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Research Article

Carbon dioxide (CO_2) gas and eDNA assessment as tools for eradicating and monitoring invasive fish in anchialine pools in Hawai‘i

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Abstract

Invasive fish can profoundly affect communities they invade. In Hawai‘i, invasive fishes have become established in many anchialine pools, threatening the persistence of resident invertebrates, including several endangered species. Tools to eradicate invasive fishes from these pools are lacking. This study tested the efficacy of carbon dioxide (CO_2) gas diffused into anchialine pool water as a method to eradicate invasive Mozambique tilapia (*Oreochromis mossambicus*), guppies (*Poecilia reticulata*), and western mosquitofish (*Gambusia affinis*). We first conducted aquarium trials to identify how these fishes were affected by elevated CO_2 and the concomitant reduction in pH. We then carried out field trials in pools containing these fish in one pool each at two national historical parks on the Island of Hawai‘i during July 2021–January 2022. We also developed environmental DNA (eDNA) protocols to detect fish that may have survived CO_2 treatments. The effect of CO_2 on fish behavior varied among species; at pH 5.3 (CO_2 = 255 mg/L) for tilapia and 5.0 (CO_2 = 488 mg/L) for tilapia, guppies, and mosquitofish, all generally lost their ability to swim, showed slow or no gill movement, and altered their position in the water column. No tilapia survived the trials (n = 4 and 6 individuals at pH 5.3 and 5.0, respectively). In contrast, 41.7% (n = 12) of adult guppies and 66.7% (n = 12) of adult mosquitofish survived treatment at pH 5.0. In the field we were unable to reduce anchialine pool water pH below 5.7. Regardless, we were able to eradicate tilapia from one pool over four sequential treatments. Post-treatment eDNA assessments supported visual surveys, confirming our results. We were not able to eradicate guppies and mosquitofish. Results from this study show that CO_2 can be an effective tool for eradicating invasive tilapia from anchialine pools, and post-treatment eDNA assessments can provide managers with a method for evaluating the success of eradication efforts.

Key words: management tools, restoration, piscicide, aquatic habitats, Mozambique tilapia, guppies, western mosquitofish

Introduction

Invasive species can alter biodiversity and ecosystem function at a global scale (Charles and Dukes 2008; Gallardo et al. 2016), and island ecosystems are particularly vulnerable to their effects (Denslow 2003; Reaser et al. 2007). Invasive plants and animals have become established in many habitats in

the Hawaiian Islands (Kueffer and Loope 2009; Matsunaga et al. 2019). Anchialine pools represent an example of an aquatic Hawaiian ecosystem that is greatly affected by invasive fish. Anchialine pools are coastal, tidally influenced brackish bodies of water that lack surface connection to the ocean (Holthuis 1973). Across the Hawaiian archipelago, at least 600 anchialine pools have been identified (Brock 2004); most are found in basaltic environments that dominate the volcanic islands, but some are in eroded limestone benches (Yamamoto et al. 2015). Hawaiian anchialine pools typically support a small but unique assemblage of invertebrates and fish (Brock 1977; Polhemus 1996; Sakihara 2012; Tango et al. 2012), including several species of arthropods that are rare or endangered (USFWS 2013, 2016). Although Hawaiian anchialine pools are experiencing a variety of threats, including loss of habitat from resort development (Hoover and Gold 2006), groundwater pollution (Brock and Kam 1997; Hoover and Gold 2006), groundwater development (Oki 1999; USFWS 2016), and rising sea level (Marrack 2016; Marrack et al. 2020), invasive fish are one of the most destructive factors (Dalton et al. 2013; Havird et al. 2013; Marrack et al. 2015; Seidel et al. 2015).

Invasive fish most prevalent in Hawaiian anchialine pools include Mozambique tilapia (*Oreochromis mossambicus*), guppies (*Poecilia reticulata*), and western mosquitofish (*Gambusia affinis*) (Nico and Walsh 2011; Marrack et al. 2015; Seidel et al. 2015). These fish have been in Hawai'i for many decades (Brock 1960) and are established in a wide range of fresh and brackish water habitats across the archipelago. On the Island of Hawai'i, one or more of these fish species was found to occupy about 25% of the nearly 400 anchialine pools surveyed (Marrack et al. 2015). Collectively, these fishes have substantial effects on the distribution, abundance, and behavior of native anchialine pool species, particularly shrimp (Capps et al. 2009; Marrack et al. 2015; Seidel et al. 2015), although damselflies (Odonata) are also likely affected (England 1999).

Restoration of anchialine pools is a high priority among coastal land managers in Hawai'i, with the eradication of invasive fish an essential element for the recovery of endangered shrimp and damselflies (USFWS 2021). A variety of piscicides have been used to remove invasive fish from ponds and streams, including antimycin A (Gresswell 1991; Finlayson et al. 2002) and rotenone (McClay 2000; Rayner and Creese 2006; Nico et al. 2022). Although these piscicides may be effective in anchialine pools, they are not registered for use in Hawai'i, largely due to environmental concerns, such as contamination of nearshore food fish. More recently, carbon dioxide (CO_2) was registered with the U.S. Environmental Protection Agency (EPA) as a new aquatic pesticide (Cupp et al. 2021). Carbon dioxide in water results in Root and Bohr effects in fish, whereby the presence of CO_2 reduces the oxygen content of blood (Pelster and Weber 1991). Sustained exposure to CO_2 can result in respiratory acidosis and oxygen debt, which can contribute to behavioral changes or mortality. Carbon dioxide is naturally occurring in

the environment, leaves behind no harmful residues, presents a low risk to human health, and naturally dissipates from water through biological uptake, effervescence, and carbonate buffering (Wurts and Durborow 1992). Several studies have demonstrated the efficacy of CO_2 as an anesthetic for harvesting or transporting fish (Bell 1987; Fivelstad et al. 1998) and to suppress aquatic invasive species (Watten 2004; Abbey-Lambertz et al. 2014; Waller et al. 2021). Current CO_2 applications in the United States are largely focused on suppressing and containing invasive carps and other nuisance fishes (Cupp et al. 2020; Cupp et al. 2021). For example, application of CO_2 gas into large earthen ponds resulted in substantial overwinter mortality of several invasive carp species (*Ctenopharyngodon idella, Cyprinus carpio, Hypophthalmichthys molitrix, Hypophthalmichthys nobilis*) (Cupp et al. 2017b, 2018). Another study found CO_2 to alter fish behavior, resulting in avoidance of the gas and loss of equilibrium (Cupp et al. 2017a). If EPA registration is obtained for diffusing CO_2 in anchialine pools, then this piscicide could be applied to this habitat, where a similar suppression or eradication of invasive fishes could help conserve important native species and historical ecosystem services.

Although CO_2 demonstrates potential as a lethal and behavioral control for invasive fish, its effectiveness against the species established in Hawaiian anchialine pools has not been tested, nor has the ability to obtain critical levels of CO_2 within anchialine pools been established. To address these knowledge gaps, we conducted aquarium studies to identify how CO_2 influences the behavior of invasive tilapia, guppies, and western mosquitofish and then tested the efficacy of CO_2 in two anchialine pools containing one or more of these fish species. During the aquarium trials, body and breathing response, position in the water column, and survival were quantified during exposure to CO_2 at concentrations reported to influence the physiology or behavior of other fish species (Cupp et al. 2017a, b, 2021). Behavioral data were considered important because CO_2 applications within anchialine pools may require manual collection of target fishes during treatments. More specifically, treatments with CO_2 could potentially impair avoidance and swimming behaviors of target fish and make them more susceptible to removal. We also investigated the potential effects of CO_2 on the survival of ʻōpae ula shrimp (*Halocaridina rubra*; Atyidae), feeble shrimp (*Palaemon debilis*; Palaemonidae), and a thiarid snail (*Thiara granifera*; Thiaridae), all common inhabitants of anchialine pools free of invasive fish in Hawaiʻi.

Efforts to eradicate invasive species are often hindered by an inability to detect the relatively few individuals that may survive removal efforts (Garciá-Díaz et al. 2017; Guillera-Arroita et al. 2014). Efforts to eradicate invasive fish from anchialine pools will likely face similar challenges, as surviving fish may hide in vegetation or under rocks, or may be too small to detect visually. We developed qPCR primers for environmental DNA (eDNA) sampling and testing protocols for detection of the targeted fish species. The objectives of this study are to evaluate CO_2 applications for the removal

of invasive fishes from anchialine pools, develop eDNA tools for monitoring, and to provide essential data for potential EPA registration for CO_2 use to control invasive species in this habitat.

Materials and methods

Aquarium trials

Study animals

Fish and invertebrates used for aquarium trials were collected using hand nets or baited traps at Kaloko-Honokōhau National Historical Park and Puʻuhonua o Hōnaunau National Historical Park on the western coast of the Island of Hawaiʻi, Hawaiʻi, USA (refer to *Field Trials* section below). Tilapia and guppies were collected from two large brackish fishponds. Western mosquitofish, ʻōpae ula shrimp, feeble shrimp, and thiarid snails were collected from anchialine pools. Animals were transferred directly from their source to the U.S. Geological Survey Pacific Island Ecosystems Research Center (PIERC), Hawaiʻi Volcanoes National Park, and allowed to acclimate for several days prior to testing. Holding tanks for test animals were 38-L aquariums filled with water that was maintained at salinity 12.0–12.5 PSU, pH 7.7–7.9, and 20–21 °C. Conditions in the holding tanks were similar to source locations. Fish were at handled at PIERC under Institutional Animal Care and Use Committee (IACUC) protocols.

