Salvinia molesta removal efforts in the Kilauea Stream, Kauai, Hawai‘i

Hawai`i DAR
Division of Aquatic Resources

Prepared By:
Division of Aquatic Resources
Aquatic Invasive Species Team
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FIELD REPORT

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Executive Summary

In 2016, community members on the Island of Kauai noted increased growth of *Salvinia molesta,* commonly known as Giant Salvinia, along the banks of the Kilauea Stream. The fast and aggressive growth of a known invasive aquatic plant species concerned residents, which prompted a manual removal effort in late 2017 and subsequent monitoring efforts from 2018 to 2019. The manual removal in 2017 was a multi-agency collaborative event with the local community and although it was not effective in completely eradicating *S. molesta* from the Kilauea Stream, attempted removal brought awareness to the presence of aquatic invasive species in local waters. Monitoring was conducted at twenty-five stream mouths along the north and east coasts, seven stream mouths on the south and west coasts, and along eighteen tributaries upstream of the north and east coasts. The presence and extent of invasion was measured and recorded using visual surveying methods at two streams most impacted by *S. molesta*. Additionally, water samples were collected and processed to record the presence of environmental DNA (eDNA) of *S. molesta*. Monitoring efforts determined that *S. molesta* eDNA was present in multiple stream systems but was most prevalent in the Kilauea and Kapa'a Streams. Although occasional positive results of eDNA were reported in other streams, ultimately no new stream systems were confirmed to contain *S. molesta* through physical observation, other than the initial streams that were reported by community members. Presence of *S. molesta* in the Kilauea and Kapa'a Streams did change drastically over time, which could have been due to changes in stream flow rates and nutrient availability. Low density of *S. molesta* may have caused the eDNA analysis to falsely report negative results in other streams. More surveys are needed to maintain the accuracy of the known distribution and to increase the confidence of the results reported as eDNA technology becomes more sophisticated. Finally, the data provided by this report can be used by community members, non-profit organizations, and resource managers to create a management plan that will guide continued monitoring and contain the spread of *S. molesta* in the watersheds of Kauai.

Introduction

Aquatic invasive plant species can disrupt native ecosystems and have devastating economic impacts (van Wilgen & De Lange 2011, Thomas et al. 2019). Natural resource managers struggle to actively monitor, manage, and contain distributions of aquatic invasive plant species due to their ability to spread through multiple vectors, quickly reproduce, and resist eradication (Luque et al. 2013, Scriver et al. 2015, Martin et al. 2018, GISD 2021). Tracking and managing invasive species can be difficult when reliant on field surveys that are limited, and the invasive species are located in areas that are not easily accessible.

The integration of analyzing environmental DNA (eDNA) to identify the presence of non-indigenous and invasive species in aquatic ecosystems has been beneficial in quickly creating

distribution maps (Thomas et al. 2019). All organisms constantly shed traces of DNA into the environment through decaying dead tissues, gametes, waste excretions, and mucous shedding (Scriver et al. 2015). Using specific molecular markers produced from standards, the presence of target eDNA belonging to aquatic invasive plant species can be recorded by processing water samples (Parrondo et al. 2018, Thomas et al. 2019). Identifying and mapping the distribution of invasive organisms down to the species level has been successfully accomplished for vertebrate, invertebrate, and plant species (Scriver et al. 2015, Parrondo et al. 2018, Thomas et al. 2019).

Salvinia molesta is a free-floating aquatic fern species native to Argentina, Brazil, Colombia, and Guyana (GISD 2021). Thriving in slow-moving and nutrient rich waters, *S. molesta* is sterile and does not reproduce sexually, however this species can quickly multiply via vegetative growth (Cilliers et al. 2003). *Salvinia. molesta* grows in three stages; primary growth is defined as single plantlets, secondary growth involves the extension of singular plantlets into long chains, and tertiary growth is the formation of thick matted clusters (GISD 2021). The spread of *S. molesta* is attributed to its use as an ornamental aquatic plant species in ponds and aquarium, mulch, compost, fodder, paper making, handcrafts, and bio-gas generation (Luque et al. 2013, GISD 2021).

Salvinia molesta can create 10-20 cm thick mats that block out sunlight to other aquatic plants and restrict water flow in waterways (Luque et al. 2013). Mats of *S. molesta* can clog dams, streams, and rivers causing both ecological and economic damage (Cilliers et al. 2003, van Wilgen & De Lange 2011). *Salvinia molesta* actively smothers and displaces native species because of its rapid growth rate (Luque et al. 2013). In 2011, through loss of water, grazing, and biodiversity, *S. molesta* was estimated to have an economic cost of ~421 million USD per year (van Wilgen & De Lange 2011). In 2013, *S. molesta* was designated as the 100th worst species on the ICUN's invasive species list, based on a survey of 652 experts from 63 countries (Luque et al. 2013, GISD 2021).

Salvinia molesta was first spotted outside of its native range in 1939 (Luque et al. 2013, Martin et al. 2018). It began invading waterways in South Africa and was deemed South Africa's worst aquatic invasive plant species by the 1960's (Martin et al. 2018). Currently, *S. molesta* is widespread and established or reported in an estimated 41 countries (GISD 2021). In Hawai'i, *S. molesta* was first reported in 1999 at Enchanted Lake on the island of O'ahu, where it was introduced as an aquatic ornamental plant (DLNR 2018, Thayer et al. 2018). *Salvinia molesta* raised concerns in Hawai'i when it completely covered the surface of Lake Wilson on the island of O'ahu in 2002 (DLNR 2018). State prompted eradication efforts took place from 2002-2003 and cost over $1 million, but *S. molesta* was successfully eradicated from Lake Wilson (DLNR 2018). Since this eradication effort, the import and sale of *S. molesta* by any persons has been banned within the state (DLNR 2018, HDOA 2015). According to Chapter §150A-6.1 in the Hawai'i State Legislature, *S. molesta* was designated as a noxious weed and importation into the state is only allowed for research purposes (HDOA 2015).

Salvinia molesta was later reported on the island of Kauai in the Kapa'a Stream in 2007, and later reported in the Kealia and Kilauea streams in 2016 (Else 2016, Thayer et al. 2018). In 2016, community members reported an increasing population of *S. molesta* in the Kilauea Stream, which prompted eradication efforts by the State of Hawai'i from 2018-2019.

I. Manual Removal

A. Goals

The goals of the manual removal performed in 2017 were to survey lower reaches of the Kilauea Stream to map the distribution of *Salvinia molesta* and measure the severity of the distribution. Once an initial survey was complete, the next goal was to assess the feasibility of removal efforts, leading to effective eradication and the promotion of cooperation and partnerships with various stakeholders.

