Vol. 126: 143–153, 2017
https://doi.org/10.3354/dao03167

DISEASES OF AQUATIC ORGANISMS
Dis Aquat Org

Published October 18

Seawater detection and biological assessments regarding transmission of the oyster parasite *Mikrocytos mackini* using qPCR

Mark P. Polinski, Gary R. Meyer, Geoffrey J. Lowe, Cathryn L. Abbott*

Fisheries and Oceans Canada, Pacific Biological Station, 3190 Hammond Bay Road, Nanaimo, BC V9T 6N7, Canada

ABSTRACT: *Mikrocytos mackini* is an intracellular parasite of oysters and causative agent of Denman Island disease in Pacific oysters *Crassostrea gigas*. Although *M. mackini* has been investigated for decades, its natural mode of transmission, mechanism for host entry, and environmental stability are largely unknown. We explored these biological characteristics of *M. mackini* using a recently described quantitative PCR (qPCR) assay. We detected *M. mackini* in the flow-through tank water of experimentally infected oysters and during disease remission in host tissues following 6 wk of elevated water temperature. Waterborne exposure of oysters to *M. mackini* further confirmed the potential for extracellular seawater transmission of this parasite and also identified host gill to have the highest early and continued prevalence for *M. mackini* DNA compared to stomach, mantle, labial palps, or adductor muscle samples. However, infections following waterborne challenge were slow to develop despite a substantial exposure (>10^6 *M. mackini* l^{-1} for 24 h), and further investigation demonstrated that *M. mackini* occurrence and infectivity severely declined following extracellular seawater incubation of more than 24 h. This study demonstrates a potential for using qPCR to monitor *M. mackini* in wild or farmed oyster populations during periods of disease remission or from environmental seawater samples. This work also suggests that gill tissues may provide a primary site for waterborne entry and possibly shedding of *M. mackini* in oysters. Further, although extracellular seawater transmission of *M. mackini* was possible, poor environmental stability and infection efficiency likely restricts the geographic transmission of *M. mackini* between oysters in natural environs and may help to explain localized areas of infection.

KEY WORDS: *Mikrocytos mackini* · Oyster parasite · Waterborne transmission · *Crassostrea gigas* · Quantitative PCR

INTRODUCTION

Each spring, a small portion of Pacific oysters *Crassostrea gigas* along the southwest coast of Canada develop focal green lesions characteristic of a pathological condition known as Denman Island disease (Quayle 1961). The disease is caused by *Mikrocytos mackini,* an obligate intracellular ascetosporean parasite in a newly defined order, Mikrocytida (Burki et al. 2013, Hartikainen et al. 2014), which can contribute to significant mortality and reduced marketability in infected oyster populations (Quayle 1961, Farley et al. 1988). Yet as annual seawater temperatures rise, both the parasite and disease appear to vanish, only to reappear the following spring in a cycle that has persisted for nearly 6 decades (Farley et al. 1988). Indeed, *M. mackini* infections in British Columbia (BC), Canada, are only detectable by histology between the months of January and June, and associated disease or mortality have only been observed between March and May in natural environs (Bower 1988, Quayle 1988). How *M. mackini* is naturally transmitted, such as through an intermediate host or directly between oysters, what becomes of the

*Corresponding author: cathryn.abbott@dfo-mpo.gc.ca

Publisher: Inter-Research · www.int-res.com

parasite during seasonal periods of remission, or how changing ocean climates will affect *M. mackini*-associated disease seasonality and distribution are largely unknown.

Hervio et al. (1996) demonstrated through injection and bath exposure challenges that Denman Island disease required prolonged (>3 mo) periods of cold (≤10°C) water temperature to develop. Holding oysters at elevated temperature (15–17°C) for 3 mo following experimental exposure extended the prepatent period but did not prevent overt infections from manifesting once oysters were returned to cold (10°C) conditions. Further, this work successfully used *M. mackini* purified from tissues of diseased oysters to initiate infections via waterborne exposure. This provided evidence that *M. mackini* could persist covertly within host tissues during periods of elevated temperature and that waterborne transmission of *M. mackini* to oysters was possible. However, whether *M. mackini* was naturally shed into the water column or what tissues could support covert infections was not investigated. It is also unclear if yet unknown intermediate hosts may play a role during the transmission and possible seasonality of *M. mackini*-associated disease or if, like other ascetosporean parasites such as *Bonamia* spp., *M. mackini* reproduce through a sporic life cycle.

In a recent study, Polinski et al. (2015) described a new quantitative detection assay for identifying *M. mackini* DNA from host tissues using real-time quantitative PCR (qPCR). This technique was exquisitely sensitive for detection of parasite DNA from both active and resolving infections. In fact, more than 30% of sampled oysters determined to be negative using the detection methods of conventional PCR, histopathology, or gross pathology tested positive by qPCR. This technique is also currently the only species-specific diagnostic test available for *M. mackini* — a feature which has become increasingly relevant in recent years due to the identification of at least 2 other *Mikrocytos* species (Abbott et al. 2014, Hartikainen et al. 2014). Further, although the qPCR assay developed by Polinski et al. (2015) was designed to detect *M. mackini* from within oyster tissues, similarly designed qPCR assays targeting other aquatic pathogens have been adapted to detect target organisms in environmental samples such as seawater. For example, a qPCR assay developed for detecting the amoebic fish parasite *Neoparamoeba perurans* from gill samples of salmon (Bridle et al. 2010) has also been used to quantitatively detect free-swimming amoebae in seawater of salmon farm net pens using filter retention methods (Wright et al. 2015). We hypothesized that if *M. mackini* is actively shed from infected oysters, the qPCR assay developed by Polinski et al. (2015) could be used to detect *M. mackini* in environmental seawater using similar filter retention methods to those described by Wright et al. (2015). This would greatly enhance our ability to study the transmission dynamics of this organism. Thus the aims of this study were to revisit and expand on the work of Hervio et al. (1996) to explore the biology and transmission of *M. mackini* via the new perspective afforded by qPCR, as well as to determine the feasibility of expanding the utility of this qPCR assay to incorporate environmental detection and identifying covert *M. mackini* infections during periods of seasonal disease remission.

