Vol. 76: 205–214, 2007 | DISEASES OF AQUATIC ORGANISMS
Dis Aquat Org | Published July 16

Spores of two fish microsporidia (*Pseudoloma neurophilia* and *Glugea anomala*) are highly resistant to chlorine

Jayde A. Ferguson, Virginia Watral, Adam R. Schwindt, Michael L. Kent*

Center for Fish Disease Research, Department of Microbiology, 220 Nash Hall, Oregon State University, Corvallis, Oregon 97331-3404, USA

ABSTRACT: *Pseudoloma neurophilia* (Microsporidia) is the most common pathogen found in zebrafish *Danio rerio* research facilities. The parasite is associated with marked emaciation. Zebrafish laboratories usually disinfect eggs to prevent transmission of pathogens, typically with chlorine at 25 to 50 ppm for 10 min. The ability of chlorine to kill spores of *P. neurophilia* and 2 other microsporidia, *Glugea anomala* and *Encephalitozoon cuniculi*, was evaluated using 2 viability stains. SYTOX® Green was used to visualize dead spores, and live spores were identified by their ability to extrude polar tubes in Fungi-Fluor™ solution following UV exposure. Results with both stains were similar at various chlorine concentrations for *P. neurophilia* and *G. anomala*, but Fungi-Fluor was not useful for *E. cuniculi*, due to the much smaller spore size. Using the SYTOX stain, we found that 5 ppm chlorine for 10 min causes 100% death in spores of *E. cuniculi*, which was similar to findings in other studies. In contrast, the spores of *P. neurophilia* and *G. anomala* were much more resistant to chlorine, requiring >100 or 1500 ppm chlorine, respectively, to achieve >95% spore death. Repeating chlorine exposures with spores of *P. neurophilia* using solutions adjusted to pH 7 increased the efficacy of 100 ppm chlorine, achieving >99% spore inactivation. We corroborated our viability staining results with experimental exposures of zebrafish fry, achieving heavy infections in fry at 5 to 7 d post-exposure in fish fed spores treated at 50 ppm (pH 9). Some fish still became infected with spores exposed to 100 ppm chlorine (pH 9.5). This study demonstrates that spores of certain fish microsporidia are highly resistant to chlorine, and indicates that the egg disinfection protocols presently used by most zebrafish research facilities will not prevent transmission of *P. neurophilia* to progeny.

KEY WORDS: SYTOX Green · Fungi-Fluor · *Pseudoloma neurophilia* · *Glugea anomala* · *Encephalitozoon cuniculi* · Chlorine toxicity · Viability staining

INTRODUCTION

Microsporidian parasites are common in captive and wild fishes, and several are important causes of disease (Dyková 1995, Shaw & Kent 1999). Zebrafish *Danio rerio* serve as an excellent model to study early vertebrate development (van der Sar et al. 2004); hence, they are used as a laboratory model in many research laboratories. The most common infectious disease of zebrafish in research facilities is microsporidiosis, caused by *Pseudoloma neurophilia* (Matthews et al. 2001, Kent & Bishop-Stewart 2003). The parasite causes encephalitis, meningitis, and occasionally severe chronic myositis. The clinical and macroscopic manifestation of the disease is emaciation, lethargy, and skeletal deformities.

Pseudoloma neurophilia commonly infects the ovaries, but we have rarely detected it inside eggs. This led us to hypothesize that there is a significant risk of transmission to progeny from spores released

*Corresponding author. Email: michael.kent@oregonstate.edu

during spawning. Most zebrafish research facilities employ quarantine procedures and disinfect eggs with chlorine before introduction of fish to their main populations (Westerfield 2000). Therefore, it is important to know whether chlorine is effective at killing spores of *P. neurophilia*. Chlorine concentrations around 2 to 5 ppm effectively kill spores of *Encephalitozoon* species (Wolk et al. 2000, Johnson et al. 2003), but there are no reports on the efficacy of chlorine for killing fish microsporidia. Shaw & Kent (1999) showed that levels of iodine routinely used to disinfect eggs in salmon hatcheries were not entirely effective for killing *Loma salmonae*, a microsporidian that is also suspected of transmission during spawning (Docker et al.1997).

Another fish commonly used in laboratory research is the three-spine stickleback *Gasterosteus aculeatus*. *Glugea anomala* (Microsporidia) is common in this fish, and causes massive xenomas in the skin and viscera, often leading to death (Shaw & Kent 1999, Dezfuli et al. 2004, Kent & Fournie 2007). Spores of *G. anomala* were initially used to design the experiments and perfect methods, as we were able to collect large numbers of spores from macroscopic xenomas. We then more thoroughly investigated the efficacy of chlorine to kill spores of *Pseudoloma neurophilia*.

There are several methods for evaluating viability of microsporidian spores. Viability of spores has been correlated with their ability to perform polar tube extrusion. This can be accomplished by exposing spores to different conditions such as pressure, chemical treatment, change in pH, or re-hydration (reviewed by Keohane & Weiss 1999). Green et al. (2000) used SYTOX® Green nucleic acid fluorescent stain to visualize dead spores of *Encephalitozoon cuniculi* that had been inactivated by boiling. Johnson et al. (2003) used cell culture infectivity assays to determine the required dose of chlorine needed to inactivate *E. cuniculi*.

We evaluated the viability of spores exposed to either chlorine or boiling with a modified version of the SYTOX method. We also developed a method to evaluate live spores using the observation of polar tube extrusion in spores exposed to Fungi-Fluor™, a high pH fluorescent stain for chitin. Furthermore, we compared our results to previous studies by evaluating *Encephalitozoon cuniculi* with our SYTOX staining. We also conducted assays with various chlorine concentrations in which the pH of the solution was adjusted to pH 7, as chlorine is less toxic to microorganisms at higher pH values (Clark et al. 1989). Finally, we conducted an experimental exposure of zebrafish larvae to *Pseudoloma neurophilia* spores treated with various concentrations of chlorine to corroborate our *in vitro* findings.

