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Capture and Amplify: Novel Approaches to Extracting and Multiplication Entomopathogenic Nematodes Effectively

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Abstract

Entomopathogenic nematodes (EPNs) are members of soil communities that play an essential role as biological control agents. Two major challenges to understanding and exploring EPNs are the difficulty in extracting from soil and the multiplication in the lab to be used for further research or biological control. Traditional methods of extraction have low recovery rates of live nematodes, while the use of past multiplication methods has resulted in bacterial and fungal contamination, and have low production. We designed a new instrument, the NEMA Device, that can be used for both extraction and multiplication. We evaluated the efficiency of our novel device by comparing it to the Baermann funnel extraction method and the White trap method commonly used for multiplying nematodes. To validate the instrument we used two nematode species (*Steinernema khoungi* and *Heterorhabditis bacteriophora*) that vary in size and are commonly used to control pest species. The NEMA Device resulted in having higher recovery rates of both *S. khoungi* and *H. bacteriophora* compared to the Baermann method. There was no difference in production rate from the NEMA Device or the White trap. This new device promises to be instrumental in improving in recovering more nematodes. This standardized, cost-effective methodology will make

studying these agriculturally important nematodes more accessible and result in better outcomes.

1. Introduction

Entomopathogenic nematodes (EPNs) are non-segmented roundworms that parasitize a wide range of insects and have been intensively studied to understand how they infect and kill their hosts. During their infective juvenile stage, they are in their non-feeding state and actively seeking their next host. After entering a host via openings, they release their symbiotic bacteria killing their host within 24-74 hours and completing their life cycle (Shapiro-Ilan et al., 2023 and Hazir et al., 2022). These nematodes have been shown to successfully control a range of pest species both above and below ground and have been mass commercialized as biological control agents (Bhat et al., 2020 and Abate et al. 2017).

Nematodes from the *Heterorhabditidae* and *Steinernematidae* families are the most commonly used as biological control agents (Georgis et al., 2006, and Kaya et al., 2004). EPN species from these families have controlled pest species in field trials including weevil pests (*Otiohynchus* spp.) on strawberry plants (Kakouli-Duarte et al., 1997) and root-knot nematodes, like *Meloidogyne incognita,* on watermelon plants (Abd-Elgaward 2019). Nematodes have also been used effectively in combination with traditional control agents such as pesticides. In field trials, Filgueiras et. al. (2023) found that *H*. *bacteriophora* and *S. feltiae* were effective in the control of onion maggots (*Delia antiqua*) in the presence of various pesticides. Chavan et al., (2018) found that most pesticides commonly used in rice ecosystems are compatible with *H. indica* except for some that lead to higher mortality rates of the species. Over the years, research into the benefits of EPNs has led to the formulation of packaging for large-scale production by various companies for agricultural applications (Abate et al. 2017).

Understanding how EPNs control pests is crucial for improving pest management strategies in agriculture and can have positive environmental impacts (Sabbahi et al., 2022 and Koller et al., 2023). To have a better understanding of EPNs, researchers must first be able to isolate them from soil samples and culture them in the lab. Numerous extraction and multiplication methods have been developed, but they all have some limitations. One difficulty lies in separating live nematode species from soil samples while avoiding contamination and mortality. There are multiple methods such as Cobb's, Erlenmeyer, Oostenbrink elutriator, or Centrifugal (Bezooijen, 2006) that have been developed to extract soil nematodes, however, many of these kill the animals in that process. Variations of the Baermann method have been used most frequently to successfully extract live EPNs from the soil (Viglierchio et al., 1983), but these are inherently prone to contamination and have relatively lower recovery rates (Filgueiras et al., 2016 and Cesarz et al., 2019). Additionally, factors such as desiccation, soil type, and temperature can affect the efficiency of extraction and multiplication of EPNs. Rearing nematodes often consists of using an insect host such as *Galleria mellonella* through the White trap method. The species *G. mellonella* is a species of lepidoptera also known as the greater wax moth. The larvae are useful in multiplying EPNs due to their availability and weak immune system, which makes them susceptible to EPN infection (Devi 2021). The White trap method uses *G. mellonella* larvae as the insect host to multiply nematodes in the lab. However, the White trap method has limitations,

such as fungal and bacteria growth leading to contamination, and studies have reported that mass culturing is expensive due to high labor costs and higher contamination at a greater scale (Rahoo et al., 2018, and Testa et al., 2017).