MATERIALS AND METHODS

Oysters and infectious material

Small Pacific oysters (~60–80 mm shell length) were sourced from a commercial farm site located in Sansum Narrows near Saltspring Island, BC, Canada, and brought to the Pacific Biological Station (PBS) in Nanaimo, BC. The collection site has had no history of gross pathology indicative of Denman Island disease, and all oysters appeared to be in good health. Once at PBS, oysters were reared in 45 × 30 cm mesh herring roe baskets (Frontier Packaging) held in 50 l rectangular tanks supplemented with 3 l min^{-1} 10°C (±1°C) sand-filtered seawater. Oysters were fed 3 times each week by re-suspending 5 ml of Instant Algae Shellfish Diet 1800® (Reed Mariculture) in 1 l of seawater, pouring the mixture into the tank, and diverting inflow for 2–3 h to ensure an adequate period for filter feeding. Prior to their use in challenge trials, a subsample of 50 oysters was screened and confirmed negative for the presence of *Mikrocytos mackini* by qPCR using the methods described by Polinski et al. (2015). An additional subset of oysters was prepared for injection challenge by using a rotary rasp to notch the edge of both shell valves in the area adjacent to the adductor muscle creating an aperture for needle insertion.

Live *M. mackini* parasites were initially sourced from 8 naturally infected adult oysters collected near DeCourcy Island, BC, which had lesions characteristic of Denman Island disease. Lesions were excised and pooled (4.0 g wet tissue weight), rinsed 3 times in 0.2 µm filtered seawater (FSW), minced with scissors, and homogenized in FSW using a Polytron probe set at low speed for approximately 30 s. The homogenate

was progressively sieved through 500, 220, 100, 40, and 20 µm Nitex nylon mesh to remove large tissue fragments and fibrous material similar to methods described by Joly et al. (2001). The final filtrate was concentrated using centrifugation (2000 × *g* for 10 min at 4°C), and the resulting pellet was re-suspended in approximately 5 ml of FSW. Forty-four naïve oysters were each injected in the adductor muscle with 100 µl of *M. mackini* suspension and maintained in dedicated seawater tanks for approximately 6 wk for the purpose of propagating the large number of *M. mackini* cells required for this study. For subsequent experiments, *M. mackini* were re-isolated and purified from a portion of these experimentally infected oysters using the same techniques.

Assessments for the seawater stability of *M. mackini*

To determine how long *M. mackini* can remain viable and infectious in seawater outside the host, *M. mackini* were purified from 8 experimentally infected oysters which showed heavy infection intensity by tissue imprint methods described by Hervio et al. (1996). Adductor muscle lesions were excised, pooled, and purified using the same techniques as indicated above. This yielded 60 ml *M. mackini* seawater filtrate that was divided equally into four 15 ml tubes and incubated at 6°C for 1, 24, 48, or 72 h intervals. Following each interval, 1 tube was centrifuged at 2000 × *g* (10 min at 4°C), the supernatant was removed, and *M. mackini* was re-suspended in 2.4 ml FSW. A 5 µl subsample was placed in an etched area of a glass slide (10 mm diameter), air dried, and stained with Hemacolor®, and 5 fields of view were visualized by microscopy at 1000× magnification to provide a rough quantitative estimate and confirmation that *M. mackini* were no longer associated with host cells. Two additional subsamples of 100 µl were removed, centrifuged as above, and the supernatant (representing environmental DNA) and pellet (representing cell-associated DNA) were stored separately at −80°C for DNA extraction and qPCR screening. A portion (100 µl) of the remaining *M. mackini* suspension at each time interval was injected into the adductor muscle of each of 20 Pacific oysters. An additional 20 oysters were administered 100 µl injections of FSW without *M. mackini* to provide experimental controls. All oysters were held for 5 wk in treatment-specific culture tanks in 10°C flow-through seawater, after which adductor muscle and mid-body section samples were preserved for histopathology and qPCR screening as described by Polinski et al. (2015). The quantity of *M. mackini* ITS2 DNA recovered by qPCR in reference to the total amount of extracted DNA was compared between treatments by 1-way ANOVA and post-hoc Tukey test following log transformation of the data.

Detection of *M. mackini* in Pacific oysters following waterborne challenge and during periods of elevated temperature

At the termination of the *M. mackini* seawater stability experiment just described (5 wk post challenge, wpc), adductor muscle lesions of 7 heavily infected oysters from the 1 h seawater incubation treatment group were used to generate purified *M. mackini* as described in the preceding section. The resulting *M. mackini* filtrate (75 ml) was conservatively estimated to contain greater than 1.5×10^7 *M. mackini* parasites as determined by microscopic observation (5 fields of view) at 1000× magnification. To investigate the infection progression of *M. mackini* through oyster tissues following waterborne challenge, 80 naïve oysters were exposed to 50 ml of filtrate mixed into a static 10 l bath of seawater containing 3 ml of Instant Algae Shellfish Diet 1800® added as a stimulus for filter feeding. Oysters were held for 24 h with continuous aeration at a temperature of 10–15°C, after which they were transferred to two 50 l tanks with 3 l m^{-1} flow-through 10°C seawater and maintained for a period of 16 wk as described above. Samples of adductor muscle, labial palp, stomach, mantle, and gill were collected for qPCR analysis and histopathology from 10 oysters at 1, 3, 9, 29, 57, 85, and 113 d post challenge as previously described (Polinski et al. 2015). Oysters were also assessed daily throughout the trial, and samples were collected from all fresh-dead and moribund specimens.

We also injected 100 µl of *M. mackini* filtrate into each of 41 additional Pacific oysters to confirm the infectivity of the filtrate and to track the effects of elevated water temperature on *M. mackini* infections over time throughout a 12 wk period. After 5 wk of 10°C incubation, tissue imprints from the adductor muscle of a moribund oyster revealed high intensity of *M. mackini.* Adductor muscle and mid-body cross sections of a further 10 injected oysters confirmed the presence of infection by identifying high levels of *M. mackini* DNA in all cases by qPCR. Water temperature was then elevated to approximately 18°C (for tanks with injected oysters only) and held for a further 6 wk. Samples of adductor muscle and mid-body

cross section were collected from all fresh-dead and moribund oysters daily throughout the trial. At 12 wk post injection, all surviving oysters (n = 7) were sampled, and adductor muscle and mid-body cross sections were assessed by both qPCR and histology as previously described (Polinski et al. 2015).

