Check for updates

Received: 27 July 2023 | Revised: 23 October 2023 | Accepted: 8 November 2023

DOI: 10.1002/edn3.496

Environmental DNA
Open Access
Dedicated to the study and use of environmental DNA for basic and applied sciences

WILEY

ORIGINAL ARTICLE

Hidden in plain sight: The invasive macroalga *Caulerpa prolifera* evades detection by environmental DNA methods

Tanner Waters[1,2] | **Kylie Langlois**[3] | **Zachary Gold**[4] | **Susanna Theroux**[3] | **Robert A. Eagle**[1,2,5]

[1]Institute of the Environment and Sustainability, University of California, Los Angeles, Los Angeles, California, USA

[2]Center for Diverse Leadership in Science, University of California, Los Angeles, Los Angeles, California, USA

[3]Southern California Coastal Water Research Project, Costa Mesa, California, USA

[4]NOAA Pacific Marine Environmental Laboratory, Seattle, Washington, USA

[5]Atmospheric and Oceanic Sciences Department, University of California, Los Angeles, Los Angeles, California, USA

Correspondence
Tanner Waters and Robert A. Eagle, Institute of the Environment and Sustainability, University of California, Los Angeles, LaKretz Hall, 619 Charles E Young Dr East, Los Angeles, CA 90095, USA.
Email: tannerwaters@g.ucla.edu, tannerawaters@gmail.com and robeagle@g.ucla.edu

Funding information
Center for Diverse Leadership in Science Fellowship; David and Lucile Packard Foundation, Grant/Award Number: 2020-70931; National Philanthropic Trust; National Science Foundation Graduate Research Fellowship; NOAA Research and Development database, Grant/Award Number: 24755; Switzer Foundation Fellowship; UCLA Cota-Robles Fellowship; NOAA 'Omics

Abstract

Environmental managers need a rapid and cost-effective monitoring tool for tracking the spread of invasive species, particularly at the onset of introduction. The macroalgae *Caulerpa prolifera* is considered an invasive species outside its native range, colonizing large patches of seafloor, reducing native species, and altering ecosystem functioning. Here, we developed a droplet digital PCR assay for detection of *C. prolifera* from environmental DNA seawater samples using the internal transcribed spacer (ITS) region. While the assay itself was confirmed to be highly efficient, we discovered concentrations of *C. prolifera* eDNA were present below detectable levels in the water column surrounding an outbreak. To understand why, we conducted tank-based experiments for two California invasive algae species, *Caulerpa prolifera* and *Sargassum horneri*. The steady-state eDNA concentration (eDNA copies/ gram of biomass detected) of *C. prolifera* was found to be two orders of magnitude lower than *S. horneri*. A meta-analysis of steady-state concentrations reported in the literature showed a remarkable range from ~10^4–10^{11} (copies/g), revealing *C. prolifera* to have the lowest recorded steady-state concentrations of eDNA of any known species. We attribute *C. prolifera's* low steady-state eDNA concentration to its unique biology as a unicellular macroscopic algae which reduces the possible modes of eDNA release compared to similarly sized multicellular organisms. Critically our results demonstrate the potential limits of eDNA approaches, the influence of shedding rates in the reliability of species detections, and the vital importance of benchmarking and validating eDNA assays in both field and laboratory settings.

KEYWORDS
benchmarking, *Caulerpa*, environmental DNA, introduced species, *Sargassum*

This is an open access article under the terms of the Creative Commons Attribution License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited.
© 2023 The Authors. *Environmental DNA* published by John Wiley & Sons Ltd. This article has been contributed to by U.S. Government employees and their work is in the public domain in the USA.

1 | INTRODUCTION

Invasive species are a threat to global marine biodiversity (Molnar et al., 2008). When these invasive species are introduced to a new environment, they can rapidly colonize the area because of their quick reproduction time, lack of natural predators, ability to outcompete native species, or a combination of all three (Havel et al., 2015). This causes both direct and indirect impacts to local ecosystems. Invasives can alter local biodiversity, impact ecosystem structure and reduce functional ecosystem services (Pimentel et al., 2000). Globally, marine invasive species have cost the economy an estimated $345 billion in damages (Cuthbert et al., 2021). This threat has only continued to rise in recent decades (Seebens et al., 2021) with the increase in globalized shipping, aquaculture, and accidental release (Bax et al., 2003; Hulme, 2009; Silva et al., 2009). For these reasons, early detection and eradication of invasive species before their spread is a top priority for environmental managers (Larson et al., 2020).

Two invasive species of particular concern are *Caulerpa taxifolia and Caulerpa prolifera*. *C. taxifolia* is one of the top 100 worst invasive species (Global Invasive Species Database, 2023) and is named on the US Federal Noxious Weed List due to its history of overtaking marine ecosystems. It received this level of scrutiny because in the first 16 years since its introduction off the coast of Monaco in 1984, it grew to cover nearly 131 km^2 of Mediterranean coastline (Meinesz et al., 2001). The algae was first seen in 2000 in California in Carlsbad and Huntington Harbor, California (Jousson et al., 2000) with DNA barcoding of the tissue showing that it likely originated from an aquarium store (Jousson et al., 2000). It took nearly 6 years and $7 million dollars (USD) to eradicate *C. taxifolia* from California (Merkel & Associates, 2006). In 2021, the first known case of *Caulerpa prolifera* was discovered off the West Coast of the United States in Newport Bay, CA (NOAA Fisheries, 2022). Species of the genus *Caulerpa* have been observed to stunt ecosystem services, reduce native biodiversity and significantly decrease species richness compared to native seagrass meadows (Parreira et al., 2021). These impacts and the species' relative ease in spreading made it a top priority for eradication efforts by local marine managers.

California has dealt with another invasive macroalgae for nearly 20 years, *Sargassum horneri*. *S. horneri* (Devil's weed) is a brown algae native to Eastern Asia and was introduced to the West Coast of North America in 2003. Since its introduction, its range has expanded from Baja California, Mexico to Point Conception, California including the Channel Islands (Marks et al., 2017). *S. horneri* often forms large mats off the coast that are anchored to rocky substrate. Researchers working on *S. horneri* have shown that removal techniques are most effective in culling population size and density if the scale of removal is sufficient to reduce propagule supply (Marks et al., 2017). Thus, environmental DNA (eDNA) could be used for early detection of *S. horneri* prior to introduction via ballast water, for detection in areas that are difficult to survey, or for detection of small populations that can be successfully eradicated. Previous work has developed an eDNA assay for *S. horneri* (Hamaguchi et al., 2022), however, robust benchmarking of this *S. horneri* assay to determine species' shedding rates is needed to validate the efficacy of eDNA as an appropriate monitoring method.

Conventional survey techniques to identify *C. prolifera* involve divers visually scanning the seafloor. This presents an issue in Newport Bay, CA, where the turbidity makes for poor visibility and difficulty identifying *C. prolifera* fragments. Survey efficacy through the use of artificial *C. prolifera* released in the bay found that nearly 20% of the fake fragments were never recovered, highlighting this challenge (Owens, 2021). Environmental DNA has the potential to offer an additional method to screen for Caulerpa invasions in aquatic systems, where early identification and removal are paramount to their eradication (Larson et al., 2020). Environmental DNA approaches have been shown to better detect rare and cryptic species and outcompete conventional survey techniques in the field, particularly in difficult to survey environments (Fediajevaite et al., 2021; Thomsen & Willerslev, 2015).

Our study evaluates the use of eDNA-based monitoring alongside conventional survey techniques for the tracking of *C. prolifera*. We developed a novel droplet digital PCR (ddPCR) assay for the in-situ identification of *C. prolifera* and characterize the first algal eDNA shedding rates in the literature for *C. prolifera* and *S. horneri* to benchmark this methodology as a monitoring tool for invasive algal species.

