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Lords of the Rings: Mud ring feeding by bottlenose dolphins in a Caribbean estuary revealed from sea, air, and space

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Bottlenose dolphins (*Tursiops* spp.) display a remarkably diverse array of individual and cooperative foraging tactics across their global distribution that typically reflect local adaptations to habitat conditions and prey types (Finn et al., 2009; Mann & Sargeant, 2003; Torres & Read, 2009). Specialized foraging methods documented to date include strand feeding (Hoese, 1971; Sargeant et al., 2005), kerplunking (Connor et al., 2000; Nowacek, 2002), cooperative foraging with fishermen (Pryor & Lindbergh, 1990), driver-barrier feeding (Gazda et al., 2005), foraging in or around trawl nets (Kovacs & Cox, 2014), and using tools, particularly sponges (Krützen et al., 2014; Smolker et al., 1997) and shells (Allen et al., 2011; Wild et al., 2020).

Mud ring feeding is a tactic used by common bottlenose dolphins (*T. truncatus*) inhabiting the interior of Florida Bay (Engleby & Powell, 2019; Torres & Read, 2009). This tactic involves a single “ring-maker” dolphin (typically in a group) that swims rapidly in a circle near the seafloor along shallow inner-basin mud banks. Strong fluke kicks against the muddy substrate create a large circular mud plume or mud ring barrier used to encircle a fish school, commonly mullet (*Mugil* spp.). Once the prey are encircled, individual dolphins wait with open mouths and lunge to catch airborne fish as they attempt to flee at the water’s surface (Engleby & Powell, 2019; Torres & Read, 2009). In the lower Florida Keys, bottlenose dolphins display mud plume feeding behavior where they individually create a semi-circular-shaped mud plume over seagrass beds and lunge into the plumes to capture prey (Lewis & Schroeder, 2003).

Novel remote-sensing techniques such as small unmanned aerial vehicles (UAVs or, commonly, drones) and the analysis of very high-resolution (VHR) satellite imagery (<1 m spatial resolution), have become increasingly affordable

and accessible to further document the ecology and behavior of marine mammals (Cubaynes et al., 2018; McMahon et al., 2014). Small UAVs provide unique insights into marine mammal behavior by improving the ability to observe epipelagic animals underwater (Landeo-Yauri et al., 2020; Ramos et al., 2018a; Torres et al., 2018), and in particular, document foraging behaviors that are challenging to detect for boat-based observers (Ramos et al., 2020b; Torres et al., 2020). Similarly, VHR imagery has proven an effective and noninvasive method for detecting and counting marine mammals (Cubaynes et al., 2018; Guirado et al., 2019; Höschle et al., 2021). The power of large data sets of publicly accessible remote-sensing imagery coupled with automated processing techniques were shown to improve the detection of the carcasses of mass stranded whales in a remote and difficult to access region (Fretwell et al., 2019).

Here, we document the occurrence of mud ring feeding in coastal bottlenose dolphins in Chetumal-Corozal Bay in Mexico and Belize using a combination of remote sensing, boat-based, and aerial methods. The distinct circular mud ring trails (observed using VHR satellite imagery) and dolphin behaviors (observed from a small boat, planes, and small UAVs) in Chetumal-Corozal Bay are similar to those reported in Florida Bay (Engleby & Powell, 2019; Torres & Read, 2009) and we hypothesize that similarities between these shallow bay habitats drive dolphins to develop convergent foraging behaviors.

Field data on dolphin foraging were collected opportunistically from 2012 to 2019 during year-round monitoring of Chetumal-Corozal Bay in northern Belize by Wildtracks and the Sarteneja Alliance for Conservation and Development (SACD). Chetumal-Corozal Bay is a large estuarine system in the Western Caribbean Sea located on the southern coastline of the Yucatán Peninsula in northern Belize and the southeastern Mexican state of Quintana Roo (Figure 1). The brackish and marine bay covers an area of 2,560 km², comprised of extensive mangrove wetlands, creeks, and lagoons with sparse seagrass beds, and a predominantly sandy/rocky and muddy/silty substrate (Castelblanco-Martínez et al., 2013). Water depths are primarily <6 m in the north and <2 m near the coast (Carrillo, 2009; Castelblanco-Martínez et al., 2013).

Coastal bottlenose dolphins are the only dolphin species known to occur in Chetumal-Corozal Bay and throughout the diverse habitats along the coast of Belize, including its bays, rivers, lagoons, around mangrove cayes, and in offshore atolls (Ramos et al., 2016; Castelblanco-Martínez et al., 2021). The use of mud ring feeding by bottlenose dolphins was confirmed through direct observations of dolphin foraging activity or inferred through the detection of circular mud ring trails (i.e., circular seabed scars left as the byproduct of ring-making) in the substrate in aerial and VHR satellite imagery.

Bottlenose dolphins were opportunistically sighted from small boats (7–12 m) in Chetumal-Corozal Bay during ongoing, year-round boat-based monitoring of the Corozal Bay Wildlife Sanctuary in Belize conducted by SACD several times a month since 2012. Boat-based photographs of dolphins were collected with a Canon 60D digital SLR camera equipped with a 100–300 mm telephoto lens to identify individual dolphins. The dorsal fin of one identified dolphin was matched to photos from an existing catalog in the region (Ramos et al., 2018b, 2020a).

Aerial observations of dolphin foraging were acquired opportunistically during two manned aerial surveys flown on multiple days within a single week from February to March each year from 2011 to 2015. The 13 m single engine Cessna 206 flew a line-transect to cover the bay between altitudes of 80 m and 120 m. Experienced spotters searched for marine megafauna; logged information on sighting locations and the number of animals detected according to a standard protocol (Morales-Vela et al., 2000); and took photos of animals when possible with digital SLR cameras equipped with telephoto lenses, to confirm species identity; count the number of animals, and document behaviors.

Furthermore, dolphin behavior was filmed in one mud ring feeding event with a UAV (DJI Phantom 4 Professional quadcopter). The UAV was hand-launched from the boat and flown between altitudes of 25–35 m above the dolphins. The aircraft was equipped with a gimbal-mounted camera that filmed high definition (3,860 × 2,870 dpi) video footage and streamed a live-feed to an iPad (Apple Inc.) mounted on the remote control. Aerial videos were reviewed in QuickTime Player 10.7 (Apple Inc.) to identify mud rings and determine dolphin behavior.

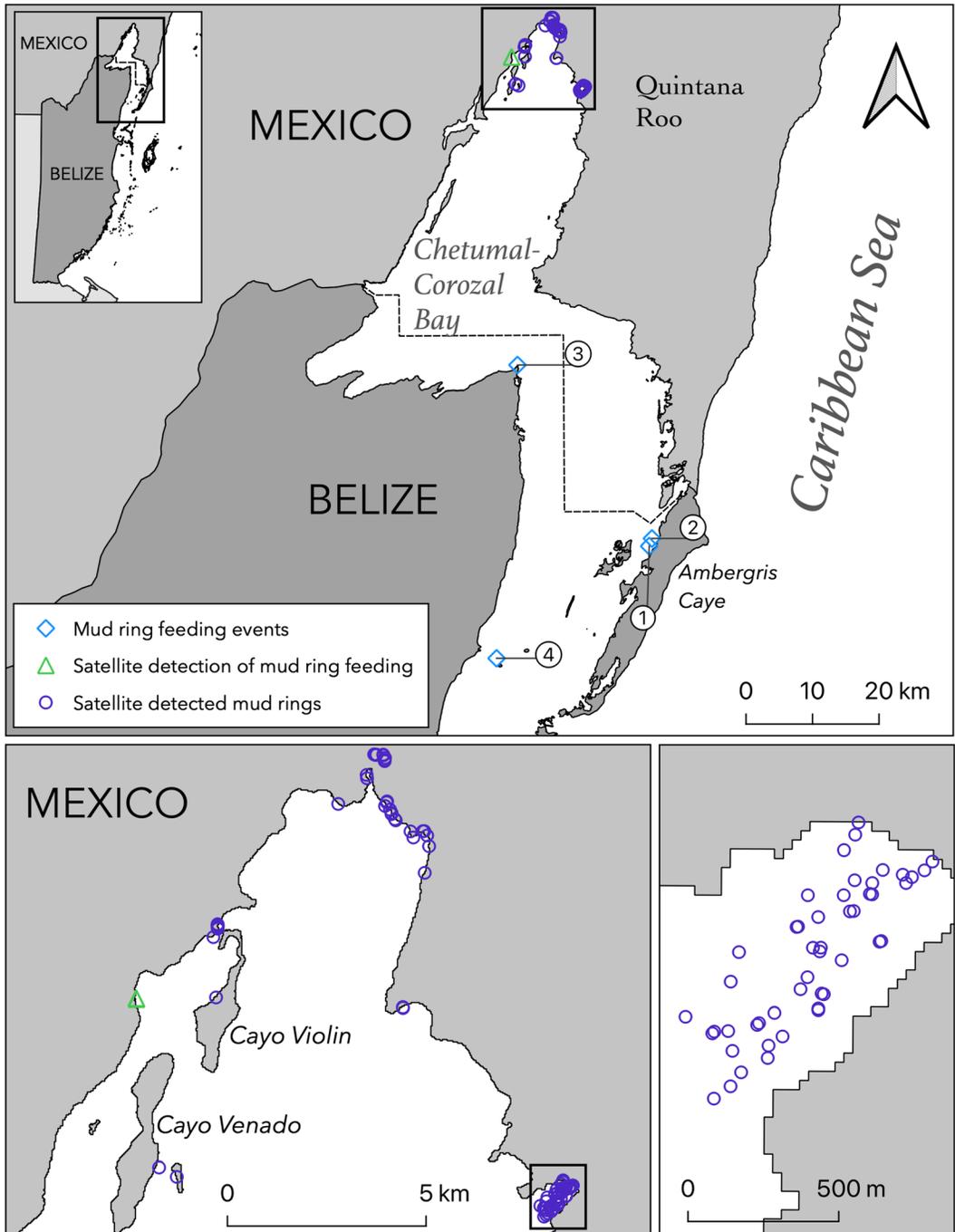


FIGURE 1 Map of Chetumal-Corozal Bay depicting the location of dolphin mud ring feeding events and mud ring trails. Mud ring feeding behavior was observed during aerial surveys, boat trips, and the flight of a small UAV. Mud ring trails ($n = 94$) and a single instance of two dolphins mud ring feeding were detected near the northwest coast of the bay in very high-resolution (VHR) satellite imagery using Google Earth. The four mud ring feeding events we documented in Belize (detailed in Table 1) are numbered by event in the top map.

TABLE 1 Details on four mud ring feeding events of bottlenose dolphins documented from 2012 to 2019 during aerial and boat-based surveys in the Belize side of Chetumal-Corozal Bay. Mud ring feeding was directly observed in three events and its occurrence was inferred from the presence of multiple mud ring trails in the seabed in imagery captured during an aerial survey. UNK = Unknown.

Event no.	Date	Time	Platform	No. dolphins present	No. dolphins foraging	No. mud ring trails
1	January 31, 2012	1000-1030	Plane	6	2	UNK
2	February 6, 2014	1000-1030	Plane	UNK	UNK	6
3	July 30, 2018	0800	Boat	20-25	UNK	UNK
4	January 19, 2019	1143	UAV	6	2	11

Publicly accessible satellite imagery was obtained and analyzed in Google Earth Pro to determine if mud ring trails and mud ring feeding behavior could be visually detected in VHR imagery of Chetumal-Corozal Bay. To identify mud ring trails and direct observations of dolphin mud ring feeding, we searched an area of 470 km² of satellite imagery covering Chetumal-Corozal Bay (including its interior and coastlines in Mexico and Belize) (Figure 1). To confirm that these behaviors were similar to those reported in Florida, we searched an area of 1.7 km² in Florida Bay, where previous reports have identified regular mud ring feeding (Engleby & Powell, 2019; Torres & Read, 2009). The Historical Imagery feature enabled review of high-resolution satellite imagery of the study area from 2006 to 2019 with minimal cloud cover and where the seabed in the bay was clearly visible. All imagery with poor resolution and excessive cloud cover was excluded from our analysis. The mosaic of satellite images in Google Earth originated from cloudless WorldView-2 satellite imagery (Maxar Technologies) including panchromatic (0.3 m spatial resolution) black and white imagery and RGB multispectral imagery (1.24 m spatial resolution).

Information on water depth and habitat type were acquired from previous studies in Chetumal-Corozal Bay (Castelblanco-Martínez, 2010; Hernández-Arana & Ameneyro-Angeles, 2011) and Florida Bay (Prager & Halley, 1997). Existing spatial data in Florida were overlaid with all detections in QGIS 3.10 to identify the bottom type at each trail. Structural characteristics of the mud ring trails were obtained by measuring the diameter of each mud ring trail by using the measurement tool in Google Earth Pro to draw a straight line between two points on opposing sides of the circular trail. The same tool was used to measure the distance of each trail to the shore by drawing a straight line between the edge of the trail closest to the shore and its nearest point on the shoreline in Chetumal-Corozal Bay. The distance of trails to mudbanks in Florida Bay was determined with the distance to hub (points) vector analysis in QGIS 3.10.

Between 2012 and 2019, mud ring feeding behavior was detected in two of 58 dolphin sightings from a small plane and two of 22 boat-based sightings within Chetumal-Corozal Bay (Table 1). Mud ring feeding behaviors were directly observed in three of these sightings, and their occurrence was inferred from the detection of multiple circular mud ring trails in the seabed in aerial imagery (Figures 2 and 3). In one boat-based sighting, a large group of dolphins was sighted foraging and creating large circular mud plumes. In the second boat-based sighting, detailed aerial observations of dolphin mud ring feeding were gathered with a small UAV (Figure 2).

In the single drone observation of mud ring feeding, an adult female (identified in 2017) and her calf were found feeding near mud ring trails (Figure 2a; see Video S1). The adult female then swam quickly and kicked her flukes against the substrate to create a mud ring (Figure 2b-d). Once the plume was nearly circular (Figure 2c), both dolphins repeatedly displayed fast swimming, pinwheels, and underwater tail slaps within the expanding sediment plume (Figure 2d). After 83 s, the dolphins exited the plume and continued to feed nearby.

