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The Use of Filters in the Sump for Monitoring the Health of Laboratory Zebrafish (*Danio rerio*)

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Early detection of pathogens is imperative for the health of laboratory zebrafish and to ensure reproducible scientific results. While most pathogens are present as subclinical or chronic infections, their presence can be a confounding factor in data collection, and some infections can affect zebrafish health and reproduction. Current methods to test for pathogens sample myriad sources, including sump swabs, detritus, water collection or filtration, and whole-fish PCR or histopathology. Sentinel mice have been used in the past for mouse health monitoring, but there has been a recent shift to replace sentinel animals with filters in rack exhausts. We wanted to determine whether such methods could be translated from mouse racks to zebrafish systems. First, we tested whether nitrocellulose filters would affect the health and behavior of adult and larval zebrafish and found no adversary effects. Next, we placed filters in the sumps of zebrafish racks to be collected and tested for pathogens at monthly intervals using PCR, and results were compared with those detected on filters in which water was actively vacuum pumped through or swabs of sump biofilm. Results suggest that the efficacy of filters may wane with prolonged use, with sensitivity being greatest at 60 d and then decreasing at 90 d. Results also showed the limitations of current testing methods for zebrafish health monitoring, with efficacy of detecting pathogens varying widely based on the method of collection. Our recommendation is to test the filters after 6 wk of exposure, and supplement with whole-fish testing of clinically ill fish.

Abbreviations and Acronyms: G, generation; PES, polyethersulfone; VMR, visual motor response; TOST, two one-sided t tests

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Introduction

Health monitoring of animals within laboratory settings has advanced significantly in the past several years. The gold standard used for mouse colonies has historically been the examination of sentinel mice exposed to soiled bedding.[1] Within the last decade evidence has supported the use of exhaust dust as an alternative form of pathogen measurement.[1] Filters applied to rack ventilation units have repeatedly been shown to be an accurate form of monitoring for several notable murine pathogens, including murine norovirus, *Helicobacter* spp., Pasteurellaceae, *Klebsiella oxytoca*, *Pneumocystis murina*, and *Proteus mirabilis*, as well as several parasites.[2] This has led to research facilities replacing sentinel animals with filters and greatly reducing the number of animals that need to be used for health monitoring purposes. The goal of our study was to test whether nitrocellulose filters placed in the sumps of zebrafish racks can be used for pathogen detection.

The zebrafish has become a popular animal model for use in research since their introduction in the 1980s.[3] They can produce large clutches that develop rapidly and are easy to manipulate genetically, making them ideal models in the field of genetics and developmental biology.[3] Due to their relatively new use as a research model, aquaculture health monitoring is not as well understood compared with health monitoring of rodents.[4] Most zebrafish housing utilizes a recirculating system: dirty water flows down to a collective sump and is then filtered and sanitized before the cleaned water is returned to the tanks.[4] This housing method is designed in such a way that water is not regularly shared between tanks, which is meant to help mitigate the spread of disease within a population.[5]

Most of the diseases within zebrafish colonies are chronic and subclinical in nature, making early detection imperative for maintaining colony health. Current health monitoring recommendations call for quarterly testing of sentinels unless fish are found to be symptomatic.[6,7] This makes early and accurate detection essential, as a false negative result could lead to proliferation of the disease. Several infectous agents that cause disease are hardy and difficult to eliminate once they have taken root in a population. *Mycobacterium* spp. can persist in the environment and replicate without fish present through the creation of biofilms, and *Pseudoloma neurophilia* is capable of horizontal and vertical transmission, allowing it to quickly propagate within a system.[8,9] Besides endangering the welfare of the animal, several pathogens that zebrafish can harbor have been shown to be zoonotic (*Mycobacterium* spp., *Aeromonas hydrophilia*, and several other bacteria and parasites[10]), and subclinical infections have been shown to potentially impair or otherwise negatively impact research data, making early detection imperative.[11]

Current health monitoring and pathogen detection in aquaculture relies on testing from multiple sources, including testing of the water, detritus/feces, tank biofilm, and sentinel fish histopathology and PCR.[12,13] Each test type has been shown to be accurate for testing of different disease pathogens: *Mycobacterium* spp. are readily picked up on PCR using environmental

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sampling, whereas *P. neurophilia* is more readily discovered with sentinel testing[14,13] The wide range of samples that need to be collected, tested, and maintained for adequate health monitoring is both time and labor intensive, and comes with the cost of animal lives. With advances in health monitoring methods for rodents based on the housing system, we wanted to see if nitrocellulose filters, placed in the sumps of zebrafish racks, would be adequately effective at detecting pathogens when compared with testing of actively filtered tank water or sump swabs, using whole-fish PCR for determining test sensitivity. Hydrophobic nitrocellulose was chosen as our filter material as recommended for use of filtering pathogens in water.[15] While nitrocellulose production plants have been known to cause toxicity to fish in nearby waterways, there has been no evidence that exposure to the material itself is toxic.[16] For this reason, prior to placing the filters into system sumps, we performed a safety study to investigate potential for toxicity, where we directly exposed fish to the filters within their tanks. Realistically, fish would have a much lower exposure to nitrocellulose via a filter in the sump. Polyethylene items were used to hold the filters in place, as polyethylene is the standard plastic used in zebrafish housing systems.

Our goal was to determine whether these filters, when left in sumps for various amounts of time, can detect the presence of several pathogens normally detectable through other means, which may help to cut down on the number of fish and environmental samples needed for health monitoring purposes in the future. We hypothesized that the filters present in the sumps would be safe for the fish, would detect more pathogens than the other methods, and may detect more pathogens over time.

Materials and Methods

Ethics statement. The study was conducted at 2 AAALAC-accredited institutions, and the protocol was approved by the Purdue University Animal Care and Use Committee.