During the aquarium trials, tilapia (adult [75–120 mm total body length; n = 10] and juvenile [14–15 mm; n = 8]), guppies (adult [17–25 mm; n = 12] and juvenile [4–5 mm; n = 10]), western mosquitofish (adult [20–33 mm; n = 12] and juvenile [4–5 mm; n = 8]), ʻōpae ula shrimp (n = 24), feeble shrimp (n = 8), and thiarid snails (n = 8) were treated with CO_2 over time.

Experimental design

Prior to treatment, collected fish and invertebrates were transferred into treatment aquariums and allowed to acclimate until normal behavior was observed – a period of approximately 15 minutes. Treatments were administered by diffusing CO_2 gas from a pressurized 9.1-kg steel cylinder (20 × 71 cm) into the water via a standard 2.5-cm aquarium air stone (Aqua Culture, Bentonville, Arkansas, USA) controlled by a pressure regulator at a rate of 3.5–4.0 mL/sec until the water reached the targeted pH.

Target pH levels were determined during pilot trials. Results indicated that water sustained at pH 5.2–5.3 (CO_2 316–255 mg/L) led to mortality of tilapia (4 of 4 mortalities for adults tested at pH 5.3 for 180 min) but had relatively little effect on the survival of guppies (0 of 4 mortalities at pH 5.3 for 180 minutes) and western mosquitofish (2 of 22 mortalities at pH 5.2–5.3 for 54–244 min). Therefore, in this study we targeted pH levels of 5.3 (± 0.1) and 5.0 (± 0.1) for tilapia, and 5.0 (± 0.1) for guppies, western mosquitofish, and non-target invertebrates that may inhabit the pools.

The behavioral responses of adult fish were recorded every 4 minutes throughout the CO_2 treatment using direct visual observations. Treatments took approximately 28 minutes to reach pH 5.3 and 44–56 min to reach pH 5.0 from pre-treatment levels. Fish were held at target pH levels for two separate 120-min periods (referred to as initial and repeated treatments). The two treatments were interrupted by a 120-min period during which the fish and invertebrates were removed from the treatment aquariums and placed into adjacent aquariums containing water maintained under ambient pre-treatment (i.e., normal) conditions. This recovery period served to conservatively mimic rising ocean tides that may dilute or flush CO_2 from the pools in the field and interrupt the treatments. Although this recovery period is shorter than high tide periods in Hawai'i (tides change at about 6-h intervals), we rationalized that test species that would survive the repeated CO_2 treatment after 120 minutes of recovery would also likely survive treatment following a longer period of recovery. Juvenile fish were exposed to CO_2 in the same manner as adults, but only juvenile mortality was recorded. Because the number of fish available, and our ability to monitor the behavior of individual fish, was constrained by COVID-19 safety protocols, we did not maintain and observe untreated fish as treatment controls. Rather, we considered the observed behaviors of acclimated fish prior to treatment as proxies for standard controls.

Response of fish and invertebrates to CO_2

Behavioral responses of fish to CO_2 treatment were categorized in three ways using visual observations. First, we identified body response as normal, erratic swimming, lethargic swimming, loss of equilibrium (i.e., loss of vertical body position), or no movement. These were expected behavioral responses from fish when exposed to anesthetics and other narcotizing compounds, such as CO_2 (Suski et al. 2007; Cupp et al. 2017a). Second, we described breathing as normal, gulping (piping at the surface), slow gill movement, or no gill movement. Finally, we divided the tank into thirds horizontally and identified the position of the fish in the water column as top, middle, or bottom. In general, these behaviors were thought to represent a progressively more severe response to changing water conditions, except for position in the water column for which the expected response was unknown. Some categories were not mutually exclusive (e.g., erratic swimming and loss of equilibrium) but we noted the behavior most strongly exhibited. For juvenile guppies and western mosquitofish, ʻōpae ula and feeble shrimp, and snails, only survival at the end of the treatments was recorded.

Water quality measures

Direct pH measurements were used as a simple proxy to calculate CO_2 concentration (mg/L) in aquarium water. The relation between CO_2 concentration (mg/L) and pH was determined using a standard curve (R Core Team 2023). Carbon dioxide was measured with a Hach digital titrator

(Model 16900) and the Hach multi-parameter meter (Model HQ40d; Hach Company, Loveland, Colorado, USA) following manufacturer protocols (HACH Method 8205). This relationship at salinity 12 PSU was represented by the function $y = 2.503e + 07 * \exp(-2.169x)$ (n = 25, P < 0.001; pH and CO_2 ranges = 7.94–4.94 and 2.1–572.0 mg/L, respectively). Based on this relationship, CO_2 concentrations were 488 mg/L at pH 5.0 and 255 mg/L at pH 5.3. Aquarium water pH and dissolved oxygen (mg/L) were recorded with the Hach multi-parameter meter at 4-min intervals throughout the treatments. Water temperature and salinity were measured with an Oakton SALT 6 + meter (Matex Corporation, Toronto, Ontario, Canada). The pH meter was calibrated prior to each trial.

Field Trials

Study sites

Field trials took place at Pu'uhonua o Hōnaunau National Historical Park (NHP) and Kaloko-Honokōhau National Historical Park. These two parks are located about 25 km southeast (19.43N; 155.91W), and 5 km northwest (19.68N; 155.03W), of the town of Kailua-Kona, respectively, on the western coast of the Island of Hawai'i, the southernmost island of the Hawaiian Archipelago. Pu'uhonua o Hōnaunau NHP contains 11 anchialine pools and preserves numerous important cultural features. Tilapia and western mosquitofish were found in three of the pools. In contrast, Kaloko-Honokōhau NHP protects more than 200 anchialine pools. This park also preserves a rich assemblage of Hawaiian archaeological features, including two large brackish Hawaiian fishponds (Kaloko Fishpond, ~ 7 ha and 'Aimakapā Fishpond, ~ 12.3 ha). Collectively, these water features provide important habitat for federally endangered shrimp (*Procaris hawaiana*), damselflies (*Megalagrion xanthomelas*), waterbirds (*Himantopus mexicanus knudseni*, *Fulica alai*) and other rare native species. Guppies (and to a lesser extent western mosquitofish) have been documented in at least 18.8% of 191 anchialine pools in this park while tilapia have been found in 1.6% of the pools (Marrack and Beavers 2011; Weijerman et al. 2015). Guppies are widespread while tilapia are restricted to 'Aimakapā Fishpond and adjacent anchialine pools that become connected to the fishpond during extreme high tide events.

At Pu'uhonua o Hōnaunau NHP, we treated the Makaloa Pool (HA_Honaun_004; about 115 m^2). The Makaloa Pool is composed of two subpools (about 25 and 90 m^2) that are connected via a narrow channel (about 1-m wide) during high tide (Figure 1). The pool substrate consists of silty sedimentation over smooth pāhoehoe lava with emergent vegetation (*Cyperus laevigatus*; Smooth Flatsedge). At Kaloko-Honokōhau NHP we treated Pool 54 (HA_Kaloko_054; Figure 2D). Pool 54 is approximately 4 m^2 and located

