Freshwater treatment of amoebic gill disease and sea-lice in seawater salmon production: considerations of water chemistry and fish welfare in Norway.

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Running head: Freshwater treatments of AGD and sealice

Abstract

Amoebic gill disease (AGD) and sea lice are two of the most significant disease issues facing the Norwegian Atlantic salmon aquaculture industry. Although both diseases respond to various extents, to freshwater treatment, the chemistry, interactions and efficacy of treatment can be variable. These variations can have significant impacts upon the success and failure of treatment and costs to the production cycle. Although it is known that soft freshwater is most effective in bathing of Atlantic salmon with AGD and that most of the freshwaters in Norway fall into the soft category, the low alkalinity and buffering capacity of such waters may impact on the pH and metal toxicity of the water source in use. Similarly dissolved organic carbon can be beneficial in treatment, although sequestration of metal ions can be reversed as the water pH drops due to high densities of fish and accumulations of carbon dioxide. Alternative treatments such as the use of oxidative disinfectants such as hydrogen peroxide used for AGD and sea lice control may have potential although the interactions in seawater with organic loads and dissolved organic carbon are unclear. Similarly the use of oxidative disinfectants in freshwater will depend upon the water chemistry and interactions with treatment chemicals, fish and water organic content. The logistics of treating large biomasses of Atlantic salmon on marine farms is challenging. The use of well boats offers potential although maintaining water quality during treatments is essential for both AGD and sea lice treatments to optimize fish welfare and treatment efficacy.

Keywords: Bath treatments; Water chemistry; AGD; *Paramoeba perurans*; sea lice *Lepeophtheirus salmonis*; Atlantic salmon; *Salmo salar*

1. Introduction

Since 2010, there has been a sharp increase in the number of amoebic gill disease (AGD) outbreaks in Western Europe with Ireland, Scotland (including the Shetland Isles) and the western coast of Norway being affected. In Norway outbreaks increased from 5 in 2012 to 56 in 2013 with a progressive advance northwards of the outbreaks in successive years. AGD is caused by the amoeba *Paramoeba perurans*, first identified as the cosmopolitan agent of AGD by Young et al., (2007). Prior to this, *Paramoeba perurans* had been identified as a potential pathogen in Norwegian salmon, found coincidentally with other causes of gill disease (Steinum et al., 2008), a feature often noted in Irish outbreaks (Birmingham and Mulchay, 2006). The pathology and immunology associated with (*Neo*)*Paramoba* sp. has been well described and subject to several reviews (Powell et al., 2008; Nowak et al., 2014). In short, infection of the gill with *Paramoba perurans* leads to a hyperplastic epithelial response of the gill accompanied by mixed inflammatory and immunological responses (the literature is often conflicting see reports by Bridle et al., 2006; Morrison et al., 2006; Pennachi et al., 2014; Nowak et al., 2014) and an acute systemic hypertension occurring in Atlantic salmon (Leef et al., 2005; 2007). The primary treatments for AGD are in the form of freshwater baths although in Europe some success using hydrogen peroxide bath treatments have been reported.

The treatment of Atlantic salmon using large-scale baths for the control of parasites is not a new concept using freshwater bath treatments for the treatment of AGD and, the control of salmon lice (*Lepeophtheirus salmonis*). Recently, the use of short-term bath treatments of Atlantic salmon during the marine phase of the production cycle has increased drastically in Norway. For example, the use of hydrogen peroxide alone has tripled from 2538 tonnes in 2012 to 8262 tonnes in 2013 (www.fhi.no). The increased occurrence of Amoebic Gill Disease (AGD) and infections with resistant/multi-resistant strains of sea-lice has caused this development. If this treatment strategy is to be developed and applied in the industry, a number of issues concerning water quality on fish welfare and treatment efficiency needs to be addressed, and knowledge-gaps identified.

Subjecting seawater (SW, hyperosmotic) adapted teleost fish to a procedure combining abrupt transfer to a hypo-osmotic freshwater (FW) environment at high fish densities, crowding and handling is a procedure likely to cause a degree of stress in the fish. Maintaining fish in these conditions also cause metabolite accumulation (CO_2/ammonia/ammonium) in the water with subsequent water quality changes that may further aggravate this stress. Thus, a fundamental knowledge about the effects of FW treatment on stress and physiology alone, and combined with water quality changes is needed to ensure fish welfare and optimal treatment effect. Treatment efficacy may also be influenced by the chemical composition of freshwater used. This review aims to summarize current knowledge on the subject.

