Extraction of Whirling Disease Myxospores from Sediments Using the Plankton Centrifuge and Sodium Hexametaphosphate

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Abstract

Myxobolus cerebralis, the causative agent of whirling disease in salmonids, has two transmission stages: the myxospore (released by salmonids) and the triactinomyxon (released by an aquatic tubificid worm, *Tubifex tubifex*). Myxospores are released into sediments after fish infected with *M. cerebralis* die and their carcasses decompose. The purpose of this study was to determine if it was possible to extract and enumerate *M. cerebralis* myxospores that were experimentally added to autoclaved sand and benthic sediments using a plankton centrifuge alone or in conjunction with sodium hexametaphosphate. The plankton centrifuge is commonly used to extract myxospores from salmonid skeletal elements and the technique involves filtration and sedimentation of myxospores. Sodium hexametaphosphate is a non-sudsing detergent that decreases soil aggregation making filtration more efficient. When the plankton centrifuge technique was used alone, we were only able to extract an average of 8 percent of myxospores inoculated into sediments. However, we were able to extract between 50-70 percent of *M. cerebralis* myxospores when sodium hexametaphosphate and the plankton centrifuge were used together. This technique could further enhance understanding of the ecology of the parasite and its association with *T. tubifex*.

Key Words: extraction, *Myxobolus cerebralis*, myxospores, plankton centrifuge, sodium hexametaphosphate, *Tubifex tubifex*, whirling disease.

Introduction

Whirling disease, the common name of a disorder of some salmonids, e.g., trout, salmon, is caused by the microscopic parasite *Myxobolus cerebralis* (Hedrick et al. 1998). The parasite has recently been implicated as the cause of severe declines in some wild rainbow trout (*Oncorhynchus mykiss*) populations in the Intermountain West (Nehring and Walker 1996, Vincent 1996). The life cycle of *M. cerebralis* alternates between fish and the tubificid, *Tubifex tubifex* (Markiw and Wolf 1983, Wolf and Markiw 1984). *Tubifex tubifex* expels the triactinomyxon form, which becomes suspended in the water column (El-Matbouli and Hoffmann 1998). Sporoplasms are injected into the fish's tissue, e.g., epithelium and gills, when triactinomyxons come in contact with salmonids (Hedrick et al. 1998). After contact the sporoplasms migrate toward the cartilage in vertebrae, gill arches, and cranium where infection develops (El-Matbouli et al. 1995). As fish die and decompose, myxospores (10-µm dia.; Hedrick et al. 1998) are released into benthic sediments where they are ingested by *T. tubifex* and the cycle begins again.

Abundances of *M. cerebralis* myxospores in benthic sediments are unknown and no technique has been developed to detect and enumerate them. However, the plankton centrifuge is routinely used to extract myxospores from skeletal elements of salmonids (O'Grodnick 1975). The technique involves filtering macerated skeletal elements and sedimenting myxospores in the plankton centrifuge. Our goal was to use the plankton

centrifuge technique to extract myxospores from sediments. However, we suspected that small soil particles (silt and clay) would promote soil aggregation and make filtration and visualization of myxospores difficult; thus, the plankton centrifuge technique might be inefficient if used alone (Buffle and Leppard 1995b). Consequently, we also tested the plankton centrifuge with aqueous sodium hexametaphosphate ($[NaPO_3]_{6aq}$), a non-sudsing detergent, that induces de-aggregation of natural soil aggregates (Bakken 1985, Buffle and Leppard 1995a, Day et al. 1995). Our objective was to ascertain the efficiency of the plankton centrifuge, alone and with $[NaPO_3]_{6aq}$, to extract *M. cerebralis* myxospores that were experimentally added to sediments.

