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**Impact of Pseudomonas H6 surfactant on all external life cycle stages of the fish parasitic ciliate Ichthyophthirius multifiliis**

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# Impact of *Pseudomonas* H6 surfactant on all external life cycle stages of the fish parasitic ciliate *Ichthyophthirius multifiliis*

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## Abstract

A bacterial biosurfactant isolated from *Pseudomonas* (strain H6) has previously been shown to have a lethal effect on the oomycete *Saprolegnia diclina* infecting fish eggs. The present work demonstrates that the same biosurfactant has a strong in vitro antiparasitic effect on the fish pathogenic ciliate *Ichthyophthirius multifiliis*. Three life cycle stages (the infective theront stage, the tomont and the tomocyst containing tomites) were all susceptible to the surfactant. Theronts were the most sensitive showing 100% mortality in as low concentrations as 10 and 13 µg/ml within 30 min. Tomonts were the most resistant but were killed in concentrations of 100 µg/ml. Tomocysts, which generally are considered resistant to chemical and medical treatment, due to the surrounding protective cyst wall, were also sensitive. The surfactant, in concentrations of 10 and 13 µg/ml, penetrated the cyst wall and killed the enclosed tomites within 60 min. Rainbow trout fingerlings exposed to the biosurfactant showed no adverse immediate or late signs following several hours incubation in concentrations effective for killing the parasite. This bacterial surfactant may be further developed for application as an antiparasitic control agent in aquaculture.

## KEYWORDS

biological control, *Ichthyophthirius multifiliis*, parasiticide, *Pseudomonas*, surfactant

## 1 | INTRODUCTION

Several chemicals are currently being tested to control white spot disease in fish, caused by the parasitic ciliate *Ichthyophthirius multifiliis* Fouquet, 1876, which elicits high morbidity and mortality in both wild and cultured freshwater fish worldwide (Ekanem, Obiekezie, Kloas, & Knopf, 2004; Fu, Zhang, Xu, Liang, & Wang, 2014; Fu, Zhang, Xu, Xia et al., 2014; Matthews, 2005). The need for novel and effective compounds arose following the ban of the organic dye malachite green which previously was used for treatment of the disease. Fish farmers were then left with a number of less effective auxiliary compounds, including formalin, sodium percarbonate, copper sulphate, formalin

and peracetic acid for control of the infection (Rowland, Mifsud, Nixon, Read, & Landos, 2008; Heinecke & Buchmann, 2009; Bruzio & Buchmann, 2010; Sudova, Straus, Wienke, & Meinelt, 2010). More specific antiparasitic drugs such as toltrazuril (Jaafar & Buchmann, 2011; Mehlhorn, Schmahl, & Haberkorn, 1988) and quinine (Schumacher, Wedekind, & El-Matbouli, 2011) with proven efficacy against other animal parasites have shown effect on *I. multifiliis* but environmental issues may limit their use in practical fish farming. Plant extracts have attracted considerable attention due to their possible role as more environmentally acceptable alternatives, and effects of several compounds on certain stages of the parasite have subsequently been documented (Buchmann, Jensen, & Kruse, 2003;

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Ekanem et al., 2004; Fu, Zhang, Xu, Xia et al., 2014). In this context purified plant-derived compounds comprising cynaratoside-C (Fu, Zhang, Xu, Liang, et al., 2014), sanguinarine (Yao et al., 2010), dihydroanguinarine and dihydrochelerythrine (Yao et al., 2011) and pentagalloylglucose (Zhang, Xu, & Klesius, 2013) have exhibited antiparasitic effects in the laboratory but despite the promising report questions on residual and toxic effects in fish and consumers remain unsolved. Other control methods involving easily biodegradable molecules are therefore being investigated and among these surfactants produced by naturally occurring bacteria such as *Pseudomonas* and *Aeromonas* have attracted interest (Carbajal-Gonzalez, Fregeneda-Grandes, Suarez-Ramos, Rodriguez-Cadenas, & Aller-Gancedo, 2011; Lategan & Gibson, 2003). Biosurfactant H6 produced by *Pseudomonas* was recently reported to suppress another fish pathogen, the oomycete *Saprolegnia diclina* (de Bruijn et al., 2007; Liu et al., 2015). The antiparasitic action against zoospores was exerted through membrane disruption, which raised the hypothesis that the membrane surrounded *I. multifiliis* would be vulnerable to the *Pseudomonas* surfactant as well. We have therefore conducted a series of in vitro studies on the effect of this biosurfactant on survival of various life cycle stages of *I. multifiliis* including theronts, tomonts and tomocysts, and we here report that the biosurfactant is highly lethal to all stages.