2. Norwegian freshwater quality

2.1 Chemistry of natural water sources

Norwegian surface waters are characterized by being of low alkalinity and soft, i.e. having a low bicarbonate buffering capacity and consequently low Ca^{2+} and Mg^{2+} content (Henriksen et al., 1989; Skjelkvåle et al., 2007; Kristensen et al., 2009) (Fig. 1). High precipitation rates and low evaporation due to the temperate climate, combined with acidic and weathering-resistant bedrock give rise to this chemical composition of the surface waters. Water pH is therefore also naturally low in many sites (Fig. 1), with additional reductions caused by acidification in the southern and south-western regions (Skjelkvåle et al., 2005). Two major concerns arise from a low buffering capacity and/or pH, namely a strong further pH decrease when CO_2 accumulates in the water and an increased gill permeability caused by low Ca^{2+} saturation of ion channels in the gills (Evans et al., 2005). The first may cause mobilization of metal ions (if present) (Fivelstad et al., 2003a), while the latter results in increased susceptibility to metals (Leivestad et al., 1980). Low pH increases the efflux of Na^+ and Cl^- across the gill surface due to an osmotic gradient of about 350 mOsm L^{-1} between the fish and the freshwater environment (Fig. 1). This problem is exacerbated by H^+ ions competing for gill binding sites with Ca^{2+} (Pagenkopf, 1983; Wilson, 2012). Additionally, metals such as Al may be mobilized to gill reactive forms (http://www.hydrolearth-syst-sci-discuss.net/4/3317/2007/hessd-4-3317-2007.pdf).

Total organic carbon (TOC) levels are, in general, relatively high in Norwegian water sources with a high degree of variability (Fig. 2). Fulvic acids in TOC of humic origin contribute to the low water pH in Norway (Lydersen et al., 2002), and also contribute to transport of associated metals. Metals bound to humic substances are generally less bioavailable than low molecular weight metal species (also denoted free metal ions), and elevated TOC may thus serve to protect fish from harmful effects of metals provided remobilization is not enhanced by decreased pH (Rosseland and Staurnes, 1994; Andren et al., 2006), and/or increased ionic strength (Bjerknes et al., 2003; Teien et al., 2006a) in the water.

Water chemistry is important in the health and physiological integrity of Atlantic salmon when stressed by other pathogens such as sea lice with the main focus studies to date being acidification of freshwater and its associated implication with the mobility of toxic metal ion species such as Al^{3+} (Finstad et al., 2007; 2012) (Fig. 3). In particular the episodic and fluctuating effects of acidified freshwater enhances the stress effects and reduced survival of post-smolts infected with sealice (Finstad et al., 2012). Thus, not all freshwater sources can be deemed suitable or optimal for the treatment of Atlantic salmon in a parasite control regime.

2.2 Metabolite accumulation: effects on water chemistry

Dissolved oxygen levels in the treatment water must be maintained by addition of oxygen gas, and it is vital to maintain levels above 80% saturation. In the following discussion on metabolites, adequate oxygenation is assumed.

Carbon dioxide (CO_2) is generated as the end product of aerobic metabolism in a theoretical molar ratio of 1-0.7 to consumed oxygen. In practice, and in an aquaculture setting, about 1.1 g CO_2 is produced for each mg O_2 consumed (Fivelstad and Binde, 1994). The solubility of CO_2 in water and body fluids is very high due to reaction with water and generation of HCO_3^- and H^+ (the bicarbonate buffering system). Reactions of the bicarbonate system is described (simplified) below (Equation 1)

$$CO_2 \leftrightarrow H_2CO_3 \leftrightarrow H^+ HCO3^- \leftrightarrow 2H^+ + CO_3^{2-} \qquad (1)$$

The amount of CO_2 dissolved in water or blood is through the generation of H^+; a strong determinant of pH. While solubility is high (30 times more soluble than O_2), the gas tension of $CO_{2(g)}$ is low in equilibrium conditions due to low atmospheric partial pressure (~0,04%, 0,04 kPa). However, the relative amount of excreted CO_2 that is converted to HCO_3^- in water is strongly dependent on pH. The pK_a of the first equilibrium-reaction of the bicarbonate system (Equation 1) is about 6.4. This means that a balance between $CO_{2(g)}$, which is the primary concern, and HCO_3^- vary substantially in the low range of pH and buffering capacity values observed. In closed aquaculture transport/treatment systems this has to be accounted for when determining safe biomass/treatment durations.