MATERIALS AND METHODS

Spore collection and purification. Spores of the neurotropic *Pseudoloma neurophilia* were harvested from several infected fish. To accomplish this, spinal cord and hindbrain were carefully removed from fish euthanized with an overdose of tricaine methanesulfonate (MS-222, Argent Laboratories). These tissues were placed in sterile double distilled (dd) water containing antibiotics (200 U ml^{-1} of penicillin G sodium and 200 µg ml^{-1} of streptomycin sulfate), and minced with sterile scalpel blades. This slurry was aspirated several times by syringe, with successively smaller gauge needles (18, 23, and 26) to further disrupt the tissue. This solution was passed through a 40 µm nylon mesh cell strainer (Becton Dickinson). Host cells were lysed by adding sterile (dd) water to dilute the sample by a ratio of 1:10 and incubated for 1 h at room temperature. The preparation was centrifuged at 1350 × *g* for 20 min. The supernatant was removed followed by a repeat of the water lysis as above for 24 h, centrifuged, and re-suspended in 0.5 to 1.0 ml sterile dd water. Spore preparations were held at 4°C for no longer than 1 wk before testing.

Glugea anomala infection occurs as large macroscopic xenomas up to about 4 mm in diameter in three-spine sticklebacks *Gasterosteus aculeatus*. Heavily infected sticklebacks were euthanized with MS-222, and xenomas were removed from the visceral cavity. Xenomas were opened in sterile dd water containing antibiotics as above, then spores were harvested with an 18 gauge needle and syringe. The spores were disrupted further by aspiration with 23 gauge needles and stored for no longer than 1 wk in sterile dd water at 4°C prior to testing.

Encephalitozoon cuniculi spore preparations grown in RK13 (rabbit kidney) cell lines in Modified Eagles Media (MEM) were obtained from L. Weiss, Albert Einstein School of Medicine, Bronx, NY, USA. The spores were concentrated and cleaned by subjecting them to overnight lysis in sterile dd water as described above for *Pseudoloma neurophilia*.

Chlorine preparation and standardization. Chlorine solutions were prepared using reagent grade (4 to 6%) sodium hypochlorite (NaOCl) (Fisher Scientific) and sterile dd water in polypropylene tubes. Chlorine concentrations were determined using the ExStik® CL200 Waterproof Total Residual Chlorine Tester (Extech Instruments), following manufacturer's instructions. Duplicate readings were taken.

Chlorine demand evaluation for the exposure containers was performed by using a freshly prepared solution of 5.70 ppm chlorine. Duplicate 20 ml samples were aliquoted into 50 ml polypropylene conical tubes for exposure to room temperature and light, and mea-

sured after 10, 30, and 60 min. The variation between duplicate readings of <0.04 ppm was acceptable, based on the instrument's manufacture specifications of an accuracy of ±10%.

Effects of pH adjustments on selected chlorine concentrations were conducted with spores of *Pseudoloma neurophilia.* Chlorine solutions were prepared at concentrations of 0 (negative control), 25, 50, 100, and 200 ppm. pH was measured using the pHep® (Hanna Instruments) instrument following manufacturer's instructions. Each chlorine solution was titrated dropwise with diluted (1:100) glacial acetic acid until a pH value of 7 was achieved.

Chlorine exposures. A preparation consisting of a final concentration of approximately 2.5×10^7 spores ml^{-1} (estimated by hemocytometer counts) was used for each chlorine test and control for each organism. Ranges in chlorine concentration for each microsporidian species were selected based on preliminary trials. For *Pseudoloma neurophilia*, we selected a range that is used by zebrafish researchers to disinfect eggs, and for *Encephalitozoon cuniculi* we chose a range based on previous reports. *Glugea anomala* spores were exposed to final chlorine concentrations (no pH adjustment) of 0 (negative control), 100, 500, 1000, 1500, and 3000 ppm in polypropylene Eppendorff microfuge tubes. Evaluations of *E. cuniculi* spores were included for the purpose of comparing our results with those of previous studies. Spores of this microsporidium were exposed to final chlorine concentrations (no pH adjustment) of 0 (negative control), 5, and 50 ppm in polypropylene Eppendorf microfuge tubes. *P. neurophilia* spores were exposed to final chlorine concentrations (no pH-adjustment) of 0 (negative control), 25, 50, 75, 100, 200, and 500 ppm in polypropylene Eppendorff microfuge tubes. pH adjusted tests were performed with *P. neurophilia* spores with exposures to final chlorine concentrations (pH 7) of 0 (negative control), 50, 100, and 200 ppm in polypropylene conical tubes. All samples were agitated at about every minute with a vortex mixer during a 10 min incubation period. Following the incubation, sodium thiosulfate was added at a final concentration twice that of the chlorine. Samples were again vigorously agitated and allowed to incubate for 5 min. Samples were then washed 3 times with 100 µl sterile dd water and centrifuged for 5 min at $9300 \times g$. After the final wash, the spore pellets were re-suspended in 100 µl sterile dd water. Spore treatments were replicated 3 to 4 times for each concentration, except where indicated.

Negative controls consisted of treating parasite spores with sterile dd water instead of chlorine. Positive controls for *Glugea anomala*, *Pseudoloma neurophilia*, and *Encephalitozoon cuniculi* (i.e. known dead spores) were derived by boiling spore solutions for 15 min, and evaluating with the SYTOX stain as described below. Boiled (10 min) and un-boiled spores of *G. anomola* were also mixed together at various percentages (0, 30, 70, and 100%) and examined with the SYTOX stain as described below.

SYTOX stain. Wet mounts of each microsporidium were prepared on microscope slides by using 5 µl of spore solution and 5 µl of 100 µM SYTOX Green nucleic acid stain (Molecular Probes). Samples were immediately examined at 650× magnification using a Lecia DMR fluorescent microscope with a FITC green filter (480 to 490 nm excitation, 527/30 emission). The morphology of the spores was observed and recorded. Killed spores were identified when the sporoplasm or entire internal region of the spore stained bright green. Bright field microscopy was periodically used to verify whether positive staining objects were spores (rather than other cells) and assisted with counting live (i.e. non-stained) spores.

Fungi-Fluor stain. Wet mounts for all 3 microsporidia were prepared by mixing 5 µl of spore solution and 5 µl of Solution A of the Fungi-Fluor stain (Polysciences) on microscope slides, with cover slips overlaid. Samples were then observed with a DAPI filter (340 to 380 nm excitation, 425 nm emission) at 1000× magnification. Spores were identified as alive if they extruded their polar tube after 10 s exposure to UV light.

Experimental exposure with *Pseudoloma neurophilia*. Spores of *P. neurophilia* were collected and prepared as above. The spore preparation was treated with the designated concentration of either 0, 50, or 100 ppm chlorine (unadjusted pH) for 10 min as described above. Inocula were then examined for viability using the SYTOX method. The larval fish exposures consisted of 40 larvae at 15 d post-fertilization. They were obtained from R. Tanguay's laboratory at Oregon State University, Corvallis, USA; we have tested this source zebrafish colony several times and found it to be uninfected with *P. neurophilia*. Groups of 10 larvae were held in 250 ml water at 27°C and exposed to 10 000 spores $fish^{-1}$ (40 spores ml^{-1}). Fish were fed 3 times daily with newly hatched brine shrimp. Approximately 50% of the water was changed twice a day. At 5 to 7 d post-exposure, fry were euthanized with MS-222; whole fish were compressed in wet mounts with cover slips overlying glass slides, and each whole fish was examined.

