2025 Environmental Practicum Project Final Report: Assessing eDNA as a tool to monitor Southern California Estuarine health and invasive species impact

Client: Southern California Coastal Water Research Project (SCCWRP)

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Table of Contents

Abstract

Caulerpa Prolifera is a highly invasive species of green algae that has infiltrated Newport Bay and threatens to outcompete native species. Newport Bay is an area of great ecological importance, so management policies for invasive *Caulerpa* outbreaks are essential. The goal of this project is to contribute to the development of an eDNA assay in order to accurately detect *C. Prolifera* in the water column. In order to accomplish this, a new assay targeting the chloroplast *tufA* gene was tested and analyzed to investigate effectiveness in comparison to a previous assay targeting the nuclear ITS gene. Archival *C. Prolifera* samples that were previously collected from outbreaks that occurred in 2021 and 2022 were analyzed using digital droplet PCR. Results showed that the new assay has been able to detect at rates 1-2 orders of magnitude higher than the previous assay. The new assay was able to detect *C. prolifera* eDNA in the water column, however the concentration of DNA was shown to be at relatively low levels. *C. Prolifera* eDNA was detected with confidence specifically in May 2022 water samples at the surface and up to 100m away from the initial outbreak. Overall, eDNA technologies are a promising tool for more efficient management and monitoring of ecosystems.

1. Introduction

Invasive species pose a threat to local and global marine ecosystems given their quick reproduction time, lack of natural predators, and ability to outcompete native species allowing them to rapidly colonize new areas (Havel et al., 2015). In California, *Caulerpa taxifolia*, *Caulerpa prolifera*, and *Sargassum horneri* are the three invasive macroalgaes that have invaded marine ecosystems over the past several decades (Marks et al., 2017). *Caulerpa prolifera* (*C. prolifera*) has notable ease of spreading and significant impacts, including reduction of native species populations and disruption of local ecosystems (Waters et al., 2023). The algae has already established colonies in the Mediterranean, Australia, and California, likely originating from aquarium stores (Waters et al., 2023). *C. prolifera* was first discovered in California marine ecosystems in 2000 and quickly established itself as a biological threat to native Californian habitats (Jousson et al., 2000). Management and reduction of *C. prolifera* outbreaks is necessary to maintain the health and function of critical southern California marine ecosystems. The recent *C. prolifera* outbreak in Newport Bay, CA monitored by the Southern California Coastal Water Resource Project (SCCWRP) has raised concerns because of the ecological importance of the Newport Bay habitat and food source (Newport Bay Conservancy, 2025). Early detection and eradication of invasive outbreaks are critical for managing the spread of invasive species (Larson et al., 2020). eDNA offers the most effective monitoring tool for management of *C. prolifera* in California waters compared to traditional surveying techniques.

In the past, *C. prolifera* detection has relied on survey techniques where divers survey the benthos for evidence of *Caulerpa* colonies or fragments. However, in the turbid waters of southern California coasts, bays, and estuaries, sight-based surveys are not reliable in locating *Caulerpa*. Additionally, dive surveys only locate existing *Caulerpa* outbreaks when early detection of *Caulerpa* before it has colonized is crucial for preventing invasion. So, environmental DNA (eDNA) offers a more appropriate monitoring method because it allows *Caulerpa* to be detected in the water column before it has colonized. eDNA monitoring is well suited for difficult to survey environments and outcompetes conventional techniques (such as dive surveys) across all environments (Fediajevaite et al., 2021; Thomsen & Willerslev, 2015). Compared to traditional surveying methods, eDNA surveys offer a far more effective monitoring tool for marine organisms, in particular, for fast colonizing *Caulerpa*.

However, *C. prolifera* has unique uni-cellular biology which contributes to low-level shedding rates that make it difficult to detect in the water column (Waters et al., 2023). Waters et al. (2023) previously developed a droplet digital PCR (ddPCR) assay for detecting *C. prolifera* that targeted the same nuclear internal transcribed spacer (ITS) gene used in previous detection of other *Caulerpa* species. *C. prolifera* was found to have low steady-state eDNA concentrations relative to any other reported species, so detection of *C. prolifera* eDNA was difficult with the existing ITS assay (Waters et al. 2023). Waters et al. (2023) conducted tank-based experiments on *C. prolifera* shedding rates and determined that *C. prolifera* lacks conventional modes of eDNA release into the water column. This pertains to its lack of shedding of gametes, mucus,

and cellular debris (Waters et al., 2023). Not only does *C. prolifera* shed significantly low levels of eDNA, but as a single-celled organism, it is also difficult to capture via water filtration methods compared to phytoplankton and bacteria. With consideration to these limitations, a different assay was required to detect limited *C. prolifera* eDNA.

In response, SCCWRP has developed an in-house assay based on a different DNA sequence found by Draisma & Sauvage, (2024). Instead of targeting the nuclear ITS gene in *C. prolifera*, the chloroplast-encoded *tufA* gene is the DNA marker barcode of choice (Draisma & Sauvage, 2024). In collaboration with SCCWRP, this project aims to evaluate the efficacy of the new *tufA* assay through analysis of samples collected by Waters et al. (2023) in their initial investigation. Samples gathered from the 2021 *Caulerpa* outbreak in Newport Bay, waters receiving *Caulerpa* eradication treatment involving containment of area from future disturbances and diver-assisted removal of *C. prolifera*, and tank-based experiments investigating the shedding rate of *C. prolifera* were initially analyzed to identify *C. prolifera* eDNA from the ITS gene. By reanalyzing previous samples and new samples with the new *tufA* assay, we will be able to expand our understanding of the efficacy of utilizing eDNA to detect *C. prolifera*. Specifically, we should be able to determine if there are higher detection rates of *C. prolifera* using the *tufA* gene compared to the ITS gene. Furthermore, we should be able to assess if higher detection rates of *C. prolifera* using the *tufA* gene arise as a result of increased sensitivity of the assay due to targeting a chloroplast gene in comparison to ITS, disparities in the size of the 2023 and 2021 outbreaks sampled by Waters et al. (2023), or due to issue of the assay erroneously identifying other species of *Caulerpa* or algae.

The leading goals of this investigation are to expand the overall understanding of *Caulerpa* detection within the water column, aiding in monitoring and mitigation efforts for future outbreaks. Our research will be a continuation of work previously done by Waters et al. (2023) and Draisma & Sauvage (2024), and will contribute to an overall better understanding of eDNA applications for *C. Prolifera.* Improving eDNA applications for *C. prolifera* monitoring will make management of the invasive algae significantly more effective. We will develop communication outreach, including presentations and a website, to communicate our findings to broader audiences outside of SCCWRP.

1.1 Characteristics of Newport Bay

Newport Bay contains Newport Beach, Lower Newport Bay, and Upper Newport Bay. The Bay is located in Orange County, California about 35 miles south of Los Angeles (Trinast, 1975). The Upper Bay is considered a estuarine bay that supports salt-marsh vegetation (Trinast, 1975). It is one of the few largest remaining natural estuaries in Southern California making it a critical stop for migratory birds, nursery for commercial and noncommercial marine species, foraging area, and breeding ground for threatened and endangered species (Sutula et al., 2006). The Upper Bay is a completely enclosed tidal channel except for a shallow inlet that allows a limited exchange of Lower Bay (harbor) waters (Trinast, 1975). Despite the singular inlet, the

Upper Bay is crucial in supporting the infrastructure of the Lower Bay and Newport Beach areas (Newport Bay Conservancy, 2025). The bottom topography and channel physiography of the Upper Bay influences the tidal current patterns; notably, mixing and recirculation processes associated with gyre formation can prevent a complete exchange of waters over a tidal current (Trinast, 1975).

The Lower Bay hosts one of the largest small boat harbors in the United States and is critical for commercial and private vessels (Newport Bay Conservancy). It is defined by a shallow coastal lagoon that runs parallel to the coast. The Lower Bay has similar, if not the same, salinity and temperature as the adjacent open coastal waters (Trinast, 1975). The Lower Bay is influenced by the open coastal water tides that move through the artificial entrance channel. Unlike the enclosed Upper Bay system, the Lower Bay mimics open ocean tides and this holds implications for the movement and presence of *C. prolifera* in Lower Bay outbreaks.

1.2. Description of the Environment

The shedding rate of *C. prolifera* eDNA in Newport Bay is likely influenced by hydrological, hydraulic, and tidal conditions, which affect both the dispersal and degradation of genetic material. Strong tidal currents and wave action may increase fragmentation of *C. prolifera* tissues, enhancing eDNA release (Barnes et al., 2014). Conversely, stagnant or low-flow conditions could lead to localized eDNA accumulation, prolonging detectability (Harrison et al., 2019). During the 2021 and 2022 outbreaks, seasonal variations in water

Figure 1.1. Locations of April 2021 and March 2022 *Caulerpa* Outbreaks

temperature and salinity may have further modulated eDNA shedding, as microbial activity and enzymatic degradation rates are temperature-dependent (Collins et al., 2018). Additionally, sediment resuspension events, often driven by boat traffic or storm surges, could temporarily elevate eDNA concentrations by releasing buried genetic material (Turner et al., 2015).

The removal and management of invasive *C. prolifera* in Newport Bay involved a combination of manual extraction, chlorine barriers, and substrate sealing to prevent regrowth (Anderson et al., 2021). Early detection via eDNA monitoring played a critical role in guiding these efforts, as rapid response is essential to prevent further spread (West et al., 2022). Hydrological modeling was also employed to predict dispersal pathways, ensuring containment strategies accounted for tidal flushing and seasonal currents (Smith et al., 2020). Despite these measures, complete eradication remains challenging due to the resilience of *C. prolifera* fragments and the dynamic nature of estuarine environments. Continued eDNA surveillance, coupled with adaptive management, will be crucial for preventing future outbreaks (Greenstein et al., 2023).

1.3. Characterization of the *Caulerpa* Outbreaks

The first *C. prolifera* outbreak in July 2021 in Newport Bay (**Figure 1.1.**) occurred during a period of moderate tidal exchange and seasonal warming, which likely influenced both eDNA shedding rates and dispersal patterns. Hydrological conditions that summer were marked by weaker currents due to regional drought, potentially leading to localized eDNA accumulation in protected coves (Harrison et al., 2019). However, increased boating activity and its geographic location at the mouth of the bay, which are know vectors for *C. prolifera* fragmentation, may have offset this effect by mechanically dispersing tissue fragments, thereby elevating eDNA concentrations in high-traffic zones (Turner et al., 2015). Water temperatures peaked near 22°C, a range that promotes *C. prolifera* growth while simultaneously accelerating microbial degradation of eDNA, shortening its detection window (Collins et al., 2018).

