Describing the diet of the yellowtail parrotfish (*Sparisoma rubripinne*) using DNA metabarcoding

A comparison of locations and seasons in the USVI

ISLA MAR

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Acknowledgements: The authors thank Rick Nemeth and the USVI dive team and Evan Tuohy for capturing the parrotfish for this project, Natalie Baez for performing the DNA extractions, Ramon Rivera for the bioinformatic analysis of the results, Agleris Santiago for the help in organizing and filtering the data and Katie Flynn for assisting in assigning species identification to the sequences.

Citation: Harms-Tuohy CA and Schizas NV. 2024. Describing the diet of the yellowtail parrotfish (*Sparisoma rubripinne*) using DNA metabarcoding. Technical Report. VI EPSCoR. 35pp

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EXECUTIVE SUMMARY

The diet of parrotfishes has been largely defined by their ecological role, whereby certain species or genera are grouped into foraging types (i.e. browsing, excavating, etc) however, this type of structuring has overlooked the finer details of what parrotfish ingest but can be clearly defined through DNA metabarcoding. Here we employ DNA metabarcoding with four genetic markers (CO1, 23S, rbcl, and ITS) to reveal the unknown and clarify the known 'prey' items of *Sparisoma rubripinne*, defining components of the epilithic algal matrix and supporting the observation of a protein-rich diet of epiphytic macroinvertebrates.

A total of 150 yellowtail parrotfish were captured using nets at four coral reef sites that vary in the level of reef degradation and sediment load over three sampling seasons in St. Thomas, USVI. The DNA extracted from the 150 parrotfish intestinal tracts was analyzed and results could be used from 126 parrotfish indicating an 84% efficiency in sequencing and prey taxa identification for this project. The use of four genetic markers was necessary to capture the dietary breadth of the yellowtail parrotfish. In some cases, certain taxa were only identified by one marker and not others but not all markers provided results for all locations or all sampling seasons. The markers rbcl and ITS were the least productive both in sequencing efficiency (i.e. few sequences retrieved) and in species identification (low diversity obtained). The other two markers, 23S and CO1, produced an abundance of sequences for all locations and sampling seasons.

The diet was composed of algae from all three major phyla (Chlorophyta, Rhodophyta, and Phaeophyta), phytoplankton like diatoms, cyanobacteria, native and invasive seagrasses, corals, sponges, juvenile fish, and small or juvenile gastropods, worms and anemones.

The highest level of taxa richness was in Reef Bay East during April while the lowest was Fish Bay during August, although no statistically significant differences were detected. Reef Bay East consistently demonstrated the highest diversity of taxa throughout the study, while Europa Bay and Reef Bay West were rather evenly spread overall but were unequal during respective sampling seasons. The most frequently observed dietary item across all locations and sampling seasons was macroalgae (coralline, filamentous and endophytic) from Rhodophyta, with *Harveylithon* coralline algae being the most frequently detected. The hypothesis of a protein-rich diet is supported by this study, where unexpected prey items like the greater pipefish (*Syngnathus acus*) and a diversity of small crabs and gastropods were identified.

The abundance of red macroalgae (both early and late-stage successional species) observed in the diet, which significantly outweighed the frequency of occurrence of brown or green macroalgae, suggests that the parrotfish in this study are visiting sites of both early and late-stage macroalgal succession. Given the diversity of both early and late-stage macroalgal colonizers found in the diet, this could indicate that the sampling locations are rather homogenous in the degradation state. In contrast, this could also indicate that the use of parrotfish as a sampling strategy to assess reef degradation may be ineffective given that the parrotfish from this study could be consuming 'prey' items throughout their migration to and from the spawning site which would mask any diet differences between locations.

INTRODUCTION

The diet of parrotfishes has been largely defined by their ecological role, whereby certain species or genera are grouped into foraging types (i.e. browsing, excavating, etc. Bonaldo et al. 2014, Adam et al. 2015). In fact, one study (Adam et al. 2015) defined parrotfishes as selective herbivores, generalizing that *Sparisoma* species tend to be browsers of macroalgae while species of the genus *Scarus* graze on turf algae. However, this type of structuring has overlooked the finer details of what parrotfish ingest. One breakthrough study by Clements et al. (2017) sought to address this issue through stable isotope analysis where their results supported a protein-rich diet comprised of cyanobacteria and other epiphytes to macroalgae, rather than a carbohydrate-rich diet based solely on macroalgae. Thus, overall, a review of the nutritional quality of parrotfishes by Choat and Clements (in Hoey and Bonaldo 2018) remarked that the parrotfish diet seems to be comprised of chlorophytes, rhodophytes, phaeophytes, seagrasses, cyanobacteria, heterotrophic bacteria, diatoms, coral, sponges and organic detritus (and see Bruggemann et al. 1994a, Bruggemann et al. 1994b, Bruggemann et al. 1994c, Bruggeman et al. 1996, Clements et al. 2017). These observations, however, were inferred from stable isotope analyses and field observations, noting that limitations apply when referencing highly heterogeneous substrates like epilithic algal matrix (EAM) as the food source for parrotfishes.