B. Methods

To determine the next management steps, the distribution of *S. molesta* was mapped. Surveyors kayaked along Kilauea Stream, noted the density of *S. molesta* along the riverbanks, and georeferenced those observations using a handheld GPS (Garmin 78). Arc GISpro was used to visualize observed densities of *S. molesta.* Density was ranked as: 0 – No Algae, 1 – Sparse, 2 – Patchy, and 3 – Dense. General observations of the site were also noted. Several different removal methods were tested, including hand removal with nets, utilizing a trash pump on a mini-barge, and cutting large mats loose to allow them to flow downstream under the assumption that they would eventually reach saltwater and perish.

C. Results

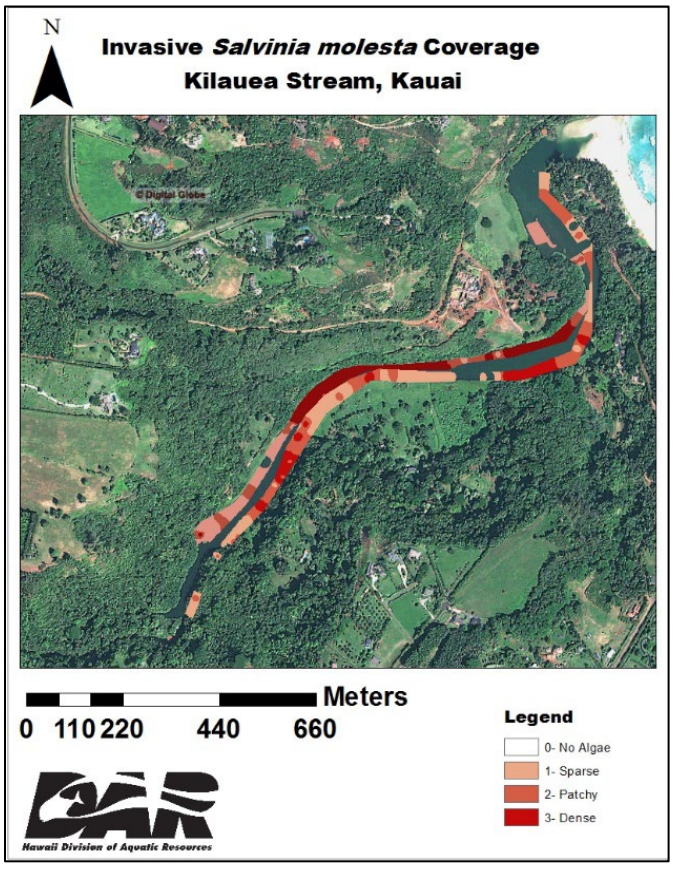

Figure 1: Map of pre-removal S*alvinia molesta* densities in the Kilauea Stream, Kauai.

When surveying the lower Kilauea Stream, the team found that it was lined with heavily vegetated banks that consist of California grass (*Urochloa maxima*) and Hau Trees (*Hibiscus tiliaceus*). *Urochloa maxima* creates a holdfast for *S. molesta* to anchor to. As *S. molesta* grows out into the stream, it was observed creating floating mats up to 0.76 m in depth that extended 4-5 m from the edge of the grass. Mats of *S. molesta* can eventually become sturdy enough to support new *U. maxima* growth from stolons, or runners, spreading further from the bank. These species seemed to create suitable habitat for one another, which expands the vegetation from the banks and reduces the amount of open stream water. Branches of *H. tiliaceus* overshade *U. maxima* and do not provide ideal growing conditions for the *S. molesta*. However, small loose rafts of *S. molesta* do get caught on branches that hang into the water. These characteristics reach from the stream's mouth to approximately 1.2 km inland. *Salvinia molesta* is found throughout this region of the stream in varying densities (Fig. 1).

Further upstream, there is more rapid water motion, which is not conducive to *S. molesta* growth.

Removal by nets was found to be the most impactful option to physically remove algae from the stream. Using the trash pump on the barge was problematic because *U. maxima* would get tangled in the trash pump. Cutting large mats off and letting them float downstream seemed to be time efficient, however, it was likely that the mats would become caught in *U. maxima* or *H. tiliaceus* further downstream and not reach the ocean.

D. Conclusions

Initial physical surveys showed large stretches of the stream edge covered in dense mats of *S. molesta*. Nearly all areas surveyed showed *S. molesta* was at least present, even if only sparsely dispersed. From testing several methods of removal and observing the distribution of the *S. molesta* on Kilauea Stream, it was determined that a large-scale removal project would be required to remove most vegetation. When *S. molesta* is physically removed, it will be important to keep it near the source to not spread it to other watersheds. Composting near the collection site is a viable option after removal. However, even if a large amount of *S. molesta* could be removed, it is unlikely that it could be fully eradicated in this manner. Small amounts of *S. molesta* left behind could quickly repopulate the stream.

The use of an herbicide that is approved for aquatic applications may be an effective management tool. However, this would need to be discussed with partners and the community on Kauai. Although the use of herbicides was successful in the eradication of *S. molesta* during the Lake Wilson outbreak, it was unclear if the community supported such use. Additionally, the continued support through community workdays using hand removal techniques, may be one option to keep *S. molesta* from expanding further. The Kauai Invasive Species Committee (KISC) created informational brochures to spread awareness of the issue and how to prevent the spread of *S. molesta* to additional watersheds.

It was determined that to create a well-informed management plan for *S. molesta*, it is crucial to understand the distribution of the species throughout all the windward watersheds of Kauai. Therefore, a new technique for detecting the presence and absence of *S. molesta* by using environmental DNA (eDNA) was established. By partnering with genetic researchers at the Oceanic Institute, a genetic marker unique to *S. molesta* was identified. If *S. molesta* is present in the stream, remnants of its DNA are discoverable. By filtering water samples and processing them, eDNA can determine whether *S. molesta* is present or absent in that watershed. Further research was conducted to determine if this survey method is an effective and efficient way to map the distribution of the *S. molesta* compared to manually surveying each stream.

II. Watershed Distribution with eDNA

A. Downstream Detection

1. Goals

The goal of this section of the project was to sample stream systems with known *S. molesta* presence using an experimental design to determine downstream detectability using eDNA sampling. Using the detectability results, detection methods can be better designed for future eDNA studies.

2. Methods

Collection

Water samples were collected by a two-person team. One team member oversaw recording data (bottle number, time, stream system), taking GPS waypoints, and taking photos at each collection point. The other surveyor collected the water samples. At each stream mouth, four 1 L water samples were taken in numbered plastic bottles and were spread out evenly along the banks that were accessible. At streams where both sides could be accessed, two samples were taken along each bank. Some of the streams near Kapa'a are channelized, creating multiple stream mouths originating from the same watershed. In this case, only two samples were taken at the smaller of the two outlets (Kawailoa). The first water sample at each stream mouth was taken furthest downstream so the flow of the water would prevent contamination and minimize the amount of suspended sediment in each sample. The bottles were rinsed three times in river water before the sample was taken. Once the four samples were collected, they were put inside a cooler with ice to prevent the DNA from degrading.