Visual searches for mud ring trails in VHR satellite imagery resulted in detections of 94 trails on March 10, 2010 (Figure 1). Mud ring trails were concentrated in a 75 km² area of the northern side of the bay in water depths <1 m. Mud ring trails were typically found clustered together (Figure 3b, c). Most trails (53.5%) were concentrated in a sheltered delta in the northeast section of the bay in depths <1 m (Figure 1). Most of the remaining trails were found in

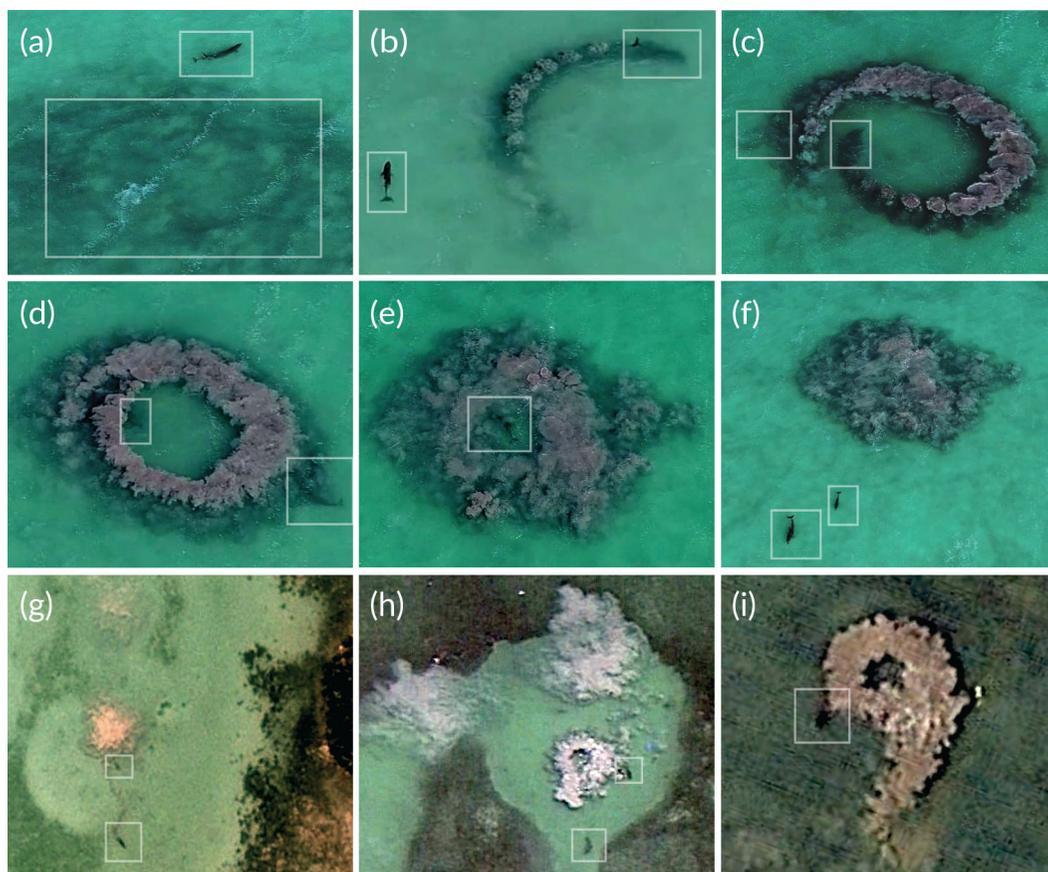


FIGURE 2 Evidence of bottlenose dolphin mud ring feeding from aerial observations with a small UAV and detections in VHR satellite imagery. The white rectangle in (a) marks the location of a mud ring trail and the dolphin nearby. In (b-i), the white rectangles mark the location of a dolphin. (a) Bottlenose dolphin mother-calf pair sighted foraging near recently made mud ring trails on 19 January 2019 (see Video S1). (b) The adult female swam in a circular pattern while hitting her flukes against the muddy substrate to create a mud ring. The calf remained outside of the plume. (c) Once the circular mud ring was complete the calf swam into the plume. (d) Both dolphins were swimming rapidly and exhibiting pinwheels and underwater tail slaps in pursuit of prey within the plume. (e) The plume expanded slowly until (f) the two dolphins emerged and began foraging in another area. (g) Two dolphins exiting a large mud plume similar to (f) in the northwest of Chetumal-Corozal Bay in VHR satellite imagery. (h & i) Bottlenose dolphins engaged in mud ring feeding near large mud plumes in Florida Bay, Florida detected in VHR satellite imagery. Image source for g-i: Google Earth, WorldView-2 satellite imagery (Maxar Technologies/DigitalGlobe).

mangrove channels in the northern tip of the lagoon and in the northwest along the coast and shores of small cays (Figure 3b, c). In imagery from the same day, one dolphin mud ring feeding event and associated circular mud plumes were detected near the northwest coast of the bay. Two bottlenose dolphins were visible in satellite imagery exiting a recently created plume (Figure 2g). The dolphin pair were similar in appearance to the mother-calf pair previously observed mud ring feeding (Figure 2f) and swimming near similarly sized, circular plumes of sediment.

Three instances of mud ring feeding were detected in VHR satellite imagery of Florida Bay, one in imagery from March 7, 2013 (Figure 2h) and two from February 13, 2017 (Figure 2i). A total of 1,817 mud ring trails were detected in an area of ~ 1 km² in Florida Bay.

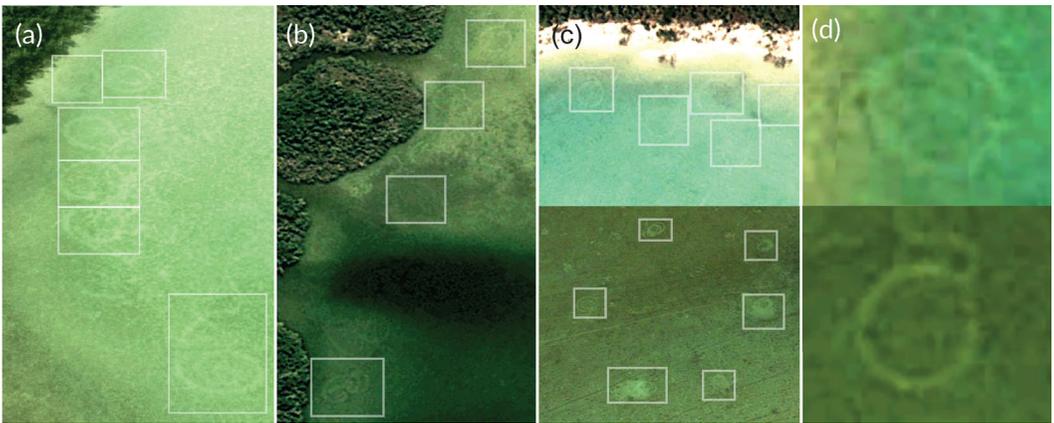


FIGURE 3 Mud ring trails in the substrate captured during aerial surveys and in searches of VHR satellite imagery of Chetumal-Corozal Bay. (a) Mud ring trails near mangrove shores photographed from a small plane in Belize. (b) Satellite detected mud ring trails in a sheltered mangrove channel in the northern tip of the bay. (c) Trails were typically found nearshore in clusters. (d) Individual mud ring trails were shaped circular or spiral and lighter in color than the surrounding substrate. Image source for b–d: Google Earth, WorldView-2 satellite imagery (Maxar Technologies/DigitalGlobe).

In Chetumal-Corozal Bay, all trails and mud ring feeding were located in muddy bottom habitats consisting of sparse algae and silt. Most trails in Florida Bay were detected in muddy substrates (50%) and seagrass beds (48.3%). The water depth at locations of all trails in Chetumal-Corozal and Florida Bays was <1 m.

The diameter of mud ring trails in Chetumal-Corozal Bay was similar (range = 4.6–14.7 m; $M = 9.7$ m; $SD = 2.2$; $n = 85$) to a random sample of trails in Florida Bay (range = 3.4–14.1 m; $M = 8.0$ m; $SD = 1.9$; $n = 100$). Mud ring trails were detected close to shore in Chetumal-Corozal Bay ($M = 106.6$ m; $SD = 97.0$; $n = 85$) and near the edges of mudbanks found within Florida Bay ($M = 221.5$ m; $SD = 286.9$; $n = 1,817$).

In this study, we report the use of mud ring feeding behaviors by coastal bottlenose dolphins in Chetumal-Corozal Bay. To our knowledge, this is the first time that satellite images have been used to document the foraging behavior of small cetaceans. In Chetumal-Corozal Bay, dolphins were directly observed mud ring feeding in small groups. Images of circular mud trails were confirmed to be the remnants of ring-making activity during Event #4 (Figure 2a) and were useful indicators of recent dolphin foraging sites that enabled us to validate the occurrence of this behavior in VHR imagery of the study site.

The mud ring feeding behavior documented in Chetumal-Corozal Bay was remarkably similar to that documented in Florida Bay (Engleby & Powell, 2019; Torres & Read, 2009), occurring nearshore in very shallow waters (<1 m) dominated by mud, sand, and silt substrates (see Video S1). We are confident that these rings have not been caused by other organisms or by boats due to their unique circular characteristics and observed associations with dolphin foraging in both regions. In Florida Bay, boat propeller scars are linear and can be easily discriminated from dolphin mud rings, particularly from the air but also from the surface. Additionally, boats do not have access to these shallow areas in Florida Bay and this is not a fishing technique that is known or used in either bay. Trails were circular- or spiral-shaped in both regions, of similar sizes, and generally found in clusters. The detection of dolphin groups engaged in mud ring feeding in Chetumal-Corozal Bay suggests these dolphins may forage cooperatively, as previously reported in Florida Bay (Torres & Read, 2009). However, our observations were limited, and more data are needed to assess the importance of mud ring feeding relative to other foraging tactics in Chetumal-Corozal Bay.

Unlike mud ring feeding reported in Florida Bay where mud ring creation is tightly associated with the capture of schools of mullet, fish were not observed leaping at the end of ring creation and there was no apparent in-air pursuit of fish in our three sightings of this behavior. Repeated foraging behaviors observed by the mother-calf dolphin pair in Chetumal-Corozal prior to ring creation, immediately following the completion of the circular plume, and

afterwards in the expanding sediment plume suggest that dolphins use the mud ring's sediment plume either as a means of prey disorientation or as a concentration zone to facilitate prey capture. Similar to mud plume feeding in the Florida Keys (Lewis & Schroeder, 2003), the plumes likely create a temporary visual and physical barrier capable of confusing and disorienting prey and possibly impeding collective antipredator responses (Abrahams & Kattenfeld, 1997). The behaviors we report were more similar to previously reported mud ring feeding behaviors (Engleby & Powell, 2019; Torres & Read, 2009) than to mud plume feeding (Lewis & Schroeder, 2003) due to the creation of a distinct circular ring-shaped mud plume. These observations, however, were of short duration and our sample size was too limited to draw robust conclusions about the regional similarities and differences in mud ring feeding or mud plume feeding between the bays.

Mud ring feeding dolphins in Florida Bay exclusively target species of mullet, such as white mullet (*Mugil curema*; Engleby & Powell, 2019; Torres & Read, 2009). The prey species hunted by bottlenose dolphins during mud ring feeding in Chetumal-Corozal Bay are as yet unknown and dolphin diets or regional prey preferences are yet to be investigated.

As all mud ring trails and the two dolphins mud ring feeding in Chetumal-Corozal Bay were detected in VHR satellite imagery on a single day in 2010 in an area of over 75 km², it was not possible to determine when these trails were created. The protected waters of the bay allow seabed disturbances to remain detectable for long periods of time, thus, we could not confirm if these trails were the result of multiple groups of dolphins foraging in different locations at the same time, or one dolphin group foraging over multiple days. Nevertheless, evidence of mud ring feeding detected suggests similarities between these behaviors in Florida and Chetumal-Corozal Bays. Satellite detections in both locations revealed instances of dolphins distinguishable against the sandy bottom as they swam near circular and spiral mud plumes. These findings suggest that VHR imagery may be of use in studies of small marine mammals in coastal habitats, as has already been demonstrated with large whales (Guirado et al., 2019).

Our study supports the idea that habitat characteristics, and/or prey types and their behavior, play a pivotal role in shaping the foraging tactics used by coastal dolphins. We have documented behavioral convergence between two geographically separate locations that share habitat characteristics, leading dolphins to develop similar strategies for prey capture. Future research should investigate the relative contributions of environmental conditions and social transmission, and their possible interplay, on convergent foraging behaviors in geographically distanced dolphin populations.

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AUTHOR CONTRIBUTIONS

Eric Ramos: Conceptualization; data curation; formal analysis; investigation; resources; writing - original draft; writing-review & editing. **Leomir Santoya:** Conceptualization; data curation; formal analysis; methodology; resources; writing - original draft; writing-review & editing. **Joel Verde:** Conceptualization; data curation; methodology; resources; writing - original draft; writing-review & editing. **Zoe Walker:** Conceptualization; data curation; formal analysis; investigation; methodology; resources; writing - original draft; writing-review & editing. **Nataly Castelblanco-Martínez:** Conceptualization; data curation; formal analysis; methodology; resources; writing - original draft; writing-review & editing. **Jeremy Kiszka:** Methodology; supervision; writing - original draft; writing-review & editing. **Guillaume Rieucou:** Methodology; supervision; writing - original draft; writing-review & editing.

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SUPPORTING INFORMATION

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Behavioural and physiological responses of captive Antillean manatees to small aerial drones

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Abstract

Context. Unmanned aerial vehicles or drones are powerful tools for wildlife research. Identifying the impacts of these systems on target species during operations is essential to reduce risks of disturbance to wildlife, to minimise bias in behavioural data, and to establish better practices for their use.

Aims. We evaluated the responses of captive Antillean manatees to the overhead flight of a small aerial drone.

Methods. We used aerial and ground videos to compare manatee activity budgets and respiration rates in three 15-min sampling periods: ‘before’, ‘during’ and ‘after’ flights with a DJI Phantom 3 Advanced. The drone was hovered stationary for 3 min at five altitudes (100 m, 40 m, 20 m, 10 m, 5 m) to determine whether manatees display behavioural responses compared with the control period, and whether they respond more at lower altitudes. Only one flight was performed per manatee group to avoid bias owing to habituation to the drone.

Key results. Manatees responded to drone flights by (1) increasing their activity levels during and after flights, therefore signalling after effects; (2) decreasing their respiration rate during flights; and (3) displaying behavioural reactions including grouping, tail-kicking, fleeing from their original position and moving under submerged structures. From the 11 individuals displaying behavioral reactions, 9 reacted in the first ~2 min of flight, preventing assessments of altitude effects and suggesting manatees responded to the drone sound at take-off.

Conclusions. Behavioural changes of responding manatees were similar to previous reports of disturbance responses to boats and drones in this species. Our use of a control period showed shifts in respiration rates and activity budgets that persisted after flights. Several manatees reacted to the drone from the time of take-off and first minutes of flight, indicating that the sound of the electric rotors could be a strong negative stimulus to manatee and highlighting the importance of establishing safe distances for take-off.

Implications. Future studies should consider that drones could elicit conspicuous and inconspicuous responses in manatees. Our results emphasise the need for control data on animal behaviour to better assess the impact of drones on wildlife and to design non-invasive protocols.

Keywords: behaviour, disturbance, unmanned aerial vehicles, *Trichechus*, UAS, UAV.

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Introduction

Unmanned aerial vehicles (UAV), or ‘drones’, are versatile remote-sensing tools now popular in ecological research, conservation, and the management of wildlife (Koh and Wich 2012; Anderson and Gaston 2013). These tools have many applications, including high-resolution mapping and habitat assessments (Messinger *et al.* 2016; Joyce *et al.* 2019), anti-poaching surveillance (Mulero-Pázmány *et al.* 2014), and wildlife monitoring (Linchant *et al.* 2015; Hodgson *et al.* 2018). Drones are relatively safe for the operator compared with manned aerial surveys (Jones *et al.* 2006) and are less costly to operate (Colefax *et al.* 2018). Additionally, these systems enable access to remote areas (Christie *et al.* 2016) and have the capacity to collect systematic data and permanent high-resolution visual records (Hodgson *et al.* 2013).

