Antiprotozoal effects of metal nanoparticles against *Ichthyophthirius multifiliis*

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SUMMARY

Ichthyophthirius multifiliis is a widespread, ciliated protozoan ectoparasite of fish. In the present study, we investigated the effects of metal nanoparticles on the reproduction and infectivity of free-living stages of *I. multifiliis*. We determined that ~50% of theronts could be killed within 30 min of exposure to either 20 ng mL^{-1} gold, 10 ng mL^{-1} silver or 5 ng mL^{-1} zinc oxide nanoparticles. Silver and zinc oxide nanoparticles at concentration of 10 and 5 ng mL^{-1} killed 100 and 97% of theronts, respectively and inhibited reproduction of tomonts after 2 h exposure. Gold nanoparticles at 20 ng mL killed 80 and 78% of tomonts and theronts 2 h post exposure, respectively. *In vivo* exposure studies using rainbow trout (*Oncoryhnchus mykiss*) demonstrated that theronts, which survived zinc oxide nanoparticles exposure, showed reduced infectivity compared with control theronts. No mortalities were recorded in the fish groups cohabited with theronts exposed to either nanoparticles compared with 100% mortality in the control group. On the basis of the results obtained from this study, metal nanoparticles particularly silver nanoparticles hold the best promise for the development of effective antiprotozoal agents useful in the management of ichthyophthiriosis in aquaculture.

Key words: *Ichthyophthirius multifiliis*, gold, silver, zinc oxide, nanoparticles, antiprotozoal activity.

INTRODUCTION

Ichthyophthirius multifiliis is a ciliated protozoan parasite with a worldwide distribution. It causes 'white spot disease' of freshwater fish, and is responsible for severe epizootics in aquaria, hatcheries, and ponds. The parasite has low host and tissue specificity, infecting body surfaces including gills, skin, eyes, and fins. Although the ciliate was well recognized in the Middle Ages, and possibly had its origin in cyprinid fishes imported from Asia, it was formally first described in carp and other freshwater fishes in central Europe (Hoffman, 1967). The parasite has worldwide distribution, which is primary correlated to human mediated introductions of alien host species (non-naive) into new environments (Hoffman, 1970), and has been reported in feral fishes in tropical and temperate regions (Nigrelli *et al*. 1976). The broad spatial distribution of the parasite is due in part, to the ability of the encysted tomonts to survive temperatures ranging from 2 to 27 °C. Factors leading to epizootic include thermal triggers, the temperature tolerance of the host fish, and degree of resistance of the host (Nigrelli *et al*. 1976). Despite the widespread and costly impacts of the parasite, few successful management strategies have been developed to control *I. multifiliis* infections (Shinn *et al*. 2012). After theronts penetrate into fish skin and gills, control of the disease becomes difficult. Inhibiting the parasite while it is still a tomont, before it replicates, is crucial to stopping its spread (Tucker & Robinson, 1990; Schäperclaus, 1991; Fu *et al*. 2014). Chemicals and drugs currently used to control *I. multifiliis* have potential environmental and host toxicities, particularly the most efficacious, malachite green (Rintamäki-Kinnunen & Valtonen, 1997). This compound is now prohibited for use in food fish due to its carcinogenic and teratogenic properties, which leaves few other chemicals that are effective (Shinn *et al*. 2012). Thus, there is a crucial need to investigate novel therapeutic agents to control *I. multifiliis*.

Nanoparticles with a diameter ⩽100 nm are now being increasingly applied for medical purposes and have attracted immense attention as an alternative approach to control infectious agents (Swain *et al*. 2014). Nanoparticles are characterized by their large surface area and high particle number per unit mass compared with bulk materials (Buzea *et al*. 2007). They have unique physical, chemical, and biological characteristics, which are currently the subject of much scientific research. In aquaculture, metal and metal oxide nanoparticles exhibit effective antimicrobial properties against fish pathogens (Swain *et al*. 2014), and have been utilized in water decontamination and as antimicrobial agents

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(Li *et al*. 2008; Rana & Kalaichelvan, 2011; Saleh *et al*. 2016; Shaalan *et al*. 2016). Due to their non-toxicity, high ability for functionalization and polyvalent effects, gold nanoparticles are promising in the development of novel antimicrobial agents (Tiwari *et al*. 2011; Zhou *et al*. 2012; Lima *et al*. 2013; Lolina & Narayanan, 2013; Saleh *et al*. 2016). The antimicrobial activity of the nanoparticles is attributed to their attachment to microbial cell membranes, followed by alteration of membrane potential, decrease of ATP level and inhibition of tRNA binding to the ribosome (Cui *et al*. 2012). However, particles can aggregate when weakly bound capping agents like citrate are used, which leads to reduced surface area and decreased interactions with the nanoparticles (Zhou *et al*. 2012). In contrast, gold nanoparticles with the same shape and size but capped with strongly bound agent may exhibit enhanced antimicrobial properties (Zhou *et al*. 2012; Dizaj *et al*. 2014). The antimicrobial activity of zinc oxide nanoparticles is due to contact between nanoparticles and pathogen cells, which alters surface charges and electrostatic interactions (Stoimenov *et al*. 2002; Neal, 2008; Zhang *et al*. 2010), leading to rupture of the cell membrane (Zhang *et al*. 2007; Jiang *et al*. 2009). In aquaculture, zinc oxide nanoparticles have been reported to affect the growth of *Aermonas hydrophila*, *Edwardseilla tarda*, *Flavobacterium branchiophilum*, *Citrobacter* species, *Staphylococcus aureus*, *Vibrio* species, *Bacillus cereus* and *Pseudomonas aeruginosa* (Ramamoorthy *et al*. 2013; Swain *et al*. 2014).

