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Filtering *Myxobolus cerebralis* Triactinomyxons from contaminated water using rapid sand filtration

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Abstract

Rapid sand filtration was explored as a means of removing Triactinomyxon actinospores (Tams), the waterborne infective stage of the salmonid parasite *Myxobolus cerebralis* that causes whirling disease, from contaminated water. Preliminarily, a batch of sand was sieved to create 12 size ranges from 180 to 2000 μm. These individual ranges were tested for their efficacy of removing Tams through sand beds either 2 cm or 4 cm deep. The critical size at which no Tams passed through the sand bed was 300 μm at 2 cm depth and 425 μm for 4 cm bed depth. Additional tests evaluated the passage of Tams through filter beds comprised of sand that had all particles smaller than 180 μm removed. With this sand, 0.2 ± 0.5% of Tams passed through a 2 cm bed, and 0.0 ± 0.0% with a 4 cm sand bed. Based on these preliminary results, small (61 cm × 15 cm) rapid sand filters were placed in-line with aquaria containing rainbow trout fry. The sand bed depth was 10 cm under which lay 10 cm of aquarium gravel. Four treatments were (1) negative control, (2) positive control, (3) sand of >180 μm, (4) sand of >300 μm. Tams were regularly introduced to the rearing systems above the sand filters. After 60 days, clinical signs of whirling behavior and black tails were seen among the positive controls. A polymerase chain reaction assay for *Myxobolus cerebralis* 1 month after exposure proved negative for negative controls and the >180 μm group, whereas 10% of the >300 μm group and 71% of the positive controls were infected. Results from the PCR assay at the study's conclusion indicated the negative controls and >180 μm group were still disease free. All positive control fish were infected, and 49% of >300 μm fish were infected. These results were mirrored by those obtained from a pepsin–trypsin digest assay, except one fish among the >180 μm group was found to be infected. These results demonstrate that sand filtration may be a viable option in treating hatchery water supplies that are contaminated with whirling disease.

Keywords: Rapid sand filtration; Whirling disease; *Myxobolus cerebralis*; Triactinomyxon

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1. Introduction

Sand filtration is a process where water is passed through a bed of sand and through mechanical and biological processes; particles including organic debris, bacteria and viruses are removed. This technology has been used for almost two centuries to treat municipal water supplies. Sand filters are still used to treat culinary water, and they have also gained acceptance as filters for swimming pools and in the aquaculture industry. Commercial sand filters are available for industrial systems that can accommodate flows up to 3700 l/min, although site-built systems can accommodate significantly higher rates. Within the concept of sand filtration there are two distinct types; slow sand filtration (SSF), and rapid sand filtration (RSF). Slow sand filtration is generally characterized by gravity fed flows in the range of 1–8 m^3/m^2/day (volume/filter surface area/time) and is further characterized by containing a significant volume of water above the filter bed and a biologically active zone of the sand bed called the Schmutzdecke (Haarhoff and Cleasby, 1991), German for dirt blanket. The Schmutzdecke must be removed every 4–6 weeks as it impedes water flow as it matures. With SSF, you therefore get mechanical filtration by restriction of particle movement through the sand bed, and biological filtration of organics by the organisms living in and adjacent to the Schmutzdecke.

Rapid sand filtration operates at much higher rates, 100–475 m^3/m^2/day, either via pumping or adequate head pressure (Droste, 1997). Because of the higher flows, RSFs accumulate more debris over a shorter period of time leading to the need for frequent backflushes. To clean the debris from the filter bed, a volume of water is flushed in the opposite direction of normal water flow. This process fluidizes the bed material and dislodges trapped material. Backflushing may be necessary one to three times daily. For both styles of filters, the filtration efficacy is determined by many variables including incoming organic load, sand composition, water temperature, and frequency of backflushing.

Sand filters are highly effective at removing disease organisms from incoming water. The use of sand filters in the late 19th century significantly reduced the effects of a cholera outbreak in Altona, Germany, and lowered the incidence of typhoid cases in Lawrence, Massachusetts (Hendricks and Bellamy, 1991). In more recent research, Schuler et al. (1991), using seasoned SSFs, were able to remove 99.9% of experimentally added cysts of *Giardia*, *Cryptosporidium*, and coliform bacteria. *Cryptosporidium* and *Giardia* fall in the size range of 1–25 μm. Slow sand filters have also demonstrated their ability to remove viruses from water supplies (Hendricks and Bellamy, 1991).