Management efforts prioritized rapid response, combining manual removal by divers with chlorine-treated barrier systems to smother colonies (Anderson et al., 2021). Early eDNA sampling guided these efforts by identifying infestation hotspots, though tidal redistribution of fragments complicated eradication. Hydraulic dredging was avoided due to benthic habitat concerns, prompting reliance on physical removal and public outreach to curb human-mediated spread (West et al., 2022). Despite these measures, residual fragments persisted in sediments, highlighting the challenge of complete eradication in dynamic estuaries.

The second outbreak in May 2022 was planted deeper into Newport Bay (Figure 1a). The timing of this outbreak coincided with stronger El Niño-driven currents and higher turbidity, which likely enhanced eDNA transport while diluting its concentration in the water column (Barnes et al., 2014). Frequent winter storms resuspended sediments, burying some fragments while exposing others, creating patchy eDNA distribution that complicated monitoring (Smith et

al., 2020). Spring temperatures averaged 18°C, which was cooler than 2021, potentially slowing microbial eDNA degradation and prolonging detectability but also suppressing *Caulerpa* metabolic rates (Collins et al., 2018). These conditions reduced regrowth post-removal but demanded extended surveillance.

Adaptive strategies in 2022 included sediment vacuuming and expanded eDNA surveillance grids (Greenstein et al., 2023). Tidal modeling informed containment, and anchor restrictions were enforced in infested zones (Smith et al., 2020). Despite these advances, the recurrence underscored *Caulerpa's* resilience, particularly in cryptic habitats. Ongoing eDNA monitoring and public reporting protocols remain critical to mitigating future outbreaks (West et al., 2022).

2. Materials and Methods

2.1 Assay Design

To create the eDNA assay utilized in this analysis, SCCWRP referenced sequences for *Caulerpa* Prolifera from Draisma et al.(2024). SCCWRP aligned the sequences and potential primer/probe sets. The primer was created for the chloroplast-coded *tufA* gene based on primers encoded by Lagourgue et al.(2024). This primer was developed to target *tufA* based on previous work done by Waters et al.(2023) and Varela-Alvarez et al.(2015), that found the ITS gene to be an ineffective gene target for *Caulerpa* detection, and suggested that *tufA* may be a more viable target based on distribution profiles. Primer specificity was tested in-silico using Eco-PCR and showed species-specific *Caulerpa* prolifera amplification. The samples described in sections 2.2 and 2.3 were tested utilizing this new assay.

2.2 Environmental Sample Testing

Samples utilized in this study were previously collected by Waters et al.(2023) and tested for *Caulerpa* using an ITS-targeting assay. We re-ran those samples using SCCWRP's new assay in order to conduct comparative analyses of the two assays. Details regarding the outbreaks and sampling are provided below and further information can be found in Waters et al. (2023).

In April of 2021, scuba diving surveys discovered a C. Prolifera patch in China Cove, Newport Bay. *Caulerpa* was removed from this region between July 7th and July 14th of that year. Seawater samples were collected on July 30th, 2021 at the surface of the bed (33.596406, −117.879731), and then at depth, from 5, 10, 50, 100 and 500 meters bay-ward from the C. Prolifera patch (Waters et al. 2023). Samples taken above the patch were collected in 1 Liter Kangaroo enteral feeding bags above the patch. Samples taken further from the patch were collected in 5 Liter Niskin bottles. These samples were then transferred into 1 Liter Kangaroo bags in triplicates. All samples were then gravity filtered through a sterile 0.22 μm Sterivex cartridge filter before being stored for analysis.

Sampling for the next outbreak was conducted on May 5th, 2022. Seawater samples were collected at the surface and depth at multiple distances from the *C. Prolifera* patch before remediation efforts took place. Samples were taken 0, 5, 10, 50, 100 and 500 meters from the patch. These samples were similarly gravity filtered and stored for analysis. Diving teams estimate the outbreak was contained in two patches, equalling a combined area less than 0.4 square meters.

2.3 Shedding Experiment Testing

Alongside his work testing environmental DNA samples, Dr. Waters additionally conducted shedding experiments of two different invasive microalgae species: *Caulerpa Prolifera* and *Sargassum horneri*(Waters et al. 2023). These experiments were conducted in order to understand the shedding rates of *Caulerpa Prolifera* in comparison to other algal species in order to further understand the dynamics of *Caulerpa Prolifera's* shedding of DNA in the water column and associated challenges with detection.

The shedding experiment began with acquiring *C. Prolifera* and *Sargassum horneri* and leaving them to acclimate in tanks for 2 days, before data collection began. For each species, three replicate tanks were filled with 20 liters of deionized(DI) water and 36g/L of Instant Ocean sea salt for aquariums. To each respective tank, 23.99g, 24.44g, and 23.39g of *C. Prolifera.* The weights of all algae were measured prior to placement into the tanks. An additional tank, containing only artificial seawater, was utilized as a control variable.

Data collection began in the replicate tanks, following the acclimation period. Samples taken at the start of the experiment(hour 0), before species were added into the replicate tanks. Samples were taken at hours 1,2,4,8,12,24,48,72 and 96. Samples were collected by collecting 1 liter of tank water, which were gravity filtered into two sterile 0.22 μm Sterivex cartridge filters, wit 500 milliliters for each filter. Following sampling,1 Liter of seawater was added back into the replicate tanks. More specificity regarding the shedding experiment can be found in Waters et al.(2023).

For the context of this analysis, only the *C. Prolifera* samples from this shedding experiment were utilized in this study.

2.4. ddPCR Overview and Relevance

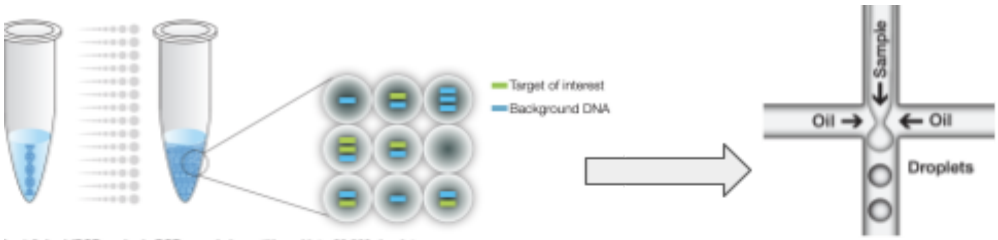
ddPCR or Droplet Digital PCR was the form of ddPCR used to run both the ITS and *tufA* assay. Polymerase chain reaction (PCR) is a technique used in molecular biology to make multiple copies of a specific DNA sequence. It works by repeatedly heating and cooling the DNA to separate the strands, attach short primers to the target sequence, and use an enzyme to build new strands. This process rapidly amplifies the DNA, making it easier to detect and

analyze specific genes or mutations (Khan Academy, 2017). Droplet digital PCR (ddPCR) is a highly sensitive form of polymerase chain reaction that detects specific target genes by dividing a DNA sample into thousands of droplets and amplifying the DNA within each droplet separately. SCCWRP chose to use ddPCR because of its unique qualities that differ from other forms of PCR, such as its degree of precision and quantification (Droplet Digital TM PCR Applications Guide). ddPCR results in absolute quantification, meaning that when the amplification process is complete, the number of target molecules are measured directly, this eliminates the need for additional analysis like using standard curves (Droplet Digital TM PCR Applications Guide). This level of quantification and precision is especially important when working with a species that sheds very little DNA, like *Caulerpa*.

Figure 2.1. shows the basic workflow of ddPCR which can be summarized by:

1. Sample is distributed into droplets when combined with oil
2. Droplets are placed in individual wells and run through the thermal cycler for amplification
3. After the amplification, each droplet is analyzed for fluorescence using the QX droplet reader
4. Droplets are spaced out in the reader to be analyzed
5. Each droplet is read as positive or negative based on fluorescence
    a. Positive = target sample present
    b. Negative = target sample is not present
    c. Ratio of positive to negative droplets reveals DNA concentration in sample

Figure 2.1. ddPCR Workflow



ig. 1.3. In ddPCR, a single PCR sample is partitioned into 20,000 droplets.

Fig. 1.4. The droplets created by the QX200

Droplet Generator are uniform in size and volume.

PCR Amplification

Droplets are transferred to a 96-well plate for PCR in a thermal cycler. We recommend the C1000 Touch™ Thermal Cycler with 96-deep well reaction module for PCR (Figure 1.5). This high-performance thermal cycler has excellent temperature uniformity and settling across all 96 wells to help ensure successful PCR.

Fig. 1.5. The C1000 Touch Thermal Cycler provides robust performance for ddPCR experiments.

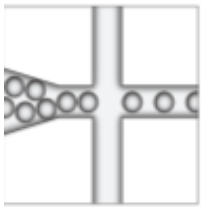
Droplet Reading

Following PCR amplification of the nucleic acid target in the droplets, the plate containing the droplets is placed in a QX100 or QX200 Droplet Reader, which analyzes each droplet individually using a two-color detection system (set to detect FAM and HEX [or VIC]).

The autosampler of the droplet reader picks up the droplets from each well of the PCR plate (Figure 1.6).

Fig. 1.6. QX200 Droplet Reader autosampler.

Droplets are spaced out individually for fluorescence reading by the droplet reader (Figure 1.7). Fluorescence in two channels is then measured for individual droplets.



ig. 1.7. Separating individual droplets in the QX100 Droplet Reader.

Positive droplets, which contain at least one copy of the target DNA molecule, exhibit increased fluorescence compared to negative droplets (Figure 1.8).

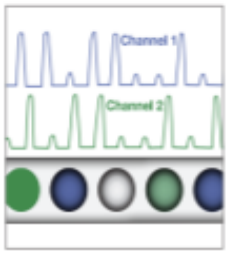


Fig. 1.8. Fluorescence readings are measured for each droplet in two channels.