Silver nanoparticles have been reported to affect microorganisms by different mechanisms (Franci *et al*. 2015). Silver nanoparticles bind to cell membrane proteins and disrupt cell membrane and induce production of reactive oxygen species leading to cell death (Lara *et al*. 2010). Intracellulary, they bind to cytochrome and interfere with nucleic acids of the pathogens, thereby inhibiting cell division and subsequent replication (Lara *et al*. 2010; Shaalan *et al*. 2016). Silver nanoparticles have been shown to affect morphology and pathogenicity of the protozoan parasite *Leishmania tropica in vitro* (Allahverdiyev *et al*. 2011).

Given the versatile characteristics and low toxicity of nanoparticles, we consider they represent a potentially useful approach to manage pathogens in aquaculture. In this study, we investigated the effects of gold, silver and zinc oxide nanoparticles on survival and reproduction of *I. multifiliis* free-living stages, and assessed the infectivity of surviving theronts to rainbow trout *in vivo*.

MATERIALS AND METHODS

Fish and parasite

The life cycle of *I. multifiliis* in the laboratory was initiated by cohabitation of juvenile rainbow trout *(Oncorhynchus mykiss)* (~5 g) obtained from a registered disease free commercial fish farm in Vienna, Austria, with naturally infected common carp (*Cyprinus carpio*) acquired from carp pond. The infected carp were kept with 10 rainbow trout in a 250 L tank for 7 day to allow infection of the rainbow trout by *I. multifiliis*. The temperature of the water was controlled at 16 ± 2 °C, and the fish were fed daily at 1% of fish weight. When rainbow trout were heavily infected with mature trophonts, they were anesthetized (150 mg L^{-1} tricaine methanesulfonate (MS-222, Sigma). Subsequently, skin was gently scraped into Petri dishes that contained 10 mL of water at 16 °C, and trophonts were allowed to escape the fish mucus. The free-swimming trophonts (protomonts) were collected and rinsed several times with dechlorinated water to eliminate residual fish mucus. All experiments were approved by the Animal Experimentation Ethics Committee of Vienna University of Veterinary medicine (BMWFW-68·205/0051-WF/V/3b/2016).

Collection of the parasite stages

Protomonts were collected and placed in batches of 50 in Petri dishes containing 10 mL filtered freshwater and either used directly or incubated at 15 ± 1 °C, until they reached either the encysted tomont stage (minimum eight cells) or the theront stage (after ~23–30 h). To obtain tomonts, the Petri dishes with trophonts were incubated for only 16 h at 15 °C so that the development of theronts was not complete. To obtain theronts, the protomonts were put into Petri dishes (approximately 50 trophonts per Petri dish) with 10 mL of dechlorinated water and incubated at 15 °C for 24 h, after which time theronts were released. To determine the number of theronts produced, 10 × 20 μL subsamples were put on slides fixed with 5 μL Roti-Histofix® (Carl Roth) and counted under a microscope; the mean count was used to assess the total number of theronts produced. A dual fluorescent staining technique using propidium iodide and fluorescein diacetate was used to differentiate between viable and damaged parasites by fluorescent microscopy (Schumacher *et al*. 2011).

Zinc oxide nanoparticles

The zinc oxide nanoparticles (~66 nm) were purchased from Sigma Aldrich, Austria, together with all reagents used for in-house synthesis of gold and silver nanoparticles.

Gold nanoparticles

We synthesized gold nanoparticles by reduction of tetrachloroauric acid ($HAuCl_4$) with sodium

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citrate (Saleh *et al*. 2016). Briefly, an aqueous solution of $HAuCl_4 \cdot 3H_2O$ was brought to boil under reflux with stirring. After rapid addition of 10 mL of 1% trisodium citrate, the colour of the solution changed from yellow to deep red. After an additional 15 min reflux, the solution was allowed to cool to room temperature, before being filtered through a 0·45 μm acetate filter, and then stored at 4 °C.

Silver nanoparticles

We synthesized silver nanoparticles by chemical reduction of silver nitrate according to the protocol of El Mahdy *et al*. (2015). Both sodium citrate tribasic hydrate and sodium borohydride were utilized as reducing agents, and polyvinyl pyrrolidone as a stabilizing agent to prevent particle agglomeration (Wang *et al*. 2005). The solution was stored in an autoclaved, dark bottle at 4 °C.

Characterization of nanoparticles

Formation of gold and silver nanoparticles was confirmed by Ultraviolet-visible spectral analysis. The absorbance spectra were recorded using (NanoDrop 2000®). Deioinized water was used as a blank. All measurements were performed at room temperature on three different days. The morphology of the synthesized gold and silver nanoparticles was investigated using TEM (EM 900, Zeiss, Oberkochen, Germany) and Image SP Viewer® software was used to calculate their mean size from 100 randomly sampled nanoparticles. We used a Zetasizer Nano ZS® (Malvern.com), to measure size distribution of the nanoparticles based on dynamic light scattering (DLS). Triplicate measurements were performed at room temperature.

In vitro exposure of protomonts to gold, silver and zinc oxide nanoparticles

Approximately 50 protomonts in 500 μL dechlorinated freshwater were placed into each well of a 24-well tissue culture plate. 500 μL of each nanoparticle solution (gold, silver and zinc) was added in triplicates, to attain final concentrations of 0 (control), 2·5, 5·0, 10·0, 20·0, 40·0, 80·0 and 160 ng mL^{-1}. The efficacy of each dose was assessed by counting the number of protomonts at 15 and 30 min, 1, 2, 4, 6, 12, 18 and 24 h until the protomonts had either died or theronts were released. Protomonts were classified as active (survival) or motionless (dead) using a microscope at 40 × or by using the dual fluorescent staining technique as described above. The numbers of encysted tomonts and released theronts were determined at 6 and 24 h post-exposure to nanoparticles, respectively.

In vitro exposure of encysted tomonts to gold, silver and zinc oxide nanoparticles

Approximately 30 encysted tomonts in 500 μL dechlorinated freshwater were placed into each well of a 24-well tissue culture plate. Only 30 encysted tomonts were used because not all of the protomonts encysted successfully. 500 μL of each nanoparticle solution was added in triplicates, to final concentrations of 160, 80, 40, 20, 10, 5, 2·5 and 0 ng mL^{-1}. Encysted tomonts were categorized as active (survival) or motionless (dead) as above. The numbers of released theronts were determined at 24 h post-exposure to nanoparticles.