2 | MATERIALS AND METHODS

2.1 | Assay design

To create our eDNA assay, we downloaded reference sequences of *Caulerpa prolifera* from GenBank (Benson et al., 2015) (https://www.ncbi.nlm.nih.gov/genbank/). Sequences were aligned in Geneious 2019.2.3 (https://www.geneious.com) and potential primer/probe sets were created using Geneious' design new primer/probes feature with guidelines based on Klymus et al., 2020. Our primers were created for the 'internal transcribed spacer' or ITS gene based on previous work which has used the ITS for *Caulerpa* sp. phylogenetics (Kazi et al., 2013; Stam et al., 2006). Primer specificity was tested *in-silico* using EcoPCR (Ficetola et al., 2010) and showed species-specific *Caulerpa prolifera* amplification. To validate the primers, we tested qPCR primer efficiency of our *C. prolifera* DNA from tank and field tissue samples using a dilution series from 5 ng/μL of genomic DNA to 0.00005 ng/μL. Primer and probe sequences for the *C. prolifera* assay are given below (Table 1).

We ran an annealing temperature gradient to optimize amplification and identify the greatest difference between positive and negative droplet fluorescence amplitudes. Based on our optimization, cycling conditions for ddPCR were 95°C for 10 min, 40 cycles of 95°C for 30 s and 58°C for 60 s, 98°C for 10 min, and a 4°C indefinite hold. Mastermix concentrations were: 14.4 μL

26374943, 2024, 1, Downloaded from https://onlinelibrary.wiley.com/doi/10.1002/edn3.496 by University of California - Los Ange, Wiley Online Library on [01/03/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

TABLE 1 Primer and probe details for the *C. prolifera* ddPCR assay.

Scientific name	Primer/probe	Sequence 5′–3′	bp
Caulerpa prolifera	Caulerpa_ITS_F	TGGCGCTATGTAATGTTGATGTTG	106
Caulerpa prolifera	Caulerpa_ITS_R	GCAATTCGCAACACCTTTCGTA	
Caulerpa prolifera	Caulerpa_Probe	56-FAM-CGGTTCCCGTGTCGATGAAGGACG-3IABkFQ	

of 4× ddPCR Multiplex Supermix (Bio-Rad, Hercules, CA, USA), 0.5184 μL of 100 μM forward primer, 0.5184 μL of 100 μM reverse primer, 0.144 μL of 100 μM probe, 20.4192 μL of water, and 12 μL of sample. This mix was partitioned into duplicate replicates of 22 μL and added to a 96-well plate. The reaction mixture was combined with Bio-Rad Droplet Generation Oil (20 μL reaction mixture + 70 μL oil) and partitioned into nanodroplets via microfluidics in the Automated Droplet Generator (Bio-Rad). This resulted in a total nanodroplet volume of 40 μL, which was transferred to a standard 96-well PCR plate for amplification using a multichannel pipettor. The plate was heat sealed with pierceable foil using a PX1 PCR plate sealer (Bio-Rad) and PCR amplification was carried out in a S1000 thermal cycler (Bio-Rad, ramping speed at 2°C per second). After PCR, the plate was read by the Bio-Rad QX200 Droplet Reader and analyzed using the Bio-Rad QX Manager (v.1.2 or v.2.0) software.

Sargassum horneri primers were chosen from Hamaguchi et al. (2022). We used AkamokuITS2-F 5′-TCGCTATATGCAGG TTTA-3′, AkamokuITS2-R 5′- GACTGCCTACCGTCAA-3′ and AkamokuITS2-P 5′- HEX-AGCCTCTAGCAACGCTCCAA-BHQ1-3′. We ran an annealing temperature gradient on the primers which showed a 56°C annealing temperature to be the optimum temperature. All other assay conditions were the same as described above for the *C. prolifera* assay.

2.2 | Field testing

A *C. prolifera* patch was found in China Cove, Newport Bay in April 2021 via scuba diving surveys and was roughly 1 foot in diameter and contained ~20 fronds. We sampled seawater on June 30, 2021, directly above the bed (33.596406, −117.879731), and then above the sea floor 5, 10, 50 100, and 500 m bay-ward from the *C. prolifera* patch, employing the eDNA collection method of Curd et al. (2019), described as follows. First, we collected seawater samples directly above the patch using divers to collect 1 L of water in a Kangaroo enteral feeding bag (Covidien, Minneapolis, MN, USA) as to not disturb the *C. prolifera*. Samples taken away from the patch were then collected using a 5 L niskin bottle. From the niskin, we transferred 1 L of seawater to a Kangaroo bag (Covidien, Minneapolis, MN, USA) in triplicate. We immediately gravity filtered 1 L of seawater through a sterile 0.22 μm Sterivex cartridge filter (MilliporeSigma, Burlington, MA, USA) for all the samples simultaneously. We capped the filters and stored them on dry ice during sampling until we returned to the lab where they were stored at −20°C. Additionally, we filtered 1 L of Milli-Q water through the same process for a negative field control (Goldberg et al., 2016). Tissue samples from the patch were taken for species verification and preserved in 70% molecular grade ethanol.

2.3 | Experimental design of shedding experiment

We tested the shedding rates of two California invasive macroalgae, *Caulerpa prolifera* and *Sargassum horneri*. We purchased the *Caulerpa prolifera* from an online aquarium store (ReefCleaners.org, Port St. Lucie, FL, USA) and divers from Cabrillo Aquarium identified and collected the *Sargassum horneri* off the coast of San Pedro, CA (33.774, −118.43). The algae were left to acclimate in tanks with artificial seawater for 2 days before the start of the experiment. We filled three replicate tanks per species with 20 L of deionized (DI) water and 36 g/L of Instant Ocean sea salt for aquariums (Instant Ocean, Blacksburg, VA, USA). Wet weights of the algae were measured and recorded before they were added to the tanks. Once added, the algae were kept alive for the length of the experiment and were free-floating for this period. We kept the tank water at ambient room temperature in the lab (20 ± 1°C) and exposed to natural, indirect sunlight through the window. An additional tank containing only artificial seawater was used as a control.

We took samples before the addition of the species (hour 0) and then subsequently at 1, 2, 4, 8, 12, 24, 48, 72, and 96 h after they were added in the same manner as the field samples. We added 23.99, 24.44, and 23.39 g of *C. prolifera* and 20.47, 23.49, and 22.36 g *S. horneri* into their respective first, second, and third tanks (Figure 1). At each timepoint, we stirred the tank gently with a sterile stirrer for a well-mixed sample and then collected 1 L of tank water into a Kangaroo enteral feeding bag (Covidien, Minneapolis, MN, USA). This 1 L bagged sample was then filtered onto two sterile 0.22 μm Sterivex cartridge filters (MilliporeSigma, Burlington, MA, USA) running 500 mL through each via gravity filtration to avoid filter clogging. We stored the filters at −20°C until they were extracted the following day. After each sample collection time point, we immediately refilled the tank with 1 L of seawater from a carboy so as to maintain consistent volume within the small tanks. At each timepoint, we collected water from the control tank and carboy in the same manner to test for contamination.