Filtering/data processing

To avoid cross contamination, a separate set of gloves were used for each stream systems' samples and all four 1 L samples from the stream were processed by one team member. An individually wrapped filtering cup with a nanopore filter was placed on a plastic Erlenmeyer flask, which acted as the catchment for the filtered water. A plastic tube connected the flask to a pump to create the negative pressure needed to pull water through the filters. Most of the 1 L samples required the use of two filters, as suspended particles in the water clogged the filter and slowed the flow of water. After the first 500 ml of the sample was passed through the filter, it was removed using sterile tweezers and replaced with a fresh filter. With separate sterile tweezers, each nanopore filter was placed into a small vial with the corresponding sample number containing Longmire's buffer, a solution that simultaneously works to lyse cells (and cellular membranes) captured on the filter while preserving the DNA that is released into solution (Renshaw et al. 2015). The two filters from each sample were placed in the same vial. Some of the water samples contained enough suspended sediment that four filters were required and were split between two vials (A and B) to be analyzed together. In total, 96 stream water samples were taken from 23 watersheds at 25 different stream mouths. Additionally, four control samples of filtered bottled water were submitted to ensure there was no cross-contamination during the filtering process. All the sample vials were given to the eDNA lab at the Oceanic Institute without the location to keep the study blind.

Detailed lab procedures (Renshaw et al. 2015)

The sample vials were sent to Mark Renshaw at the Oceanic Institute on April 9, 2018, to run the quantitative polymerase chain reaction (qPCR) process to identify *S. molesta* eDNA. In the lab, the DNA was extracted from Longmire's buffer solution using a Phenol-Chloroform-Isoamyl protocol, resulting in a clean DNA pellet that was rehydrated in sterile water (Renshaw et al. 2015).

A qPCR was then used to amplify a targeted species-specific sequence fragment from the extracted DNA. If the targeted fragment was absent, the amplification failed. To produce quantitative estimates of DNA for positive samples, a serial dilution of a synthetic standard was included on each qPCR plate. The qPCR assays included a fluorescent dye (correlated to the number of targeted DNA fragments in the sample) to visualize changes in the number of targeted DNA fragments. The level of fluorescence was

measured every qPCR cycle, since the fluorescent signal increased as amplification proceeded, and more fragments were created. The fluorescent dye was generally included in one of two ways: **[1]** in the form of a fluorescent dye additive in the PCR mix that binds to double-stranded DNA, such as SYBR or **[2]** the addition of a hydrolysis probe with a reporter molecule that fluoresces when cleaved from the DNA fragment during elongation in the PCR cycle. Hydrolysis probes are preferable for eDNA applications because the probe is a third DNA sequence fragment (like the primers) that will only bind and work if it finds a matching DNA sequence in the DNA extract. The likelihood of the PCR amplification occurring successfully on a non-targeted fragment of DNA, such as from a closely related – and genetically similar – species, becomes even less likely with the use of the probe in addition to the two primer sequences (forward and reverse).

Each eDNA sample was run in four qPCR replicates. On a single 96-well qPCR plate, there were 21 eDNA samples (four replicates each), 10 wells covering the range in serial dilutions for the synthetic standard, and two wells with sterile water (negative controls) to monitor for contamination in the qPCR reagents or technique. For every putative positive eDNA sample, a single qPCR replicate was Sanger sequenced to confirm that the amplified DNA sequence fragment was the originally intended target; in this case, a DNA fragment from the Internal Transcribed Spacer 1 (ITS1) region located between the 18S and 5.8S ribosomal genes in the *S. molesta* nuclear genome.

Downstream Detection

To address the main factors influencing eDNA accuracy, a sampling protocol was designed and used for two different stream systems with confirmed S. *molesta* populations. Using this approach, all eDNA samples were expected to result in a positive detection and false negatives could be quantified. Kapa'a and Kilauea streams had verified S. *molesta* presence through physical observation and their population densities were quantified over the course of the project through visual surveys (Figure 2 and 3). The sampling design required finding isolated patches of *S. molesta* with no individuals within 200 m downstream. This proved difficult to find on the streams where the distribution had shifted from the prior surveys in August 2018. The only patches that fit into these requirements were smaller patches (approximately 25 cm by 25 cm). Larger patches would have been more desirable for this test due to the increase of eDNA into the system, thereby increasing the likelihood of detection.

The sampling scheme consisted of three replicates of three volumes of samples (250 ml, 500 ml and 1 L) taken at four distances downstream from lone standing patches of *S. molesta* (10 m, 50 m, 100 m and 200 m). These sample sets were taken on both banks of each stream system. Additionally, four control samples of bottled water were submitted with the samples. All other sampling methods remained the same from previous surveys. All the sample vials were given to the eDNA lab at Oceanic Institute without the location, ensuring a blind study.

3. Maps

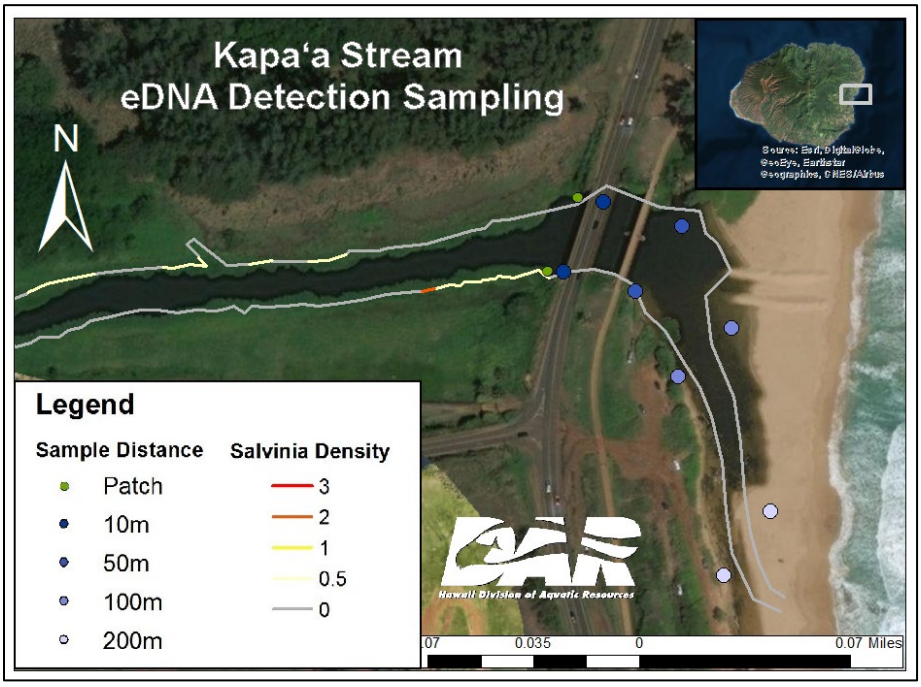

Figure 2. Downstream detection sampling locations in the Kapa'a Stream system from February 2019.

