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Short communication

Effects of anaesthetics containing eugenol on *Neoparamoeba perurans*

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ABSTRACT

Oil extracted from cloves, *Syzygium aromaticum*, consists mainly of eugenol, which has been documented to affect protozoa attachment, viability and growth. Isoeugenol (sold under the trade name AQUI-S®) is a commercially available fish anaesthetic produced by isomerisation of eugenol. Both are anaesthetics commonly employed during the husbandry and euthanasia of Atlantic salmon, *Salmo salar* L., used for amoebic gill disease (AGD) research. *Neoparamoeba perurans* (the causative agent of AGD) trophozoites were exposed to clove oil at 10, 20, 40 and 80 μL L^{-1} for 10 min and 5, 10, 20 and 40 μL L^{-1} for 120 min; AQUI-S® at 5, 10, 20 and 40 μL L^{-1} for 10 min and 2.5, 5, 10 and 20 μL L^{-1} for 120 min There were no significant differences in viability and survival of trophozoites after exposure to the anaesthetics. When trophozoites were exposed to clove oil at 80 μL L^{-1} for 10 min there was significantly ($P < 0.05$) more detachment from a plastic surface compared to the control. Continued use of clove oil and AQUI-S® at 40 μL L^{-1} or less for up to 120 min is unlikely to have a detrimental impact on amoebae that are isolated and collected from salmon with AGD to be used for downstream research such as projects involving disease challenges and *in vitro* screening of anti-*N. perurans* compounds.

Statement of relevance: We believe that the study has relevance because it establishes that there were no anti-amoeba properties of eugenol observed at concentrations commonly used and therefore interpretation of previous *in vitro* and *in vivo* work unlikely to be affected as will future work.

1. Introduction

Amoebic gill disease (AGD) is caused by *Neoparamoeba perurans* which affects Atlantic salmon, *Salmo salar* L., during the marine production phase (Crosbie et al., 2012). Research into AGD requires access to the pathogen and this is currently achieved by maintaining Atlantic salmon in an AGD infection tank from which amoebae are obtained from fish, either post-mortem, or when moribund. To ensure supply of the amoeba a clonal culture of *N. perurans* (clone 4) was established on malt yeast seawater agar and Koch's postulates fulfilled soon after (Crosbie et al., 2012). However, virulence of the clone was lost after around 3 years in culture (Bridle et al., 2015). Amoebae harvested from dead salmon are used for experimental AGD challenges, *in vitro* growth studies (Crosbie et al., 2012), and screening of potential therapeutants against the amoeba (Howard and Carson, 1994; Powell et al., 2003; Powell and Clark, 2003; Florent et al., 2010; Adams et al., 2012; Crosbie et al., 2012). Fish infected with *N. perurans* are routinely handled in the course of macroscopic gill observations (Adams et al., 2012) and to obtain gill swabs to determine disease status during *in vivo* experiments.

Exposure of *N. perurans* to anaesthetics occurs when fish are sedated during AGD experiments and when moribund fish are euthanised. Clove oil and AQUI-S® are examples of commonly used fish anaesthetics; both are eugenol-based and demonstrate good efficacy at low concentrations (Iversen et al., 2003). Around 90–95% of clove oil is eugenol (4-allyl-2-methoxyphenol) which is the active component (Briozzo et al., 1989). AQUI-S® was developed based on eugenol and contains 50% iso-eugenol (2-methoxy-4-propenylphenol) and 50% polysorbate 80 (Ross and Ross, 1999). Eugenol has been attributed with anti-bacterial properties (Kalemba and Kunicka, 2003; Rhayour et al., 2003; Walsh et al., 2003; Burt, 2004; Devi et al., 2010), anti-fungal properties (Kalemba and Kunicka, 2003; Pinto et al., 2009), anti-parasitic properties (Al-Yaqout and Azad, 2010) and anti-helminthic properties (Ueda-Nakamura et al., 2006). Unlike anaesthetics such as benzocaine and MS-222 (Tricaine Methanesulfonate), clove oil is inexpensive with no withdrawal time (Ross et al., 2008) and classified to be a substance that is generally regarded as safe (GRAS) by the FDA (Anderson et al., 1997).