2.4 | DNA extraction

All eDNA and tissue samples we extracted from the Sterivex cartridge using a modified DNeasy Blood & Tissue Kit protocol (Qiagen

26374943, 2024, 1, Downloaded from https://onlinelibrary.wiley.com/doi/10.1002/edn3.496 by University of California - Los Ange, Wiley Online Library on [01/03/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

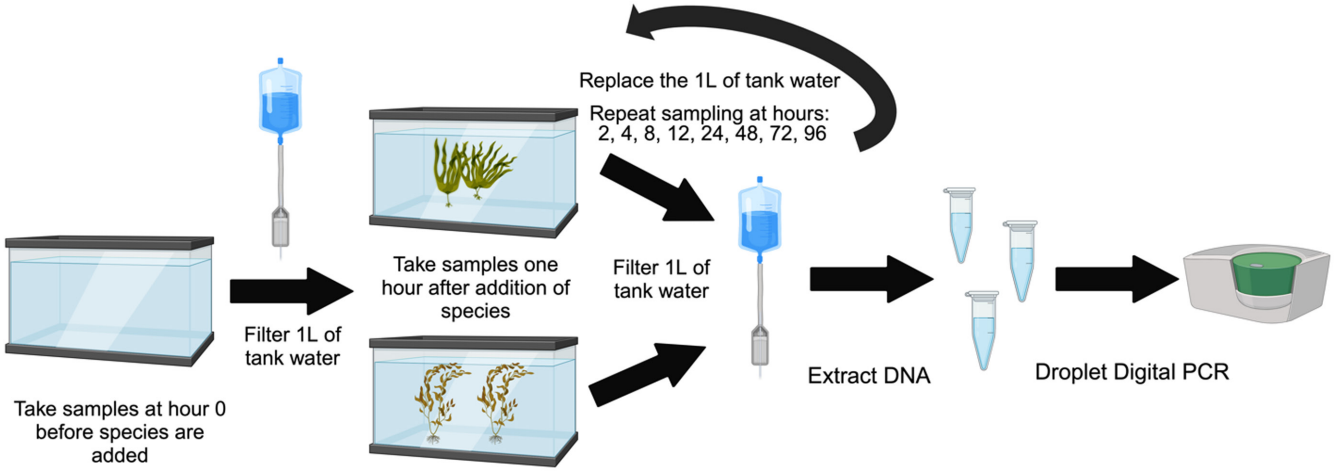

FIGURE 1 A schematic of the tank experiment setup.

Inc., Germantown, MD, USA) optimized for increased eDNA yield (Spens et al., 2017). Sterivex filters were incubated at 56°C overnight with 720 μL of ATL buffer and 80 μL of proteinase K. After incubation, equal parts AL buffer and ice-cold molecular grade ethanol were added to the ATL buffer/proteinase K mixture and spun through a spin column. AW1 and AW2 buffers were added to wash the columns. The DNA was eluted using 100 μL of AE buffer and stored at −20°C.

2.5 | Data analysis

Following recommendations in Cao et al. (2016) and Steele et al. (2018), a minimum of two reactions and a total of ≥10,000 droplets per reaction were generated per sample; samples that failed to meet the droplet requirement were reanalyzed. At least six no template control (NTC, RNA/DNA-free water; UltraPureTM, Life Technologies, Carlsbad, CA, USA) reactions were run per assay. NTC samples were required to contain less than three positive droplets. Two positive control reactions were included per assay. When samples exceeded the upper limit of quantification, these were diluted 1:100 with RNA/DNA-free water and reanalyzed.

Based on the concentrations from the QX Manager software, we back calculated the tank concentration of ITS gene copy number. Specifically, the ddPCR output in copies/μL of reaction were converted to copies/μL in the filter and then converted to copies/μL of tank water (https://github.com/kylielanglois/SCCWRP/blob/main/ddPCR/ddPCR_autofill_clean.R). Since replicates came from the same 1 L bag, they were averaged together to account for larger particles that were unevenly distributed between the two filters.

We then calculated the steady-state concentration per gram of body mass using the equation from Sassoubre et al. (2016). Briefly, $V^*dC/dt = S - kCV$, where V is the tank volume in liters, C is eDNA concentration, t is hours, S is the shedding rate, and k is the first-order decay rate constant per hour. At steady state, $dC/dt = 0$ so the shedding rate/decay rate constant would equal the concentration of eDNA multiplied by the tank volume. Since our experiment did not measure the decay rate constant, we cannot directly solve for decay and shedding rates. However, we can solve for the steady-state concentration per gram of body mass by using the 96-h concentration when our tanks reached steady state. We argue that this is still a meaningful metric as it reflects the total number of eDNA molecules per biomass (g) of an organism available to be captured in a given volume of water. We then compare steady-state concentration per gram of body bass across other previously reported values for other species (Andruszkiewicz Allan et al., 2021; Kwong et al., 2021; Maruyama et al., 2014; Nevers et al., 2018; Plough et al., 2021; Sansom & Sassoubre, 2017; Sassoubre et al., 2016; Wilder et al., 2023). When shedding and decay rates for multiple conditions in a given study for a single species were reported, we report both the lowest and highest reported steady state to show the range. All values shown are from other tank-based, single species shedding experiments allowing for comparable results within the meta-analysis.

3 | RESULTS

3.1 | Assay benchmarking

We successfully amplified extracted *C. prolifera* tissue DNA collected from the field invasion using our Caulerpa-specific primer set. We sequenced this tissue and the tank tissue using custom ITS primers on a Capillary Sequencer at Laragen Sequencing Facility (Culver City, CA). We evaluated chromatograms using Geneious. The consensus sequence is provided in the Supplement. We identified two mismatches between the field and tank consensus sequence in our forward primer and two deletions between our tank consensus sequence and reverse primer. No mismatches were found in the probe region. Our qPCR dilution series resulted in a 101.05% and 103.35% primer efficiency for the tank and field samples, respectively. These results demonstrate our primers to

26374943, 2024, 1, Downloaded from https://onlinelibrary.wiley.com/doi/10.1002/edn3.496 by University of California - Los Ange, Wiley Online Library on [01/03/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

be highly efficient and robust to small mismatches discovered in the Sanger sequences (Table S1).

3.2 | Field sampling

Despite the efficiency of our assay, none of the field eDNA samples taken directly above or away from the *C. prolifera* patch detected *C. prolifera* eDNA. Additional replicate field samples taken directly above the *C. prolifera* patch were tested for inhibition using a serial dilution and a Qiagen DNeasy PowerClean Pro Cleanup Kit and similarly showed no ddPCR amplification of *Caulerpa prolifera* DNA.

3.3 | Tank-based experiment

The *Caulerpa prolifera* and *Sargassum horneri* in the tank experiments both yielded quantifiable eDNA in the water samples (Tables S3 and S4). *C. prolifera* was characterized by a sharp increase in initial eDNA concentration in the tank to ~$10^{5.75}$ copies/L followed by slight decline and then steady plateau at ~10^5 copies/L (Figure 2). *S. horneri* instead saw a general increase in eDNA tank concentration followed by a similar plateau at ~10^7 copies/L. Our sampling method of filtering 1 L and replacing it with 1 L of water would have diluted the concentrations by 5%, which would have no bearing on the final interpretation of the results given the orders of magnitude difference in steady-state concentration observed. An ANOVA between the 48, 72, and 96 h concentrations show no statistical significance in the difference of means indicating that both tanks reached steady state by 96 h (ANOVA, $p > 0.05$). For the 20 g samples of algae in each tank, this steady state equates to roughly $10^{4.5}$–10^5 copies of DNA/L of tank water for *C. prolifera* and 10^7 copies/L for *S. horneri* (Figure 2). This equates to a nearly 100–315× greater amount of *S. horneri* eDNA concentration per gram of biomass compared to *C. prolifera*. All tank controls and PCR controls were negative.

3.4 | Steady state

Steady-state copies of DNA per gram of biomass for available species in the literature spanned over seven orders of magnitude (Table S2). *Caulerpa prolifera* had the lowest steady-state concentration while *Sargassum horneri* was one of the median reported values (Figure 3).