The algae found on coral reefs can range from endolithic (within hard substrates), epilithic (EAM including ‘turf’), and macro (i.e. *Dictyota*) and can be further classified as calcified or non-calcified. The epilithic algal matrix is often referred to as the prime parrotfish diet and contains a high diversity of bacteria, including cyanobacteria which are identified as the most important bacteria of EAM (Stal 1995, Connell et al. 2014, Echenique-Subiabre et al. 2015). However, the algae of EAM alone does not comprise the totality of the parrotfish diet, as evidenced by Clements et al (2017). Given the wide diet breadth that has now been observed or inferred for parrotfishes, the need to solidify this hypothesis of a ‘microphage that targets protein-rich epilithic, endolithic and epiphytic microscopic phototrophs’ (Choat and Clements 2018) can be supported through molecular analyses of the gut contents, or DNA barcoding and DNA metabarcoding.

DNA barcoding, or DNA metabarcoding, in gut content analyses provides a robust method to amplify known or unknown prey DNA from the guts of the 'predator' to clearly define the diet. DNA metabarcoding has been used by the authors to identify all types of fish prey of the invasive lionfish (Harms-Tuohy et al. 2016) and DNA barcoding has been used to identify the presence of known target items in the diet of other herbivores (Chelsky Budarf et al. 2011, Garcia-Robledo et al. 2013). DNA metabarcoding provides an advantage over the previously dominant method of DNA barcoding because prey items within the gut can remain a mystery rather than be known and specifically targeted. In the case of parrotfishes, DNA metabarcoding allows for the search of all known and unknown prey items within the gut, therefore examining a potentially broad diversity of the diet. Subsequently, this method also helps address the long-standing question of the actual composition of EAM and turf. To date, DNA metabarcoding has yet to be employed in the diet of Caribbean parrotfishes.

This study sought to clarify this knowledge gap by using DNA metabarcoding to define the diet of yellowtail parrotfish (*Sparisoma rubripinne*). As a member of the *Sparisoma* genus, the yellowtail parrotfish is generally described as a browser of macroalgae (Adam et al. 2015). This generalization is supported by this project's simultaneous field study, where the yellowtail parrotfish was observed to primarily target species from the genus *Dictyota* (R. Nemeth, personal observations of bites). The success of DNA metabarcoding greatly depends on the selected genetic markers (and associated primers) and their coverage of the targeted prey species. Many DNA metabarcoding studies will use one marker (i.e. CO1). Still, in the case of defining a perceived omnivorous diet, or a diet that contains items that span the diversity of life, the use of multiple genetic markers (i.e. ITS for seagrasses, CO1 for corals and metazoans) may be more appropriate (see Zou et al. 2016). Here we employ DNA metabarcoding with four genetic markers to reveal the unknown and clarify the known 'prey' items of this *Sparisoma* species, defining components of EAM and supporting the observation of a protein-rich diet of epiphytic macroinvertebrates.

METHODS

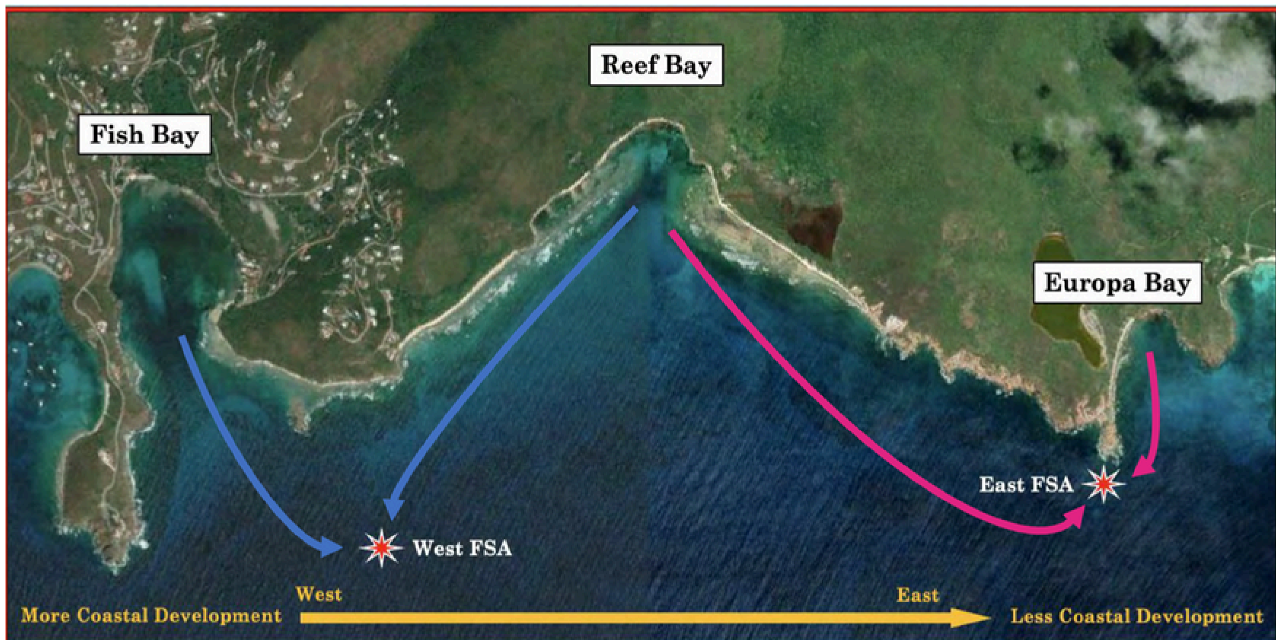


Figure 1. The sampling locations at St. Thomas. Reef Bay was divided into two sampling locations indicating the presence of the parrotfish spawning aggregations (West and East).