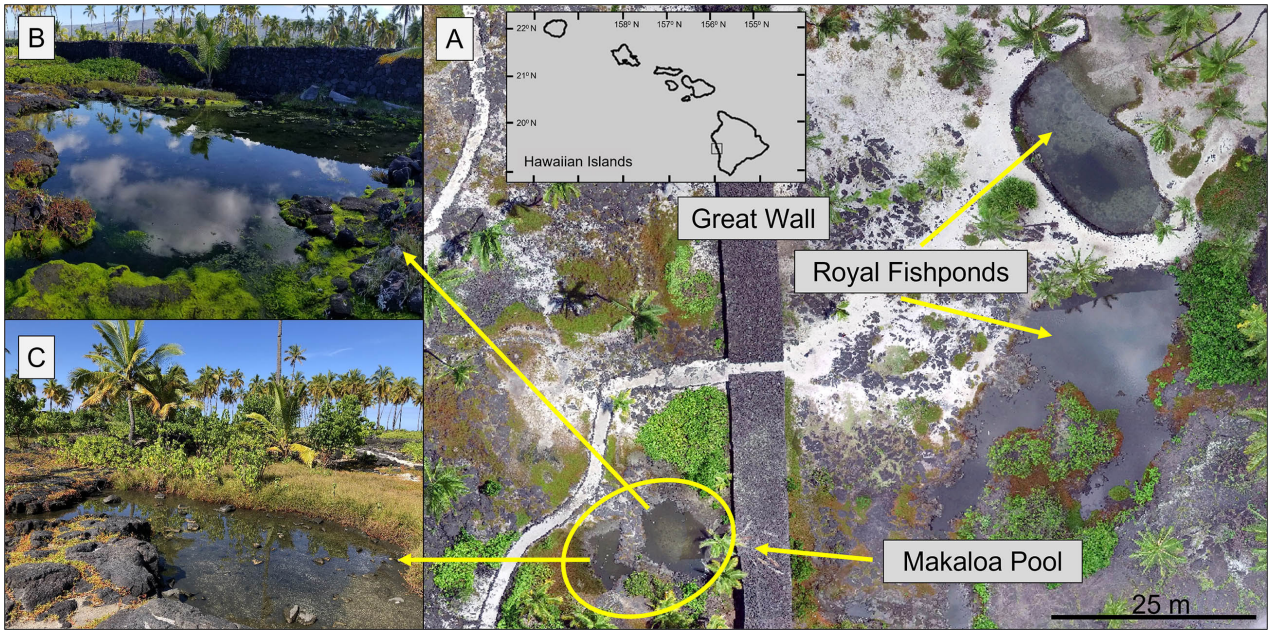

Figure 1. Location of the Makaloa Pool on the west edge of the Great Wall that bounds the pu'uhonua (Hawaiian place of refuge) at Pu'uhonua o Hōnaunau National Historical Park, Island of Hawai'i (A). The large (B) and small (C) subpools are shown at the left. Note that water from the west edge of southern Royal Fishpond and the east edge of the Makaloa Pool contact adjacent sides of the Great Wall during very high tides. The traditional dry-fit masonry of the Great Wall (about 5.5 m across) creates interstices among rocks that may allow small fish and invertebrates to move between pools. The aerial image (A) was created for the National Park Service by the Spatial Data Analysis and Visualization Labs, University of Hawai'i at Hilo.

within a largely unvegetated a'ā lava flow. The pool substrate consists of loose lava rocks with sediment or emergent vegetation. More than 40 anchialine pools exist within this unconfined lava substrate, some of which dewater during low tide, precluding them as habitat for invasive fish. Shrimp persist in these pools by following the descending water into rock interstices. Pool 54 is located about 100 m inland from the shoreline. Guppies are present in this pool.

Application of CO_2 into anchialine pools

Carbon dioxide gas (CO_2) was infused into pools from pressurized cylinders via 0.9-m long microbubble diffusers (Point Four; Pentair Aquatic Eco-Systems, Inc., Cary, North Carolina, USA) controlled by a pressure regulator (Figure 2). One pair of diffusers was attached to each CO_2 cylinder and two or three cylinders were operated concurrently in each pool. The CO_2 release rates were not monitored but approximated the maximum flow rate recommended for the diffusers (18–27 L/min). Each cylinder was stabilized in an upright position by being inserted through a hole in the lid of an 18.9-L plastic bucket. Kayak paddles were used by hand to distribute the CO_2-rich water away from the diffusers.

Dissolved oxygen and pH were monitored at several points on the bottom of the pools periodically throughout each treatment with a calibrated Hach multi-parameter meter (Model HQ40d). Salinity and temperature were measured before and after treatment with an Oakton SALT6 + meter.

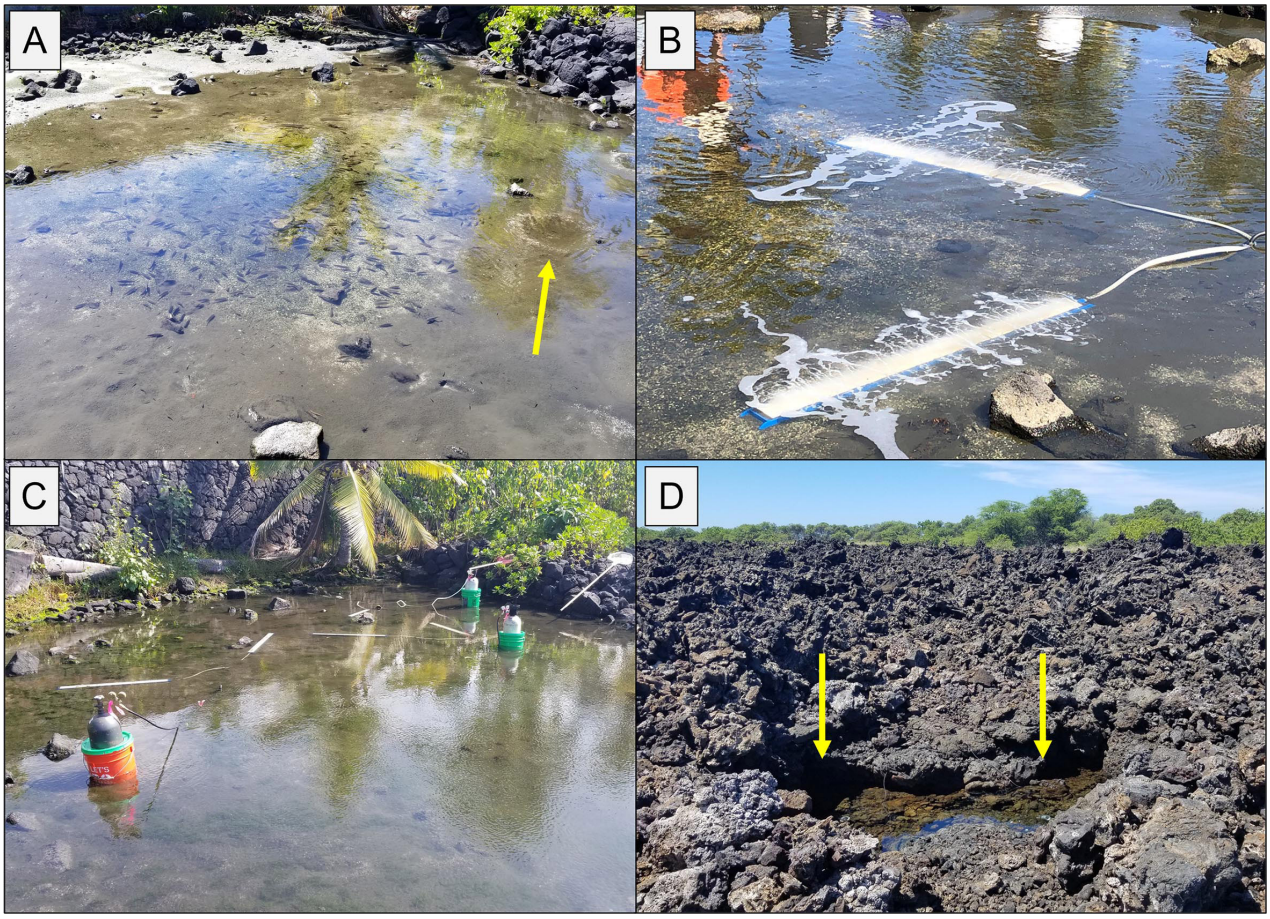

Figure 2. Treatment of anchialine pools with CO_2 to eradicate invasive fish. (A) Tilapia congregating in the center of the Makaloa Pool at Pu'uhonua o Hōnaunau National Historical Park are seen during low tide. The yellow arrow indicates a tilapia nest arena. (B and C) Carbon dioxide (CO_2) gas being released into the large subpool of the Makaloa Pool via pairs of diffusers attached to compressed gas cylinders (held in 18.9-L buckets for stability). Note the three pairs of diffusers along the Great Wall side of the pool (C); the diffusers had been moved incrementally across the pool driving the increasingly affected tilapia toward the edge where they could be concentrated and collected more easily. (D) Anchialine Pool 54 at Kaloko-Honokōhau National Historical Park. The two yellow arrows indicate locations where CO_2 diffusers were initially placed. Pool 54 contained only guppies.

Treatment of Makaloa Pool at Pu'uhonua o Hōnaunau NHP

The Makaloa Pool was treated with CO_2 on 13 July 2021, 24 August 2021, 22 November 2021, and 5 January 2022. Because we expected tilapia and western mosquitofish to retreat away from water most heavily affected by CO_2, diffusers were deployed in a manner aimed to concentrate the fish at the east side of the pool (towards the Great Wall) where they could more easily be collected by hand (Figure 2A–C). That is, diffusers were initially placed in a curvilinear array along the western edge of the pool and collectively moved towards the center, and then towards the eastern side of the pool, in a stepwise fashion over the course of treatment. During the first treatment, two pair of diffusers were placed in the large subpool and one pair was placed in the small subpool. After the first treatment, it became clear that the CO_2 had relatively little effect on the large number of western mosquitofish in the pool, and subsequent treatments focused on removing tilapia. The last three treatments were conducted in the larger subpool because the first treatment indicated a lack of tilapia in the smaller subpool.

Water treatment coincided with receding tide and generally lasted for 1.5–2.5 hrs. After discovering that tilapia took refuge from CO_2 within voids among rocks that form the southern edge of the pool, we placed a sheet of fine mesh fabric tightly against the bottom and along the wall prior to final treatment to prevent fish from moving into this area. Tilapia and western mosquitofish that were collected alive were maintained in buckets of clean water and returned to the laboratory where they were euthanized following IACUC protocols.