Filtration of spiked seawater and challenge tank culture water for environmental detection of *M. mackini*

Under light microscopy, *M. mackini* has a generally spherical morphology 1–3 µm in diameter (Farley et al. 1988). Although its deformability is unknown, we hypothesized that extracellular *M. mackini* could be captured via filtration using a 1 µm absolute pore size. To test this hypothesis, we prepared an inoculum of *M. mackini* as described above from the adductor muscle lesions of 2 experimentally infected moribund oysters collected at 5 wpc. A 3-step, 10-fold serial dilution of inoculum was prepared using FSW. One ml of each dilution was then added separately to 1 l volumes of seawater, and recovery of *M. mackini* was attempted for each 1 l of spiked seawater using a filtration apparatus consisting of 4 magnetic filter funnels (Pall 4238), glass-fiber A/C 47 mm diameter 1 µm retention filters (Pall 66215), an aluminum filter funnel manifold (Pall 15403), and a Gast 1HAB25-M100X vacuum air pump. Samples were filtered at a rate of <500 ml min^{-1} with vacuum pressure not exceeding 27 kPa. Following filtration, glass-fiber filters were removed and aseptically halved using scissors. One-half of each filter was rolled and placed in a 2 ml microtube where DNA was extracted using a DNeasy® Blood and tissue kit (Qiagen) with these minor modifications to the manufacturer's guidelines: an initial overnight incubation at 55°C in 480 µl Buffer ATL supplemented with 20 µl Proteinase K was done; samples were vortexed for 30 s and liquid (~400 µl) transferred to 1.5 ml microtubes; an equal volume of Buffer AL was added and vortexed for an additional 10 s, after which 400 µl of 100% ethanol were added and inverted 30–40 times to mix, and samples were transferred to Qiagen spin columns by 2 successive 600 µl additions with a 1 min centrifugation at 6000 × *g* to remove liquid filtrate between additions. Membrane-bound DNA was then processed following the manufacturer's instructions before being eluted in 100 µl AE buffer. The other half of the retention filter was rolled and placed in a 2 ml PowerBead tube where DNA was extracted using a Powersoil® DNA Isolation Kit (Mobio 12888-100) following the manufacturer's instructions and eluted to a final volume of 100 µl $sample^{-1}$.

To provide a reference control for all filtration-derived samples, 500 µl of each dilution of *M. mackini* spiking material were centrifuged at 2000 × *g* (10 min at 4°C). Supernatant was removed and DNA was extracted from the cell pellet using the DNeasy® Qiagen-based method described by Polinski et al. (2015). All samples were then assessed by qPCR analysis in triplicate analytical replication from which linearity and efficiency for *M. mackini* recovery was estimated for each recovery method. The mean percent recovery of *M. mackini* across the 3-step, 10-fold dilution series was compared between DNeasy® and Powersoil® extraction methods by Student's *t*-test on arcsine transformed data.

In addition to the spiked seawater recovery experiment just described, 2 attempts were also made to detect *M. mackini* from the tank water of experimentally infected Pacific oysters. One attempt was made at 5 wpc from a tank containing 40 experimentally injected oysters in 50 l of water with 3 l min^{-1} exchange rate. One liter of seawater was collected and filtered, and DNA was recovered from half of the filter by either the DNeasy® or Powersoil® extraction methods described above. A second attempt was made at 10 wpc in a tank containing 17 injected oysters. One liter of seawater was again collected during normal flow-through operations. Following this collection, flow was stopped for a period of 4 h, after which a second 1 l sample of seawater was collected. Both samples were filtered as described above, and DNA was extracted using the DNeasy® method. In all instances, a volume of 2.5 µl of eluted DNA was used as template for qPCR screening to detect *M. mackini* ITS2 DNA copies as previously described (Polinski et al. 2015).

RESULTS AND DISCUSSION

Detection of *Mikrocytos mackini* from seawater by filter retention

The filter retention methods used in this study proved effective for consistent recovery of *M. mackini* DNA from both spiked seawater samples as well as from the culture water of experimentally infected oysters (Fig. 1). These techniques proved reliable over a 1000-fold dynamic range of template (Fig. 1A) and could detect naturally shed *M. mackini* DNA from the flow-through tank water of infected oysters at both 5 and 10 wpc (Fig. 1C). Whether recovery in our experi-