Collection

A total of 150 yellowtail parrotfish were captured using nets at four locations over three sampling seasons (August, April, and November 2022; n= 50 fish per season) in St. Thomas, USVI (Figure 1). This species was selected for the study because of its predictable spawning aggregation that occurs daily at two sites in St. Thomas, USVI, where large numbers of yellowtail parrotfish migrate to and from the spawning sites allowing for ease of capture and observation. The four sampling sites are located on coral reefs of varying levels of degradation and thus contain different algal assemblages. Once caught, fish were placed on ice in the field to slow digestion. The number of fish collected varied by location, but every attempt was made to collect and analyze at least ten fish per location (Table 1).

At the University of the Virgin Islands, morphological metrics (fish size, sex) were collected, and the fish were dissected to remove the entire intestinal tract. Each tract was placed in separate, sterile plastic bags and labeled. They were immediately frozen in a -80C freezer to await transport to Puerto Rico. After each sampling session concluded, the intestinal tracts were transported frozen to the Marine Genomics Laboratory at Isla Magueyes, Department of Marine Sciences, University of Puerto Rico Mayagüez where they were immediately processed for DNA extraction of the gut contents to ensure retrieval of the highest quality DNA from the diet.

Table 1. The number of yellowtail parrotfish (*S. rubripinne*) collected from each of the sampling locations and per season.

Location	April	August	November	Total
Europa Bay	10	13	12	*35*
Fish Bay	10	12	14	*36*
Reef Bay East	20	13	11	*44*
Reef Bay West	10	12	13	*35*
Total Collected	*50*	*50*	*50*	*150*

DNA Extraction, Amplification and Marker Selection

DNA extraction occurred in a laboratory room separate from other pre- and post-polymerase chain reaction procedures. All dissection tools were flamed and sterilized between samples. The Qiagen DNEasy Power Soil Pro kit (Germany) was used to extract DNA from each parrotfish gut contents, following the manufacturer's instructions. Duplicates of each sample were created to provide a back-up copy of the DNA extraction in the event that samples were lost in transit to the sequencing facility. Thus, a total of 300 DNA extractions were performed. DNA concentration was tested using Qubit to provide known values per sample and ensure that high concentrations of DNA were submitted for downstream processing. DNA extractions were maintained in a -80C freezer until shipment.

The genetic markers used in this study were selected based on their use in other DNA barcoding and metabarcoding studies for the targeted prey items (Table 2, see Reference column). The prey items targeted by these markers are known prey items to the parrotfish diet, based on previous literature on observed consumption (Adam et al. 2015), personal observations of the researchers, and stable isotope analysis (Clements et al. 2017) but also allowed for the potential to unveil unknown diet components.

Photo: Yellowtail parrotfish caught from St Thomas USVI for this project

These markers also have useful coverage in the NCBI GenBank database, allowing for a confident comparison of our sequenced results against the reference databases. The predator-blocking primer was created specifically for this project.

Admera Health Services (New Jersey, USA) performed the DNA amplification process using the four genetic markers. The predator-blocking primer for CO1 was used to reduce the likelihood of the yellowtail parrotfish DNA overwhelming the results of other dietary items. The blocking primer was modified at the 3' end with a Spacer C3 CPG (3 hydrocarbons) in order to prevent elongation without preventing the annealing process.

The blocking primer was included at 10 times the concentration of COI primers during amplification. Admera performed the DNA amplification process according to their guidelines for the polymerase chain reaction procedure (PCR) best suited for each primer set. DNA amplification was performed using all four genetic markers on all submitted samples, aiming to produce results for each of 150 samples for each marker, targeting a total of 600 amplification reactions (one per sample, per marker).

Table 2. The markers used to target the specific known dietary items of the yellowtail parrotfish (*S. rubripinne*) diet.

Marker	Primer Sequence	Size (bp)	Targets	Reference
CO1	F = mlCOIintF-XT (5'-GGWACWRGWTGRACWITITAYCCYCC-3') R = jgHCO2198 (5'-TAIACYTCIGGRTGICCRAARAAYCA-3'	313	Coral, other invertebrates, fish, algae, cyanobacteria	Leray et al. 2013 Geller et al. 2013
23S	F = p23SrV_f1 (5'- GGACAGAAAGACCCTATGAA -3') R = p23SrV_r1 (5'- TCAGCCTGTTATCCCTAGAG -3')	410	Coral, other invertebrates, fish, algae, cyanobacteria	Sherwood and Presting, 2007
ITS	F = P674 (5'-CCTTATCATTTAGAGGAAGGAG-3') R = P675 (5'-TCCTCCGCTTATTGATATGC-3')	700	Seagrass, algae	Nguyen et al 2015
rbcl	F = P609 (5'-GTAAAATCAAGTCCACCRCG-3') R = P610 (5'-ATGTCACCACAAACAGAGACTAAAGC-3')	599	Seagrass, algae	Lucas et al. 2012
Blocking primer	5' CAAAAAATCAAAATAGATGCTGGTAAAGA 3'		Yellowtail parrotfish	

DNA Metabarcoding and Results Filtering

DNA Sequencing

Sequencing of the pooled DNA from all samples was performed on the Illumina MiSeq with the resulting output for each sample as 0.1M PE reads, and thus 25K PE reads for each marker. After quality control, any amplified samples with library concentrations less than <0.3nM were not used for sequencing. The produced sequencing reads were then subjected to strict downstream processing to trim adaptors and remove unsuccessful reads.