4 | DISCUSSION

Our results demonstrate the vital importance of lab and field validating eDNA assays prior to their adoption as a monitoring technique. Here, we created a novel eDNA assay that is able to amplify *C. prolifera* DNA in the lab and controlled mesocosms. Despite this, we were unsuccessful in identifying *C. prolifera* in-situ over a known patch of the algae. Tank-based experiments demonstrate that *C. prolifera* has the lowest observed steady-state eDNA concentration of any reported species. In contrast, *Sargassum horneri* assay shows promise as an invasive monitoring tool given the higher observed

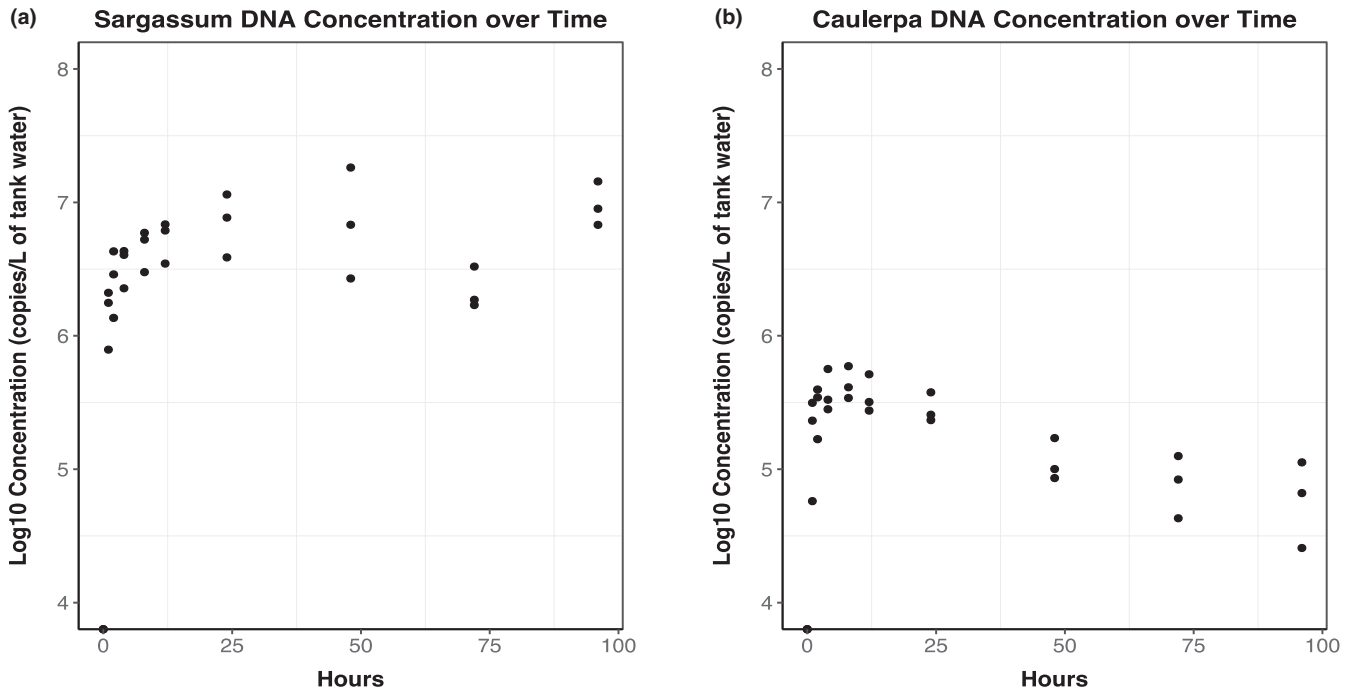

FIGURE 2 Plots of the tank eDNA concentrations over time in log form. *S. horneri* shows an initial jump in eDNA copies/L and then a steady plateau after 24 h. *C. prolifera* exhibits an initial spike in concentration before decreasing and leveling off after 48 h. Steady state for both was reached at the 96-h time point.

26374943, 2024, 1, Downloaded from https://onlinelibrary.wiley.com/doi/10.1002/edn3.496 by University of California - Los Ange, Wiley Online Library on [01/03/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

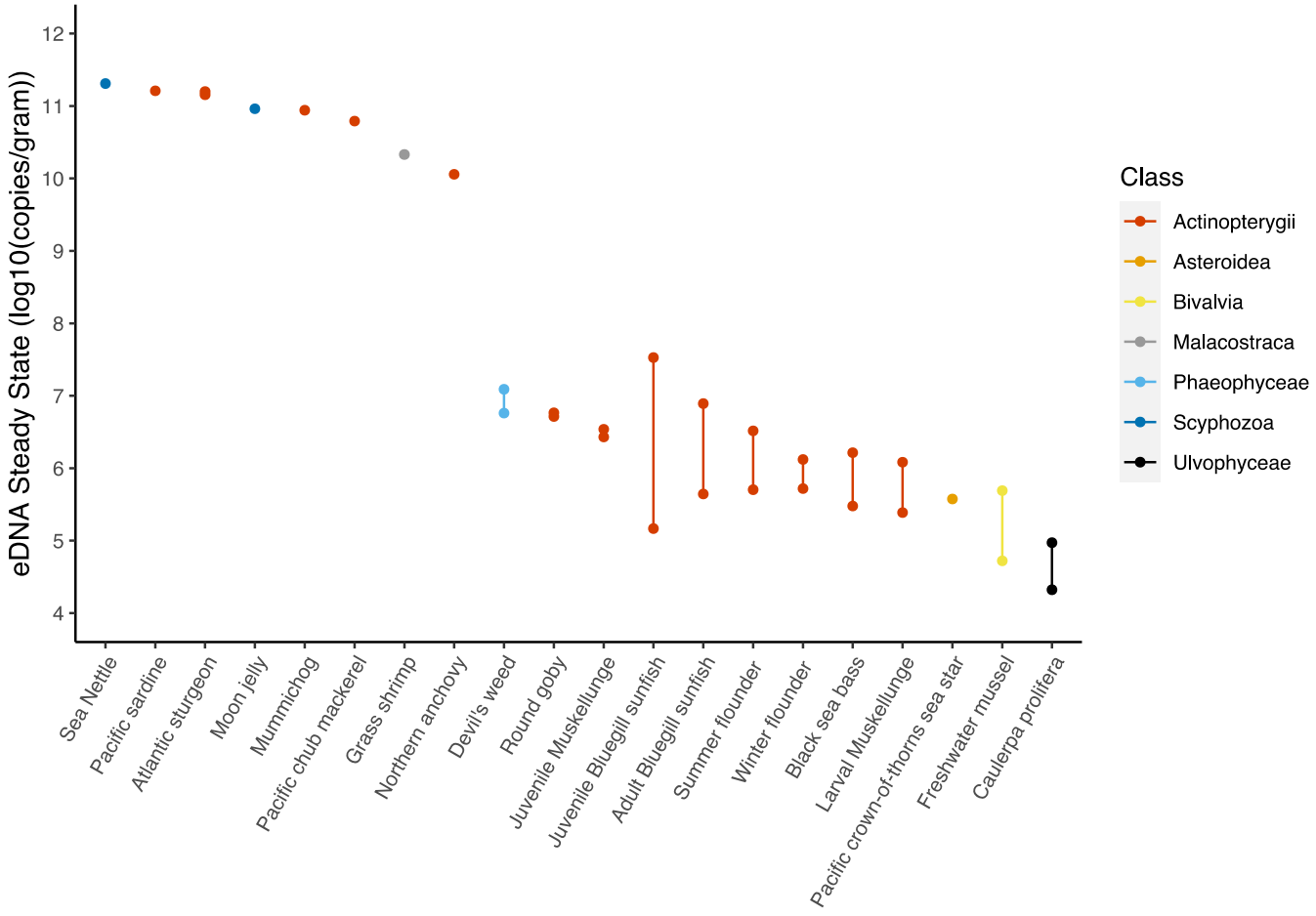

FIGURE 3 Log_{10} conversion of the steady-state concentration of eDNA by species and class. Where variable steady states were reported between experiments, we plot the lowest and highest rates reported and the range in values is indicated by the bar linking two points. Those with one steady-state rate only reported one shedding and decay rate. Scientific names given in Table S2.

eDNA steady-state concentrations. Our results have implications on the use of eDNA in the field of invasive species monitoring and on our understanding of eDNA shedding mechanisms.