Filter safety study. *Animals.* Zebrafish with different genetic background used in this study were housed in static tanks filled with reverse osmosis system water and maintained a temperature range of 23.0 to 28.4 °C, pH 7.2 to 7.4, and dissolved oxygen at 3 to 7 mg/L. The static tanks were not provided additional heat or aeration. The room was on a 14-h light/10-h dark cycle. The room temperature was 24.4 to 26.1 °C. Tank water was fully changed weekly; therefore, ammonia and conductivity were not measured. Fish aging between studies were maintained on recirculating racks under the same conditions, although the recirculating tanks were at the higher end of the ranges provided, and tanks were not changed unless algae accumulated. Adult fish were fed twice a day with brine shrimp. Larvae were fed twice a day with 0.25 mL of 1 mg/mL Otohime A (75 to 250 µm, Reed Mariculture) in system water.

Fish may have been positive for zebrafish picornavirus, *P. neurophilia*, *Mycobacterium chelonae*, *Mycobacterium fortuitum*, and/or *Myxidium streisingeri*, and they were free of other known pathogens based on annual whole-fish PCR of fish on each system. Specifically, all pathogens were shared among both institutions except *M. fortuitum*, which was only at Purdue University, and *Pseudocapillaria tomentosa*, which was only at the University of Notre Dame. Water conditions were maintained within similar parameters at both sites.

Experiment 1. Exposure of larval zebrafish. To assess whether the filters had any acute deleterious effects on fish development, the fitness of larvae was assessed via performance on a visual motor response (VMR) behavior test as well as morphology assessments comparing the size of larvae total body and head measurements. Assessments occurred after an acute exposure of 96 h.[17] At 7 d postfertilization, larval zebrafish (n = 160) were housed at a density of 20 fish/500 mL in static tanks of system water that was filtered through a 22-µm filter. Four tanks each of 7 d postferilization larval zebrafish were exposed to either 0.45-µm, 47-mm, hydrophobic, nitrocellulose filters (WHA7184004, Sigma-Aldrich, St. Louis, MO)[17] or no filter (control) for 96 h. The filters were placed within holders that were made of polyethylene mesh (Amazon, Seattle, WA) and submerged in the tank water. They were clipped to the side of the tank to stay in place with binder clips. Control tanks did not have anything added to the tank. Every 24 h the larvae were counted for survival manually, tank-side. At 96 h, 5 fish/tank were placed in a 96-well plate for VMR. VMR is a noninvasive startle reflex to light onset and offset. The assay was conducted as previously reported.[18,19] Larvae were placed into separate wells in a 96-well plate with 0.5 mL of filtered system water. The larvae were presented with the light stimulus and illuminated by infrared light, and their locomotor activity during the experimental period was recorded by an infrared-sensitive camera that acquires video in 30 frames per second. The assay consisted of 30 min of dark adaptation, a 10-min light cycle, and a 10-min dark cycle. Data for total distance traveled were collected. After VMR, the same 5 fish/tank for a total of 20 fish/group were euthanized via an ice water bath, and morphology assessments of body and head were made. Once the 96 h were over, the remaining fish were raised on a recirculating rack until 90 to 120 d of age for use in experiment 3 (generation [G]1).

Experiment 2. Acute adult exposure. Adult zebrafish (n = 36) were exposed for 96 h to nitrocellulose filters in 1-L static tanks with system water or no filter control system water (6 nitrocellulose tanks [3 males, 3 females] and 6 control tanks [3 males, 3 females]). Any mortality or abnormal appearance was recorded. At 96 h, the male and female tanks of each group were combined for mating in approximately 500 mL of water in a breeding tank. The embryos were collected in a culture dish and housed in an incubator (26.5 °C) for 96 h. At 96 h, 20 larvae/tank were euthanized via an ice water bath, and morphology assessments of body and head were made. The remaining embryos were raised under normal conditions to adults, 90 to 120 d of age for use in experiment 4 (G2).

Control fish (n = 18) for acute and chronic experiments were housed on a recirculating rack system. The same controls were used for both the acute and chronic experiments, as well as for comparison to the G2 group.

Experiment 3. Chronic adult exposure. Adult zebrafish (n = 18) from experiment 2 were continuously exposed to the nitrocellulose filters in 1-L static tanks for approximately 90 d, at which they were bred again as above. Any mortality or abnormal appearance was recorded. The embryos were collected in a culture dish and housed in an incubator (26.5 °C) for 96 h. At 96 h, 20 larvae/tank were euthanized via an ice water bath, and morphology assessments of body and head were made.

Control fish (n = 18) for acute and chronic experiments were housed on a recirculating rack system. The same controls were used for both the acute and chronic experiments, as well as for comparison to the G2 group.

Experiment 4. Assessment of next generation breeding. Ninety- to 120-d-old adult fish bred from experiments 1 and 2 (G1, G2) were paired within each group for mating. The embryos were collected in a culture dish and housed in an incubator (26.5 °C) for 96 h. At 96 h, 20 larvae/tank were euthanized via an ice water bath, and morphology assessments of body and head were made.

Control fish (n = 18) for acute and chronic experiments were housed on a recirculating rack system. The same controls were used for both the acute and chronic experiments, as well as for comparison to the G2 group.