Methods and Materials

We tested myxospore extraction from autoclaved sand and benthic sediments. We used autoclaved sand because we expected optimal myxospore recoveries when the percentage of fine sediments (silt and clay) and their associated bacteria were low. We used benthic sediments to test myxospore extraction under natural conditions and to determine if we could detect wild *M. cerebralis* myxospores. The benthic sediments were collected from the East Fork of Rock Creek and the West Fork of the Madison River, Montana, where whirling disease is epizootic (Vincent 2000). Using particle fractionation and size analysis (Day 1965), we determined that the autoclaved sand was 99 percent sand (2000 to 50 µm in diameter), 1 percent silt (50 to 2 µm in diameter) and had no clay (< 2 µm in diameter). The benthic sediments were composed of more silt and clay than the autoclaved sand (West Fork of the Madison River, 85-87% sand, 11-12% silt and 2-3% clay; East Fork of Rock Creek, 61-73% sand, 25-31% silt and 2-8% clay). For all trials, *M. cerebralis* myxospores were removed from fish by grinding heads in a blender, filtering the myxospore solution through a 50-µm sieve, and sedimenting the filtrate (the solution passing through the sieve) that contained the myxospores in a plankton centrifuge (O'Grodnick 1975). The resulting pellet was resuspended in water and myxospores were enumerated by placing an aliquot of the solution on a 0.4-mm Neubauer hemacytometer (Markiw and Wolf 1974). The numbers of myxospores counted on the hemacytometer were then extrapolated to calculate the total number of myxospores in solution. Myxospores were counted in a similar manner after extraction from sediments. In the trials with autoclaved sand without $[NaPO_3]_{6aq}$ and for two trials of autoclaved sand with $[NaPO_3]_{6aq}$, we only counted myxospores on the hemacyctometer once at the beginning and once at the end of the trials. In all other trials, we enumerated myxospores on the hemacytometer three times at the beginning of a trial and three times at the end of the trial. The mean of three replicate hermacytometer counts was used to characterize total myxospore numbers. Our method of comparing myxospore numbers assumed that any biases in enumeration techniques were similar before and after extraction and that those myxospores unaccounted for were lost during extraction.

Autoclaved sand without $[NaPO_3]_{6aq}$

In the first trial about 4.3×10^5 myxopores were placed in 100 ml of autoclaved sand and covered with water. The myxospore and sand solution was thoroughly mixed and then passed through a series of sieves (500 to 123 µm) while being flushed with water. The filtrate (solution passing through the sieves containing the myxospores) was placed in the plankton centrifuge. The resulting pellet was resuspended in water and passed through a 20-µm sieve. The number of myxospores in the filtrate was determined using the methods described above. Only one replicate hemacytometer count was used before and after the trial.

The second trial was similar to the first, except that about 3.6×10^4 myxospores were added to 25 ml of sand and then water was

added to bring the volume to 75 ml. The sieve series ran from 500 to 63 µm. The filtrate that passed through the 63-µm sieve was placed in the plankton centrifuge and the resulting pellet was resuspended in water, sieved (20 µm), and examined for myxospores as above. Testing was abandoned after two trials because of low extraction success.

Autoclaved sand with $[NaPO_3]_{6aq}$

A range of myxospore abundances ($1.4x10^4$-$2.2x10^5$) were added to 7 g of autoclaved sand and 35 ml of $[NaPO_3]_{6aq}$ in a 50-ml centrifuge tube. Aqueous $[NaPO_3]_6$ was prepared by using 6200 mg of $[NaPO_3]_6$/L. The myxospores, autoclaved sand, and $[NaPO_3]_{6aq}$ were agitated for three minutes by repeated inversion of the centrifuge tube. The sand was then allowed to settle out of solution (40 seconds; Gee and Bauder 1979, 1986). The supernatant with the silt, clay, and myxospores was removed with a macropipet and placed in a 1000-ml beaker. The remaining particles in the centrifuge tube were subsequently washed with 35 ml of $[NaPO_3]_{6aq}$. This washing process was repeated 25 times to maximize the removal of myxospores from the sediments. All of the combined supernatants (the 25 repetitions) were wet sieved through a 20-µm filter with deionized water. Myxospores were sedimented from the filtrate using a plankton centrifuge. The concentrated filtrate was diluted with deionized water and three replicate counts on the hemacytometer were made to determine the number of myxospores recovered. We regressed the mean number of myxospores recovered against the mean number of myxospores that were initially stocked into the sediments. We also examined the material collected by the filter to determine if myxospores were retained in the sediment.