To address the challenges associated with nematode recovery and multiplication methodologies, our research focuses on comparing these traditional methods to our newly developed NEMA Device The NEMA Device has been constructed to incorporate aspects of the Baermann method and the White trap to both recover live nematodes from the soil and multiply them in large quantities. We compared the effectiveness of the NEMA Device to the traditional methods using two EPN species (*Heterorhabditis bacteriophora* and *Steinernema khoungi*) that vary in size. *Steinernema spp.* have a larger body size (average ≥ 1000 µm) (Stock et al., 2019) and *Heterorhabditis spp.* have a smaller body size (520-600 µm) (Polinar, 1975). Incorporating both of these species provides a representation of the diverse range of sizes of EPNs that can evaluate the effectiveness of the NEMA Device.

We hypothesize that the NEMA Device will exhibit a higher performance in both nematode recovery and population multiplication compared to the traditional methods. Additionally, we are interested to know if there are differences in recovery rates and population production in the NEMA Device due to EPN sizes. The goal of our study is to determine the effectiveness of the NEMA Device in improving nematode methodologies, with a particular emphasis on cost-efficiency, to provide a valuable tool for advancing research in the field of nematology.

2. Methods

Figure 1. Traditional Methods Illustration: The Baermann funnel (1) for extracting nematodes from soil and the White trap (2) for multiplying/rearing nematodes.

2.1 Description of Traditional Soil Extraction and Multiplication Methods

The Baermann funnel (Fig. 1.1) is the most common technique used to extract live nematodes from soil. This technique uses funnels ranging from commercially produced funnels to upside-down halves of plastic bottles that are connected to a collecting tube. The thread of the bottle mouth was covered with plumbing tape to prevent leakage. A KimTech Science™ KimWipes™ served as filter paper and was placed within the open bottle (Fig. 3) and filled with autoclaved sand containing

nematodes. The filter paper is then folded over the top and water is poured into the funnel until the filter paper is covered and left for 24 h. These infective juveniles swim across the filter paper and migrate downwards into the collecting tube. After 24 h, the funnel is disassembled from the collecting tube containing live nematodes for collecting.

The White trap (Fig. 1.2) is commonly used to multiply populations of nematodes in a host. Inoculated, symptomatic hosts are placed on a small Petri dish lined with filter paper and housed in a medium Petri dish with water. The filter paper is kept moisten to help nematodes move across the surface. The traps were filled until the water level reached approximately halfway up the Petri dishes and covered with a lid to prevent evaporation. As the nematodes emerge from the cadaver, they migrate to the water in the medium Petri dish for collection. For the collection of the White trap, the trap is uncovered and the small Petri dish is removed and set aside, the nematode solution is collected by pouring it into a collecting tube. The volume of water collected was then replaced with more reverse osmosis water and reset the trap, allowing the collection to continue. If the filter paper was dry or semi-dry, we moistened it by adding droplets of reverse osmosis water.

2.2 Organisms: EPN rearing

We used *H. bacteriophora* and *S. khoungi* which are housed in the Natural Enemy Management and Application (NEMA) lab at UNC Asheville. We followed the standard technique from Filgueiras et al., (2021) to rear nematodes in *G. mellonella* larvae. This included adding 1 mL of EPN suspension (at 1000 IJ/mL) of each species to healthy *G. mellonella* larvae that were placed on a clean filter paper in 54 mm Petri dishes. These were monitored daily and reverse osmosis water was added to maintain a damp substrate to induce nematode release and migration. Following emergence from *G. mellonella* cadavers, nematodes were extracted and stored in 250 ml tissue culture flasks at 25^{o} C until use in assays within 7 d of emergence.

