial reefs in the nGOM. The results indicate higher species richness at reefs versus paired control sites as well as differences between seasons, reef materials, and reef positions. Our results suggest that this technique is a viable method of monitoring ray-finned fish species on artificial reefs and can provide baseline information on the fish assemblages associated with artificial reefs in the nGOM.

The northern Gulf of Mexico (nGOM) is a biologically unique ecosystem that contains a rich biodiversity of fishes (McEachran and Fechhelm 1998, 2005). Much of the fish diversity present in inshore regions of the nGOM is the result of highly productive wetland and marsh habitats, a shallow continental shelf, and the flow of nutrients into coastal environments via the Mississippi and Atchafalaya rivers (Chesney and Baltz 2001; Rabalais et al. 2002; Piazza and Peyre 2007; Bianchi et al. 2010). However, overexploitation of fish stocks, excessive nutrient loading, sea level rise, and oil spills over the past several decades have impacted catches in recreational and commercial fisheries (Ramelow et al. 1989; Murray and Beck 1990; Bianchi et al. 2010; Anderson and Alford 2014).

In recent decades, artificial reefs have become an important tool for augmenting habitat for the development, improvement, or restoration of fisheries around the globe (Svane and Petersen 2001; Relini et al. 2007; Isroni et al. 2019). The term "artificial reef" is used to describe a broad range of human-made structures, including but not

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limited to intentionally submerged ships and barges, derelict oil rigs, concrete and limestone debris, and commercially fabricated concrete reef balls (Stone 1982; Baine 2001; Svane and Petersen 2001; Lokesha and Sannasiraj 2013). The introduction of hard substrates into areas that may be devoid of such structures allows for the settlement and recruitment of epibenthic and benthic macroinvertebrate organisms, which in turn attract fish populations (Svane and Petersen 2001; Charbonnel et al. 2002; Higgins et al. 2019). Although artificial reefs are generally viewed as a positive fisheries restoration tool, others have suggested that artificial reefs may have negative impacts on fish communities by attracting fish and subsequently increasing the potential for overharvesting, as fish are concentrated at these structures and are susceptible to catch (Bohnsack et al. 1997; Grossman et al. 1997; Lindberg 1997). Despite this debate, artificial reef programs have been developed across the nGOM, resulting in the deployment of multiple artificial reef structures (Simonsen and Cowan 2013; La Peyre et al. 2014; Garner et al. 2019). Recent studies conservatively have estimated that more than 250 inshore artificial reef structures have been deployed across the nGOM (La Peyre et al. 2014; Ajemian et al. 2015).

Evaluating the success of artificial reefs after their deployment has been challenging. A variety of monitoring approaches have been employed, such as gathering recreational angler data, conducting visual SCUBA surveys, and sampling with gill nets (Plunket and La Peyre 2005; Boswell et al. 2010). Unfortunately, all of these approaches have limitations, and they cannot provide accurate species assemblage data for all species and life history stages that are of interest to resource managers (Plunket and La Peyre 2005; Boswell et al. 2010). Environmental DNA (eDNA) has arisen as a promising technique for surveying habitats and environments that are otherwise difficult to sample, such as artificial reefs. Furthermore, utilization of eDNA data can reduce the amount of physical labor and, more importantly, can increase the detectability of a large number of game and nongame fishes on artificial reefs as it has for other aquatic habitats from arctic seas to tropical lakes to temperate rivers (Foote et al. 2012; Sigsgaard et al. 2015, 2016; Thomsen and Willerslev 2015; Hänfling et al. 2016; Evans et al. 2017; Yamamoto et al. 2017; Lacoursière-Roussel et al. 2018; Valdez-Moreno et al. 2018). Although eDNA has been used to monitor fish communities across the globe (Yamamoto et al. 2017; Stat et al. 2019), its usefulness for monitoring artificial reefs has not been thoroughly tested.

The objectives of this study were twofold. First, we gathered eDNA data to describe bony fish species richness on artificial reefs that were made of different construction materials, constructed in different years, and sampled during two seasons. It is hypothesized that artificial reefs will possess greater species richness in comparison with control sites due to the fact that they provide habitat complexity and shelter in an otherwise barren landscape. In regards to the material used in reef construction, previous studies have suggested that both artificial and natural reefs with habitat complexity typically possess higher species richness than nonreef habitats (Almany 2004; Lingo and Szedlmayer 2006) due to the occurrence of heterogeneous microhabitats. As a result, concrete and limestone reefs in this study were expected to have higher diversity in comparison with the other materials. Second, we directly tested the effects of reef presence, reef construction material, season, and potential interactions (among these three main effects) on assemblage structure dynamics among sites.

METHODS

Environmental DNA sampling and capture.— Sampling sites consisted of nine artificial reefs and nine paired control sites distributed along the nGOM (Figure 1). Water samples were collected in two separate sampling events; the first effort was undertaken during March, April, and May 2018, while the second sampling event was completed during November and December 2018, for a total of 36 water samples (Table 1). Four different reef types were sampled in this study: (1) concrete rubble consisting of fragmented concrete, (2) limestone reefs comprising piles of limestone fragments/chunks, (3) human-made shell reefs consisting of oyster shells, and (4) oil and gas reefs consisting of metal supports from derelict structures. Each paired control site was located 500 m adjacent to its paired reef following the recommendation of Neves dos Santos and Zalmon (2015), who analyzed fish communities near artificial reefs to determine the relationship between distance (0–300 m) from the reef and species richness and abundance. They noted significant differences (lower abundances and lower species richness) in fish communities located 50–300 m from the reefs. We expanded this distance to 500 m and sampled controls in either an east or west direction to minimize false detection due to total or current movement of eDNA from a reef. Sampling was conducted at slack tide for each reef when possible.

Prior to sampling, multiple coolers used for transportation of water samples were sterilized with 10% bleach and rinsed twice with tap water. In addition, multiple 2-L Nalgene bottles were sterilized by autoclaving at 93°C for 60 min. At each sampling site, three 2-L water samples were taken from each reef and each control site. During each water capture event, a Van Dorn sampler (Aquatic Research Instruments, Inc.) was lowered to 0.5–1.0 m above the reef or the benthos (for controls). In addition, a negative control was taken at each sampling site. A Nalgene bottle was filled with deionized water in the laboratory and transported to each site in a sterile cooler. At each site, the negative control bottle was opened, exposed

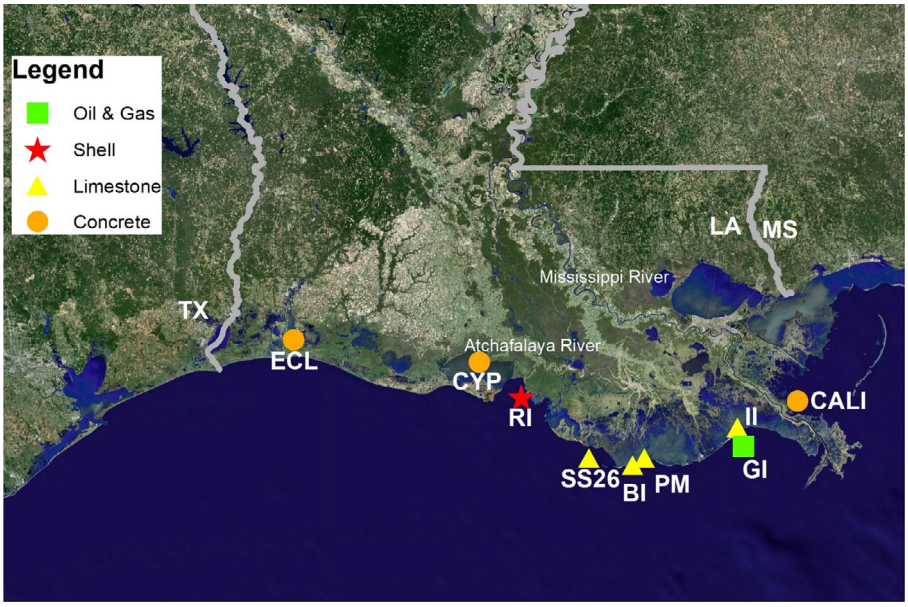

FIGURE 1. Map depicting the artificial reefs sampled in the northern Gulf of Mexico during spring and fall 2018. Abbreviations (defined in Table 1) refer to reefs mentioned in the text.

to the air, resealed, submerged under the water's surface, sterilized, rinsed, and returned to the sterile cooler with the other sampling bottles. This ensured that the sample contained water from the immediate reef or control area. Water samples were transferred to a sterile 2-L Nalgene bottle and then stored on ice in a sterilized cooler. Next, the sampler was submerged into a bucket containing 10% bleach solution and then rinsed twice with nuclease-free water between reef and control sites to prevent DNA carryover. Each water sample was stored on ice in a cooler and returned to the laboratory within 2–4 h after collection. Each water sample was filtered within 24 h of collection by using vacuum filtration with a 47-mm glass microfiber membrane filter with a 1.5-μm pore size (Whatman, catalog number 1827-047); 1 L of each sample was filtered per filter.

Extraction and amplification.— Extraction of DNA was performed using the Qiagen MoBio DNeasy Power Water Kit (Qiagen, Inc., catalog number 14900-100-NF) following the manufacturer's protocol, with the exception of the final DNA elution step, which was reduced to 75 μL of Buffer EB (elution buffer) instead of 100 μL in order to increase the DNA concentration. At the conclusion of the extraction, DNA concentration was quantified using a Qubit High-Sensitivity dsDNA (double-stranded DNA) Kit (Invitrogen, catalog number Q32854). Preparation for PCR took place in a room separate from where DNA was extracted. The reactions were prepared in an enclosed Air Clean System PCR workstation with a HEPA filter. The workstation space, tips, tubes, and water were decontaminated using the ultraviolet light in the workstation prior to setting up the reactions. A 301-bp fragment of the 12S ribosomal RNA gene was amplified using the protocol of Miya et al. (2015). The first round of PCR amplification was conducted in triplicate for each reef and each control, resulting in 18 amplifications for each reef–paired control sampling site per sampling period. Primers for the first round of PCR consisted of the priming sequence as well as an overhanging adaptor sequence for a second round of PCR. The final reaction volume for PCR was 12 μL, including 0.7 μL of each primer (10 μM), 6 μL of 2× KAPA HiFi HotStart ReadyMix (Kapa Biosystems, Inc.), 3.6 μL of deionized H_2O, and 2 μL of template. Reactions were run under the following conditions: 94°C for 2 min; then 35 cycles of 98°C for 15 s, 50°C for 30 s, and 72°C for 30 s; and finally 72°C for 5 min. All reactions were carried out in a Veriti 96-Well Thermal Cycler (Applied Biosystems, Inc.). All PCR products were visualized on a 2% agarose gel via gel electrophoresis with a 100-bp ladder (New England Biolabs, catalog number N3231L).