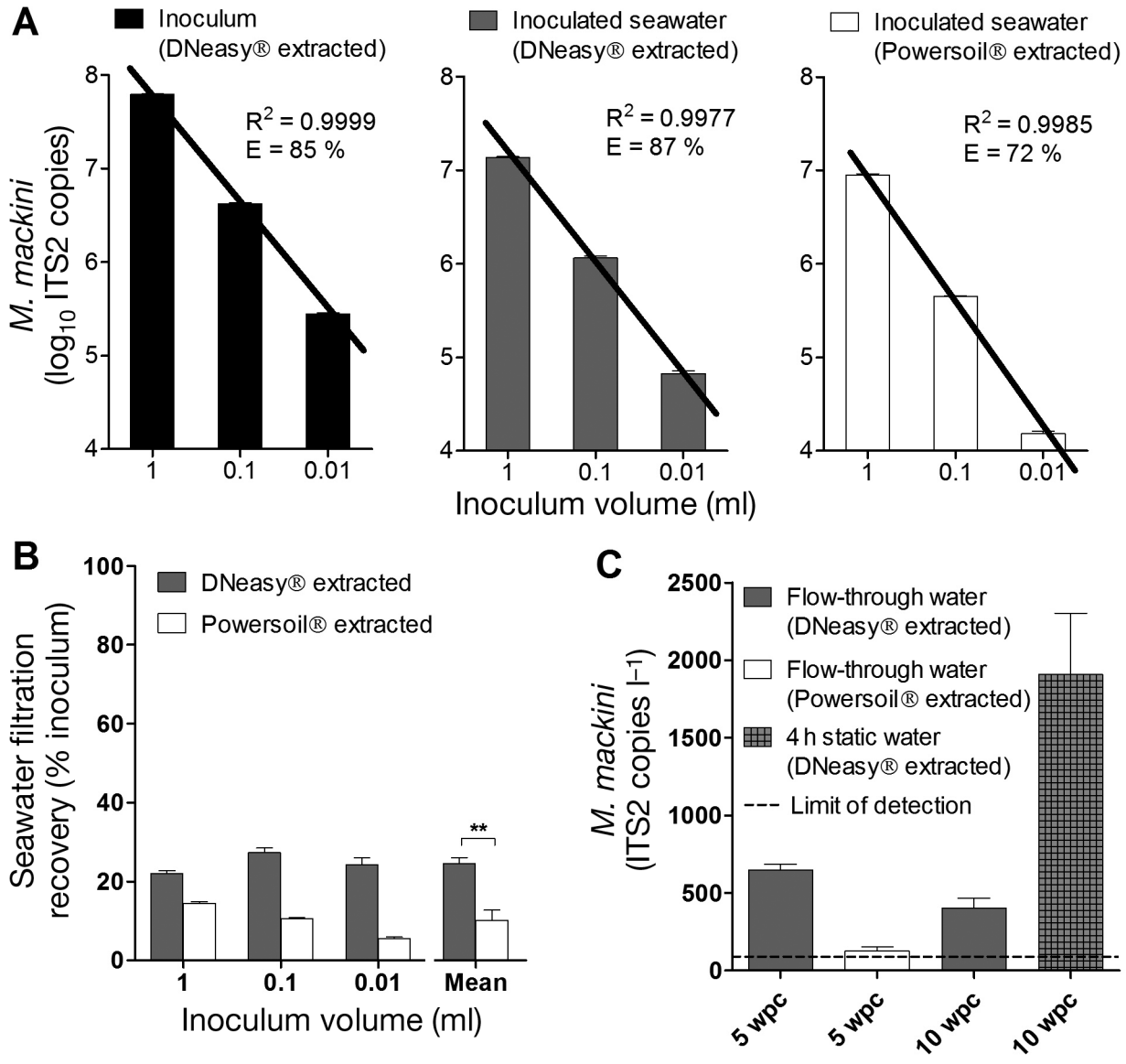

Fig. 1. Detection of *Mikrocytos mackini* DNA from seawater following 1 µm filtration. (A) qPCR detection of *M. mackini* DNA (based on yield of ITS2 copies), linearity (R^2), and efficiency (E) for a 3-step, 10-fold serial dilution of infectious material. In the black bar plot, DNA was extracted directly from seawater following centrifugation using a DNeasy® kit; in the grey bar plot, DNA was extracted using a DNeasy® kit following recovery on a 1 µm glass-fiber filter, and in the white bar plot, DNA was extracted using a PowerSoil® kit following recovery on a 1 µm glass-fiber filter. (B) Percentage of ITS2 copies recovered from 1 l of *M. mackini*-inoculated seawater using a 1 µm glass-fiber filter by either DNeasy® or PowerSoil® kits relative to DNA extracted directly from the inoculum used to spike the seawater. The mean (±SE) percent recovery between extraction methods across the 3-step, 10-fold dilution series is compared by Student's *t*-test on arcsine transformed data (**$p < 0.01$). (C) Quantity of *M. mackini* ITS2 copies recovered by 1 µm glass-fiber filtration of 1 l of seawater from a 50 l tank (3 l min^{-1} exchange rate) containing 40 Pacific oysters *Crassostrea gigas* at either 5 or 10 wk post *M. mackini* injection challenge (wpc). All samples were collected during normal flow-through operation except 1 sample at 10 wpc, which was collected following 4 h of static water conditions

ments was due to the physical barrier presented by the filter or DNA–silica binding which may have been encouraged by the high concentration of anions in seawater (Romanowski et al. 1991) is impossible to tell; however, because there was no strong chaotropic agent, we suspect that recovery observed here relied mainly on the physical barrier presented by the filter. Further, the fact that more than 98% of *M. mackini* DNA in the inoculum was cell-associated (see Fig. 2A) confirms that most of the *M. mackini* DNA recovered by filter retention originated from structurally intact organisms. As such, this technique represents the first

method for detecting *M. mackini* organisms outside the host and indicates that *M. mackini* are shed for prolonged periods during acute infection.

DNA isolation and qPCR detection of some oyster pathogens such as *Vibrio aestuarianus* have been conducted directly on small (i.e. 200 µl) seawater samples without filtration (Saulnier et al. 2009). However, this type of detection is only practical when the microorganisms and/or associated DNA are in relatively high concentrations (Longshaw et al. 2012). In situations where microorganisms are anticipated to be rare, filter retention presents one of the most straightforward methods for concentrating target organisms/DNA from large quantities of water and has been used effectively for detecting the presence of a variety of organisms in aquatic environments, such as the cells from large vertebrates (Turner et al. 2014), single-cell amoebae (Wright et al. 2015), and viruses (Jiang et al. 2001). However, seawater filtration can also concentrate planktonic, humic substances and proteinaceous material which may not only clog filter membranes but can also interfere or inhibit downstream PCR analyses (Jiang et al. 2001, Brooks et al. 2005, Sidstedt et al. 2015). As the load of non-target (possibly inhibitory) material was unknown in the seawater used in this study and can be highly variable in environments where Pacific oysters are cultured, we compared 2 different DNA isolation techniques: one modified from the DNeasy® kit designed to isolate DNA from blood and tissue samples and the other involving the Powersoil® kit designed for separating DNA from soil, stool, or other environmental samples. The DNeasy®-based method is effective for identifying *M. mackini* DNA in Pacific oyster tissues (Polinski et al. 2015) but has a limited capability for removing humic acid, and its suitability for extracting environmental DNA from glass-fiber filters was unknown. In contrast, the Powersoil® DNA isolation kit is specifically designed for environmental samples with high organic and inorganic material including humic acid, cell debris, and protein.

In this study, both DNA isolation methods were successful at recovering *M. mackini* DNA from filter membranes. However, DNeasy®-based methods had significantly higher yields than the Powersoil® kit for isolating DNA from the relatively sterile seawater used in this study (Fig. 1B). Higher recovery using DNeasy®-based methods became even more evident in conditions with low DNA abundance, as evidenced by the reduced efficiency observed in serial-diluted template using Powersoil® relative to DNeasy®-based methods (Fig. 1A). This indicates that the Powersoil® DNA isolation kit is less suitable than the DNeasy®-based methods employed in this study for recovering *M. mackini* DNA from clear oligotrophic water samples. Nevertheless, in turbid copiotrophic conditions, such as those sometimes observed in the shallow benthic environments where Pacific oysters are cultured, we believe the Powersoil® DNA isolation methodology would be more suited. Indeed, similar methods using a QIAamp DNA Stool Mini Kit have proved more effective for detecting the oyster parasite *Perkinsus marinus* from benthic environmental seawater samples than DNeasy® methods (Audemard et al. 2004), and we suspect this would also be true for benthic seawater screening of *M. mackini* in turbid environments.