## 2 | MATERIALS AND METHODS

### 2.1 | Parasites

A Danish strain of *I. multifiliis* was established in a laboratory population of rainbow trout originally raised in a disease-free recirculated system (Xueqin, Kania, & Buchmann, 2012). Parasites were collected from infected rainbow trout reared in a Danish commercial trout farm (Jutland, western part of Denmark) as previously described (Aihua & Buchmann, 2001). Infected live fish were transported to the University of Copenhagen, and *I. multifiliis* parasites were isolated at room temperature by placing fins and gills, recovered from a fish killed with 300 mg/L of tricaine methanesulfonate (MS222, Sigma-Aldrich, Denmark), into Petri dishes with freshwater (22°C). This induced release of trophonts residing in the fish tissues. The resulting free swimming tomonts were isolated and used directly for surfactant exposure studies. Other tomonts were incubated further allowing their transformation into tomocysts each containing several hundreds of tomites (24 hr). A subpopulation of these were used for exposure, and others were incubated further until they released theronts within 24–30 hr. These were isolated and similarly used for in vitro evaluation of surfactant effects.

### 2.2 | *Pseudomonas* H6 surfactant (PS)

An extract of *Pseudomonas* H6 lipopeptide surfactant was prepared and lyophilized (Liu et al., 2015) and stored at –20°C until initiation of experiments. A stock solution of 10 mg/ml was prepared by

dissolving the product in sterile distilled water whereafter a dilution series was prepared for parasite exposures.

### 2.3 | In vitro incubation and exposure

Glass plates (thickness 6 mm) each with 30 concave wells (diameter 25 mm, depth 3 mm, maximum water capacity 2,000 µl) were used for incubation of parasite stages (theronts, tomont and tomocysts). The final concentrations of the surfactant in the wells were 1,000, 100, 20, 13, 10, 7, 5, 2.5, 2 and 1 µg/ml, and all concentrations were tested in triplicate for theronts and six replicates for tomonts and tomocysts. The number of parasites in each well was for theronts 10, for tomonts 2 and for tomocysts 2. The volume added into each well was 100 µl composed by mixing 50 µl of surfactant solution with 50 µl of fresh water containing parasites. The experiment was performed at room temperature 22°C and parasite motility recorded at 0, 15, 30, 45 and 60 min.

### 2.4 | Monitoring motility of parasites

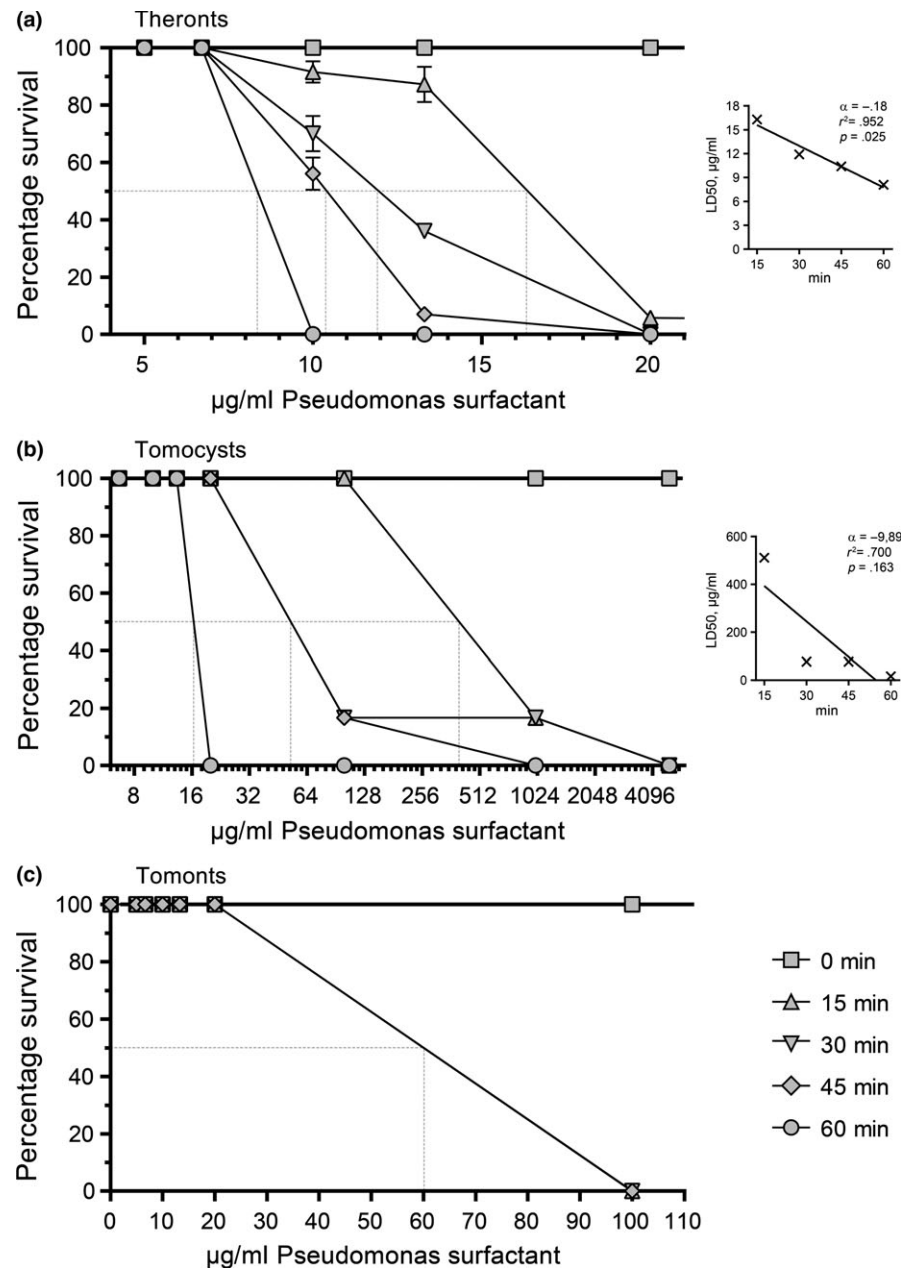
A Leica MZ 9<sub>s</sub> dissection microscope (magnification 6–40X) was used for monitoring motility of tomonts, tomocysts and theronts. Motility was recorded as presence of ciliary activity and cell movements of free theronts, free tomonts and tomites enclosed in tomocysts. Non-motile and lysed tomites, theronts and tomonts were considered dead.