Whirling disease, caused by the salmonid parasite *Myxobolus cerebralis*, has established itself in the waters of Utah over the past decade, and in the past several years, two state hatcheries have become infected with the disease. In both hatcheries, the water source became infected or at least served as a conduit to the hatcheries for *M. cerebralis* Triactinomyxon actinospores (Tams), the fish-infective stage of the disease. Tams are shaped like a grappling hook and are generally 125 μm long, but their style and processes are only 10 μm wide. Filters constructed from 20 μm NITEX® mesh are the standard for Tam collection in the field (Thompson and Nehring, 2000) and the laboratory, but the 20 μm size is not 100% efficient. Tam recovery work conducted in our laboratory can result in 70–80% Tams recovered when a known number of Tams are added to clean water and subsequently filtered, but the confidence intervals may range from 0 to 100% (unpublished data).

Both SSF and RSF designs seem to be reasonable means of removing Tams from hatchery water systems. The sand bed and underlying support bed represent a tortuous 3-D path through sharp-edged substrate that may trap and destroy waterborne Tams. The 10 μm Tam size also falls within the size range of 99.9% removal efficacy established by Schuler et al. (1991). We conducted multiple tests to determine first the appropriate size range of sand particles that were most effective at trapping Tams in a sand bed, and second, the ability of two different sand sizes in a gravity fed sand filter to remove Tams from a simulated infected water supply feeding into a fish culture system.

2. Methods

2.1. Preliminary tests

To determine the sand grain size range required for effective Tam removal, a series of sieves were used to separate masonry grade sand purchased from a local landscaping supply business. The sieve sizes ranged from 180 μm to 2 mm mesh and provided sand of 12 different sizes (180–211, 212–249, 250–299, 300–354, 355–424, 425–499, 500–599, 600–709, 710–849, 850–999, 1000–1999, and >2000 μm). Sand recovered on each of the sieves was kept separate for each recovery test in which Tams were added to a small filter apparatus (Gelman filter funnel, 49 mm diameter) with either 2 or 4 cm of sand. Prior to testing, the sand in each filter was rinsed with hatchery well water until there was no turbidity in the filtrate. This first batch of sand had an effective size of 130–180 μm and a uniformity coefficient of 1.65.

Tam collection and enumeration from worm collection were conducted according to the methods outlined by Arndt et al. (2002). The worm cultures used for Tam production contained a mixed collection of oligochaete species, which had been fed *M. cerebralis* myxospores to induce infection. A typical mixed culture may consist of 16% *T. tubifex*, 9% *Quistodrilus multisetosus*, 8% *Limnodrilus hoffmeistri*, and 64% immature, unidentified worms. Tams freshly harvested from worm cultures were diluted in 200 ml of hatchery well water. This was carefully added to the filter to avoid disturbance of the sand layer. The total Tams added varied among the tests due to availability, ranging from 10,226 to 66,400 per treatment. An additional 200 ml of well water with no Tams was added as a rinse. The filtrate was subsequently filtered through a 20 μm mesh to concentrate the Tams. A control group consisted of Tams filtered through the Gelman funnel without sand present and recovered on the 20 μm mesh filter. Three slides of 50 μl each were made for each treatment counting the total number of Tams. The filtration process was repeated twice for each sand depth (2 cm or 4 cm) and sand size.

Additional tests were conducted in which all sizes of sand combined (no sorting) were compared, with respect to Tam removal and flow rates, to filtration using sand for which particles less than 180 μm had been removed. The removal of the smaller sizes was of interest to improve the filtration speed. The same process described above was used for these tests except that three replicates were conducted instead of two.

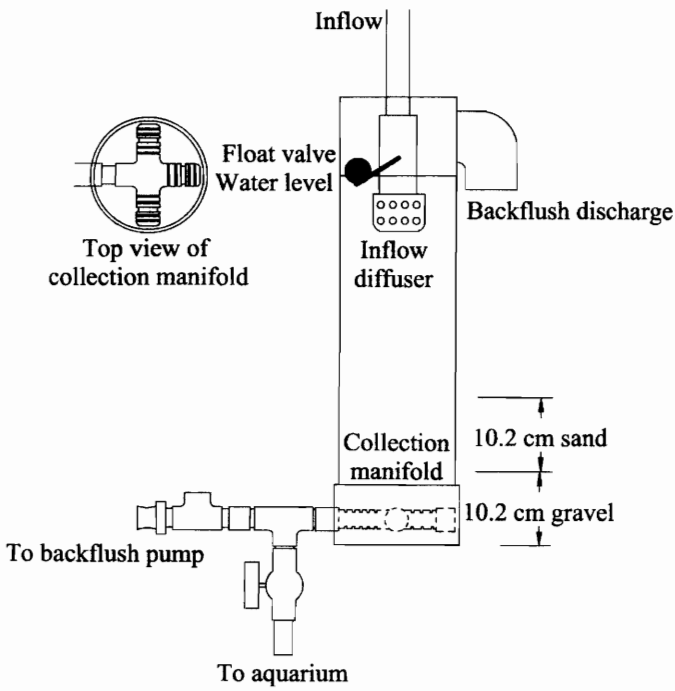

Fig. 1. Side view schematic diagram of the sand filter used. Included is a top view of the water collection manifold. See the text for a more detailed description of dimensions and materials used.