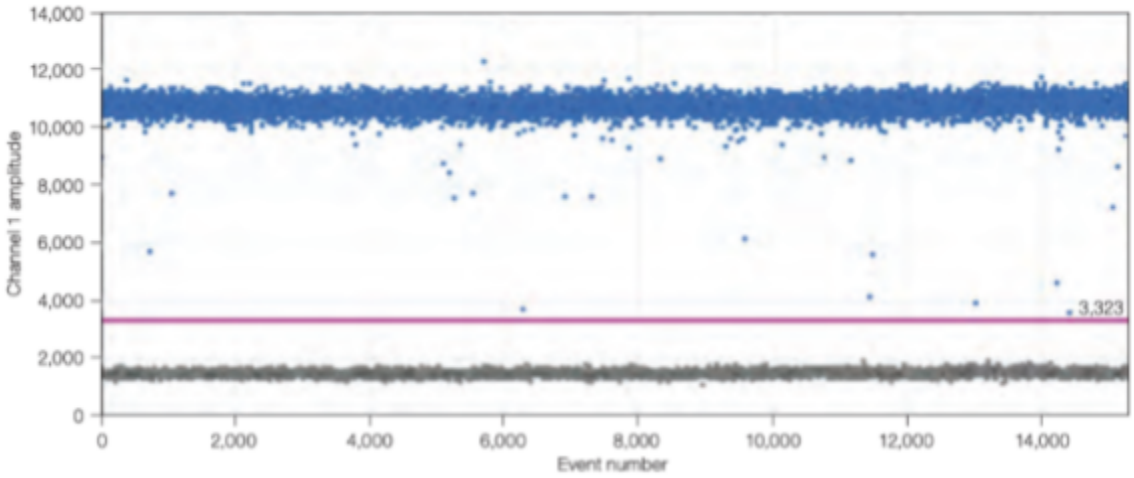


Figure 2.2. ddPCR fluorescence graph with threshold.

In **Figure 2.2.,** you can see a graphical representation of the results, the pink line represents the threshold, the points in blue above are positive and the grey points below the threshold are negative (Droplet Digital TM PCR Applications Guide). The threshold will vary depending on the experiment. For Sccrwp's *tufA* assay, the threshold of detection is set at 1 positive droplet but the limit of quantification (LOQ) is set at 3 positive droplets. The LOQ is set on an individual well basis (Limit of detection is done on an individual well). This is calculated per sample based on the total number of droplets and uncertainty is considered below 3 positive droplets. These thresholds are based on a combination of calculations from BioRad and from SCCWRP.

3. Results

3.1 Field Samples

2021 Outbreak

From the 2021 outbreak, none of the field samples taken directly above or from a distance away from the *C. Prolifera* outbreak detected *C. Prolifera* eDNA. This was consistent in both surface and depth samples. As can be seen in **Figures 3.1. - 3.3.,** while *tufA* concentrations were higher than ITS concentrations, the values remained below the Limit of Quantification(LOQ). This is consistent with Dr. Waters' findings with the ITS assay, as his assay was unable to detect *C. Prolifera* eDNA.

LOQ, in the context of the *tufA* assay, was set to three positive droplet detections. The Limit of Quantification is the lowest concentration at which *Caulerpa* can be accurately and

precisely measured with a level of confidence. This means that while a sample may be below the LOQ, the sample may have positive droplet detections under lower confidence.

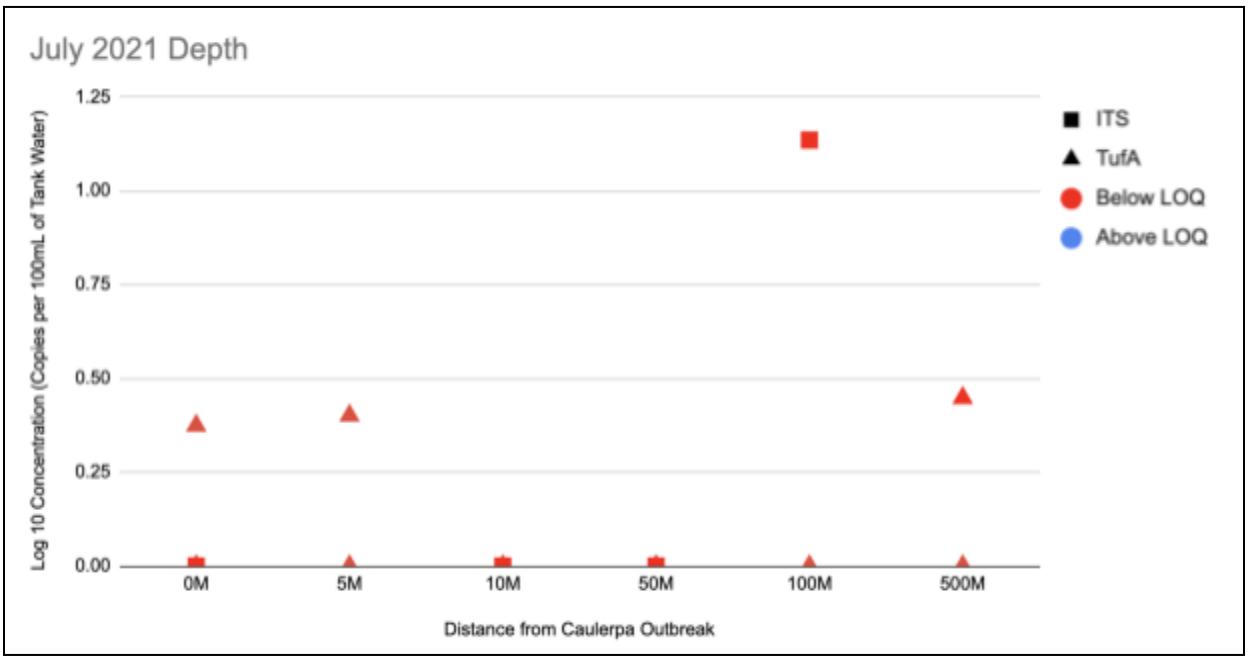


Figure 3.1. July 2021 Depth Sample Detection. Limit of Quantification or LOQ refers to the lowest concentration that can accurately and precisely be measured with an adequate level of confidence. This does not mean that the samples above did not include positive droplet detections, simply that these cannot be relied upon with a level of confidence.

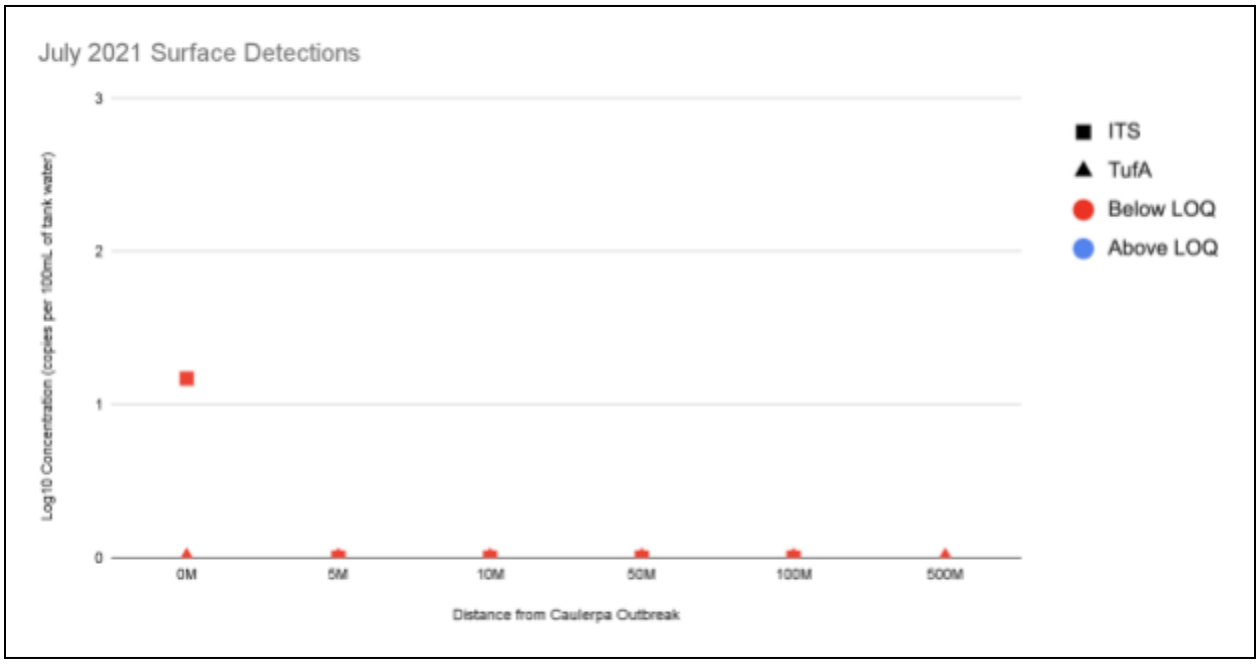


Figure 3.2. July 2021 Surface Detections. None of the data points were above the limit of quantification. This does not indicate negative results, simply reflects low confidence in the results meaning they cannot be relied upon as a detection.

2022 Outbreak

From the 2022 outbreak, field eDNA samples were able to detect *C. Prolifera* in surface samples away from the patch. While higher concentrations of *C. Prolifera* were detected using this assay, only a handful of samples exceeded the LOQ. There were no detections of *C. Prolifera* in eDNA samples taken at depth.