Although consistent seawater recovery of *M. mackini* DNA was achieved in this study, the amount recovered during spiking experiments was consistently less than 28% of the quantity added prior to filtration (Fig. 1B). As the deformability and integrity of *M. mackini* cellular membranes are unknown, it is possible that some *M. mackini* may have passed directly through the filter or that a portion of retained *M. mackini* were ruptured and the DNA subsequently lost. However, imperfect DNA extraction efficiency from the glass-fiber filters also likely contributed considerably to the reduced recovery in these experiments. This was particularly evident for extractions conducted using Powersoil® DNA isolation methods, as DNA quantity was significantly less than those obtained using a modified DNeasy®-based extraction approach (Fig. 1B). Nevertheless, even the DNeasy®-based methods did not recover all *M. mackini* DNA from the retention filters, as not all liquid could be recovered from the filters during extraction. Further, the presence of the chaotropic sodium dodecyl sulfate (SDS) in the DNeasy® lysis buffer strongly enhances DNA-silica binding and likely prevented the elution of at least some of the DNA. Therefore, if improved sensitivity is required, consideration should be given to employing filters with a lower DNA-binding affinity (e.g. cellulose acetate), a smaller absolute pore size (e.g. 0.5 µm) or using a reduced suction pressure (e.g. <20 kPa) to possibly improve *M. mackini* DNA recovery from seawater.

Extracellular stability for *M. mackini* in seawater

Following 1 h of seawater exposure, the shortest period necessary to prepare and assess inoculums

prior to injection, *M. mackini* morphology appeared comparable to previous intra-host observations with only minor irregularity (data not shown). Visual quantitative estimation via microscopic examination of 5 fields of view at 1000× magnification identified roughly 2.5×10^6 cells per 100 µl injection dose following inoculum preparation, which corresponded to 2.6×10^7 ITS2 DNA copies identified by qPCR. It should be noted that visual assessments provide only a rough quantitative estimation; differentiation of *M. mackini* from host cell nuclear debris can be difficult in some instances, and this method has not been validated for consistency across multiple dilutions or sample replicates. Nevertheless, the estimated copy number of ITS2 copies identified here (approximately 10 copies cell^{-1}) is consistent with other single-celled eukaryotic parasites (Torres-Machorro et al. 2010) and gives a first tentative demonstration for using qPCR to estimate putative *M. mackini* number in certain circumstances.

Although qPCR detection does not guarantee viability or even whole-cell presence in the sample material, nearly all (>98%) of the DNA which was detected in the experimental inoculum was cell-

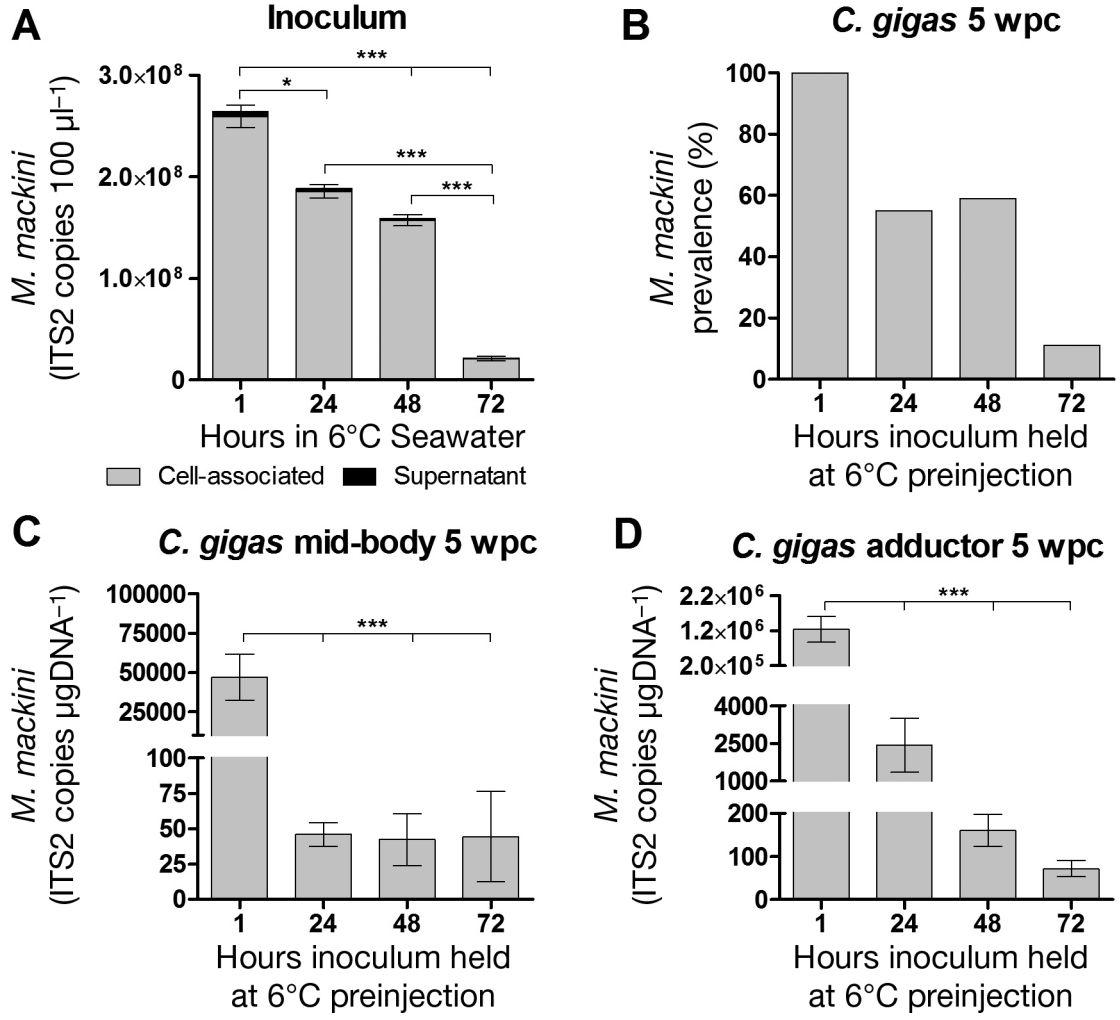

Fig. 2. Extracellular persistence and infectivity of *Mikrocytos mackini* in seawater. (A) ITS2 copies of *M. mackini* recovered from inoculation material (20 µm-filtered adductor muscle lesion homogenate in sterile seawater) following a variable 6°C incubation period. DNA recovered from pelleted material (grey) is distinguished from supernatant (black) following centrifugation at 2000 × *g* (10 min at 4°C). Mean (±SE) recovery in each instance is compared by 1-way ANOVA and post-hoc Tukey test following log transformation (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$). (B) Prevalence of *M. mackini* qPCR detection in Pacific oysters *Crassostrea gigas* at 5 wk post challenge (wpc) with seawater inoculum incubated for 4 discrete durations at 6°C prior to injection (n = 20 treatment^{-1}). The mean (±SE) quantity of *M. mackini* ITS2 copies recovered from infected oyster (C) mid-body cross section or (D) adductor muscle at 5 wpc is also provided in reference to the quantity of total genomic DNA extracted from these tissues in each instance

associated (Fig. 2A). Recovery was achieved following only low-speed (2000 × *g*) centrifugation, which is otherwise insufficient to recuperate eluted DNA from an environmental seawater medium. Further, a significant portion of this material was not only structurally intact but also highly infectious, as evidenced by the large amounts of *M. mackini* DNA subsequently detected in adductor muscle and mid-body cross section samples of challenged oysters 5 wk following injection (Fig. 2B–D). Thus, despite the rather substantial osmotic variation experienced by *M. mackini* in transitioning from host cells to seawater, short (1 h) environmental exposure does not appear to inhibit the infectivity of *M. mackini*.