The second round of PCR used a 1:10 diluted aliquot of the first-round PCR products as a template for a second reaction. The second PCR consisted of primers containing the amplification primer, dual-indexed sequences, and adaptors that bind to the Illumina flow cell. The final reaction volume was 12 μL, including 6 μL of 2× KAPA HiFi HotStart ReadyMix, 0.7 μL of each primer (10 μM), 3.6 μL of deionized H_2O, and 1 μL of template. The reaction was run using a thermal cycle profile with an initial 3 min at 95°C; then, 12 cycles of 98°C for 20 s and 72°C for 15 s; and finally, 72°C for 5 min. All reactions were carried out in a Veriti 96-Well Thermal Cycler. All products were

TABLE 1. Names, locations, year of construction, and sampling dates for reefs in the northern Gulf of Mexico.

Reef site name	Abbreviation	Water body	Reef material	Year of reef construction	Latitude	Longitude	Depth of reef (m)	Spring 2018 sampling date	Fall 2018 sampling date
California Point	CALI	Breton Sound	Concrete	2013	29°29.024'	89°29.049'	2.53	Mar 16	Nov 19
Grand Isle 9	GI	Barataria Bay	Oil and gas	1999	29°11.2211'	89°53.3357'	12.53	Mar 25	Nov 20
Independence Island	II	Barataria Bay	Limestone	2012	29°18.450'	89°56.002'	2.02	Mar 25	Nov 20
Point Mast	PM	Terrebonne Bay	Limestone	2017	29°06.444'	90°38.143'	2.83	May 8	Dec 18
Bird Island	BI	Lake Pelto	Limestone	2009	29°3.5635'	90°43.3929'	2.24	May 8	Dec 18
Ship Shoal 26/Pickets	SS26	Terrebonne Bay	Limestone	2014	29°6.4361'	91°3.2458'	2.64	May 8	Dec 18
Rabbit Island	RI	Cote Blanche Bay	Shell	2013	29°30.567'	91°33.867'	3.10	Mar 9	Nov 16
Cypremort Point 2	CYP	Vermilion Bay	Concrete	2013	29°44.328'	91°52.764'	1.99	Mar 9	Nov 16
East Calcasieu Lake	ECL	Calcasieu Lake	Concrete	2017	29°53.114'	93°16.763'	1.88	Mar 9	Dec 6

visualized on a 2% agarose gel via gel electrophoresis. The appropriately sized PCR band was excised from the gel. After excision, PCR amplicons from the second round of PCR were cleaned up using the Qiagen QIAquick Gel Extraction Kit (catalog number 28706). Standard procedures were followed except for the last step, in which 40 μL of Buffer EB were used instead of 50 μL to increase the final concentration of the DNA.

Illumina sequencing.— At the conclusion of the second round of PCR amplification, the concentrations of all products were sequenced on an Illumina HiSeq platform (Caporaso et al. 2012; Shokralla et al. 2012). The recovered sequences were demultiplexed and then input into the MiFish analytical pipeline (Sato et al. 2018). Within the pipeline, sequences were subjected to an initial quality check using FastQC; any sequences with low-quality scores had their 3' tails trimmed by DynamicTrim.pl (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/ ; Cox et al. 2010). All paired-end reads were merged by FLASH along with erroneous merged reads that contained either uncalled nucleotides or reads without typical lengths (Magoč and Salzberg 2011; Sato et al. 2018). Primer sequences and those with a maximum 3-bp mismatch were removed by the program TagCleaner (Schmieder et al. 2010). Uclust and NCBI Blast+ were used to assign species and their taxonomic assignment (Camacho et al. 2009; Edgar et al. 2011). The conclusion of the pipeline yielded two data sets: 80–96% and ≥97% similarity matrices. All analyses were conducted using the ≥97% data set as it provided the most confident species identification.

Data analysis.— Two analytical approaches were conducted to assess fish assemblages from the nine artificial reefs and their paired controls. First, we examined variation in species richness among and between the reefs and paired controls. In addition, we described variation in fish species richness on artificial reefs of different ages (year of construction), consisting of different construction materials, and sampled across two seasons. Species richness was chosen as a measure of diversity because of its universal importance in biology (Stirling and Wilsey 2001; Spellerberg and Fedor 2003; Olds et al. 2016). In addition to raw richness, we ran rarefaction and extrapolation using the Hsieh et al. (2016) procedure for all comparisons presented. Second, we analyzed the eDNA data in a multivariate (assemblage structure) context. We used permutational multivariate ANOVA (PERMANOVA) based on the Sorensen index resemblance matrix to examine the influences of three main effects (reef presence, season, and reef material) on fish assemblage structure. We used the Sorensen index because it handles incident data (i.e., presence/absence data) in a similar manner as Bray–Curtis with abundance data (Legendre and Legendre 2012). We employed a three-way crossed PERMANOVA using type III sums of squares (partial) and 9,999

permutations of residuals under a reduced model. This design allowed for the examination of potential interactions between the three main effects. Reef construction year was not included in the model due to a lack of necessary replication within reef material groups (i.e., numerous "empty cells" present in the model). Nonmetric multidimensional scaling (NMDS) based on the same previous Sorensen index matrix was used to visually depict relationships among sites, and similarity percentage (SIMPER) analysis was used to identify the fish species that were most responsible for assemblage structure patterns. The statistical model was run using PERMANOVA+ in PRIMER version 7 (Anderson et al. 2008), and NMDS visualizations and SIMPER analyses were conducted using PRIMER version 6.1.10.

RESULTS

Total Species Richness

In total, 32 fish species were detected across both sampling periods for all reefs and control sites. Twenty-six species were detected at both reef and control sites, three species were unique to control sites, and three species were unique to artificial reefs (Appendix Tables A.1.1−A.1.4). The rarefaction and extrapolation of richness did not differ much from the raw richness results so only the raw analyses will be presented here. The rarefaction and extrapolation data are presented in Appendix 2. The data are available from datadryad (https://doi.org/10.5061/dryad.xpnvx0kh3).

Species Richness: Spring 2018 Sampling

Based on the ≥97% sequence similarity matrix, 24 species were detected across all reef sites during the spring 2018 sampling period, with the California Point (CALI) reef having the greatest number of species ($N = 18$) and the Point Mast (PM) reef having the fewest species ($N = 7$; Figure 2A). The average number of species detected across all reefs was 12.0. Gulf Menhaden *Brevoortia patronus* accounted for the majority of reads (90.55%) recovered across all reefs, followed by Striped Mullet *Mugil cephalus* (4.00%) and Red Drum *Sciaenops ocellatus* (2.62%). All other species comprised less than 1% of the total reads. Nineteen species were detected across all paired control sites, with Rabbit Island (RI) and East Calcasieu Lake (ECL) both having the greatest number of species ($N = 15$), while the paired control site for the Grand Isle 9 (GI) reef had the fewest species detected ($N = 6$). Again, Gulf Menhaden accounted for the most reads (98.37%) across all control sites. Mean species richness values, although higher on average for reefs, were not significantly different than those for control sites ($t = 0.98$, df = 8, $P = 0.170$).

Different numbers of species were detected at reefs comprised of different construction materials. Across reef materials (spring 2018), limestone reefs (Bird Island [BI], Independence Island [II], PM, and Ship Shoal 26/Pickets [SS26]) had the largest number of total species, with 21 ($\overline{x} = 13.1$, range = 9–18); followed by concrete reefs (CALI, Cypremort Point 2 [CYP], and ECL), with 14 species ($\overline{x} = 12.3$, range = 10–14); the shell reef (RI), with 9 species; and the oil and gas reef (GI), with 8 species (Figure 3A).

Across reef construction years, the reef that was constructed in 2009 (BI) possessed the largest total number of species, with 18, whereas the ECL and PM reefs (constructed in 2017) possessed the fewest total species, with 11 ($\overline{x} = 11.0$, range = 9–10; Figure 3B). Other reef ages had total species richness values that were intermediate, with 13 (SS26, 2014) and 14 ($\overline{x} = 13.4$, range = 13–14) for the GI (1999), II (2012), CALI (2013), and CYP (2013) reefs.

Species Richness: Fall 2018 Sampling

Based on the ≥97% sequence similarity matrix, 22 species were detected during the fall 2018 sampling period, with the CALI reef having the greatest number of species ($N = 18$) and the PM and GI reefs having the fewest species ($N = 8$; Figure 2B). The average number of species detected across all reefs was 12.7. Gulf Menhaden, Striped Mullet, and Red Drum accounted for the majority of the reads (97.17%) across all reef sites. Twenty-four species were detected across all paired control sites, with the most species detected at RI ($N = 16$), while the GI control site had the fewest species ($N = 6$). Gulf Menhaden accounted for the majority of reads (98.11%) across all control sites. Mean species richness values, although higher on average for reefs, were not significantly different than those of control sites ($t = 0.23$, df = 8, $P = 0.410$).

Differences in species richness (fall 2018) were detected for reefs comprised of different construction materials. In total, 19 species were detected across all reef materials in fall 2018. Concrete reefs ($\overline{x} = 16$ species, range = 14–18; CALI, CYP, ECL) and limestone reefs ($\overline{x} = 10.8$ species, range = 8–13; II, PM, BI, SS26) had the most total species, with 18; followed by the shell reef (RI), with 15 species; and the oil and gas reef (GI), with 8 species (Figure 3A). Across reef construction years, reefs constructed in 2013 (CALI, CYP, RI) possessed the largest total number of species, with 19 ($\overline{x} = 16.3$, range = 15–18; Figure 3B), whereas the GI reef, constructed in 1999, possessed the fewest total species ($N = 8$). Other reef ages had total species richness values that were intermediate, with 10 (II, 2012), 11 (SS26, 2014), 13 (BI, 2009), and 15 (ECL, PM; $\overline{x} = 12.0$, range = 8–14) species.