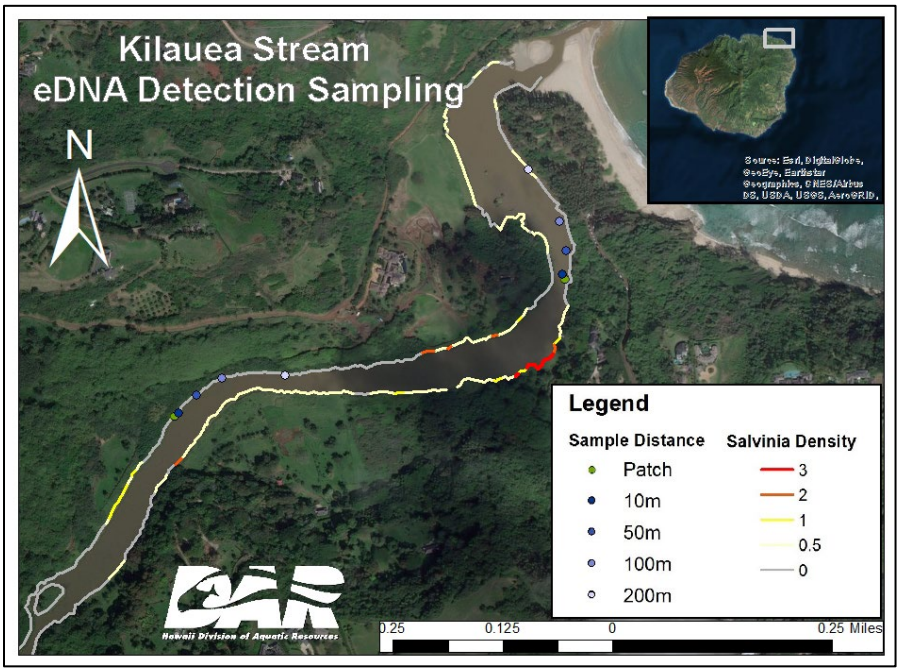

Figure 3. Downstream detection sampling locations in the Kilauea stream system from February 2019.

4. Results

One important result was that this experiment showed false negatives are possible when performing eDNA sampling for *S. molesta.* The results also showed that using 1 L sample size provides the most accurate results, although the larger water volume takes more time to filter. Also, it is possible that in some areas, increasing the sample volume can increase ambient chemicals which could confound the lab results. Results determining the accuracy of samples from different distances from source DNA, were inconclusive. Surprisingly, samples taken 10 m from a known source of visually confirmed *S. molesta*, were less accurate than 50 m or 100 m for Kapa'a Stream and the 10 m distance was the same or only slightly better than 50 m or 100 m in the Kilauea Stream. Additionally, results from samples taken 200 m from a source site were less accurate, indicating that eDNA may quickly dissipate in the water and samples may only be reliable when taken from closer proximity to a source patch of *S. molesta*.

There were large differences in successful detection between the banks within the Kilauea and Kapa'a streams (Table 1). Overall, Kilauea had a higher rate of detection at all sample distances and volumes. This could be due to the larger amount of *S. molesta* biomass in the Kilauea Stream system compared to the Kapa'a system, which was reflected in our visual survey data conducted in each stream system (Fig. 1). This indicates that eDNA surveying may not be reliable when trying to detect presence of *S. molesta* at lower densities and could result in false negative results.

Table 1. Results of eDNA detection surveys conducted in Kilauea and Kapa'a streams in February 2019. Percentages shown are percent of positive tests. Example: 33.3% means only one test out of three came back positive. Dark green represents a 100% detection rate and red highlights no detection.

Kilauea	10m		50m		100m		200m	
	Left Bank	Right Bank	Left Bank	Right Bank	Left Bank	Right Bank	Left Bank	Right Bank
250ml	33.3%	66.7%	33.3%	66.7%	0.0%	66.7%	66.7%	66.7%
500ml	33.3%	100.0%	33.3%	100.0%	33.3%	66.7%	0.0%	100.0%
1L	66.7%	100.0%	100.0%	100.0%	100.0%	100.0%	33.3%	100.0%
Kapa'a	10m		50m		100m		200m	
	Left Bank	Right Bank	Left Bank	Right Bank	Left Bank	Right Bank	Left Bank	Right Bank
250ml	0.0%	0.0%	100.0%	0.0%	33.3%	33.3%	66.7%	0.0%
500ml	33.3%	0.0%	100.0%	33.3%	33.3%	100.0%	33.3%	33.3%
1L	100.0%	33.3%	100.0%	66.7%	66.7%	100.0%	100.0%	66.7%

One issue that should be considered is that lab results indicated that one of the field sample blanks contained a trace amount of *S. molesta* eDNA. Meaning that somewhere along the sample collection, storage, or filtering process there was contamination. The blanks were taken from bottled water for human consumption, so the possibility that the water source for the blanks was contaminated is minimal. Although the manifold allowed the filtering process to be more time efficient, the ability to process multiple samples at once increased the risk of cross-contamination. All previous sampling efforts have not resulted in any positive results from the field blanks and each sample was filtered individually.

Preventing contamination and the use of sterilization wipes are needed to continue the time-saving benefits of the manifold setup.

B. Northeast Perennial Stream Surveys

1. Goal

The goal of this section of the project was to take water samples at the mouth of all perennial streams on the North and East side of Kauai to determine the presence or absence of *S. molesta* in each watershed. This information would help to develop an island wide management plan with a local task force.

2. Methods

Previously described methods for sample collection and processing were followed. Once the samples were processed by the Oceanic Institute, the results were received and subsequently mapped using ArcMap 10.7.1 (GIS) to create Figure 4.