4.1 | *C. prolifera* sheds negligible amounts of DNA

We demonstrate *C. prolifera* to have the lowest recorded eDNA steady-state concentration of any currently reported species. We hypothesize that the low steady-state eDNA concentration of *C. prolifera* can be attributed to the algae's unique organismal and cellular biology. The genus *Caulerpa* is home to some of the largest single-celled organisms in the world (Jacobs, 1994). *C. prolifera* is a multinucleated single-celled macroalgae which spreads primarily through asexual reproduction (Jacobs, 1994). Thus *C. prolifera* lacks conventional modes of eDNA release including shed cells via gametes, mucus, and other cellular debris etc., which account for a considerable amount of total eDNA release for other species (Klymus et al., 2015; Thomsen & Willerslev, 2015). *C. prolifera* is unique in that as a single-celled organism it may not shed small cell-like units into the environment but, at the same time, cannot be easily captured via water filtration as is the case for bacteria and phytoplankton.

This leaves only a few potential modes of eDNA shedding, namely cellular leakage of mitochondria or free-floating DNA. Previous work has demonstrated that cellular leakage accounts for only a small proportion of total environmental DNA, and thus we would expect low *C. prolifera* shedding rates from this fact alone (Zhao et al., 2021). However, given the unique physiology of *C. prolifera* there are two possible reasons for further reduced cellular leakage in this species. The first is the thick cell wall surrounding the algae which acts to prevent regular shedding of cellular material into the water column (Jacobs, 1994). The second is *C. prolifera's* unique cellular organization that allows for dramatic morphological differentiation within a single cell. Previous studies have shown dramatic differences in gene expression across the organism despite being a single cell with shared cytoplasm, helping explain the unique morphology of fronds, rhizomes, etc., of the organism (Arimoto et al., 2019; Ranjan et al., 2015). This dramatic differentiation of distinct parts of the single cell, suggest additional cellular mechanisms to limit DNA and RNA activity and transport within the shared cytoplasm. It is currently unknown what mechanisms allow for such differentiation within the organism despite shared cytoplasm. However, one potential explanation may be a high degree of RNAse and DNAse activity which would limit the spread of transcription and translation to

26374943, 2024, 1, Downloaded from https://onlinelibrary.wiley.com/doi/10.1002/edn3.496 by University of California - Los Ange, Wiley Online Library on [01/03/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

specific regions of the cell, allowing for the substantial phenotypic differentiation observed across the organism. Such a mechanism may also act to reduce the amount of free-floating DNA available within the cytoplasm, and thus reduce the amount of eDNA shed via cellular leakage.

Together, these factors strongly suggest that the unique physiology and morphology of *C. prolifera* contribute to low shedding rates. Unfortunately, our results indicate that this invasive species is uniquely equipped to evade detection through eDNA surveying, indicating the need for alternative non-molecular detection methods in low visibility and difficult to survey environments.

We note that the other reported values from previously reported shedding and decay experiments also underestimate the amount of eDNA release that occurs in-situ. During tank-based trials, animal species are restricted from food before and during the experiment to minimize the amount of eDNA introduction to the tanks from sources such as feces. This means in the wild, when species have access to food and likely higher metabolic rates, eDNA release rates are expected to be higher. Previous work has demonstrated that sea stars in tank experiments that were given food released roughly 7× more eDNA than when not given food, strongly supporting this hypothesis (Kwong et al., 2021). Thus, given that the majority of steady-state concentrations were generated from heterotrophic species, we expect their relative eDNA shedding rates to be even higher than the photosynthetic *C. prolifera*, providing further evidence of distinctly lower steady-state eDNA concentrations of this invasive species.

Furthermore, a large number of aquatic plant and animal species also introduce eDNA through the release of gametes during spawning events. However, *C. prolifera's* predominantly asexual mode of reproduction limits shedding rates compared to broadcast spawning organisms (Smith & Walters, 1999). Thus, we are confident that values presented for *C. prolifera* in this study appropriately capture the expected steady-state concentration this species would exhibit in the wild.

We note that *C. prolifera* steady-state concentrations at 96 h were 5%–20% of the maximum concentration (Figure 2). However, previous tank experiments results show an initial spike in DNA concentration as a result of stress to the organism, leading to greater cellular degradation, and thus more free-floating cells and materials (Klymus et al., 2015; Nevers et al., 2018) followed by a decline in production. All values used in our comparisons of steady-state concentrations were reached within 24–48 h into their respective experiments so as to avoid any differential physiological effects of initial stress influencing our comparisons.

Additionally, the steady-state values calculated were normalized by grams of biomass so that the values were comparable across taxa. This metric undervalues the difficulty in detecting *C. prolifera* in-situ compared to other species in this list. The next lowest mean steady-state concentrations are from the freshwater mussel and Pacific crown-of-thorns sea star (Kwong et al., 2021; Sansom & Sassoubre, 2017). These species weigh roughly 100 and 3000 g, respectively, whereas a single *C. prolifera* frond weighs just a fraction of a gram. A colony of *C. prolifera* that consists of 100–3000 g of biomass would make environmental DNA as a tool unnecessary because the patch, likely to be multiple square meters in size depending on its density, would be easily visible to conventional visual surveys.

4.2 | eDNA as a tool to track *Sargassum horneri*

Previous *S. horneri* research found that removal efforts have considerable challenges to success once the algae has been established (Marks et al., 2017). This emphasizes the importance of early detection to the protection of our coastal ecosystems. This study calculated *Sargassum horneri*'s steady-state concentration to be roughly 10^7 copies/g which placed it well within the range of previously recorded fish and invertebrates. We demonstrate here that because of its relatively high steady-state concentration, and large biomass in the wild, *S. horneri* is an ideal candidate for environmental DNA detection as evidenced by previous detections via metabarcoding (Ely et al., 2021; Gold et al., 2022). Specifically, the use of this ddPCR assay in areas with low abundance before species establishment, environments of high turbidity and low visibility, locations that are difficult to dive in, and in ballast water of ships would allow for higher sensitivity monitoring and earlier intervention for this invasive species. State and federal agencies such as CDFW, NOAA, USGS, and USFWS that are tasked with monitoring and stopping invasive species would particularly benefit from the use of eDNA to monitor *Sargassum horneri* populations.

4.3 | Implications for environmental DNA studies

The results of this study show a multiple order of magnitude difference in species' eDNA steady-state concentrations. The differential steady-state values highlight the influence various eDNA release modes play in detection probabilities and the difficulty in ascribing quantitative metrics to eDNA data between species. Notably, fish species show a significant range of steady-state concentrations from 10^6–10^{11} copies/g. Fish with a higher steady-state concentration are expected to have higher probabilities of being detected in the wild. Furthermore, we expect that all else being equal, species with five orders of magnitude higher steady-state concentrations will be overrepresented in environmental DNA surveys. As the field of eDNA moves to be more quantitative, accounting for differences in such biases will be critical (Harrison et al., 2019). Importantly, we find that relative shedding rates operate on similar orders of magnitude as amplification efficiencies, and thus controlling for both biases will likely be critical for deriving quantitative metabarcoding approaches (Shelton et al., 2023). Our meta-analysis also highlights the limited number of non-fish species in eDNA shedding and decay experiments. As eDNA aims to become a holistic monitoring tool for biodiversity, characterizing shedding, and decay relationships across a broad diversity of taxa and not just those that are commercially important, will be crucial to understanding the applicability of this

26374943, 2024, 1, Downloaded from https://onlinelibrary.wiley.com/doi/10.1002/edn3.496 by University of California - Los Ange, Wiley Online Library on [01/03/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

TABLE 2 Summary of best practices for eDNA ddPCR invasive species assays before field deployment.