Assemblage Structure Analyses

Across all samples collected, two main effects (Season and Reef Material) each explained significant (Season:

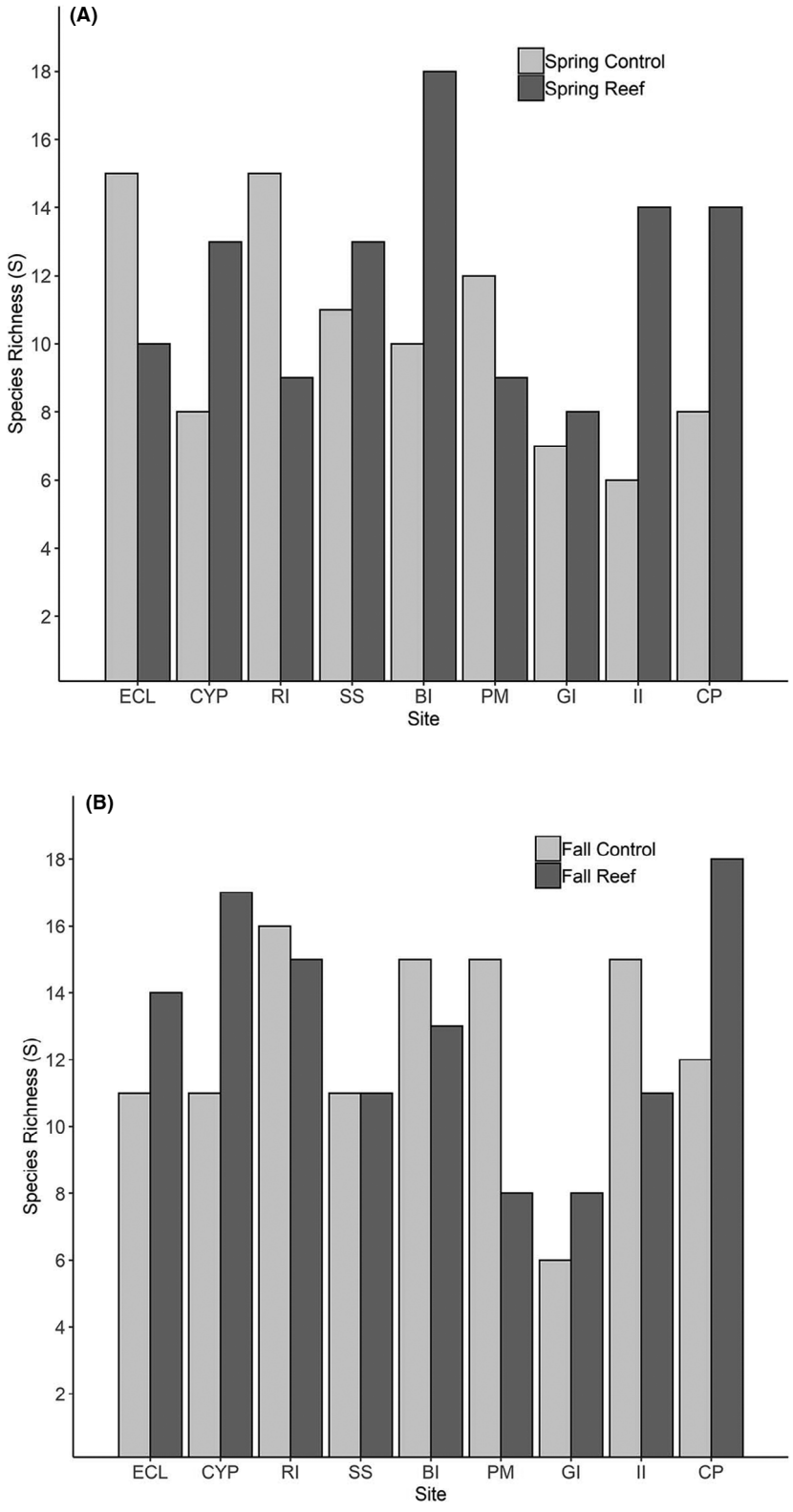

FIGURE 2. Species richness (*S*) based on environmental DNA for artificial reefs and control sites in the northern Gulf of Mexico for **(A)** spring 2018 and **(B)** fall 2018 sampling events. Site abbreviations correspond to those in Table 1 (SS = SS26; CP = CALI).

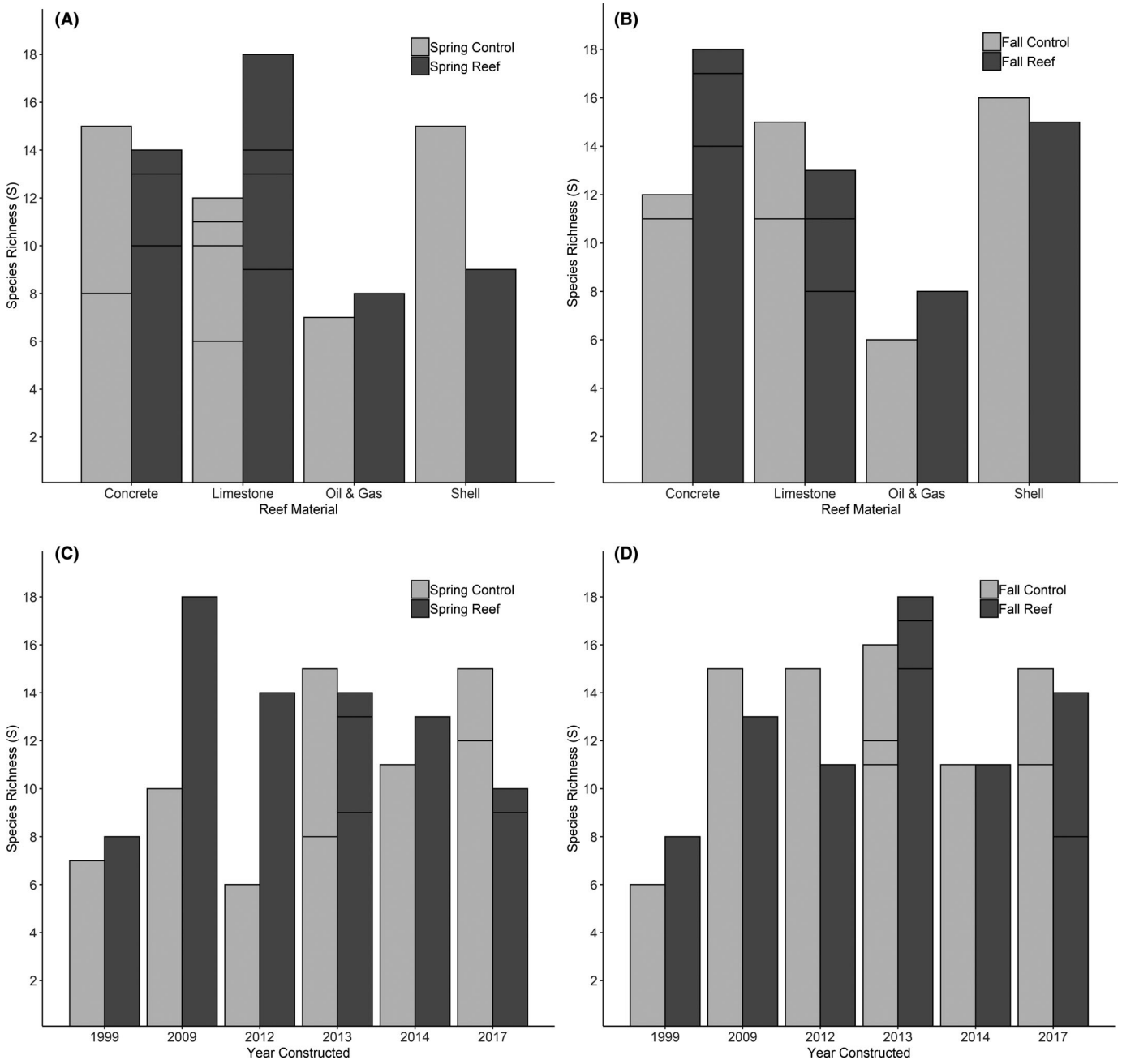

FIGURE 3. Species richness (*S*) based on environmental DNA for spring and fall 2018 sampling events at artificial reef and control sites in the northern Gulf of Mexico: **(A)**, **(B)** artificial reef material and **(C)**, **(D)** reef construction year. Horizontal lines represent replicate samples within each treatment.

pseudo-$F = 23.986$, df = 1, $P = 0.0001$; Reef Material: pseudo-$F = 2.763$, df = 3, $P = 0.0044$) assemblage structuring based on the PERMANOVA model (Table 2). In addition, there were two significant interactions uncovered by the model: Reef Material × Reef or Control (pseudo-$F = 2.148$, df = 3, $P = 0.021$) and Season × Reef Material × Reef or Control (pseudo-$F = 2.459$, df = 3, $P = 0.016$). The Reef Material × Season interaction and the Season × Reef or Control interaction were not significant in the initial model run (P-values > 0.4 for each), so they were removed from the model one at a time (starting with the lowest sum of squares value) and pooled with model residuals. This resulted in the model presented in Table 2. The NMDS visualizations of the main effects Season and Reef Material are depicted in Figures 4 and 5, respectively. The SIMPER analysis of the seasons indicated an average dissimilarity of

TABLE 2. Permutational multivariate ANOVA model showing significant influences of the main effects Season and Reef Material (ReefMat) on fish assemblage structure (ReCon = Reef or Control). Additionally, there were two significant interactions (ReefMat × ReCon; and Season × ReefMat × ReCon). Bold italics indicate significant *P*-values.

Source	df	Sum of squares	Mean square	Pseudo-*F*	*P*(perm)	Unique permutations
Season	1	9,224.1	9,224.1	23.986	***0.0001***	9,947
ReefMat	3	3,188.1	1,062.7	2.7634	***0.0044***	9,939
ReCon	1	459.31	459.31	1.1944	0.3443	9,957
ReefMat × ReCon	3	2,478	825.99	2.1478	***0.0212***	9,916
Season × ReefMat × ReCon	3	2,836.5	945.49	2.4586	***0.0162***	9,942
Pooled	24	9,229.6	384.57			
Total	35	27,059				

44.34%; the top-three species contributing to the seasonal assemblage differences were Chain Pickerel *Esox niger*, Longear Sunfish *Lepomis megalotis*, and Threadfin Shad *Dorosoma petenense* (Table 3).

The relationship among sites based on reef materials is depicted in the NMDS (Figure 5). The only significant relationships, as determined by running pairwise PERMANOVA on Reef Material (Table 4), were (1) between the oil and gas reef (GI) and the concrete reefs (CALI, CYP, ECL; $t = 2.501$, $P = 0.005$) and (2) between GI and the limestone reefs (II, PM, BI, SS26; $t = 1.710$, $P = 0.028$). Based on the SIMPER analysis (Tables 5, 6) between the oil and gas reef and the concrete reefs, the average dissimilarity was 40.48%, with the top-three species contributing to dissimilarities being primary freshwater species: Bigmouth Buffalo *Ictiobus cyprinellus*, Chain Pickerel, and

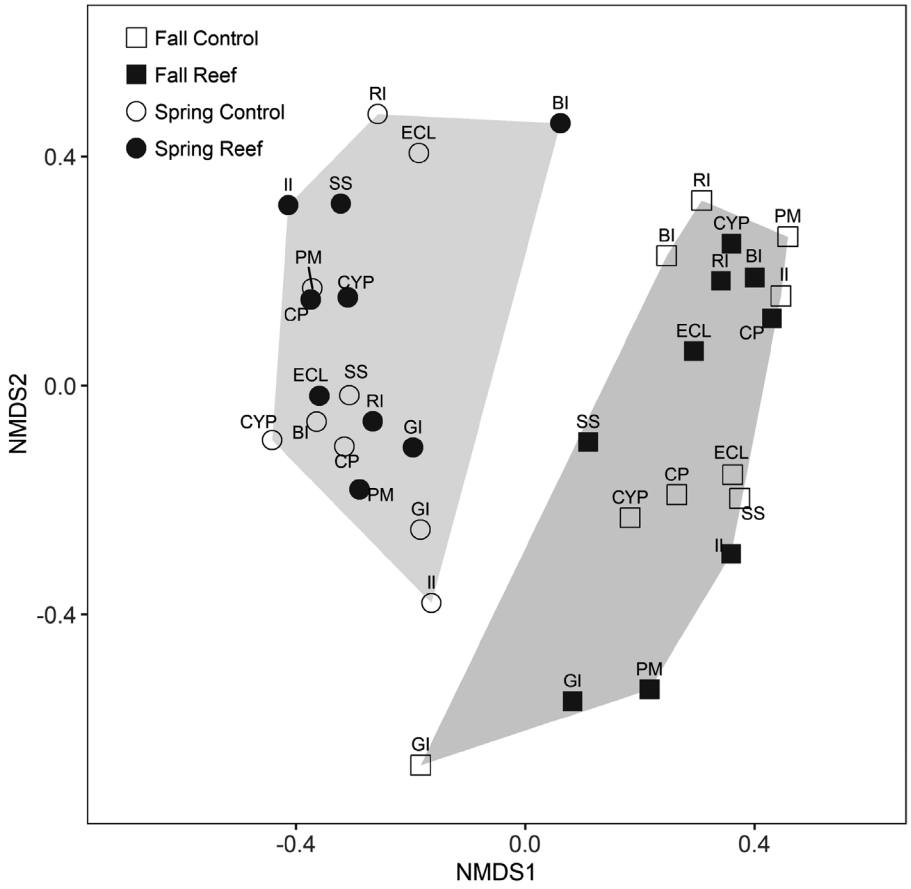

FIGURE 4. Nonmetric multidimensional scaling (NMDS) plot depicting the fish assemblage relationships between seasons in two-dimensional space. Shaded hulls illustrate fall and spring samples. Site abbreviations correspond to those in Table 1 (SS = SS26; CP = CALI).