3. Map

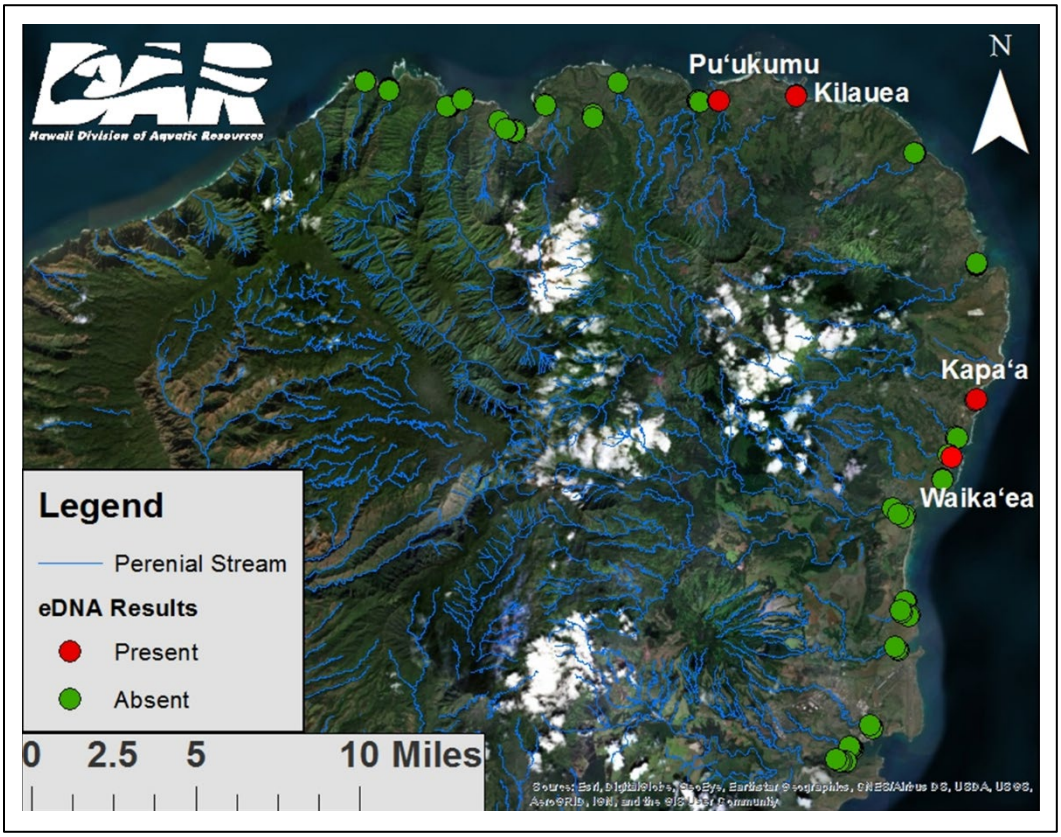

Figure 4. *Salvinia molesta* presence/absence map of Northeast perennial streams in April 2018.

4. Results

The results were returned on April 18, 2018, with a total of nine samples testing positive for the presence of *S. molesta* DNA. Six of the positive results were from streams where *S. molesta* was visually observed to be present: two of the four samples taken from Kilauea Stream and all four samples from

Kapa‘a Stream. Two of the samples taken at Pu‘ukumu Stream had positive results and the other two were negative. Pu‘ukumu Stream forks near the coast and the right fork was not sampled due to inaccessibility. Two samples taken upstream of the fork tested positive, while samples taken from the left fork tested negative. The final positive result came from Waika‘ea Boat Ramp. This is the only stream without a fork that had only one positive result and the other negative. The positive sample was the closest sample taken next to the boat ramp and the ocean. The stream prior to this sample site (Moikeha) came back negative, so cross contamination from the previous sampling location is not likely. It is possible that a boat or trailer that was contaminated with *S. molesta* could have recently used the ramp and was the source for the DNA. The main conclusion from this study, since Kilauea and Kapa‘a Streams already had a known presence of *S. molesta* from physical surveys and the sample taken near the boat ramp was unreliable, is that Pu‘ukumu Stream would be a logical site for a physical survey to confirm the eDNA results.

C. Surveys of Southwest Streams and Resurvey of Northeast Streams

1. Goals

The goals of this section of the project were to conduct eDNA surveys of perennial streams on the southwest side of the island and to collect eDNA samples in upstream tributaries of watersheds that had positive results in previous eDNA sampling of northeast perennial streams in April 2018.

2. Methods

The same water sample collection, filtering, and lab methods used in April 2018 surveys were used for eDNA testing in August 2018. Within the NE tributaries, the following were sampled:

NE Tributary Sampling (Fig. 5)

- Kilauea: 1 site, 2 samples
- Kapa‘a: 8 sites, 18 samples
- Waika‘ea: 7 sites, 20 samples
- Pu‘ukumu: 1 site, 4 samples
- Kalihiwai: 1 site, 2 samples

On the SW side of Kauai, four samples were taken at the stream mouths from the following sites:

SW Sampling (1 site, 4 samples) (Fig. 6)

- Waimea
- Waipao
- A‘akukui
- Hanapepe
- Lawai
- Waiawa
- Waikomo

A total of 12 streams were sampled for eDNA with 25 sample locations distributed throughout the 12 stream systems (Fig. 5 and 6). From all 25 sample locations, 74 individual water samples were

collected and processed to determine the presence/absence of eDNA. Figures 5 and 6 were created using ArcMap 10.7.1 (GIS), metadata collected in the field, and the results of the eDNA surveys.

3. Maps

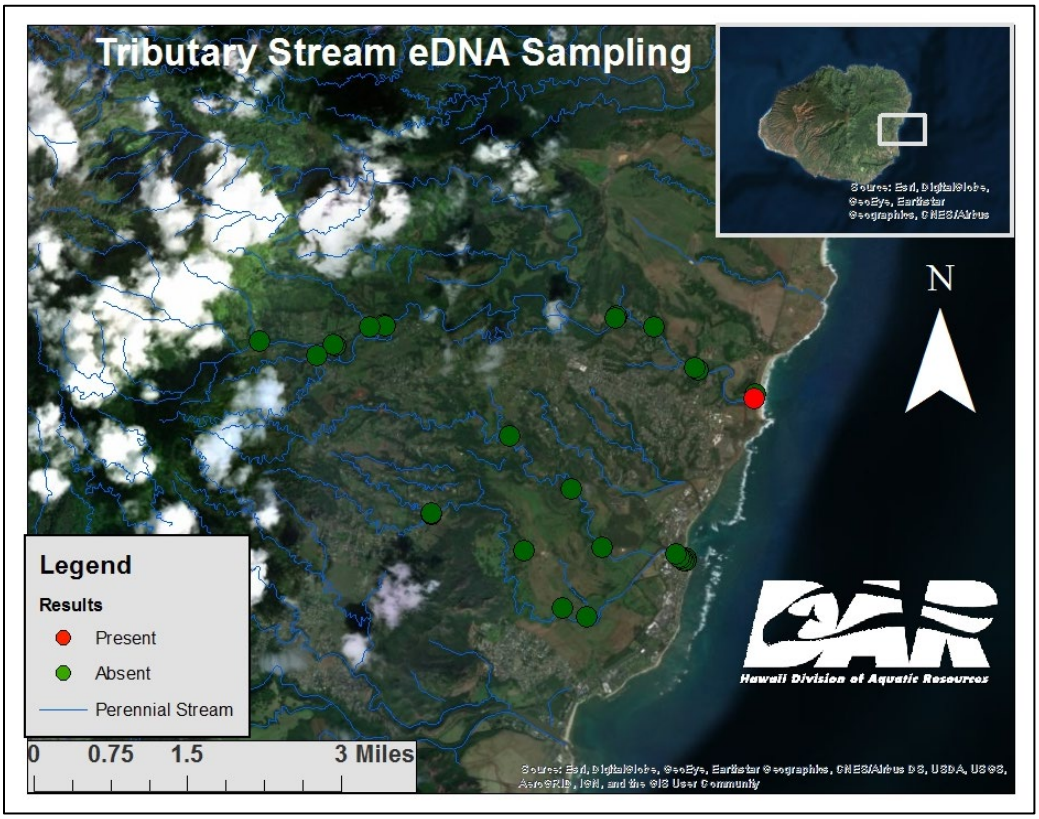

Figure 5. *Salvinia molesta* presence/absence map of northeast tributaries retested in August 2018.