Measurements of head and body length and statistical analysis. Assessment of larval morphology involved measuring the head or total body length of larvae[20,21,22] using a microscope (Olympus SZX16) with camera (AmScope MU203-BI) at 2× and 8× magnification and ImageJ. Head and body length can be used as indicators of larval health and toxicity of substances to which larvae are exposed.[23,24,25] Equivalences of sample means were tested via an individual-samples two one-sided t tests (TOST) with an α threshold of 0.05 for significance. TOST is a statistical analysis wherein equivalence is assessed, in contrast to standard t tests where differences are assessed. That is, a significant result in a TOST indicates significant equivalence of the sample means as determined by their alignment within an equivalence range. The equivalence range is simply an upper and lower bound of mean difference representing the smallest effect size that would be interesting to the researchers.[26] Equivalence ranges were set to ±0.5 mm for body length, ±0.05 mm for head length, and ±1 cm for VMR distance traveled. Animal numbers were determined by those used in standard zebrafish toxicology tests.[17]

Filter study. Nitrocellulose filters (pore size 0.45 µm, diameter 47 mm) were placed in the zebrafish sumps at Purdue University and the University of Notre Dame. Multiple different housing systems were used. Rooms with one sump had 3 filters placed in the sump. Rooms with multiple sumps had one sump with 3 filters (defined as system filters) and the rest with one sump (defined as sump filter). Mesh polyethylene biofilter bags (Amazon, Seattle, WA) were placed inside the sumps. Each biofilter bag contained nitrocellulose filters housed in polyethylene mesh plastic holders to keep the filters flat and in place, as well as aquarium rocks (PetSmart, Lafayette, IN) to ensure that the bags remained submerged (Figure 1). Polyethylene fishing line (Amazon, Seattle, WA) was used to anchor the bags in place. A total of 15 systems and 58 sumps (n = 58) were tested, from racks of various makes and models. From the system filters, one filter was collected every 30 d starting at 1, 2, and 3 mo from placement. Sump filters were collected at 92 d. In addition, at the time of each filter collection, sumps were swabbed for biofilm and 3 L of sump water was vacuum filtered through a 0.45-µm polyethersulfone (PES) filter (referred to from here as water test) (Fisher Scientific, Pittsburgh, PA) (Figure 2). Nitrocellulose filters, swabs, and PES filters were sent to VRL Diagnostics (Gaithersburg, MD) for PCR testing, and results were compared with whole-fish PCR to conduct a sensitivity analysis for each detection method.

Statistical analysis. For each testing method (filter, swab, water) we calculated sensitivity of detection for each of 6 pathogens. Sensitivity is calculated as the number of true positive detections divided by the sum of true positives and false negatives (that is, standard sensitivity analysis). True positive and false negative outcomes were assessed based on comparison to the results of that year's whole-fish PCR sentinel monitoring results. That is, if a positive result for a pathogen was detected in a given room via whole-fish PCR as part of our annual sentinel monitoring, we assumed that the pathogen was present in that room and therefore a positive result would be expected from every testing method for that room. Thus, a positive result from the novel detection methods for rooms where the pathogen was previously detected with whole-fish PCR represents a true positive, and a negative result represents a failure to detect, that is a false negative. Sensitivity therefore represents the ability of a given detection method to detect pathogens when they are present.

Figure 1. Example of filter assemblies placed in fish rack sumps. Depicted is a nitrocellulose (Nitro) filter, stabilized with a black, polyethylene plastic holder apparatus contained within a mesh polyethylene biofilter bag retrieved from a sump after its submersion period.

Sensitivity is therefore agnostic toward rooms where a given pathogen was not detected with whole-fish PCR, and those cases would be covered under an analysis of specificity, which is the ability of a detection method to produce a negative result when the sample was truly negative, that is, avoiding false positives. Specificity was also calculated, but these results are not presented herein because the primary aim of this study was to determine whether the novel tests produced a comparable sensitivity to whole-fish PCR. That is, we were more concerned with avoiding the scenario of failing to detect a pathogen when it is actually present, and less concerned about preventing false positive results. Once sensitivity is assessed for each of the filter, swab, and water methods, we were than able to draw conclusions as to whether any of the given methods produced results sufficient to replace the whole-fish PCR method. These results are specific for each pathogen type, and other pathogens are not considered within each assessment, thus the detection of anything other than the given pathogen would not produce a false positive.

Results

Filter safety. ***Experiment 1. Exposure of larval zebrafish.*** Body and head lengths were compared between control and nitrocellulose groups. Significant results for equivalence were obtained for body length ($t(38) = -7.73$, $P = 0.0$) and head length

Figure 2. PES vacuum filter apparatus.

($t(38) = 3.02$, $P = 0.0022$) (Figure 3A, B). That is, we conclude that body length and head length were both equivalent between control and nitrocellulose groups. Mortality was 0.03% for both groups. In the VMR assay, nitrocellulose larvae showed less distance traveled than did the control group during the first 5 s of the light cycle ($t(38) = -3.01$, $P = 0.9977$) (Figure 4A). Distance traveled was significantly equivalent during the 10 min of the dark cycle ($t(38) = 6.59$, $P = 0.0$) (Figure 4B), but this is due to the expected low overall movement during the dark cycle.

Experiment 2. Acute adult exposure. Body length and head length were compared between control and nitrocellulose groups. Significant results for equivalence were obtained for both body length ($t(118) = 15.15$, $P = 0.0$) and head length ($t(118) = -7.03$, $P = 0.0$) (Figure 5A, B). No mortality was observed in either group.

Note that the same control data were used for experiments 2 and 3.