Stage	Recommendation	Explanation
Primer/probe creation	Sanger sequence invasive tissue	The reference sequences available on NCBI might not be the same sequence as your target invasive's sequence. Sanger sequencing ensures the researchers primers and probe have no mismatches
Primer/probe creation	Follow eDNA primer/probe guidelines	For high-efficiency and high-sensitivity assays, follow established guidelines for primer and probe creation, such as Klymus et al. (2020)
Primer/probe creation	qPCR for primer efficiency	When working with ddPCR, testing primers using traditional qPCR is important for measuring primer efficiency (Ramón-Laca et al., 2021)
Pre-field deployment	Tank experiment	Running a tank experiment allows researchers to establish a species, eDNA shedding signature to better contextualize field results (Thalinger et al., 2021)

methodology for biodiversity monitoring efforts. Thus, additional studies to characterize shedding and decay rates, particularly for species of interest like invasives are clearly warranted.

Previous work has highlighted the utility of eDNA as a complement to conventional survey techniques (Bohmann et al., 2014; Kelly et al., 2017). In the case of invasive species, eDNA can aid in early detection of areas of concern given the sensitivity of qPCR/ddPCR assays; however, there is always value for 'boots on the ground' confirmation, especially when there are significant management implications (Gold et al., 2022). A strong advantage of eDNA is the ability to reduce the complexity of the field logistics by narrowing the range of visual surveys and the time it would take to complete them. Our study demonstrates than an eDNA approach is not equally effective for all species and was particularly ineffective in capturing the *C. prolifera* signal in the field using standard eDNA collection protocols. We demonstrate the value in benchmarking eDNA assays both in the lab and in the field prior to its deployment as a monitoring tool. Best practices in method validation should be adopted for all eDNA assays to ensure that results in the field, such as the negative results obtained in this study, are properly scrutinized and validated. These practices are summarized below (Table 2).

With any new methodology, it is important to understand its strengths and limitations. Understanding the value and shorting comings of eDNA is especially important so that researcher and managers can maintain reasonable expectations when deploying novel molecular assays. As demonstrated here, there may be applications where eDNA-based approaches cannot adequately replace traditional methods. Researchers must use caution and conduct rigorous validation of eDNA assays in the field and lab to understand the efficacy of this tool within a given system. Our study here presents a cautionary tale for eDNA applications and we expect there to be dozens if not hundreds of more taxa that similarly cannot be readily detected because they exhibit low steady-state eDNA concentrations. Future limitations for the detection of *C. prolifera* eDNA may be ameliorated with the development of a more sensitive assay (perhaps targeting a chloroplast or mitochondrial marker gene which may be more robust than a ribosomal target) or the filtration of larger water volumes using tangential flow filtration to acquire hundreds to thousands of liters of water. Ultimately, we demonstrate the limitations of eDNA as a survey tool as it relates to the invasive algae *Caulerpa prolifera* and demonstrate the importance of contextualization and validation of eDNA assays for biomonitoring applications.

AUTHOR CONTRIBUTIONS

Conceptualization: TW, ZG, ST, RE. Formal Analysis: TW, KL, ZG. Funding Acquisition: TW, ST, RE. Investigation: TW, KL. Methodology: TW, KL, ZG. Supervision: ST, RE, ZG. Writing—Original Draft Preparation: TW. Writing—Review and Editing: All authors.

ACKNOWLEDGMENTS

Tanner Waters is supported by the UCLA Cota-Robles Fellowship, the National Science Foundation Graduate Research Fellowship, the Switzer Foundation Fellowship, and the UCLA Center for Diverse Leadership in Science Fellowship. Environmental DNA work in the Eagle lab is supported by a grant from the David and Lucile Packard Foundation (no. 2020-70931) to the Center for Diverse Leadership in Science at UCLA. Robert Eagle also acknowledges support from the Pritzker Endowment to UCLA IoES. Susanna Theroux acknowledges support from the National Philanthropic Trust. Zachary Gold was supported by the NOAA 'Omics program and NOAA Research and Development database project ID 24755. This is PMEL contribution number 5530. The authors acknowledge Dr. Jeana Drake and the Jacobs lab for the assistance in the use and access to the tanks used in the experiment. We thank Dr. Joshua Steele for his help in answering primer/probe design questions, and Dr. Kim Parsons and Ana Ramón-Laca for their help in assay design and PCR troubleshooting. We thank Jose Bacallao and the Cabrillo Marine Aquarium for the collection of the *Sargassum horneri* species. We also want to acknowledge Brian Owens, Christopher Potter, Terri Reeder, Keith Merkel, Dr. Robert Mooney, and others at California Department of Fish and Wildlife, the California Water Board, and Merkel & Associates for their help in identifying, surveying, and removing the Caulerpa from Newport Bay.

CONFLICT OF INTEREST STATEMENT

The authors have no conflicts of interest to declare.

DATA AVAILABILITY STATEMENT

Data from this study will be included in the supplemental file for this manuscript (Tables S1–S4).

26374943, 2024, 1, Downloaded from https://onlinelibrary.wiley.com/doi/10.1002/edn3.496 by University of California - Los Ange, Wiley Online Library on [01/03/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

ORCID

Tanner Waters https://orcid.org/0000-0002-7013-1837
Kylie Langlois https://orcid.org/0000-0002-1541-2919
Zachary Gold https://orcid.org/0000-0003-0490-7630
Susanna Theroux https://orcid.org/0000-0002-9812-7856
Robert A. Eagle https://orcid.org/0000-0003-0304-6111

REFERENCES

Andruszkiewicz Allan, E., Zhang, W. G., Lavery, A. C., & Govindarajan, A. F. (2021). Environmental DNA shedding and decay rates from diverse animal forms and thermal regimes. *Environmental DNA*, *3*(2), 492–514. https://doi.org/10.1002/edn3.141

Arimoto, A., Nishitsuji, K., Narisoko, H., Shoguchi, E., & Satoh, N. (2019). Differential gene expression in fronds and stolons of the siphonous macroalga, *Caulerpa lentillifera*. *Development, Growth & Differentiation*, *61*(9), 475–484. https://doi.org/10.1111/dgd.12634

Bax, N., Williamson, A., Aguero, M., Gonzalez, E., & Geeves, W. (2003). Marine invasive alien species: A threat to global biodiversity. *Marine Policy*, *27*(4), 313–323. https://doi.org/10.1016/S0308-597X(03)00041-1

Benson, D. A., Clark, K., Karsch-Mizrachi, I., Lipman, D. J., Ostell, J., & Sayers, E. W. (2015). GenBank. *Nucleic Acids Research*, *43*(Database issue), D30–D35. https://doi.org/10.1093/nar/gku1216

Bohmann, K., Evans, A., Gilbert, M. T. P., Carvalho, G. R., Creer, S., Knapp, M., Yu, D. W., & de Bruyn, M. (2014). Environmental DNA for wildlife biology and biodiversity monitoring. *Trends in Ecology & Evolution*, *29*(6), 358–367. https://doi.org/10.1016/j.tree.2014.04.003

Cao, Y., Raith, M. R., & Griffith, J. F. (2016). A duplex digital PCR assay for simultaneous quantification of the Enterococcus spp. and the human fecal-associated HF183 marker in waters. *Journal of Visualized Experiments*, *109*, 53611. https://doi.org/10.3791/53611

Curd, E. E., Gold, Z., Kandlikar, G. S., Gomer, J., Ogden, M., O'Connell, T., Pipes, L., Schweizer, T. M., Rabichow, L., Lin, M., Shi, B., Barber, P. H., Kraft, N., Wayne, R., & Meyer, R. S. (2019). Anacapa toolkit: An environmental DNA toolkit for processing multilocus metabarcode datasets. *Methods in Ecology and Evolution*, *10*(9), 1469–1475. https://doi.org/10.1111/2041-210X.13214