Gaseous CO_2 accumulation over time in closed treatment systems causes pH depression through H^+/HCO_3^- generation and elevated $CO_{2(g)}$ tension, termed hypercapnia. External hypercapnia forces blood $CO_{2(g)}$ and HCO_3^- levels to increase in order to maintain excretion by diffusion across the gills (Wood and Jackson, 1980; Perry and Gilmore, 2006). Where proliferative gill disease occurs, the accumulation of $CO_{2(g)}$ in the blood is already increased due to diffusion limited CO_2 excretion (see Powell and Perry, 1999; Powell, 2006; 2007), thus under conditions of hypercapnia, the resulting respiratory acidosis (drop in blood pH due to accumulations of $CO_{2(g)}$) is even further enhanced. The rate of CO_2 accumulation is dependent on water volume to biomass ratio, and the metabolic rate of the fish. Metabolic rate depends on temperature, fish size and the state (stress, active swimming) of the fish. For practical purposes, a maximal metabolic rate at a given temperature and fish size should be assumed in bath treatments to provide a safety factor when calculating biomass loading. Equations given in Thorarensen and Farrell (2011) are recommended used for this purpose.

Atlantic salmon is ammonitelic, i.e. excreting the bulk of nitrogenous waste from deamination of proteins as ammonia ($NH_{3(g)}$). A pH dependent equilibrium exists between NH_3 and ionized ammonium NH_4^+ with a pK_a of about 9,2 (Emerson et al., 1975). NH_3 is equilibrated between body compartments while NH_4^+ is distributed

according to pH (Randall and Wright, 1995). The gills are the main site of ammonium and ammonia excretion (Evans, 2005; Terjesen, 2008). Ammonia is excreted through passive diffusion, and ammonium by NH_4^+/Na^+ exchange (Randall and Wright, 1995). The two forms are collectively measured in water as total ammonia nitrogen (TAN) and NH_3/NH_4^+ ratio calculated as a function of pH and temperature (Emerson et al., 1975). Unionized NH_3 is regarded as the main toxic form of the two, and toxicity is therefore highly pH dependent (Thorarensen and Farrell, 2011). The primary toxic effect is regarded to be disruption of oxidative metabolism and draining of energy stores in the brain. Acute responses include disruption of enzyme activity, reduced swimming capacity, increased gill irrigation rate and osmoregulatory disturbances, while chronic exposure reduces growth and disease resistance (reviewed by Thorarensen and Farrell, 2011). Recommended safe levels for salmonids range from 12 to 30 µg NH_3-N L^{-1} (Thorarensen and Farrell, 2011). In closed transport or treatment systems, pH depression will help detoxify ammonia. The main concern arises if aeration is applied to remove CO_2 such that unionised ammonia build-up may cause problems, and if sudden shifts to a higher pH occur, i.e. is seawater is added to the treatment/transport water. Equations in Thorarensen and Farrell (2011) may provide the tools to calculate TAN accumulation. However, the risk of abrupt pH changes must be also taken into account to determine risk for toxic effects.

3. **Amoebic Gill Disease**

Amoebic Gill Disease (AGD) of marine Atlantic salmon is caused by the amoeba *Paramoeba perurans*. *Paramoeba perurans* appears to be a facultative parasite of fish, having been identified to infect a number of different marine and euryhaline species (Nowak et al., 2002) including rainbow trout (*Oncorhynchus mykiss*), brown trout (*Salmo trutta*) (Leef et al., 2005), chinook salmon (*O. tsawyschta*) (Zilberg and Munday 2006), turbot (*Psetta maxima*)(Dykova et al., 1995), Ballan wrasse (*Labrus bergylta)* (Karlsbakk et al., 2013), sharpsnout sea bream (*Diplodus puntazzo*)(Dykova and Novoa, 2001), seabass (*Dicentrachus labrax*)(Dykova and Novoa 2001; Santos et al., 2010), ayu (*Plecoglossus altivelis*) (Crosbie et al., 2010), blue warehou (*Seriolella brama*) (Adams et al., 2008), lumpfish (*Cyclopterus*

lumpus)(L. Andersen pers. comm). It is speculated to have a relatively simple lifecycle, although this has yet to be confirmed. The impact of this pathogen has been seen in Tasmania, Australia since the late 1980s where cost estimates of 10-15% of the value of production being attributed to its control. However, the agent *Paramoeba perurans* and indeed AGD has been recognized and diagnosed in many other countries besides Australia including Chile (Bustos et al., 2011); Ireland, Japan, New Zealand, Norway, USA, Scotland and Spain (Nowak et al., 2002; Young et al., 2007; 2008; Steinum et al., 2008; Nylund et al., 2008).