2.2. *Sand filter and fish culture systems*

The efficacy of filters containing either >180 or >300 μm sand beds was tested with fish reared in four independent recycle systems, one for each treatment. Negative and positive controls completed the four groups.

The main bodies of the filters were constructed from a 61 cm section of 15 cm (i.d.) PVC sewer pipe (Fig. 1). A 5.1 cm 90° elbow was fitted through the main filter body and it served as the discharge point for back-flush water and was also connected to a common discharge line that connected all filters. The lower edge of the elbow was placed through the filter body at a point such that if water backed up within the filter, due to float valve malfunction, it would discharge when it reached 7.6 cm of the top. A standard 1.3 cm, brass float valve was used to control water flow into the sand filter. Due to the narrowness of the filter, a 6 cm diameter plastic fishing float was attached directly to the valve and was used to actuate the valve. The filter base was constructed from a 15.2 cm end cap. A 1.9 cm couple fitting passed through the wall of the base cap at 5.1 cm on center from the upper edge of the cap. Inside the filter this couple connected to the collection manifold, and on the outside it lead to the aquarium (Fig. 1). The collection manifold was constructed from 1.9 cm PVC pieces including a 4-way cross plugged on three sides by caps, and on the fourth side leading through the coupler out to the aquarium. On a horizontal plane the manifold

had four, 3 mm-wide cuts made across the capped ends to allow for flow collection through the filter bed to the aquarium. A section of 200 μm Nitex mesh was wrapped around each section of cuts to prevent passage of larger materials to the aquaria.

On the outside of the base cap, water flowed through a 1.9 cm tee and then from the downward oriented branch, through a ball valve. The valve was used to control flow through the filter, to the aquarium. The outward oriented branch of the tee served as the connection point of the back-flush system, which consisted of a one-way valve and a quick disconnect fitting used to allow for easy connections to the back-flush pump. The one-way valve was used to direct water through the filter to the aquarium while under normal operations. When in back-flush mode, the one-way valve allowed pressure to be directed up through the filter bed after the valve to the aquarium was closed.

From week 1–20, sand filters were back-flushed on a weekly basis, however beginning on week 21 through the end of the study, filters were back-flushed twice weekly. The extra back-flushing was required due to decreased water flow through the >180 treatments filters. Back-flushing was accomplished by pumping 20 l of well water from a container through the sand filter in the direction opposite of normal water flow, and out a discharge line. For pumping, we used a JABCO® self-priming pump (1/4 H. P., 115 V, JABCO Products, Costa Mesa, CA, USA) attached to a section of clear vinyl tubing (1.7 cm i.d.), which ended in a quick connect fitting. This fitting could then be mated with a quick connect fitting attached to the back-flush line on the sand filter (see Fig. 1). Midway in the line between the pump and the sand filter was a brass ball valve which was used to control the flow through the filter. The full pressure of the pump was used to completely fluidize the sand filter bed, but as soon as the bed was fluidized, the flow was cut back, using the valve, so that the sand was not washed from the filter. During the first back-flush event, the flushed water was captured and filtered through a 125 μm sieve and the retentate was collected and dried in a drying oven for 24 h at 105 °C to determine whether or not sand was being lost during the back-flush process.

The filter bed consisted of 10.6 cm of support media, which surrounded the collection manifold. The support media was aquarium gravel which on average, had a major axis of 7.2 ± 1.6 mm and a minor axis of 5.2 ± 0.7 mm ($N = 124$). On top of this lay the 10.6 cm of sand, either >180 μm or >300 μm depending on the treatment. The sand used for the filters was a masonry grade product purchased from a local landscape supplier. Before use, this sand was sifted through a series of decreasing sieve sizes, using the sieves mentioned previously, to characterize the sand's composition. The percent by weight of sand trapped on each sieve was recorded and used to calculate effective size and uniformity coefficient. The effective size is defined as the size fraction at which only 10% of smaller particles remain (Wheaton, 1985). The effective size is important because the smaller size fractions reduce flow rate. When the value where 60% (by weight) of remaining sand has a smaller diameter is calculated, the uniformity coefficient of the sand batch can be determined. The uniformity coefficient characterizes the amount of size variation within a batch of sand (i.e., a larger uniformity coefficient indicates less uniformity in size). After the sand was characterized, batches were produced with either all sand smaller than 180 μm removed, or all sand smaller than 300 μm removed. The sand for the >180 μm treatment had an effective size of 180 μm and a uniformity coefficient of 1.14, and the sand for the >300 μm treatment had an effective size of 250–300 μm and a uniformity coefficient of 0.81.

