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Mortality of *Cryptocaryon irritans* in sludge from a digester of a marine recirculating aquaculture system

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ABSTRACT

Cryptocaryon irritans, a marine protozoan fish parasite with a life cycle that includes cyst formation, constitutes a major concern in intensive recirculating aquaculture systems (RASs), due to its potential propagation in a system with high fish density and minimal water change. In this study, the survival of *C. irritans* in sludge collected from an up-flow anaerobic sludge blanket (UASB) reactor of a RAS system was studied using a PCR assay. The detection sensitivity of the different life stages in sludge was determined to be 50 tomonts, 5 theronts and 5 trophonts. Dead *C. irritans* DNA was not detectable after 24 h of inoculation. The survival of the different *C. irritans* life stages in anaerobic conditions, as present in the UASB reactor, was evaluated. Theronts died within 3 h post-exposure to anaerobic conditions. Protomonts encysted within a day post-exposure, but unlike in aerobic conditions, the resulting tomonts did not hatch. Excystment of theronts from tomonts was delayed in anaerobic conditions, and occurred only after transfer to an aerobic environment. However, the tomonts did not die after 96 h of such exposure and the release rate of theronts did not decrease, compared to the control. The results suggest that infective theronts cannot originate from the UASB reactor and protomonts or theronts that reach the UASB do not survive. However, tomonts appear to be able to survive the reactor's conditions, and if they are released from it with the water outflow, they could hatch in aerobic parts of the RAS.

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1. Introduction

In order to ease pressures on wild fishery stocks and meet the growing global demand for seafood, farming of marine species is becoming increasingly prevalent. Fish production in intensive land-based recirculating aquaculture systems (RAS) are being developed as eco-friendly alternatives to the potentially polluting marine cage farming (Naylor et al. 2000; Naylor 1998). To reduce water exchange and to better recycle solids and carbon, near-zero-discharge RASs typically include, in addition to solid filtration and nitrification reactors (standard for "traditional RASs"), a denitrification stage and sludge treatment system (Van Rijn et al. 2006; Van Rijn 2013).

One major concern in the development of near-zero-discharge RASs is the potential amplification of disease-causing agents and their persistence in the different treatment units. The high fish stocking density, combined with a concomitant high organic load in the treatment units, increases the potential threat of a high pathogen load and disease outbreaks. Recently, an upflow anaerobic sludge blanket (UASB) reactor was suggested as an efficient treatment for saline sludge (Tal et al. 2009; Mirzoyan et al. 2008; Mirzoyan et al. 2010). During the digestion process, sludge is biodegraded, ammonia and other elements are released into the supernatant, and biogas containing 50–65% methane, 50–35% CO_2 and other trace gases, such as H_2S and ammonia, is produced and can be collected and used as an energy source.

Cryptocaryon irritans is a marine ciliated protozoan parasite causing "marine white spot disease". It has a wide host range and can infect almost all marine teleosts (Colorni 1985). Its life cycle is temperature dependent and comprises four morphologically distinct developmental stages (Colorni 1987). The trophont or parasitic stage resides within the epidermis of the fish and feeds on the epithelial layer of the skin, fins and gills. It leaves the fish host upon maturity as a free-swimming prototomont, and settling on a suitable substrate, it then transforms into the tomont or cyst stage by secreting a double-layered cyst wall. Inside the tomont, numerous small theronts are produced and released in the free-swimming infective stage; they contact the fish skin and penetrate into the epidermis, where they settle as trophonts and complete the life cycle. Elevated numbers of trophonts will cause mass mortalities due to disruptions in osmotic balance, asphyxiation and secondary bacterial infection (Diamant et al. 1991; Dickerson 2006).

With the growth of the mariculture industry, *C. irritans* has become one of the most common and persistent disease-causing agents, leading to significant economic losses (Dickerson 2006). In closed recirculating systems, *C. irritans* has the potential to proliferate, even more so in a

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near-zero-discharge RAS, in which sludge, which contains many of the parasite's tomonts (encysted stage), is not removed from the system.

The aims of this study were to develop a DNA-based detection method for *C. irritans* in sludge and analyze the survival of *C. irritans* in sludge originating from an active UASB. The potential spread of *C. irritans* infection in a RAS through outflow from the UASB is discussed.

2. Materials and methods

2.1. Isolation and culture of Cryptocaryon irritans

C. irritans that had been isolated from cultured sea bass (*Dicentrachus labrax*), from the Israel Oceanographic and Limnological Research (IOLR), Eilat, Israel, in 2011 was kindly provided to us by Dr. Angelo Colorni (ILOR). The organism was initially identified by its characteristic morphological features (Colorni 1985; Dickerson 2006) and subsequently by PCR analysis, as described below. Infection was maintained in our laboratory by serial passage through seawater-adapted guppies (*Poecilia reticulata*), held in 10-l glass aquaria equipped with biological filters and aeration. Temperature was maintained at 26 ± 2 °C. Seawater was prepared at 25 ppt using de-chlorinated tap water through the addition of 50 mg/l of sodium thiosulphate pentahydrate, to which sea salt (Red Sea Fish Pharm Ltd., Eilat, Israel) was added. The mass production of *C. irritans* life stages was achieved by infecting juvenile barramundi (*Lates calcarifer*) of about 20 g in 12-l glass aquaria supplied with moderate aeration. Salinity and temperature were maintained as above. Fish were treated in compliance with the Ben-Gurion University's Committee for the Ethical Care and Use of Animals, authorization number IL-78-10-2012.