The pathology of AGD specifically (Powell et al., 2008) and related infectious and non-infectious gill disorders have been widely reviewed in the past (Mitchell and Rodger, 2011; Rodger et al., 2010). Specific to AGD is the interaction of the gill epithelium with *Paramoeba perurans* whereby attachment of the parasite, results in acute cellular necrosis (Powell et al., 2008) and filamental epithelial cell hyperplasia giving rise to a compensatory plaque of tissue infiltrated with inflammatory immune cells and specifically eosinophils (Lovy et al., 2006) that essentially prevents further damage to the gill. The filamental hyperplasia, reduces the functional gill surface area and the associated accumulation in mucus production causes inhibition of carbon dioxide excretion across the gill leading to a persistent respiratory acidosis (Powell et al., 2000) or increases in circulating total CO_2 levels (Leef et al., 2005; Table 1). However, respiratory disturbances are only part of the pathology. In Australian Atlantic salmon, an acute cardiovascular compromise occurs whereby systemic hypertension develops causing to circulatory collapse (Powell et al., 2002; Leef et al., 2005; 2007) and finally death – particularly in fish susceptible to stress (eg. triploid fish, Powell et al., 2008). Indeed, AGD resistence (as determined by gill score of the severity of gill damage) and survival are not related to systemic antibody responses (Taylor et al., 2009a).

This means that assessment of the success of treatments for AGD can either focus upon:

1. The presence or absence of the parasite
2. The presence or absence of gill lesions

3. Fish survival, mortality or other indicators of performance such as appetite or growth

In reality, all three approaches make for a holistic assessment of treatment success. In recent years tools have been developed to assess the presence or absence of *Paramoeba perurans* using real-time PCR of gill swabs or tissue samples (Young et al., 2008). This test has since been commercialized by a number of diagnostic companies. Although exquisitely sensitive; good correlations between actual amoeba numbers, pathology caused and treatment success have not been established.

3.1 Approaches to control of AGD – practical limitations

In commercial farm situations the number of fish to be treated (upto 200 000 individuals), size of the fish to be treated (0.2-7.0 kg), and location of net pens pose significant logistical limitations. The experience from sea lice treatments have given Norwegian farmers extensive experience in handling cage-based bath treatments although the time and expense for treatment of large numbers of Atlantic salmon impose significant constraints upon the frequency, speed and success for treating an entire AGD affected site. Important with any form of bathing treatments, cages of Atlantic salmon need to be confined either by tarpaulin, cage skirt or else transferred to a vessel such as a well boat for treatment. This imposes a handling effect, resulting in acute stress on the fish with the implications to water quality (see discussion above) and fish welfare (see discussion below). Some treatments are likely to be relatively less stressful than other, although even a cage skirt (open at the bottom but enclosing the water at the surface of the cage) can have significant effects on water circulation, oxygenation and stress effects on the fish (Stein et al., 2012). Indeed other studies have suggested that the oxygenation of any treatment is likely to be a constraint to bath treating Atlantic salmon under commercial conditions (Treasurer et al., 2000).

Current treatment practices are to either treat at cage-side or else to remove the cage (or fish) from the main production site to a designated treatment site. In Australia, a country with extensive experience of this practice, cages are towed to a treatment barge prior to treatment. This handling of fish means that feeding is

stopped 24h prior to scheduled treatment, and fish are not fed again until after treatment and replacement of the cage onto the production site. In some cases this may represent a non-feeding period of over 48h with the associated loss of growth. The other implication of this treatment strategy is that typically one cage at a time is treated and then the clean cage is replaced alongside a potentially heavily infected cage. The risk of cross contamination and a re-infection is therefore magnified. Indeed the epidemiology and infection risk associated with the movement of cages, treatment and use of fallowing have been explored (Douglas-Helders et al., 2004) and although clear benefits were seen by placing newly bathed fish onto virgin and partly fallowed sites, the occurrence of AGD was delayed and not prevented – subsequently this practice has been all but abandoned in Australia, citing the lack of non-AGD affected sites or available sites to allow fallowing to occur.