In marine mammal research, drones serve as powerful tool to supplement and enhance the capacity to collect important data on wild populations (Hodgson *et al.* 2017; Colefax *et al.* 2018; Raoult *et al.* 2020). The increasing use of drones for wildlife research (Linchant *et al.* 2015) emphasises the need for regulations guiding the safe use of these systems to avoid potential impacts on the target species during operations. Disruption of wildlife can affect vital activities, such as, for example, causing reductions in feeding time (Williams *et al.* 2006), changes in spatial use (Buckingham *et al.* 1999), increases in energetic expenditure (Lusseau and Bejder 2007), and eliciting physiological stress (French *et al.* 2010). Moreover, animal responses to the observation platform create biases in data collection, such as, for instance, skewing detection accuracy during individual counts (Brisson-Curadeau *et al.* 2017) or causing behavioural shifts that can bias studies of natural behaviour. Thus, guidelines and protocols for the use of drones are needed to reduce the risks they pose to wildlife and to minimise bias in data collection (Smith *et al.* 2016; Raoult *et al.* 2020).

A critical component for evaluating the effects of disturbance from drones is to report and understand the spectrum of responses displayed across a range of species (Bevan *et al.* 2018) and in different environments. Raoult *et al.* (2020) reviewed and outlined operational protocols for using drones to study marine megafauna and identified inter-specific variation in marine animal physiology (e.g. auditory capacity) and ecology (e.g. predation by aerial animals) as relevant factors in determining the impact of drones on these species. Numerous studies have directly assessed the risk of disturbance of drones on marine mammals such as grey seals (*Halichoerus grypus*, Pomeroy *et al.* 2015; Arona *et al.* 2018), blue whales (*Balaenoptera musculus*, Domínguez-Sánchez *et al.* 2018), humpback whales (Fiori *et al.* 2020), bottlenose dolphins (*Tursiops truncatus*, Ramos *et al.* 2018; Fettermann *et al.* 2019), and Antillean manatees (*Trichechus manatus manatus*, Ramos *et al.* 2018). However, most studies investigating behavioural responses of marine mammals to drones lack control data where animal behaviour can be observed without the presence of the platform, data essential for determining the risk of disturbance of different drone systems and flight methods (Bevan *et al.* 2018). Thus, experiments quantifying disturbance using captive animals, where control data are less challenging to obtain, are ideal for developing species-specific protocols (Hodgson and Koh 2016).

Sirenian research using aerial drones has been conducted only with dugongs (*Dugong dugon*, Hodgson *et al.* 2013) and West Indian manatees (*Trichechus manatus*; Jones *et al.* 2006; Ramos *et al.* 2018; Landeo-Yauri *et al.* 2020). Of the two subspecies of the West Indian manatee, namely, the Florida manatee (*T. m. latirostris*) and the Antillean manatee (*T. m. manatus*), the latter is distributed throughout at least 19 countries across the Caribbean Sea, and Central and South America (Reynolds 1999). Most of these nations are small, low-income countries that would benefit from the development and adaptation of inexpensive tools to monitor and study wild manatee populations. With the expanded accessibility to drone technology, the use of these systems for manatee research will likely increase in these areas. Thus, exploring the effect of drones on manatees and using this information to design protocols for non-invasive data collection is necessary to mitigate their potential negative effects (e.g. physiological and behavioural stress responses, changes in group cohesion, among others).

Only one study has attempted to assess the disturbance responses of free-ranging Antillean manatees to small drones. Manatees responded to the overhead flight and vertical approach of a small quadcopter (DJI Phantom 4 Pro) in a quarter of all flights, with more responses occurring at lower flight altitudes. Given no vessels or other plausible sources of disturbance were near any of the responding manatees, the reactions of the individuals were likely caused by the sound or visual stimulus associated with drone flight (Ramos *et al.* 2018). In the present study, we tested whether captive manatees respond to experimentally controlled drone flights at different altitudes. We recorded ground-based footage before, during, and after the flights, and aerial footage during flights, to compare activity budgets and respiration rates across sampling periods.

Methods

Drone flights

All experiments were performed with 25 captive manatees (12 females and 13 males) of different age classes (calves, juveniles, adults) housed at eight pools located in six different aquarium facilities in the state of Quintana Roo, Mexico (Table 1, Fig. 1). A DJI Phantom 3 Advanced quadcopter (P3A, DJI Technology Co., San Diego, CA, USA) was flown for drone exposure experiments in the daytime (from 0900 hours to 1830 hours). The aircraft was equipped with a 12.4 MP camera (FOV 94°, 20 mm, f/2.8 lens) filming in 2700 dpi. The drone was flown manually with a remote control with a mounted iPad tablet (Apple Inc., Cupertino, CA, USA) and visually monitored via the live video stream to correctly orient over aquarium pools ($n = 8$, Table 1).

The pilot (SL) positioned the drone over each manatee pool, starting at an altitude of 100 m and descending the drone with stationary hovering for approximately 3 min at altitudes of 100 m, 40 m, 20 m, 10 m and 5 m. Aerial videos were recorded for the 15-min duration of each flight. Only one flight was performed per pool to avoid introducing bias due to the possible habituation or sensitisation of the manatees to the presence of the drone. Seven flights were conducted in total, six flights over one pool each, and one flight was conducted over two adjacent pools (G and H).

Table 1. Study locations and manatee groups used to test for responses to small aerial drones
 Sex: M, male; F, female; Age class: A, adult; J, juvenile; C, calf; R, rescued, C, captive-born

Flight date	Facility	Pool	Manatees per pool	Individuals
2 May 2017	DD. Puerto Aventuras	A	8	Pablo (MAR), Quijote (MAC), Nohoch (MCC), Julieta (FAR), Dorothy (FAR), Conchis (FJC), Claudia (FJC), Bombon (FCC)
26 July 2017	DD. Dreams	B	2	Roberto (MAR), Lorenzo (MJC)
27 July 2017	DD. Isla Mujeres	C	4	César (MAR), Fabián (MAC), Sabina (FAR), Africa (FCC)
9 May 2017	DD. Cozumel	D	3	Yoltzin (MAR), Angel (MAR), Edgar (MAC)
26 July 2017	Xel-Há	E	2	Tunich (FJC), Nikté (FJC)
		F	3	Baxal (FAR), Dayami (FJR), Pompom (FAR)
25 July 2017	Xcaret ^A	G	2	Mach (MAR), Buul (MAC)
		H	1	Nohoch (MAR)

^AAt Xcaret, two flights were performed: one over the female pool (F) and one over the male pools (G and H).



Fig. 1. Aerial view of the facilities and pools where our drone tests were conducted. Pools are outlined in yellow and labelled according to facility in Table 1.

To determine the effect of the drone on manatees and to rule out alternative variables possibly influencing their behaviour (e.g. pool structure, vegetation presence, number of manatees present), we used the same sampling periods across all pools. In that way, uncontrolled variables were maintained constant across sampling periods within each pool.

Each experimental exposure involved a pre-exposure period of video-recorded ground observations of manatees, followed by a single flight, and a post-exposure period of ground observations. Ground video and drone-based observations were used to examine the activity of manatees. One to two ground observers filmed manatee behaviour with digital video cameras (Sony

DSC-HXA50 cyber-shot and Fujifilm FinePix XP130). Three 15-min videos were recorded in each pool corresponding to the three sampling periods: ‘before’, ‘during’, and ‘after’ the drone flights. The pre-exposure ‘before’ period was considered the control. Aerial drone videos also provided information in the ‘during’ sampling period. All behaviour events considered by observers as a visible response displayed from drone take-off through drone flights (i.e. behaviour reactions) were noted and described, as well as the flight altitude during which they occurred.

Data analysis

Aerial and ground videos were reviewed in VLC Media Player to determine individual activity budget (i.e. time each manatee spent in different behaviours) and the respiration rate (number of breaths per minute) of each manatee. The following behavioural states were used to sample the behaviour of each focal manatee with-continuous sampling: locomotion (*L*), which includes swimming movement displayed at any level of the water column and diving; bottom resting (*BR*), which includes lying near or at the bottom, and exhibiting minimal movements, and/or rotations along their axes; and surface resting (*SR*), which includes resting near the surface and exhibiting minimal movements and/or rotations along the axes. Videos of manatee behaviour throughout experiments were reviewed to detect behavioural reactions to the drone that could be interpreted as avoidance, fear or stress.

Manatees were considered out-of-sight (OS) when the target individual was not visible in the video. The proportion of time each manatee spent in different behavioural states was calculated to determine individual activity budgets. OS periods were discarded to standardise observations among individual manatee activity budgets.

Manatee respiration rates and activity budgets were compared among sampling periods (‘before’, ‘during’ and ‘after’ drone flight) using the Wilcoxon matched-pairs tests in STATISTICA 0.7 (StatSoft, Inc. Tulsa, Oklahoma, USA, 2004). Individuals with more than 10 min of OS time in each sampling period were not included in this analysis. We considered that manatees responded to the drone if changes were detected in their respiration rate and activity budgets from the ‘before’ sampling period compared with the ‘during’ or ‘after’ sampling periods. Flight altitude was not considered as a category for manatee respiration rate or activity budget comparisons, because each flight altitude was maintained for no more than 3 min. This time lapse is insufficient for adequate comparisons regarding activity budgets, or to assess the breathing interval of manatees (2–3 min for low-energy activities according to Hartman 1979). The flight altitude at which behavioural events were detected was identified to determine whether lower flights altitudes had a higher likelihood of causing responses than did higher altitudes.

Results

In total, 660 min of video observations were analysed, including 105 min from aerial videos and 555 min of ground-based videos. Of all focal manatees evaluated, 18 individuals (72%) met the condition of having less than 10 min of OS time to compare their

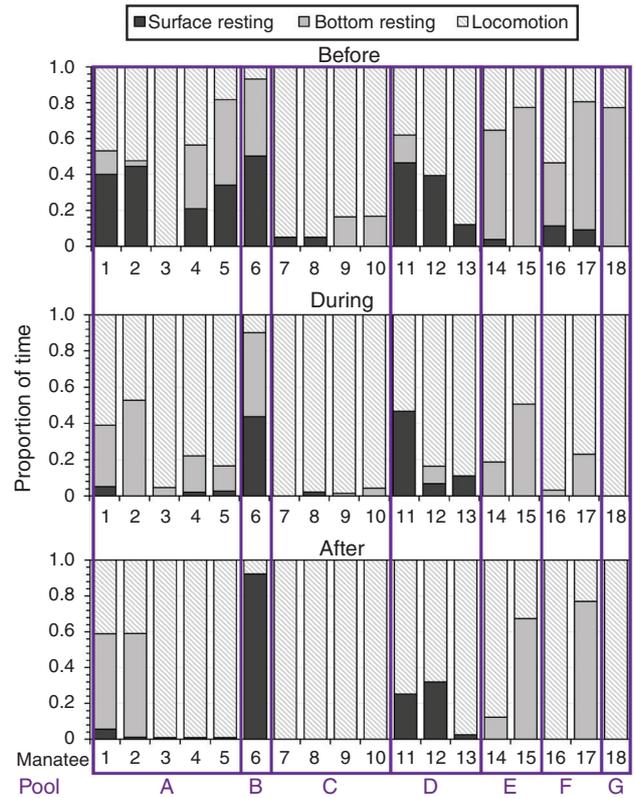


Fig. 2. Activity budgets of exposed Antillean manatees (n = 18) for the three sampling periods. Individual numbers correspond to 6 = Lorenzo, 7 = Sabina, 8 = Africa, 9 = César, 10 = Fabian, 11 = Angel, 12 = Yoltzin, 13 = Edgar, 14 = Nicté, 15 = Tunich, 18 = Nohoch. We were not able to identify individuals 1–5, 16 and 17.

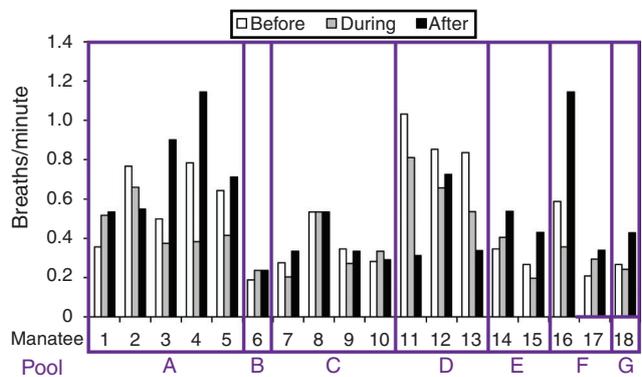


Fig. 3. Respiration rates of exposed Antillean manatees (n = 18) grouped by pool for each of the three sampling periods. Individual numbers correspond to 6 = Lorenzo, 7 = Sabina, 8 = Africa, 9 = César, 10 = Fabian, 11 = Angel, 12 = Yoltzin, 13 = Edgar, 14 = Nicté, 15 = Tunich, 18 = Nohoch. We were not able to identify individuals 1–5, 16 and 17.

respiration rate and activity budget among sampling periods. Individual data recorded from these are presented in Fig. 2 (activity budgets) and Fig. 3 (respiration rates).