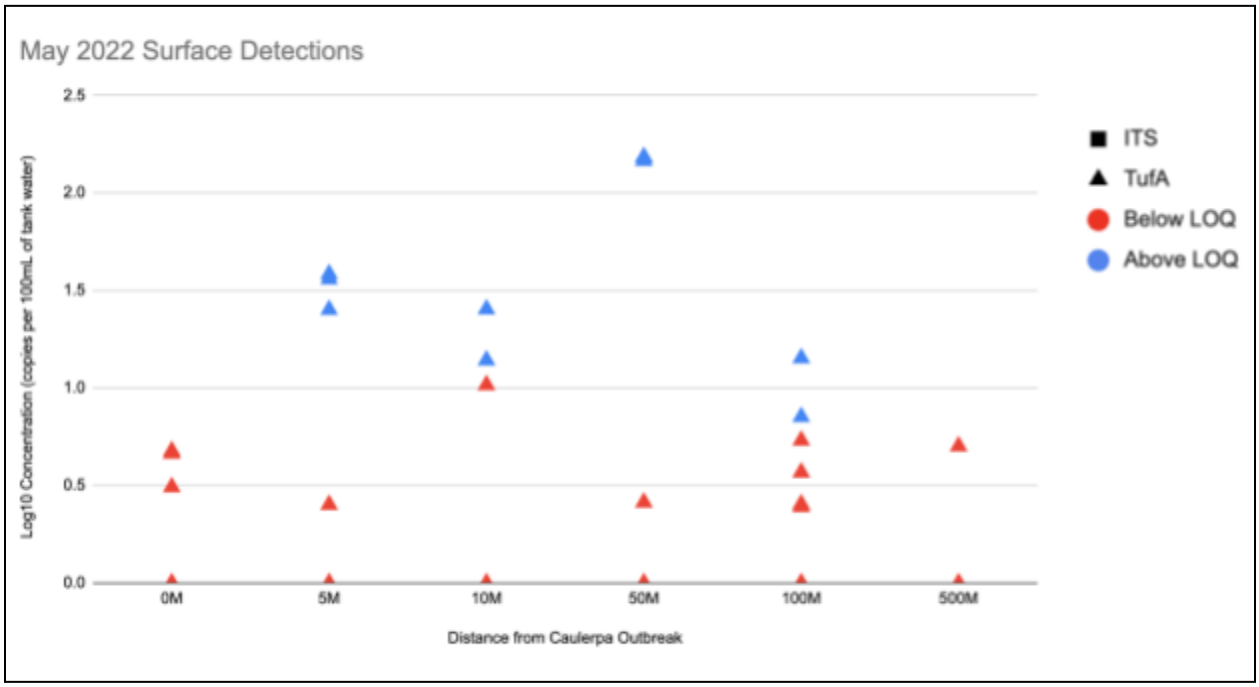


Figure 3.3. May 2022 Surface Detections Note: Some of the data points were above the limit of quantification. This does not indicate negative results, simply reflects low confidence in the results meaning they cannot be relied upon as a detection.

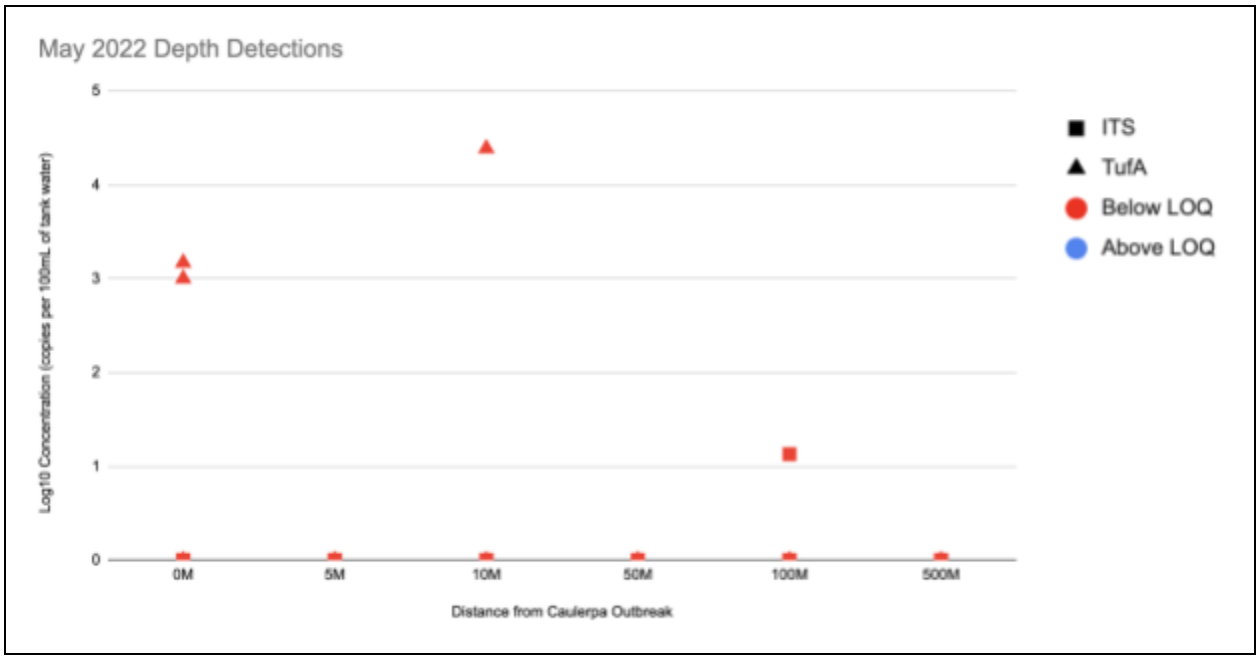


Figure 3.4. May 2022 Depth Detections. Note: none of the data points were above the limit of quantification. This does not indicate negative results, simply reflects low confidence in the results meaning they cannot be relied upon as a detection.

3.2 Tank-Based Experiment.

Comparative analysis of the ITS results from Dr. Waters' assay and our new assay, found drastic improvements in detection of *Caulerpa* of magnitudes of 1-2 (**See Figure 3.3.**). In comparing this Tank-based experiment with the Sargassum results from Dr. Waters' assay, while accounting for differences in units, we can see (**Figure 3.5.**) has similar values to the results from Waters et al albeit with higher detection rates. This suggests that *Caulerpa* shedding rates, in comparison to other species, are still low.

Caulerpa detection rates between both *tufA* and ITS assay provided similar trends regarding eDNA concentration over time. *C. Prolifera* is characterized by a high initial increase in initial eDNA concentration, gradually decreasing with time. At around hour 96, the DNA concentrations begin to plateau. The tank based experiment overall, provides evidence that in a controlled environment, the *tufA* assay sees higher rates of detection in relation to the ITS assay. However, in comparison to Sargassum and other similar species, the eDNA steady-state concentrations of *Caulerpa* still remain lower than other species.

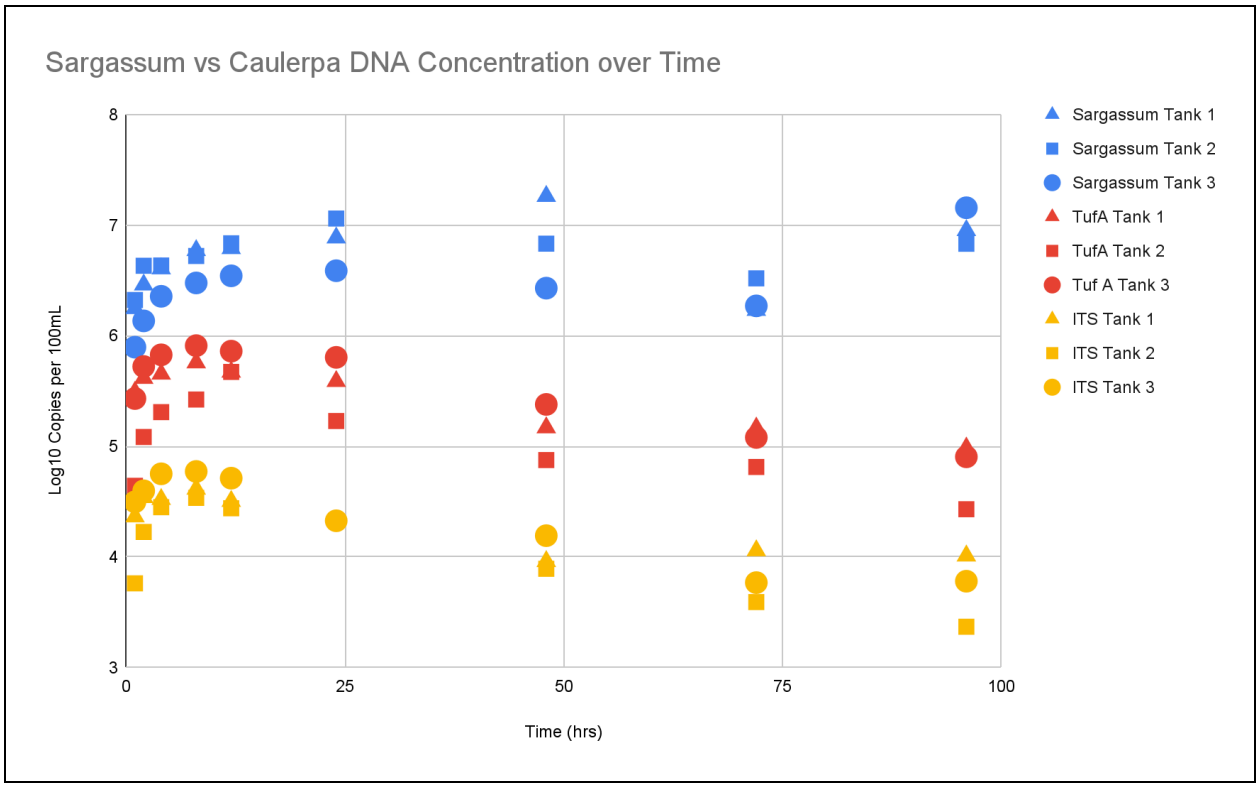


Figure 3.5. Comparison plots of tank eDNA concentrations over time in log form. This figure shows a comparison of eDNA concentrations overtime for Sargassum and *Caulerpa* (for both ITS and *tufA* genes). Note: Calculations utilized copies per 100mL, while Waters et al. utilized copies per L.