Prior to the first treatment we attempted to remove native shrimp by deploying six baited traps for 30 min. Trapping was not performed during subsequent treatments because no shrimp were collected. Native fishes (two mullet [Mugilidae] and five unidentified gobies [Gobiidae]) and several unidentified crabs were collected by hand during the treatments and released in the Royal Fishponds.

The Makaloa Pool was visually searched for surviving fish on 18 January 2022, 8 and 17 February 2022, and 28 March 2022. We collected water to analyze for tilapia eDNA on 13 July 2021, 24 August 2021, 25 January 2022, 28 February 2022, 28 March 2022, and 28 June 2022.

Treatment of Pool 54 at Kaloko-Honokōhau NHP

Pool 54 was treated with CO_2 on 11 August 2021 using the procedure described above. Because of its small size, two pair of diffusers were placed on opposite ends of the pool for much of the treatment period (Figure 2). The pool was treated for 120 min during the receding tide.

The U.S. Fish and Wildlife Service required Pool 54 to be monitored for the endangered shrimp *P. hawaiana* and damselfly *M. xanthomelas* before application of CO_2 because they had been observed in or around nearby anchialine pools. Monitoring consisted of 30 min visual surveys for both species and the deployment of six baited shrimp traps for 60 min. Neither endangered species were detected during the surveys, but 40–50 ʻōpae ula shrimp were observed, and 66 guppies were collected in the shrimp traps (11 juveniles [<15 cm total body length], 38 adult females and 17 adult males), possibly excluding the ʻōpae ula. The guppies were removed from the pool, sedated with MS-222 (50 mg/L), and euthanized.

Primer design and testing

Species specific qPCR probes and primers were developed for Mozambique tilapia, western mosquito fish, and guppies by aligning representative mitochondrial DNA sequences for each species from Genbank (NC004388, NC024238, AY597335) with Geneious version 8.1.9 (Biomatters, Auckland, New Zealand). Alignments of the 12S and 16S RNA, cytochrome oxidase II (COI) and cytochrome B (CytB) genes were manually searched for short segments with high dissimilarity that would allow selection of highly specific hydrolysis probes and primers for each species. Primer design software

Table 1. Primer and probe sequences for qPCR eDNA assays to detect fishes invasive to anchialine pools in Hawai'i. ZEN™ double quenched probes were used for each assay (IDT, Coralville, Iowa, USA) to reduce background signals.

Species	Target	Name	Sequence	Amplicon length
Tilapia	COI[1]	F1	CCC TGC CAT CTC TCA ATA TC	125
		R1	GGT TTC GGT CTG TTA GAA GTA	
		Tilapia Probe 1	FAM/ATT ACT CCT/ZEN[2]/ACT ATC CCT GCC CGT/BHQ[3]	
Guppy	COI	F13	CGA TAT TCT GAT TAC CCA GAC G	127
		R8	CAC GTT TTG CTG CGA AA	
		Guppy Probe 4	FAM/ATC CCT AAT/ZEN[2]/CTC CCT CGT AGC AGT/BHQ[3]	
Mosquitofish	16S RNA	F6	AAA GCA GCC ACC CTT AAG AAA GCG	199
		R6A	AAT TGC CGG TGG GTG GTC CG	
		Gambusia Probe 6	FAM/AGC TCA GAC/ZEN[2]/ACA CCC CTT CCC ACC TCA AAT CCC G/BHQ[3]	

[1]Cytochrome Oxidase.
[2]ZEN™ Quencher.
[3]Iowa Black®Fluorescent Quencher.

within Geneious was used to design candidate primer – probe combinations for promising regions. These were tested in silico for cross reactivity to other aquatic organisms that may be encountered in anchialine pool environments. Finally, the most promising primer probe combinations (Table 1) were screened with 10-fold serial dilutions of genomic DNA from each fish species for cross reactivity. These combinations were then tested with serial two-fold dilutions of double stranded, synthetic Gblocks standards (IDT, Coralville, Iowa, USA) to determine efficiency and limits of detection for each species-specific assay. Each serial dilution was tested 8 times, and results were analyzed by Probit analysis (MedCalc® Statistical Software version 20.116, Ostend, Belgium) to determine the minimum copy number that can be detected with a 99% probability of detection. Genomic DNA from each species was purified from skeletal muscle collected from frozen fish archived at Pu'uhonua o Hōnaunau NHP or from fish that were netted, sedated with MS-222 (50 mg/L), and euthanized.

Collection of eDNA

Up to four, 1-L water samples were collected from multiple locations within individual anchialine pools in 1-L plastic bottles, transported to PIERC on wet ice and filtered through 1.5-µm glass fiber filters (47 mm; Whatman 934AH, Cytiva, Marlborough, Massachusetts, USA) using a glass filtration flask and peristaltic pump (Masterflex, Cole Parmer, Vernon Hills, Illinois, USA). Filters were cut in half with a sterile scalpel. Each half was transferred to a 2-mL screw cap tube containing ceramic beads (six, 3-mm beads and 0.3-g, 800-µM ceramic beads; OPS Diagnostics, Lebanon, New Jersey, USA), ATL buffer (180 µL) and Proteinase K (20 µL) from a Qiagen dNeasy Blood and Tissue Kit (Qiagen, Germantown, Maryland, USA). Tubes were placed in a FastPrep 5G bead mill (Mp Biomedicals, Cleveland, Ohio, USA) and agitated at 6.0 m/sec for 40 sec to pulverize the glass fiber filter and disrupt particulates filtered from the water. Samples were digested overnight at 55 °C and then extracted using a dNeasy Blood and Tissue Kit (Qiagen, Germantown,

Maryland, USA) following manufacturer instructions. Purified DNA was eluted from spin columns in 80 µL of AE buffer and tested with a Nanodrop 2000 spectrophotometer (Thermofisher, Waltham, Massachusetts, USA) to evaluate DNA concentration and purity.

qPCR Assays

Purified DNA was tested in each assay by qPCR with species specific primers and probes. Each assay was run in plate format with up to eight replicates per sample on a CFX96 thermocycler (Bio-Rad, Hercules, California, USA) with 15-µL reaction volumes containing 5 µL of DNA template. Purified DNA from water samples was diluted 1:10 with molecular grade water to reduce concentration of PCR inhibitors that may have carried over from DNA extractions. Each reaction volume also contained SSOAdvanced Universal Probe Supermix (Bio-Rad, Hercules, California, USA), 0.6 µM of forward and reverse primers, and a 0.4-µM probe. Samples were tested on the CFX96 with positive genomic controls and negative no template controls containing molecular grade water at an initial incubation at 95 °C for 3 min, followed by 50 cycles of 95 °C for 10 sec and 60 °C for 30 sec.

Field evaluation of primers and probes

Environmental DNA isolated from water samples from 10 pools with known populations of invasive fish, and 7 pools with no records of invasive fish, were tested by qPCR assay for each invasive fish species to evaluate accuracy and specificity of the assays. Presence or absence of invasive fish in each pool were based on historical records and visual surveys conducted by National Park Service staff prior to collection of water samples (Hudgens et al. 2022; Marrack and Beavers 2011; Weijerman et al. 2015).

Results

Aquarium trials

Tilapia

Behaviors of tilapia prior to treatments at pH 5.3 (n = 4) and 5.0 (n = 6) consisted of normal body and breathing responses for all fish, and positions at the bottom of the aquarium for 9 of 10 fish (Figure 3). During the initial 28 min of CO_2 infusion to achieve pH 5.3, tilapia body response exhibited loss of equilibrium or lethargy (3 and 1 individuals, respectively), gill movement slowed and gulping was observed (3 and 1 individuals, respectively), and one fish moved to the top of the water column. All four fish displayed no movement after 16 min during the initial 120-min treatment. Two of the four fish changed their body response slightly during the recovery period and the first 8 min of the repeated treatment, but again there was no body movement during the remaining 112 min of treatment. Slow gill movement