In contrast, both the prevalence and infectivity of *M. mackini* significantly declined following 24 h or more of seawater incubation (Fig. 2). Morphology of *M. mackini* at 24 h was comparable to the observations at 1 h; however, a 30% reduction in *M. mackini* DNA was observed and only 55% of challenged oysters developed detectable infections. After 48 h, *M. mackini* cell and nuclear membranes had developed a necrotic polymorphic appearance, although prevalence and infectivity appeared comparable to 24 h observations. By 72 h of seawater incubation, *M. mackini* were barely recognizable by microscopy, and only 8% of the initial 1 h quantity of *M. mackini* DNA could be recovered from inoculum. Challenge of Pacific oysters using this material resulted in only a small portion (11%) of oysters developing detectable infections at 5 wpc.

Interestingly, although infection prevalence declined with respect to increasing periods of seawater incubation, only the shortest exposure of *M. mackini* to seawater (1 h) manifested into acute high-load infections or disease within 5 wk of injection (Fig. 2C,D). Seawater incubation of *M. mackini* for 24 h or more failed to produce anything but low-level systemic infections over the 5 wk period as indicated by mid-body cross section load which was less than 100 *M. mackini* ITS2 copies μg^{-1} host DNA in all cases (Fig. 2C). This suggests that although *M. mackini* has a short environmental stability, injection of even a relatively small quantity of viable organisms appears sufficient to develop persistent low-level infections, which agrees well with field observations where *M. mackini* is often subclinical (Polinski et al. 2015). However, these data also suggest that a relatively high quantitative threshold of *M. mackini* may be required to overpower host immune defenses and manifest acute disease.

M. mackini prevalence in tissues of Pacific oysters following waterborne exposure

Waterborne exposure of Pacific oysters to *M. mackini* confirmed the potential for the extracellular seawater transmission of this parasite previously demonstrated by Hervio et al. (1996). Infections were slow to develop and almost undetectable in the first week following exposure. *M. mackini* DNA was detected by qPCR in only 2 of 10 individuals at both 1 and 3 d post challenge and with loads very near the limit of detection (≤7 ITS2 copies μg^{-1} host DNA). By 2–3 wpc, prevalence had increased to 45%, although intensity remained low (data not shown). At 4 wpc, loads had increased to >100 ITS2 copies μg^{-1} host DNA in a few individuals, and both prevalence and intensity continued to increase through the 16 wk trial. By the end of the study, 85% of the challenged oysters had detectable *M. mackini* infections, which in some cases were very severe (>10 000 ITS2 copies μg^{-1} host DNA; Fig. 3).

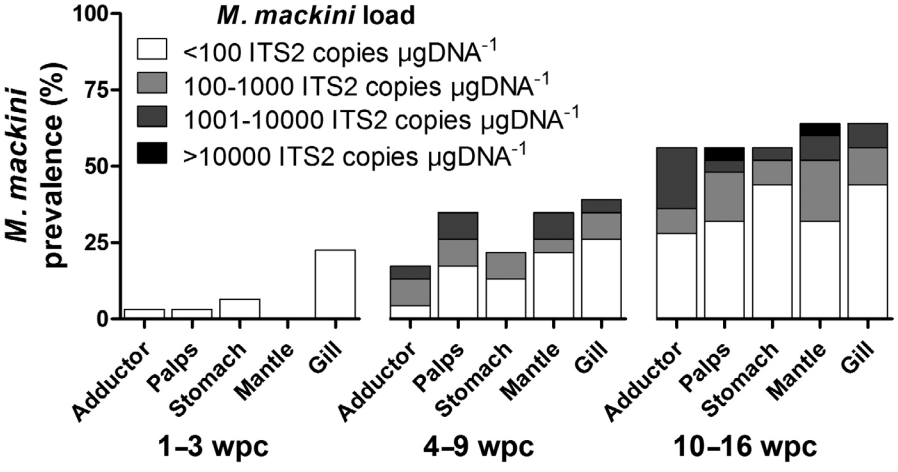

Fig. 3. Infectivity of *Mikrocytos mackini* to Pacific oysters *Crassostrea gigas* following waterborne exposure. Prevalence and comparative load of *M. mackini* copies detected by qPCR in adductor muscle, labial palp, stomach, mantle, or gill tissues during 1–3 (n = 31), 4–9 (n = 23), or 10–16 (n = 26) wk post challenge (wpc). Challenge was initiated by exposure of 80 oysters to $>10^7$ *M. mackini* in 10 l of sterile seawater for 24 h

The slow temporal increase of *M. mackini* in oyster tissues following waterborne exposure in this study is comparable to those previously observed by Hervio et al. (1996) and is thought to be similar to natural seasonal infections of beach-cultured oysters in BC (Quayle 1988, Hervio et al. 1996). In contrast, oysters injected with the same material used for waterborne exposure all developed severe infections within 5 wpc (data not shown), indicating that waterborne infection efficiency is relatively poor for this parasite yet reinforces that only a relatively small quantity of organisms need to breach host defenses to establish persistent infections.

Of the 5 sample types screened by qPCR, gill demonstrated the highest early and continued prevalence of parasite DNA (Fig. 3). For the more well-studied oyster parasite *P. marinus*, pallial organs such as mantle and gill are considered the primary sites for parasite entry (Perkins 1988, Dungan et al. 1996, Bushek et al. 1997, Allam et al. 2013). Given that the highest early prevalence of *M. mackini* was observed in gill tissues in this study, we suspect that the gill presents a primary entry site for *M. mackini* during waterborne transmission. Nevertheless, oysters are known to use gill ctenidia to preferentially sort particles prior to ingestion and are able to reject particles deemed biochemically unsuitable via mucus excreted as pseudofeces (Ward et al. 1998). This includes the majority of *P. marinus* following seawater exposure (Allam et al. 2013), and a similar type of mucosal elimination of *M. mackini* may help to explain the relatively poor infection efficiency observed in this study by seawater transmission even when oysters were exposed to a considerable waterborne infectious dose ($>10^6$ *M. mackini* l^{-1}).