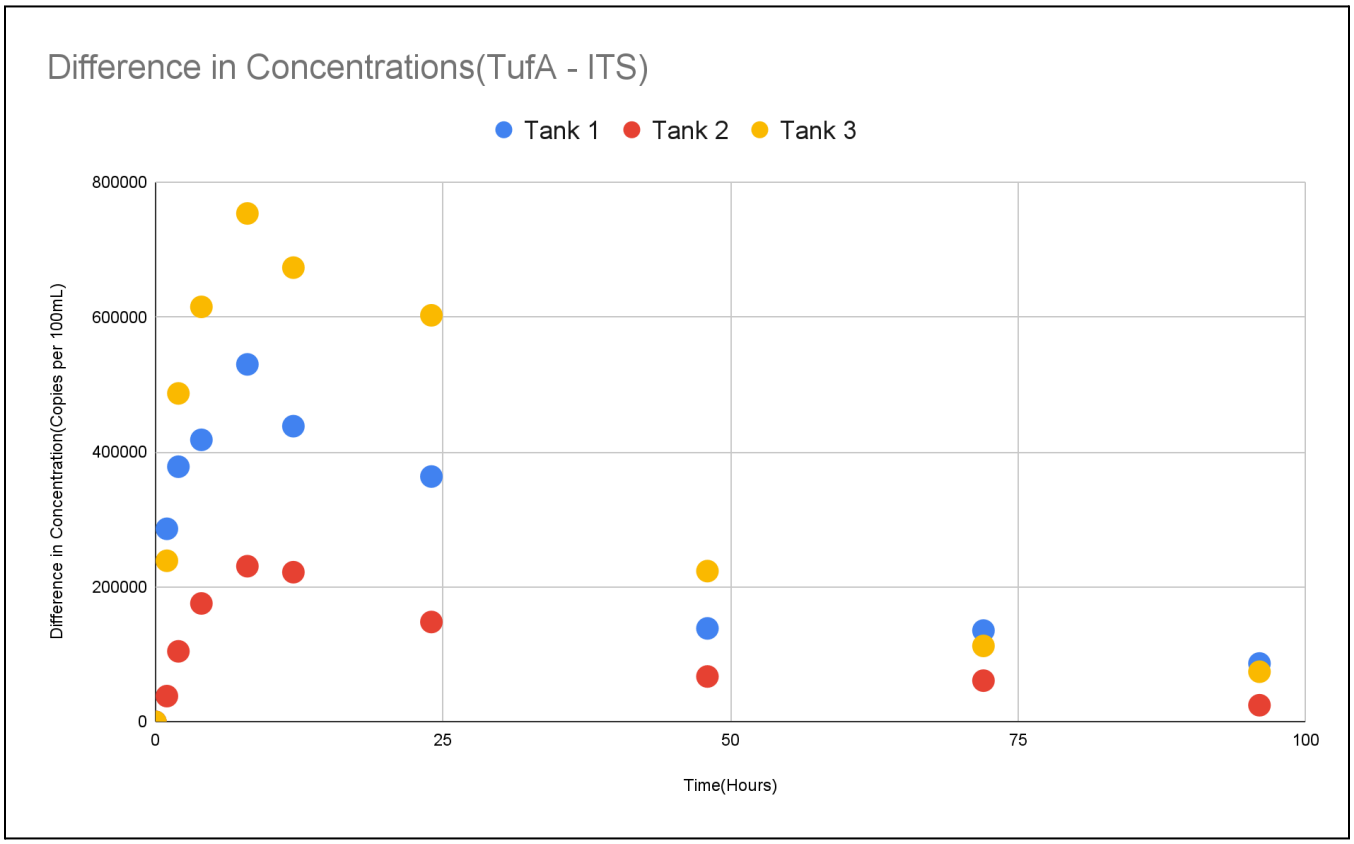


Figure 3.6. Differences in Concentrations Between *tufA* and ITS from the Tank samples. This figure shows the difference between concentrations found in *tufA* and ITS from the tank samples. This is not adjusted for LOQ, but simply detection concentrations in copies per 100mL.

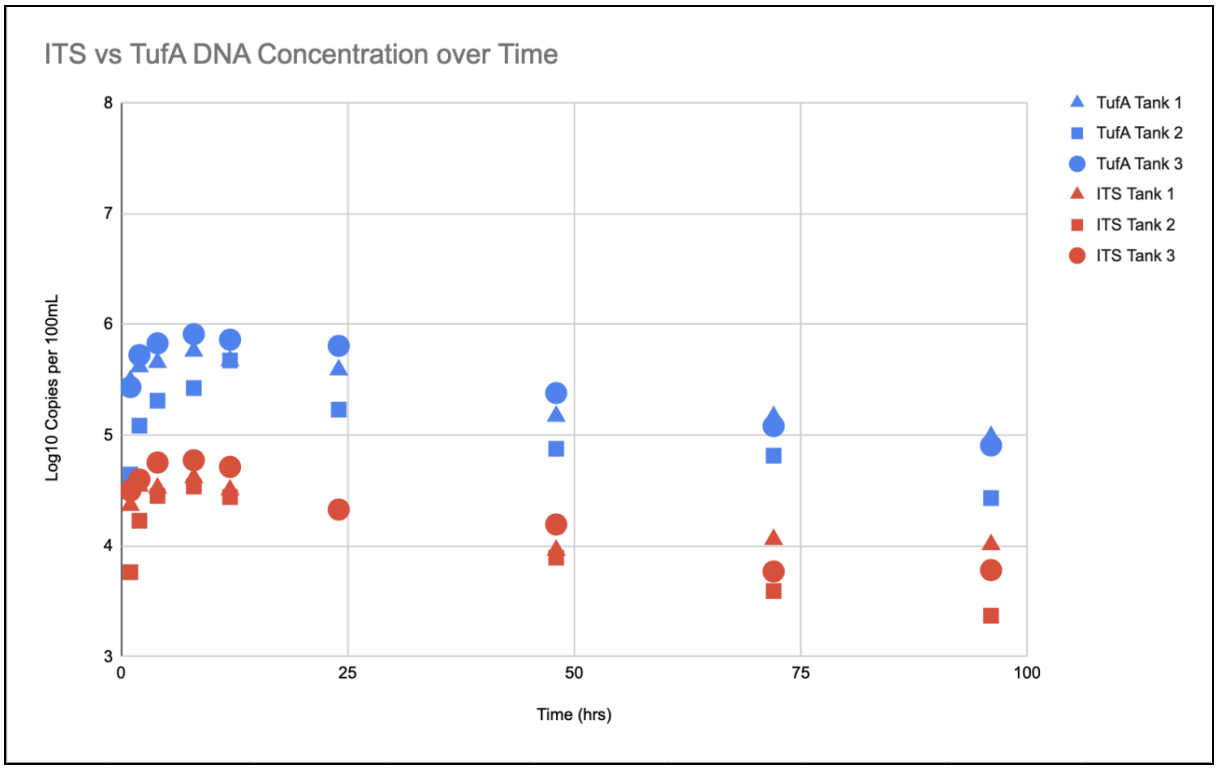


Figure 3.7. ITS vs *tufA Caulerpa* DNA Concentrations over time This figure compares detected concentrations of *Caulerpa* between the *tufA* assay and the ITS assay from the Tank-based experiment. Some points on the graph overlap, specifically ITS samples overlap at hour 24 and at hour 72.

4. Discussion

4.1. SCCWRP lab samples/Tanner lab samples analysis

The tank-based experiments yielded quantifiable eDNA results for both assays, characterized by the similar trends in increases and decreases in detected concentrations. Both assays see an initial increase in concentration, before a gradual decline and plateau to its steady state. The ITS assay plateaus at roughly 10^4 copies per 100 mL, while the *tufA* assay plateaus at roughly 10^5 copies per 100mL. This indicates that the *tufA* assay detects tank-based samples at a magnitude of roughly 1 higher than the ITS assay.

4.1.1 Shedding Rates

This increase in detection is rather notable, given that when comparing the steady-state concentrations of *C. Prolifera* under this new assay, with previous literature on steady-state concentrations, the new assay finds steady-state concentrations closer to the values of other species.

With the results from the tank-based experiments, we were able to plot an updated figure that conveys the shedding rate of *Caulerpa* with the *tufA* target gene against the ITS target gene (**Figure 3.5.**). The tank-based experiments using the *tufA* gene conveyed that steady state was reached by 96 hours, which displayed a concentration range of 10^5 copies per 100mL. First, copies per 100 mL were scaled to copies per liter (copies/L) by multiplying by 10. This was then divided by the wet weight (g) of *Caulerpa* in each tank (20.47g, 23.49g, and 22.36g, respectively) to account for tissue mass. This yielded steady-state eDNA shedding rates in copies/g, enabling cross-species comparisons. For logarithmic scaling (log10(copies/g)), values were transformed after normalization. This resulted in *Caulerpa* detection of about 2.9 - 3.7 log10(copies of DNA/gram) with the ITS target assay, whereas the *tufA* target assay detected about 4 - 4.7 log10(copies of DNA/gram). In other words, the *tufA* target assay had improved *Caulerpa* eDNA detection in the tank experiments by nearly 2 orders of magnitude. The data used to calculate the steady state shedding results from the ITS gene marker was provided by SCCWRP. Refer to **Appendix D** for the specific data and calculations used for these results.

It is important to note that the results we calculated for the ITS steady state concentrations, based on the raw data provided by SCCWRP, are different from the results that Waters had reported in his own report. Due to time constraints we were unable to resolve this discrepancy, and it may be in SCCWRP's interest to address this discrepancy to further validate these results.