In vitro exposure of I. multifiliis theronts to gold, silver and zinc oxide nanoparticles

Wells of 24-well plates in triplicates were loaded with 500 μL theront suspension containing ~150 theronts enumerated as described above. Then, 500 μL of each nanoparticle solution was added to wells to reach final concentrations of 160, 80, 40, 20, 10, 5, 2·5 and 0 ng mL^{-1}. The number of theronts surviving in each well was determined at 15 and 30 min, 1, 2, 4, 6, 12, 18 and 24 h post exposure. Triplicates were set up for each concentration and each time point, each triplicate originating from the same group of parasites to reduce possible different survival rates among different cohorts.

The per cent inhibition of *I. multifiliis* free-living stages was calculated as per cent inhibition = 100−[(mean number of viable parasites counted in exposed wells/mean number of parasites counted in non-exposed wells) × 100]. The differences between nanoparticles exposed and non-exposed parasites were analysed using *t* tests with Bonferroni α-correction. For all statistical tests, a P value <0·05 was regarded as significant. Statistical analyses were conducted with SPSS V.20 software.

The ability of theronts surviving exposure to subsequently infect fish

To determine whether theronts treated with nanoparticles had the ability to infect fish, nine Petri dishes (three triplicates) were prepared, each containing 25 mL of theronts suspension (~150 theronts mL^{-1}) drawn from one pool of theronts. Subsequently, 25 mL of silver, zinc oxide nanoparticles or filtered freshwater (control) were added to each dish to a final volume of 50 mL and a final concentration of 10 (silver), 5 (zinc) or 0 (control) ng mL^{-1}nanoparticles. Dishes were incubated at 15 °C for 18 h.

The number of live *vs* dead theronts in three separate 1 mL aliquots taken from each Petri dish was determined. The remaining 47 mL was then added

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to separate tanks of rainbow trout to determine if any surviving theronts could infect fish.

For the infection trial, triplicate 10 L tanks were maintained in a constant temperature at 15 °C. Each tank contained 10 ~5 g *O. mykiss* fingerlings. The fish in each tank were exposed to the relevant batches of theronts for 3 h under static conditions, in the dark with aeration.

Fish were maintained for 10 days at 15 °C on a 2% body weight day^{-1} ration of commercial feed. Fish were then killed using an overdose of 150 mg L^{-1} tricaine methanesulfonate (MS-222, Sigma, Austria). The total number of trophonts on the fins, gills and entire body surface was recorded.

The ability of trophonts collected from fish after infection with theronts surviving zinc nanoparticles exposure to subsequently encyst into tomonts and release theronts

Trophonts were collected from infected fish 10 day post-exposure (from above) and placed into Petri dishes, which contained 10 mL water at 15 °C. Their ability to encyst into tomonts and release theronts was observed at 6 and 24 h, respectively.

RESULTS

UV–Vis analysis of gold and silver nanoparticles showed maximum absorption at 523 and 395 nm, which matched expected values for gold and silver nanoparticles, respectively. TEM revealed both gold and silver nanoparticles to be spherical, with mean diameters of 18 and 21 nm, respectively. DLS measurements showed one peak at 23 nm for gold nanoparticles, and two peaks (at 8·3 and 44·5 nm) for silver nanoparticles. Parasite stages were categorized as active (survival, Fig. 1A, C, E) or motionless (dead, Fig. 1B, D, F). For samples where the dual fluorescence staining technique was used, intact parasites fluoresced green, while dead or damaged stages fluoresced red.

Protomonts: Exposures to 20, 10, 5 ng mL^{-1} gold, silver or zinc oxide nanoparticles for 30 min resulted in ~50% mortality (Table 1). About half of the protomonts stopped moving after exposure, and displayed slow ciliary movement with some developing small projections over their surface and rupturing after 30 min (Fig. 1B, D). The surviving protomonts showed asymmetric division, and released theronts after 72 h. Negative control protomonts encysted successfully and released theronts after ~24 h. Exposure to doses lower than 5 ng mL^{-1} for 30 min were less successful in killing *I. multifiliis* protomonts, however, longer exposure time (⩾2 h) increased protomont mortality (Table 1).

Encysted tomonts: Approximately 50% of tomonts exposed to 20, 10, 5 ng mL^{-1} gold, silver or zinc oxide nanoparticles were killed by a 30 min exposure, and for survivors, speed of development was affected, and subsequent release of theronts was delayed. Exposure to doses lower than 5 ng mL^{-1}was less effective, and tomonts released theronts after ~24 h, similar to the control unexposed encysted tomonts. Exposure of encysted tomonts to 20, 10, 5 ng mL^{-1} gold, silver and zinc oxide nanoparticles for 2 h resulted in 100% mortality (Table 1).

In vitro exposure of I. multifiliis theronts: survival of theronts after exposure to 20, 10, 5 ng mL^{-1} gold, silver and zinc oxide nanoparticles for up to 24 h demonstrated a dose and time dependent response, with survival decreasing with increased concentrations of nanoparticles (Table 1). However, increasing concentration above the 20, 10, 5 ng mL^{-1} gold, silver and zinc oxide nanoparticles did not affect mortality significantly. With 10 ng mL^{-1} silver nanoparticles, theront survival decreased with exposure time. After 2 h, no theronts survived in the silver nanoparticle exposure group, compared with 100% survival in the control; after 24 h, all exposed theronts had died. Theront survival after exposure to 5 ng mL^{-1} zinc oxide nanoparticles also showed a reduction over time, however, after 24 h ~3% of theronts still survived.