Although *in situ* hybridization has previously identified occasional presence of *M. mackini* in gill tissues of infected oysters (Meyer et al. 2005), pathology appears restricted to the adductor muscle, labial palps, and mantle where the parasite is usually observed in the vesicular connective tissue cells (Abbott & Meyer 2014). The highest *M. mackini* DNA loads in this study were also observed in adductor muscle, labial palps, and mantle following waterborne exposure (Fig. 3), which supports the conclusion that *M. mackini* has a tropism for these organs. Nevertheless, the continued high prevalence of *M. mackini* in gill tissues throughout the 16 wk culture period of this trial is suggestive that gills may be involved in parasite shedding and/or re-entry. For other microcell parasites of oysters, namely *Bonamia* sp., the gill has been considered a primary site of shedding at certain times of the year (Hine 1991), and the presence of *M. mackini* in gill tissues late during infection may signify a similar circumstance for Pacific oysters infected with *M. mackini*. Alternatively, the slow but steady increase in prevalence of *M. mackini* in cultured oysters in concert with the continual occurrence of low-level infections during each progressive sampling event in this trial supports the supposition that the increasing prevalence may have resulted more from horizontal transmission than a delayed onset from primary exposure. Further work will be required to definitively address the role that gill infections play in the transmission of *M. mackini* in oyster populations; however, this study provides an important foundation for suggesting that direct horizontal transmission of *M. mackini* is possible and even likely to some degree in certain natural environments.

Detection of *M. mackini* during covert infections following elevated water temperature

As previously mentioned, a portion of *M. mackini* filtrate not used during waterborne exposure was injected into 41 oysters to confirm the infectivity of the filtrate and to assess the effects of elevated water temperature on *M. mackini* infections. During the fifth week of permissive (10°C) incubation, tissue imprints of the adductor muscle from a moribund oyster revealed high intensity of *M. mackini.* Adductor muscle and mid-body cross sections of a further 10 oysters sampled at 6 wpc confirmed severe infections with high levels of *M. mackini* DNA in all cases by qPCR (Fig. 4) as well as microscopic visualization of the parasite from histological sections (data not shown). Water temperature was then elevated to 18°C, and in the subsequent 2.5 wk parasite loads remained high and mortality/morbidity was common (Fig. 4). By the third week of increased water temperature, mortality had subsided, and after 6 wk (12 wpc), no evidence of disease or *M. mackini* could be identified by histopathology. Nevertheless, low-level persistence of *M. mackini* DNA was identified from all individuals (n = 7) at 12 wpc by qPCR, indicating the continued presence of low-level covert infections (Fig. 4).

The detection of *M. mackini* in oyster tissues for at least 6 wk of elevated water temperature in this study and the previous demonstration for at least 3 mo of prepatant persistence within oysters held at

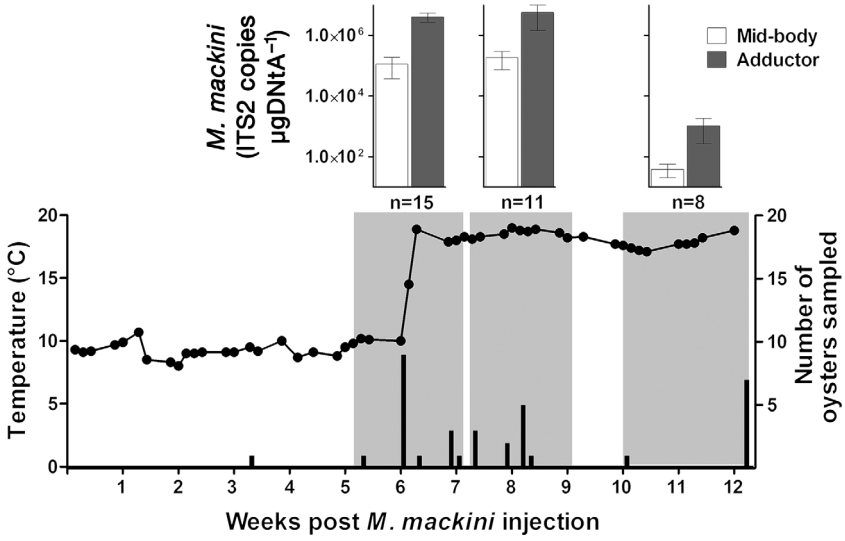

Fig. 4. Consequences of elevated water temperature on *Mikrocytos mackini* tissue loads in experimentally injected Pacific oysters *Crassostrea gigas*. A time course of recorded water temperature (line) and number of sampled oysters (bars) are presented in reference to weeks post challenge. Mean (±SE) quantities of *M. mackini* ITS2 copies detected by qPCR in a mid-body section (representing systemic load) relative to adductor muscle (site of injection) are also presented for each discrete shaded area encompassing 5–7, 8–9, and 10-12 wk post challenge. The total number of oysters sampled during each shaded interval is indicated

similar temperatures (Hervio et al. 1996) gives strong support that low levels of *M. mackini* can remain viable in oyster populations throughout the summer season in BC. Future qPCR screening of oysters during warm summer months in areas prone to experiencing Denman Island disease may therefore prove insightful, as the knowledge that *M. mackini* may be establishing continual rather than transient infections within cultured populations could improve management strategies concerning this parasite.

CONCLUSIONS

This study provides validation for the high sensitivity and accuracy afforded by qPCR to detect *M. mackini* in oyster tissues and has expanded the utility of this assay to include seawater detection of this elusive microcell parasite. We also confirm that seawater transmission of *M. mackini* between Pacific oysters is possible and that *M. mackini* DNA is naturally shed from living oysters during infection. During seawater transmission, gill tissues likely provide a primary site for parasite entry; however, the low infection efficiency and short environmental stability of *M. mackini* observed in this study would geographically restrict the effectiveness for this mode of transmission in natural environs if and when it takes place. Nevertheless, these and previous exposure studies have also demonstrated that *M. mackini* can persist for long periods once inside the host, and it is not unfathomable that modest amounts of seawater transmission in conjunction with long-term persistence could be the cause of the sporadic localized areas where Denman Island disease is currently observed. This possibility will be important to consider when assessing biosecurity and diagnostic screening measures with relation to this parasite.