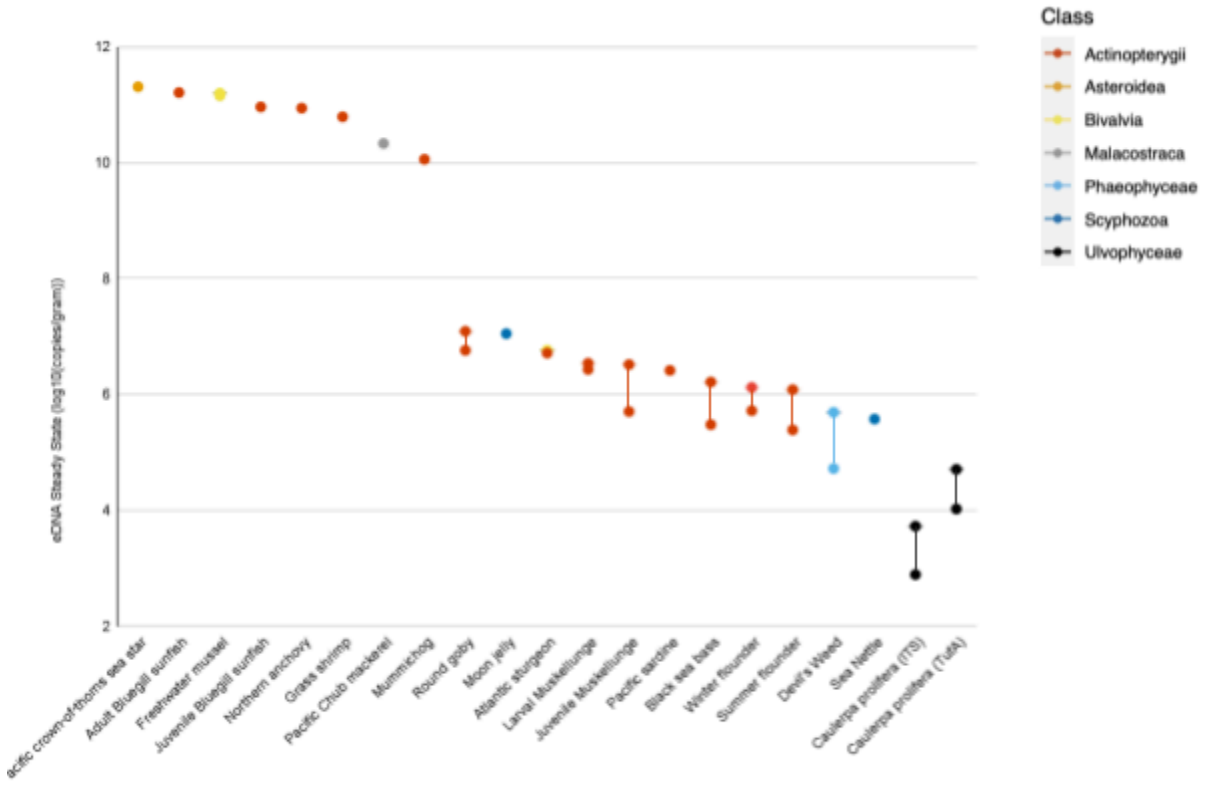


Figure 4.1. Shedding Rate Comparison Graph. Log10 conversion of the steady-state concentration of eDNA by species and class. Where there were multiple, varied steady states reported, we plot the lowest and highest rates reported and placed a bar along the points to indicate the range. Those with one steady-state rate only reported one shedding and decay rate.

It is important to note that tank-based experiments are not representative of real-oceanographic conditions given the lack of hydrological conditions and the controlled environment under which the experiments were performed. Furthermore, in comparison to field sampling, the concentrations within the tank samples would be drastically higher given the known presence of *Caulerpa* and visual detectability of *Caulerpa* within samples. These tank-based experiments, while useful in comparing the efficacy of the two assays, are not representative of how these assays perform in the field.

Furthermore, in comparison to other species, specifically animals, tank-based experiments do not include the feeding of animals. This means that animals tested within tank-based experiments likely exhibit higher metabolic rates and shedding rates in the field due to higher releases of fecal matter into the water column.

4.1.2 Efficacy of tufA Assay

The July 2021 sampling results indicate that both assays were unable to successfully detect *C. Prolifera* eDNA samples both above and away from the outbreak patch. Although we currently have limited information regarding the sampling event, it is speculated that Waters et al. mislabelled his samples from the April 2021 outbreak in his report based on correspondence with the dive team and labelling of the samples. In his prior report, Waters et al. described sampling to occur within included a sampling date of June 30th, however dive teams discuss that sampling occurred in July of 2021, after remediation efforts occurred. This may explain why these results are low.

The May 2021 sampling results indicate that the *tufA* assay was successfully able to detect *C. Prolifera* eDNA samples above the LOQ at surface but not at depth. This provides positive signs that may indicate an improved efficacy of this new assay, however more investigation must be conducted in order to provide more conclusive evidence.

4.2. Validation of appropriate baseline for what is considered positive detection in eDNA research

For eDNA research, an appropriate baseline of positive hits in ddPCR analysis is required to determine what can be considered positive detection of *C. prolifera* eDNA. Justification of an appropriate baseline allows us to more accurately interpret what the data is indicating about eDNA detection in our and Waters et al. (2023) sample analysis. In the relatively new area of eDNA investigation, universal guidelines are needed to determine a limit of detection (LOD) in PCR-based analysis of low-concentration DNA (Hunter et al., 2016). *C. prolifera* has unique low levels of eDNA shedding and, therefore, requires careful consideration of what is defined as a positive hit. Limit of detection and limit of quantification (LOQ) need to be defined in order to refrain from overestimating DNA concentrations. Limit of detection is defined as the lowest possible analyte concentration likely to be reliably distinguished from background noise indicating if the signal can be reliably detected (Ambruster & Pry, 2008). Limit of quantification is defined as the lowest concentration that can be accurately and precisely measured which may be equivalent or higher than the determined LOD (Ambruster & Pry, 2008).

4.2.1. Rule of Three

The company Bio-Rad partners with test-kit providers to develop ddPCR assays. For estimating theoretical LOD, Bio-Rad recommends the Rule of Three. Based on Poisson's law of small numbers, if there is a random distribution of quantifiable, independent events, predictions can be made on which event is more likely to occur (Bio-Rad). Poisson predicts the degree of spread (95% confidence interval) around a known average rate of occurrences, so in order to reach 95% confidence that a sample frequency is 1 in 1,000, it is necessary to identify 3 in 3,000

events, hence, the Rule of Three (Bio-Rad). For determining if samples are a true positive, Bio-Rad recommends the use of the Rule of Three and determine the false positive rate (FPR) from the no template controls. By multiplying the number of positive droplets per well by three, one can determine if the positive samples have at least three times the number of positive droplets than the FPR (Bemis et al., 2016).

4.2.2. Waters et al. (2023) positive detection benchmark

In the Waters et al. (2023) paper, the samples analyzed with the ITS assay followed recommendations from Cao et al. (2016) and Steele et al. (2018) where a minimum of two reactions and a total of more than 10,000 droplets per reaction were generated per sample (Waters et al., 2023). At least six no template control reactions were run per assay, these samples were required to contain less than three positive droplets, and two positive control reactions were included per assay (Waters et al., 2023). If samples exceeded the upper limit of quantification, these were diluted 1:100 with RNA/DNA-free water and reanalyzed. Based on tank concentrations from the ddPCR process QX Manager software, ITS gene copy numbers were back calculated: (https://github.com/kylielanglois/SCCWRP/blob/main/ddPCR/ddPCR_autofill_clean.R).

4.2.3. SCCWRP LOQ interpretation

At SCCWRP, Dr. Susanna Theroux developed the in-house chloroplast-based *tufA* assay from the Draisma & Sauvage (2024) DNA barcode sequence. This assay used an LOQ and LOD set on an individual well basis. The LOQ was calculated per sample based on the total number of droplets and roughly translated to 3 positive droplets. The LOD was considered to be 1 positive droplet. Anything below 3 positive droplets was considered uncertain. Summarized briefly, the LOQ is the lower limit of what is considered a quantifiable result and the LOQ can be lowered by running multiple wells and merging the data. For low-shedding *C. prolifera*, this is important for increasing sensitivity of the analysis. At SCCWRP, the general rule is using 3 positive droplets as the LOQ. While there technically can be detection below the LOQ, these detections are flagged and the sample is often re-run to verify positive hits.

4.2.4. Three positive droplets as positive detection

Both Waters et al. (2023) and the SCCWRP *tufA* assay analysis use a LOQ of three positive droplets. This baseline is based on the statistical concept of the Rule of Three derived from the Poisson distribution where, if you expect 1 positive droplet in 1000, you need 3 in 3,000 to be 95% confident that it is a true positive droplet. Adjustments to accommodate

individual wells and total number of droplets per well allow for the Rule of Three to be applied to low-shedding and small samples of *C. prolifera*. Using three positive droplets as the LOQ for *C. prolifera* eDNA research ensures the most accurate determination of present concentrations of eDNA. The LOQ used by Waters et al. (2023) and Dr. Theroux at SCCWRP allows for reliable interpretation of assay analysis for both the ITS and *tufA* assays for old and new samples.

4.3. Evaluation of two different primer effectiveness is detecting Caulerpa prolifera eDNA

The basis for our work, the study completed by Water's evaluating the effectiveness of the ITs gene as an assay target for detecting *Caulerpa*, reveals that despite the efficiency and accuracy of an assay design, the low-shedding rate of *Caulerpa* makes identification difficult. Water's lack of results in detecting *C. prolifer*a in the field was not due to the assay itself. The ITS assay was benchmarked, and primer efficiency was found to be 101.05% and 103.35% for both the tank and field samples (Waters et al., 2023). The ITS primers were also tested in silico via EcoPCR, demonstrating species-specific *C. Prolifera* amplification (Waters et al., 2023), all of which further proves that the issue in detection stemmed from the biology of Caulerpa in the water column (See comparison of ddPCR assay conditions in **Appendix A**).

The ITS sequence comes from nuclear DNA, which may influence the shedding rate and detection capability. A previous study evaluating eDNA detection using the ITS-1 sequence in the Japanese Jack Mackerel found that the shedding rate of nuclear DNA was affected by biomass and water temperature, with higher temperatures and biomasses increasing nuclear decay (Jo et al., 2019). Given the size and unicellularity of *Caulerpa*, the nuclear shedding rate very well could be minimal. Additionally, details about the oceanic and atmospheric conditions at the time of sampling may have affected the presence of nuclear DNA in the samples (NOAA, 2024).

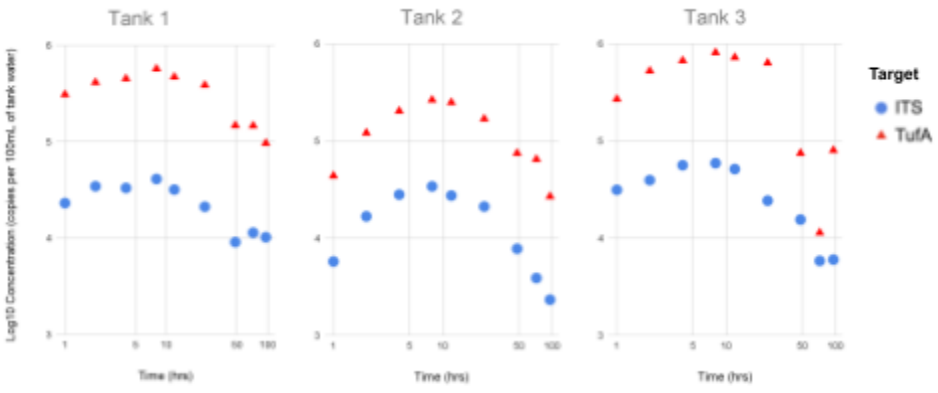


Figure 4.2. Gene copies per 100ml vs hour markers 1-96 for all three tanks samples. *tufA* detection vs ITS detection.