Figure 2. NEMA Device Illustration: New multi-dimensional system for both extraction (1b) and multiplication (1a) of nematodes.

2.3 NEMA Device

The NEMA Device has three nested PVC components (Fig.2), combining elements of the Baermann funnel and White trap method. The initial PVC component (Fig.2 1a & b) is a screen system that serves as a dual function. First, for the extraction method, the screen component is upward, serving as a reservoir to place a KimTech Science™ KimWipes™ to hold autoclaved sand containing nematodes. Reverse osmosis water is poured onto the autoclaved sand and filtered paper until fully covered and left for 24 h. Second, for the multiplication, when the screen is flipped, it serves as a platform for a small Petri dish containing inoculated *G. mellonella* larvae. Reverse osmosis water filled until the water level reached approximately halfway up the small Petri dish and covered the device with a lid to prevent evaporation. The next component (in which the screen is placed) is a modified PVC cap with a hole drilled through the center functioning as a drainage system. This component sits on a PVC coupler (diameter = 12.7cm) and functions as a base. A silicon tube is connected from the bottom of the modified cap and then passes through a hole in the PVC coupler that serves as an exit channel. A valve is connected to the silicon tube to control the flow of water, allowing nematodes to pass through when open and preventing all drainage when closed. To collect from the NEMA Device, the nematodes settle near the bottom and we secure a collecting tube at the end of the silicon tube and open the valve to release the nematodes.

Figure 3. Bioassay 1 Illustration: The Baermann funnel (left), consists of a half-plastic bottle connected to a collecting tube and containing a single KimTech Science™ KimWipes™ serving as a filter paper. On the right is the NEMA Device, imitating the Baermann funnel, with the screen facing upwards to hold the filter paper and soil.

2.4 Bioassay 1: Soil Extraction

In the first bioassay, we compared the Baermann funnel method of extraction (*S.k.* n=4, *H.b.* n=10) with the NEMA Device (*S.k.* n=5, *H.b.* n=10). For the initial set of five replicates of *H. bacteriophora,* we added 3 mL of *H. bacteriophora* stock into each treatment, applying 2100 IJ/mL. For the following five additional replicates of *H.bacteriophora*, we added 3 mL into each treatment, applying 2400 IJ/mL. For the five replicates of *S. khoungi,* we added 4 mL of *S. khoungi* stock into each treatment, applying 2800 IJ/mL. During this process, any funnels that leaked were removed. For each collected sample, they were transferred onto a marked Petri dish with a grid pattern, where each nematode was counted individually (Fig. 5).

Figure 4. Bioassay 2 Illustration: On the left, the White trap method is shown, with a small Petri placed inside a medium Petri dish filled with reverse osmosis water. On the right, this aspect of the NEMA Device uses aspects of the White trap method, with the screen facing downwards and a small Petri dish placed on top.

2.5 Bioassay 2: Multiplication

In the second bioassay, we compared the White trap method of multiplication (n=5 for both species) with the NEMA Device (n=5 for both species). The same protocol was followed for multiplying EPNs by inoculating *G. mellonella* before being transferred to either the White trap or the NEMA device. Three symptomatic *G. mellonella* cadavers were randomly distributed to each replicate and EPNs were collected until no more nematodes emerged from the cadavers. During this process, contaminated cadavers were removed. Samples were reduced to 10 mL. From this, 1mL was pipetted and the number of nematodes was counted.