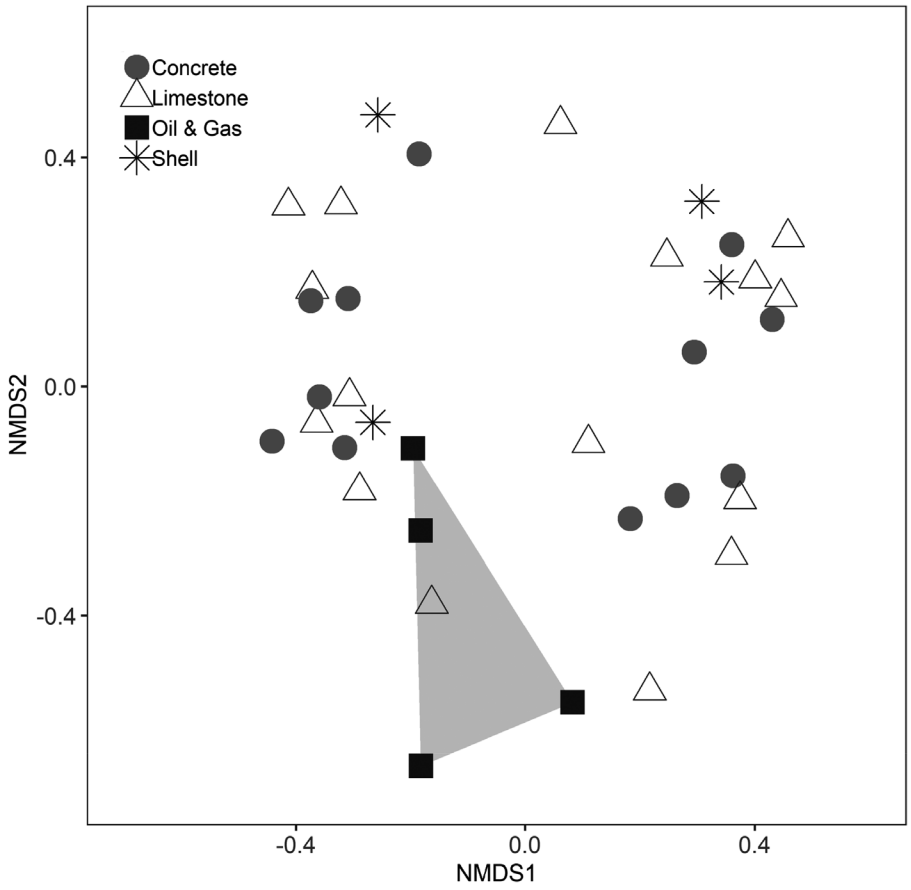

FIGURE 5. Nonmetric multidimensional scaling (NMDS) plot depicting the fish assemblage relationships among reef types in two-dimensional space.

Longear Sunfish. For the oil and gas versus limestone reef comparison, the average dissimilarity was 40.11%, with Grass Carp, Chain Pickerel, and Bigmouth Buffalo (primary freshwater species) being the top-three contributors to reef material assemblage dissimilarity.

DISCUSSION

Assessing artificial reef assemblages has long been challenging due to a suite of logistical sampling constraints (Ajemian et al. 2015). In many cases, researchers have had to take targeted gear approaches to sample a subset of the fishery associated with these structures (Neves dos Santos and Zalmon 2015; Bollinger and Kline 2017; Streich et al. 2017). Unfortunately, many of the sampling gears that have been used to survey artificial reefs have limitations. The inclusion of eDNA data from this study, despite its limitations, indicates that it is a viable approach for monitoring these types of structures as it can provide a snapshot of an entire fish assemblage from a single sampling event, as demonstrated in this study and in other situations (Yamamoto et al. 2017; Ahn et al. 2020; Nester et al. 2020).

Many studies have shown a link between habitat/structure and fish species presence (Friedlander et al. 2003; Pradella et al. 2013). The results from this study indicate that the artificial reefs generally had a positive impact on fish assemblages based on species richness. We hypothesized that artificial reefs would possess greater species richness in comparison to control sites due to the habitat and structure provided by the reefs. Generally, the mean species richness of reef sites (within each season) was greater than control species richness; however, this was not always the case. During the spring 2018 sampling, species richness values for the control sites were lower than or equivalent to those of the paired artificial reefs, with the exception of ECL, RI, and PM. The same pattern was recovered for the fall sampling, with the exception of the RI, BI, PM, and II control sites, which had higher species richness than the artificial reef sites.

In the comparison of reef and control sites, several species were present at both reef and control sites within seasons. Many of the species recovered at both control and reef sites are generalists (i.e., Red Drum, Striped Mullet, and Gulf Menhaden) and are often associated with open-

TABLE 3. Similarity percentage analysis results showing the fish species driving the seasonal distinction in two-dimensional space depicted in Figure 4.

Species	Spring average abundance	Fall average abundance	Average dissimilarity	Dissimilarity/ SD	Contribution (%)	Cumulative percentage
Chain Pickerel *Esox niger*	0.83	0.00	3.52	2.02	7.94	7.94
Longear Sunfish *Lepomis megalotis*	0.72	0.00	3.10	1.51	6.99	14.94
Threadfin Shad *Dorosoma petenense*	0.00	0.72	3.08	1.52	6.96	21.89
Alligator Gar *Atractosteus spatula*	0.06	0.61	2.56	1.17	5.78	27.68
Grass Carp *Ctenopharyngodon idella*	0.56	0.50	2.22	0.95	5.01	32.69
Gafftopsail Catfish *Bagre marinus*	0.06	0.56	2.21	1.08	4.99	37.68
Southern Flounder *Paralichthys lethostigma*	0.28	0.56	2.17	1.03	4.88	42.57
Jack *Caranx* sp.	0.33	0.50	2.11	0.96	4.77	47.33
Redspotted Sunfish *Lepomis miniatus*	0.06	0.50	2.10	0.97	4.73	52.06
Darter Goby *Ctenogobius boleosoma*	0.00	0.44	1.92	0.87	4.33	56.39
Least Puffer *Sphoeroides parvus*	0.17	0.44	1.85	0.91	4.17	60.56
Skipjack Herring *Alosa chrysochloris*	0.06	0.44	1.84	0.86	4.15	64.71
Largemouth Bass *Micropterus salmoides*	0.06	0.44	1.78	0.89	4.01	68.72
Western Mosquitofish *Gambusia affinis*	0.00	0.39	1.76	0.78	3.98	72.70

TABLE 4. Pairwise permutational multivariate ANOVA results confirming that fish assemblages on the oil and gas reef differed significantly from assemblages on limestone and concrete reefs. Bold italics indicate significant *P*-values (*P*[MC] = Monte Carlo *P*-values).

Comparison	*t*	*P*(perm)	Unique permutations	*P*(MC)
Limestone vs. concrete	1.4626	0.0994	9,959	0.1109
Limestone vs. oil and gas	1.7102	***0.0279***	9,950	0.0366
Limestone vs. shell	1.1716	0.2785	9,959	0.2707
Concrete vs. oil and gas	2.5009	***0.0048***	9,942	0.0056
Concrete vs. shell	1.2683	0.2248	9,933	0.2316
Oil and gas vs. shell	1.7211	0.1308	799	0.1592

water habitats and/or habitats with structure. Therefore, it not surprising to detect such species at both reef and control sites. Although we attempted to sample control sites during low tide and at a 500-m distance from the reefs, we have no assurance that DNA from reef and control sites had not drifted into other areas for the species shared at reef and control sites. Kelly et al. (2018), however, noted that most eDNA recovered in nearshore marine waters is endogenous in nature and does not seem to change with the tides. These results and the distance between reef and control habitats suggest that the eDNA recovered in our study is likely from the movement of fish at or around reef and control sites rather than from tidal movements and is representative of the reef community.

Reef Material

In regards to the material used in reef construction, previous studies using visual surveys have shown that both artificial as well as natural reefs with greater habitat complexity typically possess higher species richness (Charbonnel et al. 2002; Almany 2004; Lingo and Szedlmayer 2006). The hypothesized reason for such observations was an increased heterogeneity of microhabitats through the creation of crevices and open spaces of varying sizes that provide shelter and cover for various prey items (Bohnsack 1991; Sherman et al. 2002; Hunter and Sayer 2009). As a result, concrete and limestone reefs in this study were expected to have higher diversity in comparison to the other reef materials. Our results indicate that concrete, limestone, and shell reefs possessed similar species richness values, followed by the oil and gas reef, thereby supporting our hypothesis. On a broader scale, a meta-analysis of 39 studies from across the globe showed that there was no relationship between artificial reef material and species richness (Paxton et al. 2020). Another study, which examined colonization of three different reef types, noted no significant difference in species richness among reef ball, rock pile, and layered cake reefs, but significant differences were observed among artificial reefs and bare sand control sites (Hylkema et al. 2020), indicating that reefs do provide attractive cover and shelter for fishes.

TABLE 5. Similarity percentage analysis results showing the fish species driving the significant distinctions between the oil and gas reef and the concrete reef type (average dissimilarity = 40.11) in the pairwise analysis (Table 4).