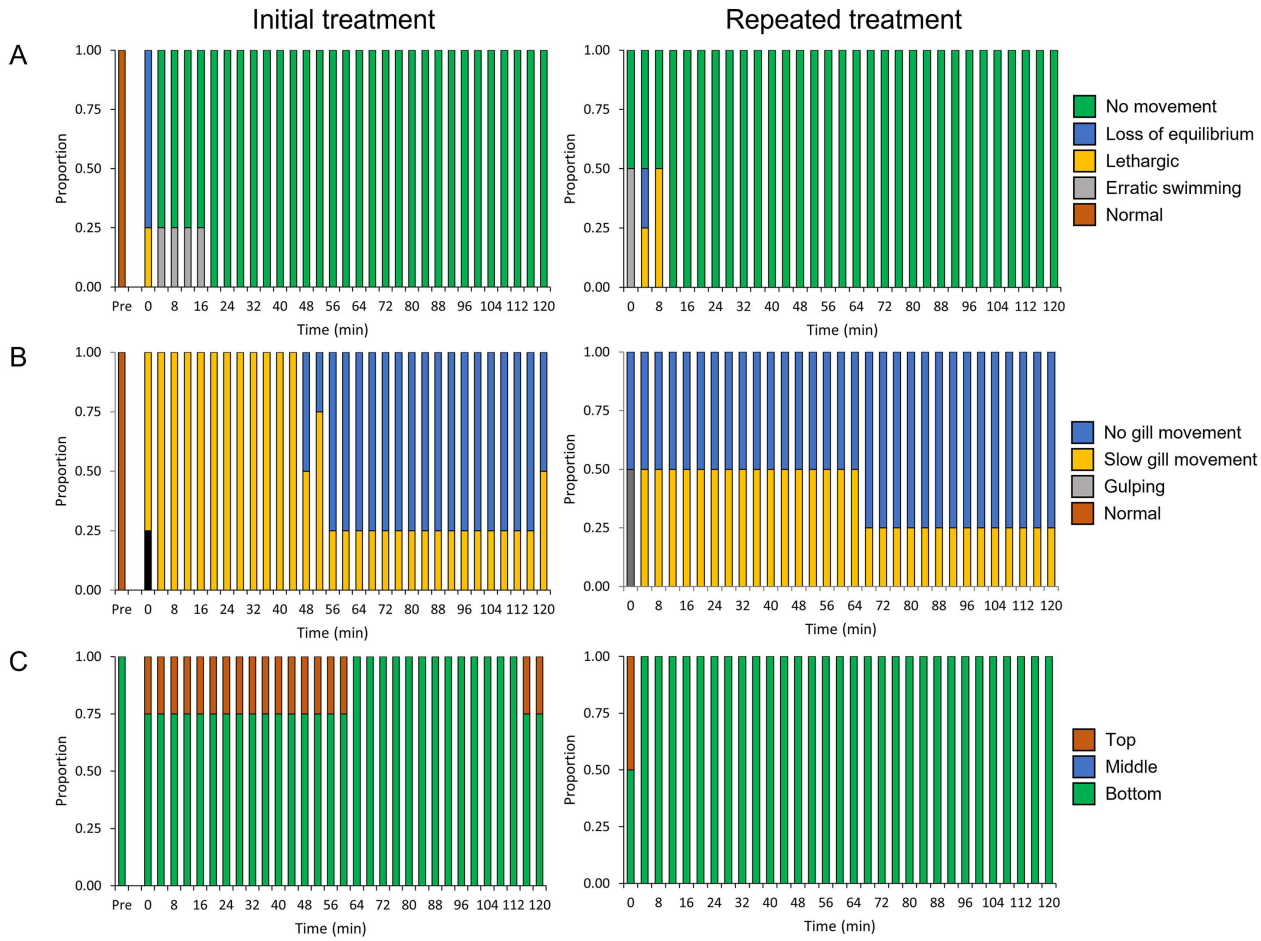

Figure 3. Behavioral response of adult tilapia (n = 4) in aquarium water held at pH 5.3 for 2 hours on two occasions. Water was reduced from pH 7.8 (normal conditions) to 5.3 (treatment conditions) using CO_2. Behavioral variables measured include (A) body response, (B) breathing response, and (C) position in the water column. The repeated treatment was similar to the initial treatment but followed a 120-min recovery period during which the fish were placed in aquarium water held at pre-treatment conditions. The behavior of the fish prior to the initial treatment ("Pre" for pre-treatment) is shown by the first bar in the figure.

was the primary breathing response for all four tilapia for the first 44 min of the initial treatment, after which three individuals displayed no gill movement during most of the remaining 68 min. No gill movement was observed for two individuals during the first 64 min of the second treatment, and three individuals during the remaining 52 min. Tilapia were primarily found on the bottom of the aquarium throughout both treatments except for one individual observed at the top during the first 60 min, and last 8 min, of the initial treatment. We did not determine whether the two fish that showed no body or gill movement were alive after initial treatment but none of the four survived both treatments.

Behavioral responses of tilapia exposed to CO_2 treatments at pH 5.0 were generally similar to those at pH 5.3 during the first treatment (data not shown). No body movement was observed for the six fish after 30 min, breathing generally transitioned from slow gill movement during the first 28 min (for 5 or 6 individuals) to no gill movement for all six fish after 56 min, and all six fish were at the bottom of the aquarium throughout this

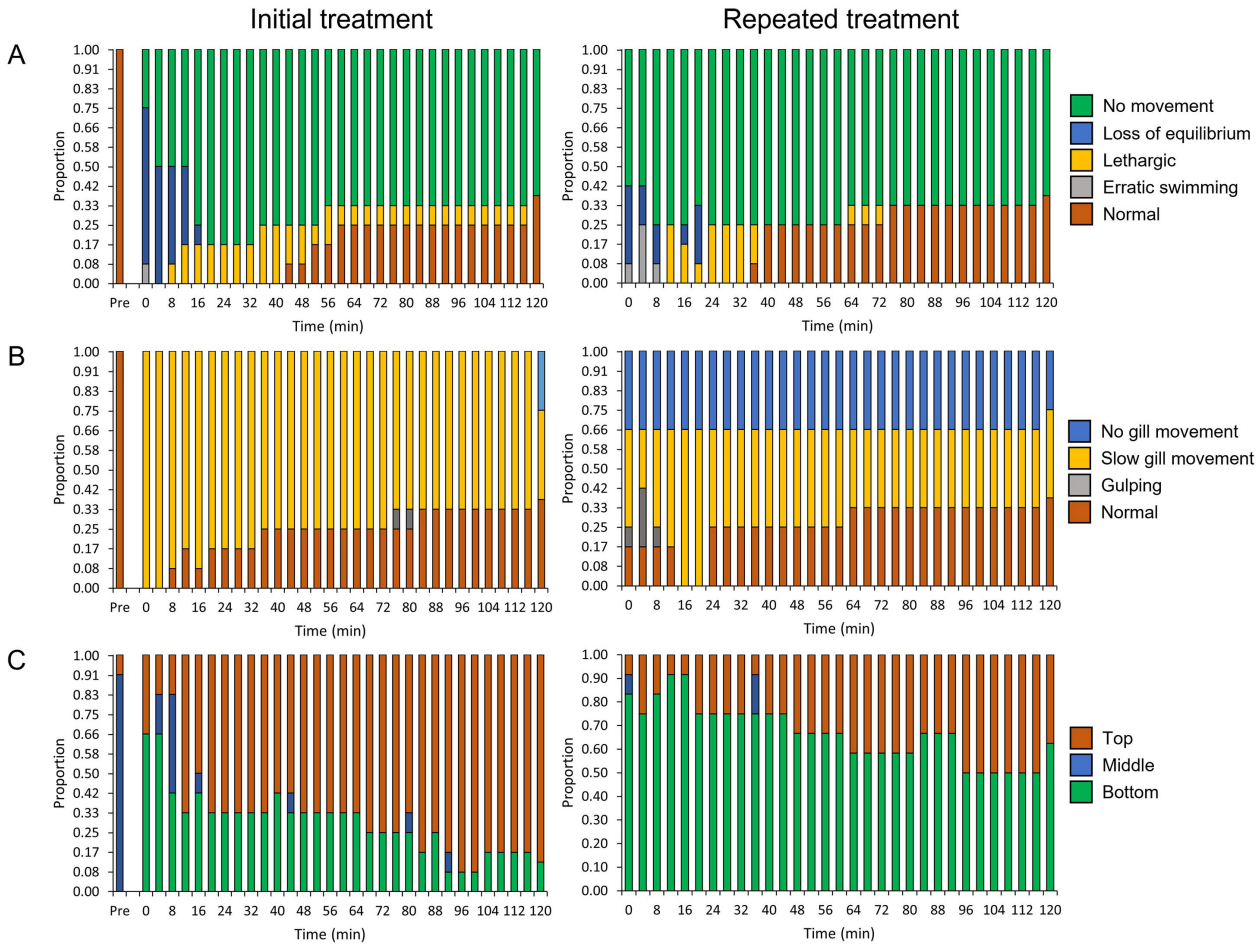

Figure 4. Behavioral response of guppies (n = 12) in aquarium water held at pH 5.3 for 2 hours on two occasions. Water was reduced from pH 7.8 (normal conditions) to 5.3 (treatment conditions) using CO_2. Behavioral variables measured include (A) body response, (B) breathing response, (C) and position in the water column. The repeated treatment was similar to the initial treatment but followed a 120-min recovery period during which the fish were placed in aquarium water held at pre-treatment conditions. The behavior of the fish prior to the initial treatment ("Pre" for pre-treatment) is shown by the first bar in the figure.

treatment. All six adults died after the first treatment so no repeated treatment was conducted. Additionally, the eight juvenile tilapia challenged at pH 5.0 also died following the initial treatment.