Acknowledgements. We thank Eliah Kim for technical assistance with oyster necropsy and histological processing. Funding for this work was provided by a Centre for Aquatic Animal Health Research and Diagnostics grant (within Fisheries and Oceans Canada) to C.L.A. and M.P.P.

LITERATURE CITED

Abbott CL, Meyer GR (2014) Review of *Mikrocytos* microcell parasites at the dawn of a new age of scientific discovery. Dis Aquat Org 110:25–32

Abbott CL, Meyer GR, Lowe G, Kim E, Johnson SC (2014) Molecular taxonomy of *Mikrocytos boweri* sp. nov. from Olympia oysters *Ostrea lurida* in British Columbia, Canada. Dis Aquat Org 110:65–70

Allam B, Carden WE, Ward JE, Ralph G, Winnicki S, Espinosa EP (2013) Early host–pathogen interactions in marine bivalves: evidence that the alveolate parasite *Perkinsus marinus* infects through the oyster mantle during rejection of pseudofeces. J Invertebr Pathol 113:26–34

Audemard C, Reece KS, Burreson EM (2004) Real-time PCR for detection and quantification of the protistan parasite *Perkinsus marinus* in environmental waters. Appl Environ Microbiol 70:6611–6618

Bower SM (1988) Circumvention of mortalities caused by Denman Island oyster disease during mariculture of Pacific oysters. Spec Publ Am Fish Soc 18:246–248

Bridle A, Crosbie P, Cadoret K, Nowak B (2010) Rapid detection and quantification of *Neoparamoeba perurans* in the marine environment. Aquaculture 309:56–61

Brooks HA, Gersberg RM, Dhar AK (2005) Detection and quantification of hepatitis A virus in seawater via real-time RT-PCR. J Virol Methods 127:109–118

Burki F, Corradi N, Sierra R, Pawlowski J, Meyer GR, Abbott CL, Keeling PJ (2013) Phylogenomics of the intracellular parasite *Mikrocytos mackini* reveals evidence for a mitosome in rhizaria. Curr Biol 23:1541–1547

Bushek D, Allen S, Alcox KA, Gustafson RG, Ford SE (1997) Response of *Crassostrea virginica* to *in vitro* cultured *Perkinsus marinus*: preliminary comparisons of three inoculation methods. J Shellfish Res 16:479–486

Dungan C, Hamilton R, Burreson E, Ragone-Calvo L (1996) Identification of *Perkinsus marinus* portals of entry by histochemical immunoassays of challenged oysters. J Shellfish Res 15:500

Farley CA, Wolf PH, Elstons RA (1988) A long-term study of 'microcell' disease in oysters with a description of a new genus, *Mikrocytos* (g. n.), and two new species, *Mikrocytos mackini* (sp. n.) and *Mikrocytos roughleyi* (sp. n.). Fish Bull 86:581–593

Hartikainen H, Stentiford GD, Bateman KS, Berney C and others (2014) Mikrocytids are a broadly distributed and divergent radiation of parasites in aquatic invertebrates. Curr Biol 24:807–812

Hervio D, Bower SM, Meyer GR (1996) Detection, isolation, and experimental transmission of *Mikrocytos mackini*, a microcell parasite of Pacific oysters *Crassostrea gigas* (Thunberg). J Invertebr Pathol 67:72–79

Hine P (1991) The annual pattern of infection by *Bonamia* sp. in New Zealand flat oysters, *Tiostrea chilensis.* Aquaculture 93:241–251

Jiang S, Noble R, Chu W (2001) Human adenoviruses and coliphages in urban runoff-impacted coastal waters of Southern California. Appl Environ Microbiol 67:179–184

Joly JP, Bower SM, Meyer GR (2001) A simple technique to concentrate the protozoan *Mikrocytos mackini,* causative agent of Denman Island disease in oysters. J Parasitol 87:432–434

Longshaw M, Feist SW, Oidtmann B, Stone DM (2012) Applicability of sampling environmental DNA for aquatic diseases. Bull Eur Assoc Fish Pathol 32:69–76

Meyer GR, Bower SM, Carnegie RB (2005) Sensitivity of a digoxigenin-labelled DNA probe in detecting *Mikrocytos mackini*, causative agent of Denman Island disease (mikrocytosis), in oysters. J Invertebr Pathol 88:89–94

Perkins FO (1988) Parasite morphology, strategy and evolution. Spec Publ Am Fish Soc 18:93–111

Polinski M, Lowe G, Meyer G, Corbeil S, Colling A, Caraguel C, Abbott CL (2015) Molecular detection of *Mikrocytos mackini* in Pacific oysters using quantitative PCR. Mol Biochem Parasitol 200:19–24

Quayle D (1961) Denman Island oyster disease and mortality, 1960. Manuscript Report Series (Biological) No. 713. Fisheries Research Board of Canada, Ottawa

Quayle D (1988) Pacific oyster culture in British Columbia. Can Bull Fish Aquat Sci 218:1–241

Romanowski G, Lorenz MG, Wackernagel W (1991) Adsorption of plasmid DNA to mineral surfaces and protection against DNase I. Appl Environ Microbiol 57:1057–1061

Saulnier D, De Decker S, Haffner P (2009) Real-time PCR assay for rapid detection and quantification of *Vibrio aestuarianus* in oyster and seawater: a useful tool for epidemiologic studies. J Microbiol Methods 77:191–197

Sidstedt M, Jansson L, Nilsson E, Noppa L, Forsman M, Radstrom P, Hedman J (2015) Humic stubstances cause fluorescence inhibition in real-time polymerase chain reaction. Anal Biochem 487:30–37

Torres-Machorro AL, Hernández R, Cevallos AM, López-Villaseñor I (2010) Ribosomal RNA genes in eukaryotic microorganisms: witnesses of phylogeny? FEMS Microbiol Rev 34:59–86

Turner CR, Barnes MA, Xu CC, Jones SE, Jerde CL, Lodge DM (2014) Particle size distribution and optimal capture of aqueous macrobial eDNA. Methods Ecol Evol 5: 676–684

Ward J, Levinton J, Shumway S, Cucci T (1998) Particle sorting in bivalves: in vivo determination of the pallial organs of selection. Mar Biol 131:283–292

Wright DW, Nowak B, Oppedal F, Bridle A, Dempster TD (2015) Depth distribution of the amoebic gill disease agent, *Neoparamoeba perurans*, in salmon sea-cages. Aquacult Environ Interact 7:67–74

Editorial responsibility: Stephen Feist, Weymouth, UK

Submitted: March 14, 2017; Accepted: August 11, 2017
Proofs received from author(s): September 19, 2017