In vivo fish-infection trials: Fish cohabited with control theronts (no nanoparticle exposure) had 100% mortality, with a mean of 110 ± 20 trophonts attached to the skin of each fish (Fig. 2A). After 24 h, no theronts survived exposure to silver nanoparticles, and no infections were recorded in fish cohabited with this group (Fig. 2B). Fish cohabited with the theronts after exposure to zinc oxide nanoparticles became infected, with 1–7 trophonts attached to each fish (Fig. 2C–F). These trophonts were collected, then failed to successively encyst and release theronts. However, the infection level in this group was significantly lower than that observed in the control group. No mortalities were recorded in the fish groups exposed to either silver or zinc oxide nanoparticles.

DISCUSSION

Due to the complex life cycle of *I. multifiliis* and the scarceness of effective treatments, novel therapeutics are being investigated for disease management (Dickerson and Findly, 2014). Some success against aquatic fish pathogens has been shown with therapeutic strategies that utilize metal nanoparticles, specifically gold and silver nanoparticles (Soltani *et al.* 2009; Vaseeharan *et al.* 2010; Umashankari *et al.* 2012; Antony *et al.* 2013; Mahanty *et al.* 2013; Velmurugan *et al.* 2014; Saleh *et al.* 2016; Shaalan *et al.* 2016) and zinc oxide nanoparticles (Ramamoorthy *et al.* 2013; Swain *et al.* 2014). Based on these results, in the present study we investigated the antiprotozoal

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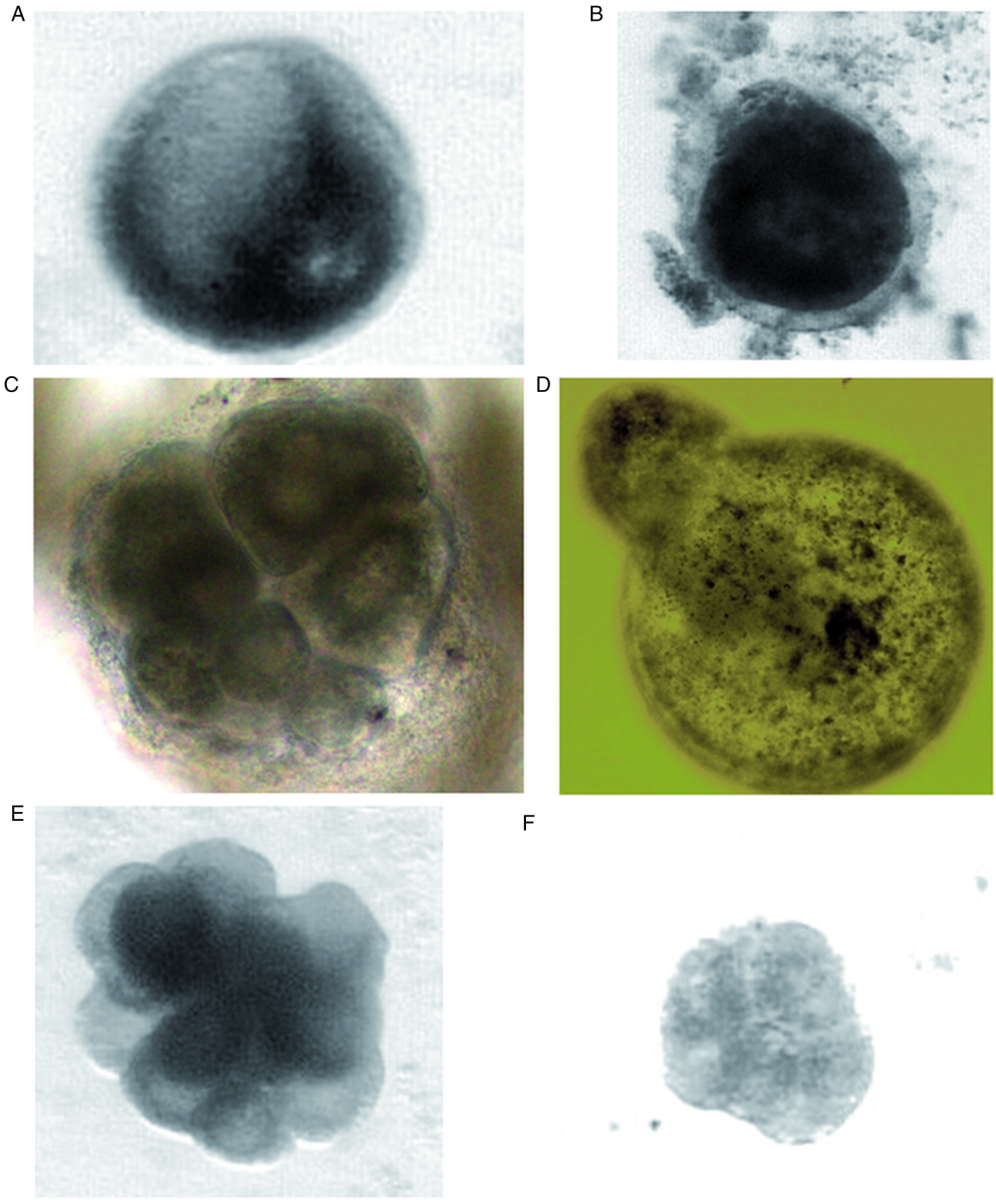

Fig. 1. Parasties were categorized as either active (alive; A, C, E) or motionless (dead; B, D, F) using microscopy.

activity of gold, silver and zinc oxide nanoparticles against free-living stages of *I. multifiliis*.

Exposure of the free-living theront stages of *I. multifiliis* to 20, 10 and 5 ng mL^{-1} gold, silver and oxide nanoparticles killed 48, 52 and 50% of the parasites, respectively, and ~50% of both protomonts and encysted tomonts after 30 min exposure. Protomonts surviving exposures successfully transformed into encysted tomonts; however, these tomonts showed asymmetric cell division and/or delayed development time to release theronts (72 h compared with 24 h for control). Exposure of encysted tomonts to the same concentrations of nanoparticles killed 50% within 30 min and the rest over the subsequent 48 h. Lower concentrations of nanoparticles were less effective in killing encysted tomonts, although they affected the parasite metabolism and delayed theronts release.