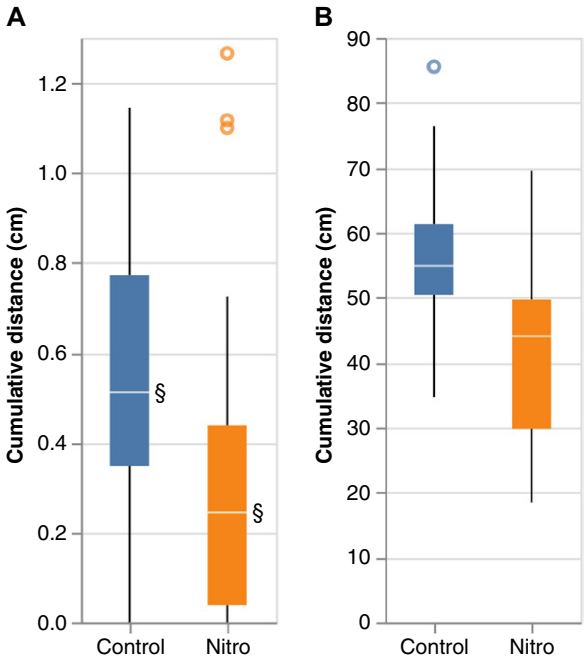

Figure 4. Box-and-whisker plots of larva performance on visual motor response assay for light cycle (A) and dark cycle (B) measured during experiment 1. For each measurement, larva exposed to nitrocellulose (Nitro) filters are compared with controls, which were not exposed to any filter. Significant equivalence results (where present) are represented as follows: §, $P \leq 0.0001$.

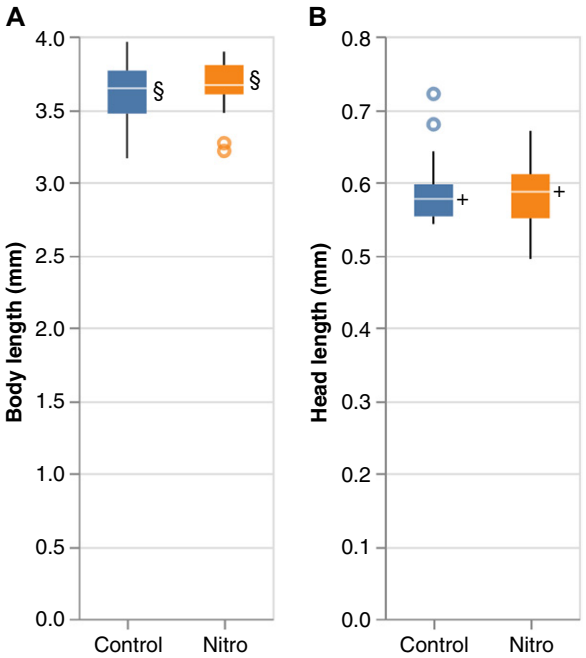

Figure 3. Box-and-whisker plots of larva body length (A) and head length (B) measured during experiment 1. For each measurement, larva exposed to nitrocellulose (Nitro) filters are compared with controls, which were not exposed to any filter. Significant equivalence results (where present) are represented as follows: §, $P \leq 0.0001$; +, $P \leq 0.005$.

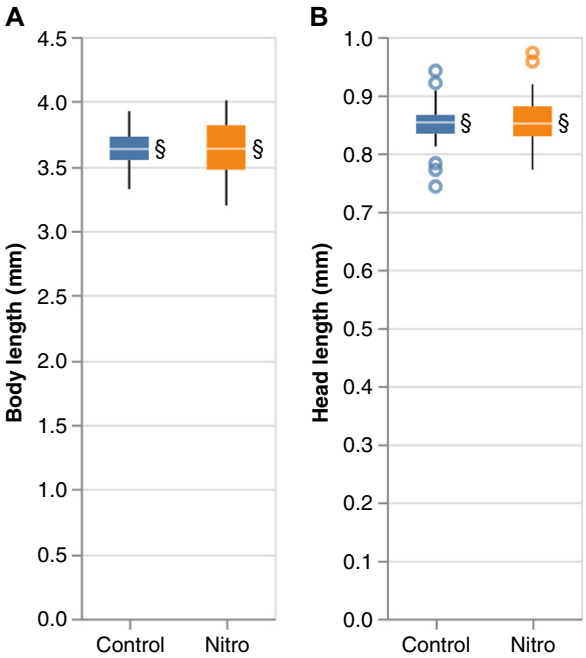

Figure 5. Box-and-whisker plots of larva body length (A) and head length (B) measured during experiment 2. For each measurement, larva exposed to nitrocellulose (Nitro) filters are compared with controls, which were not exposed to any filter. Significant equivalence results (where present) are represented as follows: §, $P \leq 0.0001$.

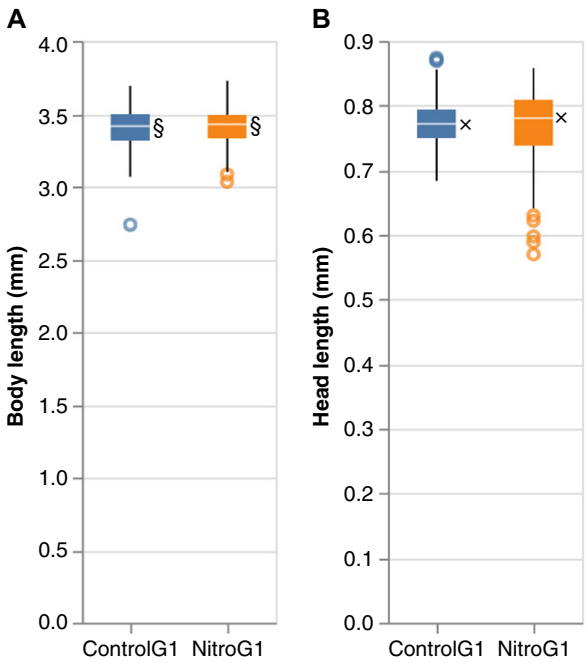

Figure 6. Box-and-whisker plots of larva body length (A) and head length (B). In this case, larva exposed to nitrocellulose (Nitro) filters on a chronic basis were compared with the G2 of control larva, which were collected at a comparable time since birth and not exposed to any filters. Significant equivalence results (where present) are represented as follows: §, $P \leq 0.0001$; ×, $P \leq 0.0005$.