Manatees (n = 18) spent most of their time in locomotion (Fig. 4). During drone flights, manatees increased their activity

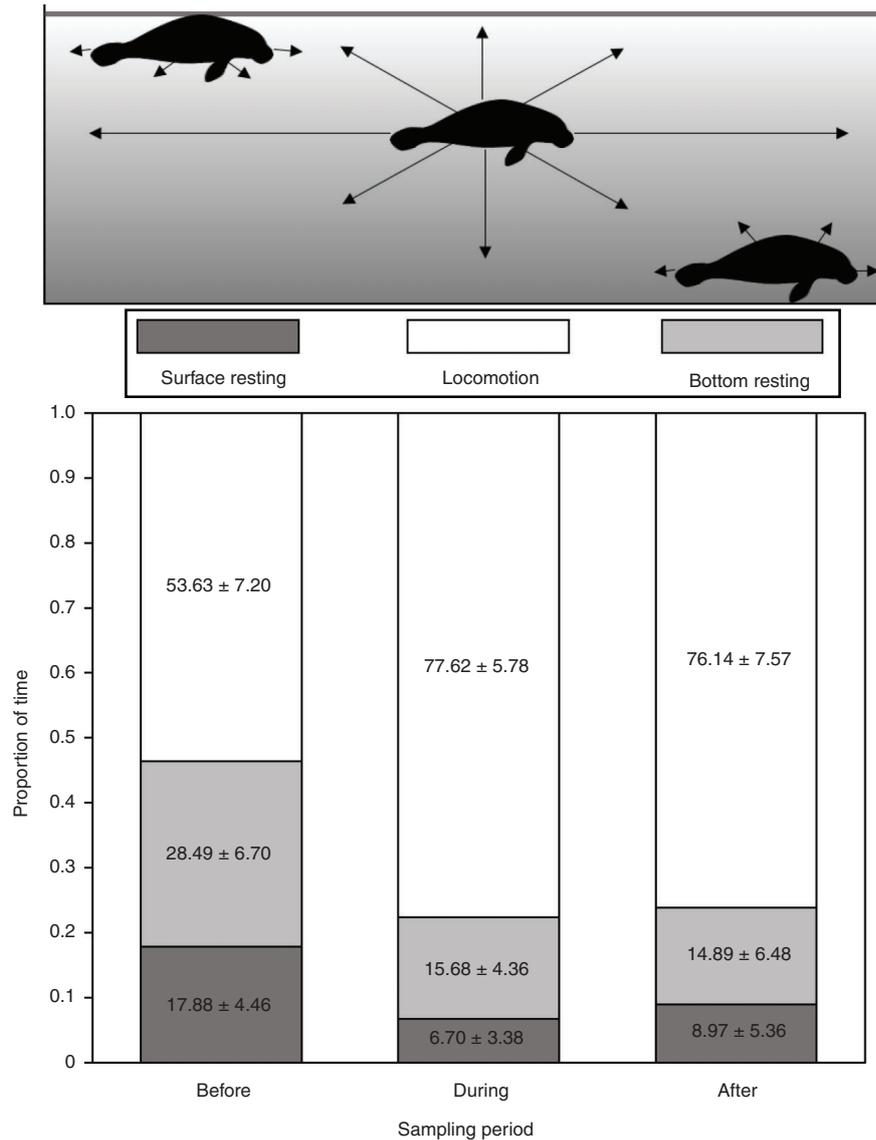


Fig. 4. Average proportion of time (\pm standard error) Antillean manatees ($n = 18$) spent in different behavioural states (surface resting, locomotion, bottom resting) for all three sampling periods.

levels and spent less time at the surface than before drone exposure (locomotion: $t = 10$, $Z = 3.288$, $P = 0.001$; surface resting: $t = 1$, $Z = 3.110$, $P = 0.002$). Increased activity levels were also observed after flights when comparing to before flights (locomotion: $t = 17$, $Z = 2.983$, $P = 0.003$; surface resting: $t = 14$, $Z = 2.417$, $P = 0.016$). There were no significant differences among sampling periods in time spent bottom resting (before vs during: $t = 28$, $Z = 1.817$, $P = 0.069$; before vs after: $t = 21$, $Z = 1.712$, $P = 0.087$). The average respiration rate of manatees ($n = 18$) decreased ‘during’ the drone flight when compared with ‘before’ and ‘after’ flight sampling periods (Fig. 5). Manatee respiration rate increased significantly during flight compared with before flights ($t = 28$, $Z = 2.296$, $P = 0.022$), but not between ‘before’ and ‘after’ periods ($t = 52$, $Z = 1.159$, $P = 0.246$), or between ‘during’ and ‘after’ periods ($t = 36$, $Z = 1.917$, $P = 0.055$).

Four behavioural events detected in manatees were considered reactions to drone. *Grouping* involved several manatees aggregating in a small area, sometimes close enough to be in physical contact. *Take cover* involved individuals going under submerged structures, where the body can be entirely or partly out of sight, but head is hidden; *Fleeing* involved individuals swimming away from previous position; and *Tail-kick* was a rapid tail flap, generally as a result of startling. These reactions to the drone were observed at three different aquarium facilities during experiments and/or confirmed during the review of videos. Manatees responded to the drone in three of the seven flights (43% of total), including the flight over two adjacent pools. Of the 25 manatees exposed to overhead drone flight, at least 11 (44%) displayed visible reactions.

Most reactions (e.g. *fleeing*, *grouping*) started within the first 2 minutes of the flight and continued throughout the flight,

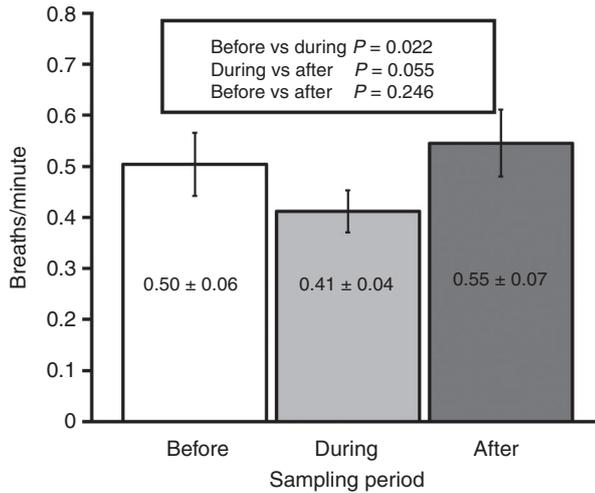


Fig. 5. Average respiration rate of individual Antillean manatees ($n = 18$) and standard error for sampling time periods: before, during and after drone flight. Results of the Wilcoxon matched pair tests between sampling periods are shown. Respiration rate was significantly lower 'during' the flights compared with the 'before' sampling period ($P = 0.022$). No differences were found between during and after ($P = 0.055$) nor between before and after ($P = 0.246$).

making it infeasible to examine the disturbance effects of drone flight at a specific altitude owing to the responses that occurred. Lower flight altitudes did not elicit more responses than did higher flight altitudes (Fig. 6, Table 2).

Fleeing was observed in one manatee in pool G, where at the beginning of test (flight altitude of 100 m), the male 'Mach' fled from its previous position and remained in the opposite end of the enclosure during the flight. After the flight ended, 'Mach' returned to his previous position. *Tail-kick* was observed in the same facility during the drone take-off, but the responding individual was not identified. *Take cover* was observed in two manatees in pool E, where females 'Tunich' and 'Nikté' went under structures (platform and stairs) unused by the animals in the sampling period prior the flight. This behaviour lasted less than a minute and was performed by the females on at least five occasions during the drone flight. *Grouping* was observed in pool A, during which all individuals left their previous positions and aggregated in close proximity to each other at the beginning of flight (at a flight altitude of 100 m). During the flight, the group separated into two subgroups, which regrouped three times at flight altitudes of 40 m, 20 m and 10 m. During this process, the male 'Pablo' circled the group(s) multiple times. When the drone was closer to the water surface at a flight altitude of 5 m, the subgroups grew separated.

Discussion

Our experimental design allowed us to compare the behaviour of captive Antillean manatees before, during and after drone flights. Ground behavioural observations prior launching the drone ('before') were critical to ensure that manatees were not evidently stressed before the experiment, and therefore, that the behaviours suggesting stress observed during and after flight were likely to have been triggered by the presence of the drone.

Hence, in the present study, we demonstrated that drones have the potential to affect manatee behaviour and cause shifts in their behavioural activity and physiological responses such as their respiration rate. Manatee respiration rate is reported to increase with the activity level (Hartman 1979); thus, we expected that the increased activity during drone flights would correlate with an increased respiration rate. In contrast, the average respiration rate of manatees decreased during drone flights. Decreased respiration rates may represent a stress response to a negative stimulus. For example, during the capture and handling of wild manatees and dugongs, animals can enter apnoea or decrease their respiration rate (Lanyon *et al.* 2010; Wong *et al.* 2012). Additionally, the manatees in the present study decreased their time at the surface during and after drone flights. Reduced blow rates and time at the surface are documented as an avoidance strategy on fin whales (*Balaenoptera physalus*) responding to watercraft (Jahoda *et al.* 2003). In manatees, this avoidance response may result in animals spending extended periods of time hidden underwater from a source of disturbance, causing decreases in respiration rate.

Changes in manatee activity budgets resulted in increased energetic expenditure (more time spent in locomotion) during drone flights. This effect lasted for at least 15 min after exposure. In contrast, average respiration rates after the drone flight did not differ from those before flight. This suggests that drones can elicit physiological changes, but these changes may have shorter recovery times than do behavioural changes of manatees. These findings closely parallel those of Ditmer *et al.* (2015) who reported median recovery times of less than 16 min for the increased heart rates of black bears (*Ursus americanus*) in response to drone flight.

Most of the manatee reactions reported during our drone flights (*fleeing*, *grouping*, *take cover*, *tail-kick*) involved avoidance and the seeking of refuge following disturbance. The exception is *tail-kick*, which seems to be a typical reaction of manatees when startled, a movement facilitating propulsion before fleeing (Hartman 1979). *Fleeing* and *tail-kick* were the primary reaction behaviours observed by Ramos *et al.* (2018) in wild manatees exposed to a multirotor drone. *Grouping* and *take cover* behaviours are probably reactions influenced by the presence of other individuals and/or the characteristics of their enclosure. Particularly, *take cover* is a response that depends on the presence of structures in the manatee pools. However, regardless of the environmental characteristics, this reaction suggested that manatees try to avoid a disturbance by seeking refuge (Nowacek *et al.* 2004; Ramos *et al.* 2018). In natural habitats, this behaviour may involve diving into deeper areas or using submerged vegetation so as to evade other manatees or boats (Hartman 1979). *Grouping* could have been influenced by the enclosure size and the manatees' inability to flee from it, thus associating in a safer space, seeking refuge. In a sense, the reaction of grouping involves fleeing previous positions and associating, likely as threat avoidance.

Studies of sources of disturbance for manatees have primarily focussed on the effects of motorised watercraft as the primary anthropogenic stressor to manatees. Responses of manatees to boat disturbance include increasing their swimming speed and orienting to deep waters (Nowacek *et al.* 2004) and an increased variability in respiration rates during vessel approaches (Miks-

Grouping



Fleeing



Take cover

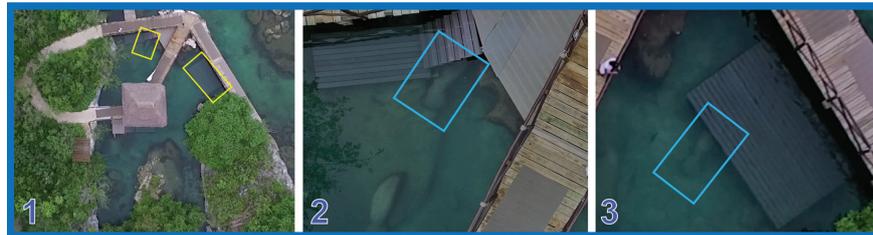


Fig. 6. Aerial imagery of the reactions of captive Antillean manatees to the small drone (DJI Phantom 3). Blue squares point to manatees' position. Grouping: Panel 1 shows manatee's starting to leave the previous area towards reference object and grouping (recording at 1:00 min), panel 2 shows manatees still grouping near reference object around middle of flight (recording at 7:52 min), panel 3 shows in detail the 8 manatees near reference object. On a yellow square reference object. Fleeing: From 1 to 3, sequence of positions of the manatee during the first 150 s of recording, panel 3 shows the position the individual occupied during the rest of drone flight. Take cover: Panel 1 shows yellow squares framing panel 2 and 3 positions within pool, panel 2 shows manatees under stairs and panel 3 shows manatee under platform.

Table 2. Individual manatee reactions to drone in relation to flight altitude

Transitions involved steady vertical descents to the next lower altitude. The unknown manatee could not be identified to an individual. X represents a response behaviour detected in each listed manatee. Age Class: A, adult; C, calf; J, juvenile. T, transition

Manatee name	Sex	Age class	Pool	Reaction	Flight altitude (m)									
					100	T	40	T	20	T	10	T	5	
Julieta	F	A	A	Grouping	X		X		X		X			
Dorothy	F	A	A	Grouping	X		X		X		X			
Conchis	F	J	A	Grouping	X		X		X		X			
Claudia	F	J	A	Grouping	X		X		X		X			
Bombon	F	C	A	Grouping	X		X		X		X			
Pablo	M	A	A	Grouping	X		X		X		X			
Quijote	M	A	A	Grouping	X		X		X		X			
Nohoch	M	C	A	Grouping	X		X		X		X			
Tunich	F	J	E	Take cover		X						X		
Nikté	F	J	E	Take cover			X	X						X
Mach	M	A	G	Fleeing	X									
Unknown	M	A	G or H	Tail kick	^A									

^AThe individual that tail-kicked during drone take-off could not be identified.

Olds *et al.* 2007). The responses displayed by manatees in our experiment can be interpreted as stress responses, and agree with observations on wild manatee behaviour under situations of disturbance such as boat approaches (Nowacek *et al.* 2004; Miksis-Olds *et al.* 2007), human presence (Abernathy 1995) and drone exposures (Ramos *et al.* 2018).

Some of the manatees did not visibly react to the drone exposure. This was also reported by Ramos *et al.* (2018) who detected visible manatee responses to drone on 24% of all exposures. Moreover, they noticed differences in responsiveness of individuals during repeated drone exposures. This highlights that the individual response can be influenced by an array of factors, including a manatee's personality and life experience, and how manatees differ across individuals (Sorice *et al.* 2003; Ramos *et al.* 2018). Furthermore, animals of the same species may respond to drones on different ways depending on their age, sex, and biological state (e.g. breeding; Pomeroy *et al.* 2015).

The absence of visible behavioural reactions in manatees does not rule out an effect over the animals: differences in activity budgets and respiratory rates with and without the presence of drone are also relevant evidences of the drone's effect. The captive manatees with which we conducted our experiments are individuals under constant monitoring and sometimes trained to interact with visitors, so their personalities are well known by their trainers. The animal caretakers noticed that some individuals were 'distracted' and 'uncooperative' to instructions after drone flights. Although these observations are anecdotal, they support the fact that some effects of disturbance are not visible or may not be easily measurable using short-term behavioural observations.

The ability to detect the visual and acoustic disturbance of an aerial drone, and the degree to which these are associated with a threatening stimulus (e.g. predator), is related to a given species disturbance threshold (Bevan *et al.* 2018). Manatees do not have any natural predators and there are no reports of manatees being attacked or harassed by flying animals such as seabirds. Thus, it is possible that the sound of the approaching drone is the main source of disturbance to manatees.

The noise perceived by manatees and the disturbance level associated can be influenced by physical factors. Environmental noise has the potential for masking drone noise (Christiansen *et al.* 2016) and it is likely that it was variable among experiment facilities (e.g. mechanical noise from nearby buildings). Additionally, the received noise levels underwater from an overhead aircraft typically decrease as aircraft flight altitude increases. The reception of the sound also depends on the orientation and position of the animal in relation to the source because physical factors from the environment can cause sound interference (Erbe *et al.* 2017). Finally, because of the structure of facilities we conducted our work at, in most cases, the drone was launched near manatee enclosures. The *tail-kick* reaction was reported during a drone take-off, when drones are typically louder (Arona *et al.* 2018). Interestingly, this response occurred when the drone was in audible range but not visible to the manatee, thus, strongly pointing to the drone's noise as the disturbance stimulus. *Grouping* and *fleeing* also occurred during the first 2 min of the flight recording. This suggests that some individuals were already aware of the drone and modified their behaviour from its launch or when the drone was at 100 m above them.

Manatee responses to take-off operations made it infeasible to isolate an effect of flight altitude on manatee reactions, but indicated that the noise of aircraft rotors represents a strong negative stimulus to manatees. Ramos *et al.* (2018) reported that lower flight altitudes increased the likelihood of disturbance in wild Antillean manatees exposed to drones. However, in some cases, animals continued to respond to the aircraft at >100 m. The reactions of manatees to the sound of drone take-off in our study indicated that increasing the distance of the launch site to the animals is a key consideration to reduce possible impacts and tests for responses.