Quality check and adapter removal

Sequencing reads were processed with fastp (v0.20.1; Chen et al., 2018) using a minimum reads quality for filtering of 25 and minimum read length of 75. Forward and reverse adapters were removed using cutadapt (v4.1; Martin 2011).

Assembly, dereplication, clustering, chimera detection

All samples were processed using VSEARCH (v2.15.2; Rognes et al., 2016). Paired-end fastq reads were assembled using the merge option of VSEARCH and converted to fasta format. The minimum read length cutoff was at least 150bp. Merging strictly identical sequences was carried out with the dereplication option and abundance annotations were calculated for each dereplicated sequence. Clustering of sequences and generation of count tables was performed using the centroid-based clustering method. Clustered sequences were denoise according to the UNOISE algorithm implemented in VSEARCH. Chimera detection and removal were done to the sequences with sorted decreasing abundance annotations. All sequences that remained from each sample were used for the species identification analyses.

Photo: Yellowtail parrotfish stomach cavity opened to expose intestinal tract.

BLAST and species identification

The preformatted NCBI NR BLAST database was downloaded after each batch of samples were processed with the script developed by NCBI (i.e., update_blastdb.pl). A nucleotide BLAST (blastn v2.13.0; Camacho et al., 2009) run was performed using the entire NCBI database. Using the taxonomy browser of NCBI (Schoch et al., 2020), each corresponding Taxonomic ID was assigned to each species. When species taxonomic data was not available, the Taxonomic ID from a higher classification was used instead. Blast results from all samples were processed using a custom bash script to divide sequences into individual fish gut contents and sampling sessions. For each sequence, the top species match was identified.

Sorting and filtering

Following the demultiplexing step (the assignment of sequences to the digestive system of each respective parrotfish), all sequences with a 95% or greater match to a species reference in the NCBI database (Hatfield et al. 2020) were used for further processing. Each species was validated for presence in the USVI using AlgaeBase or the World Register of Marine Species. Expert opinion was also consulted where necessary. Thus, all sequences that met the above guidelines were used for analyses.

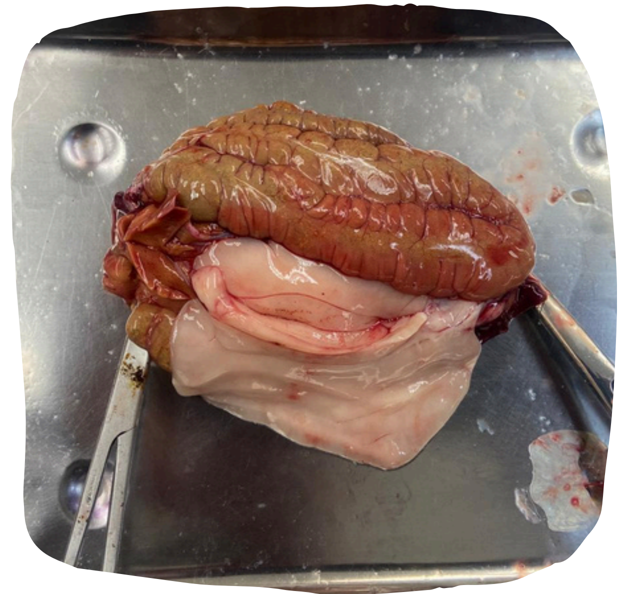


Photo: Yellowtail parrotfish entire intestinal tract and ovaries removed for analysis.

Analyses

Results were prepared in an Excel spreadsheet, organized by fish ID, location collected and sampling season. Pivot tables were created to sort and arrange the results into a filtered selection of identified prey taxa. The results were then sorted by taxa frequency of occurrence to produce a species list for each location per sampling season, a list of the most frequently observed species per location per season, and a comparison of distinct prey counts by fish sex. A two-factor ANOVA was used to compare the taxa richness of the diet between locations and seasons.

RESULTS

Sequencing Efficiency

Of the 150 yellowtail parrotfish gut content samples that were sequenced, results could be used from 126 parrotfish indicating an 84% efficiency in sequencing and prey taxa identification for this project (Table 3). The inability to produce results for all sampled fish began during DNA library preparation before sequencing where some samples did not provide the required DNA concentration for specific genetic markers (namely rbcl and ITS) after DNA amplification steps. Thus, those samples were not ultimately sequenced. Following sequencing, some additional samples were removed from downstream analysis after the filtering steps described in the "Methods: DNA Metabarcoding and Results Filtering: Sorting and filtering" disqualified many sequences from inclusion. Overall, the distribution of samples across locations and within sampling seasons was skewed to favor Reef Bay East while Europa Bay and Reef Bay West were even, and Fish Bay was consistently the lowest represented (Table 3).