Experiment 3. Chronic adult exposure. Body length and head length were compared between control and nitrocellulose groups for chronic exposure groups. Significant results were obtained for both body length ($t(118) = -9.03$, $P = 0.0$) and head length ($t(118) = 3.59$, $P = 0.0002$), in that body length was larger for the nitrocellulose group than the control group (Figure 6A, B). No mortality was observed in either group.

Experiment 4. Assessment of next generation breeding. Body length and head length were compared between control and nitrocellulose groups for G1 and G2 groups. In the G1 groups, significant results for equivalence were obtained for both body length ($t(118) = -16.96$, $P = 0.0$) and head length ($t(118) = 3.63$, $P = 0.0002$) (Figure 7A, B). Adult mortality at 90 d was 11.1% for the nitrocellulose group.

In the G2 groups, significant results were obtained for body length ($t(118) = 14.39$, $P = 0.0$) but not head length ($t(118) = 0.42$, $P = 0.3362$) (Figure 8A, B). No issues with breeding these fish were observed.

Limitations. Original control fish did not produce sufficient numbers of eggs to completely fill all groups or have any to allow for G2 breeding, so a separate control group was used for acute, chronic, and G2 comparisons. In this case, these fish were only bred once, and the same data were used for comparison. In addition, to get the fish to breed, they were housed on the recirculating rack. This still represents individual samples for the purposes of the TOST test, but it is notable that the acute, chronic, and G2 fish in the nitrocellulose groups and the control group were not bred at the same time and were housed differently.

Filter sensitivity. Complete sensitivity data are shown in Table 1.

Overall sensitivity (calculated across all collection timepoints) was low for all pathogens, with the highest sensitivity (approximately 50% to 55%) obtained for *M. chelonae* from the filter and swab tests. The sensitivity for *M. chelonae* from the water testing was only 21%. The only other pathogens that were detected at all with these alternative tests were zebrafish picornavirus, which was detected at a rate of approximately 17% to 26%, and *M. streisingeri*, which was detected at a rate of approximately 6% to 11%. *M. fortuitum*, *P. neurophilia*, and *P. tomentosa* were never successfully detected by any of the novel tests.

Filters collected after 30 d of submersion yielded a sensitivity rate of 100% for *M. chelonae* and approximately 21% for zebrafish picornavirus, but 0% for *M. fortuitum*, *M. streisingeri*,

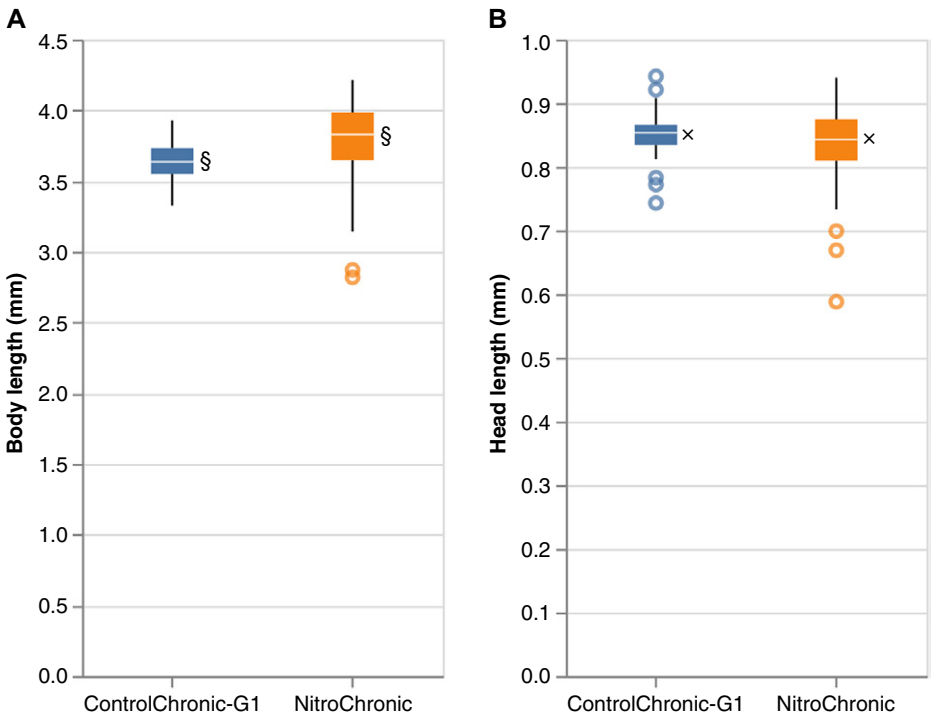

Figure 7. Box-and-whisker plots of larva body length (A) and head length (B). In this case, G1 offspring of fish that were chronically exposed to nitrocellulose (Nitro) filters were compared with the G1 offspring of control fish, which were not exposed. Significant equivalence results (where present) are represented as follows: §, $P \leq 0.0001$; ×, $P \leq 0.0005$.