2.6 Bioassay 1:Soil Extraction Data Analysis

Analysis was conducted in R (4.3.1) using RStudio as an IDE and supporting packages that were used include: tidyverse, car, emmeans, lmtest, multcomp, MASS, AER, foreach, and doParallel. The analysis aimed to assess the efficiency of the NEMA Device compared to the Baermann funnel in recovering EPNs. To compare the recovery rates between treatments we subtracted the applied nematode before the bioassay from the number of nematodes counted after for both *H. bacteriophora* and *S. khoungi*. The normality of the data distribution was assessed using the Shapiro-Wilk test (W=0.955, P=0.243), indicating a normal distribution. A Levene's Test was performed to evaluate the variability in recovery rate across species and treatments, revealing non-significant differences (df= 3, F= 1.586, P=0.218), assuming equal variances. A linear model was created to investigate the relationship between the recovery rate and species and treatments. Best fit models were chosen after considering residual analysis, interactions, and likelihood ratio tests. This was followed by Tukey's tests and a comparison pair test using the mean recovery test in combination with species and treatment. To compare the differences in recovery rates between the Baermann funnel and NEMA Device within each nematode species, a bootstrap analysis was conducted to replicate statistical data in mean recoveries (Fig. 7). A Permutation test was analyzed for significant differences in mean recoveries between the *S. khoungi* and *H. bacteriophora* between devices. Visualization was created to illustrate the recovery rate in species and treatments.

2.7 Bioassay 2: Multiplication Data Analysis

Data analyses for the second bioassay were conducted using RStudio with the packages used in the first bioassay. The analyses aimed to assess the efficiency of the NEMA Device compared to the White trap in multiplying EPNs. The number of EPNs per mL was counted. The number of *G. mellonella* cadavers was considered to calculate the total number of EPNs per sample (total amount of nematodes divided by the number of cadavers). Data normality was assessed using the Shapiro-Wilk test (W=0.287, P<0.001) and Levene's Test for EPN counts per mL (df= 3, F= 0.759, P=0.518). The Shapiro-Wilk test indicated a non-normal data distribution resulting in doing data transformation. Best fit models were also chosen after considering residual analysis, possible interactions, and likelihood ratio tests followed by Tukey's tests. Visualization was created to demonstrate the EPN counts per mL in species and treatments.

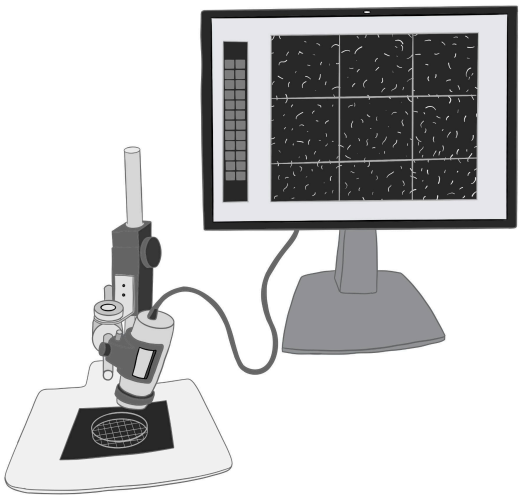

Figure 5. EPN Counting Illustration: All samples that were prepped for counting were placed within a grid marked Petri and using the DinoLite camera projected onto the computed screen for easier counting.

3. Results

3.1 Bioassay 1

H. bacteriophora and *S. khoungi* had a significantly higher (t=3.6, df=26, P=0.001) recovery rate in the NEMA Device compared to the Baermann funnel (Fig. 6). *S. khoungi* had a 72% (ranging from 62% to 83%; 95% CI) recovery rate in the NEMA Device compared to the Baermann funnel recovery rate of 53% (ranging from 42% to 64%; 95% CI). Similarly, *H. bacteriophora* had a 62% (ranging from 53% to 70%; 95% CI) recovery rate compared to the Baermann funnel recovery rate of 42% (ranging from 33% to 52%; 95% CI). However, there were no significant differences in recovery rate between species (t=-1.9, df=26, P=0.07).