Manatee behaviours we documented in response to the drone could have been influenced by the presence of the other individuals within the enclosure. For example, one startled animal could have driven the *Grouping* response observed in multiple individuals. However, this was not always the case, because in one response, *fleeing* was observed only on one of the two manatees sharing the same pool. This is similar to reports by Ramos *et al.* (2018) who observed multiple manatees in one group flee in response to drone flight, whereas in other groups, only one animal responded. Individual experiences and personalities are likely to influence the response we observed, and future experiments conducted with isolated individuals would be needed to determine whether there are group effects in manatee responses to drones.

Recommendations for using drones with manatees

Our study has provided evidence of small drones causing disturbance in Antillean manatees, by eliciting behavioural and physiological responses (i.e. change in respiration rate), likely signalling temporary stress. It is important to balance the quality and type of data needed with the potential level of disturbance inflicted (Bevan *et al.* 2018).

Visible reactions to drone flight, such as those observed during our experiments (e.g. tail-lick, fleeing), can be used as an indicator of stress. These indicators can lead the operator to suspend, redirect or continue the approach on a case-by-case basis. These measures may be feasible when collecting data for photogrammetry, photo-ID and behaviour studies, in which the operator manually controls the drone and is able to observe the manatee's reactions in real time. Precautionary principles should be adopted for individuals sensitive to disturbance impacts, such as mother-calf pairs, because they could separate during fleeing. For detection and density estimation studies, the high flight altitudes in which drones typically operate (>100 m) should prevent the disturbance of individuals (Ramos *et al.* 2018; Raoult *et al.* 2020).

Behavioural studies of manatees using drones should consider the potential bias owing to the effect of the method on manatee behaviour. For example, studies aiming to assess behavioural budgets would not be recommended because of important potential effects of the drone on activity rates. The increases in manatee activity levels observed after drone flights should be considered when recording further data after flights. Including no-fly periods (30–60 min) after each drone flight could help minimise biased assessments when recording behaviour-related information (e.g. bioacoustics, ground observations) after these flights. Also, limiting the number of low flights per day on the study area should reduce the possible

pressures on targeted individuals. Future studies should perform additional drone-exposure experiments to obtain more information regarding the after-effects and recovery times of manatees exposed to drones.

The manatees in our study exhibited physiological responses to the drone, with changes in their respiration rate. Thus, we advise caution when using drones for precise data recording of manatee respiration rates unless associated information such as surface behavioural observations or animal-borne tag data is available to identify changes in respiration rate related to drone operations. If control data are available, respiration rates during drone operations could be monitored to detect stress in manatees.

The behavioural and physiological responses we detected in captive manatees in response to small drones support the validity of previous recommendations for the use of drones in wildlife research (Hodgson and Koh 2016; Mulero-Pázmány *et al.* 2017) and emphasises the need for careful guidance in their use with manatees. An important first step is the selection of smaller and quieter drones with suitable data-capture capabilities (e.g. DJI Mavic Pro) to minimise visual and auditory stimuli (Hodgson and Koh 2016). New commercial drone models are emerging regularly with a smaller visual and acoustic profile than in previous models, likely reducing the probability of manatees detecting the aircraft. Data acquisition needs and preliminary assessments of the disturbance levels caused by different systems should be considered during the conceptual stages of a study, at each study site and with each study population (Fettermann *et al.* 2019). For example, testing behavioural responses of the same and multiple species with different types of drones is important because each may differ in their level of noise output (Erbe *et al.* 2017). In general, pilots should fly drones at the highest altitudes feasible for obtaining sufficient-quality data (Ramos *et al.* 2018) and permitted according to local law. Aircrafts should not directly approach animals in vertical descents and target-oriented flight patterns (Mulero-Pázmány *et al.* 2017; Ramos *et al.* 2018). Finally, minimum distances for a launch site should be implemented because multirotor aircrafts are louder during take-off (Brisson-Curadeau *et al.* 2017). The manatee responses we observed at take-off and within minutes of the first flight position at 100 m suggest that the drone operators should not launch aircrafts near manatees.

In conclusion, our results have provided support for previous findings about manatee reactions to drones, demonstrating responsiveness to drone flights in the species, and present additional evidence for the behavioural and physiological responses of manatees to overhead drone flights. Further studies are needed to better understand the physiological responses of manatees to drones and the effect of other sources of behavioural differences, including manatee group interactions, age, sex, personality and habituation.

Conflicts of interest

The authors declare no conflicts of interest.

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Isotopic composition of aquatic and semiaquatic plants from the Mexican Caribbean: A baseline for regional ecological studies

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ABSTRACT

Primary producers in the aquatic community structure are fundamental elements because they are the first link in the trophic network as occur in most ecosystems. They produce oxygen and biomass, act as a shelter for several species, and provide food for a wide variety of megaherbivore species like manatees and sea turtles. Physico-chemical processes taken place in primary producers can be determined through stable isotope analysis (SIA) as natural tracers from elements like carbon and nitrogen, applied in ecological, and physiological studies. In the Mexican Caribbean ecosystems, SIA has been little applied in aquatic plants, where *Thalassia testudinum* is the main seagrass species studied. Here, we present the isotopic composition ($\delta^{13}\text{C}$ and $\delta^{15}\text{N}$) from 95 aquatic and semiaquatic plant species of four vegetation types, from three different environments, hydroclimatic seasons, and geographical zones in the Mexican Caribbean. Main statistical differences in $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ were found according to vegetation type and environment. Besides, for $\delta^{13}\text{C}$ were also found statistical differences among seasons, while for $\delta^{15}\text{N}$ differences were found among zones (H-Test, $p < 0.05$). This study provides an isotopic baseline for further ecological studies in the region. This information can contribute to understanding the structure of aquatic food webs and infer the diet and feedings habits of aquatic species, as well as to detect possible changes related to anthropogenic activities that can affect the survival of these plant species, and the fauna depending on them.

1. Introduction

Aquatic environments along the coastline of the Mexican Caribbean harbor a great diversity of aquatic and semiaquatic plant species. There are more than 540 types of macroalgae (Aguilar Rosas et al., 1998; González-Solis et al., 2018; Vilchis et al., 2018), seven varieties of seagrasses (Rioja-Nieto et al., 2019), and four mangrove species (Agraz-Hernández et al., 2006), among others. Macroalgae, seagrasses and

mangroves provide important benefits to ecosystems because they are photosynthetic organisms, and therefore, key elements in the ocean community structure. They are often the first link in the trophic chain of aquatic ecosystems as they produce oxygen and biomass, provide shelter for several animal species, and represent a food source for many others (León Álvarez et al., 2012). Macroalgae are considered good aquatic bioindicators since they fully depend on water and substratum to complete their physiological processes. Thus, any disturbance of physical and

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chemical characteristics of the environment leads to changes in their composition, abundance, and diversity (Valdez-Cruz et al., 2015). Seagrasses and mangroves play a significant ecological role in coastal marine zones, because they are an important source of carbon in the detritus cycle (Duarte et al., 2007). Furthermore, they help to mitigate impacts from natural phenomena like hurricanes by providing a natural protective barrier for the coastline, reducing the effects of erosion and catching and storing nutrients (Hemminga and Duarte, 2000; Torres and Rivera, 1989). These ecosystems act as habitat and breeding sites for fishes and invertebrates (Conabio, 2009; Green and Short, 2003) and provide food for a wide variety of megaherbivores (Castelblanco-Martínez et al., 2009; Green and Short, 2003).

Stable isotope analysis (SIA) applied to aquatic plants can provide knowledge about ecological changes in primary producers and modification of natural sources in a food web which could have significant impacts on herbivorous animals (Ehleringer and Cerling, 2002), like manatees (Alves-Stanley et al., 2010) and sea urchins (Cabanillas-Terán et al., 2019). Isotopes are natural tracers of physicochemical processes and application of SIA has mainly focused on ecological, physiological and paleontological studies (Clementz et al., 2007; Guerrero and Berlanga, 2000; Newsome et al., 2010). For the present study, we analyzed $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ in four aquatic and semiaquatic vegetation types (seagrasses, macroalgae, mangroves and "other vascular plants"). $\delta^{13}\text{C}$ of primary producers provide information about the way plants adjust their metabolism during the gaseous exchange, identify resource strategies (Farquhar et al., 1989), and infer the efficiency of water use during photosynthesis (Farquhar et al., 1982). Differences among primary producers are predicted by the photosynthetic pathway. For

instance, $\delta^{13}\text{C}$ values of C_4 plants are less impoverished in ^{13}C (Peterson and Fry, 1987), range from -20‰ to -10‰ , whereas $\delta^{13}\text{C}$ in C_3 plants, usually fluctuates between -33‰ and -22‰ (Bender, 1971). Moreover, $\delta^{15}\text{N}$ is useful to identify biogeochemical processes and nitrogen sources of the trophic net (Kohl et al., 1973), which can be affected by natural events (e.g. phytoplankton bloom, denitrification and nitrification processes, etc), or anthropogenic factors (e.g. domestic or industrial wastewater discharges) (Peterson, 1999). The $\delta^{15}\text{N}$ values on plant tissues vary between -5‰ and $+10\text{‰}$ (Mariotti, 1983). Nitrogen isotopic fractionation in plants occurs as a result of nitrate (NO_3^-) or ammonium (NH_4^+) assimilation, translocation toward the leaves, and nitrogen metabolism in the cytoplasm (Mariotti et al., 1980).

The most common aquatic plant species studied using SIA are seagrasses and macroalgae (Ciotti, 2012; Fry, 1984; Loneragan et al., 1997; Reich and Worthy, 2006). *Thalassia testudinum* is the most studied species in the Mexican Caribbean (Camacho-Cruz et al., 2019; Carruthers et al., 2005; Mutchler et al., 2007, 2010; Rodríguez Juárez, 2011; Sánchez et al., 2013). The aim of this research was to analyze $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values of aquatic and semiaquatic plants collected in the Mexican Caribbean, in order to identify possible isotopic differences among vegetation type (seagrasses, macroalgae, mangroves, and "other vascular plants"), environment (marine, estuarine and freshwater), geographic distribution (north, center, and south zone), and hydroclimatic season (rainy, dry, and cold-fronts). Results will provide isotopic baseline information for 95 aquatic and semiaquatic plant species along the Mexican Caribbean coast, which can be used for further ecological research in the region.

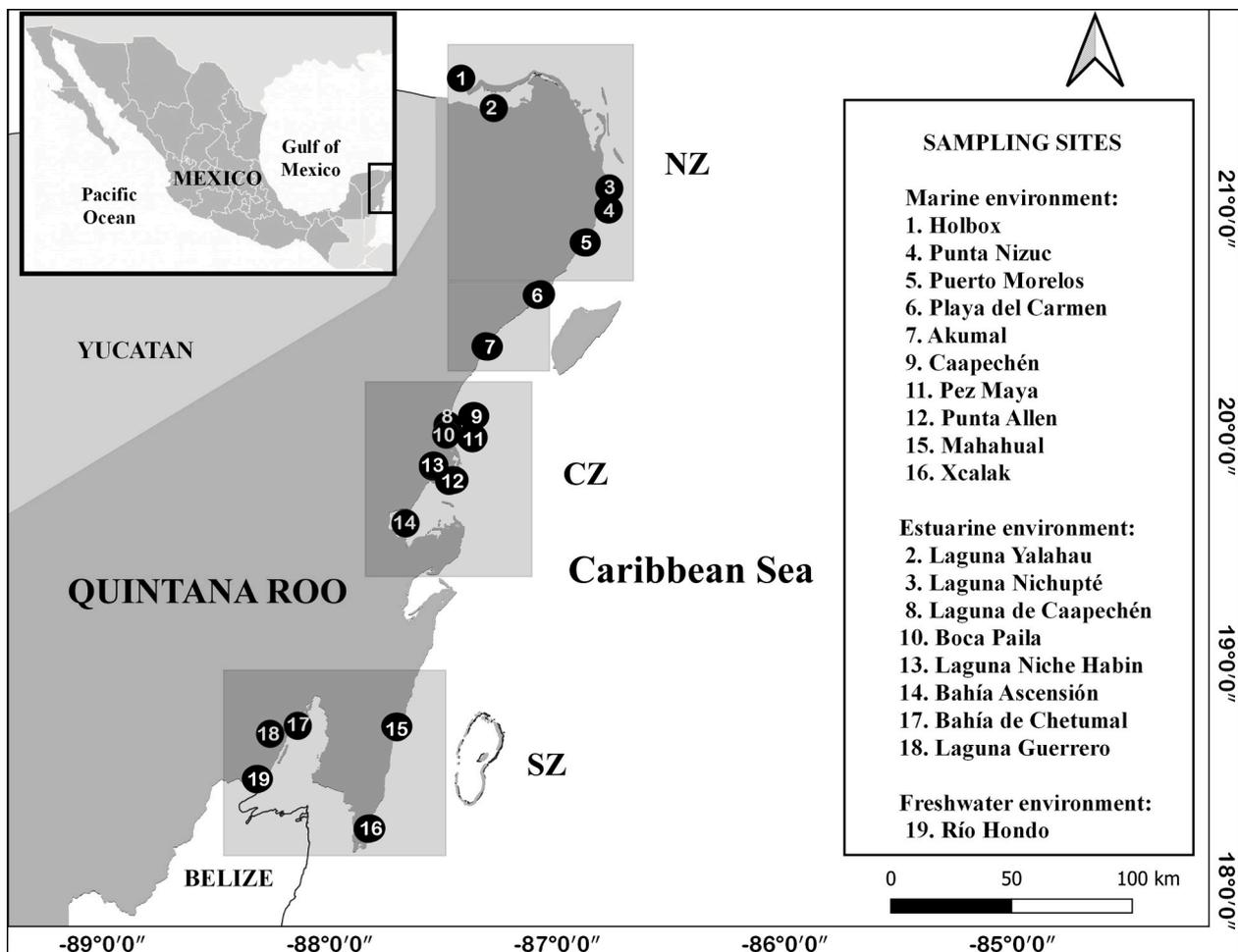


Fig. 1. Sampling sites along the Mexican Caribbean where aquatic and semiaquatic plants were collected. NZ = north zone; CZ = center zone; SZ = south zone.

2. Materials & methods

2.1. Study area

The Mexican Caribbean is located in the state of Quintana Roo, which is delimited to the north by Holbox Island (21° 31' N; 87° 23' W), and to the south by Chetumal Bay (17°52'-18°50'N, 87°50'-88°25'W) (Fig. 1). This region has a coastal system of karstic origin, including a great heterogeneity, high infiltration rate, and fast flux, making it a vulnerable ecosystem (Aranda-Cirerol et al., 2011; Bakalowicz, 2005). In addition, it belongs to the second most important coral reef barrier worldwide, the megadiverse Mesoamerican Barrier Reef System (Aguilar et al., 2008b). One of the most important vegetation communities in the coastal zone are mangroves, which typically border lagoons, estuaries and bays (De la Lanza Espino, 2004). The predominant climate in this region is warm and subhumid, with an annual mean temperature of 26 °C (De La Lanza Espino et al., 2013). The Mexican Caribbean has three different seasons (rainy, wet, and cold-fronts), and has frequent tropical storms and hurricanes (Carrillo et al., 2009). Three important bays exist in Quintana Roo: Ascensión, Espíritu Santo, and Chetumal (De La Lanza Espino et al., 2013). The Hondo river is the only superficial river in the State, which flows into Chetumal Bay (Herrera-Sansores and Heredia-Escobedo, 2011).