It is unknown if eugenol based fish anaesthetics exert any anti-microbial effect on *N. perurans* despite regular usage in AGD experiments. Therefore, the aim of the present study was to determine the effects of clove oil and AQUI-S® on *N. perurans*, both those isolated from dead fish (host-associated) and clone 4 *in vitro* in terms of survival and growth post exposure and the ability to attach to a surface.

2. Materials and methods

Amoebae were obtained from donor fish from the experimental AGD infection tank at the University of Tasmania's aquaculture centre. All fish in this trial were approved for experimentation by the University

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of Tasmania, Australia (Animal Ethics Committee Permit No. A13840). Host-associated *N. perurans* trophozoites were isolated from salmon according to Morrison et al. (2004). Trophozoites of clone 4 were obtained by dislodgement from agar with gentle stream of 0.2 μm filtered seawater (FSW) and then the cell suspension was poured onto clean empty Petri dishes, covered and allowed to adhere for 1 h at 18 °C. Plates were then washed with FSW and cells that had adhered to the plates were dislodged with 0.05% trypsin-EDTA (Gibco). Cells were washed and concentrated by centrifugation (450 ×*g* for 5 min, Eppendorf Centrifuge 5810 R).

Both host-associated and clone 4 *N. perurans* trophozoites were exposed to anaesthetic concentrations which are in routine use for euthanasia and anaesthesia with recovery (Tables 1 and 2).

2.1. Ability to attach to a surface post anaesthetic exposure

To determine the impact on attachment post anaesthetic exposure on both host-associated and clone 4 trophozoites, 100 amoebae in 100 μL of 0.2 μm FSW were inoculated on 96-well cell culture plates (Corning®) in triplicate wells and allowed to attach for 1 h at 18 °C. The initial 100 μL of FSW was then removed and replaced with 100 μL of 0.2 μm FSW containing the various concentrations of anaesthetics and incubated at 18 °C as shown in Table 1. After incubation, the 100 μL of FSW containing anaesthetics and any detached amoebae were removed and replaced with the 100 μL of 0.2 μm FSW. Amoebae that remained attached were counted and the mean numbers of detached amoebae calculated as a percentage of the initial total. FSW only was used as control. Viability of detached cells was assessed as outlined in Section 2.2.

2.2. Anaesthetic exposure and assessment of amoebae viability and growth

For each trial a suspension of 1000 amoebae in 500 μL was placed into triplicate 1 mL microcentrifuge tubes and incubated with 500 μL of either clove oil (CL032 85% eugenol Chem-supply) or AQUI-S® solutions as outlined in Table 2. To remove the majority of the anaesthetic after the designated incubation period, the amoebae were concentrated by centrifuge at 13,000 ×*g* for 15 s, after which 900 μL of supernatant was removed and replaced with 900 μL of 0.2 μm FSW and the suspension was mixed by vortex for 5 s, and then concentrated again by centrifuge as described above. Thereafter the entire supernatant was removed, leaving behind the pellet which was re-suspended in 100 μL of 0.2 μm FSW. Viability of amoebae was then assessed by inclusion of a vital dye (neutral red; Sigma). A 20 μL aliquot of the amoeba suspension was added to 20 μL of neutral red solution (50 μg mL^{-1} in phosphate buffered saline) in a 1 mL microcentrifuge tube and incubated at room temperature for 20 min. Excess dye was then removed by adding 900 μL of 0.2 μm FSW, mixing by vortex for 10 s and centrifugation at 13,000 ×*g* after which 900 μL of supernatant was removed, leaving behind the amoebae. Cells were examined using a haemocytometer and 10 μL of cell suspension and those that had taken up the dye into vacuoles were considered viable. Percentage viability was calculated by dividing total viable count by total number of cells. Viability of amoebae was expressed as percentage viable amoebae.