3.2 Freshwater treatment of AGD

In Australia, the treatment of choice for the control of AGD is freshwater bathing. This treatment was quickly identified as the primary control option in the early 1990s. Subsequently, the efficacy of such treatments was characterized (Parsons et al., 2001a and b) and a demonstrated positive physiological effect on the fish shown (Powell et al., 2001). The effects of treatment on re-infection showed that although gill lesions (hyperplastic patches) were removed, amoebic re-colonisation of the gill occurred quickly with amoeba numbers equalling pre-treatment levels in as few as 10 days with the most aggressive infections at the height of the Tasmanian summer (Clark et al., 2003).

The process of freshwater bathing involves the filling of a large plastic tarpaulin (approximately 1 ML) with water piped to the bathing site. Under the tarpaulin is a clean net cage. Fish to be treated are transferred (typically by air-lift pump) to the freshwater filled tarpaulin and maintained for 3-4 h with additional oxygenation. Oxygen levels are targeted at 120-150% air saturation. Following the bathing period the tarpaulin is removed by winch and the fish fall into the awaiting cage. The process takes approximately 1 working day to complete.

Amoebae isolated from the gills of AGD affected salmon (specific diagnostics for *Paramoeba perurans* were not available at the time) were used in a number of short-term, *in vitro* bioassays that allowed determination of some of the key chemical characteristics that favoured survival or killed gill amoebae. Isolated gill amoebae showed resilience to freshwater treatment where the Na^+ concentration was increased, however, the effects of increased concentrations of Mg^{2+} and Ca^{2+} ions significantly promoted survival over 3 hours of freshwater exposure. Even when Na^+ concentrations were relatively high (250 mg L^{-1}), low Ca^{2+} concentrations (10 mg L^{-1}) were as effective as unionized freshwater after 3 hours of freshwater exposure (Powell and Clark, 2003). This suggested that soft freshwater sources were more favourable for the control of AGD in bathing compared to hard water sources. This was confirmed under field conditions where artificially softened freshwater (ion exchange with Na^+) (Roberts and Powell, 2003a) and the selection of soft freshwater sources produced increased efficacy of bathing and a 113 degree day (13% increase in the inter-bath interval) delay in subsequent bathing (Powell et al., 2005). Other approaches of removing Ca^{2+} from freshwater and seawater were also examined using the ionic chelator Calgon T™ although at effective concentrations (6 mg L^{-1} per mg L^{-1} of hardness) to soften hard water (225 mg L^{-1} $CaCO_3$ equivalents) the resultant discharge of phosphate and cost would be prohibitive under commercial conditions (Powell et al., 2005).

Dissolved organic carbon (in the form of humic and tannic acid) has been shown to enhance the efficacy of freshwater at killing Paramoebae (Green et al., 2005). In combination with different concentrations of Ca^{2+}, a combination of soft water with high concentrations of organic acids resulted in the best conditions for killing amoebae in freshwater both in vitro as well as in an experimental freshwater bath (Green et al., 2005). The effects of DOC also resulted in the decrease in the number of hyperplastic gill lesions following the bath. The mechanism by which this effect acts is unclear but it is possible that the organic acids (tannic and humic) resulted in chelation of divalent cations so enhancing the efficacy of the freshwater treatment. Alternatively, the organic acid load may have had a direct toxic effect on the amoebae (Green et al., 2005).

The effects of combining freshwater with oxidative disinfectants have been investigated with some limited success. It was found that chloramine-T and hydrogen peroxide both enhanced the efficacy of freshwater baths, although the benefits were small (Powell and Clark, 2002). However, the variability in treatment success may have been as a result of different water qualities. Combined treatments offer the advantage of ensuring that amoebae are killed once removed from the gills of affected fish so reducing the chance of re-infection once the bath is ended.

3.3 Oxidative disinfectants for AGD control in seawater

Alternatives to freshwater bathing have included the investigation of a number of oxidative disinfectants. In general, most oxidative disinfectants work through the release of reactive oxygen or chlorine species thus destabilizing or permeating cell membranes. Two main oxidative disinfectants have been the focus of significant investigation for the control of AGD: Chlorine-based Chloramine-T (*n*-sodium-*n*-chloroparatoluenesulphonamide) and oxygen-based hydrogen peroxide (H_2O_2). Chloramine-T, hydrogen peroxide and chlorine dioxide were all toxic *in vitro* to isolated gill amoebae from AGD-affected salmon primarily *Neoparamoeba pemaquidensis* (Howard and Carson, 1993; Powell and Clark, 2002; 2003) and specifically hydrogen peroxide with *Paramoeba perurans* (Adams et al., 2012). Preliminary tests with fish suggested that AGD was reduced when added to enhance the efficacy of freshwater baths (Powell and Clark, 2002).