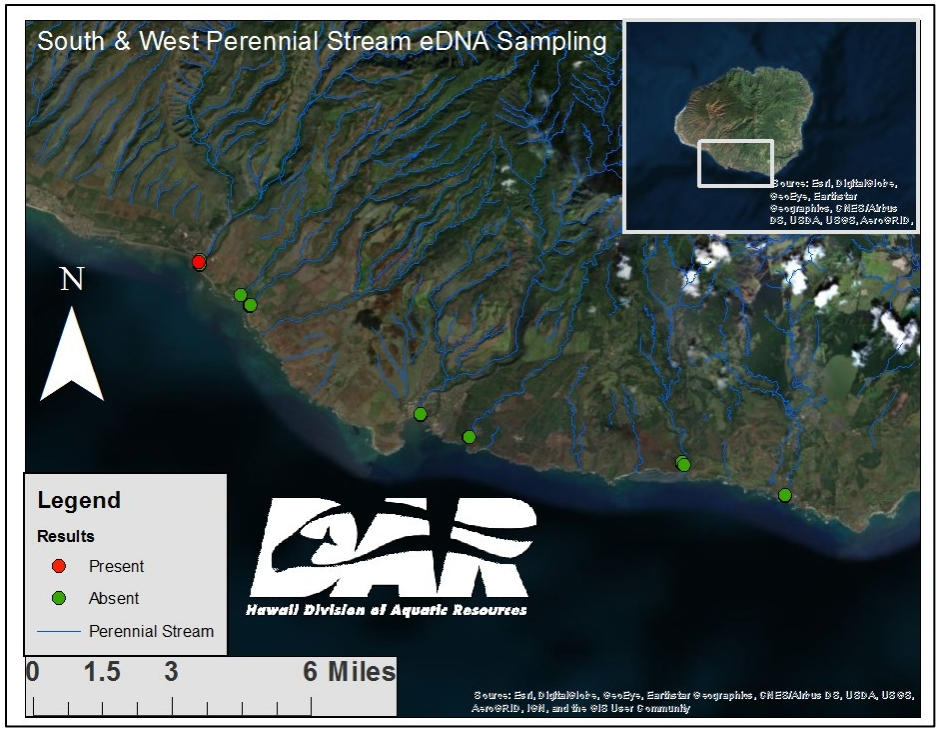

Figure 6. *Salvinia molesta* presence/absence map of southwest perennial streams in August 2018.

4. Results

The upstream tributary samples were negative for *S. molesta* in Kapa'a, Kilauea, and Waika'ea. This was expected as upstream water flow is stronger and not suitable habitat for *S. molesta.* However, one of the two samples from the mouth of the Kapa'a stream came back positive (Fig. 5). One out of four samples from the Waimea River came back positive, but all other samples from the SW stream mouths were negative (Fig. 6). These results were encouraging as they showed that the invasion of *S. molesta* was limited to a few watersheds on Kauai and had not spread further upstream. However, the results did conflict with previous surveys, showing no presence of *S. molesta* in Kapa'a, Kilauea, and Waika'ea, streams that previously had positive eDNA results. It was also unclear as to why Waimea Stream only showed one positive test of the four samples taken.

D. Resampling of Unclear Results

1. Goal

The goal of this section of the project was to sample areas with past partial positive eDNA results, meaning the results were from the same stream in which one or more outcome was positive and the rest were negative, to verify *S. molesta* presence.

2. Methods

Methods used for sample collection, filtration, and lab processing were repeated for stream systems with past partial positive eDNA results.

Resurveyed stream systems:

- Waimea: 1 site, 6 samples
- Kalihiwai: 1 site, 4 samples
- Pu‘ukumu: 1 site, 4 samples
- Waika‘ea: 1 site, 4 samples

3. Results

Analysis of the samples taken at stream mouths of Waimea, Kalihiwai, Pu‘ukumu, and Waika‘ea all came back negative. The previous positive eDNA results in 2018 were attributed to false positives. This is possible through a number of ways, including contamination during sampling, eDNA persistence (DNA remaining present in a water source after the source species has left), and sampling independent contamination (e.g. eDNA from target species being present due to bird or predator feces or ballast water; Burian et al. 2021). All mentioned scenarios are possible in this study and may explain the singular positive results from Waimea, Kalihiwai, Pu‘ukumu, and Waika‘ea Streams.

Therefore, as of February 2019, Kilauea and Kapa‘a streams are the only stream systems with verified populations of *S. molesta.*

III. Visual Surveys of Density and Extent

A. Goals

The goals of this section of the project were to conduct visual surveys to confirm the presence, absence, and extent of invasion of *Salvinia molesta* temporally in the Kilauea and Kapa‘a Streams, as well as to quantify the extent and the density of the *S. molesta* population in stream systems and understand temporal changes.

B. Methods

Visual surveys were conducted in the Kilauea Stream system in April 2018, August 2018, and February 2019, and in the Kapa‘a Stream system in August 2018 and February 2019. The surveys were conducted with a two-person crew on a kayak, where one person took data and operated the GPS, while the other paddled the kayak along the bank. Both team members visually scanned the bank and riparian vegetation, noting the presence of *S. molesta*. *Salvinia molesta* density was broken down into 5 categories: 0) absent, 0.5) sparse patches and small mats less than 1 m wide, 1) floating mats 1-2 m wide, 2) floating mats 2-3 m wide, and 3) large floating mats greater than 3 m wide. The beginning and end of each category were marked by a GPS point along the entire lower portion of the streams and on both banks. The points were then entered into ArcMap 10.7.1 and polylines following the bank were created. Each category was summed to get the total amount of each density per stream and the total length of the bank with *S. molesta* growth. Graphs and charts below were created using the data from these surveys (Fig. 7, 8, and 9).

C. Results

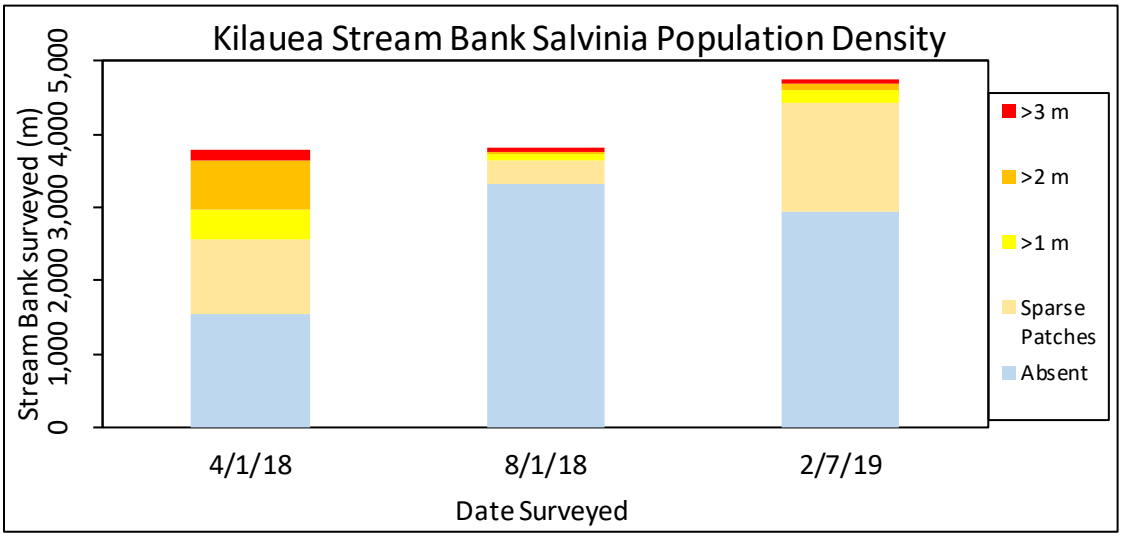

Figure 7. Results from visual surveys conducted along the bank of the Kilauea Stream from 2018-2019.