Guppies

Pre-treatment behaviors of adult guppies (n = 12) consisted of normal body and breathing responses for all fish and a position within the middle of the aquarium for 11 individuals (Figure 4). Behaviors changed during the 52–56 min it took for water to reach pH 5.0, as 11 fish exhibited loss of equilibrium or no movement (8 and 3 individuals, respectively), all fish had slowed gill movements, and fish moved to the bottom or top of the water column (8 and 4 individuals, respectively). The body responses of guppies were generally similar during both treatments. During most of the treatment periods, no movement was observed for 8–9 individuals, while lethargic or normal behavior was observed for the remaining 3–4 individuals. Breathing response during the initial treatment was dominated by normal or slowed gill movement (generally 3–5 and 7–9 individuals, respectively). Breathing

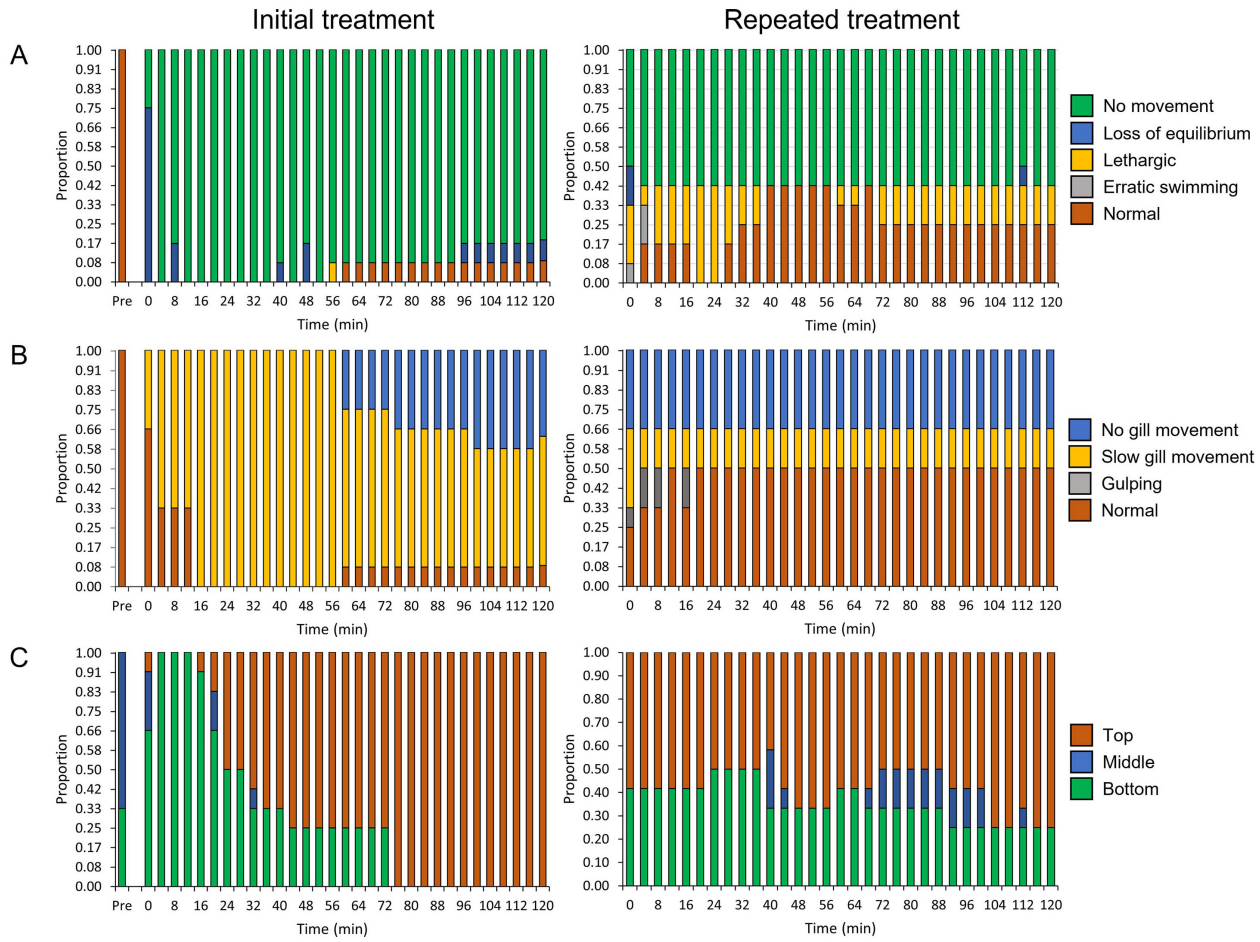

Figure 5. Behavioral response of western mosquitofish (n = 12) in aquarium water held at pH 5.3 for 2 hours on two occasions. Water was reduced from pH 7.8 (normal conditions) to 5.3 (treatment conditions) using CO_2. Behavioral variables measured include (A) body response, (B) breathing response, and (C) position in the water column. The repeated treatment was similar to the initial treatment but followed a 120-min recovery period during which the fish were placed in aquarium water held at pre-treatment conditions. The behavior of the fish prior to the initial treatment ("Pre" for pre-treatment) is shown by the first bar in the figure.

responses were relatively uniform throughout the repeated treatment, as 2–4 fish showed normal breathing, 4–5 fish displayed slow gill movement, and 4 fish showed no gill movement. Guppy position in the water column followed a similar trend during both treatments with fish generally moving from the bottom to the top of the aquarium. By the end of the first treatment 2–3 individuals were at the bottom whereas 5–6 individuals were at the bottom after the repeated treatment. Overall, 7 of 12 (58.3%) adult guppies and 6 of 10 (60.0%) juvenile guppies failed to survive both treatments.

Western mosquitofish

Pre-treatment behaviors of adult western mosquitofish (n = 12) consisted of normal body and breathing responses for all individuals, and four and eight fish at the bottom and middle of the water column, respectively (Figure 5). At the end of the 40–52 min it took for pH to decrease to 5.0, all fish were experiencing loss of equilibrium or no movement (9 and 3 individuals, respectively), four fish transitioned to slow gill movement, and fish were positioned in the bottom, middle, and top sections of the water

column (8, 3, and 1 individuals, respectively). No body movement was found for most (10–11 individuals) fish during the initial treatment. In contrast, five fish displayed lethargic or normal movements during the repeated treatment, while the other seven fish showed no body movement. Breathing responses were dominated by slow gill movement during the first 56 minutes of the initial treatment, while one fish showed normal breathing, and 2–4 individuals showed no gill movement during the last 60 minutes of that treatment. Breathing response was consistent during the repeated treatment with six fish breathing normally, two displayed slow gill movement, and four had no gill movement. Position in the water column during the initial treatment gradually shifted from fish being primarily at the bottom during the first 20 minutes, to all individuals being at the top during the last 48 minutes. Water column position during the second treatment was generally stable with 7–8 individuals at the top and 4–6 at the bottom. Overall, 4 of 12 (33.3%) adult and 100% of juvenile western mosquitofish tested failed to survive the two treatments.

Shrimp and snails

Exposure to pH 5.0 during the two 120-min treatment intervals had differing effects on invertebrates: 6 of 20 (30.0%) ʻōpae ula, 8 of 8 feeble shrimp, and 0 of 8 thiarid snails died during the experiments.

Field Trials

Makaloa Pool at Puʻuhonua o Hōnaunau NHP

We were unsuccessful reaching pH limits as low as those targeted during aquarium trials. Regardless of placement of diffusers, or the length of time diffusers remained in place, pH was never measured below 5.7 during any of the treatment days. In general, pH declined during the first 30–60 min of CO_2 application but stabilized, or increased, as diffusers were moved across the pool.

A total of 539 adult or near-adult tilapia were removed from the Makaloa Pool during the four treatments; the number of fish removed decreased sequentially during each treatment: 439 (not measured individually but all > 8 cm total body length), 77 (body length [cm] = 6.0 ± 0.3 SE; range 1.1–13.8), 20 (body length [cm] = 12.6 ± 0.7 SE; range 6.0–7.4) and three (body length [cm] = 12.0 ± 0.4 SE; range 11.5–12.8) fish were removed during each treatment, respectively. During the initial treatment, 50–100 tilapia eggs and hatchlings were also collected from two clusters, apparently abandoned by mouth-brooding females; additionally, one adult female carrying a clutch of fry was observed, but none of the fry were collected. During the repeated treatment, 15–20 juvenile tilapia (< 2 cm) were collected by hand. During the initial treatment, 1040 western mosquitofish were removed manually using dip nets; many western mosquitofish

remained in the pool after treatment, indicating poor efficacy of CO_2, and none were collected during subsequent treatments.

Tilapia and western mosquitofish eDNA were detected in Makaloa Pool on 13 July 2021 immediately before the first CO_2 treatment. We were not able to detect tilapia eDNA in Makaloa Pool in water samples collected 21, 88, and 175 days after the last treatment, respectively (25 January 2022, 28 March 2022 and 28 June 2022). One of eight qPCR replicates from samples collected on 28 February 2022 (55 days after the last treatment) was positive for tilapia eDNA in the absence of visually detectable fish. Water was not tested for western mosquitofish eDNA after efforts to remove that species were suspended.

Pool 54 at Kaloko-Honokōhau NHP

Water pH at Pool 54 was reduced to 6.88 and 5.71 in the east and west end of the pool, respectively, after 30 min, and to 5.68 and 5.72 after 60 min. The lowest pH obtained in the pool was 5.34 in the west end after 105 min. The CO_2 tanks were turned off after 120 min; pH increased to 8.10 and 7.29 in the east and west ends, respectively, 15 min after CO_2 diffusion ceased.