Once mature, trophonts exited the heavily infected fish (protomont stage), and allowed to settle on removable plates that lined the bottom of the aquaria. The plates were carefully removed and the protomonts were collected, washed in seawater, placed in 6-well plates (Corning Inc., USA) containing 5 ml artificial seawater (25 ppt) and allowed to encyst. The tomonts were incubated at 30 °C, and the water was changed daily until theront excystment commenced. After excystment, the theronts were collected and counted. When small numbers of theronts were required, they were allowed to chill on ice and then collected individually using a pipette under a dissecting microscope (Zeiss Stemi 2000-C).

2.2. DNA extraction

Genomic DNA was extracted from theronts and trophonts in sludge using a commercial kit (QIAamp DNA Stool Mini Kit, Qiagen). DNA extraction from tomonts was carried out in a similar manner but with a prior freeze-thawing protocol (2 min in liquid nitrogen and 2 min at 100 °C, repeated five times). DNA concentration and purity were measured spectrophotometrically at 260/280 nm (NanoDrop 1000, ThermoScientific, Wilmington, USA).

2.3. PCR analysis of C. irritans

2.3.1. Plasmid construction

A plasmid was constructed to serve as a positive control in the PCR assay. DNA was extracted from 300 to 400 theronts, using the phenol-chloroform method as detailed by Aruety et al. (2016). PCR amplification was performed in a 50-μl reaction volume containing a DNA template (80 ng) using CI-120f and CI-120r primers (Taniguchi et al. 2011). The PCR mix contained 20 nM of each primer, 0.5 mM of each deoxy nucleotide triphosphate (Origolab, Rehovot, Israel), 2 mM of $MgCl_2$, and 4 U of Bio-X-Act short DNA polymerase (Bioline, Taundton, MA) in a 1 × reaction buffer. The PCR thermal cycling program was as follows: denaturation at 95 °C for 5 min, followed by 40 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 20 s. The reaction was completed with an additional step of 5 min at 72 °C. A sample of the PCR product was separated on a 1% (w/v) agarose gel containing ethidium bromide and visualized under UV to ensure the presence of a single specific band sized 150 bp; then 1 μl of the PCR reaction was used for direct cloning using a Clonejet PCR cloning kit (Thermo Scientific, Waltham, MA) according to the manufacturer's instructions. Plasmids were transformed into competent *E. coli* DH5α bacteria and plated on LB plates containing ampicillin (100 μg/ml) for selecting colonies that contained the insert. Plasmids were extracted from bacteria using the *AccuPrep* Plasmid Mini Extraction Kit, according to the manufacturer's instructions (Bioneer, Daejeon, South Korea), and were sequenced by Hylabs (Rechovot, Israel) using an ABI 3730xl DNA Analyzer.

2.3.2. Determining the detection sensitivity of C. irritans in sludge

The detection sensitivity of the different life stages of *C. irritans*, i.e., theronts, trophonts and tomonts, in sludge from the UASB reactor of an RAS system was determined using a PCR assay. The UASB sludge contained 0.7% solids, of which 40% were volatile; dissolved O_2 and H_2S were 0 and 10 mg/l, respectively; pH was 6.9 and the redox potential was −286 mV (Aruety et al. 2016).

Sludge was prepared as follows: 50 ml of sludge was centrifuged at 3000 × *g* for 20 min, the supernatant was removed and replaced with 40 ml of 25 ppt artificial seawater, vortexed and centrifuged again as before, and the supernatant was removed. Known numbers of theronts (0, 1, 5, 10, 50), trophonts (0, 1, 5, 10) and tomonts (1, 5, 10, 50) were collected as described above and spiked into 220 mg of concentrated sludge in a 1.5-ml Eppendorf tube. DNA extraction was carried out as described above, and extracted DNA was amplified using primers specific for *C. irritans* CI-120f and CI-120r (Taniguchi et al. 2011), under the same amplification conditions as detailed above.

2.3.3. Detection of DNA from dead C. irritans

Since the aim of the PCR analysis was to evaluate the presence of infective parasites in the sludge, it was essential to confirm that the DNA detected was from live parasites, and therefore, the detection of DNA from dead parasites was evaluated. Theronts were killed and fixed by being placed in 70% ethanol. Concentrated sludge (220 mg in 1 ml of seawater (25 ppt)) was added to 1.5-ml Eppendorf tubes, spiked with 10 and 50 ethanol-fixed theronts and incubated at room temperature (RT). Samples were collected at 24 and 48 h, DNA was extracted, and the PCR was carried out as described above.

2.4. Determining the effect of anaerobic conditions on C. irritans life stages

The effect of an anaerobic environment, simulating the conditions in the USAB reactor, on the survival of *C. irritans* was analyzed. For this purpose, serum bottles were filled with the sample to be tested in seawater or sludge (at a concentration of 1 g concentrated sludge, by centrifugation, per 50 ml of seawater). Sludge was collected from a UASB that treated saline aquaculture sludge in a near-zero-discharge RAS. To ensure anaerobic conditions, bottles were sparged with nitrogen for ca. 30 min and then sealed. Controls were not sparged and were left with open caps. All experiments were carried out in triplicates.

Cryptocaryon irritans theronts, protomonts (collected as trophonts from infected fish) and tomonts were added to SW and sludge at a concentration of 1000, 50 and 50 ciliates per 10 ml, respectively, in 50-ml serum bottles. Theronts and protomonts in serum bottles were directly observed under an inverted light microscope (Axiovert 40 CFL, Zeiss, Germany). Theronts were observed for motility over 24 h, every 30 min for the first 6 h and every 30 min for 4 h in the next day. Protomonts were similarly directly observed 2–3 times a day for 8 days for attachment to the serum bottle surface, cyst formation, the occurrence of theronts in the cyst and then their excystment. Tomonts were exposed to anaerobic conditions for different time periods by opening the serum bottle cap at the end of the designated anaerobic exposure. Tomonts were observed daily for hatchability (release of theronts) as described above and the % of hatchability determined.

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