Species	Concrete: average abundance	Oil and gas: average abundance	Average dissimilarity	Dissimilarity/ SD	Contribution (%)	Cumulative percentage
Grass Carp	0.83	0.25	3.41	1.35	8.50	8.50
Chain Pickerel	0.50	0.25	2.68	0.96	6.69	15.18
Bigmouth Buffalo	1.00	0.50	2.63	0.96	6.55	21.73
Threadfin Shad	0.50	0.00	2.42	0.97	6.02	27.75
Longear Sunfish	0.42	0.00	2.39	0.82	5.95	33.70
Jack *Caranx* sp.	0.42	0.25	2.26	0.88	5.63	39.33
Inland Silverside *Menidia beryllina*	0.50	0.00	2.25	0.98	5.60	44.93
Alligator Gar	0.33	0.25	2.07	0.82	5.15	50.08
American Eel	0.25	0.25	1.95	0.75	4.85	54.94
Darter Goby	0.25	0.25	1.91	0.76	4.76	59.70
Southern Flounder	0.42	0.00	1.90	0.83	4.74	64.43
Skipjack Herring	0.17	0.25	1.70	0.68	4.25	68.68
Western Mosquitofish	0.33	0.00	1.68	0.69	4.19	72.87

TABLE 6. Similarity percentage analysis results showing the fish species driving the significant distinctions between the oil and gas reef and the limestone reef type (average dissimilarity = 40.48) in the pairwise analysis (Table 4).

Species	Limestone: average abundance	Oil and gas: average abundance	Average dissimilarity	Dissimilarity/ SD	Contribution (%)	Cumulative percentage
Bigmouth Buffalo	0.88	0.50	2.69	0.97	6.65	6.65
Longear Sunfish	0.44	0.00	2.27	0.86	5.60	12.25
Chain Pickerel	0.38	0.25	2.26	0.86	5.57	17.83
Jack *Caranx* sp.	0.38	0.25	2.24	0.85	5.53	23.36
Grass Carp	0.31	0.25	2.22	0.80	5.49	28.85
Alligator Gar	0.38	0.25	2.22	0.86	5.47	34.32
Least Puffer	0.44	0.00	2.13	0.86	5.26	39.58
Gafftopsail Catfish *Bagre marinus*	0.44	0.00	2.09	0.86	5.16	44.74
Southern Flounder	0.44	0.00	2.00	0.87	4.95	49.69
Threadfin Shad	0.38	0.00	2.00	0.76	4.95	54.64
Skipjack Herring	0.25	0.25	1.97	0.75	4.87	59.51
Redspotted Sunfish	0.38	0.00	1.81	0.76	4.46	63.97
Darter Goby	0.13	0.25	1.60	0.66	3.96	67.93
American Eel *Anguilla rostrata*	0.06	0.25	1.51	0.61	3.73	71.66

The lowest species richness—albeit with one reef sampled across two seasons—was recovered at the oil and gas reef (GI). In addition, we also detected significant differences in the fish assemblages between the oil and gas reef and the other reefs by using multivariate approaches. Although our sampling was limited, these results are intriguing as there has been a push to develop "rigs-to-reefs" programs in many Gulf Coast states (Macreadie et al. 2011; Ajemian et al. 2015). Relative to limestone and concrete reefs, the lack of structural heterogeneity for oil and gas reefs may limit colonization of these reefs by marine and estuarine fishes. Alternatively, the oil and gas reef also possessed the highest salinity values for any reef in this study (>24.0‰) and occurred in the deepest water (>13.0 m), suggesting that salinity and depth may also play a role in the fish assemblage associated with this artificial reef. Many of the freshwater species detected at other, lower-salinity reefs in shallow water were not present at the GI reef. Removal of the GI reef from the PERMANOVA eliminated the statistical significance of reef

material, suggesting that other factors may have a greater influence on assemblage structure and that more replicates are truly needed to address the fish assemblage distinctions associated with oil and gas structures, as well as the impact of reef depth and salinity.

This study recovered seasonal differences in assemblage structure for both the reefs and the control sites. Reefs and controls within seasons were more similar to one another in the NMDS plots than they were to the same reefs and controls across seasons, and this was supported with the PERMANOVA. The lack of fish assemblage stability is relevant but not surprising because we expected instability in the fish assemblages among control sites and between seasons as the open-water controls provide few resources to attract and maintain fish assemblages across sampling periods. In addition, several other studies have noted a lack of stability for reef fish assemblages across seasons for natural and artificial reefs as well as seamounts (Godoy et al. 2002; Jorgensen et al. 2016; Vaughan et al. 2020). Although sampling was conducted for only two seasons in our study, fish assemblages associated with artificial reefs also showed a large degree of instability across sampling periods. The lack of seasonal stability for both reef and control sites suggests that other components, particularly environmental variables and life history traits, may more strongly impact fish assemblages than reef materials (Akin et al. 2003; Vaughan et al. 2020). These results are relevant because postconstruction reef monitoring has been identified as a weakness of many artificial reef programs (Baine 2001; Seaman 2002), although this is due in part to the logistical difficulties of sampling these structures. For continued monitoring of artificial reef structures, the single-snapshot sampling approach may not appropriately capture reef fish assemblage variation due to the impacts of environmental variation across seasons.

Reef Position

Reef location also played an important role in the species composition at some reefs. The two reefs that consistently had high species richness values (CALI and RI) were located near the mouths of the two largest rivers (based on discharge) in the nGOM (Atchafalaya and Mississippi rivers). The CALI and RI reefs are 7.63 and 12.08 km, respectively, from the mouths of the Mississippi and Atchafalaya rivers, which discharge large volumes of freshwater and suspended sediment into the nGOM. Salinity values at these reefs during both sampling events were low (<0.50‰), whereas other sampled reefs had salinity values from 3.16‰ to 25.02‰. As a result, eDNA was detected from many freshwater species, such as Paddlefish *Polyodon spathula*, Bigmouth Buffalo, and Longear Sunfish, which were either absent or represented by very few reads at the other reefs in the nGOM. In addition, positive detections also were recovered for Threadfin Shad and Largemouth Bass—primary freshwater fishes that one might not expect to be associated with inshore artificial reefs in the nGOM.

Other studies have shown that eDNA can be transported via river currents for several kilometers. This is due to the ability of eDNA to persist for several days to weeks after the initial discharge/shedding event and its ability to bind to molecules, such as suspended sediments (Thomsen et al. 2012; Pilliod et al. 2013; Goldberg et al. 2016, 2018; Chambert et al. 2018). During the spring and fall 2018 sampling events, river discharge values for both the Mississippi and Atchafalaya rivers were substantially higher than normal, as both rivers were above flood stage. However, because of proximity, it is equally plausible that these species physically migrated to the inshore artificial reefs as several of these species are relatively mobile (Ross 2001). Without physically sampling the fishes on the artificial reefs, it is difficult to unravel these two scenarios. Ultimately, it is important to understand river and tidal flows in relation to reef location, as future nearshore artificial reefs are planned and deployed to target or attract specific fish assemblages.

In contrast to the CALI and RI reefs, which were predominantly freshwater during our sampling, the GI reef consistently had the lowest species richness but the highest salinity values (>24‰). This reef was dominated by pelagic, euryhaline species, such as Gulf Menhaden, Red Drum, and Striped Mullet, as well as Gizzard Shad *Dorosoma cepedianum*, a species with a wide salinity tolerance (2.0–33.7‰; Gunter 1945). These results indicate that salinity levels in nearshore habitats may be as important as reef material and should be considered if a particular type of reef assemblage (i.e., marine fishes versus estuarine fishes) is desired. However, the lack of multiple replicates of deepwater, marine artificial reefs in our study suggests that more detailed information is needed to truly assess the fish communities on these types of artificial reefs. If natural resource agencies are targeting the development of a particular type of fish community (i.e., marine or freshwater species assemblage), then environmental conditions, such as salinity, must be taken into consideration.

Summary: Artificial Reefs and Environmental DNA

To date, there has been a limited amount of information available on the fish assemblages of inshore artificial reefs in the nGOM (Strelcheck et al. 2005; Ajemian et al. 2015; Streich et al. 2017), and data on the impact of reef materials and reef position have been even rarer. Initially, our results indicated that reef material, which was marginally significant, was an important driver of reef fish diversity. However, other factors, including depth and salinity —particularly for the oil and gas reef—also likely played an important role in artificial reef fish diversity, indicating

that reef position should be taken into consideration as different types of reef fish communities are targeted and developed in the future.

Despite the lack of information, many natural resource agencies have prioritized reef construction without the development of comprehensive monitoring programs (Seaman 2002), but this is due in part to the challenges of monitoring reef fish assemblages. Our data indicate that eDNA sampling is an effective and informative approach that can and should be used to monitor assemblages associated with these types of structures. Although the eDNA approach is imperfect at present, it provides a comprehensive picture of the fish assemblages associated with reef habitats. For example, Hollweg et al. (2020) reported the presence of more than 50 fish species associated with oyster reef habitats in freshwater and brackish-water areas of the nGOM. In our study, we detected 32 species, representing a high proportion of species known to occur in the inshore regions of the nGOM, especially in comparison to other sampling approaches that have been used at reef habitats (La Peyre et al. 2019; Hollweg et al. 2020). Furthermore, the detection of 32 species in our study is likely an underestimate of the true species diversity at the artificial reefs due to the lack of a complete reference database (discussed below). In addition, eDNA approaches offer a high degree of species-level resolution in comparison to visual surveys, as demonstrated in other studies (Thomsen et al. 2012), since accurate visual species identification in highly turbid waters in inshore habitats of the nGOM is challenging at best, even for the most skilled observer. Finally, the field sampling effort required for eDNA is significantly lower in comparison to traditional approaches, as shown in other studies (Rees et al. 2014; Barnes and Turner 2016; Evans et al. 2016; Goldberg et al. 2016; Valentini et al. 2016; Lacoursière-Roussel et al. 2018), although laboratory work is an additional time requirement that can vary considerably depending on the laboratory, personnel, and resources.

Relative to other fishery sampling approaches, eDNA monitoring is in its infancy, with protocol modifications and technique improvements constantly occurring. In spite of the benefits provided by eDNA, there is one significant issue that should be considered. The accurate genetic identification of a taxon requires the existence of a comprehensive reference database. In the case of the nGOM fishes, the lack of a comprehensive 12S mitochondrial DNA reference database is a substantial issue as the existence of incomplete reference databases can lead to underestimates of biodiversity in eDNA studies given that recovered DNA sequences are assigned to known reference sequences (Kocher et al. 2017; Liu et al. 2019).

In our study, several species in the families Gobiidae, Blenniidae, and Gobiesocidae (McEachran and Fechhelm 1998, 2005; Hollweg et al. 2020), which are known to commonly occur in inshore regions of the nGOM, were rarely recovered in the ≥97% eDNA similarity matrix. The inability to detect these species in our study is unlikely to be the result of their absence from the reefs. Instead, sequence data (eDNA) from these families, and in many cases the same genera, were present in the 80–96% matrix and likely represent nGOM species, but they were bioinformatically assigned to a different species not present in the nGOM because of the lack of nGOM species' sequences in the reference database. This suggests that the artificial reef diversity results presented in our study likely represent an underestimate of the true species diversity at both reef sites and control sites (Porter et al. 2014; Curry et al. 2018). For our data, if the ≥97% matrix recovered a large number of species, the 80–96% matrix also recovered a large number of species, and this pattern was statistically supported ($R^2 = 0.316$, $r = 0.562$, $P < 0.005$; Appendices 3–5).