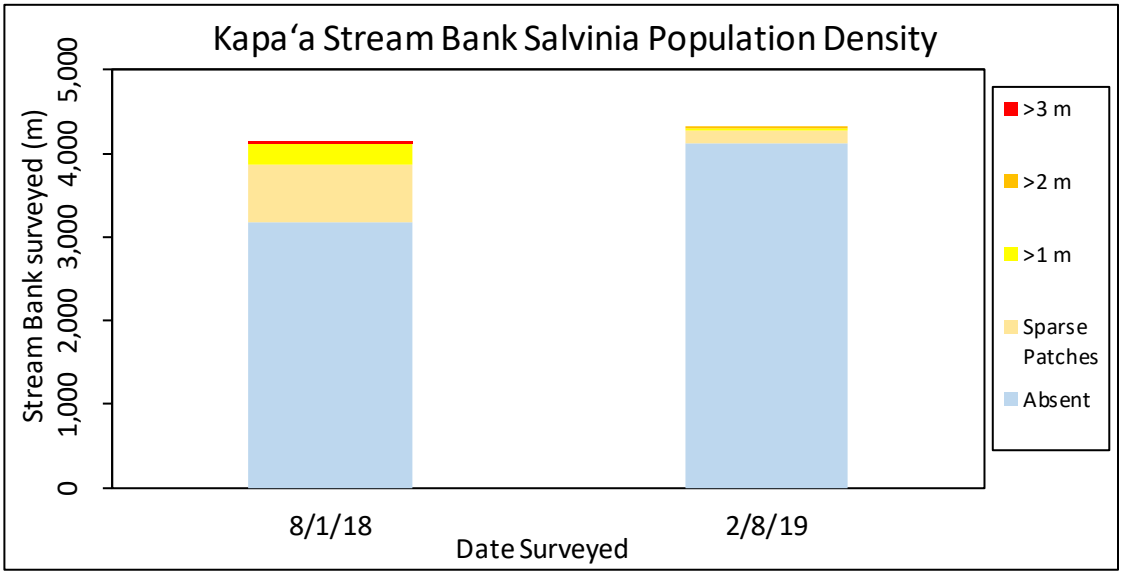

Figure 8. Results from visual surveys conducted along the bank of the Kapa‘a Stream from 2018-2019.

From the visual data collected along the banks of both the Kilauea and Kapa‘a Stream systems, the percentage of the banks that were clear of *S. molesta* varied greatly between survey dates. The first visual survey of the Kilauea Stream bank, on April 1, 2018, revealed that the area was heavily infested with *S. molesta*, with 58.8% of the survey area covered had *S. molesta* present. Of the areas with *S. molesta* present, 26.4% had only sparse patches, 11.0% had patches greater than 1 m thick, 17.5% had patches greater than 2 m thick, and 3.9% had patches greater than 3 m thick (Fig. 7 and 9A). During the subsequent survey four months later, the *S. molesta* cover along the bank had drastically reduced to 13.3% of surveyed area having *S. molesta* present (Fig. 7 and 9B). From the last visual survey, on February 7, 2019, more of the stream bank was surveyed, along with the same portions previously

monitored, which resulted in an increase in *S. molesta* cover (37.7% present), mostly in the form of sparse patches (31.1%) (Fig. 7 and 9C).

On August 1, 2018, *S. molesta* was present along 23.6% of the Kapa'a Stream bank, with 16.8% being in the form of sparse patches, 5.7% >1 m thick patches, 0.2% >2 m thick patches, and 0.9% >3 m patches (Fig. 8 and 9D). In the visual survey conducted the following year, on February 8, 2019, *S. molesta* cover decreased to 4.4%, where 3.9% was in the form of sparse patches, 0.1% was >1 m thick patches, and 0.4% was >2 m thick patches (Fig. 8 and 9E).