We observed significantly higher survival (~22%) of theronts after exposure to ⩾20 ng mL^{-1} gold nanoparticles than to silver and zinc oxide nanoparticles, and attributed this to higher aggregation of the gold nanoparticles due to the weakly bound capping citrates used in the synthesis (Zhou *et al.* 2012), and thus gold nanoparticles were not used in subsequent *in vivo* trials. We found using *in vitro* assays that zinc oxide nanoparticles reduced the number of theronts over time, however, those that survived exposure were still able to infect fish.

Previous work has shown silver nanoparticles to be effective against *I. multifiliis* trophonts using a bath exposure at 10 ng g^{-1} body weight of silver nanoparticles, which left fish infection free for more than 12 months (Daniel *et al.* 2016). However, prior to our current study, no assessment had been made with silver nanoparticles on the

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Table 1. Effects of gold, silver and zinc oxide nanoparticles on *Ichthyophthirius multifiliis*

Nanoparticle type	Nano particle concentration (ng mL^{-1})	Mean number of killed protomonts/ well after 30 min (each well started with 50) ±S.D.	Mean % dead protomonts after 30 min (%)	Mean number of killed encysted tomonts/ well after 30 min (each well started with 30) ±S.D.	Mean % dead encysted tomonts after 30 min (%)	Mean number of killed theronts/ well after 30 min (each well started With ~150) ±S.D.	Mean % dead theronts after 30 min (%)	Mean number of killed protomonts/ well after 2 h (each well started with 50 protomonts) ±S.D.	Mean % dead protomonts after 2 h	Mean number of killed encysted tomonts/well after 2 h (each well started with 30 tomonts)±S.D.	Mean % dead encysted tomonts after 2 h	Mean number of killed theronts/well after 2 h (each well started with ~150 theronts) ±S.D.	Mean % dead theronts after 2 h
Gold nano particles	0 ng mL^{-1}	2 ± 1·3	4	0	0	0	0	3 ± 2·3	6	0	0	0	0
	2·5 ng mL^{-1}	10 ± 3·7	20	5 ± 3·7	16·7	31 ± 3·7	20·7	15 ± 2·7	30	11 ± 3·8	36·6	41 ± 3·1	27·3
	5 ng mL^{-1}	14 ± 3·4	28	9 ± 3·1	30	47 ± 3·1	31·3	21 ± 3·1	42	17 ± 3·3	56·6	57 ± 3·7	38
	10 ng mL^{-1}	20 ± 3·0	40	10 ± 2·9	33·3	61 ± 2·9	40·7	34 ± 2·9	68	20 ± 2·5	66·7	99 ± 3·9	66
	20 ng mL^{-1}	25 ± 2·3	50	15 ± 1·1	50	72 ± 1·1	48	40 ± 1·3	80	24 ± 2·1	80	117 ± 1·5	78
	40 ng mL^{-1}	25 ± 1·9	50	15 ± 2·1	50	72 ± 2·1	48	40 ± 1·1	80	24 ± 1·9	80	117 ± 2·1	78
	80 ng mL^{-1}	25 ± 1·1	50	15 ± 1·9	50	72 ± 1·9	48	40 ± 1·5	80	24 ± 2·3	80	117 ± 1·7	78
	160 ng mL^{-1}	25 ± 2·1	50	15 ± 1·3	50	72 ± 1·3	48	40 ± 1·3	80	24 ± 1·5	80	117 ± 1·1	78
Silver nanoparticles	0 ng mL^{-1}	3 ± 2·3	6	0	0	0	0	3 ± 2·3	6	0	0	0	0
	2·5 ng mL^{-1}	15 ± 3·6	30	8 ± 3·6	26·7	57 ± 3·7	38	23 ± 3·2	46	17 ± 4·6	56·7	119 ± 3·7	79·3
	5 ng mL^{-1}	20 ± 3·1	40	12 ± 3·1	40	60 ± 2·9	40	29 ± 2·5	58	21 ± 2·3	70	137 ± 2·9	91·3
	10 ng mL^{-1}	25 ± 1·6	50	15 ± 2·9	50	78 ± 2·7	52	50 ± 1·9	100	30 ± 2·6	100	150 ± 2·7	100
	20 ng mL^{-1}	25 ± 2·5	50	15 ± 1·5	50	78 ± 2·5	52	50 ± 1·5	100	30 ± 1·7	100	150 ± 2·3	100
	40 ng mL^{-1}	25 ± 1·5	50	15 ± 1·3	50	78 ± 2·3	52	50 ± 1·7	100	30 ± 1·9	100	150 ± 1·1	100
	80 ng mL^{-1}	25 ± 1·7	50	15 ± 1·4	50	78 ± 2·5	52	50 ± 1·6	100	30 ± 1·7	100	150 ± 2·2	100
	160 ng mL^{-1}	25 ± 0·9	50	15 ± 1·2	50	78 ± 3·1	52	50 ± 1·9	100	30 ± 2·4	100	150 ± 1·7	100
Zinc oxide nanoparticles	0 ng mL^{-1}	3 ± 2·3	6	0	0	0	0	2 ± 2·7	4	0	0	0	0
	2·5 ng mL^{-1}	21 ± 3·6	42	13 ± 3·1	43·3	55 ± 3·2	36·6	35 ± 2·9	70	23 ± 2·3	76·7	121 ± 3·2	80·7
	5 ng mL^{-1}	25 ± 3·8	50	15 ± 3·5	50	75 ± 2·5	50	50 ± 2·1	100	30 ± 1·7	100	146 ± 2·5	97
	10 ng mL^{-1}	25 ± 2·7	50	15 ± 2·9	50	75 ± 2·9	50	50 ± 3·4	100	30 ± 2·9	100	146 ± 2·9	97
	20 ng mL^{-1}	25 ± 2·3	50	15 ± 1·1	50	75 ± 1·3	50	50 ± 2·3	100	30 ± 2·5	100	146 ± 1·3	97
	40 ng mL^{-1}	25 ± 1·4	50	15 ± 1·3	50	75 ± 1·7	50	50 ± 3·1	100	30 ± 2·4	100	146 ± 1·7	97
	80 ng mL^{-1}	25 ± 1·7	50	15 ± 1·4	50	75 ± 1·5	50	50 ± 2·1	100	30 ± 2·7	100	146 ± 1·5	97
	160 ng mL^{-1}	25 ± 1·1	50	15 ± 1·2	50	75 ± 1·9	50	50 ± 1·7	100	30 ± 1·9	100	146 ± 1·9	97

Mean of 6 wells each trial (±S.D.) numbers after 30 min and 2 h exposure to different concentrations of nanoparticles.