Cuthbert, R. N., Pattison, Z., Taylor, N. G., Verbrugge, L., Diagne, C., Ahmed, D. A., Leroy, B., Angulo, E., Briski, E., Capinha, C., Catford, J. A., Dalu, T., Essl, F., Gozlan, R. E., Haubrock, P. J., Kourantidou, M., Kramer, A. M., Renault, D., Wasserman, R. J., & Courchamp, F. (2021). Global economic costs of aquatic invasive alien species. *Science of the Total Environment*, *775*, 145238. https://doi.org/10.1016/j.scitotenv.2021.145238

Ely, T., Barber, P. H., Man, L., & Gold, Z. (2021). Short-lived detection of an introduced vertebrate eDNA signal in a nearshore rocky reef environment. *PLoS ONE*, *16*(6), e0245314. https://doi.org/10.1371/journal.pone.0245314

Fediajevaite, J., Priestley, V., Arnold, R., & Savolainen, V. (2021). Meta-analysis shows that environmental DNA outperforms traditional surveys, but warrants better reporting standards. *Ecology and Evolution*, *11*(9), 4803–4815. https://doi.org/10.1002/ece3.7382

Ficetola, G. F., Coissac, E., Zundel, S., Riaz, T., Shehzad, W., Bessière, J., Taberlet, P., & Pompanon, F. (2010). An in silico approach for the evaluation of DNA barcodes. *BMC Genomics*, *11*, 434. https://doi.org/10.1186/1471-2164-11-434

Gold, Z., Wall, A. R., Schweizer, T. M., Pentcheff, N. D., Curd, E. E., Barber, P. H., Meyer, R. S., Wayne, R., Stolzenbach, K., Prickett, K., Luedy, J., & Wetzer, R. (2022). A manager's guide to using eDNA metabarcoding in marine ecosystems. *PeerJ*, *10*, e14071. https://doi.org/10.7717/peerj.14071

Goldberg, C. S., Turner, C. R., Deiner, K., Klymus, K. E., Thomsen, P. F., Murphy, M. A., Spear, S. F., McKee, A., Oyler-McCance, S. J., Cornman, R. S., Laramie, M. B., Mahon, A. R., Lance, R. F., Pilliod, D. S., Strickler, K. M., Waits, L. P., Fremier, A. K., Takahara, T., Herder, J. E., & Taberlet, P. (2016). Critical considerations for the application of environmental DNA methods to detect aquatic species. *Methods in Ecology and Evolution*, *7*(11), 1299–1307. https://doi.org/10.1111/2041-210X.12595

Hamaguchi, M., Miyajima, T., Shimabukuro, H., & Hori, M. (2022). Development of quantitative real-time PCR for detecting environmental DNA derived from marine macrophytes and its application to a field survey in Hiroshima Bay, Japan. *Water*, *14*(5), Article 5. https://doi.org/10.3390/w14050827

Harrison, J. B., Sunday, J. M., & Rogers, S. M. (2019). Predicting the fate of eDNA in the environment and implications for studying biodiversity. *Proceedings of the Royal Society B: Biological Sciences*, *286*(1915), 20191409. https://doi.org/10.1098/rspb.2019.1409

Havel, J. E., Kovalenko, K. E., Thomaz, S. M., Amalfitano, S., & Kats, L. B. (2015). Aquatic invasive species: Challenges for the future. *Hydrobiologia*, *750*(1), 147–170. https://doi.org/10.1007/s10750-014-2166-0

Hulme, P. E. (2009). Trade, transport and trouble: Managing invasive species pathways in an era of globalization. *Journal of Applied Ecology*, *46*(1), 10–18. https://doi.org/10.1111/j.1365-2664.2008.01600.x

Invasive Species Specialist Group ISSG. (2023). *Global invasive species database*. http://www.iucngisd.org/gisd/100_worst.php

Jacobs, W. P. (1994). Caulerpa. *Scientific American*, *271*(6), 100–105.

Jousson, O., Pawlowski, J., Zaninetti, L., Zechman, F. W., Dini, F., Di Guiseppe, G., Woodfield, R., Millar, A., & Meinesz, A. (2000). Invasive alga reaches California. *Nature*, *408*, S6809. https://doi.org/10.1038/35041623

Kazi, M. A., Reddy, C. R. K., & Jha, B. (2013). Molecular phylogeny and barcoding of *Caulerpa* (Bryopsidales) based on the tufA, rbcL, 18S rDNA and ITS rDNA genes. *PLoS One*, *8*(12), e82438. https://doi.org/10.1371/journal.pone.0082438

Kelly, R. P., Closek, C. J., O'Donnell, J. L., Kralj, J. E., Shelton, A. O., & Samhouri, J. F. (2017). Genetic and manual survey methods yield different and complementary views of an ecosystem. *Frontiers in Marine Science*, *3*, 283. https://doi.org/10.3389/fmars.2016.00283

Klymus, K. E., Ramos, D. V. R., Thompson, N. L., & Richter, C. A. (2020). Development and testing of species-specific quantitative PCR assays for environmental DNA applications. *Journal of Visualized Experiments*, *165*, e61825. https://doi.org/10.3791/61825

Klymus, K. E., Richter, C. A., Chapman, D. C., & Paukert, C. (2015). Quantification of eDNA shedding rates from invasive bighead carp *Hypophthalmichthys nobilis* and silver carp *Hypophthalmichthys molitrix*. *Biological Conservation*, *183*, 77–84. https://doi.org/10.1016/j.biocon.2014.11.020

Kwong, S. L. T., Villacorta-Rath, C., Doyle, J., & Uthicke, S. (2021). Quantifying shedding and degradation rates of environmental DNA (eDNA) from Pacific crown-of-thorns seastar (*Acanthaster* cf. *solaris*). *Marine Biology*, *168*(6), 85. https://doi.org/10.1007/s00227-021-03896-x

Larson, E. R., Graham, B. M., Achury, R., Coon, J. J., Daniels, M. K., Gambrell, D. K., Jonasen, K. L., King, G. D., LaRacuente, N., Perrin-Stowe, T. I., Reed, E. M., Rice, C. J., Ruzi, S. A., Thairu, M. W., Wilson, J. C., & Suarez, A. V. (2020). From eDNA to citizen science: Emerging tools for the early detection of invasive species. *Frontiers in Ecology and the Environment*, *18*(4), 194–202. https://doi.org/10.1002/fee.2162

Marks, L. M., Reed, D. C., & Obaza, A. K. (2017). Assessment of control methods for the invasive seaweed *Sargassum horneri* in California, USA. *Management of Biological Invasions*, *8*, 205–213. https://doi.org/10.3391/mbi.2017.8.2.08

Maruyama, A., Nakamura, K., Yamanaka, H., Kondoh, M., & Minamoto, T. (2014). The release rate of environmental DNA from juvenile and adult fish. *PLoS One*, *9*(12), e114639. https://doi.org/10.1371/journal.pone.0114639

26374943, 2024, 1, Downloaded from https://onlinelibrary.wiley.com/doi/10.1002/edn3.496 by University of California - Los Ange, Wiley Online Library on [01/03/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

Meinesz, A., Belsher, T., Thibaut, T., Antolic, B., Mustapha, K. B., Boudouresque, C.-F., Chiaverini, D., Cinelli, F., Cottalorda, J.-M., Djellouli, A., El Abed, A., Orestano, C., Grau, A. M., Ivesa, L., Jaklin, A., Langar, H., Massuti-Pascual, E., Peirano, A., Tunesi, L., … Zuljevic, A. (2001). The introduced Green alga *Caulerpa taxifolia* continues to spread in the Mediterranean. *Biological Invasions*, *3*(2), 201–210. https://doi.org/10.1023/A:1014549500678

Merkel & Associates, Inc. (2006). *Final report on eradication of the invasive seaweed Caulerpa taxifolia from Agua Hedionda Lagoon and Huntington Harbour, California*. Merkel & Associates, Inc.