The use of four genetic markers for this study was necessary to capture the dietary breadth of the yellowtail parrotfish. In some cases, certain taxa were only identified by one marker and not others. Not all markers provided results for all locations or all sampling seasons (Table 4). In April, two locations (Europa Bay and Reef Bay East) had gut samples with results obtained from all four markers. In August, no locations were defined by all four markers and only Reef Bay East had samples defined by at least three markers. In November, only Europa Bay had results from all four markers while the other locations were defined by the other three. This coverage is relatively well distributed for most locations in most seasons, except for Fish Bay which only had results from one marker in both April and August. This indicates a loss of data for this location during both of these sampling seasons and is considered in the final discussion of diet. The markers rbcl and ITS were the least productive both in sequencing efficiency (i.e. few sequences retrieved) and in species identification (low diversity obtained). The other two markers, 23S and CO1, produced an abundance of sequences for all locations and sampling seasons.

Table 3. The number of yellowtail parrotfish (*S. rubripinne*) that were ultimately analyzed after DNA amplification steps. The number represents the number of fish for which results were obtained from the diet that could be used in these analyses. In parentheses is distribution by sex (female/male/unknown or both).

Location	April	August	November	Total Analyzed
Europa Bay	10 (3/7)	11 (7/4)	10 (9/1)	31
Fish Bay	6 (5/1)	7 (5/2)	7 (6/1)	20
Reef Bay East	20 (9/10/1)	12 (8/4)	12 (7/4/1)	44
Reef Bay West	8 (6/2)	12 (11/1)	11 (10/1)	31
Total Analyzed	***44***	***42***	***40***	***126***

Table 4. The number of genetic markers that produced results for the diet study. A total of four markers were processed. The colors are used to group the same for a visible representation of the spread across locations and seasons.

Location	April	August	November
Europa Bay	4	2	4
Fish Bay	1	1	3
Reef Bay East	4	3	3
Reef Bay West	3	2	3

Diet Classification

The diet of the yellowtail parrotfish was composed of algae from all three major phyla (Chlorophyta, Rhodophyta, and Phaeophyta), phytoplankton like diatoms, cyanobacteria, native and invasive seagrasses, corals, sponges, juvenile fish, and small or juvenile gastropods, worms and anemones. For this study, 'taxa richness' is used to refer to the traditional 'species richness' or the number of different taxa/species observed. The term 'taxa' was chosen given that some diet items could not be identified to the species level and thus were represented by a higher classification (usually genus).

The highest level of taxa richness was in Reef Bay East (n=68 different taxa identified) during April while the lowest was Fish Bay (n=9) during August (Table 5). Reef Bay East consistently demonstrated the highest diversity of taxa throughout the study, while Europa Bay and Reef Bay West were rather evenly spread overall but were unequal during respective sampling seasons. A two-factor ANOVA indicated no significant difference in the diversity of taxa identified by location (df=3, F=3.082, p=0.07) and sampling season (df=2, F=0.11, p=0.89).

Table 5. The taxa richness was identified from the gut contents of yellowtail parrotfish (*S. rubripinne*) from four locations over three sampling seasons.

Location	April	August	November
Europa Bay	60	42	31
Fish Bay	28	9	21
Reef Bay East	68	52	51
Reef Bay West	53	39	42

Table 6. The most frequently encountered species observed in the intestinal tract of the yellowtail parrotfish (*S. rubripinne*) are separated by location and season (light green). If more than one species is listed, it was tied for most frequent. The bottom portion (light brown) of the graph defines the species by common description.

Location	April	August	November
Europa Bay	*Harveylithon munitum*	*Colaconema* sp.	*Harveylithon munitum*
Fish Bay	*Centroceras micracanthum*	*Mithraculus coryphe* *Tedania ignis*	*Syngnathus acus*
Reef Bay East	*Gayliella* sp.	*Colaconema* sp.	*Colaconema* sp.
Reef Bay West	*Harveylithon munitum* *Jania* sp.	*Harveylithon munitum*	*Harveylithon munitum*
	April	**August**	**November**
Europa Bay	Red coralline macroalgae	Red endophytic macroalgae	Red coralline macroalgae
Fish Bay	Red filamentous macroalgae	Crab Sponge	Fish
Reef Bay East	Red filamentous macroalgae	Red endophytic macroalgae	Red endophytic macroalgae
Reef Bay West	Red coralline macroalgae Red coralline macroalgae	Red coralline macroalgae	Red coralline macroalgae

The most frequently observed dietary item across all locations and sampling seasons was macroalgae (coralline, filamentous and endophytic) from Rhodophyta (Table 6). Fish Bay was the only location to present a different frequently observed species each season which was also not dominated by macroalgae but represented instead by invertebrates, sponges and juvenile fish.

Some organisms identified in the diet were observed in all three sampling seasons at each location (Table 7) representing two macroalgal phyla, invertebrates, and seagrass. Seagrasses were only observed in all sampling seasons from Reef Bay East. The brown macroalgae, *Sargassum*, was only observed in Reef Bay West across sampling seasons, but other fleshy brown macroalgae were found in Reef Bay West and Europa Bay.

Table 7. Taxa that appeared in the diet across all sampling months in each location. The lower portion (light brown) describes the species listed for that location in common terms, in alphabetical order. The highlighted colors in the top portion refer to the description in the bottom portion.