Statistics. Differences in spore mortality with corresponding increases in chlorine concentrations (dose-response curves) were determined by ANOVA followed with the Tukey Honestly Significant Difference post hoc test for pairwise differences. Levene's test was used to determine whether any unequal variance

existed. To determine the effect of pH on chlorine-induced spore mortality, Student's *t*-tests were used at the indicated chlorine concentrations following arcsine square-root transformations. Transformation was necessary to correct for unequal variances. Untransformed data are presented in the figures. We compared the intercepts of the dose-response curves between SYTOX and Fungi-Fluor stains by the extra sums of squares *F*-test following arcsine square-root transformation. Statistical significance was set at $p < 0.05$ and p-values are 2-tailed.

RESULTS

The SYTOX stain was found to be very effective at identifying dead spores of all 3 microsporidan species, and Fungi-Fluor was very useful for identifying live spores of *Pseudoloma neurophilia* and *Glugea anomala* (Fig. 1).

Glugea anomala in vitro assays

SYTOX staining showed that even 3000 ppm chlorine (pH 11.3, performed 4 times) was ineffective at killing over 90% of the *Glugea anomala* spores (Fig. 2). In a fifth replicate, spores were stained either with SYTOX Green or Fungi-Fluor to compare the 2 staining methods. Stains correlated well, with similar spore mortality within chlorine concentrations (ppm) of 0 (pH 5.5), 100 (pH 9.5), and 500 (pH 10.1) (Fig. 3). Although not statistically different (extra sums of squares *F*-test), Fungi-Fluor staining consistently showed more death than SYTOX (Fig. 3) at the higher chlorine concentrations of 1000 (pH 10.7), 1500 (pH 11.0), and 3000 (pH 11.3) ppm.

SYTOX results derived from mixing boiled spores (10 min) with untreated spores showed the following: 100% untreated, 4.2% dead; 30% boiled + 70% untreated, 32.6% dead; 70% boiled + 30% untreated, 72.6% dead; and 100% boiled, 97.0% dead. Boiling of

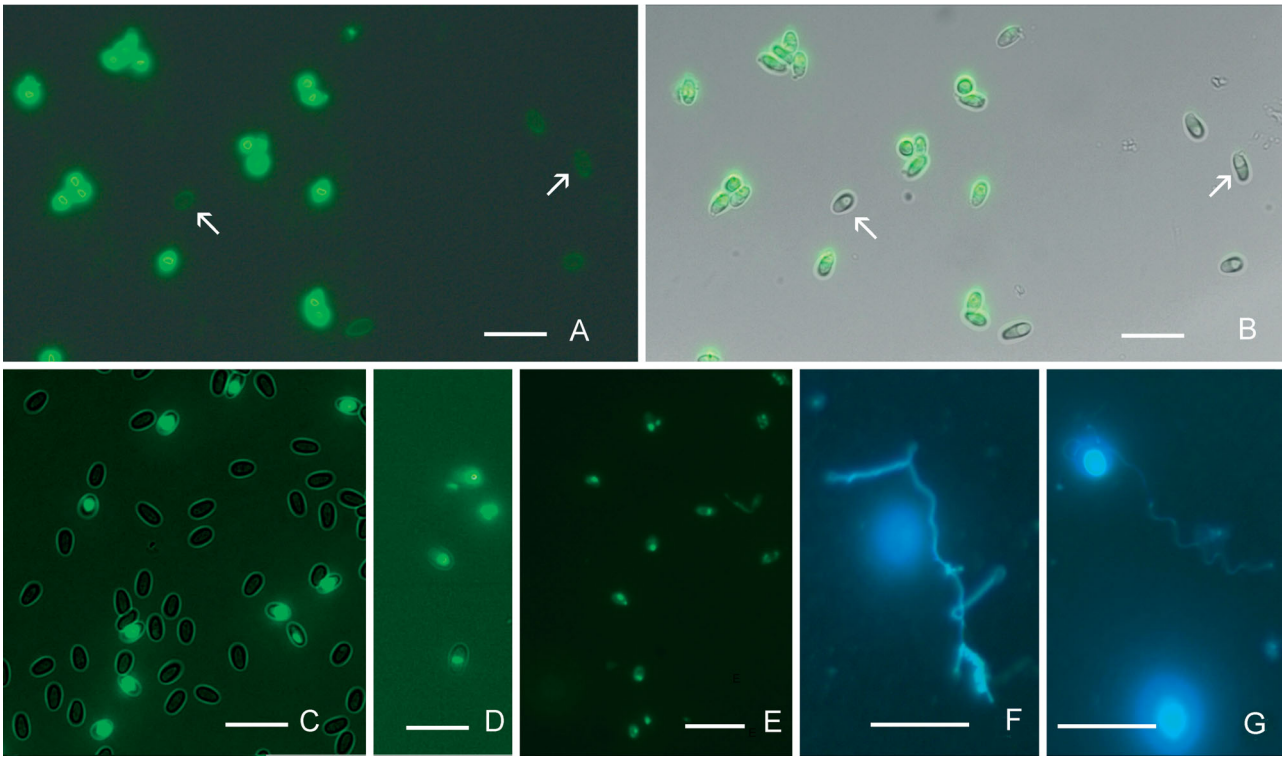

Fig. 1. *Glugea anomala*, *Pseudoloma neurophilia,* or *Encephalitozoon cuniculi* spores stained with (A–E) SYTOX or (F,G) Fungi-Fluor after treatment with chlorine without pH adjustment, or boiled (control). Scale bars = 10 µm. (A) Mixture of live and dead (boiled) *G. anomala* spores examined with FITC UV filter. Live spores exhibit very faint green outlines with no internal staining (arrows). Dead spores exhibit bright green internal staining. (B) Same field of view as (A) with bright field image overlaid. Note absence of stain within live spores (arrows). (C) *G. anomala* treated with 500 ppm chlorine (pH 10.1). Note numerous live spores demarked by green halo and no internal staining. (D) *P. neurophilia*; 3 spores scored as dead, treated at 50 ppm chlorine (pH 9.0). (E) *E. cuniculi* spores scored as dead following treatment with chlorine at 5 ppm (pH 7.7). Well-defined spherical sporoplasms stained green. (F,G) Fungi-Fluor treated spores of *G. anomala* scored as live with extruded polar tubes after treatment with 100 ppm chlorine (pH 9.5)