Sequence data gaps can be bridged via intensive barcoding of individuals from throughout the nGOM, thus allowing eDNA to become an even more viable and comprehensive tool for conservation and management (Porter et al. 2014). The upside of augmenting reference sequences from any study region is that once databases are updated, the original metabarcode sequence data generated in any study can be resubmitted to the analytical pipeline. The results can be reanalyzed to recover a much more comprehensive picture of an eDNA-based fish assemblage using the more complete database.

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Appendix 1: Environmental DNA Presence/Absence Matrices

TABLE A.1.1. Presence/absence matrix for the spring control samples. Site abbreviations are defined in Table 1 (reef material is given in parentheses: L = limestone; C = concrete; OG = oil and gas; S = shell). Year of construction for each reef is also provided in Table 1.

Species	BI (L)	CALI (C)	CYP (C)	ECL (C)	GI (OG)	II (L)	PM (L)	RI (S)	SS26 (L)
Skipjack Herring *Alosa chrysochloris*	0	0	0	0	0	0	0	0	0
American Eel *Anguilla rostrata*	0	0	0	0	1	0	1	1	0
Alligator Gar *Atractosteus spatula*	0	0	0	0	0	0	0	0	0
Alosa sp.	0	0	0	0	0	0	0	0	0
Gafftopsail Catfish *Bagre marinus*	0	0	0	0	0	0	0	0	0
Gulf Menhaden *Brevoortia patronus*	1	1	1	1	1	1	1	1	1
Darter Goby *Ctenogobius boleosoma*	0	0	0	0	0	0	0	0	0
Grass Carp *Ctenopharyngodon idella*	1	0	1	1	0	1	0	0	1
Spotted Seatrout *Cynoscion nebulosus*	1	1	1	1	1	1	1	1	1
Jack *Caranx* sp.	0	0	0	1	0	0	1	1	0
Gizzard Shad *Dorosoma cepedianum*	1	1	1	1	1	1	1	1	1
Threadfin Shad *Dorosoma petenense*	0	0	0	0	0	0	0	0	0
Chain Pickerel *Esox niger*	1	1	1	1	0	0	1	1	1
Engraulidae	0	0	0	0	0	0	0	0	0
Western Mosquitofish *Gambusia affinis*	0	0	0	0	0	0	0	0	0
Eastern Mosquitofish *Gambusia holbrooki*	0	0	0	0	0	0	0	0	0

TABLE A.1.1. Continued.

Species	BI (L)	CALI (C)	CYP (C)	ECL (C)	GI (OG)	II (L)	PM (L)	RI (S)	SS26 (L)
Bigmouth Buffalo *Ictiobus cyprinellus*	1	1	1	1	1	0	1	1	1
Blue Catfish *Ictalurus furcatus*	0	0	0	0	0	0	0	0	0
Channel Catfish *Ictalurus punctatus*	0	0	0	1	0	0	0	1	0
Bluegill *Lepomis macrochirus*	0	0	0	1	0	0	0	1	0
Longear Sunfish *Lepomis megalotis*	1	1	1	0	0	0	1	0	1
Redspotted Sunfish *Lepomis miniatus*	0	0	0	0	0	0	0	0	0
Gar *Lepisosteus* sp.	0	0	0	1	0	0	1	1	0
Leuciscid sp.	0	0	0	1	0	0	0	1	0
Inland Silverside *Menidia beryllina*	0	0	0	1	0	0	1	1	0
Striped Mullet *Mugil cephalus*	1	1	1	1	1	1	1	1	1
Largemouth Bass *Micropterus salmoides*	0	0	0	0	0	0	0	0	0
Southern Flounder *Paralichthys lethostigma*	0	0	0	1	0	0	0	1	0
Paddlefish *Polyodon spathula*	1	0	0	0	0	0	0	0	1
Red Drum *Sciaenops ocellatus*	1	1	0	1	1	1	1	1	1
Least Puffer *Sphoeroides parvus*	0	0	0	0	0	0	0	0	1
Sciaenid sp.	0	0	0	0	0	0	0	0	0

TABLE A.1.2. Presence/absence matrix for the spring reef samples. Site abbreviations are defined in Table 1 (reef material is given in parentheses: L = limestone; C = concrete; OG = oil and gas; S = shell). Year of construction for each reef is also provided in Table 1.

Species	BI (L)	CALI (C)	CYP (C)	ECL (C)	GI (OG)	II (L)	PM (L)	RI (S)	SS26 (L)
Skipjack Herring	0	0	0	0	0	0	0	0	1
American Eel	0	1	1	1	0	0	0	0	0
Alligator Gar	1	0	0	0	0	0	0	0	0
Alosa sp.	0	0	0	0	0	0	0	0	0
Gafftopsail Catfish	1	0	0	0	0	0	0	0	0
Gulf Menhaden	1	1	1	1	1	1	1	1	1
Darter Goby	0	0	0	0	0	0	0	0	0
Grass Carp	0	1	1	1	1	0	0	1	0
Spotted Seatrout	1	1	1	1	1	1	1	1	1
Jack *Caranx* sp.	1	1	1	0	0	0	0	0	0
Gizzard Shad	1	1	1	1	1	1	1	1	1
Threadfin Shad	0	0	0	0	0	0	0	0	0
Chain Pickerel	1	1	1	1	1	1	0	1	1
Engraulidae	0	0	0	0	0	0	0	0	0
Western Mosquitofish	0	0	0	0	0	0	0	0	0
Eastern Mosquitofish	0	0	0	0	0	0	0	0	0
Bigmouth Buffalo	1	1	1	1	1	1	1	1	1
Blue Catfish	0	0	0	0	0	0	0	0	0
Channel Catfish	1	0	0	0	0	1	0	0	1
Bluegill	1	0	0	0	0	1	0	0	1
Longear Sunfish	1	1	1	1	0	1	1	1	1
Redspotted Sunfish	1	0	0	0	0	0	0	0	0
Gar *Lepisosteus* sp.	1	1	1	0	0	1	0	0	1
Leuciscid sp.	0	0	0	0	0	0	0	0	0
Inland Silverside	0	1	1	0	0	0	0	0	0
Striped Mullet	1	1	1	1	1	1	1	1	1

TABLE A.1.2. Continued.

Species	BI (L)	CALI (C)	CYP (C)	ECL (C)	GI (OG)	II (L)	PM (L)	RI (S)	SS26 (L)
Largemouth Bass	1	0	0	0	0	0	0	0	0
Southern Flounder	1	0	0	0	0	1	0	0	1
Paddlefish	0	1	0	0	0	1	1	0	0
Red Drum	1	1	1	1	1	1	1	1	1
Least Puffer	1	0	0	0	0	0	1	0	0
Sciaenid sp.	0	0	0	0	0	1	0	0	0

TABLE A.1.3. Presence/absence matrix for the fall reef samples. Site abbreviations are defined in Table 1 (reef material is given in parentheses: L = limestone; C = concrete; OG = oil and gas; S = shell). Year of construction for each reef is also provided in Table 1.

Species	BI (L)	CALI (C)	CYP (C)	ECL (C)	GI (OG)	II (L)	PM (L)	RI (S)	SS26 (L)
Skipjack Herring	0	1	1	0	0	0	0	1	0
American Eel	0	0	0	0	0	0	0	0	0
Alligator Gar	1	1	1	0	1	1	0	1	0
Alosa sp.	0	0	0	1	1	0	0	0	0
Gafftopsail Catfish	1	1	1	1	0	0	0	0	1
Gulf Menhaden	1	1	1	1	1	1	1	1	1
Darter Goby	0	0	0	1	1	0	0	1	1
Grass Carp	0	1	1	1	0	0	0	1	1
Spotted Seatrout	1	1	1	1	1	1	1	1	1
Jack *Caranx* sp.	1	1	1	0	0	0	0	1	0
Gizzard Shad	1	1	1	1	1	1	1	1	1
Threadfin Shad	0	1	1	1	0	1	1	0	1
Chain Pickerel	0	0	0	0	0	0	0	0	0
Engraulidae	0	0	0	0	0	0	0	0	0
Western Mosquitofish	0	1	0	0	0	1	1	0	0
Eastern Mosquitofish	0	0	0	0	0	0	1	0	0
Bigmouth Buffalo	1	1	1	1	0	1	0	1	1
Blue Catfish	0	0	0	0	0	0	0	0	0
Channel Catfish	0	0	0	0	0	0	0	0	0
Bluegill	0	0	0	0	0	0	0	0	0
Longear Sunfish	0	0	0	0	0	0	0	0	0
Redspotted Sunfish	1	1	0	0	0	1	0	1	0
Gar *Lepisosteus* sp.	0	0	0	0	0	0	0	0	0
Leuciscid sp.	0	0	0	0	0	0	0	0	0
Inland Silverside	0	1	1	1	0	0	0	0	1
Striped Mullet	1	1	1	1	1	1	1	1	1
Largemouth Bass	1	1	1	1	0	0	0	1	0
Southern Flounder	1	1	1	1	0	0	0	1	0
Paddlefish	0	0	0	0	0	0	0	0	0
Red Drum	1	1	1	1	1	1	1	1	1
Least Puffer	1	1	1	0	0	0	0	1	0
Sciaenid sp.	0	0	1	0	0	1	0	0	0

TABLE A.1.4. Presence/absence matrix for the fall control samples. Site abbreviations are defined in Table 1 (reef material is given in parentheses: L = limestone; C = concrete; OG = oil and gas; S = shell). Year of construction for each reef is also provided in Table 1.