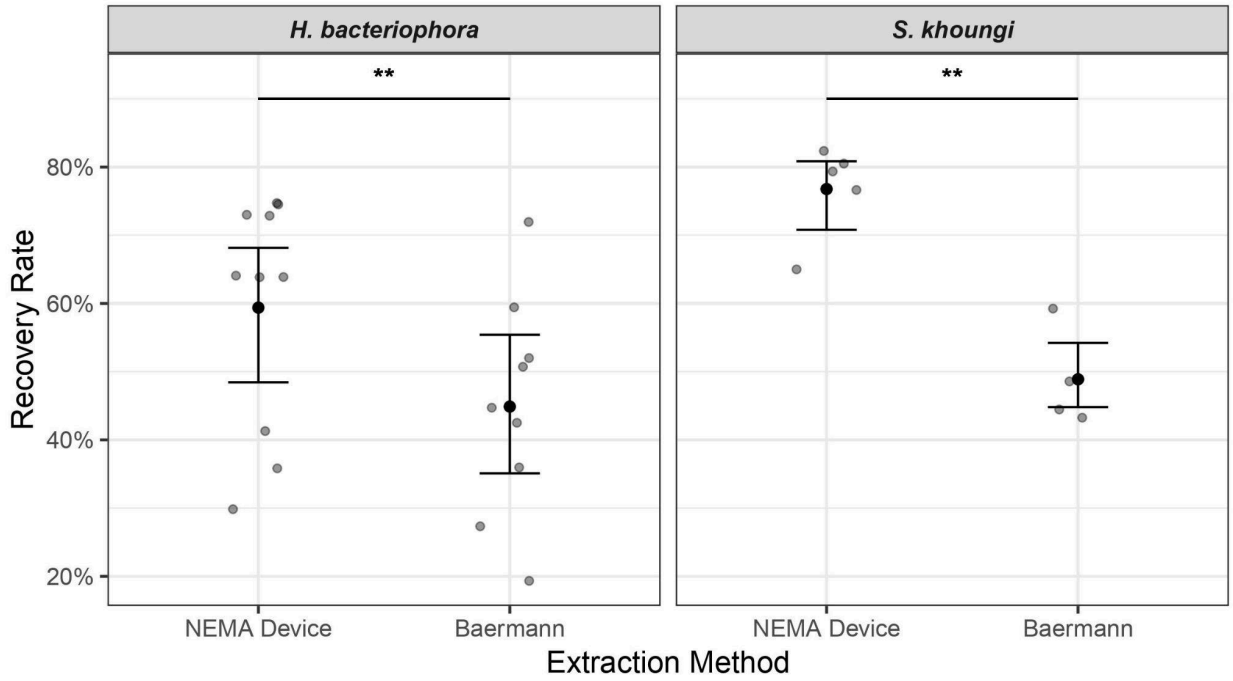

Figure 6. Nematode recovery rate from extraction methods. Solid dots and error bars denote the mean and 95% CI, respectively. Gray points denote observed values. Double asterisks denote significance at P=0.001.

There was a significant difference between *S. khoungi* and *H, bacteriophora* in the mean difference in recovery rates between the devices from the Bootstrap summaries (P=0.046, Permutation). *S. khoungi* had a significantly higher increase in recovery rate using the NEMA Device compared with *H. bacteriophora* (Fig. 7).