Europa Bay	Fish Bay	Reef Bay East	Reef Bay West
Amphiroa sp.	*Amphiroa fragilissima*	*Aglaothamnion boergesenii*	*Bacillaria paxillifer*
Amphiroa fragilissima	*Mithraculus coryphe*	*Bacillaria paxillifer*	*Bacillariophyceae sp.*
Bacillaria paxillifer		*Bacillariophyceae sp.*	*Colaconema sp.*
Bacillariophyceae sp.		*Centroceras clavulatum*	*Corallinales sp.*
Centroceras clavulatum		*Ceramiales sp.*	*Dictyota guineensis*
Ceramiales sp.		*Coelothrix irregularis*	*Galaxaura rugosa*
Chroodactylon ornatum		*Colaconema sp.*	*Gelidiella sp.*
Colaconema sp.		*Corallinales sp.*	*Griffithsia sp.*
Harveylithon sp.		*Exaiptasia diaphana*	*Harveylithon munitum*
Harveylithon munitum		*Dictyota humifusa*	*Harveylithon sp.*
Laurencia sp.		*Gayliella sp.*	*Jania rubens*
Laurencia obtusa		*Harveylithon munitum*	*Jania sp.*
Lobophora variegata		*Harveylithon sp.*	*Mithraculus coryphe*
		Hypnea musciformis	*Padina gymnospora*
		Laurencia sp.	*Peyssonnelia inamoena*
		Lobophora canariensis	*Peyssonnelia sp.*
		Millerella sp.	*Sargassum natans*
		Margalefidinium polykrikoides	*Sargassum platycarpum*
		Thalassia testudinum	
red macroalgae, calcified and non-calcified	red macroalgae, calcified	anemone	diatoms
brown macroalgae	crab	diatoms	red macroalgae, calcified and non-calcified
diatoms		red macroalgae, non-calcified	brown macroalgae, calcified and non-calcified
		brown macroalgae	crab
		dinoflagellate	
		seagrass	

Many organisms were unexpected and previously unreported for the parrotfish diet, including the greater pipefish (*Syngnathus acus*) and invertebrates like anemones (*Exaiptasia diaphana*), fireworms, feather duster worms and various crabs.

The diet was also assessed in relation to the sex of the fish. The distribution of females to males was roughly 70:30 for specimens caught, which was also heavily skewed towards females in those analyzed as well. Females consistently consumed a larger diversity of prey items compared to males (Table 8). In some cases, the number of prey items is equal to the number of fish analyzed, such as with males in Fish Bay for August and November indicating that only one species was consumed by that male.

Table 8. Number of different prey items consumed by males and females of the yellowtail parrotfish (*S. rubripinne*) at each location and sampling season.

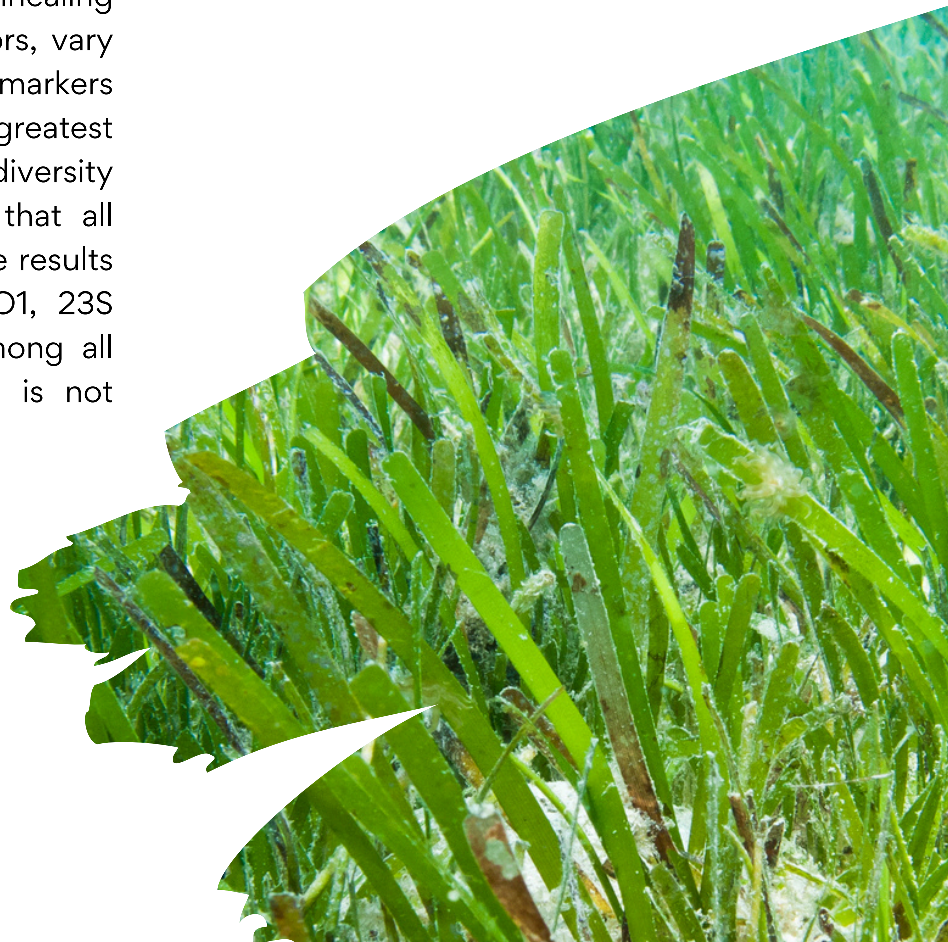
Males	**April**	**August**	**November**
Europa Bay	49	18	13
Fish Bay	12	2	1
Reef Bay East	42	25	31
Reef Bay West	20	2	1
Females	**April**	**August**	**November**
Europa Bay	25	32	23
Fish Bay	16	14	21
Reef Bay East	44	35	26
Reef Bay West	48	41	42