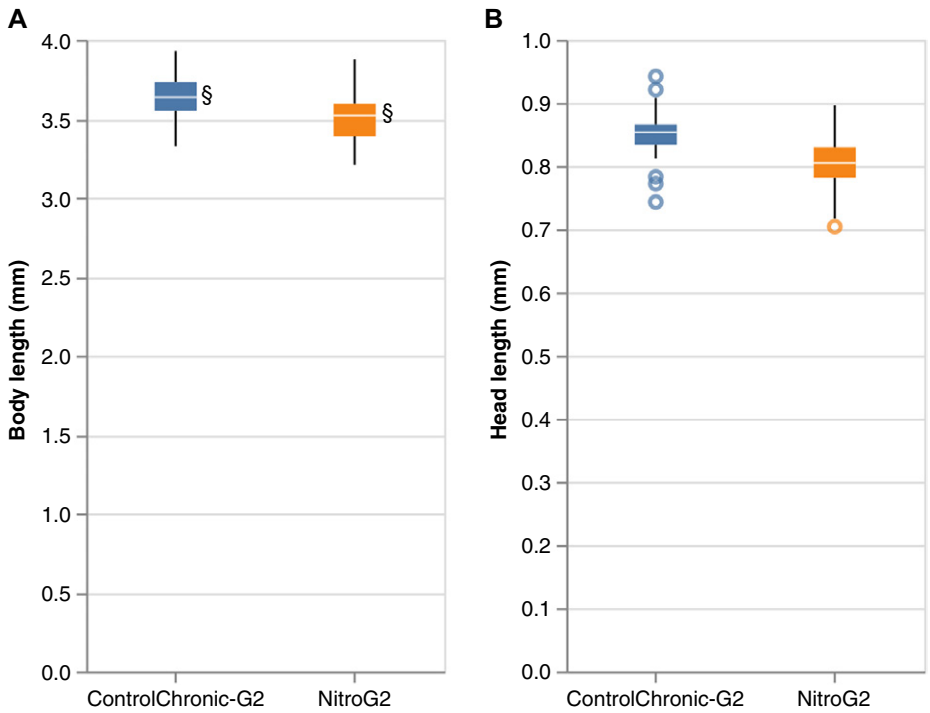

Figure 8. Box-and-whisker plots of embryo body length (A) and head length (B). In this case, G2 offspring of fish that were chronically exposed to nitrocellulose (Nitro) filters were compared with the G2 offspring of control fish, which were not exposed. Significant equivalence results (where present) are represented as follows: §, $P \leq 0.0001$.

P. neurophilia, and *P. tomentosa*. This is compared with swab and water tests collected at the same time. Both swab and water tests yielded a sensitivity of 50% for *M. chelonae*. Water tests yielded a sensitivity of 29% for zebrafish picornavirus, whereas swab tests yielded 0%. Both water and swab testing also yielded 0% for all other pathogens. Collections of filters after 60 d of submersion yielded our highest sensitivity results, with rates of 100% for *M. chelonae*, 37% for *M. streisingeri*, and 37% for zebrafish picornavirus. The filters yielded 0% sensitivity for *M. fortuitum*, *P. neurophilia*, and *P. tomentosa*. The swab test from this timepoint yielded a sensitivity of 100% for *M. chelonae*, 29% for zebrafish picornavirus, and 0% for all other pathogens. The water test from this timepoint yielded a sensitivity of 75% for *M. chelonae*, 55% for *M. streisingeri*, 58% for zebrafish picornavirus, and 0% for all other pathogens.

Each of these results should be contextualized by the data presented in Table 2, which presents the detection results from whole-fish PCR for each of the tested rooms. Table 2 shows whole-fish PCR results from approximately 5 randomly chosen fish per system in each room. System sizes varied greatly, with some only having one rack of fish and others having many racks on the same system. The sensitivity of each testing method can be conceptualized as the percentage of the total prevalence that was detected by the given method. For example, the total prevalence of *M. chelonae* as detected by whole-fish PCR was 4, and the sensitivity for the filter method for the detection of *M. chelonae* at 30 d was 1, meaning that all 4 instances of *M. chelonae* presence were successfully detected by the filter method. In comparison, the sensitivity of the swab method at the same timepoint was 0.5, meaning that

Table 1. Sensitivity of PCR

		M. chelonae	*M. fortuitum*	*M. streisingeri*	*P. neurophilia*	*P. tomentosa*	Zebrafish picornavirus
Filter	Overall	0.54	0.00	0.08	0.00	0.00	0.26
	30 d	1.00	0.00	0.00	0.00	0.00	0.21
	60 d	1.00	0.00	0.36	0.00	0.00	0.36
	90 d	0.29	0.00	0.04	0.00	0.00	0.25
Swab	Overall	0.52	0.00	0.06	0.00	0.00	0.19
	30 d	0.50	0.00	0.00	0.00	0.00	0.00
	60 d	1.00	0.00	0.00	0.00	0.00	0.29
	90 d	0.40	0.00	0.08	0.00	0.00	0.21
Water	Overall	0.22	0.00	0.11	0.00	0.00	0.18
	30 d	0.50	0.00	0.00	0.00	0.00	0.29
	60 d	0.75	0.00	0.55	0.00	0.00	0.57
	90 d	0.00	0.00	0.04	0.00	0.00	0.05

Sensitivities of the filter, swab, and water methods of pathogen detection for the pathogens *M. chelonae*, *M. fortuitum*, *M. streisingeri*, *P. neurophilia*, *P. tomentosa*, and zebrafish picornavirus are shown. Results were assessed at 3 time periods (30, 60, and 90 d) based on how long the filter was submerged in the sump. In addition, the overall sensitivity was calculated based on the full data pooled across each timepoint. The sensitivity was calculated as a direct comparison of the testing method to a gold standard (full-fish PCR).

Table 2. Positive pathogen detections via whole-fish PCR in 2023

Room	*M. chelonae*	*M. fortuitum*	*M. streisingeri*	*P. neurophilia*	*P. tomentosa*	Zebrafish picornavirus
1	0	0	0	0	0	1
2	1	1	1	1	0	1
3	1	0	1	1	1	1
4	0	0	1	1	1	1
5	0	0	1	0	1	1
6	0	0	0	0	0	1
7	0	0	0	0	0	0
8	0	0	0	0	0	1
9	0	0	1	1	0	1
10	0	0	1	1	0	1
11	0	0	1	0	1	1
12	1	0	1	0	0	1
13	1	0	1	0	0	1
14	0	0	1	0	0	1
15	0	0	1	1	0	1
Total	**4**	**1**	**11**	**6**	**4**	**14**

Detection of each pathogen via full-fish PCR per room from an annual sentinel monitoring program for the year 2023 (the same year as the study) is shown. A positive detection in a given room is denoted as a 1, whereas a negative result is denoted as a 0. The sum of detections for each given pathogen is recorded in the total row.

this method only successfully detected 2 out of the 4 instances where *M. chelonae* was present.