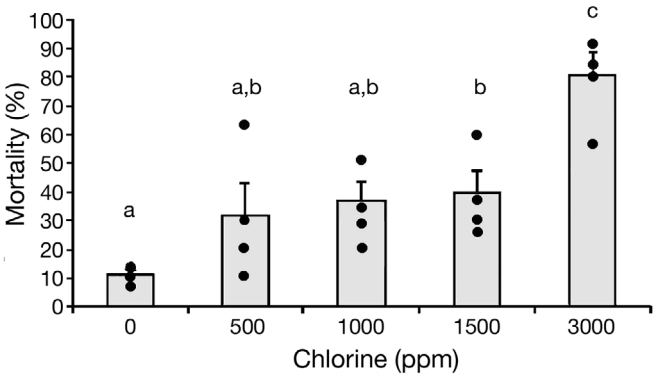

Fig. 2. *Glugea anomala*. Average (+SE) mortality of spores treated with chlorine without pH adjustments. Tests were conducted in 4 replicates and were evaluated with the SYTOX stain. Unadjusted pH values for chlorine in ppm were as follows: 0 (pH 5.5), 100 (pH 9.5), 500 (pH 10.1), 1000 (pH 10.7), 1500 (pH 11), and 3000 (pH 11.3). ●: individual data points. Different letters indicate significantly different average mortality, ANOVA $F_{4,15} = 16.90$, $p < 0.0001$, Tukey's honestly significant difference (HSD) at 95% confidence post hoc

spores for 15 min resulted in 100% death as indicated by the SYTOX stain.

Live spores of *Glugea anomala* were large and pyriform with a prominent posterior vacuole (Fig. 1A). Spores stained by SYTOX exhibiting a faint green external fluorescence with no internal staining were recorded as alive. Spores scored as dead were morphologically similar, but had in their centers large internal fluorescing bands or spheres. In addition, the posterior vacuole was often not evident in dead spores. Occasionally, the entire internal area of the spore stained bright green. At very high concentrations of chlorine, some spores that were scored as alive (i.e. no internal staining) were actually found to be empty spore walls with no sporoplasms or internal structures when

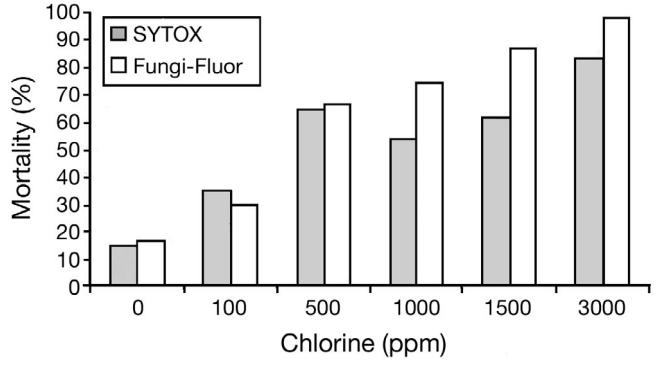

Fig. 3. *Glugea anomala*. SYTOX (stain for dead spores) and Fungi-Fluor (stain for viable spores) methods. Unadjusted pH values of chlorine in ppm were as follows: 0 (pH 5.5), 100 (pH 9.5), 500 (pH 10.1), 1000 (pH 10.7), 1500 (pH 11), and 3000 (pH 11.3)

observed with bright field microscopy. Empty spores were not seen at low concentrations where nearly all spores had a visible posterior vacuole.

When viewed with the 100× microscope objective, live spores stained with Fungi-Fluor extruded polar tubes within a few seconds following exposure to UV light (DAPI filter) (Fig. 1F,G). Spores twitched rapidly at the instant when the polar tubes extruded.

Encephalitozoon cuniculi in vitro assays

Evaluation of 500 spores of this microsporidium with SYTOX showed the following: 73.8% of untreated spores were dead, 100% were dead at 5 and 50 ppm chlorine. All spores were recorded as dead by SYTOX staining when spores were boiled for 15 min. The original material from cell culture, before water lysis, showed about 50% viability. A bright, green sphere in the region of the sporoplasm was seen in spores that were scored as dead (Fig. 1E). Evaluation of spores of this species with Fungi-Fluor was unsuccessful, i.e. we did not detect extruded polar tubes, even in untreated spores.

Pseudoloma neurophilia in vitro assays

SYTOX showed that 25 ppm chlorine (pH 8.5) did not dramatically increase the spore death of *Pseudoloma neurophilia* compared to controls (pH 5.5). Treatment of spores with 50 ppm chlorine (pH 9) caused an increase in spore death compared to controls, but complete death was not achieved (Fig. 1D, Fig. 4). Treatment of spores with chlorine concentrations (ppm) of 100 (pH 9.5) and 200 (pH 10) resulted in about 82 and 97% mortality of spores, respectively. Sub-samples of each preparation from the latter 2 trials were also stained separately with SYTOX Green and Fungi-Fluor. Differences between the 2 methods when applied to the same sample revealed a range of 0.3 to 30% in mortality, with Fungi-Fluor consistently showing higher mortality. Discrepancies were highest at 100 ppm chlorine (pH 9.5), where the Fungi-Fluor indicated 93% spore death, while SYTOX showed only 63% in one trial (Fig. 5). Nevertheless, throughout the entire range of chlorine concentrations, the percent mortality was not different between SYTOX and Fungi-Fluor (*F*-test for intercepts $p = 0.22$). Treatment of spores with 500 ppm chlorine (pH 10.1) showed 100% death with both tests.

Adjustment to pH 7 of the chlorine treatment solution used for *Pseudoloma neurophilia* caused significantly higher mortality of spores as determined by SYTOX for the mid range treatments (Fig. 4). At

25 ppm chlorine, there was an average spore death of 73%, in contrast to only 40% at pH 8.5 (T_5 = 14.44, p = 0.00003). At 50 ppm, an average of 83% spore mortality occurred, versus 63% at pH 9 (T_5 = 2.86, p = 0.03). At 100 ppm 99 to 100% average spore death was achieved, compared to 83% at pH 9.5 (T_5 = 3.37, p = 0.02). Exposure at 200 ppm chlorine killed all spores.