Species	BI (L)	CALI (C)	CYP (C)	ECL (C)	GI (OG)	II (L)	PM (L)	RI (S)	SS26 (L)
Skipjack Herring	1	0	0	0	1	1	1	1	0
American Eel	0	0	0	0	0	0	0	0	0
Alligator Gar	0	0	1	1	0	1	1	0	1
Alosa sp.	0	0	0	0	0	0	0	0	0
Gafftopsail Catfish	1	0	0	0	0	1	1	1	1
Gulf Menhaden	1	1	1	1	1	1	1	1	1
Darter Goby	1	1	1	0	0	0	0	1	0
Grass Carp	1	1	1	0	0	0	0	1	0
Spotted Seatrout	1	1	1	1	1	1	1	1	1
Jack *Caranx* sp.	1	0	0	0	1	1	1	1	0
Gizzard Shad	1	1	1	1	0	1	1	1	1
Threadfin Shad	1	1	1	1	0	1	0	1	1
Chain Pickerel	0	0	0	0	0	0	0	0	0
Engraulidae	0	0	0	0	0	0	1	0	0
Western Mosquitofish	0	1	1	1	0	0	0	0	1
Eastern Mosquitofish	0	0	0	0	0	0	0	0	0
Bigmouth Buffalo	1	1	1	1	0	1	1	1	1
Blue Catfish	0	0	0	0	0	0	0	1	0
Channel Catfish	0	0	0	0	0	0	0	0	0
Bluegill	0	0	0	0	0	0	0	0	0
Longear Sunfish	0	0	0	0	0	0	0	0	0
Redspotted Sunfish	0	1	0	1	0	1	1	0	1
Gar *Lepisosteus* sp.	0	0	0	0	0	0	0	0	0
Leuciscid sp.	0	0	0	0	0	0	0	1	0
Inland Silverside	1	0	0	0	0	0	0	0	0
Striped Mullet	1	1	1	1	1	1	1	1	1
Largemouth Bass	0	1	0	0	0	1	1	0	0
Southern Flounder	1	0	0	1	0	1	1	1	0
Paddlefish	0	0	0	0	0	0	0	0	0
Red Drum	1	1	1	1	1	1	1	1	1
Least Puffer	1	0	0	0	0	1	1	1	0
Sciaenid sp.	0	0	0	0	0	0	0	0	0

Appendix 2: Rarefaction and Extrapolation Graphs Following the Approach of Hsieh et al. (2016) Using the R Package iNEXT

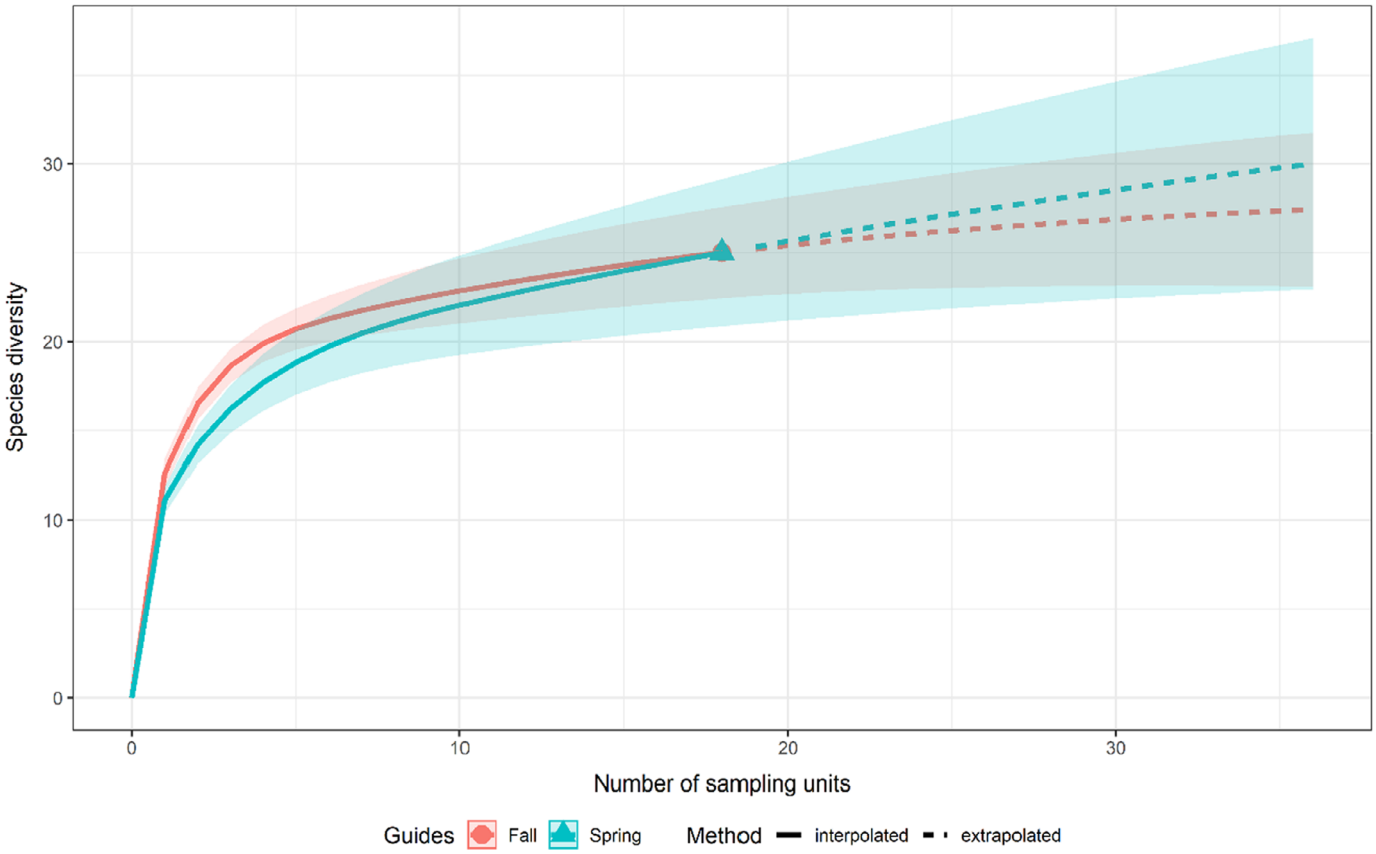

FIGURE A.2.1. Rarefaction (interpolation) and prediction (extrapolation) of species richness, comparing pooled fall samples and pooled spring samples. The 95% confidence intervals are indicated by color bands surrounding interpolated and extrapolated lines.

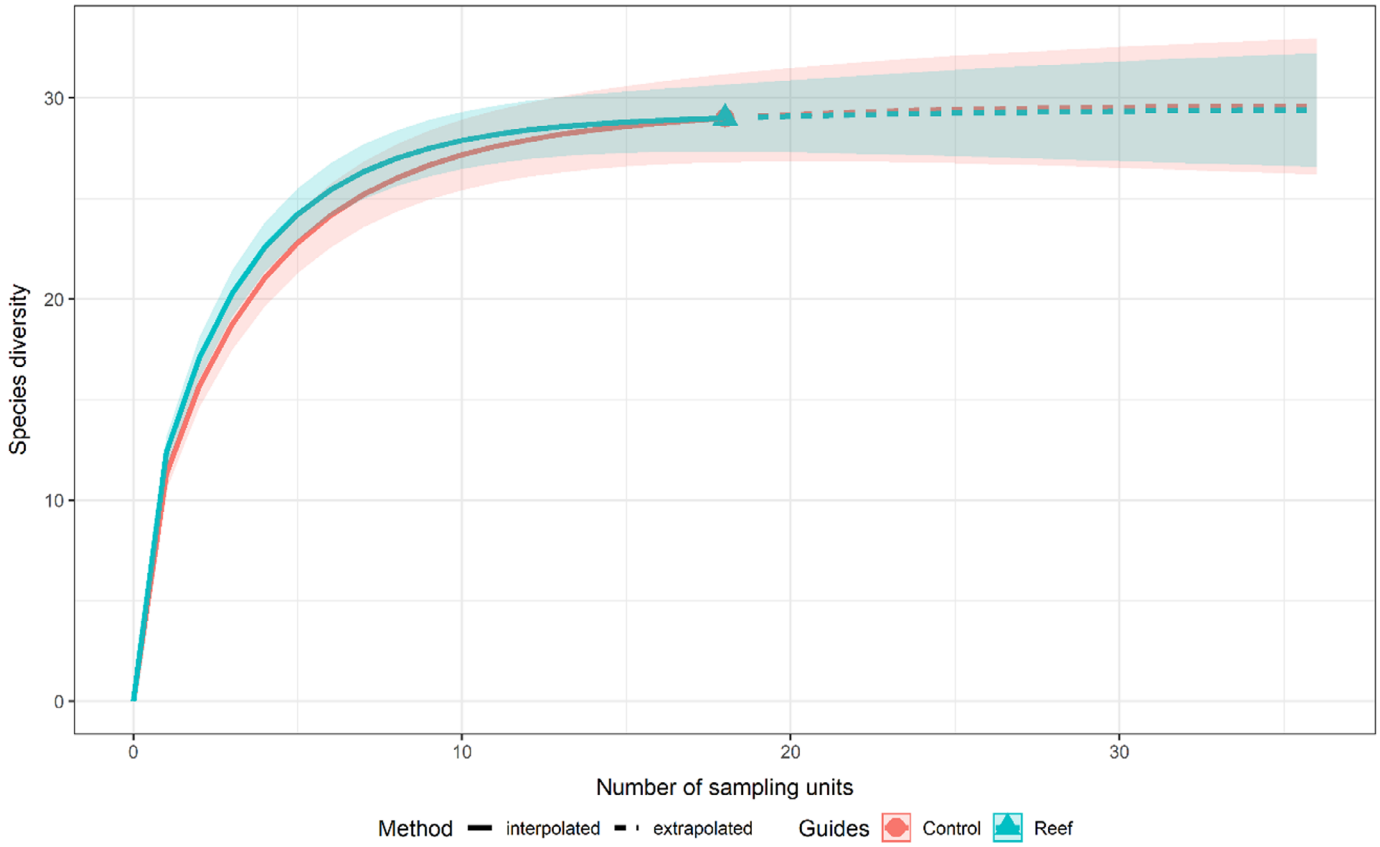

FIGURE A.2.2. Rarefaction (interpolation) and prediction (extrapolation) of species richness, comparing pooled control samples and pooled reef samples.