### 2.5 | Sensitivity of fish to surfactant

Rainbow trout (2 × 3) were exposed in plastic fish tanks (total volume 3 L), each containing 1 L of surfactant solution and three rainbow trout to concentrations of surfactant which were found effective for all tested parasite stage within 90 min (10 and 13 µg/ml). Control fish (2 × 3) were kept similarly but without surfactant. Fish were monitored in the surfactant solution for 3 hr after exposure whereafter they were transferred to 80-L tanks containing only pure water and observed for any adverse behavioural signs (balance disturbances, lethargy, anorexia) for 7 days.

### 2.6 | Data analysis

As no significant differences in the triplicate wells with theronts (10 parasites per well) were observed with regard to parasite survival, data from these were pooled. Total mortality of 12 tomonts and 12 tomocysts (six replicates of two parasites per well) was recorded for each concentration of surfactant. Survival rates of the different parasite stages exposed to different concentrations over 60 min were expressed in a Kaplan–Meier plot and tested by Dunn's multiple comparison test with a probability level of 5% (Figure 1). LD50 for each concentration and exposure time was calculated and plotted whereby Pearson's correlation coefficient  $r^2$ , linear regression with slope ( $\alpha$ ) and probability level ( $p$ ) could be calculated and plotted (Figure 1 insets). All the statistical analyses were performed using Graph Pad Prism version 5.