Benthic sediments with $[NaPO_3]_{6aq}$

The procedures for benthic sediments were similar to those with the autoclaved sand, except myxospores were stained before the initial enumeration to distinguish them from wild myxospores (if present) in the benthic sediments. Only nonviable myxospores will stain so myxospores were killed by heating to 90°C for 10 minutes (Hoffman and Markiw 1977). Nonviable myxospores were stained for 10 minutes with 0.25 percent aqueous methylene blue (18 µl 0.25% aqueous methylene blue/37 µl myxospore solution). After staining, the solution was centrifuged at 4000 revolutions/minute for 20 minutes to remove residual stain in solution and the supernatant with the residual stain was discarded. The pellet was resuspended with deionized water and centrifuged again (4000 revolutions/minute for 20 minutes) for further removal of stain. The resulting pellet containing the myxospores was resuspended in water. We added between $7.5x10^3$ and $4.7x10^5$ myxospores to 7 g of benthic sediment. The extraction protocol and statistical analysis used in the autoclaved sand experiment with $[NaPO_3]_{6aq}$ were then followed.

Results and Discussion

We extracted no myxospores from our first trial and only 5834 myxospores (16 %) from the second trial when the plankton centrifuge was used alone with sand. We were able to extract *M. cerebralis* myxospores from both sediment types when aqueous $[NaPO_3]_6$ was used with the plankton centrifuge. Mean percent myxospore recovery was higher in sand (70.9 ± 7.6 [1 SE], $n = 7$) than in benthic sediment (59.4 ± 15.3 [1 SE], $n = 9$). Slopes of the regressions of myxospore recovery in sand and benthic sediments were statistically significant (Fig. 1), suggesting that percent recovery was similar across myxospore doses within a sediment type. The regressions relating number of myxospores recovered to initial number of myxospores fit very well ($r^2 > 0.96$). Consequently, it would be possible to predict the number of myxospores in sediments based on the number recovered.