Collections of filters at 90 d are different in that every sump in a room was assessed at this timepoint. In this case, a positive whole-fish PCR result for a given room would indicate that every sump should return a positive result. The higher number of assessments at this timepoint therefore increased the granularity of the sensitivity analysis but does not change the overall interpretation. The samples from this timepoint yielded rates of 28% for *M. chelonae*, 4% for *M. streisingeri*, and 25% for zebrafish picornavirus. The filters yielded a 0% sensitivity for *M. fortuitum*, *P. neurophilia*, and *P. tomentosa*. The swab test from this timepoint yielded a sensitivity of 40% for *M. chelonae*, 7% for *M. streisingeri*, 21% for zebrafish picornavirus, and 0% for all other pathogens. The water test from this timepoint yielded a sensitivity of 4% for *M. streisingeri*, 5% for zebrafish picornavirus, and 0% for all other pathogens.

Discussion

This study is a follow up to a pilot study that sought to compare the pathogen detection abilities of nitrocellulose filters with active filtration through a PES filter (water test), sump swabs, and whole-fish PCR data collected from the year of testing. The pilot study was conducted during the summer of 2022 as an initial attempt to look at how filters would fare as a method of health monitoring for zebrafish. Twelve filters were placed in tanks filled with diverted sump water. A filter was collected from each tank weekly for 12 wk, and results were compared with water filtration and sump swab results. These modalities of testing were in line with FELASA-AALAS recommendations for zebrafish health monitoring, which include testing a mix of environmental, water, and whole-fish samples using PCR and histopathology.[6] Diseases tested for were those considered SMOP (screen more often pathogens) such as *P. neurophilia*, *Mycobacterium* spp., and *P. tomentosa* and then SLOM (screen less often microbes) that we knew were present in our system.[6] The whole-fish PCR data review, sump swabs, and active filtration were done identically to our current study. However, at the time of the pilot study it was unknown if there would be possible toxic effects from exposure to the nitrocellulose filters in the sumps. As such, during the pilot study, filters were kept in a diversion tank that received sump water but was then connected by a hose to overflow drainage. The filters were collected weekly over 12 wk and tested for pathogens in a similar manner to the ones in this experiment. Nitrocellulose filters were chosen for their known pathogen detection properties.[17] We used a hydrophobic variety to ensure that the material would not break down over time in the water.

The filters in the pilot study consistently identified *Mycobacterium* spp., Zebrafish picornavirus, and *M. streisingeri*, but they were unable to detect *P. neurophilia*. *P. neurophilia* was detected on whole-fish PCR; however, this result was inconsistent, as systems testing positive would later test negative. These initial results served as the basis for our experiments. In research settings, most zebrafish are housed in some form of recirculating system.[5] Because of this, the filters would need to be placed in the sumps of an active, recirculating system. Given the nature of a recirculating system, and that the fish would be exposed to possible toxins from the nitrocellulose filters, a safety study was conducted to determine whether there were any toxic effects related to exposure to the filters.

Our results from the toxicology study (Figures 3–8) indicated that there would be no toxic effects from filter exposure to the fish. While the control fish failed to produce an adequate number of eggs to populate the chronic group, it did not impact the results of our study. Due to the low numbers, the initial control group was used in comparison for both the acute and chronic trials and were housed in a different environment (recirculating compared with static). Because the control fish lacked any exposure to the filters, it can be assumed that any changes through generations would be the effect of some other influence. Furthermore, the fish in the toxicology study had direct access to the filter, whereas the fish in the sump filter study would only be exposed through the recirculating water. Statistically, there was little evidence of any toxic changes due to filter exposure. From the toxicology study, most body lengths were comparable between the control group and the filter groups trial but not for the head trials. The results for the safety study occasionally failed to produce significant levels of equivalence for head measurements (experiments 1 and 3);

however, the head measurements were the least reliable given the difficulty of taking these measurements and the high level of precision involved. The presence of equivalency in most of the head length comparisons, as well as all of the body length comparisons, suggests a lack of any detrimental effects of the filters on fish growth.

There was also no issue with breeding fish exposed to the nitrocellulose, as the fish exposed to the filters still produced clutches. In contrast, despite maintaining the same conditions, we had much difficulty breeding control fish. This could have been due to the water temperature and/or dissolved oxygen content being lower than ideal for breeding since the static tanks were not provided with additional heat or aeration. We also did not test for ammonia or conductivity levels, as the tanks received complete water changes weekly, which aligns with our institutional policy for maintaining water quality. While these may be limitations of this study, these parameters did not affect the breeding of the nitrocellulose groups that were housed in the same conditions. We even tried repeating with new fish and the controls still did not produce, which led us to having to house them on the recirculating rack to complete the study. The original fish had been randomly assigned to groups from the same tanks, so it is unknown why there were breeding problems. It is possible that the filter acted as enrichment that encouraged breeding in the nitrocellulose groups.

Morphology is a common way to evaluate toxicity in zebrafish. Various parameters can be measured; however, total body length and head length are good measurements to evaluate effects in growth, especially when overt signs of toxicity were not expected to be seen.[20,21,22] The head measurements had a low confidence interval; however, this could be due to the difficulty in gathering accurate data with regard to head length. Positioning and precision of measurements are essential for obtaining accurate readings, and the landmarks for measuring head length in fish are hard to accurately measure. There also tends to be morphologic variability within clutches for individual fry, with size potentially varying significantly between individuals from the same clutch. Nevertheless, given the increased exposure to filter material of the trial fish, the fact that they still produced clutches, and the body/head measurements, we believe that the filters were safe for use in the filter sump study.