Table 1
Anaesthetics concentration and exposure time for determination of growth and viability of *N. perurans* trophozoites.

Exposure time	Anaesthetic	Exposure concentration (μL L^{-1})
20 min	AQUI-S®	25
	Clove oil	80
	Control	0.2 μm filtered seawater
120 min	AQUI-S®	5, 10
	Clove oil	20, 40
	Control	0.2 μm filtered seawater

Table 2
Anaesthetics concentration and exposure time for determination of detachment of *N. perurans* trophozoites after anaesthetics exposure.

Exposure time	Anaesthetic	Exposure concentration (μL L^{-1})
10 min	AQUI-S®	5, 10, 20, 40
	Clove oil	10, 20, 40, 80
	Control	0.2 μm filtered seawater
120 min	AQUI-S®	2.5, 5, 10, 20
	Clove oil	5, 10, 20, 40
	Control	0.2 μm filtered seawater

To determine growth, 15 amoebae based on volume of amoebae suspended in FSW from each treatment were inoculated into individual wells of a 96-well cell culture plate (Corning®). Viability of amoebae was determined as described above. Each well was filled with 100 μL of malt yeast agar (0.01% malt, 0.01% yeast, 2% Bacto Agar, 0.2 μm FSW at 35‰ salinity, 18 °C) in triplicate. Each well was then overlayed with 100 μL of 0.2 μm FSW. Total amoebae counts in each well were performed every 24 h for 4 days. Counts were performed using an inverted microscope at 40× magnification; a grid pattern was marked onto bottoms of individual wells to aid counting. Growth was expressed as the mean number of amoebae in each treatment.

2.3. Statistical analysis

One-way ANOVA (SPSS® Version 20; IBM®) was used to determine the differences in amoeba viability, growth and attachment for each treatment. Tukey's HSD was used for comparisons of means where assumptions of normality (Shapiro–Wilk test) and homogeneity (Levene's test) were met. $P < 0.05$ was adopted for rejection of the null hypothesis.

3. Results

Attachment of both host-associated and clone 4 trophozoites was affected when exposed to clove oil at 80 μL L^{-1} for 10 min but not AQUI-S (Fig. 1). Significantly more amoebae had detached after exposure to

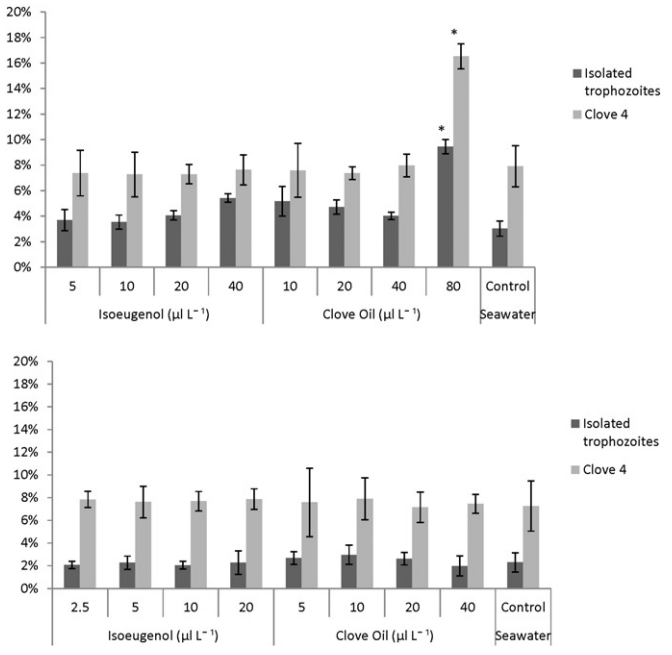

Fig. 1. Percentage of isolated trophozoites and clone 4 trophozoites that detached after anaesthetic exposure for 10 and 120 min. Asterisk indicates significant difference between treatments ($P < 0.05$). Results are presented as mean percentage of amoebae that had detached after anaesthetics exposure ± standard error.