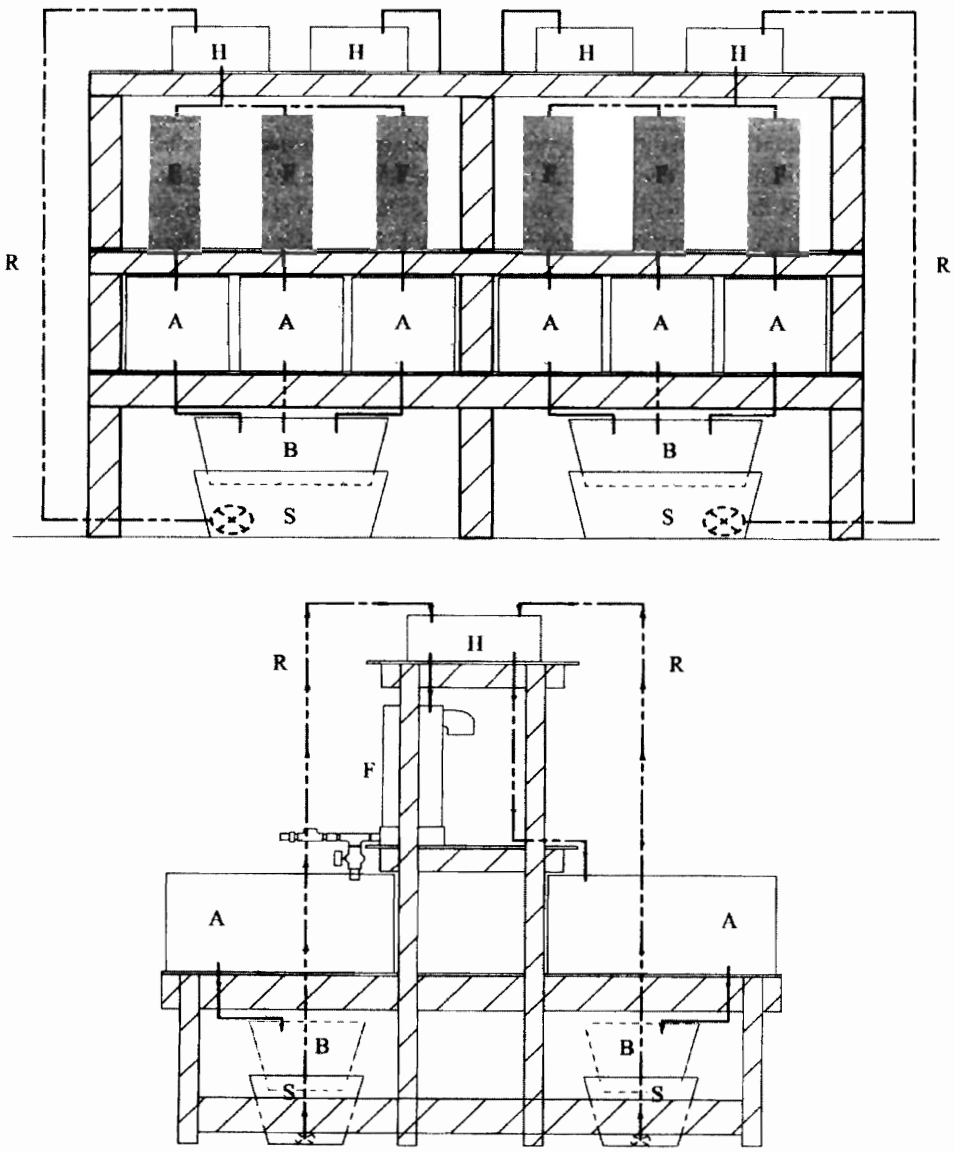

Fig. 2. Front and side view schematics of the recirculation systems used during the tests. See the side view for individual identification of system components (R: return line; H: head box; F: sand filter; A: aquarium; B: biofilter; and S: sump).