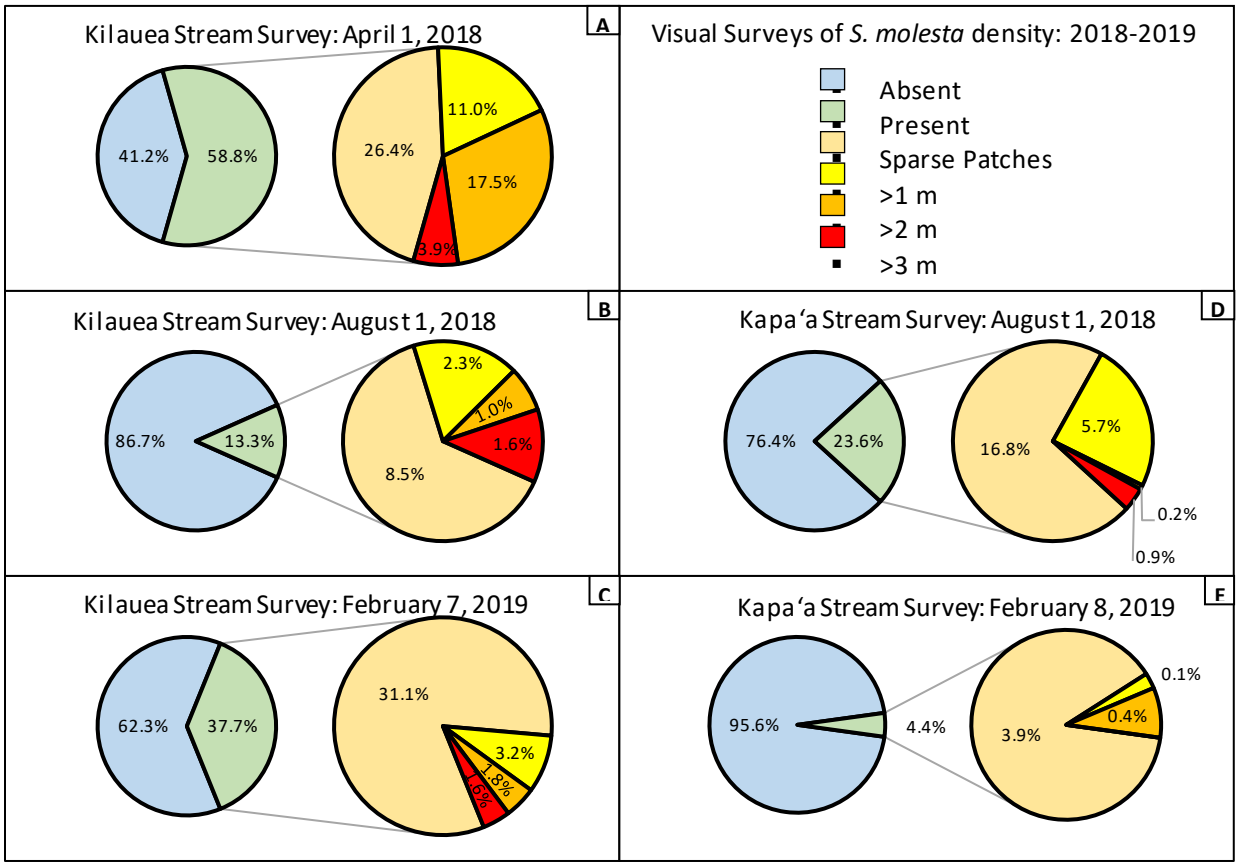

Figure 9: Percentage of *Salvinia molesta* density categories observed in the Kilauea and Kapa'a stream systems from 2018-2019. **A-C**: Percentage break down of the results from the visual survey conducted along the Kilauea Stream bank on April 1, 2018, August 1, 2018, and February 7, 2019. **D-E**: Percentage break down of the results from the visual survey conducted along the Kapa'a Stream bank on August 1, 2018 and February 8, 2019.

D. Stream Flow Data and Effect On Visual Surveys of *Salvinia molesta*

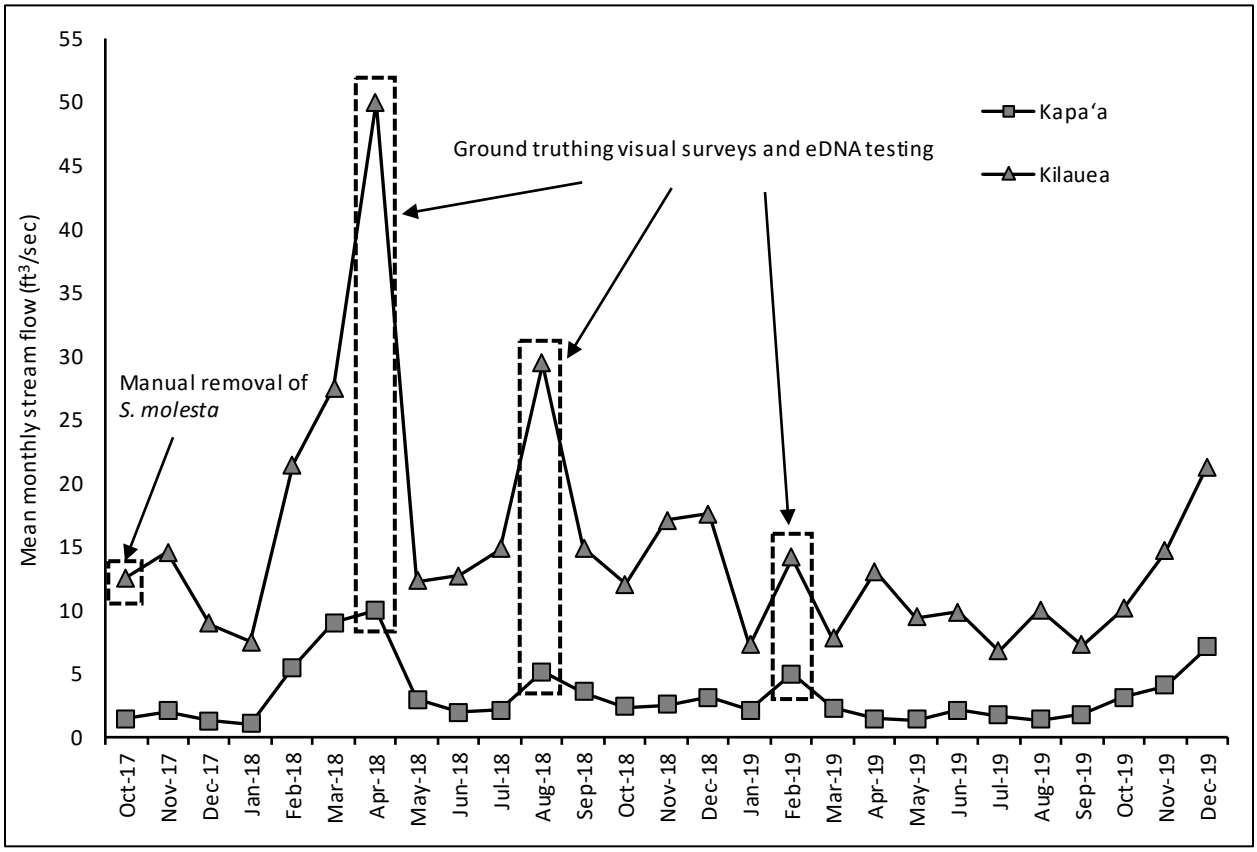

Figure 10: Graph of the mean monthly stream flow, from data obtained from USGS 2021a and USGS 2021b, at the Kilauea and Kapa'a Stream systems from October 2017 to December 2019.

E. Conclusions

In the months preceding the eDNA and visual surveys in April 2018, mean stream flow increased substantially, reaching its peak at 50 ft^3/sec in April 2018 (Fig. 10). In the months between April and August 2018, stream flow fell to between 10-15 ft^3/sec, but increased again to 29.4 ft^3/sec in August 2018 (Fig. 10). In February 2019, the stream flow was 14.1 ft^3/sec; however, the month before the eDNA and visual survey had half the mean stream discharge rates (Fig. 10). Higher flow rates were likely due to increased precipitation and could have influenced the eDNA and visual survey results. During times of low mean stream flow, *S. molesta* can grow and multiply quickly, once mean stream flow increases the *S. molesta* is flushed down the stream system. If samples were collected at stream mouths during times of high mean stream flow, it is more likely that *S. molesta* eDNA will be in higher concentrations.

The Kapa'a Stream system tended to follow the same mean stream flow pattern as the Kilauea Stream. In April 2018, August 2018, and February 2019, the mean stream flow was much higher in these months compared to the months before and after (Fig. 10). Therefore, the four positive results of the April 2018 surveys from the four water samples may have been influenced by high mean stream flow rates. With the negative results in the upper Kapa'a tributary and the false positive from the stream

mouth in August 2018, *S. molesta* may have been washed out of the stream during the high flow months from February-April 2018. The positive eDNA results from the February 2019 resurvey of the Kapa'a Stream mouth could indicate that not all *S. molesta* was washed out of the stream in 2018 and was able to multiply in the months after the August 2018 survey.

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