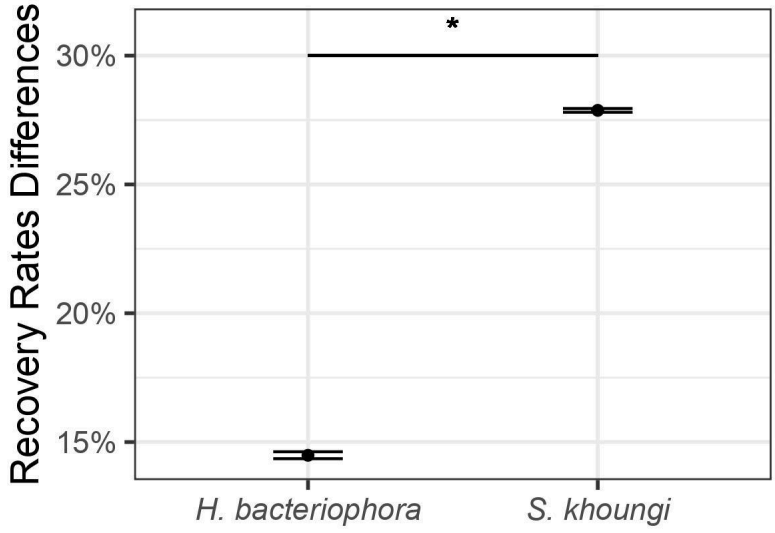

Figure 7. Comparison of mean differences in recovery rates between the NEMA Device and Baermann funnel using Bootstrap summaries. Solid dot and error bars denote

mean recoveries and 95% CI, respectively. A single asterisk denotes significant differences between the mean difference in recovery rates and devices at P=0.046.

3.2 Bioassay 2

H. bacteriophora and *S. khoungi* did not have a significant difference of (t=0.411, df=12, P=0.689) EPN counts in the NEMA Device and White trap (Fig. 8). However, a significant difference was observed between the two species in the number of individuals produced (t=4.56, df=12, P=0.001). *H. bacteriophora* had higher EPN counts from both devices compared to *S. khoungi*. Though not measured, we observed more contamination in the White trap compared to the NEMA Device.