The fish culture systems used for each treatment consisted of a common head box (volume = 7.0 l), followed by a manifold that directed water to each of the triplicate aquaria that comprised a treatment (Fig. 2). In the case of the negative controls, water flowed directly from the head box to the aquaria. In the case of the positive controls, water

occasions exposures were skipped. The actual average dose for the course of the study was 13,092 ± 269 Tams per exposure, and the cumulative dose was 798,706 Tams per treatment. The Tams were diluted with 2 l of well water, placed into a 3.8 l chicken water feeder, which discharged its contents into a treatment's head box over a 44 min (±13) period. Tams were collected and enumerated from worm cultures as previously outlined (Arndt et al., 2002). However, in addition to enumeration, Tam viability was established with vital staining techniques using propidium iodide and fluorescein diacetate (Markiw, 1992). With this technique, Tams that stain green are considered viable, while those that stain red are not. This procedure was conducted on Tams from one exposure a week for the first 4 weeks of the study, after which a Tam qualification step was incorporated into the enumeration process. During enumeration, all Tams viewed under the microscope were classified as either viable or non-viable. Viable Tams were classified as only those Tams that had intact polar capsules and tightly compacted spore bodies with no loose sporoplasms. Non-viable Tams were those with fired polar capsules; those that lacked sporoplasms or had loosely packed sporoplasms within the style; or those Tams that had damaged or missing caudal processess. To verify this classification technique, on two occasions Tams that had been screened as viable or non-viable were also analyzed by the vital staining technique. The actual fish exposures were then carried out using a calculated amount of Tams based only on the number of viable Tams or those that stained green.

One month after the start of the study, 10 fish were sampled from each replicate tank of the >180 μm treatment, >300 μm treatment group, and the negative controls to check for infection. An average of seven fish per tank were sampled from the positive controls due to fewer available fish resultant from the mortalities experienced during the first 2 weeks of the study. The sample fish were euthanized with an overdose of tricaine methanesulfonate, heads removed immediately posterior to the operculum, and individual heads were placed into plastic bags and frozen.

After the heads were frozen, they were sent to an independent laboratory (Pisces Molecular LLC) for assaying of *M. cerebralis* via polymerase chain reaction (PCR) using heat shock primers. The PCR assay used was a single-round test (Baldwin and Myklebust, 2002) with the modification of using the heat shock protein gene (Hsp70) as the region targeted as opposed to the 18S rRNA gene. Epp, Wood, and Mitton presented their modified assay at the 8th Annual Whirling Disease Symposium, and have developed their assay around the Hsp70 gene and have determined it to be sensitive and specific to *M. cerebralis*. In order to quantify the degree of infection, the PCR results were scored according to Schisler et al. (2001) based on the intensity of the DNA banding pattern. The categories used were: negative (−), weakly positive (w+), positive (+), strongly positive (++), and very strongly positive (+++).

At the end of the 24-week study, the remaining fish were euthanized, and their heads removed. A scalpel was brushed and disinfected with a 50% chlorox solution (2.6% sodium hypochlorite) between each fish, and a clean paper towel was used for each head and served as the cutting surface. The heads were then processed by the pepsin–trypsin digest (PTD; Markiw and Wolf, 1974). After digestion, the spore preparation was divided with part being used for continued PTD methodology, and part for PCR analysis.

2.4. Statistical analysis

All statistical tests were conducted with an alpha value of 0.05. With the preliminary, sand grain size Tam recovery research, Tam recovery data were arcsine transformed prior to analysis. When comparisons were made between sand bed depth (2 cm or 4 cm) and using various sand grain sizes, differences in Tam recovery were made by a two-tailed *t*-test. A one-way ANOVA with a LSD post hoc test was used when bed depth was held constant, and Tam recovery was analyzed across the various sand grain sizes and combinations thereof.

Percent mortality data were arcsine transformed and analyzed by one-way ANOVA with multiple comparisons by the Tukey Test. Data obtained from the PCR and PTD assays were not analyzed in depth statistically because the study was not designed to determine to what degree sand filters reduced whirling disease, but whether or not they could prevent it. However, percent prevalence data defined as positive or negative for infection by the PTD assay were cursorily analyzed by chi-square analysis.

3. Results

3.1. Preliminary tests

For the preliminary tests looking at sand grain size and Tam removal, the use of sand significantly reduced TAM recovery from the filtrate. Higher post-sand filtration Tam recovery numbers were found in the larger sand size groups (Table 1). The sand size for which no Tams were recovered was 355 μm at 2 cm depth and 425 μm at 4 cm of sand depth. The size

Table 1
Triactinomyxon actinospore recovery (%) in filtrate after passage through sand grain size fractions at bed depths of 2 and 4 cm

Sand size (μm)	Recovery (%)	
	2 cm	4 cm
Control—no sand	74.0 ± 28.5	87.1 ± 24.8
Sand sizes combined	0.6 ± 1.3	0 ± 0
Combined sizes >180	0.2 ± 0.5	0 ± 0
180–211	0 ± 0	0 ± 0
212–249	0 ± 0	0 ± 0
250–299	0 ± 0	0 ± 0
300–354	0 ± 0	0 ± 0
355–424	0 ± 0	0 ± 0
425–499	1.8 ± 1.8	0 ± 0
500–599	4.7 ± 2.5*	0.2 ± 0.5
600–709	25.5 ± 12.2*	1.8 ± 1.7
710–849	14.3 ± 6.1*	5.1 ± 1.8
850–999	38.4 ± 12.2	23.0 ± 10.9
1000–1999	44.3 ± 14.1	30.0 ± 11.3
>2000	23.5 ± 7.7	