In vitro and medium scale treatment investigations with chloramine-T demonstrated that chloramine-T was acutely toxic to isolated gill amoebae (Powell and Clark, 2003). In small scale field studies, exposure of AGD affected Atlantic salmon to chloramine-T at 10 mg L^{-1} for 1 hour in seawater were moderately successful with significant reductions in gill amoeba numbers (Harris et al., 2004; Harris et al., 2005). Furthermore, treatment of Chinook salmon (*Onchorynchus tsawhytcha*) smolts was also successful at removing gill amoebae with minimal adverse effects, although AGD in this species is not believed to be a significant health issue (Powell personal observations). However, the toxicity of chloramine-T to Atlantic salmon smolt is enhanced in seawater and further enhanced when oxygen levels are increased to

200% air saturation (Powell and Harris, 2004). Moreover, attempts to upscale chloramine-T treatment to commercial operations proved challenging with mixed results and have not been developed further (Powell personal observations).

Hydrogen peroxide has been tested in salt water laboratory bath treatments of AGD affected Atlantic salmon smolts at 10 and 15^{o}C with post-treatment survival dependent upon exposure duration. Toxicity increased with exposure time at both temperatures at concentrations exceeding 1000 mg L^{-1}. However, total gill amoebae counts were not significantly reduced compared to untreated controls and the variability between fish was high (Powell et al., 2005). More recently this approach has been re-examined suggestion that at 12^{o}C hydrogen peroxide (1250 mg L^{-1}) for 15 min reduced the number of gill lesions (Adams et al., 2012). However, as with the previous study (Powell et al., 2003) the results were variable with slightly longer durations of exposure resulted in a highly variable efficacy (Adams et al., 2012). Observations from hydrogen peroxide treatments in Norway against AGD indicate a similar result with the persistence of lesions and varied efficacy against controlling AGD (T.A. Mo unpublished report 2014).

Oxidative disinfectants (such as chloramine-T and hydrogen peroxide) have a number of well documented patho-physiological effects on healthy and damaged gill tissue of salmonids in both fresh (Powell and Perry, 1995; 1997a, b and c) and marine conditions (Powell and Harris, 2004). In general, the effects of acute oxidative disinfectant exposure include an acute congestion of the gill filament and central venous sinus, most typically as a result of an increased vascular pressure caused by elevated cardiac output or intra-branchial pressure increased due to adrenergic responses induced by the release of adrenalin and noradrenalin. The gill lamellar epithelium is often either crenated or denuded, often associated with epithelial cell necrosis. The consequences of this in freshwater is a net influx of water (and efflux of plasma electrolytes), increased vascular volume, haemolysis and eventual cardiovascular collapse due to increase vascular viscosity and ultimately haemostasis. Under marine conditions, haemo-concentration occurs with an apparent efflux of water, and potential influx of Na^{+} and Cl^{-}, an associated hyper-

natriuremia and ultimately electrolytic imbalance resulting in death. Sublethal effects of oxidative disinfectants result in permeablisation of epithelial cell membranes and trans-cellular efflux (freshwater) or potentially influx (seawater) of Na^+ and Cl^- ions. This process results in acid-base disturbances (Powell and Perry, 1997d;1998). These ionic disturbances are often manifest in hypertrophy of chloride and mitochondrial rich cells in the gill (Powell and Harris, 2004).

4. Sea lice

The issue of sea lice infestation of farmed Atlantic salmon (primarily by *Lepeophtheirus salmonis* in the Northern hemisphere and *Caligus rogercresseyi* in the Southern hemishere) has been a constant challenge for commercial farm production of the species for many decades. The primary issue is that the salmon is infected by a free swimming phototactic nauplius stage that moults and attaches onto a host as the attached chalimus stage, after successive moults, the motile copepodite stages and adult stages move over the epithelial surface of the salmon grazing upon the skin and mucus. The subsequent result is acute erosive lesions leading to osmoregualtory stress. Although much has been discussed regarding the immune responses to infestation, control measures still rely mostly on chemical de lousing and disinfection to control the level of infestation of fish on commercial farms.