Molnar, J. L., Gamboa, R. L., Revenga, C., & Spalding, M. D. (2008). Assessing the global threat of invasive species to marine biodiversity. *Frontiers in Ecology and the Environment*, *6*(9), 485–492. https://doi.org/10.1890/070064

Nevers, M. B., Byappanahalli, M. N., Morris, C. C., Shively, D., Przybyla-Kelly, K., Spoljaric, A. M., Dickey, J., & Roseman, E. F. (2018). Environmental DNA (eDNA): A tool for quantifying the abundant but elusive round goby (*Neogobius melanostomus*). *PLoS One*, *13*(1), e0191720. https://doi.org/10.1371/journal.pone.0191720

NOAA Fisheries. (2022). *Caulerpa species on the West Coast|NOAA Fisheries (West Coast)*. NOAA. https://www.fisheries.noaa.gov/west-coast/habitat-conservation/caulerpa-species-west-coast

Owens, B. (2021). *Caulerpa prolifera* identified in Newport Bay. *Cal-IPC symposium*. https://www.cal-ipc.org/wp-content/uploads/2022/01/Cal_IPC_Symposium_2021_Brian_Owens_Caulerpa_prolifera_in_Newport_Bay.pdf

Parreira, F., Martínez-Crego, B., Lourenço Afonso, C. M., Machado, M., Oliveira, F., dos Santos, M., Gonçalves, J., & Santos, R. (2021). Biodiversity consequences of *Caulerpa prolifera* takeover of a coastal lagoon. *Estuarine, Coastal and Shelf Science*, *255*, 107344. https://doi.org/10.1016/j.ecss.2021.107344

Pimentel, D., Lach, L., Zuniga, R., & Morrison, D. (2000). Environmental and economic costs of nonindigenous species in the United States. *Bioscience*, *50*(1), 53–65. https://doi.org/10.1641/0006-3568(2000)050[0053:EAECON]2.3.CO;2

Plough, L. V., Bunch, A. J., Lee, B. B., Fitzgerald, C. L., Stence, C. P., & Richardson, B. (2021). Development and testing of an environmental DNA (eDNA) assay for endangered Atlantic sturgeon to assess its potential as a monitoring and management tool. *Environmental DNA*, *3*(4), 800–814. https://doi.org/10.1002/edn3.186

Ramón-Laca, A., Wells, A., & Park, L. (2021). A workflow for the relative quantification of multiple fish species from oceanic water samples using environmental DNA (eDNA) to support large-scale fishery surveys. *PLoS One*, *16*(9), e0257773. https://doi.org/10.1371/journal.pone.0257773

Ranjan, A., Townsley, B. T., Ichihashi, Y., Sinha, N. R., & Chitwood, D. H. (2015). An intracellular transcriptomic atlas of the Giant coenocyte *Caulerpa taxifolia*. *PLoS Genetics*, *11*(1), e1004900. https://doi.org/10.1371/journal.pgen.1004900

Sansom, B. J., & Sassoubre, L. M. (2017). Environmental DNA (eDNA) shedding and decay rates to model freshwater mussel eDNA transport in a river. *Environmental Science & Technology*, *51*(24), 14244–14253. https://doi.org/10.1021/acs.est.7b05199

Sassoubre, L. M., Yamahara, K. M., Gardner, L. D., Block, B. A., & Boehm, A. B. (2016). Quantification of environmental DNA (eDNA) shedding and decay rates for three marine fish. *Environmental Science & Technology*, *50*(19), 10456–10464. https://doi.org/10.1021/acs.est.6b03114

Seebens, H., Bacher, S., Blackburn, T. M., Capinha, C., Dawson, W., Dullinger, S., Genovesi, P., Hulme, P. E., van Kleunen, M., Kühn, I., Jeschke, J. M., Lenzner, B., Liebhold, A. M., Pattison, Z., Pergl, J., Pyšek, P., Winter, M., & Essl, F. (2021). Projecting the continental accumulation of alien species through to 2050. *Global Change Biology*, *27*(5), 970–982. https://doi.org/10.1111/gcb.15333

Shelton, A. O., Gold, Z. J., Jensen, A. J., D'Agnese, E., Andruszkiewicz Allan, E., Van Cise, A., Gallego, R., Ramón-Laca, A., Garber-Yonts, M., Parsons, K., & Kelly, R. P. (2023). Toward quantitative metabarcoding. *Ecology*, *104*(2), e3906. https://doi.org/10.1002/ecy.3906

Silva, S. S. D., Nguyen, T. T. T., Turchini, G. M., Amarasinghe, U. S., & Abery, N. W. (2009). Alien species in aquaculture and biodiversity: A paradox in food production. *AMBIO*, *38*(1), 24–28. https://doi.org/10.1579/0044-7447-38.1.24

Smith, C. M., & Walters, L. J. (1999). Fragmentation as a strategy for *Caulerpa* species: Fates of fragments and implications for Management of an Invasive Weed. *Marine Ecology*, *20*(3–4), 307–319. https://doi.org/10.1046/j.1439-0485.1999.2034079.x

Spens, J., Evans, A. R., Halfmaerten, D., Knudsen, S. W., Sengupta, M. E., Mak, S. S. T., Sigsgaard, E. E., & Hellström, M. (2017). Comparison of capture and storage methods for aqueous macrobial eDNA using an optimized extraction protocol: Advantage of enclosed filter. *Methods in Ecology and Evolution*, *8*(5), 635–645. https://doi.org/10.1111/2041-210X.12683

Stam, W. T., Olsen, J. L., Zaleski, S. F., Murray, S. N., Brown, K. R., & Walters, L. J. (2006). A forensic and phylogenetic survey of *Caulerpa* species (caulerpales, Chlorophyta) from the Florida coast, local aquarium shops, and E-commerce: Establishing a proactive baseline for early detection1. *Journal of Phycology*, *42*(5), 1113–1124. https://doi.org/10.1111/j.1529-8817.2006.00271.x

Steele, J. A., Blackwood, A. D., Griffith, J. F., Noble, R. T., & Schiff, K. C. (2018). Quantification of pathogens and markers of fecal contamination during storm events along popular surfing beaches in San Diego, California. *Water Research*, *136*, 137–149. https://doi.org/10.1016/j.watres.2018.01.056

Thalinger, B., Deiner, K., Harper, L. R., Rees, H. C., Blackman, R. C., Sint, D., Traugott, M., Goldberg, C. S., & Bruce, K. (2021). A validation scale to determine the readiness of environmental DNA assays for routine species monitoring. *Environmental DNA*, *3*(4), 823–836. https://doi.org/10.1002/edn3.189

Thomsen, P. F., & Willerslev, E. (2015). Environmental DNA – An emerging tool in conservation for monitoring past and present biodiversity. *Biological Conservation*, *183*, 4–18. https://doi.org/10.1016/j.biocon.2014.11.019

Wilder, M. L., Farrell, J. M., & Green, H. C. (2023). Estimating eDNA shedding and decay rates for muskellunge in early stages of development. *Environmental DNA*, *5*(2), 251–263. https://doi.org/10.1002/edn3.349

Zhao, B., van Bodegom, P. M., & Trimbos, K. (2021). The particle size distribution of environmental DNA varies with species and degradation. *Science of the Total Environment*, *797*, 149175. https://doi.org/10.1016/j.scitotenv.2021.149175

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Waters, T., Langlois, K., Gold, Z., Theroux, S., & Eagle, R. A. (2024). Hidden in plain sight: The invasive macroalga *Caulerpa prolifera* evades detection by environmental DNA methods. *Environmental DNA*, *6*, e496. https://doi.org/10.1002/edn3.496

26374943, 2024, 1, Downloaded from https://onlinelibrary.wiley.com/doi/10.1002/edn3.496 by University of California - Los Ange, Wiley Online Library on [01/03/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License