An asterisk (*) indicates a significant difference between sand bed depths within a size fraction ($P < 0.05$).

fraction of 500–599 μm was sufficient to remove 99% of Tams. The deeper sand bed was also significantly better at removing Tams than the 2 cm bed. With a sand bed of 4 cm and size fractions of 500–599, 600–709, and 710–849, significantly more Tams were trapped by the filter than with the 2 cm depth (Table 1). By removing the smallest sand fractions (<180 μm), water flow rates were significantly increased ($P < 0.001$). With gravity flow through 2 cm of sand, the time required to filter 200 ml of clean well water through all sand sizes combined was 155 ± 36 s ($N = 6$). This time was reduced to 33 ± 1 s when the sand bed consisted only of sand >180 μm. When using a 4 cm filter bed the same trend was apparent: sand of >180 μm improved flow (39 ± 8 s) compared to sand of all sizes combined (160 ± 19 s, $N = 6$). Within a given sand size, there was no significant difference in filtration time between sand depths of 2 cm or 4 cm (t-test, $P > 0.05$).

3.2. Sand filter and fish culture systems

The debris collected and dried from the first back-flush event was entirely comprised of organic debris that had been trapped in the filter; no sand was found. This indicated the rate of flushing was adequate to remove trapped debris and restore good flow, without removing sand from the filter bed. On the two occasions that back-flush rate was monitored, the first rate, averaged for all six filters, was 14.1 l/min (50.5 m/h), and the second time the average was 13.3 l/min (47.1 m/h). There were no significant differences in filter flow-through rate between treatments, which averaged 2.4 l/min (8.7 m/h) at the beginning and the conclusion of the trial.

The fish from all treatments grew reasonably well, however because feeding levels were not quantified, treatment effects on growth could not be ascertained. By day 35 of the test, fish in the positive control tanks were larger (1.6 g) than the other treatments (mean = 1.1 g). At the end of study, positive controls were the largest (8.3 g), followed by the negative controls (7.5 g), the >300 group (6.8 g), and the >180 group (6.2 g). Although the fish were on a relatively restricted ration, specific growth rate, averaged across treatments was 2.29 ± 0.10%. All treatments experienced mortalities, however the positive control fish had a significantly ($P < 0.001$) higher mortality rate (62 ± 5%) than any other group. The >300 group experienced a mortality rate of 14 ± 5%, followed by the negative controls, 12 ± 2%, and 7 ± 3% for the >180 fish. All mortality among the >180, >300, and negative controls, can be attributed to the fish jumping through the outer drain standpipe where they became stranded on top of the biofilter splash plate. Various attempts were made to rectify this design flaw, but none were entirely successful. It is interesting to note that no positive control fish were lost through standpipes. With the exception of one fish, all mortality within the positive control was experienced during the first 15 days of the trial, during which time they experienced six Tam exposure events.

With the exception of unionized ammonia, overall water quality was similar to that considered good for the culture of trout. Beginning on the third day of tests, unionized ammonia for all four systems averaged 0.018 mg/l, which is above the acceptable upper limit of 0.013 for continuous exposure as outlined by Piper et al. (1992). Elevated unionized ammonia levels continued for approximately 6 weeks and averaged 0.025 (Table 2), after which it dropped to almost undetectable levels.

Table 2
Summary of water quality parameters measured over the course of the study

Parameter	Average	S.D.	*N*	Minimum	Maximum
Temperature (°C)	15.7	1.1	38	12.6	20.2
Dissolved oxygen (mg/l)	8.6	0.9	9	6.0	12.0
Alkalinity (mg/l $CaCO_3$)	210.3	30.6	29	136.8	256.5
Hardness (mg/l $CaCO_3$)	228.8	23.4	22	153.9	273.6
pH	8.0	0.2	26	7.2	8.4
Nitrite-nitrogen (mg/l)	0.17	0.33	25	0.00	2.00
Total ammonia nitrogen (mg/l)	0.64	0.50	21	0.01	1.80
Unionized ammonia (mg/l)	0.0193	0.0188	23	0.0001	0.0805

Values were averaged for each tank within a treatment's recirculation system and averages calculated from each recirculation system.