2.2. Aquatic plants sampling

From July 2017 to May 2018, samples of aquatic and semiaquatic plants were manually collected using freediving equipment in shallow waters (depth < 3 m) (Caricomp, 2001). The sampling was conducted along to the Mexican Caribbean coast, from the northernmost point at Holbox, to Chetumal Bay south of Quintana Roo. Three environments were covered (marine, estuarine and freshwater), during three hydroclimatic seasons (rainy, dry, and cold-fronts). The region was divided into three zones (north, center and south) according to the distribution of several aquatic and semiaquatic plants reported as food items for manatees in the study area (Castelblanco-Martínez et al., 2009; Espinoza-Avalos, 1996; Flores-Cascante et al., 2013), as well as based on previous studies related to manatee habitat use (Morales-Vela and Olivera-Gómez, 1997; Morales-Vela and Padilla-Saldívar, 2009). Moreover, 19 randomly selected sampling sites were defined (Fig. 1), according to SIA studies applied in aquatic plants from the Mexican Caribbean (Carruthers et al., 2005; Mutchler et al., 2007, 2010; Sánchez et al., 2013), and principal manatee habitats (coastal lagoons, estuaries, and rivers) (Morales-Vela and Padilla-Saldívar, 2001). In most cases, three specimens of each morphotype were collected and stored in labeled paper bags to keep them dry for further morphological identification and SIA. At the laboratory of Ecology and Molecular Biology of Quintana Roo University (UQROO), vegetation specimens were identified when able to species level using common keys (Agraz-Hernández et al., 2006; Guterres et al., 2008; Littler and Littler, 2000; Van Tussenbroek et al., 2010), stereoscopic and compound Olympus microscopes. The collected samples were grouped according to vegetation type in four groups: seagrasses, macroalgae, mangroves, and “other vascular plants”.

2.3. Sample preparation for SIA

Aquatic and semiaquatic plant samples were rinsed with distilled water. A piece of leaf was cut and submerged into hydrochloric acid (10%) to eliminate associated carbonates, followed by a second distilled water rinsing process. Each sample was then oven-dried at 60 °C for 24 h. Samples were grained in an agate mortar until a fine powder was obtained (Sánchez et al., 2013), and subsequently stored into 2 ml vials. Approximately 2 mg of powder per sample were encapsulated into micro tin cups. Later, nitrogen and carbon isotopic composition of samples was analyzed in a Carlo Erba NC1500 elemental analyzer coupled with a Delta Plus XP (ThermoQuest, Bremen, Germany) mass spectrometer

(EA-IRMS). Isotope measurements were carried out at the Stable Isotope Laboratory of the Instituto Andaluz de Ciencias de la Tierra (CSIC-UGR, Granada, España).

2.4. Data analysis

Stable isotope ratios were expressed as δ (delta) in parts per thousand (‰), using the equation:

$$\delta X = (R_{\text{sample}}/R_{\text{standard}} - 1) \times 1000 \quad (1)$$

where X is ^{13}C or ^{15}N , R_{sample} and R_{standard} represent the $^{13}\text{C}/^{12}\text{C}$ or $^{15}\text{N}/^{14}\text{N}$ ratio of the sample and standard, respectively. The precision of the measurement was calculated after correction of the mass spectrometer daily drift. The analytical error for the $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ determinations was <0.1‰. The reference standard for reporting $\delta^{13}\text{C}$ carbon measurements was Vienna Pee Dee Belemnite (V-PDB) and for $\delta^{15}\text{N}$ the atmospheric nitrogen (AIR). For carbon, 22 internal standards (organic and inorganic material) ranging between -49.44 and +28.59‰ (V-PDB), contrasted with the IAEA international references NBS-28, NBS-29, NBS-20 (carbonates) and NBS-22, IAEA-CH-7, IAEA-CH-6 (organic material), are used in relation to the isotopic range of samples to be analyzed. This study comprised two internal standards of -30.63‰ and -11.65‰ (V-PDB). For nitrogen, nine internal standards (organic and inorganic material) ranging between -1.94 and +16.01‰ (AIR) served as contrasts to the IAEA international references IAEA-N-1, IAEA-N-2, IAEA-NO-3, USGS32, USGS34 and USGS35. This study also resorted to the two internal standards of -1.02‰ and +16.01‰ (AIR).

2.5. Statistical analysis

Data were tested for normality using the Shapiro-Wilk test, as well as Levene's test of homogeneity of variances, in order to find the proper test to determine differences in populations' distribution (Zar, 2010). Since the variables $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ did not present a normal distribution ($W p = 0.00$ and $p = 0.01$, respectively) the non-parametric Kruskal-Wallis test using the H statistic (Zar, 2010) was performed for each factor: vegetation type, environment, zone, and season. A post-hoc Dunn test with Bonferroni correction was used to determine statistical differences among levels into each group. Regarding zones, “other vascular plants” and freshwater samples were excluded from the statistical analysis, because these samples were only collected in the south zone (SZ). A descriptive analysis from the samples was performed with violin graphics in order to represent the distribution of the sample data. We used the mean as a measure of central tendency in order to describe and compare the data with other studies. All statistical analyses were performed using basic functions from R language (R Core Team (2019), and a $p < 0.05$ was considered statistically significant in all cases.

3. Results

A total of 560 samples of aquatic and semiaquatic plants were collected, including 73 algal taxa, five seagrass species, three mangrove species, and 14 taxa of “other vascular plants”, along the Mexican Caribbean (Appendix 1). Stable isotope analysis revealed that seagrasses contained the lesser negative values of $\delta^{13}\text{C}$, while “other vascular plants” contained the most negative values. On the other hand, “other vascular plants” had the highest values of $\delta^{15}\text{N}$, while seagrasses had the lowest values. When comparing environments, the marine environment had the lesser negative $\delta^{13}\text{C}$ values while freshwater environments had the highest $\delta^{15}\text{N}$ values. Stable isotope ratios also differ according to season and geographic distribution. Rainy season had less -negative $\delta^{13}\text{C}$ values than dry and cold-fronts, and displayed the lowest $\delta^{15}\text{N}$ values, when compared to the center and north zone (Table 1).

Table 1
 $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values according to vegetation type, environment, hydroclimatic season and geographical distribution.

Vegetation type	$\delta^{13}\text{C}$ (‰)			$\delta^{15}\text{N}$ (‰)		
	Mean \pm SE	Min	Max	Mean \pm SE	Min	Max
Macroalgae (n = 351)	-16.67 \pm 0.20	-29.64	-6.92	3.85 \pm 0.18	2.46	10.33
Seagrass (n = 130)	-10.65 \pm 0.33	-21.79	-2.31	1.85 \pm 0.28	-7.89	10.63
Mangrove (n = 37)	-28.63 \pm 0.24	-31.29	-25.75	3.35 \pm 0.59	-2.55	9.06
Other vascular plants (n = 42)	-29.90 \pm 0.48	-40.17	-21.72	4.05 \pm 0.48	-3.44	9.06
Environment						
Marine (n = 318)	-14.36 \pm 0.26	-29.91	-2.31	3.01 \pm 0.15	-4.21	12.46
Estuarine (n = 184)	-18.28 \pm 0.44	-30.70	-6.11	3.27 \pm 0.32	-7.89	14.24
Freshwater (n = 58)	-27.99 \pm 0.70	-40.17	-14.16	5.53 \pm 0.45	-3.44	13.37
Hydroclimatic season						
Rainy (n = 354)	-16.05 \pm 3.14	-31.61	-2.97	3.30 \pm 1.87	-6.80	14.24
Dry (n = 134)	-18.18 \pm 0.61	-34.60	-2.31	3.61 \pm 0.32	4.03	7.26
Cold-fronts (n = 72)	-20.02 \pm 1.00	-40.17	-5.41	3.24 \pm 0.37	-2.95	10.63
Geographical distribution						
North zone (n = 243)	-15.95 \pm 0.33	-29.91	-2.97	3.24 \pm 0.23	-7.89	14.24
Center zone (n = 105)	-15.31 \pm 0.55	-30.70	-6.11	3.64 \pm 0.31	-4.55	13.98
South zone (n = 149)	-19.23 \pm 0.54	-40.17	-2.31	2.61 \pm 0.26	-5.71	12.46

3.1. Changes in $\delta^{13}\text{C}$ y $\delta^{15}\text{N}$ from the aquatic and semiaquatic plants

Bulk sample $\delta^{13}\text{C}$ values ranged from -40.17‰ to -2.31‰ with an average of -17.07‰ and a median of -16.01‰ . Statistical differences were found among vegetation type ($H = 319.22$, $df = 3$, $p < 0.00$), environment ($H = 161.19$, $df = 2$, $p < 0.00$), and season ($H = 16.84$, $df = 2$, $p = 0.00$) (Fig. 2). On the other hand, the $\delta^{15}\text{N}$ values fluctuated between -7.89‰ and 14.24‰ , with an average of 3.37‰ , and a median of 3.14‰ . Statistical differences were found among vegetation type ($H = 32.70$, $df = 3$, $p = 0.00$), environment ($H = 27.03$, $df = 2$, $p = 0.00$), and zone ($H = 13.08$, $df = 2$, $p = 0.00$) (Fig. 3).

According to vegetation type (seagrasses, macroalgae, mangroves and "other vascular plants"), $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values are differentiated by three principal groups (Fig. 4). Significant statistical differences in $\delta^{13}\text{C}$ were between macroalgae ($-16.67 \pm 0.2\text{‰}$), and other vegetation types (seagrasses = $-10.65 \pm 0.33\text{‰}$, mangroves = $-28.63 \pm 0.24\text{‰}$, and "other vascular plants" = $-29.9 \pm 0.48\text{‰}$) ($p = 0.00$), and seagrasses vs. mangroves and "other vascular plants" ($p = 0.00$). For $\delta^{15}\text{N}$ values, statistical differences were obtained between seagrasses ($1.85 \pm 0.28\text{‰}$), and macroalgae ($3.85 \pm 0.18\text{‰}$), and "other vascular plants" ($4.05 \pm 0.48\text{‰}$) ($p = 0.00$) (Fig. 4).

On the other hand, significant statistical differences were found for $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values when compared among environments (Fig. 5). $\delta^{13}\text{C}$ values were different among environments (marine = $-14.36\text{‰} \pm 0.26\text{‰}$, estuarine = $-18.28\text{‰} \pm 0.44\text{‰}$ and freshwater = $-27.99\text{‰} \pm 0.7\text{‰}$) ($p = 0.00$). $\delta^{15}\text{N}$ values in freshwater ($5.53\text{‰} \pm 0.45\text{‰}$) was significantly different from the other two environments: marine ($3.01\text{‰} \pm 0.15\text{‰}$), and estuarine ($3.27\text{‰} \pm 0.32\text{‰}$) ($p = 0.00$) (Fig. 5).

4. Discussion

4.1. Changes in $\delta^{13}\text{C}$ of aquatic and semiaquatic plants

The $\delta^{13}\text{C}$ results found among vegetation type, coincide with the values reported by previous studies (Ciotti, 2012; Fry, 1984, 2006; Fry and Sherr, 1989; Mutchler et al., 2010; Ramírez Palomeque, 2013), where the lesser negative $\delta^{13}\text{C}$ values belong to seagrasses ($-10.65 \pm 0.33\text{‰}$). While all aquatic plants follow C_3 photosynthesis pathways, our values seem to be closer to those of C_4 plants (Hemminga and Mateo, 1996). This can be due to a limitation of dissolved CO_2 in water, which results in a decrease of ^{13}C discrimination in the plant coupled with the use of bicarbonate (HCO_3^-) as a source of carbon (Anderson and Fourqurean, 2003; García-Sánchez et al., 2016). This characteristic distinguishes between marine and continental species (Berry, 1989). On the other hand, the most negative $\delta^{13}\text{C}$ values obtained for mangroves ($-28.63 \pm 0.24\text{‰}$), and "other vascular plants" ($-29.90 \pm 0.48\text{‰}$) are typical values of atmospheric CO_2 sources and photosynthesis type C_3 , and similar to reports in previous studies (Ciotti, 2012; Del Río Salas, 2014; Loneragan et al., 1997; Rao et al., 1994; Rodelli et al., 1984). Likewise, macroalgae usually present intermediate $\delta^{13}\text{C}$ values ($-16.67 \pm 0.2\text{‰}$) between seagrasses and the group formed by mangroves and "other vascular plants" (Ciotti, 2012; France, 1995; Loneragan et al., 1997; Mutchler et al., 2010). Variations in $\delta^{13}\text{C}$ are determined by the isotopic composition of the dissolved inorganic carbon (DIC). As DIC is assimilated (CO_2 or HCO_3^-), isotopic discrimination of the enzyme is responsible for the carbon fixation, and intracellular concentration of CO_2 or HCO_3^- (Farquhar et al., 1982; France, 1995; Keeley and Sandquist, 1992). Some types of aquatic vegetation use HCO_3^- (which is less negative in $\delta^{13}\text{C}$ values than CO_2 in approximately 8‰ at 25°C) in addition to dissolved CO_2 (Mook et al., 1974), and as a result, have $\delta^{13}\text{C}$ values between -11.03‰ and -21.4‰ (Maberly et al., 1992). This was observed in $\delta^{13}\text{C}$ values of seagrasses and macroalgae obtained in our study. Also, more negative $\delta^{13}\text{C}$ values are present in species for which their principal source of carbon is CO_2 , varying between -30‰ and -34.5‰ , (Maberly et al., 1992), which are similar to those obtained for the group of mangroves and "other vascular plants" in the present study.

Similar to other studies comparing $\delta^{13}\text{C}$ values plants of marine, estuarine and freshwater environments, freshwater samples showed the most negative $\delta^{13}\text{C}$ values (Alves-Stanley et al., 2010; Reich and Worthy, 2006). These $\delta^{13}\text{C}$ differences are principally attributed to intrinsic physicochemical and biological conditions of each environment (Mateo et al., 2004). In freshwater and estuarine environments, the pool of inorganic carbon is influenced by the input of carbon depleted in ^{13}C derived from organic matter decomposition (respiration) from land (Hemminga and Mateo, 1996). In general, seawater has higher $\delta^{13}\text{C}$ values of DIC, and as consequence, marine primary production is less negative than continental aquatic production (Fry, 2002). The current study found similar results, since marine vegetation had the less negative $\delta^{13}\text{C}$ values.