DISCUSSION

Sequencing Efficiency

Using four genetic markers to identify the dietary components of the yellowtail parrotfish proved useful and necessary for this project. Despite this, each marker had varying levels of efficiency, with CO1 and 23S being the most efficient and providing the greatest level of diversity in the diet. The two markers that traditionally target seagrasses (rbcl and ITS) were low in efficiency, and even failed to produce results for entire sampling seasons in some cases. Determining the reasons behind low efficiency in this study is difficult because the primers used to target these genetic markers cannot be easily compared. The specificity of the primers, GC content, the position of the target region, and the annealing temperature, among other factors, vary significantly. The four genetic markers were selected to provide the greatest potential to identify the widest diversity of prey. It was not expected that all markers would indicate the same results because these genes (rbcl, CO1, 23S and ITS) are not all shared among all potential 'prey' items (i.e. rbcl is not present in corals).

Regardless, in many cases, some ecologically relevant prey items were only identified by one of the four markers (i.e. *Halophila stipulacea* the invasive seagrass by ITS) while other markers identified previously unknown and unsuspected components of the diet (i.e, *Syngnathus acus*, the greater pipefish). Therefore, using all four markers allowed this study to capture a new range of diversity for the diet beyond simply identifying the previously known dietary items.



Diet Classification

The diet of the yellowtail parrotfish has been classified based on field observations of bites taken on the substratum, indicating that they primarily consume and target brown fleshy macroalgae (Bruggemann et al. 1994a-c). Over time, other studies that make use of different approaches like stable isotope analysis have revealed that parrotfishes must be consuming a protein-rich diet of cyanobacteria and epiphytic macroinvertebrates given that a typical macroalgal carbohydrate-rich diet was not observed (Clements et al 2017). In a complementary component of this project, field observations of the yellowtail parrotfish revealed that this species consumes brown macroalgae, turf, seagrass, corals, and sponges among other benthic invertebrate community members typical of EAM like cyanobacteria (R. Nemeth, personal obvs). In this study, the use of molecular methods unveiled a much wider dietary breadth and suggests that the yellowtail parrotfish is primarily targeting a variety of red macroalgae (both calcified and non-calcified) but predominantly the reef-building coralline algae *Harveylithon* sp., and also secondarily consuming brown and green macroalgae, seagrass, cyanobacteria and other non-target prey items such as invertebrate epiphytes to EAM like polychaete worms, crabs, and urchins. Beyond this, they are also ingesting anemones, urchins (*Arbacia punctulata*), tunicates (*Pyura vittata*), sea hares (*Bosellia mimetica*) and fish which have not previously been identified as part of the diet of this herbivore.

This study supports the hypothesis of a protein-rich diet, identifying unexpected prey items like the greater pipefish (*S. acus*) and a diversity of small crabs and gastropods. The greater pipefish is small and slender and found among seagrass beds or rocky rubble areas with terrigenous leaf litter and organic detritus. To become part of the parrotfish diet, this ‘prey’ item is likely being ingested incidentally, as is the likely case with the many epiphytic macroinvertebrates.

The abundance of red macroalgae (both early and late-stage successional species) observed in the diet, which significantly outweighed the frequency of occurrence of brown or green macroalgae, suggests that the parrotfish used in this study are visiting sites of both early and late-stage macroalgal succession (Fricke et al. 2011). The early colonizers of a degraded coral reef, such as the green macroalgae *Bryopsis* and *Ulva* sp. were not frequent dietary items. Additionally, highly competitive and easily detected species like the orange boring sponge (*Cliona delitrix*), *Ramicrusta textilis* and the encrusting alga *Peyssonnelia* sp. are also early colonizers and were observed in the diet, but not in high frequency.

However, the reef-building coralline algae, *Harveylithon* sp. (related to but different from an earlier identified coralline genus, *Hydrolithon*), was the most frequently observed dietary component and is representative of both early and late-stage succession on coral reefs. The other most frequently observed macroalgae were representatives of late colonizers like the red macroalgae *Jania* sp., *Griffithsia* sp., *Centroceras* sp., and brown macroalgae like Dictyotales. In fact, multiple species of *Dictyota* were identified in each location across all sampling seasons. Given that this genus is representative of late colonizers (Figure 2), we can predict that all sites indicate signs of degradation regardless of the level of coastal development.