3.3. Fish exposures and disease diagnosis

After 48 days of Tam exposures, whirling behavior became evident in fish from all three tanks of the positive controls. After 60 days, black tails were observable on several fish from each of the same tanks. The PCR assay of preliminary samples taken 1 month after Tam exposures began indicated that fish from the negative controls and the >180 μm treatment group were negative for *M. cerebralis* infection, however 10% ($N = 30$) were positive in the >300 μm treatment group, and 71% ($N = 21$) were infected in the positive control group (Table 3). The remaining fish that were sampled by both PTD and PCR at the test's conclusion exhibited similar levels of infection. The PCR data indicated that all 54 fish from the >180 μm treatment group were negative. In the >300 μm treatment group, 49% were infected with *M. cerebralis*. Of that 49%, 4 of 47 were weakly positive, 12 were strongly positive, and 3 were very strongly positive. All 29 of the positive control fish were infected. From the positive controls, 5 were positive, 17 were strongly positive, and 7 were very strongly positive. All of the negative control fish ($N = 49$) were negative for infection.

The results from the PTD assays were very similar to those of the PCR, with a few discrepancies (Table 3). With the PTD methodology there was a single spore (1111 spores per head) found for one fish ($N = 54$) in the >180 μm group. In the >300 μm group, 53%

Table 3
Summary of percent of *M. cerebralis* infection for rainbow trout reared in systems supplied with Tam-contaminated water filtered by sand filters containing different sizes of sand

Treatment	Initial PCR (*N*)	Conclusion PCR (*N*)	Conclusion PTD (*N*)
Negative control	0 (30)	0 (49)	0 (49)
>180 μm sand	0 (30)	0 (54)	2 (54)
>300 μm sand	10 (30)	49 (47)	53 (47)
Positive control	71 (21)	100 (29)	100 (29)

Initial samples were taken 1 month after the study started and were assayed by PCR only. Samples taken at the conclusion of the study were processed by both PCR and PTD. Percent values shown for a given treatment represent the sample size of the entire treatment, not an average of replicates within a treatment. Values within parenthesis are the total sample size within a treatment.

of fish were infected ($N = 47$), with an average of 14,222 ± 23,612 spores per head. All of the positive control fish were heavily infected ($N = 29$), averaging 2.41 ± 1.48 million spores per head. With the PTD assay, no infected fish were found within the negative control group.

4. Discussion

These tests demonstrated that sand beds consisting of grains >180 μm were very effective at filtering Tams out of infected water. Over the course of the study over 300,000 Tams had been introduced into water flowing to each treatment filter. Within the >180 μm treatment, fish were disease free after 24 weeks of Tam exposure according to PCR results. When viewing the PTD results for the >180 μm fish, only one fish was positive at the study's conclusion. The finding that a sand filter containing >180 μm sand is effective at removing water-borne Tams is reinforced by research presented at the 6th (2000) and 8th (2002) Annual Whirling Disease Symposia by Barrows and his coworkers (unpublished data). They concluded that columns containing 200 μm diameter glass beads were effective at trapping the Tams within the filter bed. Nehring et al. (2002; 8th Annual Whirling Disease Symposium; unpublished data) demonstrated that the actinospore load in contaminated water could be greatly reduced when it was passed through a sand filter containing a 40 cm deep bed of washed playground sand.

Our fish exposure test also demonstrates that at some point, filtering efficiency is lost, and Tams pass through the sand bed. This possibility is reinforced when comparing the 1 month samples to the end of study PCR results for the >300 μm fish. After 1 month, 10% were infected compared to 49% at the conclusion. On the other hand this data may be interpreted as representing a cumulative infection caused by a low, yet constant level of Tams passing through the >300 μm sand bed.

The results from this study conducted at the FES are reinforced by a similar, yet unreplicated preliminary test conducted at the Midway State Fish Hatchery, Midway, Utah. Midway's water supply is contaminated with *M. cerebralis* actinospores. In an attempt to test sand filters on a large scale, a commercially produced filter (Baker Hydro, 91 cm diameter) was fitted to a contaminated spring. The filter was filled with 18 cm of pea gravel and topped with 17 cm of sand which was >180 μm. The filter was gravity fed and run at approximately 57 l/min, and was back-flushed thrice weekly at a rate of 51 l/min. Test fish were reared in filtered water and control fish were reared in spring water without filtration. Samples taken after 4 months (March 13, 2002) and analyzed by the PCR methodology showed fish reared in filtered water to be uninfected while the unfiltered controls had a single individual ($N = 20$) that tested positive. The remaining fish were held an additional 5 months, and after that period 28 of 60 (47%) unfiltered controls were positive and 3 of 60 (5%) of fish reared in filtered water were positive. Once again, the sand filter was functioning, but over a 9-month span, enough Tams passed through to cause infection. The sand bed composition (i.e., sand grain size and/or multiple size layers), sand bed depth, or back-flushing protocol may be at fault.