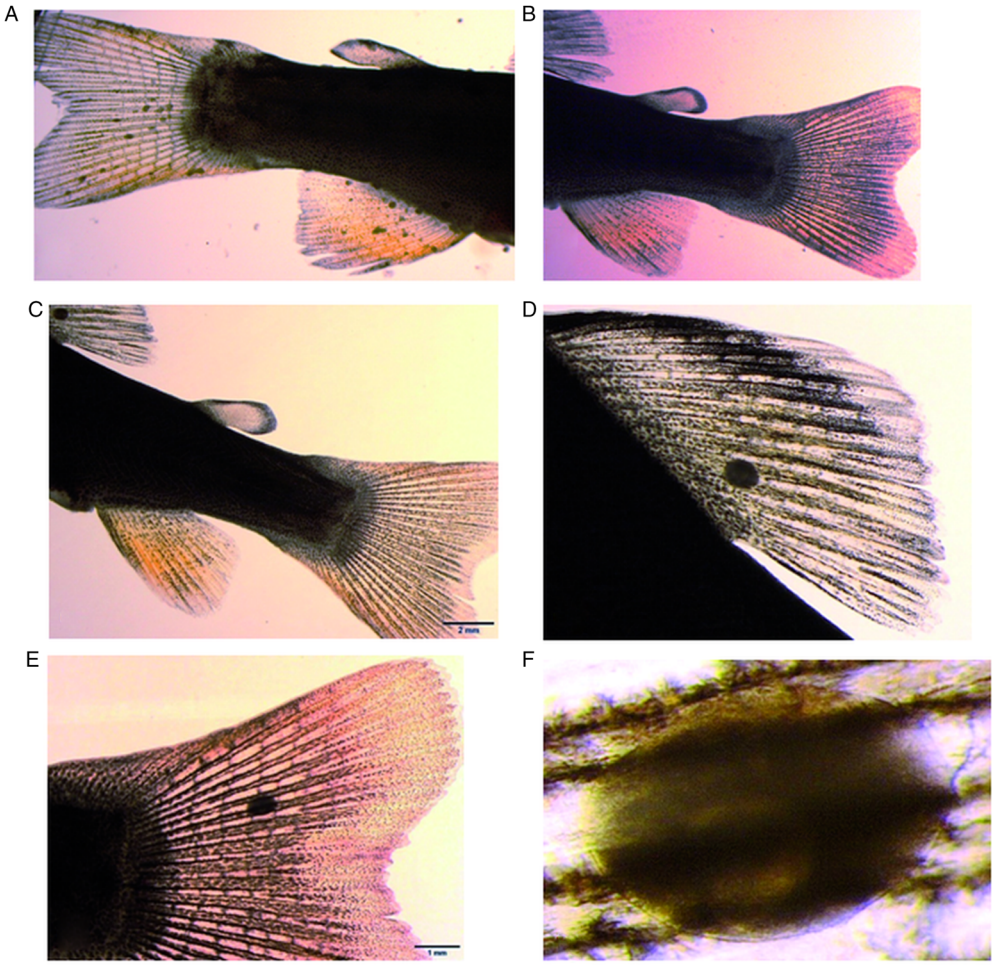

Fig. 2. Fish exposed to theronts that had been incubated for 24 h with silver nanoparticles did not become infected, whereas the control fish group showed 100 and 50% infection and mortality rates, respectively (Fig. 2A, B). Theronts survived exposure to zinc oxide nanoparticles were able to infect 50% of the fish, but at a low intensity (1–7 attached trophonts) (Fig. 2C–F).

different free-living developmental stages of *I. multifiliis*, which are considered major factors for emergence and spread of the disease.

We found that silver nanoparticles killed all theronts *in vitro*, no fish became infected by the end of the experiment, and none died within 10 days (the end of the trial). In contrast, all fish in the control group became heavily infected and died by the end of the experiment. Silver nanoparticles are reported non-cytotoxic (Arora *et al*. 2008), however at higher concentrations, rainbow trout hepatocytes cells can be affected (Farkas *et al*. 2010). Also, cytotoxic and genotoxic effects have been observed on fish cell lines and zebrafish at higher concentrations of silver nanoparticles (Kim & Ryu, 2013). We have observed that a concentration of 16 $\mu g\ mL^{-1}$ did not affect the viability of EK-1 cells assessed using an MTT assay, and silver nanoparticles did not cause mortality (within the 30-day experiment) in rainbow trout exposed via either an immersion bath at 100 $\mu g\ L^{-1}$, or intra peritoneal injection (1 $\mu g\ g^{-1}$) (El-Matbouli, unpublished data).

In the present study, we demonstrated that 10 $ng\ mL^{-1}$ silver nanoparticles were effective against all *I. multifiliis* free-living stages, and did not appear to be cytotoxic towards the fish. The precise mechanism by which silver nanoparticles exhibit their action is unknown, but likely involves disruption of the *I. multifiliis* membranes, which would lead to release of immature theronts from encysted tomonts, which are unable to infect fish.