In comparison to the ITS assay, the new assay designed in-house by SCCWRP targeted the *tufA* gene as a marker for *Caulerpa* detection, which is a chloroplast gene sequence rather than a nuclear one, like the ITS sequence. Plastid genomes, which include chloroplast genomes, have been found to vary significantly from nuclear genomes, which, based on the ITS results, could mean improved eDNA monitoring via chloroplast sequences (Ness et al. 2015). In other eDNA studies, chloroplast sequences have been selected due to the higher copy number of chloroplast markers in comparison to genomic markers (d'auriac et al. 2019). A study into the morphology of various *Caulerpa* types also identified the *tufA* gene as the preferred marker for identification of green algal taxa (Kazi et al. 2013).

Given these factors, SCCWRP hypothesized that the rate of *Caulerpa* detection may improve with the use of the *tufA* sequence. The efficacy of the new assy was confirmed through in-silico testing and Genbank. It was also validated in the field and confirmed to be very specific toward Caulerpa Prolifera. The new assay was used on the same samples used for the ITS assay, field samples from Newport Bay outbreaks in 2021 and 2022, and tank samples. In evaluating the results of both assays, the *tufA* sequence demonstrates more success than the ITS sequence. **Figure 4.2.** displays a side-by-side comparison of the effectiveness of the *tufA* and ITS sequences in detecting *Caulerpa* in the tank samples. Across all tank samples and hour markers, *tufA* has a detection of 1-2 magnitudes of gene copies per 100 mL than the ITS sequence does. In these graphs, it is also clear that the concentrations of gene copies were higher at earlier hour markers, potentially signifying that stress levels of the samples in the tanks may have affected eDNA shedding. According to a literature review of eDNA, stress can increase the rate of eDNA shedding 100-fold (Harrison et al., 2019). Another study assessing eDNA shedding of commercially harvested fish found higher levels of eDNA when fish were moved or removed from tanks, theorizing that the stress of movement resulted in greater shedding (Kirtane et al., 2021)**.** Both of these studies present evidence contrary to the shedding rates displayed in figure 1a, which means there may have been an additional factor affecting the shedding rate over time.

Figure 4.3. represents the side-by-side comparison of the effectiveness of the *tufA* and ITS sequences in detecting *Caulerpa* in the field samples. In the field, detection rates across the board are significantly lower. In the surface samples from the July outbreak, both assays had relatively zero detection. In the depth samples from July and May, the *tufA* sequence displays low detection rates close to the outbreak, with relatively zero detection at greater distances from the outbreaks, and the ITS sequence displays relatively zero detection across all distances. Our data from the surface samples of the May outbreak reveal the most positive results, with the *tufA* sequence revealing detection above the limit of quantification for multiple replicates. The *tufA* sequence also reveals detection below the level of quantification for multiple replicates across all distance markers. The ITS sequence had zero detection in the May surface samples.

Based on these results, it is clear that the *tufA* sequence was more effective in detecting the presence of *C. Prolifera* in the water column. The most concrete detection is demonstrated in the tank samples, where *tufA* displays detection of gene copies per 100 ml at 1-2 magnitudes greater than the ITS sequence. The field samples provide less concrete results. The limited detection across the July (surface and depth) and May (depth) samples could be attributed to oceanic and atmospheric conditions like temperature, salinity, depth, currents, tides, pH and organism size, age, maturity, species, all of which are known to affect eDNA dispersal (NOAA, 2024) (Allan et al. 2020). Overall, while the *tufA* assay demonstrates better detection and efficacy in the tank samples, the field samples do not provide significant evidence to support this, thus, further analysis of the field samples is necessary.

It is also important to note that while the *tufA* sequence proved to be a more effective primer than the ITS sequence, this was not due to any failures in assay design or verification. To view the specific primers and sequences for each assay, see appendix A. To further demonstrate that the differing results of the two assays in *Caulerpa* detection was influenced by the choice of target DNA (ITS vs *tufA)*, the conditions of both dPCR assays were kept almost exactly the same. See **Appendix A** for PCR conditions.

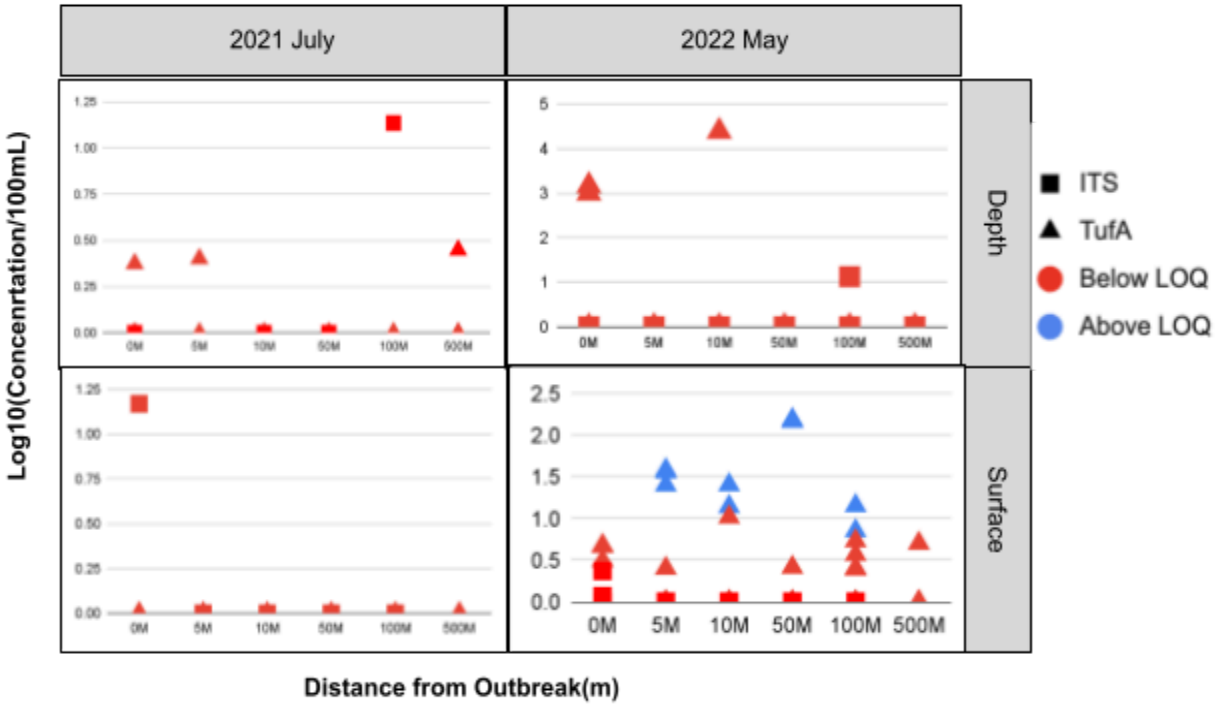


Figure 4.3. Gene copies per 100ml vs distance from outbreak for field samples at depth and surface (July 2021 and May 2022 outbreaks). TufA detection vs ITS detection.

Given the proven effectiveness of both assays and the standard PCR conditions, we hypothesize that the difference in detection results was likely due to the unicellularity and low shedding rate of *Caulerpa* (Waters et al., 2023), the effectiveness of the assay was determined by the abundance of the gene target sequence that was chosen; nuclear vs chloroplast.

4.4. Investigation into hydrological and environmental influence on eDNA detection of Caulerpa Prolifera

From Dr. Waters paper, we know that Caulerpa have very low shedding rates compared to other macroalgae. Using our assay design, we were able to find higher detection rate in our tank samples signifying its potential for field monitoring. Although when used in the field samples, the results were quite shocking. The smaller patch in the inner harbor had more concentration hits compared to the larger outbreak patch in the channel entrance which were about zero. This very well can be influenced by the location and hydrological conditions that take place. Although, after further discussion with the team, the samples taken in July of 2021 might have been taken after the removal of the outbreak. To our understanding the outbreak was removed in June of 2021 so it would make sense that our concentration hits would be minimal or close to none. Without knowing the exact residence time of *Caulerpa* eDNA, we can assume there was no eDNA present in the water column during the July sampling mission. It's still important we understand the hydrological and environmental influence on eDNA for the best results.

The physical processes that transport and mix water are well understood and for the most part many models are able to depict the environmental conditions in real time. The only issue with using models is that they require large computational equations and are not cheap. When SCCWRP did their sampling they were assisted by the use of a hydrological model in order to know when and where the most logical particle distribution would be located. This model looks promising but it's currently a prototype. With as little eDNA shedding *Caulerpa* does, it's important we can pinpoint a more accurate location for sampling. While many hydrological models do not take into consideration anthropogenic activities, in an area like Newport Bay with high amounts of boat traffic, human activity can very well influence water activity in the area. [more on anthropogenic activity influence on water bodies]

In order to ensure the success of eDNA, we must understand the production and persistence of genetic material in the water column from our species of interest. Even though *Caulerpa's* primary form of reproduction is through clonal fragmentation, when a frond breaks off and is able to become its own plant, *Caulerpa* are still able to reproduce sexually. In a study investigating the reproductive capabilities of *C.racemosa* a cousin of *C. prolifera,* researchers discovered that these plants are monoecious, meaning they produce both male and female sex cells. Even though fertilization was not common, the importance of releasing these gametes into the water column is great for eDNA detection.They were also able to confirm that *Caulerpa* try to reproduce sexually when environmental conditions change. They found that specifically in the mediterranean sea, *Caulerpa* release their sex cells 14 minutes before sunrise, this was

confirming another study in the caribbean that claimed they released them 32 minutes after sunrise. (Panayotidis & Zuljević, 2001) The difference can be attributed to the changes in dawn time. If we can find out how persistence their DNA is in the water column and the time they release these sex cells, we can have more successful sampling missions.

5. Broader Context and Future Steps

The early detection and management of *Caulerpa prolifera* is critical to maintaining the health of marine ecosystems in Southern California and around the world. As this project demonstrates, eDNA monitoring holds significant potential in addressing the limits of traditional survey techniques, especially in environments where monitoring visually is difficult. Our work, building on the important findings by Dr. Waters', has indicated the viability of eDNA assays to detect *Caulerpa* despite its low shedding rate & the lack of success seen in Waters' experiment with the ITS gene. By demonstrating the *tufA* assay leads to consistently higher detection rates in both tank and field samples, the project provides evidence that chloroplasts or other more abundant genes could be effective targets for invasive unicellular organisms. This framework could extend to the detection of other low shedding organisms globally. Furthermore, our project highlights the need for nuanced definitions of positive detection thresholds for ddPCR with organisms that do exhibit these low shedding rates. Overall, the preliminary success demonstrated by the *tufA* assay is promising, though some key next research steps to consider do exist.