It is possible that the back-flush process was the weak link in our laboratory system as well as at the Midway hatchery. When the media bed is fluidized during a back-flush, trapped

Tams should be freed from the filter and discharged from the rearing system. Perhaps some Tams are only partially liberated from the filter bed by the time back-flushing is complete. When normal flow is restored through the filter, these Tams may be able to pass through the filter before the sand bed has completely seated itself after being fluidized.

Specific recommendations are given for back-flush rates using different sand sizes and at different water temperatures (Huisman et al., 1983). Under FES and Midway conditions, at water temperatures of 10–20 °C and using sand grains 400–500 μm, Huisman et al. (1983) recommended back-flush rates of 12–20 $m^3/m^2/h$. The FES back-flush rate averaged 47 $m^3/m^2/h$. Although this rate is more than twice that recommended, samples taken and observations made did not indicate the back-flush rate was so high that sand particles were discharged. It seems likely that the higher rate would have ensured an adequate removal of trapped Tams from the sand bed. The problems experienced at Midway could be explained by the back-flush rate used, which was 5 $m^3/m^2/h$, well below the recommended level. This incomplete back-flushing may have contributed to the passage of Tams through the sand bed. In their work presented at the 8th (2002) Annual Whirling Disease Symposium, Barrows and his coworkers (unpublished data) determined that Tams trapped in their bead bed were incompletely removed from their bed after back-flushing was completed if the discharge rates or duration were too low.

Another avenue of pursuit may be the role of bacterial biofilms that develop on the sand grains, trapping Tams due to reduced interstitial space. Biofilms can serve as consumers of water-borne viruses and bacteria (Hendricks and Bellamy, 1991). These same biofilms may act as "flypaper" for Tams. It is entirely possible the biofilms associated with the biofilters used for our trials did trap Tams, however sufficient number passed though to infect fish as exhibited by the infected fish among the positive controls. Biofilms can also be associated with the Schmutzdecke of slow sand filters and have shown to be effective removers of organisms smaller than Tams such as *Giardia* and *Cryptosporidium* cysts, viruses, and coliform bacteria (Bellamy et al., 1985; Hendricks and Bellamy, 1991; Schuler et al., 1991). Future research may aim at developing a hybrid sand filter which incorporates the biological activity of the slow sand filter with the higher flow rates of a rapid sand filter.

Research into the depth and composition of the sand bed also warrant further research. The sand bed and support bed depth of the FES filters was 10 cm each. At Midway the support bed was 18 cm and the sand bed 17 cm. Suggested depths for the sand bed are 60–70 cm, and 60–90 cm for the support bed (Baylis et al., 1971). Perhaps deeper sand beds for our experiments would have been more effective at Tam removal. Unfortunately, when sand size is held constant, and sand bed depth is increased head loss increases (Wheaton, 1985). Other potential unknowns are the effects of water flow. For example would increased water flows push Tams deeper into the bed to the point they pass from the filter?

Hopefully these shortcomings may be overcome, because rapid sand filtration appears to be an inexpensive, low maintenance alternative to costly, maintenance intensive, and potentially user harmful alternatives such UV and ozonation techniques. The sand filters designed for the FES trial allowed for a reasonable flow (0.7 gpm, 2.8 l/min, 8.6 $m^3/m^2/h$) and fell within the range that defines rapid sand filters (4–20 $m^3/m^2/h$). The commercial filter used at Midway was rated for flows up to 543 l/min, but the actual flow of 57 l/min, which resulted in a rating of 5.2 $m^3/m^2/h$. The "Catch-22" of the sand filter's application to Tam removal is that a deeper sand bed of smaller sand may be needed to achieve 100%

Tam removal, however such a design necessitates a loss of head pressure. By determining the appropriate depth and size of sand, it seems probable that sand filters could be scaled up to accommodate the flows necessary to supply a large fish hatchery.

5. Conclusions

Our rapid sand filter design, containing sand with all particles less than 180 μm removed, was able to remove whirling diseases Tams to the extent that only one fish of 54 (final sample) became infected over a period of 24 weeks of regular exposure. The filters are easily adaptable to a gravity fed system and accommodated flow rates of 8.6 $m^3/m^2/h$. The sand bed was only 10 cm deep which could be considered too shallow for effective filtration. Future designs may need to incorporate a deeper filter bed, possibly comprised of different levels of sized media. With slight modifications, the sand filter design could be an attractive alternative to other costly and maintenance intensive filtration systems for decontaminating hatchery water that is enzootic for *M. cerebralis* actinospores.

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