In conclusion, we demonstrated that while all tested nanoparticles inhibited the development of *I. multifiilis*, silver nanoparticles were most effective. Silver nanoparticles adversely affect the survival of all free-living stages of *I. multifiliis* (protomonts, tomont and theronts) and could be applied in disease management. However, we propose future investigations aimed at increasing the specificity of nanoparticles towards particular pathogens, through modification of the nanoparticle surfaces by conjugation with ssRNA or siRNA that binds to known pathogen sequences (silencing/knocking down the target gene). This could lead to

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pathogen-specific nanoparticle treatments for disease management in aquaculture.

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REFERENCES

Allahverdiyev, A. M., Abamor, E. S., Bagirova, M., Ustundag, C., Kaya, C., Kaya, F. and Rafailovich, M. (2011). Antileishmanial effect of silver nanoparticles and their enhanced antiparasitic activity underultraviolet light. *International Journal of Nanomedicine* **6**, 2705–2714.

Antony, J. J., Nivedheetha, M., Siva, D., Pradeepha, G., Kokilavani, P., Kalaiselvi, S., Sankarganesh, A. and Balasundaram, A. (2013). Antimicrobial activity of *Leucas aspera* engineered silver nanoparticles against *Aeromonas hydrophila* in infected *Catla catla*. *Colloids and Surfaces B: Biointerfaces* **109**, 20–24.

Arora, S., Jain, J., Rajwade, J. M. and Paknikar, K. M. (2008). Cellular responses induced by silver nanoparticles: *in vitro* studies. *Toxicology Letters* **179**, 93–100.

Buzea, C., Blandino, I. I. P. and Robbie, K. (2007). Nanomaterials and nanoparticles: sources and toxicity. *Biointerphases* **2**, 17–172.

Cui, Y., Zhao, Y., Tian, Y., Zhang, W., Lü, X. and Jiang, X. (2012). Themolecularmechanismof action ofbactericidal gold nanoparticles on Escherichia coli. *Biomaterials* **33**, 2327–2333.

Daniel, S. C. G. K., Sironmani, T. A. and Dinakaran, S. (2016). Nano formulations as curative and protective agent for fish diseases: studies on red spot and white spot diseases of ornamental gold fish *Carassius auratus*. *International Journal of Fisheries and Aquatic Studies* **4**, 255–261.

Dickerson, H. W. and Findly, R. C. (2014). Immunity to Ichthyophthirius infections in fish: a synopsis. *Developmental & Comparative Immunology* **43**, 290–299.

Dizaj, S. M., Lotfipour, F., Barzegar-Jalali, M., Zarrintan, M. H. and Adibkia, K. (2014). Antimicrobial activity of the metals and metal oxide nanoparticles. *Materials Science and Engineering C* **44**, 278–284.

El Mahdy, M. M., Eldin, T. A. S., Aly, H. S., Mohammed, F. F. and Shaalan, M. I. (2015). Evaluation of hepatotoxic and genotoxic potential of silver nanoparticles in albino rats. *Experimental and Toxicologic Pathology* **67**, 21–29.

Farkas, J., Christian, P., Gallego-Urrea, J. A., Roos, N., Hassellov, M., Tollefsen, K. E. and Thomas, K. V. (2010). Effects of silver and gold nanoparticles on rainbowtrout (*Oncorhynchus mykiss*) hepatocytes. *Aquatic Toxicoogy* **96**, 44–52.

Franci, G., Falanga, A., Galdiero, S., Palomba, L., Rai, M., Morelli, G. and Galdiero, M. (2015). Silver nanoparticles as potential antibacterial agents. *Molecules* **20**, 8856–8874.

Fu, Y. W., Zhang, Q. Z., Xu, D. H., Liang, J. H. and Wang, B. (2014). Antiparasitic effect of Cynatratoside C from *Cynanchum atratum* against *Ichthyophthirius multifiliis* on Grass Carp. *Journal of Agricultural and Food Chemistry* **62**, 7183–7189.

Hoffman, G. L. (1967). *Parasites of North American Freshwater Fishes*. University California Press, Berkeley, Los Angeles, p. 486.

Hoffman, G. L. (1970). Intercontinental and transcontinental dissemination and transfaunation of fish parasites with emphasis on whirling disease (*Myxosoma cerebralis*). *A Symposium on Diseases of Fishes and Shellfishes* 69–81.

Jiang, W., Mashayekhi, H. and Xing, B. (2009). Bacterial toxicity comparison between nano- and micro-scaled oxideparticles. *Environmental Pollution* **157**, 1619–1625.

Kim, S. and Ryu, D. Y. (2013). Silver nanoparticle-induced oxidative stress, genotoxicity and apoptosis in cultured cells and animal tissues. *Journal of Applied Toxicology* **33**, 78–89.

Lara, H. H., Ayala-Núnez, N. V., Turrent, L. D. C. I. and Padilla, C. R. (2010). Bactericidal effect of silver nanoparticles against multidrug-resistant bacteria. *World Journal of Microbiology and Biotechnology* **26**, 615–621.

Lima, E., Guerra, R., Lara, V. and Guzmán, A. (2013). Gold nanoparticles as efficient antimicrobial agents for *Escherichia coli* and *Salmonella typhi*. *Chemistry Central Journal* **7**, 1–7.

Li, Q. L., Mahendra, S., Lyon, D. Y., Brunet, L., Liga, M. V., Li, D. and Alvarez, P. J. J. (2008). Antimicrobial nanomaterials for water disinfection and microbial control: potential applications and implications. *Water Research* **42**, 4591–4602.

Lolina, S. and Narayanan, V. (2013). Antimicrobial and anticancer activity of gold nanoparticles synthesized from grapes fruit extract. *Chemical Science Transactions* **2**, 105–110.

Mahanty, A., Mishra, S., Bosu, R., Maurya, U. K., Netam, S. P. and Sarkar, B. (2013). Phytoextracts-synthesized silver nanoparticles inhibit bacterial fish pathogen *Aeromonas hydrophila*. *Indian Journal of Microbiology* **53**, 438–446.