The ultimate goal by leveraging eDNA to detect caulpera is to create an assay and method of sampling that is consistently accurate, and to understand under what parameters it works best. In our project, we came across several future research questions to inform this overarching goal, including:

1. Why do certain field sampling locations exhibit higher detection rates than others?
2. Why does the *tufA* assay consistently perform better than the ITS assay in the tank experiment but not consistently in the field experiments?
3. How can we validate and refine the assay?
4. Understanding eDNA persistence and degradation

First, field sampling in May of 2022 exhibited much higher detection rates than July of 2021, despite being taken the same distance from the patch. However, the May sampling was taken in the bay, and the July sampling was taken in the channel, indicating that hydrological factors may be at work, such as tides and currents. Further, in the May samples, the surface sampling detected more *Caulerpa* than the depth sampling, which is puzzling because the *Caulerpa* grows at depth. It is possible that hydrological conditions could have affected these results as well, and developing an understanding of hydrologics in the area, combined with understanding tides and currents at the time of sampling would help us answer this question. This question is crucial to understand in order to assay in what circumstances the assay works best

and to mitigate conditions that would lead to a lack of detection. We recommend more experiments being done to specifically address the differences in detection at high versus low tide and in different locations, such as bay versus channel.

Second, more research should be done on why the *tufA* assay consistently performs better than ITS in tanks, but not always so in the field. This is closely related to question 1 and requires a more in-depth understanding of oceanographic conditions and substances in the field that may affect detection. Other factors that need to be considered include temperature, nutrient levels, and mechanical disturbances.

Third, we would like to further validate and refine the assay. Additional validation beyond our work should be conducted across multiple environments with varying salinity, turbidity, ecosystems, etc. to see if it continues to perform better than the ITS assay. The assay should also be tested against a broader spectrum of *Caulerpa* species to assess specificity and eliminate potential for false positives due to genetic similarities.

Fourth, continued research should investigate the residence time of eDNA in the environment. By creating experiments which examine DNA degradation under various conditions (light, temperature), we would more accurately be able to interpret field eDNA detection.

Monitoring and detecting *Caulerpa* with eDNA analysis is an iterative process, with future research building on the progress we have made. Looking forward, we hope that these research questions will create a more robust understanding of assay effectiveness and lead to further refinement & detection rates.

6. Outreach and Education

To complement our investigation into eDNA monitoring as an effective tool for detecting and controlling *Caulerpa* outbreaks, we produced several outreach and communication deliverables. We also presented our findings to different audiences in order to spread awareness of the issue and the potential benefits of eDNA monitoring.

We presented our findings to the Southern California *Caulerpa* Action Team (SCCAT) in order to update them on potential options for the expansion of *Caulerpa* monitoring. We also presented the issue of *Caulerpa* as an invasive species across Southern California at the Newport Bay Conservancy's Earth Day event.

To serve as documentation of our work and to provide further context into the guiding questions and research focus of this capstone project, we put together a documentary detailing our research process, experience working on the team, and our conclusion and recommendations for future steps. In order to provide visuals to support our research, we also produced an arcgis story map that includes the location of each recorded *Caulerpa* outbreak with its coordinates, patch characteristics and treatment methods. Both our documentary and storyboard will be provided as visual deliverables and linked on our capstone project page for viewers to browse. Our outreach presentations and visual aids were all developed to spread awareness of the threat

of *Caulerpa* outbreaks and provide an explanation into possible monitoring solutions and techniques: eDNA. See appendices for direct links.

7. Conclusion

This investigation highlights the challenges of detecting *Caulerpa* using traditional eDNA methods due to its low shedding rate and unicellular structure, based on the work of Tanner Waters. SCCWRP's new assay leveraging the *tufA* chloroplast gene developed in-house, demonstrates a marked improvement in *Caulerpa* detection compared to the ITS gene.

Results from both the tank and field samples suggest that the *tufA* assay consistently detected *Caulerpa* more often, by factors of 1-2 orders of magnitude than the ITS assay. In addition to confirming the low shedding rate of *Caulerpa*, the increase in sensitivity demonstrated by the *tufA* assay contributes to progress towards early detection of invasive species.

Our findings demonstrate that continued validation and refinement of the *tufA* assay, including experiments comparing different environmental parameters and hydrological conditions in the field, needs to be done. Further, the work reinforces the need for establishing clear detection thresholds for species with low shedding rates. Overall, these findings can push forward *Caulerpa* detection & response strategies, helping to preserve crucial ecosystems in Southern California and around the globe.

Our team thanks SCCWRP and our client, Dr. Susanna Theroux, as well as Dr. Kylie Langlois, Dr. Jill Tupitza, and Dr. Faycal Kessouri for their support and scientific expertise. We also thank our advisor, Dr. Robert Eagle, for his guidance & leadership, and Dr. Tanner Waters for his critical research into *Caulerpa's* low shedding rate and eDNA detection evasion.

Appendix A - Assay Sequence Information and Conditions

1a. tufA Primer Sequence

Scientific Name	Primer/probe	Sequence 5'-3'
Caulerpa Prolifera	Caulerpa_14F	GAGAAACATTAGATCGTTA
Caulerpa Prolifera	Caulerpa_15R	GAATCTTTGGAAAGTGCTTC
Caulerpa Prolifera	Caulerpa_16Probe	TCTGAAATACCTATTATAAG

The *tufA* sequence was designed in-house at the Southern California Coastal Water Research Project (SCCWRP) by Susanna Theroux.

1b. ITS Primer Sequence

Scientific Name	Primer/Probe	Sequence 5'-3'	bp
Caulerpa Prolifera	Caulerpa_ITS_F	TGGGCGCTATGTAATGTTGATGTTG	106
Caulerpa Prolifera	Caulerpa_ITS_R	GCAATTCGCAACACCTTTCGTA	
Caulerpa Prolifera	Caulerpa_Probe	56-FAM-CGGTTCCCGTGTCGATGAAGGACG-3IA	

The ITS sequence was designed for Tanner Water's *Caulerpa* detection project in 2023 by his team. Reference sequences were sourced from GenBank and aligned in Geneious 2019.2.3.

1c. Comparison of ddPCR assay conditions for ITS vs *tufA* analysis

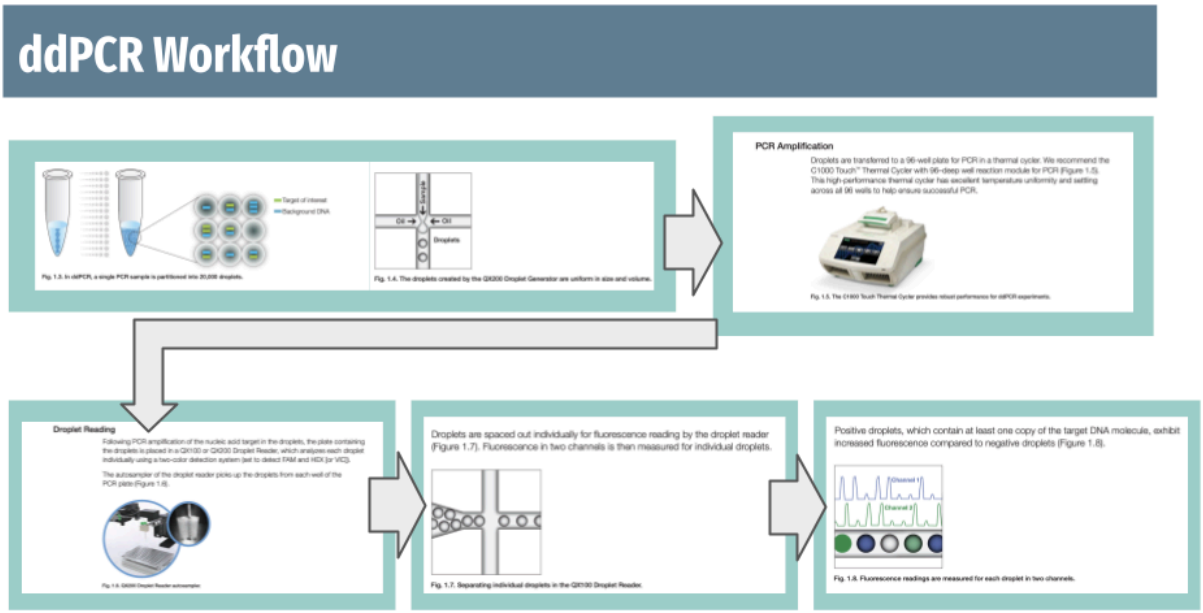
ITS Assay:

- Cycling conditions for ddPCR:
- 95°C for 10 min
- 40 cycles of 95°C for 30 s
- 58°C for 60 s
- 98°C for 10 min
- 4°C indefinite hold.

tufA Assay:

- Cycling conditions for ddPCR:
- 95°C for 10 min
- 40 cycles: 94°C 30 sec
- 46°C 60 sec
- annealing + elongation step (1 step) only changed in temperature from ITS conditions
- 98°C for 10 min

ddPCR assay conditions for both targets (ITS & *tufA)* were very similar. ITS assay was run in 2023 and the *tufA* assay was used in 2025.



Appendix B - Progress and Final Presentations

1**a. Final Presentation,** IOES Senior Practicum, (6/7/2025) - Practicum Final Presentation

1b. **Findings and Interpretations Presentation** to the Southern California Caulerpa Action Team (5/14/2025) - SCCAT_SCCWRP - Sp2025 Presentation

1c. **Mid Quarter Progress Report**, IOES Senior Practicum (5/2/2025) - SCCWRP - Sp2025 Presentation

Appendix C - Outreach Deliverables

ArcGIS *Caulerpa* Storymap - Comprehensive storymap documenting history of *Caulerpa* outbreaks, characteristics, and clean up methods.

Documentary (in progress) - A behind-the-scenes look at our research project, highlighting our goals, methods, challenges, and progress to date.

Appendix D - Data & Calculations

***Caulerpa* Concentration Data & Calculations** - Datasheet containing raw data collected from SCCWRP of *C. Prolifera* ddPCR analysis from both experiments using the ITS and *tufA* assays. Steady state concentration calculations and field/tank sample graphical analyses included in the datasheet.

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