During treatment, 145 guppies (111 subadults, 18 adult females and 16 adult males), representing most of the fish observed, were captured using dip nets or traps that remained in the pools during treatment. Fish were active throughout the treatment and remained at the bottom or mid-level of the pool. Several remaining guppies were observed moving into two deep horizontal cavities (at least 25 and 50 cm, respectively) in the rocks at the eastern end of the pool (Figure 2). A diffuser was placed in each cavity for approximately 10 min in an attempt to drive the guppies from the cavities; however, this action was unsuccessful. Within approximately 15 min of the diffusers being turned off at the end of the experiment, at least 10 fish were observed in the vicinity of the cavities. A total of 211 guppies were removed from the pool, including the 66 guppies collected in traps during pre-treatment monitoring for endangered shrimp and damselflies.

Three of the 40–50 ʻōpae ula shrimp seen in the pool prior to treatment were observed swimming erratically during treatment, an apparent response to CO_2 in the water. The affected shrimp were collected and placed into a non-insulated bucket containing about 8 L of untreated water, but all three died by the end of the treatment. Although our focus was on collecting fish, no other shrimp observed during the treatment displayed behaviors that would indicate that they were affected by the CO_2. Water temperature in the holding bucket may have been a factor in the shrimp mortality and holding protocol shifted to use of insulated coolers with lids to better maintain cooler pool temperature.

Guppy eDNA was detected in water samples collected both the day prior to (10 August 2021) and 195 days after CO_2 treatment (22 February 2022).

Specificity, Sensitivity and Limits of Detection for eDNA qPCR Primers

All three qPCR assays were both sensitive and specific for amplifying DNA from each of the three species, with no detectable cross reactivity among species using serially diluted genomic DNA. The tilapia assay (efficiency = 100.1%, R^2 = 0.964, slope = −3.319, y intercept = 39.612) had a 0.99 probability of detecting 4.9 or more copies of a Gblocks standard per reaction (X^2 = 48.115, df = 1, P < 0.0001). The western mosquitofish assay (efficiency = 96.1%, R^2 = 0.992, slope = −3.418, y intercept = 40.387) had a 0.99 probability of detecting 0.7 or more copies of a Gblocks standard per reaction (X^2 = 55.072, df = 1, P < 0.0001). The guppy assay (efficiency = 93.6%, R^2 = 0.978, slope = −3.485, y intercept = 40.952) had a 0.99 probability of detecting 21 or more copies of a Gblocks standard per reaction (X^2 = 40.526, df = 1, P < 0.0001).

Field evaluation of qPCR assays

All three assays correctly identified presence or absence of fish populations present in 15 of 17 pools at Kaloko-Honokōhau and Puʻuhonua o Hōnaunau, with the exception of two small pools (Pool 4 [HA_Kaloko_004] and Pool 6 [HA_Kaloko_006] at Kaloko-Honokōhau) that were positive for guppy eDNA but had no visual observations of these fish (Table 2).

Data Availability

Data and metadata associated with this paper are available at https://doi.org/10.5066/P9AEQWX0 (Peck et al. 2023).

Discussion

Results from this study indicate that CO_2 diffused into water can be an effective tool for eradicating invasive fish from anchialine pools. We also found eDNA monitoring of pool water to be an effective way to survey for surviving fish that are visually difficult to detect. By combining these two tools, we were able to declare eradication of the highly destructive tilapia from the Makaloa Pool at Puʻuhonua o Hōnaunau NHP over the course of four treatments. We were not able to attain CO_2 levels that our aquarium trials indicated would be sufficient to kill tilapia, but CO_2 affected tilapia behavior in a manner that contributed to our ability to catch the fish using nets. Although CO_2 proved effective for removing tilapia from this pool, we failed to eradicate western mosquitofish present in the same pool, and guppies from a small pool at Kaloko-Honokōhau NHP, demonstrating that challenges using CO_2 to eradicate these invasive poeciliid species from anchialine pools remain. Regardless, these data may prove useful for obtaining EPA registration of CO_2 for the removal of invasive fishes from anchialine pools in Hawaiʻi.

Table 2. Comparison of visual and eDNA detections of invasive fish in 17 anchialine pools from Pu'uhonua o Hōnaunau National Historical Park (PUHO) and Kaloko-Honokōhau National Historical Park (KAHO).

Park	Pool	Date	Fish detection method[1]	Tilapia	Guppy	Western mosquitofish
PUHO	HA_Honaun_004 (Makaloa Pool)	7/13/2021	Visual	Yes	No	Yes
			eDNA	Yes (4/8)	No (0/8)	Yes (8/8)
PUHO	HA_Keokea_003	8/7/2019	Visual	No	No	No
			eDNA	No (0/8)	No (0/8)	No (0/8)
PUHO	HA_Honoun_005	2/17/2022	Visual	No	No	No
			eDNA	No (0/8)	No (0/8)	No (0/8)
PUHO	HA_Honoun_007	8/7/2019	Visual	No	No	No
			eDNA	No (0/8)	No (0/8)	No (0/8)
KAHO	HA_Kaloko_004	8/2/2019	Visual	No	No	No
			eDNA	No (0/8)	Yes (6/8)	No (0/8)
KAHO	HA_Kaloko_005	8/15/2019	Visual	No	No	No
			eDNA	No (0/8)	No (0/8)	No (0/8)
KAHO	HA_Kaloko_006	8/2/2019	Visual	No	No	No
			eDNA	No (0/8)	Yes (7/8)	No (0/8)
KAHO	HA_Kaloko_018	2/22/2022	Visual	Yes	Yes	No
			eDNA	Yes (8/8)	Yes (8/8)	No (0/8)
KAHO	HA_Honoko_037	2/22/2022	Visual	Yes	Yes	No
			eDNA	Yes (8/8)	Yes (6/8)	No (0/8)
KAHO	HA_Honoko_038A	2/28/2022	Visual	Yes	Yes	No
			eDNA	Yes (8/8)	Yes (7/8)	No (0/8)
KAHO	HA_Honoko_038B	2/28/2022	Visual	Yes	Yes	No
			eDNA	Yes (8/8)	Yes (8/8)	No (0/8)
KAHO	HA_Honoko_038C	2/28/2022	Visual	Yes	Yes	No
			eDNA	Yes (8/8)	Yes (7/8)	No (0/8)
KAHO	HA_Kaloko_054	8/10/2021	Visual	No	Yes	No
			eDNA	No (0/8)	Yes (1/8)	No (0/8)
KAHO	HA_Kaloko_119	2/22/2022	Visual	No	No	Yes
			eDNA	No (0/8)	No (0/8)	Yes (8/8)
KAHO	HA_Kaloko_123	2/22/2022	Visual	No	No	Yes
			eDNA	No (0/8)	No (0/8)	Yes (8/8)
KAHO	HA_Kaloko_128	2/22/2022	Visual	Yes	No	No
			eDNA	Yes (8/8)	No (0/8)	No (0/8)
KAHO	HA_Kaloko_129	8/15/2019	Visual	No	No	No
			eDNA	No (0/8)	No (0/8)	No (0/8)

[1]Visual detection based on day of sample observation and historical records. Genetic detection of fish is indicated in the species columns that follow by presence (Yes) or absence (No) of eDNA in the water, followed by the number of positive qPCR replicates/total replicates sampled.

The three qPCR assays were highly sensitive and specific for fish species, and successfully identified presence or absence of fish from 88% (n = 17) of pools tested with known fish status. The two exceptions (Pools 4 and 6 at Kaloko-Honokōhau) were approximately 50 meters from the population of guppies in Pool 54. Given the small size of these pools and repeated visual surveys that were negative, the detections may provide evidence of subterranean movement of eDNA between pools. Pools 4 and 6 were located on the ocean side of Pool 54, and it is possible that downslope movement of freshwater, or saltwater receding with the outgoing tide, may have carried eDNA through cracks and crevices of the lava substrate. Despite this result, eDNA monitoring appears to be a useful tool if hydrological relationships of pools can be established through dye tracer studies and potential sources of eDNA can be identified.

Although the number of fish treated during our aquarium trials was small, we found complete mortality of adult and juvenile tilapia when exposed to pH 5.3 (CO_2 = 255 mg/L) for 120 min on two occasions and pH 5.0 (CO_2 = 488 mg/L) for 120 min on one occasion. Further research identifying lethal dose responses is warranted and would better inform efforts to register CO_2 against this species in Hawai'i. The field trials underscored the difficulty reaching lethal CO_2 levels in anchialine pools, even for short periods of time. Anchialine pools are unconfined, hydrologically dynamic systems with saltwater and freshwater moving into and out of the pools in complex ways. CO_2 can be applied to take advantage of outgoing tides, and outgoing tides would be expected to draw affected water into interstices in the bottom of the pool where fish may seek refuge. Both of our field treatments sought to take advantage of the hydrology during outgoing tides. In the Makaloa Pool, this approach may have contributed to the effectiveness of the treatment, as many tilapia moved into bottom sediments and under rocks during treatment. Western mosquitofish behaved differently, generally remaining near the surface of the water. In contrast to tides, spatial and temporal inputs of freshwater into pools are more difficult to predict, and likely had a negative effect on our treatments in both pools. In the Makaloa Pool, a constant flow of freshwater into the pool may have diluted the concentration of CO_2 and helped drive it out of solution. Similarly, a freshwater input was apparent in Pool 54 where our salinity meters identified freshwater flowing into the east end of the pool, the area where guppies were taking refuge from CO_2. An avoidance of CO_2 like this has been observed in red swamp crayfish (*Procambarus clarkii*) (Abdelrahman et al. 2021). Placing an impermeable barrier on the surface of the water during treatment, such as a sheet of plastic, may be an effective way to reduce the rate at which CO_2 is diffused from water (Cupp et al. 2017b), but it was not used during our trials because it would have made collecting fish more difficult.