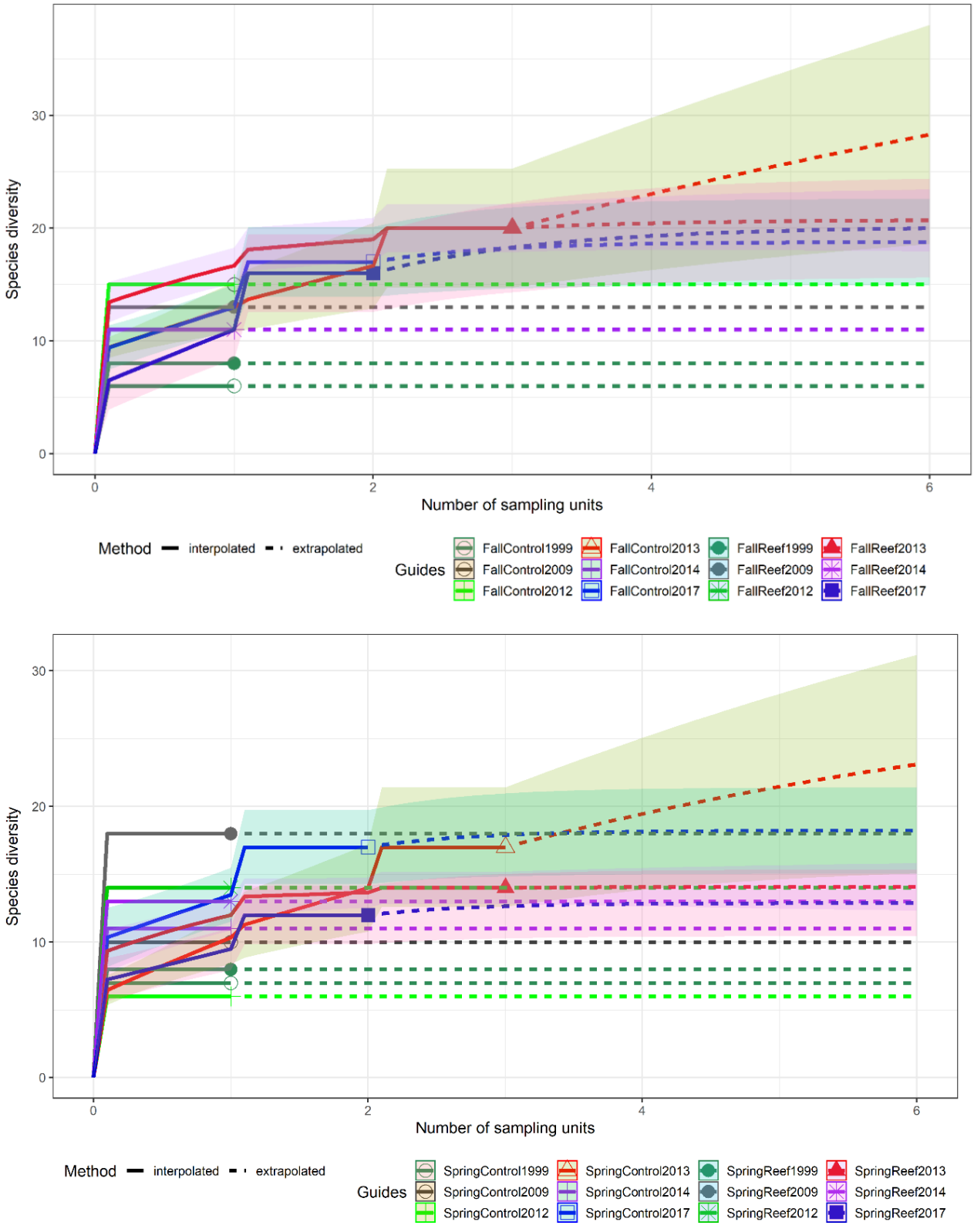

FIGURE A.2.3. Rarefaction (interpolation) and prediction (extrapolation) of species richness, comparing fall control and fall reef samples (upper panel) or spring control and spring reef samples (lower panel) for each reef construction year represented in the study.

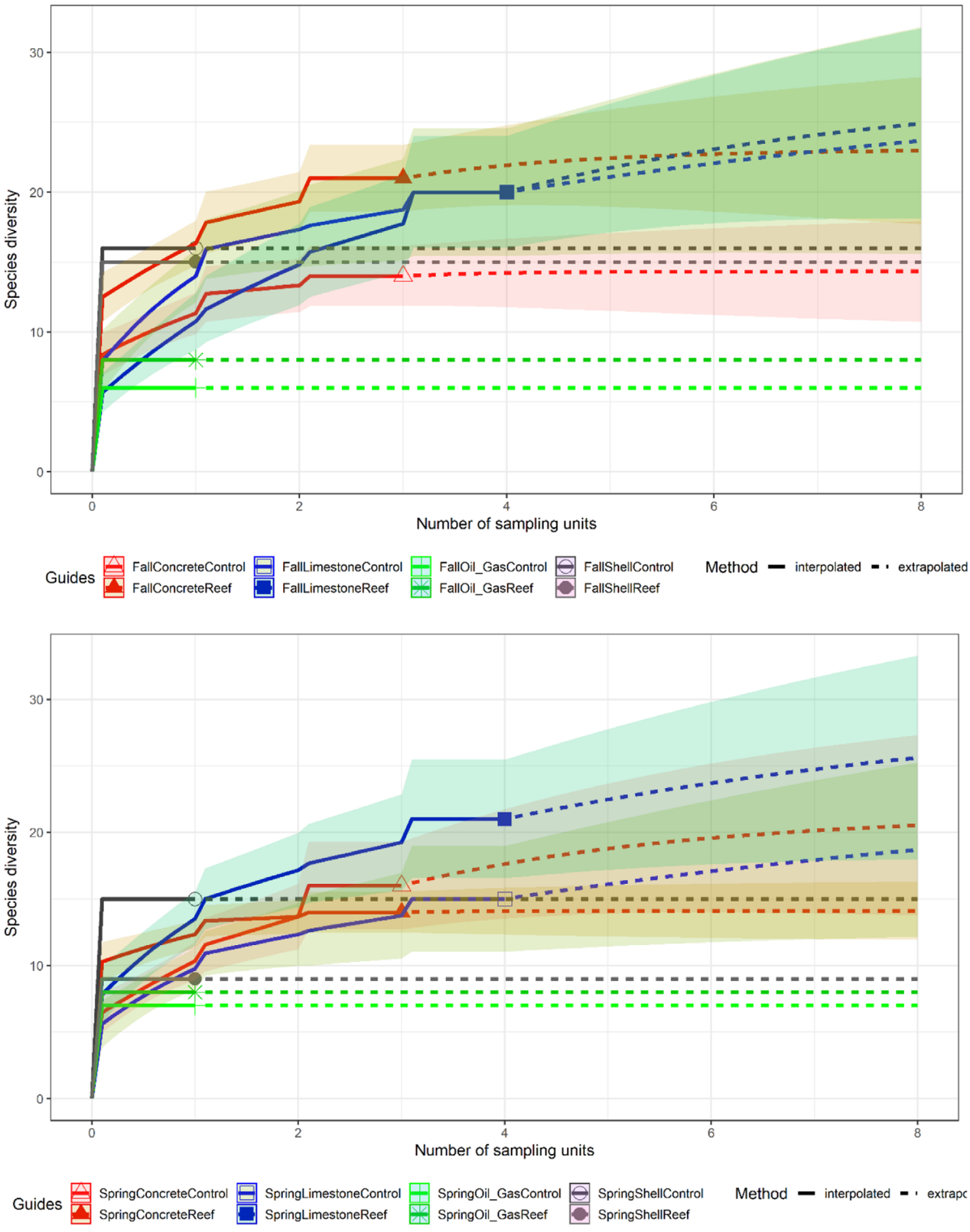

FIGURE A.2.4. Rarefaction (interpolation) and prediction (extrapolation) of species richness, comparing fall control and fall reef samples (upper panel) or spring control and spring reef samples (lower panel) for each construction material examined in the study.

Appendix 3: Linear Regression of Species Richness

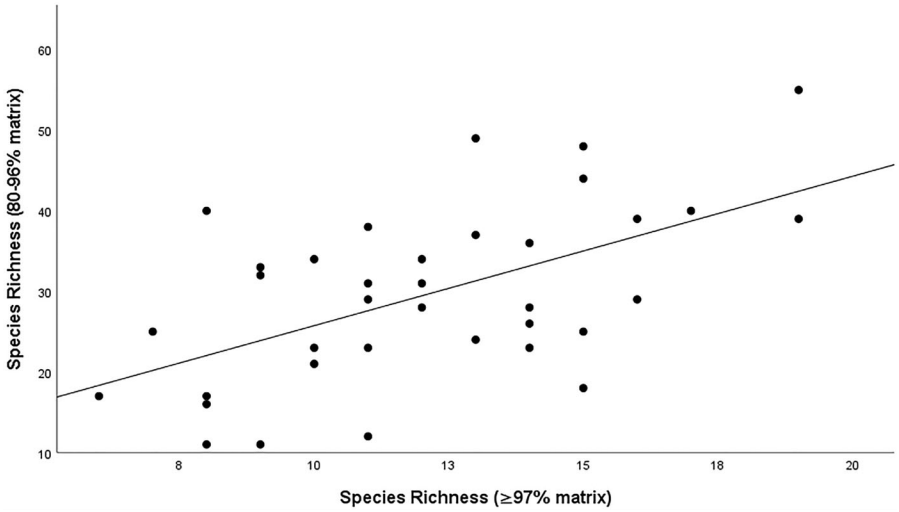

FIGURE A.3.1. Linear regression of species richness values for the ≥97% matrix versus the 80–96% matrix ($R^2 = 0.316$, $r = 0.562$, $P < 0.005$).

Appendix 4: Mean Species Richness for Data Matrices

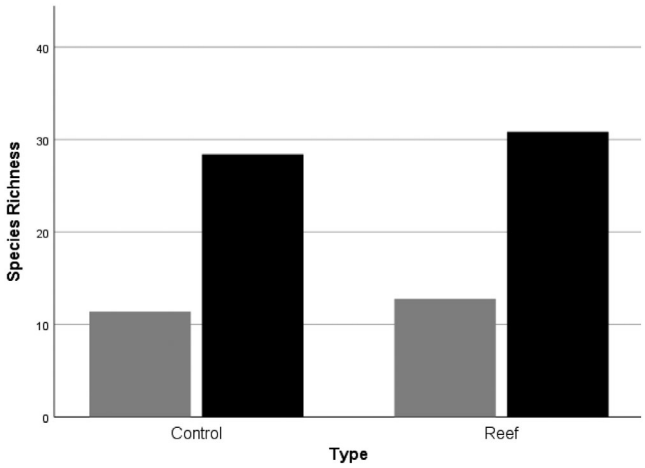

FIGURE A.4.1. Mean species richness values across reef and control sites based on the ≥97% matrix (gray bars) and the 80–96% matrix (black bars).

Appendix 5: Mean Species Richness of the Data Matrices by Locality

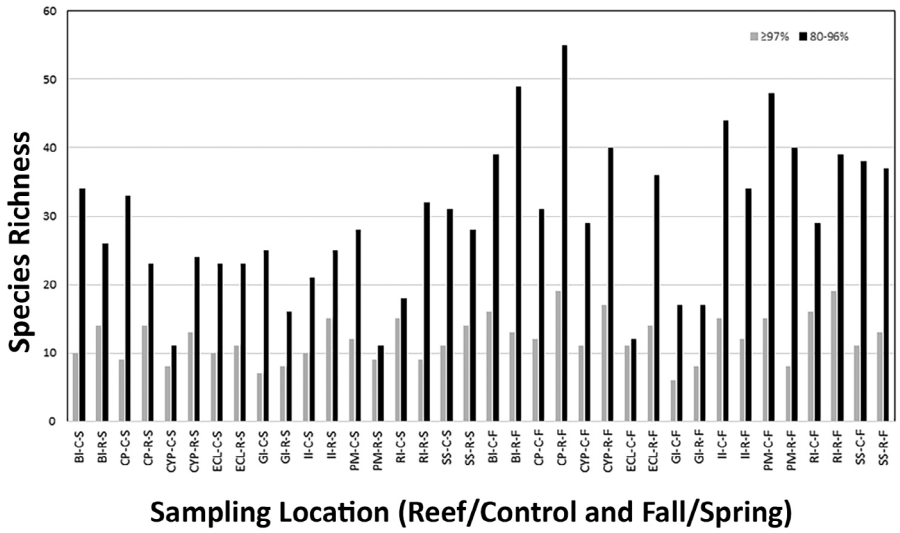

FIGURE A.5.1. Mean species richness values across reef (R) and control (C) sites and seasons fall (F) and spring (S) by locality based on the ≥97% matrix (gray bars) and the 80–96% matrix (black bars). Site abbreviations are defined in Table 1.