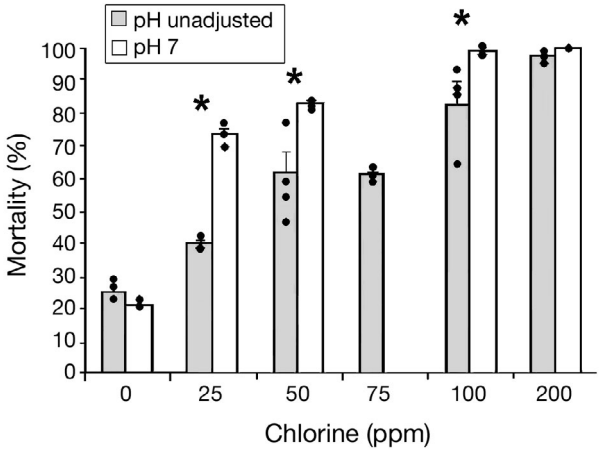

Fig. 4. *Pseudoloma neurophilia.* Average (+SE) spore mortality based on SYTOX staining at different chlorine concentrations and pH. The germicidal property of chlorine significantly increased with adjustment to pH 7. Unadjusted pH tests were conducted 4 times and adjusted pH trials were conducted 3 times, except at 200 ppm, which was performed once. Unadjusted pH values of chlorine in ppm were as follows: 0 (pH 5.5), 25 (pH 8.5), 50 (pH 9.0), 75 (pH 9.2), 100 (pH 9.5), 200 (pH 10.0). Student's *t*-test: asterisks indicate significant differences, $p < 0.05$. ●: individual data points. Note: 75 ppm was not performed with an adjusted pH of 7

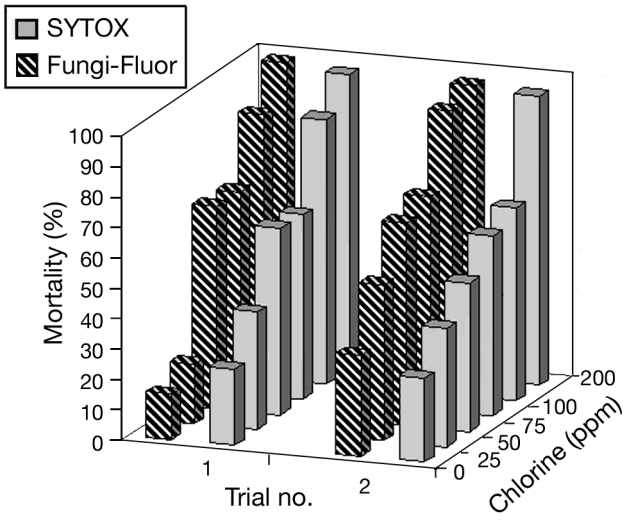

Fig. 5. *Pseudoloma neurophilia.* SYTOX (stain for dead spores) and Fungi-Fluor (stain for viable spores) methods were not significantly different (extra sums of squares *F*-test). Unadjusted pH values of chlorine in ppm were as follows: 0 (pH 5.5), 25 (pH 8.5), 50 (pH 9.0), 75 (pH 9.2), 100 (pH 9.5), 200 (pH 10.0)

Spores of *Pseudoloma neurophilia* scored as dead with SYTOX staining usually exhibited a small, spherical positive staining region, presumably the sporoplasm (Fig. 1D). Live spores appeared similar to, but smaller than *Glugea anomala* spores, with a prominent posterior vacuole visible by bright field microscopy, and faint green staining on the surface with SYTOX. Boiling spores for 15 min resulted in 100% death as judged by the SYTOX method. These spores under fluorescent microscopy appeared the same as the chlorine-killed spores, exhibiting a distinct internal dot that stained bright fluorescent green. Spores scored as alive with the Fungi-Fluor stain behaved similarly to those of *G. anomala*, with rapid twitching at the instant of polar tube extrusion within a few seconds following exposure to the UV light.

Experimental exposure with *Pseudoloma neurophilia*

The inocula used for exposing larval fish had 94.4% viability in the untreated spores, 60% in spores treated with 50 ppm, and 3.1% in the 100 ppm chlorine treatment, based on evaluation with SYTOX stain. Larval fish were first examined at 5 d post-exposure when 2 fish from the untreated spore group and one from the 50 ppm chlorine group died. These fish were heavily infected with *Pseudoloma neurophilia*, exhibiting numerous aggregates of spores throughout the spinal cord, somatic muscle, and dermis (Fig. 6). The remaining fish were euthanized and examined at 7 d post exposure. Results were as follows (number positive of number examined): no chlorine, 8 of 8; 50 ppm, 8 of 9; 100 ppm, 2 of 6; control (no spores), 0 of 8. Fish in the first 3 groups exhibited heavy infections, with numerous aggregates of spores in the central nervous system and skeletal muscle (Fig. 6), while the 2 positive fish from the 100 ppm treatment had relatively light infections. Fish in all groups varied in size and development, with the smaller fish generally exhibiting more severe infections.

DISCUSSION

Microsporidia are well-recognized, often serious, pathogens of many fishes (Lom & Dyková 1992, Dyková 1995, Shaw & Kent 1999). With the increased use of fish in biomedical research, some microsporidia are now recognized as important pathogens in laboratory fishes (Kent & Fournie 2007). An important method to control these infections is surface disinfection of eggs. This is