4.1 Approaches to control of sea lice – practical limitations

Sealice control under commercial farm conditions in some countries is highly regulated. Typically the number of gravid female lice is the primary treatment trigger and the issue of surveillance and accurate enumeration has recently been in focus (Heuch et al., 2011; Revie et al., 2007). Of particular importance, as with bathing fish for AGD treatments, cages of Atlantic salmon need to be confined either by tarpaulin, cage skirt or else transferred to a vessel such as a well boat for treatment. The consequent handling effects result in acute stress on the fish (see discussion below) with implications for water quality (see discussion above). Some treatments are likely to be relatively less stressful than other, although even a cage skirt as discussed above.

4.2 Freshwater treatment of sea lice

Exposure of sea louse infested Atlantic salmon to salinities below 29 ppt results in the gradual loss of sea lice. *Lepeophtheirus salmonis* is rapidly killed in freshwater (Connors et al., 2008), and the survival of free-swimming copepodids was severely compromised at salinities below 29 ppt, irrespective of their attachment to a host (Bricknell et al., 2006). However, earlier work by Stone et al. indicated that short duration (3 h) bath exposure was likely ineffective treatment for controlling sea lice (Stone et al., 2002). Little work has been undertaken with respect to commercial treatments for sea lice with freshwater where the water chemistry and its implications have been examined. However, it should suffice that similar constraints of water quality and chemistry as those discussed above should remain valid.

Recent studies to assess the potential for using freshwater to remove attached sea lice from infected Atlantic salmon under commercial conditions using well-boats have been undertaken. Exposing lice infected salmon to freshwater in laboratory-scale tests resulted in a significant reduction of both mature male and female *Lepeophtheirus salmonis* after 3 h. In parallel *in vitro* bioassays using freshwater, after 1 hour of exposure to freshwater, 10% of mature females but 90.9% of mature males were dead (P. Reynolds unpublished). Further studies under commercial conditions using well-boats assess the potential for using freshwater under commercial-scale scenarios with initially a total biomass of 15 tonnes and subsequently 110 tonnes of fish were exposed to freshwater. These studies showed that a significant biomass of Atlantic salmon (up to 110 T) could be successfully deloused with freshwater (Fig. 4). The reductions in attached stages recorded immediately after the fish were pumped from the cage and before exposure to freshwater for study two can be attributed to mechanical action (e.g. physical contact from crowding, contact with the inner surface of the pipes used to pump the fish and contact with the grading platform). The percentage reductions recorded for chalimus, pre-adult and mature female stages were 77%, 30% and 14% respectively, giving a total reduction for all stages of 39%. These data mirror previous observations where transferring fish from one cage to another or crowding the fish resulted in reduction of up to 40% of sea lice compared to pre-count levels of infestation. It appears that the

chalimus stages are more likely to be removed from salmon than the other later developed stages. This may be partially explained due to the site of attachment as pre-adults and particularly mature female lice seem to preferentially choose attachment sites on areas where they are subjected to less mechanical and/or environmental perturbations (behind the dorsal, pectoral and anal fins). It may also be partially attributed to the fact that the later developed stages are more robust and can withstand greater mechanical stressors compared to chalimus stages.

During freshwater bathing treatments for sea lice, initial oxygen concentrations equivalent to air saturation of 124.0% were achieved at the start of the bath treatment, and declined to 84.0% at which point oxygen was added and levels increased to 101.0% quickly thereafter for the 3 h duration of the treatment. However, pH levels steadily decreased to 6.08 during the exposure period and CO_2 concentrations of between 16.0 and 17.0 mg L^{-1} at the end of the treatment. The recommended safe limits for CO_2 used for the Norwegian production of Atlantic salmon smolts is 15 mg L^{-1} (Fivelstad, 2013) provided dissolved oxygen concentrations are high. However, constant fish respiration can raise carbon dioxide levels high enough to interfere with oxygen intake by fish, in addition to lowering the pH of the water. Maintaining a sufficient pH and minimising the pH effects of CO_2 in solution requires buffering or neutralisation of the acidic protons. Since many Norwegian freshwaters have low alkalinity (approximately 70% have less than 100 µM Fig 1), a potential option to prevent fluctuations in pH is to add sodium hydroxide (NaOH) to neutralise the acidity. Available commercially as a 50% (by weight) saturated solution, it is commonly used to titrate the acidity in the water at smolt facilities which use recirculation systems to help maintain safe pH levels throughout production. Alternately, the addition of buffering agent such as calcium carbonate or hydroxide as a lime slurry could be used.