Significant differences found among hydroclimatic seasons (rainy, dry and cold-fronts) could be attributed to the increased freshwater inputs proceeding from runoff and groundwater during rainy season. Thus, less negative $\delta^{13}\text{C}$ values in primary production are expected in dry season compared to other two seasons since contributions of freshwater during the dry season from runoff or groundwater are scarce due to decrease in precipitation rates. Therefore, decrease in precipitation rates would contribute to higher isotopic values in the tissues of the plant grown in this season. In contrast, $\delta^{13}\text{C}$ values should be most negative during the rainy season as freshwater contribution leads to most negative DIC (more heterotrophic systems with abundant C_3 biomass respiration), an important nutrient and land organic matter input into water bodies. This can also be due to the increased influence of DIC depleted in ^{13}C which in turn originates more negative $\delta^{13}\text{C}$ values in aquatic and semiaquatic vegetation (Chanton and Lewis, 2002). Despite Quintana Roo is one of the Mexican states with highest

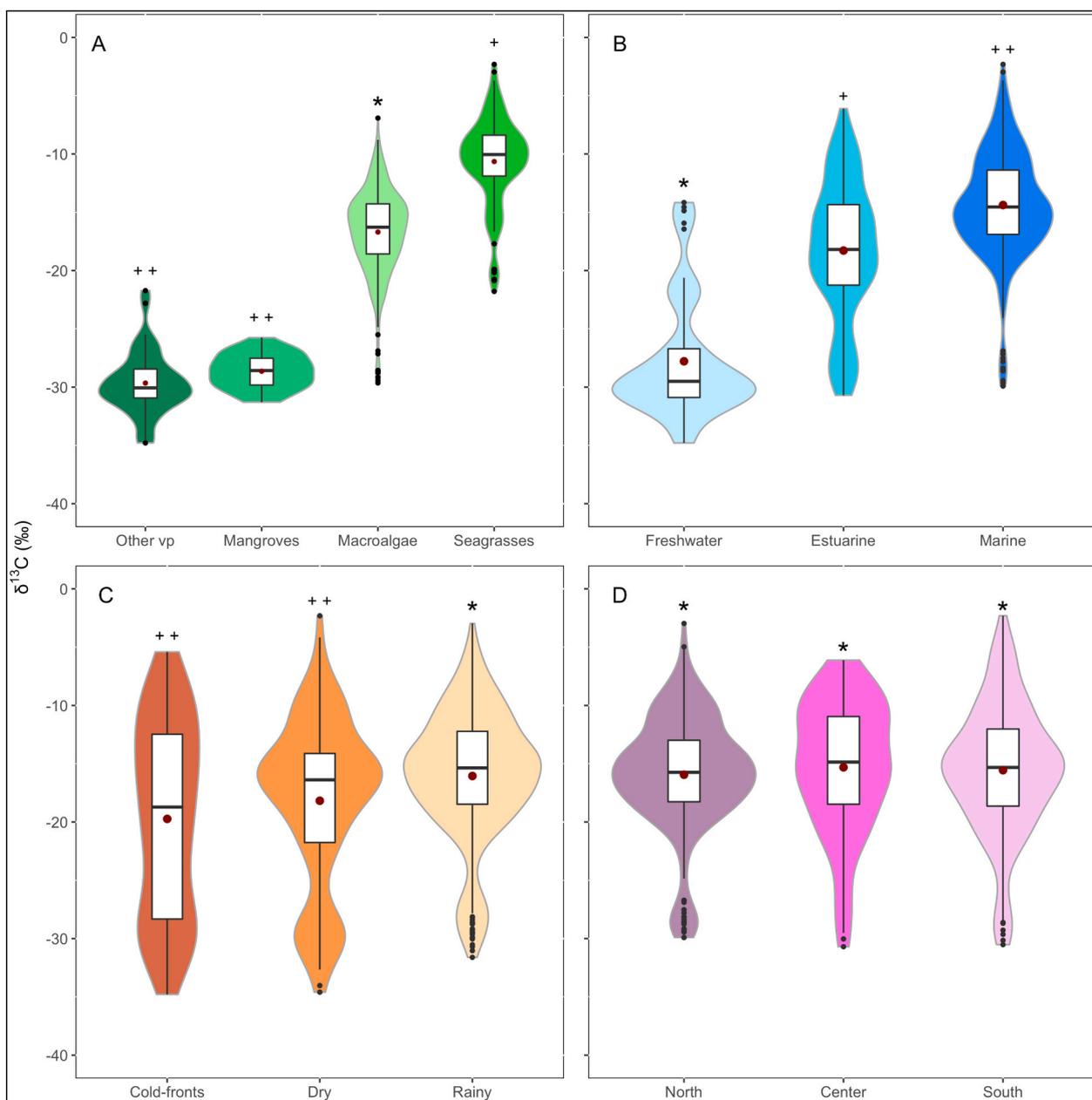


Fig. 2. Violin plots showing the variation of $\delta^{13}\text{C}$ values in aquatic and semiaquatic plants from the Mexican Caribbean according to several factors. The boxplot inside represent the three quartiles, with a median represented by a central horizontal line, and the mean is the red point. Significant differences among categories within each factor are shown with different symbol. **A.** Vegetation type; **B.** Environment; **C.** Hydroclimatic season; **D.** Zone. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

precipitation rates ($>1000 \text{ mm year}^{-1}$), there are no large rivers or streams, except for the Hondo river (Herrera-Sansores and Heredia-Escobedo, 2011). However, it is important to consider the karstic nature of the region which is characterized by underground drainage systems and very permeable soils (Aranda-Cirerol et al., 2011), as a result, the coastal areas receive important inputs of underground, dissolved inorganic and organic carbon.

As reported by other authors in other temperate places, they relate the changes in seasonal isotopic composition of seagrasses with temperature and light (Fourqurean et al., 2005). Also, with increasing temperatures, a decrease in solubility of CO_2 can lead to a smaller CO_2 pool and less isotopic discrimination with heavier $\delta^{13}\text{C}$ values which happens in marine plants (Francois et al., 1993). Otherwise, during cold-fronts, the temperature is lower compared to rainy and dry seasons. Lower temperatures are associated with less rain and less surface runoff

or underground contributions, sources characterized by a contribution of DIC with more negative values (Aguirre, 2002; De La Lanza Espino et al., 2013; Herrera-Sansores, 2011). Consequently, DIC will be less negative in $\delta^{13}\text{C}$ values as we found in the present study.

4.2. Changes in $\delta^{15}\text{N}$ of aquatic and semiaquatic plants

The differences founded in $\delta^{15}\text{N}$ values according to vegetation type provide evidence that the “other vascular plants” group showed the highest $\delta^{15}\text{N}$ values with an average of $4.05 \pm 0.48\text{‰}$. Our values are similar to results reported in several studies for some vascular plants like *Thypha* sp. ($4 \pm 3\text{‰}$), *Pontedeira crassipes* (4.1 ± 0.3 and $5.9 \pm 0.1\text{‰}$), and *Vallisneria americana* ($4.7 \pm 0.1\text{‰}$) (Reich and Worthy, 2006), *Cabomba* spp. (4.7‰), *Salvinia* spp. (3.3‰), *Coccoloba* spp. (3.3‰), and from Cyperacea family (3.2‰ and 4.6‰) (Carvalho Crema et al., 2019).

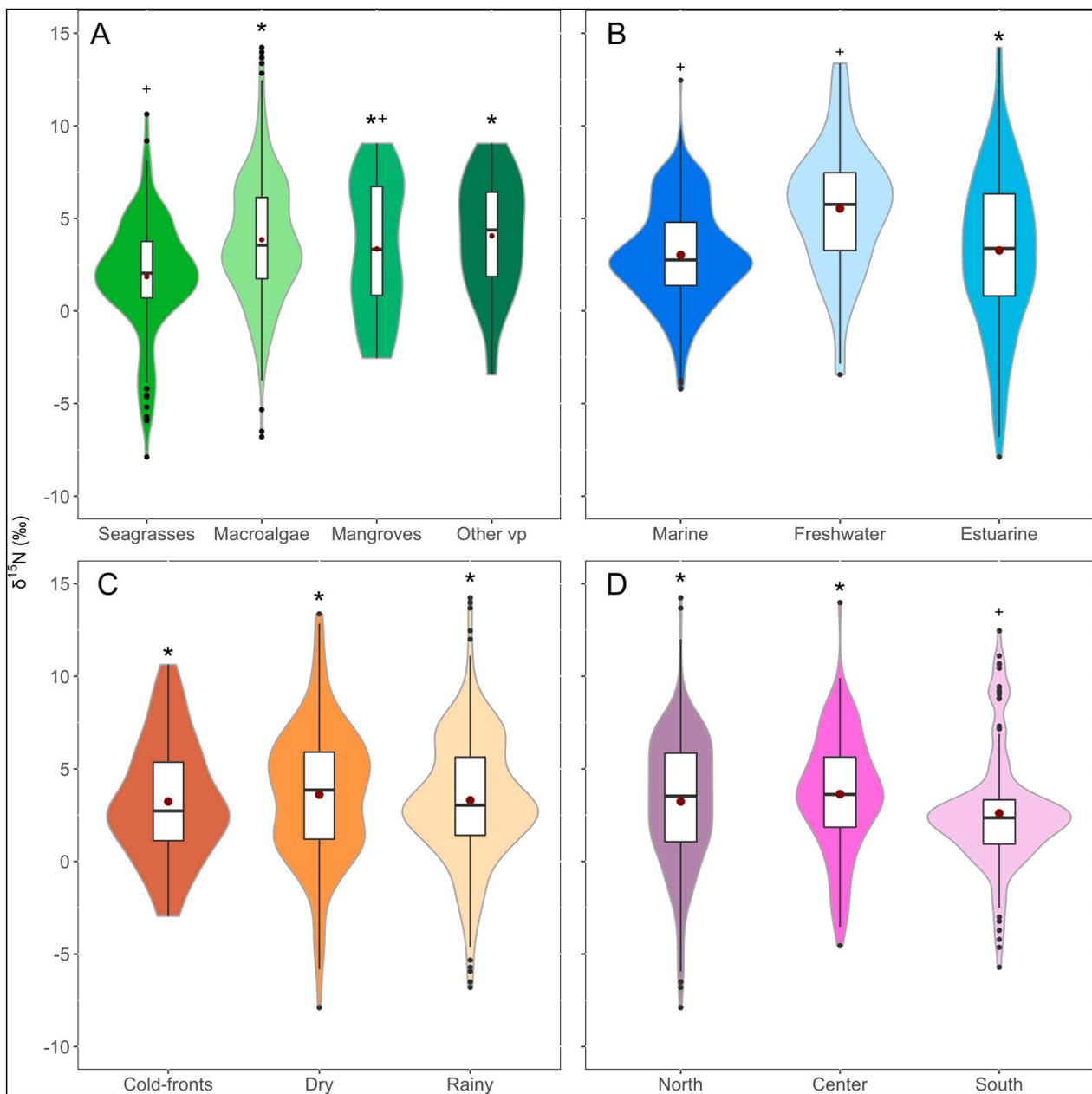


Fig. 3. Violin plots showing the variation of $\delta^{15}\text{N}$ values in aquatic and semi-aquatic plants from the Mexican Caribbean according to several factors. The boxplot inside represent the three quartiles, with a median represented by a central horizontal line, and the mean is the red point. Significant differences among categories within each factor are shown with different symbol. **A.** Vegetation type; **B.** Environment; **C.** Hydroclimatic season; **D.** Zone. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Moreover, the “seagrasses” group had the lowest $\delta^{15}\text{N}$ values with an average of $1.86 \pm 0.27\text{‰}$ which agree with previously reported values for this group (Alves-Stanley et al., 2010; Anderson and Fourqurean, 2003; Duarte et al., 2018; Mutchler et al., 2010; Reich and Worthly, 2006). Variations in $\delta^{15}\text{N}$ could be due to diverse factors, such as different metabolic pathways used for the nitrogen assimilation, changes in assimilated nitrogen compounds (nitrates, ammonium, etc.), and their concentration, changes in the relative activity from the nitrogen fixation, and bacterial denitrification (Handley and Raven, 1992). For instance, seagrasses incorporate nutrients from the water column and sediment, while macroalgae only depends on the surficial DIN (generally low in concentration and enriched in ^{15}N due to uptake processes) from the water column to supply their nitrogen requirements and are more enriched in ^{15}N in comparison to seagrasses (Dillon and Chanton, 2008; Kuramoto and Minagawa, 2001). A similar occurrence was reported in

the present study. Natural populations of macroalgae are usually enriched in ^{15}N relative to the baseline, and often utilize their N source completely, reflecting in their isotopic composition (Wada and Hattori, 1978). Since isotopic fractionation in primary producers are induced by N uptake processes (Kuramoto and Minagawa, 2001), and seagrasses incorporate nutrients from water column and sediment, it is possible that they have different strategies and speed for nutrient uptake and assimilation, preferring to incorporate it from the sediments than the water column, with a higher fractionation resulting in a ^{15}N depleted compared to macroalgae (Owens, 1988).

Our results show the highest $\delta^{15}\text{N}$ values in plants collected from freshwater environments compared to those obtained from marine and estuarine environments are possibly due to the input increase of nutrients, which enriches ^{15}N values (Fry, 2002; Olin et al., 2013). Overall, changes in $\delta^{15}\text{N}$ are related to biogeochemical processes that have

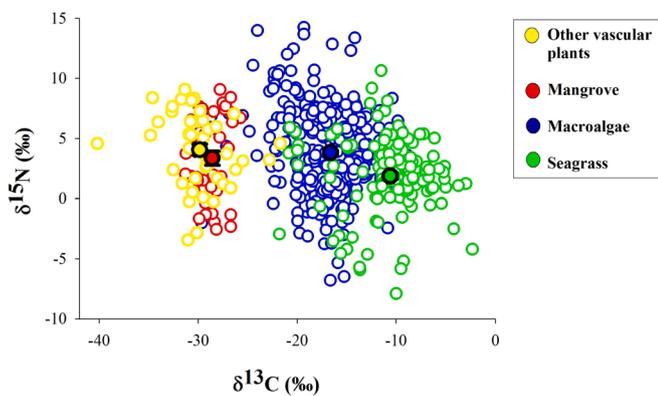


Fig. 4. $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values according to vegetation type, integrated with mean values and standard error (SE).

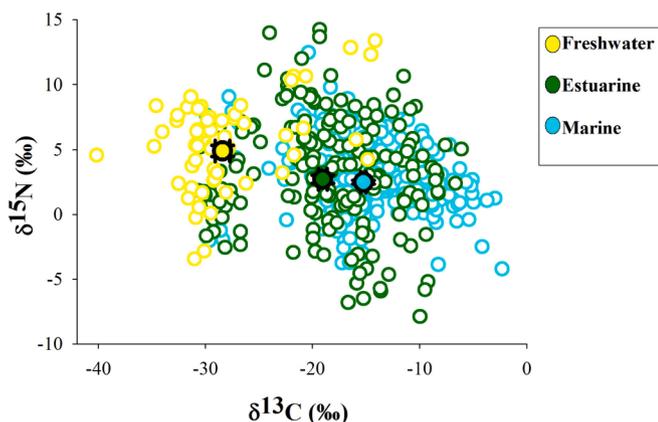


Fig. 5. $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values according to environment, integrated with mean values and standard error (SE).

affected bioavailable nitrogen (fixed nitrogen): denitrification, nitrification, anammox. Additionally, the distribution of the DIN (changes in the proportion of nitrate and ammonium) are related to isotopic variability. The first process is the isotopic composition from dissolved inorganic nitrogen (DIN), which can change as proportions of nitrate (NO_3^-) and ammonium (NH_4^+) change. The second one is the fractionation happening during the absorption and transformation (McCusker et al., 1999). In rivers and brackish environments, the supply of nutrients from terrestrial materials generate high production and $\delta^{15}\text{N}$ values are higher (Kuramoto and Minagawa, 2001). By contrast, marine plants usually have lower $\delta^{15}\text{N}$ values than the nitrate source used by the plant (Anderson and Fourqurean, 2003), and this is related to the availability and use of nutrients (Fourqurean et al., 2005).