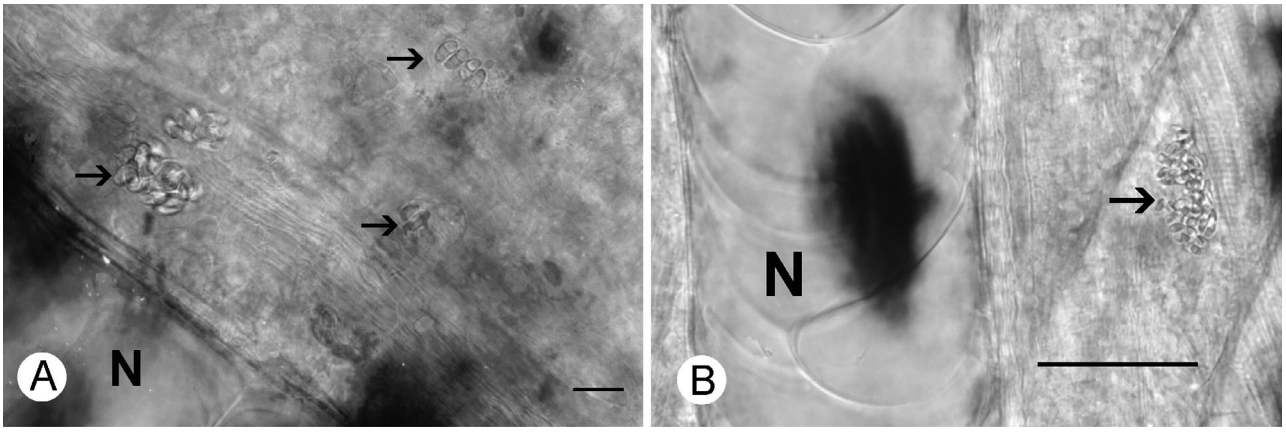

Fig. 6. *Danio rerio*. Larval fish infected with *Pseudoloma neurophilia* treated with chlorine, without pH adjustments. Arrow = spore, N = notochord. (A) Spores in developing spinal cord and surrounding tissue, exposed to spores that were not treated with chlorine (control). Scale bar = 10 µm. (B) Aggregates of spores in muscle of larval fish exposed to spores treated with 50 ppm (pH 9.0) chlorine. Scale bar = 50 µm

particularly important for newly hatched fry or eggs of zebrafish and for eggs of salmonids as they are transported globally. Indeed, disinfection of eggs has long been a primary tool in the control of the geographic spread of disease through salmonid eggs (Kent & Kieser 2003). With salmonids, iodophors are typical disinfectants, while the zebrafish research community has relied on chlorine (typically 25 ppm for 10 min). Shaw et al. (1999) showed that spores of *Loma salmonae* could survive at 150 ppm iodine for 25 min, a dose typically used for disinfecting salmon eggs.

The efficacy of chlorine and other disinfectants to kill microsporidia have been evaluated using direct exposure to animals (Shaw et al. 1999, Khalifa et al. 2001, Johnson et al. 2003), infectivity of cell cultures (Wolk et al. 2000 and papers cited therein, Santillana-Hayat et al. 2002, John et al. 2003, Leiro et al. 2004, Jordan 2005, Li & Fayer 2006), viability of spores detected by polar tube extrusion (He et al. 1996, Keohane & Weiss 1999 and papers cited therein, Shaw et al. 2001), or with stains that demonstrate either live or dead spores (Green et al. 2000, Hoffman et al. 2003). Green et al. (2000) developed a method for evaluating viability of *Encephalitozoon cuniculi* using SYTOX Green in conjunction with Calcofluor White M2R™. They verified their results by showing a strong correlation with ability to infect cell cultures using mixtures of killed (boiled) and live spores. Chlorine efficacy on spore inactivation of microsporidia has only been evaluated on species that infect humans, such as *E. cuniculi* and *E. hellem* (Wolk et al. 2000, Johnson et al. 2003, Jordan 2005). Hoffman et al. (2003) used the detection methods of Green et al. (2000) to evaluate the efficacy of chlorine to inactivate *E. intestinalis* spores which was corroborated by the absence of infectivity in rabbit kidney cells.

The modified SYTOX method used in our study differed in a few procedures from those of previously cited protocols. We examined spores in wet mounts, rather than as methanol-fixed, air-dried spore preparations. Also, the incorporation of light microscopy coupled with UV was used, rather than just staining strictly for chitin to identify the spores. This was done because the fish microsporidia examined are considerably larger than *Encephalitozoon* spp., so visualization of structures was easier and more useful. We verified that our modifications were suitable, as we observed 100% death of *E. cuniculi* with the SYTOX stain when spores were exposed to 5 ppm chlorine, as had previously been established (Wolk et al. 2000, Johnson et al. 2003, Jordan 2005).

Hoffman et al. (2003, p. 4969) concluded, 'While exclusion of these dyes (e.g. SYTOX) cannot confirm viability, the penetration of the dyes into the organisms can only occur if the membrane has been compromised'. Therefore, we also evaluated spores exposed to chlorine with a method that counts live spores, i.e. based on the ability of spores to extrude their polar tubes. This was evaluated by exposing spores to Fungi-Fluor and then exciting the spores with UV light exposure. Fungi-Fluor is a high pH solution (0.1% KOH), which may account for the ability of viable spores to extrude their polar tubes. We estimate that the pH of the final solution (50% Fungi-Fluor) was 13.5, whereas pH values of 8 to 9.5 induce polar tube extrusion in other microsporidia (reviewed by Keohane & Weiss 1999). Interestingly, exposure of spores to Fungi-Fluor and examination of them under bright field or Nomarksi phase interference microscopy did not cause polar tube extrusion. The Fungi-Fluor results generally agreed with those obtained by SYTOX staining, and there was no statistical difference between

the 2 detection tests. However, at higher chlorine levels, percent death measured using Fungi-Fluor was consistently higher than with SYTOX. For a spore to be scored as 'dead', the sporoplasm or other spore structures must stain green with SYTOX. Extremely high doses of chlorine occasionally displayed intact spores with no internal contents in bright field microscopy, and these clearly dead spores would be scored as live with STYOX. This could have accounted for the difference between the 2 detection methods.

Fungi-Fluor was not useful for detecting live spores of *Encephalitozoon cuniculi*, and no extruded filaments were observed with this method among spores not treated with chlorine. An explanation for differences in the usefulness of this test for the different species could be explained by differences in polar tube diameters. Those of *E. cuniculi* tubes are much thinner (about 100 to 150 nm, Canning & Lom 1986) than those of the fish species and would be below the limit of resolution by light microscopy. *Pseudoloma neurophilia* polar tubes are about 250 to 300 nm (Matthews et al. 2001) and our measurements of those from *Glugea anomala* were 400 to 500 nm. Indeed, it was much easier to detect extruded polar tubes of the latter than those of *P. neurophilia.* This indicates that the Fungi-Fluor method may not be as reliable for spores with thin polar tubes.

Adjusting the pH of the chlorine solutions for the SYTOX assay with *Pseudoloma neurophilia* spores showed interesting results. There is a general phenomenon that occurs as pH increases, viz. the germicidal activity of chlorine decreases (Clark et al. 1989). Using the formula developed by Clark et al. (1989), adjustment of pH from 9 to 7 should double toxicity (based on results with *Giardia*, Health Canada 2004). The most germicidal form of chlorine is HOCl, and above pH 7.5 less exists as this active form, while most becomes the less active form OCl^-. By adjusting the pH of the chlorine solution, average percent spore mortality increased greatly for all treatments, except at the highest dose, when close to 100% mortality occurred with unadjusted pH. We observed 99 to 100% spore death at 100 ppm when the pH was adjusted to pH 7. In addition, there was less variation between trials when the pH was adjusted.