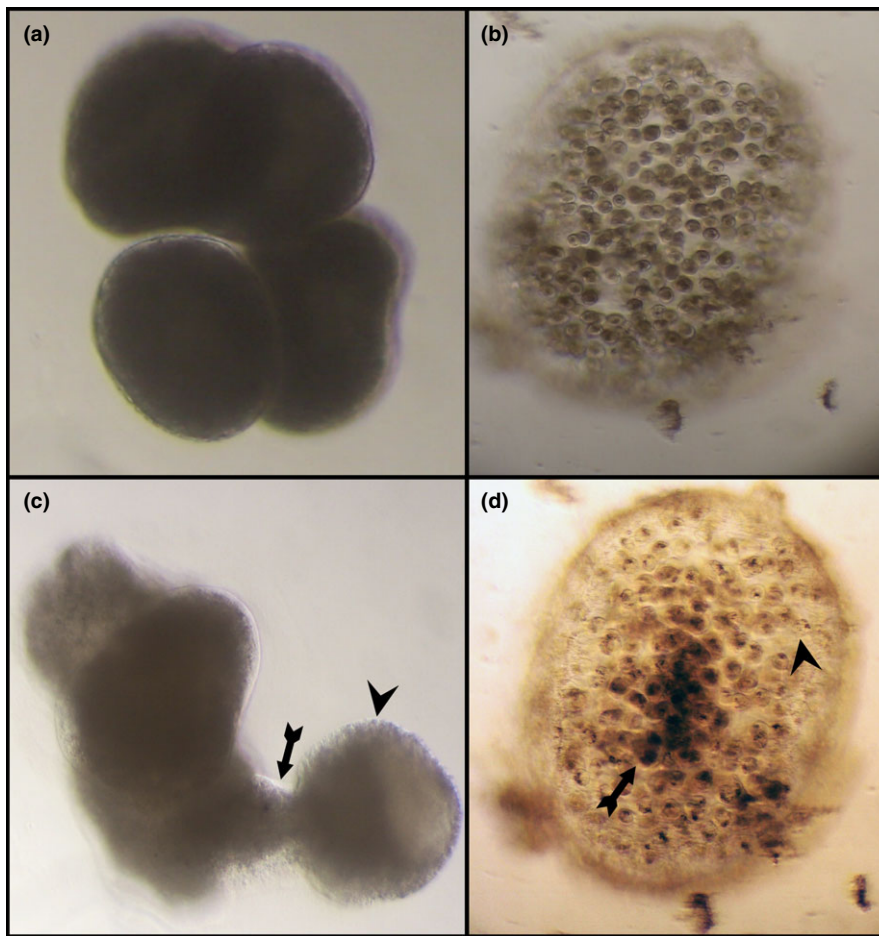
**FIGURE 1** Effects of *Pseudomonas* H6 surfactant (PS). LD50 and LD100 values based on survival rates of three stages of free-living *Ichthyophthirius multifiliis* incubated with a series of *Pseudomonas* H6 surfactant (PS) concentrations. Parasite mortality was recorded at various time points up to 60 min for theronts and tomites and up to 90 min for tomocysts. (a) Theronts, (b) Tomocysts, (c) Tomonts. Insets show correlation (Pearson) between LD50 and exposure time.  $\alpha$ , slope coefficient;  $r^2$ , Pearson's correlation coefficient;  $p$ , probability level

### 3 | RESULTS

#### 3.1 | In vitro effects *Pseudomonas* H6 surfactant (PS)

Theronts were highly sensitive to PS, and when exposed to 1,000 and 100 µg/ml PS, they displayed 100% mortality within 5 min (Figure 1a). When exposed to 20 µg/ml PS, less than 20% survival was seen at this time point and the remaining theronts were killed at 30 min. Concentrations of 13 and 10 µg/ml PS were lethal for theronts within 60 min whereas concentrations of 7 µg/ml PS and lower had no effect even after 90 min (data not shown). Tomocysts, with their enclosed tomites, showed a different sensitivity to the surfactant when compared to tomites (Figure 1b). At the highest

concentration, 1,000 µg/ml PS, the majority of tomites in the tomocysts (83%) were immotile at 15 min after exposure (Figure 1b). When exposed to 100 µg/ml PS, 83% immobilization stage was reached after 30 min and all tomocysts with their content of tomites were dead within 60 min. The effects of 13 and 10 µg/ml PS were less severe and tomocysts were not affected by 0 and 7 µg/ml PS. Tomonts were only sensitive towards the two highest concentrations (1,000 and 100 µg/ml *Pseudomonas* surfactant (PS)), which killed all parasites within 15 min (Figure 1c). Lower PS concentrations had no effect on this parasite stage within the observation period. The sensitivities of the different parasite stages were reflected by microscopic changes. Cytoplasmic movements inside the tomites (Figure 2a, Video S1) initially increased when exposed to 100 µg/ml of PS. Shortly before full inactivation of tomites, disruption of the



**FIGURE 2** Visualization of *Pseudomonas* H6 surfactant (PS) effects on tomonts and tomocysts with enclosed tomites of *Ichthyophthirius multifiliis*. (a) Tomonts without PS. (b) Tomocysts with enclosed tomites after 30-s exposure to 10 µg/ml PS with minor disturbance of peripheral tomites within the cyst wall. (c) Tomont showing membrane disruption (arrow) and release of cytoplasm (arrow head) following 15-min exposure to 100 µg/ml PS. (d) Tomocyst with enclosed dead tomites in the cyst centre after 15-min exposure to 100 µg/ml PS [Colour figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]

membrane was noted whereafter cytoplasm was released to the surroundings (Figure 2b, Video S1). Peripheral tomites in the tomocysts were similarly but slightly affected (moving faster) when exposed 30 s (Figure 2b, Video S2). Longer PS exposure showed initial inactivation of peripheral tomites which moved towards the tomocyst centre but eventually full inactivation of all tomites in the cysts was noted (Figure 2d, Video S2, Video S3).