According to geographic distribution, we found that the SZ had the lowest $\delta^{15}\text{N}$ mean values ($2.61 \pm 0.26\text{‰}$; min: 5.71‰ , max: 12.46‰) in comparison to NZ ($3.24 \pm 0.23\text{‰}$; min: 7.89‰ , max: 14.24‰), and CZ ($3.64 \pm 0.31\text{‰}$; min: 4.55‰ , max: 13.98‰), suggesting that the N source in this zone is different than the other two zones. Quintana Roo is a region with great tourist demand, and tourist activities can modify habitat, increase pollution and have an intense use of marine resources (Aguilar et al., 2008a). Several studies applying nitrogen stable isotopes analysis in macroalgae and seagrasses in the Mexican Caribbean found that $\delta^{15}\text{N}$ values are related to human activities and development (Camacho-Cruz et al., 2019; Mutchler et al., 2007, 2010; Sánchez et al., 2013, 2020; Umezawa et al., 2002), showing higher $\delta^{15}\text{N}$ values in NZ compare to SZ. However, the study area has a variety of plant communities that could contribute with N input to the system (Pacheco Ávila and Cabrera Sansores, 2003) due to plants decomposition, increase in nitrates, and an enrichment in ^{15}N from primary producers, which

would increase $\delta^{15}\text{N}$ values (Medina-Gómez and Herrera-Silveira, 2003). Also, denitrification of an N source, and N limitation during periods of rapid growth could induce high $\delta^{15}\text{N}$ values in marine plants (Fourqurean et al., 2005).

Our results, specifically for macroalgae and seagrasses, showed a high variability between -7.89‰ and 14.24‰ influenced possibly by both natural process and anthropogenic activities. We found $\delta^{15}\text{N}$ values $> 10\text{‰}$ in different stations from our three zones, without a visible relation between the high $\delta^{15}\text{N}$ values and the areas with more development and human activities, coinciding with other studies in the zone (Null et al., 2014). Nevertheless, Sánchez et al. (2013) suggest that high $\delta^{15}\text{N}$ values are a result of wastewater discharges derived from anthropogenic activities. Our highest values (between 12‰ and 14.24‰) found in Nichupté lagoon (NZ), are consistent with the results of other authors, for this specific station (Camacho-Cruz et al., 2019; Carruthers et al., 2005; Sánchez et al., 2020), suggesting pollution by sewage discharges in groundwater, with high nitrate concentration ($8.56 \mu\text{M}$) as found in this lagoon by other authors (Herrera-Silveira and Morales-Ojeda, 2010). However, in other places like Boca Paila at Sian Ka'an Biosphere Reserve (SKBR) (CZ) values $> 10\text{‰}$ could be due to the decomposition process of organic matter from aquatic vegetation and mangroves contributing an increase in nitrate, producing an enrichment in ^{15}N in primary producers (Medina-Gómez and Herrera-Silveira, 2003). It is also plausible that high ^{15}N values in areas far from anthropic effects are due to natural processes, such as denitrification occurring in oxygen-poor areas/depths.

On the other hand, $\delta^{15}\text{N}$ values between -4‰ and 4‰ could be the result of fertilizer runoff into the wetlands (Heaton, 1986). Hence, the presence of crop areas within the SKBR (CZ) (Bello et al., 2009) would explain the obtained $\delta^{15}\text{N}$ values in samples from some stations of this reserve. Nevertheless, those particular agricultural areas are located further than 50 km from the sampling points and have relatively low coverage area (Vázquez-Lule et al. (2009), therefore more studies are needed to investigate if this zone is polluted by fertilizers.

5. Conclusions

This study provides an isotopic baseline information from a wide variety of aquatic and semiaquatic plant species from the Mexican Caribbean. Our results in $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values show a wide variation according to vegetation type (seagrasses, macroalgae, mangroves, and "other vascular plants", and aquatic environment (marine, estuarine, and freshwater). This information can contribute to future ecological studies aiming to understand the structure of aquatic food webs and possible changes related to anthropogenic activities affecting aquatic and semiaquatic vegetation. Additionally, it can be used to infer diet and feeding habits of endangered aquatic megaherbivores, like manatees and sea turtles, as well as to investigate spatial and temporal changes in feeding habits of these species, since isotopic composition of an animal is primarily determined by the isotopic composition of its food. All this information is critical to understand the impacts of habitat modification, pollution and intense use of marine resources on species and natural ecosystems, which is particularly relevant for Quintana Roo as this megadiverse state has shown a steady growth of tourism and other human activities.

Credit author statement

Natalia Garcés-Cuartas: Conceptualization, Methodology, Formal analysis, Investigation, Writing – original draft, Visualization. Carlos Alberto Niño-Torres: Conceptualization, Methodology, Investigation, Resources, Writing – review & editing, Supervision. Delma Nataly Castelblanco-Martínez: Conceptualization, Methodology, Investigation, Visualization, Writing – review & editing, Supervision. Antonio Delgado-Huertas: Resources, Writing – review & editing. Neidy Pauline Cetz-Navarro: Investigation, Writing – review & editing. Raúl Ortiz-

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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APPENDIX

Appendix 1

Aquatic and semiaquatic plant species collected in the Mexican Caribbean, indicating year, hydroclimatic season, environment, and collection zone.

#	Species	Year	Season	Environment	Zone
Seagrasses					
1	<i>Halodule wrightii</i> (n: 49)	2017/2018	R, D, CF	M, E	N, C, S
2	<i>Ruppia maritima</i> (n: 7)	2017	R, CF	M, E	C, S
3	<i>Ruppia</i> sp. (n: 4)	2017/2018	D, CF	M, E, FW	C, S
4	<i>Syringodium filiforme</i> (n: 24)	2017/2018	R, D, CF	M	N, C, S
5	<i>Thalassia testudinum</i> (n: 46)	2017/2018	R, D, CF	M, E	N, C, S
Macroalgae					
6	<i>Acanthophora spicifera</i> (n: 1)	2017	D	M	N
7	<i>Acanthophora muscoides</i> (n: 1)	2018	D	M	S
8	<i>Acetabularia crenulata</i> (n: 1)	2017	R	M	S
9	<i>Acetabularia schenckii</i> (n: 9)	2017	R	E	N, C
10	<i>Acetabularia</i> sp. (n: 4)	2017	R, CF	M, E	N, C
11	<i>Avrainvillea nigricans</i> (n: 3)	2017	R	E	N
12	<i>Avrainvillea</i> sp. (n: 6)	2017/2018	R, D	M, E	N, C
13	<i>Batophora</i> sp. (n: 20)	2017/2018	R, D, CF	M, E, FW	C, S
14	<i>Bostrychia moritziana</i> (n: 1)	2017	D	M	N
15	<i>Canistrocarpus cervicornis</i> (n: 3)	2017/2018	R	M, E	C, S
16	<i>Caulerpa brachypus</i> (n: 2)	2017	R	E	N
17	<i>Caulerpa mexicana</i> (n: 2)	2017	R	M	S
18	<i>Caulerpa paspaloides</i> (n: 4)	2017/2018	R, D	M, E	N
19	<i>Caulerpa prolifera</i> (n: 3)	2017/2018	D	M, E	N
20	<i>Caulerpa sertularioides</i> (n: 1)	2018	D	M	S
21	<i>Caulerpa sertularioides f. longiseta</i> (n: 1)	2017	R	E	C
22	<i>Caulerpa</i> sp. (n: 16)	2017	R	M, E	N, S
23	<i>Centroceras clavulatum</i> (n: 2)	2017	R	M	C
24	<i>Ceramium nitens</i> (n: 6)	2017/2018	R, D	M, E	N, C
25	<i>Chaetomorpha cf. crassa</i> (n: 2)	2017	R	M	S
26	<i>Chaetomorpha clavata</i> (n: 2)	2018	D	M	N
27	<i>Chaetomorpha gracilis</i> (n: 4)	2017	R	M, E	C, S
28	<i>Chara</i> sp. (n: 10)	2017/2018	R, D, CF	E, FW	S
29	<i>Chondria capillaris</i> (n: 1)	2017	R	M	C
30	<i>Chondria cnicophylla</i> (n: 6)	2017	R	E	C
31	<i>Chondria collinsiana</i> (n: 1)	2017	D	M	N
32	<i>Chondria floridana</i> (n: 2)	2017	D	M	N
33	<i>Chondria</i> sp. (n: 1)	2018	D	E	N
34	<i>Cladophora laetevirens</i> (n: 2)	2018	D	E	N
35	<i>Cladophora prolifera</i> (n: 1)	2017	R	M	C
36	<i>Cladophora</i> sp. (n: 4)	2017/2018	D	M	N, S
37	<i>Cladophoropsis macromeres</i> (n: 1)	2017	R	M	S
38	<i>Cladophoropsis membranacea</i> (n: 1)	2017	D	M	N
39	<i>Cladophoropsis</i> sp. (n: 1)	2017	D	M	N
40	<i>Dasycladus vermicularis</i> (n: 3)	2017	R	E	S
41	<i>Dasycladus</i> sp. (n: 3)	2017	R	E	N
42	<i>Dictyota bartayresiana</i> (n: 1)	2018	D	M	S
43	<i>Dictyota menstrualis</i> (n: 1)	2017	D	M	N
44	<i>Dictyota pulchella</i> (n: 2)	2017	R	M	S
45	<i>Dictyota</i> sp. (n: 7)	2017	R, D, CF	M	N, S
46	<i>Digenea simplex</i> (n: 2)	2017	R	E	C
47	<i>Gelidiopsis</i> sp. (n: 2)	2018	D	E	N
48	<i>Gracilaria blodgettii</i> (n: 1)	2017	R	M	S
49	<i>Gracilaria bursa-pastoris</i> (n: 1)	2017	D	M	N
50	<i>Halimeda incrassata</i> (n: 8)	2017/2018	R, D	M, E	N, S
51	<i>Halimeda monile</i> (n: 5)	2017	R	M	N, S

(continued on next page)

Appendix 1 (continued)

#	Species	Year	Season	Environment	Zone
52	<i>Halimeda opuntia</i> (n: 2)	2017	R	M	S
53	<i>Halimeda</i> sp. (n: 20)	2017/2018	R, D, CF	M	N, C, S
54	<i>Hypnea spinella</i> (n: 1)	2017	R	M	S
55	<i>Laurencia</i> sp. (n: 7)	2017	R	M, E	N
56	<i>Padina</i> sp. (n: 5)	2017/2018	R, D, CF	M, E	N, S
57	<i>Palisada perforata</i> (n: 1)	2017	R	M	C
58	<i>Penicillus capitatus</i> (n: 26)	2017/2018	R, D	M, E	N, C, S
59	<i>Penicillus dumetosus</i> (n: 8)	2017	R, D	M, E	N
60	<i>Penicillus</i> sp. (n: 18)	2017/2018	R, D, CF	M	N, S
61	<i>Rhypocephalus oblongus</i> (n: 3)	2017	R	M	S
62	<i>Rhypocephalus phoenix</i> (n: 3)	2017/2018	D	M	N
63	<i>Rhypocephalus</i> sp. (n: 9)	2017	R	M	N, S
64	<i>Sargassum fluitans</i> (n: 26)	2017/2018	R, D, CF	M, E	N, C, S
65	<i>Sargassum natans</i> (n: 9)	2017/2018	R, D	M	N, C, S
66	<i>Sargassum</i> sp. (n: 1)	2017	CF	M	C
67	<i>Spyridia filamentosa</i> (n: 2)	2017	R	M	S
68	<i>Turbinaria tricostrata</i> (n: 3)	2017	R	M	C
69	<i>Turbinaria turbinata</i> (n: 3)	2017/2018	D	M, E	N
70	<i>Turbinaria</i> sp. (n: 4)	2017/2018	D, CF	M	S
71	<i>Udotea fibrosa</i> (n: 2)	2017	R	M	S
72	<i>Udotea flabellum</i> (n: 8)	2017	R	M	N, C, S
73	<i>Udotea luna</i> (n: 3)	2017	R	E	N
74	<i>Udotea spinulosa</i> (n: 1)	2017	D	M	N
75	<i>Udotea wilsonii</i> (n: 1)	2017	D	M	N
76	<i>Udotea</i> sp. (n: 19)	2017/2018	R, D, CF	M, E	N, C, S
77	<i>Yuzurua poiteaui</i> (n: 4)	2017	R	M, E	C, N
78	<i>Yuzurua poiteaui</i> var. <i>gemmaifera</i> (n: 1)	2017	D	M	N
Mangroves					
79	<i>Conocarpus erectus</i> (n: 8)	2017/2018	R, D, CF	FW	S
80	<i>Laguncularia racemosa</i> (n: 16)	2017	R	M, E	N, C, S
81	<i>Rhizophora mangle</i> (n: 13)	2017/2018	R, D, CF	M, E, FW	N, S
Other vascular plants					
82	<i>Bucida buceras</i> (n: 4)	2018	D	FW	S
83	<i>Cabomba palaeformis</i> (n: 6)	2017/2018	D, CF	FW	S
84	<i>Chrysobalanus icaco</i> (n: 6)	2017/2018	R, D, CF	FW	S
85	<i>Cladium jamaicense</i> (n: 5)	2017/2018	R, D	E, FW	S
86	<i>Coccoloba uvifera</i> (n: 1)	2018	CF	E	S
87	<i>Crinum erubescens</i> (n: 1)	2018	CF	FW	S
88	<i>Cyperus</i> sp. (n: 1)	2018	CF	FW	S
89	<i>Pontedeira crassipes</i> (n: 4)	2018	D, CF	FW	S
90	<i>Manilkara zapota</i> (n: 2)	2018	CF	E	S
91	<i>Nymphaea ampla</i> (n: 3)	2018	D, CF	FW	S
92	<i>Salvinia</i> sp. (n: 2)	2017	CF	FW	S
93	<i>Thrinax radiata</i> (n: 4)	2017/2018	D, CF	FW	S
94	<i>Typha domingensis</i> (n: 1)	2018	CF	FW	S
95	<i>Vallisneria</i> sp. (n: 2)	2018	D	FW	S

Season (R: rainy; D: dry; CF: Cold-fronts). Environment (M: marine; E: estuarine; FW: freshwater). Zone (N: north zone; C: center zone; S: south zone).

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