Our moderate reduction in pH in the Makaloa Pool affected tilapia in a way that made them conducive to capture. During exposure to pH of about 5.8 we found tilapia to swim lethargically and lose equilibrium, behaviors observed during the aquarium trials. It was clear that tilapia avoided the sources of CO_2 as we were able to manipulate their behavior in a manner that concentrated many individuals on one side of the pool by moving the diffusers stepwise across the pool. Avoidance of CO_2 has been observed in other fish and shows promise for managing invasive species (Donaldson et al. 2016; Cupp et al. 2017a). Carbon dioxide also proved to be an effective barrier to fish movement, presumably due to the presence of a gradient of affected water (Dennis et al. 2016). On only one occasion did a tilapia breach the CO_2 barrier established by a row of six diffusers, and that individual was collected with a net. Future studies could assess the efficacy of CO_2 as a tool to manipulate fish into nets or areas of pools where they are more easily collected. A challenging behavior displayed by many tilapia during

our study was that they sought shelter in the thick sediment layer at the bottom of the pool and under or between rocks when disturbed by the treatment. This behavior made them difficult to locate and capture using a net and required searching for the remaining few fish by hand.

Because we failed to reach CO_2 levels lethal to tilapia in the Makaloa Pool, four treatments were required to capture and remove all tilapia. With greater collecting effort it may have been possible to eradicate this fish with fewer treatments, but it was difficult to capture juvenile (< 1 cm) tilapia because they hid in small places and were hard to distinguish in the turbid water from the many western mosquitofish that were also present in the pool. To better collect remaining tilapia, we timed our treatments to allow surviving juveniles to grow to a size that would distinguish them from western mosquitofish but before they reached reproductive age. Little is known about the breeding of tilapia in anchialine pools, but they can reach maturity at small size (9–10 cm body length) and breed at only a few months of age during adverse environmental conditions (Hutchison et al. 2011). Following the first treatment, no male mating arenas (Figure 2A) were observed in the sediment indicating that reproduction was not taking place. A better understanding of breeding biology and seasonality in Hawai‘i may help refine a treatment strategy that minimizes the likelihood of reproduction. The fact that three of four water samples collected after the final treatment were negative for tilapia eDNA, coupled with absence of visual observations of fish, supports the conclusion that the eradication was successful. Given that the single positive eDNA detection was bracketed by negative tests in January, March, and June, sediment containing eDNA may have been resuspended during the sampling process or some subterranean movement of eDNA from the Royal Fishponds, approximately 25 meters away, may have occurred.

Why the poeciliids were less affected by CO_2 than tilapia is unclear, but it may be due to greater ability to assimilate oxygen into blood during treatment, rather than better response to reduced levels of dissolved oxygen in the water. Dissolved oxygen (DO) can be slightly suppressed when CO_2 is added to water at high concentrations. In this study, we saw a slight suppression of DO during aquarium trials when comparing pre-treatment values to those after CO_2 was added, generally decreasing DO 2–3 mg/L. However, DO values in tanks typically remained above 5 mg/L and did not result in hypoxic or anoxic conditions that would potentially contribute to organism mortality (Piper et al. 1982). Our DO data were less robust for field trials due to a malfunction with the optical DO probe and potentially a higher rate of suspended solids. Of the limited DO values collected during field trials, DO ranged from 6.6–8.4 mg/L and did not indicate any notable DO suppression. Regardless, most guppies and western mosquitofish were observed near the surface during treatment indicating that they may have

been responding to decreased concentrations of DO (Kramer and Mehegan 1981; Cech et al. 1985). Guppies may have been under more physiological stress than mosquitofish during the second aquarium treatment as a larger proportion of individuals were observed being lethargic, not moving, and at the bottom of the aquariums once pH dropped below about 6.

It is unlikely that CO_2 levels lethal to poeciliids can be obtained in anchialine pools, but behavioral responses to treatment may facilitate capture of these fish. During treatment of the Makaloa Pool, western mosquitofish appeared to avoid the CO_2 in a manner like tilapia, with large numbers of individuals becoming concentrated along the pool margin away from the diffusers. In Pool 54, CO_2 did appear to reduce guppy activity allowing most to be collected using nets. Due to the short interbrood interval of guppies and western mosquitofish (about 19–26 days depending upon environmental conditions; Vonracek et al. 1988; Dowdall et al. 2012), repeated treatments at shorter intervals than for tilapia may be beneficial.

Our aquarium study indicates that CO_2 may have negative effects on native shrimp that reside in anchialine pools during treatment. Feeble shrimp appear to be particularly sensitive as all eight individuals tested died when exposed to pH 5.0 (CO_2 488 mg/L). ʻŌpae ula fared better during the aquarium trials although approximately one-third perished. Combined, these results highlight a concern for potential effects on the endangered anchialine pool shrimp *P. hawaiana* and *Vetericaris chaceorum* (both Procarididae) if they are thought to be present during treatment. Further work would be useful to identify risks posed to these shrimps by the more moderate CO_2 levels expected during treatment of anchialine pools. However, because invasive fish, particularly tilapia, have such strong negative effects on native shrimp (Havird et al. 2013; Marrack et al. 2015), pools that contain these fish can be expected to support few if any of these invertebrates. Furthermore, these species are hypogeal and have the capacity to move through voids in the pool substrate and may be able to minimize their exposure to affected water. The endangered shrimp appear to occupy a small number of anchialine pools in Hawaiʻi, none of which currently support invasive fish (Sakihara 2012). Pre-treatment surveys, particularly using eDNA detection protocols, would be an important way to mitigate concern for affecting native shrimp.

During removal of tilapia from the Makaloa Pool, an unexpected bloom of a native benthic macroalgae (*Ulva* sp.) occurred after the second CO_2 treatment. This alga covered much of the pool bottom and provided potential refuge for surviving tilapia to evade capture. Much of the algae was removed from the pool prior to the third and fourth treatments. This alga likely existed in the pool prior to the initial treatments but was uncommon and had gone unnoticed. It is unknown if removal of tilapia triggered the outbreak, but it is plausible that decreased grazing pressure, combined with a surge in nutrients from fish cadavers, contributed to growth of this alga (Anderson et al. 2002).

In summary, we found CO_2 diffused into water to be an effective tool for eradicating invasive tilapia from the Makaloa Pool. The CO_2 altered the behavior of tilapia, guppies, and western mosquitofish, and was fatal, particularly to tilapia, following prolonged exposure at pH 5.3 or below. In contrast to aquarium trials, we were unable to reduce pH of water in anchialine pools below 5.7, but we were still able to eradicate a population of tilapia exceeding 550 individuals in the one pool. Our success was largely attributed to the anesthetizing effects of CO_2 on tilapia and their congregation near pool margins in avoidance of CO_2 sources, thereby facilitating removal with dip nets. Eradication of this population was confirmed using eDNA assessment of pool water at several points in time up to nearly six months following the fourth and final treatment. However, we were unable to eradicate guppies and western mosquitofish during similar treatments, primarily because pH levels were not low enough to affect their behaviors sufficiently to allow capture. Although we treated only two anchialine pools, it was clear that pool morphology and hydrology restricted our ability to diffuse and maintain CO_2 to reach targeted concentrations. Regardless, this study showed that CO_2 has potential as an effective tool for eradicating tilapia from anchialine pools and may provide data that could be used to expand the EPA's current registration for CO_2 to include its application in Hawaiian anchialine pools. Research that investigates more effective techniques to diffuse and maintain CO_2 in pools, determines how local hydrology influences treatments, and identifies ways to manipulate fish avoidance of CO_2, such as driving them into nets, may further advance this promising management alternative.

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Authors' contribution

All authors participated in research conceptualization, sample design and methodology, and review and editing of the draft manuscript; data were collected by RWP, MJM, MAH, CTA and SCB; data were analyzed and interpreted by RWP, MJM, CTA and ARC. RWP addressed reviewers' comments and prepared the final draft.

Ethics and permits

This study was conducted in accordance with IACUC Protocols No. 18-2975-2 and 18-2943. Research was carried out at Pu'uhonua o Hōnaunau NHP under research permits PUHO-2018-SCI-0007 and PUHO-2021-SCI-0002; and at Kaloko-Honokōhau NHP under research permits KAHO-2018-SCI-0016, KAHO-2020-SCI-0004, KAHO-2021-SCI-0008 and USFWS Biological Opinion 01EPIF00-2021-F-0259. An experimental use permit to apply CO_2 in anchialine pools was not required by Hawai'i Department of Agriculture due to the small scale of application and research focus of the work.

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