### 3.2 | Sensitivity of fish to *Pseudomonas* surfactant

Rainbow trout showed no immediate or late adverse reactions when exposed for 3 hr to PS concentrations of 10 and 13 µg/ml.

## 4 | DISCUSSION

Bacteria within the genus *Pseudomonas* produce surfactants (PS), which can be isolated and used for neutralization of fish pathogens. It was recently shown that the fish pathogenic oomycete *Saprolegnia diclina* is sensitive to a viscosin-like surfactant from *Pseudomonas* H6 (Liu et al., 2015). The PS lipopeptides interrupt the membrane of the zoospore (Liu et al., 2015), an action which suggests that PS also may interact with one of the major parasitic diseases (white spot disease, ichthyophthiriasis) in freshwater fish

culture caused by the membrane carrying ciliate *I. multifiliis*. The life cycle of *I. multifiliis* includes a trophont stage in the fish epidermis followed by three free-living stages (termed tomont (freely swimming released trophont), tomocyst (attached and with cyst wall surrounding internally produced tomites) and theront (released tomites ready for infection of fish)). We performed a series of in vitro tests documenting that PS was 100% effective against theronts and tomocysts when applied for 60 min at concentrations of 10 and 13 µg/ml, respectively. When theronts, tomonts and tomocysts were exposed to PS, morphological changes of the parasite cells appeared. When theronts were exposed, their cell morphology gradually became spherical, which indicated that PS affected the integrity of the membrane and allowed uncontrolled inflow of water into the cytoplasm. In addition, exposed theronts lost the swimming ability and rotated at the same location until death (data not shown). The same effects were observed with the tomites enclosed within tomocysts, which suggests that PS is able to penetrate the jelly-like tomocyst surrounding and directly kill the tomites before they escape from the cyst as theronts. The mode of action is not fully elucidated but PS lipopeptides may reduce surface tension of the plasma membrane of the cell and solubilize membrane constituents (Vandyke, Lee, & Trevors, 1991). Tomonts were not susceptible to low PS concentrations as only high concentrations of 1,000 and 100 µg/ml could kill this stage. This corresponds to a



series of other studies showing that tomons compared to theronts in general are more resistant to plant-derived parasiticides (Schumacher et al., 2011; Yi, Lu, Hu, Ling, & Wang, 2012; Zhang et al., 2013) or chemicals (Buchmann et al., 2003; Heinecke & Buchmann, 2009; Schumacher et al., 2011). The impact of PS on theronts is comparable to the activity found for a range of other antiparasitic compounds. Thus, in the same concentration range potassium ferrate (Ling et al., 2010), extracts from *Magnolia officinalis* and *Sophora alopecuroides* (Yi et al., 2012), garlic extract (Buchmann et al., 2003) and nystatin isolated from *Streptomyces griseus* (Yao et al., 2015) show full effect on the infective theronts within few hours. However, the killing activity of PS against tomocysts appears to be higher when compared to other compounds. Tomocysts are equipped with a cyst wall and considered more resistant to the action of various disinfectants and chemicals (Ling, Wang, Wang, & Gong, 2011; Picon-Camacho, Marcos-Lopez, Bron, & Shinn, 2012; Song et al., 2015). It is therefore noteworthy that the *Pseudomonas* H6 surfactant in this study showed a strong effect on tomocysts, which suggests that the product will have a high potential for control of *I. multifiliis* in aquaculture facilities. Further, biosurfactants have been shown to be degraded relatively fast in aquatic systems and non-sterile soil (Abd-Allah & Srorr, 1998), which will prevent problems related to residues in fish products from treated fish farms. Our preliminary studies on PS and rainbow trout sensitivity gave no indications that the surfactant should have any toxic or adverse effect on this fish host. In conclusion, this study has shown that PS has a potential as a biodegradable effective parasiticide which can control white spot disease caused by *I. multifiliis* in rainbow trout. The lethal effect of PS on tomocysts is noteworthy as this stage is not readily attacked by other chemicals applied in aquaculture. It is therefore expected that PS treatment would provide a longer lasting effect as reinfection may be prevented. Investigations under practical fish farm conditions should confirm this notion. In addition, the low toxicity of PS demonstrated for rainbow trout should be further confirmed for a range of relevant aquaculture and ornamental fish. With regard to the safety of PS—if used in open or semi-enclosed aquaculture facilities—the compound must be investigated for any environmental impact (toxicity to algae and free-living invertebrates).

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## SUPPORTING INFORMATION

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