Neal, A. L. (2008). What can be inferred from bacterium nanoparticle interactions about the potential consequences of environmental exposure to nanoparticles? *Ecotoxicology* **17**, 362–371.

Nigrelli, R. F., Pokorny, K. S. and Ruggieri, G. D. (1976): Notes on *Ichthyophthirius multifiliis*, a ciliate parasitic on fresh-water fishes, with some remarks on possible physiological races and spieces. *Transactions of the American Microscopical Society* **95**, 607–613.

Ramamoorthy, S., Kannaiyan, P., Moturi, M., Devadas, T., Muthuramalingam, J. and Natarajan, L., Arunachalam, N. and Ponniah, A. G. (2013). Antibacterial activity of zinc oxide nanoparticles against *Vibrio harveyi*. *Indian Journal of Fisheries* **60**, 107–112.

Rana, S. and Kalaichelvan, P. T. (2011). Antibacterial activities of metal nanoparticles. *Advanced Biotechnology* **11**, 21–23.

Rintamäki-Kinnunen, P. and Valtonen, E. T. (1997). Epizootiology of protozoans in farmed salmonids at northern latitudes. *International Journal of Parasitology* **27**, 89–99.

Saleh, M., Kumar, G., Abdel-Baki, A. A., Al-Quraishy, S. and El-Matbouli, M. (2016). In vitro antimicrosporidial activity of goldnanoparticles against *Heterosporis saurida*. *BMC Veterinary Research* **12**, 44.

Schäperclaus, W. (1991). Diseases caused by ciliates. In *Fish Diseases* (ed. Schäperclaus, W., Kulow, H. and Schreckenbach, K.), pp. 702–725. Amerind Publishing Co. Pvt. Ltd., New Delhi.

Schumacher, I. V., Wedekind, H. and El-Matbouli, M. (2011). Efficacy of quinine against ichthyophthiriasis common carp *Cyprinus carpio*. *Diseases of Aquatic Organisms* **95**, 217–224.

Shaalan, M., Saleh, M., El-Mahdy, M. and El-Matbouli, M. (2016). Recent progress in applications of nanoparticles in fish medicine: a review. *Nanomedicine: Nanotechnology, Biology, and Medicine* **12**, 701–710.

Shinn, A. P., Picón-Camacho, S. M., Bron, J. E., Conway, D., Yoon, G. H., Guo, F. C. and Taylor, N. G. (2012). The anti-protozoal activity of bronopol on the key life-stages of *Ichthyophthirius multifiliis* Fouquet, 1876 (Ciliophora). *Veterinary Parasitology* **186**, 229–236.

Soltani, M., Ghodratnema, M., Ahari, H., Ebrahimzadeh Mousavi, H. A., Atee, M., Dastmalchi, F. and Rahmanya, J. (2009). The inhibitory effect of silver nanoparticles on the bacterial fish pathogens. *Streptococcus iniae*, *Lactococcus garvieae*, *Yersinia ruckeri* and *Aeromonas hydrophila*. *International Journal of Veterinary Research* **3**, 137–142.

Stoimenov, P. K., Klinger, R. L., Marchin, G. L. and Klabunde, K. J. (2002). Metal oxide nanoparticles as bactericidal agents. *Langmuir* **18**, 6679–6686.

Swain, P., Nayak, S. K., Sasmal, A., Behera, T., Barik, S. K., Swain, S. K., Mishra, S. S., Sen, A. K., Das, J. K. and Jayasankar, P. (2014). Antimicrobial activity of metal based nanoparticles against microbes associated with diseases in aquaculture. *World Journal of Microbiology and Biotechnology* **30**, 2491–2502.

Tiwari, P. M., Vig, K., Dennis, V. A. and Singh, S. R. (2011). Functionalized gold nanoparticles and their biomedical applications. *Nanomaterials* **1**, 31–63.

Tucker, C. S. and Robinson, E. H. (1990). *Channel Catfish Farming Handbook*. Van Nostrand Reinhold, New York.

Umashankari, J., Inbakandan, D., Ajithkumar, T. T. and Balasubramanian, T. (2012). Mangrove plant, *Rhizophora mucronata* (Lamk, 1804) mediated one pot green synthesis of silver nanoparticles and its antibacterial activity against aquatic pathogens. *Aquatic Biosy*stems **8**, 11.

Vaseeharan, B., Ramasamy, P. and Chen, J. C. (2010). Antibacterial activity of silver nanoparticles (AgNps) synthesized by tea leaf extracts against pathogenic *Vibrio harveyi* and its protective efficacy on juvenile *Feneropenaeus indicus*. *Letters in Applied Microbiology* **50**, 352–356.

Velmurugan, P., Iydroose, M., Lee, S. M., Cho, M., Park, J. H., Balachandar, V. and Oh, B. (2014). Synthesis of silver and gold

nanoparticles using cashew nut shell liquid and its antibacterial activity against fish pathogens. *Indian Journal of Microbiology* **54**, 196–202.

Wang, H., Qiao, X., Chen, J., Wang, X. and Ding, S. (2005). Mechanisms of PVP in the preparation of silver nanoparticles. *Materials Chemistry and Physics* **94**, 449–453.

Zhang, L., Jiang, Y., Ding, Y., Povey, M. and York, D. (2007). Investigation into the antibacterial behaviour of suspensions of ZnO nanoparticles (Nanofluids). *Journal of Nanoparticle Research* **9**, 479–489.

Zhang, L., Jiang, Y., Ding, Y., Daskalakis, N., Jeuken, L., Povey, M., O'Neill, A. J. and York, D. W. (2010). Mechanistic investigation into antibacterial behaviour of suspensions of ZnO nanoparticles against *E. Coli*. *Journal of Nanoparticle Research* **12**, 1625–1636.

Zhou, Y., Kong, Y., Kundu, S., Cirillo, J. D. and Liang, H. (2012). Antibacterial activities of gold and silver nanoparticles against *Escherichia coli* and bacillus Calmette-Guérin. *Journal of Nanobiotechnolology* **10**, 19.

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