The filter efficacy study evaluated the nitrocellulose filters with actively filtered water as performed in Crim and colleagues, and sump swabs, as one of the FELASA recommended methods of health monitoring.[27,13] Results showed that filters were able to reliably detect pathogens that are either detected mainly by swabs (*M. chelonae*) or water filtration (Zebrafish picornavirus, *M. streisingeri*). Whole-fish PCR was used to determine the sensitivity of the environmental tests and was able to detect *M. fortuitum*, *P. tomentosa*, and *P. neurophilia*, which did not show up on filter testing in this study. However, we noted that *M. chelonae* was detected in more sumps than what our whole-fish PCR had detected. These might be considered false positives with the method of evaluation we used in this study, although in reality, these are likely true positives and show the weakness of whole-fish PCR in this area. Previous studies conducted by Miller and colleagues reported similar results from their experiment comparing environmental and fish PCR.[14] They noted in their study that *Mycobacterium* spp. were more readily tested from environmental PCR as compared with whole-fish PCR, and they were able to detect subspecies (*Mycobacterium marinum*) previously thought removed from their systems.[14] They attribute this to the fact that *Mycobacterium* is capable of persisting in the environment in resistant biofilms, and it is overall a slow growing bacterium once a fish is infected.[14] Miller and colleagues also came to the conclusion that a multimodal health monitoring system should be incorporated for fish health monitoring, as whole-fish PCR was able to detect *P. neurophilia*, whereas it could not be detected in the environment.[14]

While not evaluated as part of this study, our whole-fish PCR results have also been hit or miss in identifying *P. neurophilia* over the past few years, which shows a limitation of this method of testing as well. Histology may be a better way of identifying *P. neurophilia*, but this was not performed as part of this study. This shows the limitation to current methods of health monitoring, as there does not seem to be a catch-all that can detect all pathogens. This does not mean that the filters were ineffective. Across the 3 trials (30, 60, and 90 d), the filters consistently detected *M. chelonae* and zebrafish picornavirus and were able to detect *M. streisingeri* at the 60-d mark. *P. neurophilia* is notoriously hard to detect. Often the presence of the pathogen is not known until the fish begin to exhibit symptoms (emaciation, scoliosis, behavioral changes), and by that point the disease has already entered an advanced stage.[8] Several methods can be used to aid in early detection of *P. neurophilia*, including sonication of DNA. Sonication is the process in which ultrasound is used to agitate material, creating more DNA fragments to be used for PCR.[28] Sonication has been shown to be an effective way of disrupting the spores formed by *P. neurophilia*, and it helps to increase the sensitivity of PCR.[28] This process was used by VRL Diagnostics to increase sensitivity of *P. neurophilia* detection. However, sonication has been shown to be effective only with samples taken directly from zebrafish tissue. Extra measures, such as gel DNA extraction, are necessary to detect *P. neurophilia* samples from eggs or water.[28] Moreover, detection of spores is greatly reduced in systems with flowing water.[29] For environmental detection to be accurate, the fish need to be housed in great numbers and/or kept in static water, such as when placed in spawning tanks.[29]

Despite the difficulty, it may be possible to detect parasitic pathogens through filter testing of the water. One vendor has developed a filter testing system known as InterZebTEC(TM), which contains 3 filters of varying pore size in a system that attaches to the sump flow pipe before the water can be filtered.[30]According to the white paper published on the product, the InterZebTEC is capable of detecting multiple organisms from the IDEXX zebrafish PCR panel, including *P. neurophilia* and *P. tomentosa*.[30] However, they did note that these pathogens were detected "all together," but they did not specify how many of the filters tested positive for the pathogens.[30] They did note that efficacy for pathogen detection dropped between 8 and 12 wk, a similar drop off to what we have seen in our study, and in our pilot study.[30] It is unclear why this drop occurred in all of these studies. The cause may be that there were potentially enough biofilm forms that it might start falling off over time, but this is only speculation. By attaching our filters to the flow pipe, we may have a better chance at detecting the pathogens that were not found in our study. We would need to determine a way to engineer a device to do so, which could be done in the future.

Overall, while there may be some shortcomings when it comes to pathogen detection, we believe that evaluation of the filter represents an accurate way to monitor zebrafish infectious pathogen status. The filter was able to consistently detect *Mycobacterium* spp., Zebrafish picornavirus, and *M. streisingeri* with a marked drop in efficacy when left in the sump for prolonged amounts of time (greater than 60 d). In comparison to the water filtration and sump swabs, the filters performed better in overall sensitivity for both *M. chelonae* and zebrafish picornavirus, with water filtration slightly better at detecting *M. streisingeri*. The filters were easy to

place and retrieve, while the water filtration method was very time-consuming. The filter's ability to detect multiple pathogens could allow it to act as a replacement for both the swabs and the PES water filtration filter. By replacing these 2 methods with a filter that can be left in the sump for up to 60 d, it should be possible to decrease the labor and time required obtaining samples, allowing a more streamlined sample collection process. It is noteworthy that the filter is not a catch-all: it still was not able to detect *P. neurophilia* or *P. tomentosa*. As FELASA recommends multiple testing types, use of the filters may consolidate some of the testing required but should still be combined with testing of clinically ill fish, such as those exhibiting scoliosis or emaciation.[6] Timing is also an important consideration as far as health monitoring modality is concerned. Because the efficacy of filters is best between 30 and 60 d, they are not a good test method if results are needed rapidly. It is recommended to use the water filtration method in cases where results are needed quickly, despite the extra labor and time that is required. In this regard, we have started implementing the use of these filters, placing them 6 wk prior to our intended collection date. In conclusion, we recommend that the filter monitoring system described here be used in conjunction with whole-fish PCR.

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Conflict of Interest

The authors have no conflicts of interest to declare.

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