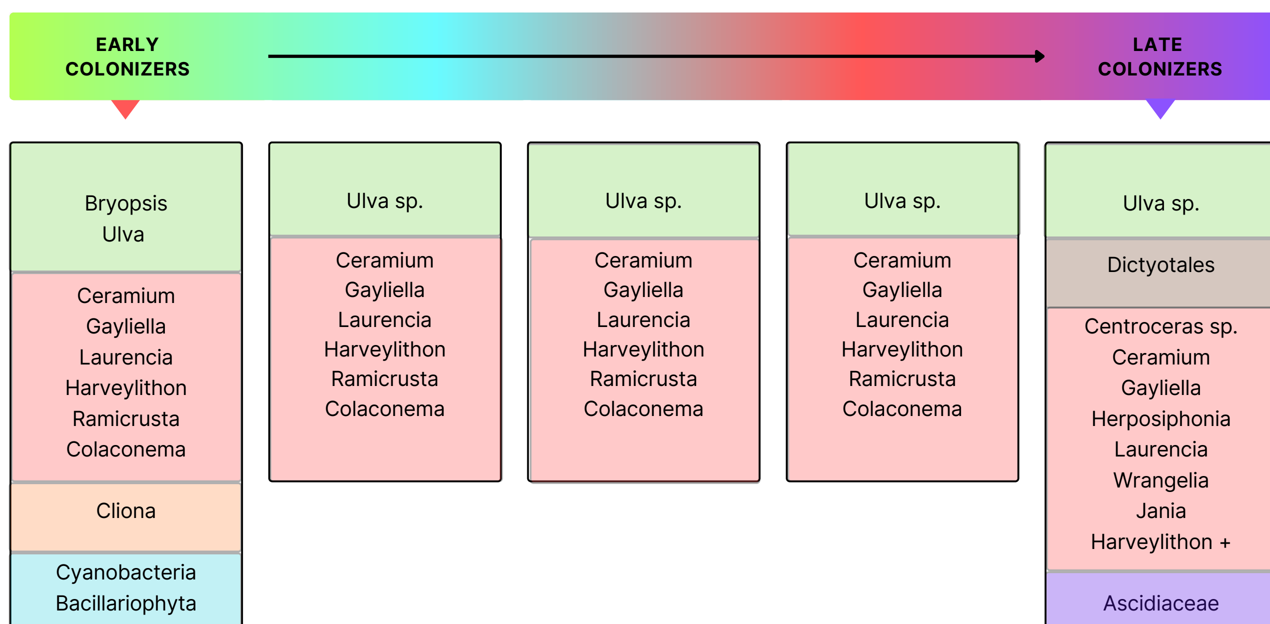


Figure 2. Visualization of the successional stage of some organisms found in the yellowtail parrotfish diet from this study, based on Fricke et al. 2011. The “+” at Late Colonizers by *Harveylithon* indicates that other Rhodophyta found in the diet would also belong here.

Given the diversity of both early and late-stage colonizers found in the diet, this could indicate that the sampling locations are rather homogenous in the degradation state. In contrast, this could also indicate that the use of parrotfish as a sampling strategy to assess reef degradation may be ineffective given that the parrotfish from this study could be consuming 'prey' items throughout their migration to the spawning site, which would mask any diet differences between locations.

When comparing diet by sex, females consumed a greater diversity of prey items compared to males. However, these results are heavily impacted by the uneven distribution of the sexes analyzed whereby more females were captured and analyzed versus males. In this sense, we cannot definitively state whether this trend is based on sex. For this study, an effort was made to capture fish of relatively the same size class (sexually mature) and of both sexes, but ultimately was a skewed distribution towards a greater number of females available for analysis. As parrotfishes are protogynous, changing sex from female to male, they undergo significant metabolic demands for reproduction both during spawning events and during the transition from female to male.

In a study of a similar-sized species, *Sparisoma viride*, initial-phase females and territorial terminal-phase (TP) males that were spawning daily showed the lowest growth rates of the species, whereas juveniles and sexually inactive TP males had the fastest growth rates (van Rooij et al 1995). In this study, the size distribution ranged from 17 – 28cm, which would correspond similarly to *S. viride* as initial phase and terminal phase fish. While energy is being directed toward gamete production (and possibly sex change, as evidenced by a few individuals with both ovaries and testes present), it could be inferred that a greater diversity of prey items in the diet (ranging from proteins to carbohydrate-rich items) would be necessary to maintain the high metabolic demands of daily reproduction. Overall, a more even distribution of the sexes within the analysis would yield a better understanding of any trends of sex on the diet.

CONCLUSIONS

This study successfully identified a broad dietary diversity of the yellowtail parrotfish, *Sparisoma rubripinne,* providing the first DNA metabarcoding analysis of the gut contents for this species.

No. 01

The most frequently occurring dietary item was a variety of the most speciose algal phyla, Rhodophyta, and was primarily represented by coralline algae of the *Harveylithon* genus.

No. 02

While the most frequent 'prey' items were represented by both early and late-stage colonizers of degraded reefs, there was also evidence of many species of *Dictyota* which are purely late-stage colonizers.

No. 03

Additionally, the use of multiple genetic markers allowed this study to define dietary items that were only identified by one marker.

The results support the hypothesis of a protein-rich diet of invertebrate epiphytes to macroalgae, EAM, and turf, among other more cryptic items like tunicates and unexpected items like urchins and fish. There were no significant differences in the diet when analyzed by location and season, which suggests that these sites may already be rather homogenous in their algal communities despite the differences in coastal development.

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