Our study indicates that fish microsporidia are much more resistant to chlorine than *Encephalitozoon cuniculi*. For *Pseudoloma neurophilia*, concentrations of ≥100 ppm were required to kill most of the spores, and spores of *Glugea anomala* required at least 1500 ppm. In contrast only 5 ppm chlorine was capable of absolute (100%) spore death for *E. cuniculi*. Furthermore, the Fungi-Fluor method showed that even at 3000 ppm, about 1% of the spores of *G. anomala* were still alive. Although the spore walls of all 3 microsporidian species have similar thickness, the greater overall size of the aquatic species correlates to a much larger volume. The sizes for all 3 microsporidian species were obtained from current literature and are as follows: *G. anomala* is 2.3 × 4.5 µm (Lom & Dyková 1992), *P. neurophilia* is 2.7 × 5.4 µm (Mathews et al. 2001), and *E. cuniculi* is 1.5 × 2.5 µm (Canning & Lom 1986). Based on these measurements and an equation for the volume of egg-shaped objects (Narushin 2005), we were able to estimate the spore volume for each species. *G. anomala* and *P. neurophilia* spores had volumes of 64.4 and 127.7 μm^3, respectively. The spore volume of *E. cuniculi* was much smaller, estimated to be only 8.5 μm^3. Furthermore, the surface areas were also estimated by these methods and were found to be 32.3 and 37.8 μm^2 for *G. anomala* and *P. neurophilia*, respectively, versus 11.8 μm^2 for *E. cuniculi*. The values of these physical parameters indicate a drastic surface area to volume ratio difference. Because the smaller spores have a larger surface area to volume ratio, they would be more readily exposed to the chlorine solution during treatments. Perhaps this is an important factor in the dramatic differences in chlorine susceptibility. Even in the absence of chlorine, our water lysis preparation killed many of the *E. cuniculi* spores, suggesting that this species is more fragile in general. It is conceivable that the aquatic microsporidian species may have adapted by becoming larger for survival of long durations in aqueous habitats with constant fluctuations of environmental factors. Of course factors other than spore morphology may also play a role in chlorine susceptibility, as *G. anomala* spores were 10 times more resistant than the morphologically similar *P. neurophilia* spores.

This study and previous unpublished exposure tests in our laboratory show that larval fish are extremely susceptible to *Pseudoloma neurophilia*, developing massive infections with complete sporulation within a week of exposure. Results from this current experimental exposures study of zebrafish larvae agreed with those obtained by vital staining. As predicted, fish exposed to 50 ppm exhibited almost the same level of infection as those exposed to untreated spores. Although not statistically valid, due to lack of replicates, larval fish exposed to spores at 100 ppm had a lower prevalence of infection and it was less severe. SYTOX staining indicated that only 3.1% of spores were viable at 100 ppm chlorine, an estimated exposure of only 310 spores $fish^{-1}$. This elevates the risk of transmission at this stage of development, because even if only a few larvae were infected, they would provide a source of infection to their tank mates.

Moreover, in female zebrafish infected with *Pseudoloma neurophilia*, we have found as many as 10 000 spores $ovary^{-1}$ (authors' unpubl. data). Thus, infection

via transovarial or sexual products contamination may also play an important role in spread of this disease. Disinfection of eggs to avoid transmission of pathogens between generations is an important tool in aquaculture, but this is predicated on the ability of the agents to kill all or most of the pathogens of concern. Additionally, at least 4 microsporidia of fishes are suspected to be transmittable either in eggs or with sexual products during spawning (Vaney & Conte 1901, Summerfelt & Warner 1970, Docker et al. 1997, Kent & Bishop-Stewart 2003). Our findings indicate that the protocol currently used by most zebrafish laboratories (25 to 50 ppm chlorine for 10 min, with unadjusted pH) is inadequate for killing spores of *P. neurophilia*. Future experiments should be conducted to determine the toxicity of 100 ppm chlorine (or slightly higher) at pH 7 for zebrafish embryos. Indeed, this concentration of chlorine with the increased germicidal activity, at pH 7, may be highly toxic to zebrafish larvae.

Considering the resistance of these fish microsporidia to chlorine, other disinfectants that do not injure fish embryos within eggs should be evaluated for killing spores. Hydrogen peroxide has been used to kill resistant stages of other protozoa, such as *Cryptosporidium* species (Weir et al. 2002). Dilute formalin (e.g. 1.5%) is used to disinfect salmonid eggs (Barnes et al. 2003), but it is unknown how this would affect these spores or zebrafish embryos. Induction of premature extrusion of spores that are outside unhatched eggs would be another strategy to prevent infections, and relatively low concentrations (1.5 to 5%) of hydrogen peroxide causes microsporidian spores to extrude their polar tubes (Keohane & Weiss 1999, Shaw et al. 1999). Salmonid eggs can tolerate at least 0.7% hydrogen peroxide (Barnes et al. 2003), but how these agents may affect zebrafish eggs and embryos is unknown.

Acknowledgements. This work was supported by NIH Grant NCRR 5R24RR017386-02 and a teaching assistantship to J.A.F. by the Department of Microbiology, Oregon State University (OSU). Special thanks to R. Tanguay (OSU) for providing *Pseudoloma neurophilia*-free zebrafish larvae, and to C. Whipps (OSU) for editing and assistance. J. Matthews (ZIRC, University of Oregon) was the first to discover that FungiFluor induced polar tube extrusion in microsporidian spores.

LITERATURE CITED

Barnes ME, Stephenson H, Gabel M (2003) Use of hydrogen peroxide and formalin treatments during incubation of landlocked fall Chinook salmon eyed eggs. N Am J Aquacult 65:151–154

Canning CU, Lom J (1986) Introduction to the Microsporidia; general characteristics and development. In: Canning CU, Lom J (eds) The Microsporidia of vertebrates. Academic Press, Orlando, FL, p 11–12

Clark RM, Member ASCE, Eleanor RJ, Hoff J (1989) Analysis of inactivation of *Giardia lamblia* by chlorine. J Environ Engineer 115(1):80–90

Dezfuli BS, Giari L, Simoni E, Shinn AP, Bosi G (2004) Immunohistochemistry, histopathology and ultrastructure of *Gasterosteus aculeatus* tissues infected with *Glugea anomala*. Dis Aquat Org 58:193–202

Docker MF, Devlin RH, Richard J, Khattra J, Kent ML (1997) Sensitive and specific polymerase chain reaction assay for detection of *Loma salmonae* (Microsporea). Dis Aquat Org 29:41–48