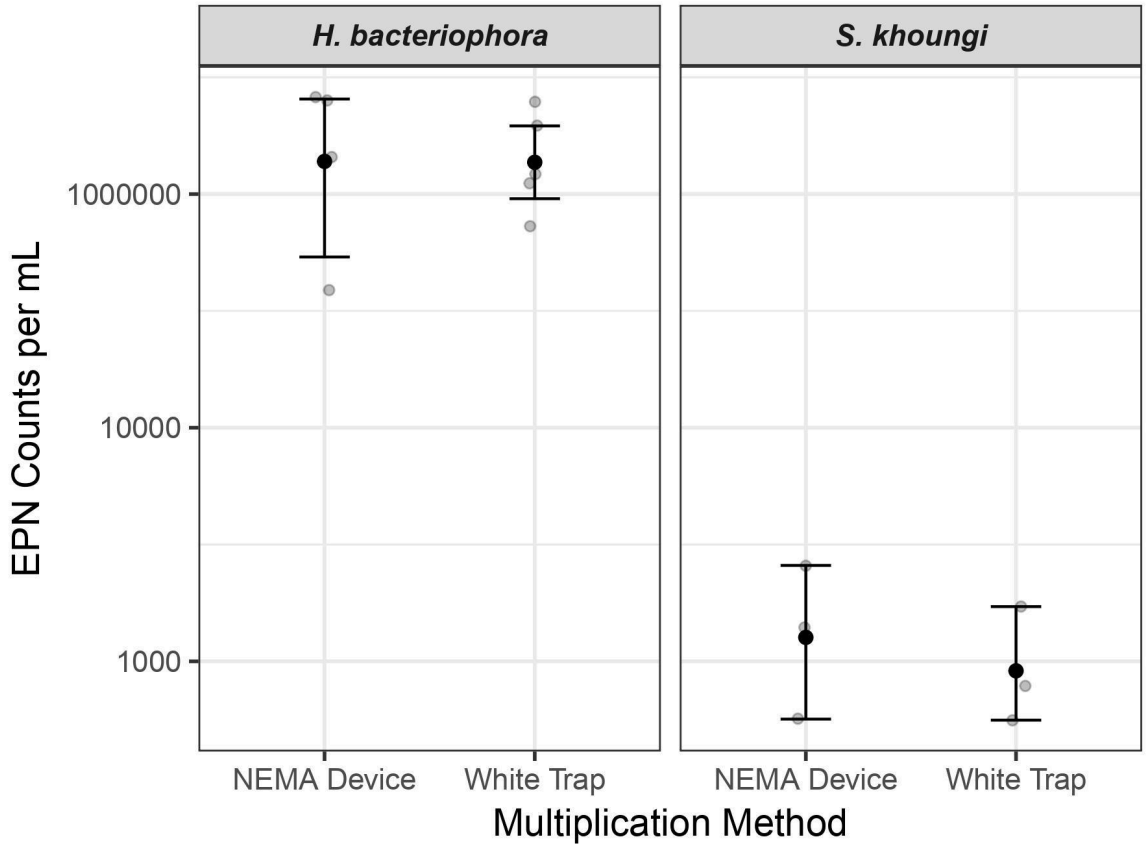

Figure 8. Nematode counts per mL from the multiplication methods. Solid dots and error bars denote the mean and 95% CI respectively. Gray points denote observed values.

4. Discussion

Our novel NEMA Device is a practical method that can be used to extract nematodes efficiently. This device is a modification of the Baermann funnel (Baermann, 1917) to extract nematodes from soil samples and a modified White trap (White, 1927) used to multiply nematode populations. A study by Filgueiras et al. (2016) stated that the Baermann funnel extraction efficiency is 13.1 ± 1.4% from soil samples, and the

extraction efficiency in the NEMA Device is 62-83% of *S. khoungi*. During multiplication, both devices had no differences in the EPN population.

We found that the NEMA Device had a significantly higher recovery rate of both *S. khoungi* and *H. bacteriophora* compared to the Baermann funnel (Fig. 6). The NEMA Device successfully extracted two different EPN species. Similar results were seen in Viglierchio et al. (1983), where the Baermann funnel was able to extract different nematode species. *S. khoungi* had a greater difference in percent recovery than *H. bacteriophora* (Fig.6), similarly Shamseldean et al., (1995) extracted a higher recovery *Steinernema spp* (larger size) than *Heterorhabditis spp* (smaller size). This difference in recovery rate between the two species could be attributed to the large size of *S. khoungi* and the large diameter of the NEMA Device in comparison to the Baermann funnel. The small diameter of the Baermann funnel could have restricted the movement of *S. khoungi* while allowing the smaller *H. bacteriophora* to move more easily into the collection device. The NEMA Device had no leakage and collection was simpler compared to the Baermann funnels.

Our study found that *H. bacteriophora* nematodes had a higher EPN production in both devices compared to S. khoungi nematodes (Fig. 8). In relation to nematode size, Kaya et al., (1987) noted the progeny of nematodes depends on size, *H. bacteriophora* being smaller has the advantage of an abundance of food resources allowing them to emerge later and in greater quantities. *H. bacteriophora* had a greater variation in EPN counts than *S. khoungi*. Similar results were found using the White trap in Rahoo et al., (2018) in the same genera; we observed higher recovery rates for *H. bacteriophora* than the other EPN in our study. The NEMA Device retained more moisture on the filter paper than the White trap which provided a suitable environment for EPN reproduction. Additionally, the White trap exposes the host during collection; the NEMA Device allows nematodes to pass through the tube and get collected without exposing the host. The NEMA Device has a larger component that allows for nematodes to swim freely and not be constricted compared to the Petri dish size in the White trap method.

The evaluation of the NEMA Device's effectiveness has demonstrated an increase in recovery rate in both *H. bacteriophora* and *S. khoungi*. The Baermann funnel methods' effectiveness was optimized through the multi-component structures of the NEMA Device, which is composed of a PVC system. Furthermore, the NEMA Device has proven to be a valuable tool for minimizing labor-intensive procedures, time investment, and the risk of contamination. It is a practical tool that has been designed for accessibility, inexpensive construction, and utilization for both nematode extraction and production.

Our study demonstrates the effectiveness of the NEMA Device in achieving a higher recovery rate for two genera of entomopathogenic nematodes. The NEMA Device has been designed to enhance extraction at a low cost and be used to continue research on the importance of EPNs as biological control agents in agricultural systems. Implementing more biological control more efficiently will potentially reduce the amount of chemical pesticides that are harmful to environmental and human health.

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