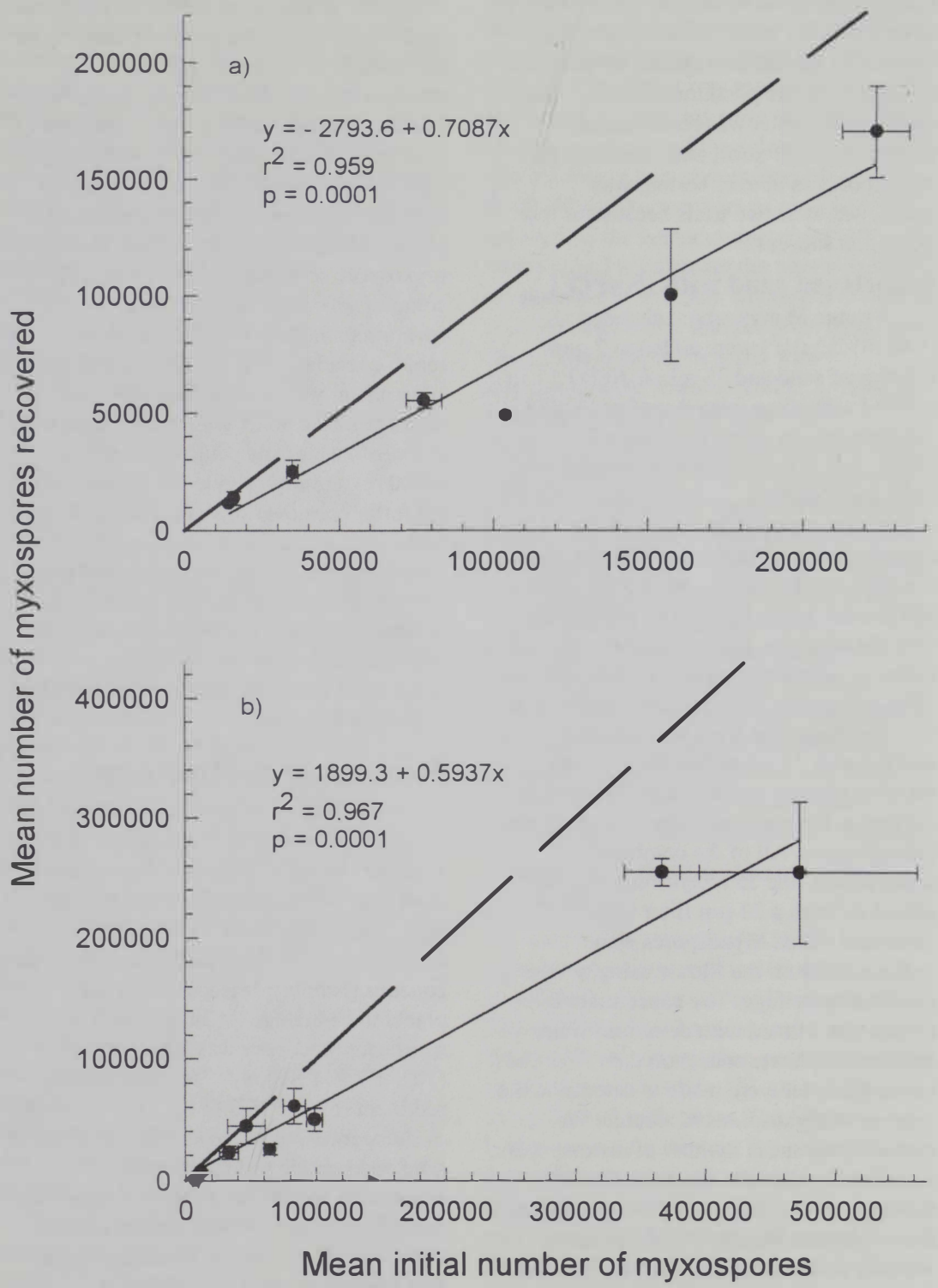

Figure 1. Number of *M. cerebralis* myxospores recovered from a) autoclaved sand and b) benthic sediments plotted against the number of myxospores initially stocked into the sediments. Trials in these experiments used a plankton centrifuge and aqueous sodium hexametaphosphate . Solid circles indicate means and error bars are ± 1 SE. Solid lines indicate the fitted regressions. Dashed lines indicate 100 percent recovery.

When aqueous $[NaPO_3]_6$ was used, the lower myxospore recovery in benthic sediments in comparison to autoclaved sand probably was the result of the higher amount of silt, clay, and organic matter present in the natural sediments. Moreover, the low myxospore recovery in sand when the planktonic centrifuge was used without aqueous $[NaPO_3]_6$ suggested that only a small percentage of silt can negatively affect myxospore extraction. Small soil particles, or organic material, increase aggregation and may reduce myxospore recovery. As small silt and clay particles increase in abundance, the size of the interstitial spaces decreases, thereby reducing fluid movement and extraction efficiency (Buffle and Leppard 1995b). Natural organic matter promotes bacterial growth, which also causes the soil to aggregate (Buffle and Leppard 1995b). Myxospores may be trapped within the aggregates and get deposited on the 20-µm sieve, resulting in reduction of flow and drastic underestimation of myxospore densities. We found only one occurrence of a myxospore in sediments collected on the 20-µm sieve from the autoclaved sand. However, we suspect more myxospores to be present in this residual material in both sediment types because our search was limited by poor visibility.

Myxospores recovered from benthic sediments were all stained. This suggests that wild *M. cerebralis* myxospores were either not present or below our detection limit. Because *M. cerebralis* occurs in these streams, myxospores should be present. However, their distribution may be patchy and our benthic samples may have been collected in patches where myxospore abundances were low. Moreover, temporal variability in myxospore abundance is unknown; therefore, we may have collected sediments when myxospore abundances were below our detection limits. Perhaps if we increased the quantity of sediments processed we would increase the probability of detecting wild myxospores.

Conclusions

This protocol using $[NaPO_3]_{6aq}$ effectively recovered myxospores in both sediment types. The procedure could be useful in laboratory assessments with *T. tubifex*. For example, reduction in myxospore numbers over the period of time it takes for *T. tubifex* to produce triactinomyxons could be monitored. Moreover, with some modification (specifically, analysis of increased volumes of sediment), this technique could provide researchers with insight on myxospore loads in the wild and add to our understanding of the ecology of the parasite.

Acknowledgements

Special thanks Dr. T. R. McDermott of Land Resources and Environmental Science of Montana State University for suggestions and information on sodium hexametaphosphate and to R. Krueger, Department of Ecology, Montana State University for determining particle sizes of our sediments. We also acknowledge M. Gangloff, and R. Stevens for their ideas. The manuscript was improved by the comments of an anonymous reviewer, R. Barrows, A. Zale, C. Cada, B. Marshall, R. Krueger and J. Jannot.

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Received 5 May 2000

Accepted 1 November 2001

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