Dyková I (1995) Phylum Microspora. In: Woo PTK (ed) Fish diseases and disorders, Vol 1, Protozoan and metazoan infections. CAB International, Wallingford, p 149–179

Green LC, LeBlanc PJ, Didier ES (2000) Discrimination between viable and dead *Encephalitozoon cuniculi* (Microsporidian) spores by dual staining with Sytox Green and Calcofluor White M2R. J Clin Microbiol 38:3811–3814

He Q, Leitch GJ, Visvesvara GS, Wallace S (1996) Effects of nifedipine, metronidazole, and nitric oxide donors on spore germination and cell culture infection of the microsporidia *Encephalitozoon hellem* and *Encephalitozoon intestinalis*. Antimicrob Agents Chemother 40: 179–185

Health Canada (2004) Guidelines for Canadian drinking water quality: supporting documentation — Protozoa: *Giardia* and *Cryptosporidium*. Water Quality and Health Bureau, Healthy Environments and Consumer Safety Branch, Health Canada, Ottawa

Hoffman RM, Marshall MM, Polchert DM, Jost BH (2003) Identification and characterization of two subpopulations of *Encephalitozoon intestinalis*. Appl Environ Microbiol 69:4966–4970

John DE, Nwachuku N, Pepper IL, Gerba CP. (2003). Development and optimization of a quantitative cell culture infectivity assay for the microsporidium *Encephalitozoon intestinalis* and application to ultraviolet light inactivation. J Microbiol Meth 52:183– 196

Johnson CH, Marshall MM, DeMaria LA, Moffet JM, Korich DG (2003) Chlorine inactivation of spores of *Encephalitozoon* spp. Appl Environ Microbiol 69:1325–1326

Jordan CN (2005) *Encephalitozoon cuniculi*: diagnostic test and methods of inactivation. MSc thesis, Virginia Polytechnic Institute and State University, Blacksburg, VA

Kent ML, Bishop-Stewart JK (2003) Transmission and tissue distribution of *Pseudoloma neurophilia* (Microsporidia) of zebrafish *Danio rerio.* J Fish Dis 26:1–4

Kent ML, Fournie JW (2007) Parasites of fishes. In: Baker DG (ed) Flynn's parasites of laboratory animals. Blackwell, New York, p 69–117

Kent ML, Kieser D (2003) Avoidance of introduction of exotic pathogens with Atlantic salmon reared in British Columbia. In: Lee CS, O'Bryen PJ (eds) Biosecurity in aquaculture production systems: exclusion of pathogens and other undesirables. World Aquaculture Society, Baton Rouge, LA, p 43–50

Keohane EM, Weiss LM (1999) The structure, function, and composition of the microsporidian polar tube. In: Wittner M, Weiss LM (eds) The Microsporidia and microsporidiosis. American Society of Microbiology Press, Washington, DC, p 196–224

Khalifa AM, El Temsahy MM, Abou El Naga IF (2001) Effect of ozone on the viability of some protozoa in drinking water. J Egypt Soc Parasitol 31:603–616

Li X, Fayer R (2006) Infectivity of microsporidian spores exposed to temperature extremes and chemical disinfectants. J Eukaryot Microbiol 53:S77–S79

Leiro J, Cano E, Ubeira FM, Orallo F, Sanmartin L (2004) *In vitro* effects of resveratrol on the viability and infectivity of microsporidian *Encephalitozoon cuniculi*. Antimicrob Agents Chemother 48:2497–2501

Lom J, Dyková I (1992) Protozoan parasites of fishes. Developments in aquaculture and fisheries science, Vol 26. Elsevier Science Publishers, Amsterdam

Matthews JL, Brown AMV, Larison K, Bishop-Stewart JK, Rogers P, Kent ML (2001) *Pseudoloma neurophilia* n.g., n.sp., a new genus and species of Microsporidia from the central nervous system of the zebrafish (*Danio rerio*). J Eukaryot Microbiol 48:229–235

Narushin VG (2005) Egg geometry calculations using the measurements of length and breadth. Poult Sci 84:482–484

Santillana-Hayat M, Sarfati C, Fournier S, Chau F, Porcher R, Molina JM, Derouin F (2002) Effects of chemical and physical agents on viability and infectivity of *Encephalitozoon intestinalis* determined by cell culture and flow cytometry. Antimicrob Agents Chemother 46:2049–2051

Shaw RW, Kent ML (1999) Fish Microsporidia. In: Wittner M (ed) Microsporidia and microsporidiosis. American Society of Microbiology Press, Washington, DC, p 418–446

Shaw RW, Kent ML, Adamson ML (1999) Iodophor treatment is not completely efficacious in preventing *Loma salmonae* (Microsporidia) transmission in experimentally challenged Chinook salmon, *Oncorhynchus tshawytscha* (Walbaum). J Fish Dis 22:311–313

Shaw RW, Kent ML, Adamson ML (2001) Viability of *Loma salmonae* (Microsporidia) under laboratory conditions. Parasitol Res 12:978–981

Summerfelt RC, Warner MC (1970) Geographical distribution and host parasite relationships of *Pleistophora ovariae* (Microsporida, Nosematidae) in *Notemigonus crysoleucas.* J Wildl Dis 6:457–465

van der Sar AM, Appelmelk BJ, Vandenbroucke-Grauls CM, Bitter W (2004) A star with stripes: zebrafish as an infection model. Trends Microbiol 12:451–457

Vaney C, Conte A (1901) Sur une nouvelle microsporidie, *Pleistophora mirandellae*, parasite de l'ovaire d'*Alburnus mirandella* Blanch. C R Acad Sci 133:644–646

Weir SC, Pokorny NJ, Carreno RA, Trevors JT, Lee H (2002) Efficacy of common laboratory disinfectants on the infectivity of *Cryptosporidium parvum* oocysts in cell culture. Appl Environ Microbiol 68:2576–2579

Westerfield M (2000) The zebrafish book: a guide for the laboratory use of zebrafish (*Danio rerio*), 3rd edn. University of Oregon Press, Eugene, OR

Wolk DM, Johnson CH, Rice EW, Marshall MM, Grahn KF, Plummer CB, Sterling CR (2000) A spore counting method and cell culture model for chlorine disinfection studies of *Encephalitozoon* syn. *Septata intestinalis*. Appl Environ Microbiol 66:1266–1273

Editorial responsibility: Dieter Steinhagen, Hannover, Germany

Submitted: January 25, 2007; Accepted: May 7, 2007
Proofs received from author(s): June 22, 2007