Application of NaOH to maintain pH when freshwater treating a large biomass of salmon in a commercial well boat showed that initially there was a small decrease in pH in both wells once fish transfer had been complete and prior to the addition of NaOH. The addition of NaOH approximately I.5 h after the fish had been transferred

to both wells at a rate of 0.25 L h^{-1}. The decline in pH slowed after the addition the decrease in pH continued however, despite increases in the rate at which NaOH was added. Although the titration of the pH was affective under commercial conditions, further investigation is required to elucidate flow rates and how much to add to maintain safe levels throughout a desired treatment period of approximately three hours.

4.3 Oxidative disinfectants for control of sea lice in seawater

Among a number of chemical treatments for sealice (for example see reviews by Torrisen et al., 2013; Burridge et al., 2010; Robertson et al., 2009), hydrogen peroxide has been identified as a potential disinfectant (Toovey et al., 2000; Treasurer and Grant, 1997; Johnson et al., 1993). Primarily, hydrogen peroxide is believed to act by either killing the copepod directly through oxidation of cell membranes, or else causing it to detach from the skin surface (Torrisen et al., 2013). Although having a relatively narrow window of safety, treatments up to 1500 mg L^{-1} for up to 20 min (depending upon temperature) are reported. The challenge remains to introduce, distribute and mix sufficient quantities of chemical into a bath treatment and eliminate the residual peroxide following the treatment, to prevent overdosing the salmon. Thus it is generally considered more effective against the motile stages of the parasite rather than the attached chalimus stages. However, recently the success of using hydrogen peroxide has been questions with evidence of resistance developing in sea louse populations Treasurer et al., 2000) and limited success of this treatment option with other species besides *Lepeophtheirus salmonis* such as against *Caligus rogercresseyi* in Chile (Bravo et al., 2010).

5. Implications for fish welfare

Atlantic salmon are a euryhaline species and post-smoltification exhibit a high capacity for ion and osmoregulation. The transfer of AGD affected post-smolt salmon into freshwater results in an acute net efflux of Na^+ and Cl^- ions that peaks at 2 h post-transfer and is quickly reduced by 3 h exposure (Roberts and Powell, 2003b). The net titrateable alkalinity flux was significant for both AGD affected and non-affected salmon with AGD affected fish having a larger net titrateable alkalinity

efflux (Roberts and Powell, 2003b). Up to a gill score of 2 (according to the Taylor et al., 2009b criteria) ionic disturbances associated with AGD are relatively minor (Fig. 4). However, with more severe AGD, more severe disturbances occur with increases in blood sodium being pronounced (Table 1). A hypo-osmotic challenge under these conditions, where ion-regulatory capacity of the gill may be compromised, may result in significant osmotic stress for the fish during treatment. However, one of the favourable effects of freshwater bathing is the enhanced break-up of the hyperplastic gill lesions (Roberts and Powell, 2003a) and physiological disturbances caused by AGD including respiratory acidosis and accumulations of CO_2 (Table 1) are reduced (Powell et al., 2001). Additionally, hyperoxic freshwater bathing also reduced the systemic hypertension in AGD–affected Atlantic salmon (Powell et al., 2002).

Despite acute short-term physiological effects of freshwater treatments for treating AGD and de lousing of sea lice there may be advantages compared to current chemical methods (e.g. H_2O_2) used in the industry at present. Results from blood analysis undertaken during a pilot-scale freshwater delousing study indicated that handling in freshwater resulted in minor physiological disturbances consistent with a stress response with an elevation in blood glucose, CO_2 and reduction in blood pH (Table 2). Further handling and replacement of fish back into seawater resulted in an increase in blood Na^+ concentrations consistent with acute hyperosmolality stress.

6. **Conclusions**

Freshwater treatments of Atlantic salmon smolts affected by AGD and sea lice (although the beneficial effects are still under investigation) are effective and generally pose low risk to overall fish health with a widely accepted large margin of safety, despite some short term physiological effects. However, there are a number of potential risks and large gaps in the knowledgebase surrounding bath treatments for this disease. Despite the work carried out so far as presented in this review and summarized in a number of reports (Powell and Clark, 2002, Powell et al., 2005; Powell et al., 2007), the water characteristics of Norwegian freshwater sources have not been previously considered prior to undertaking bath treatments. Similarly, the scale of Norwegian production and logistics of handling and treating large volumes of

fish has not been taken into account in attributing best practice for freshwater or H_2O_2 bathing AGD and, to a limited extent, sea lice treatments. In particular, the interactions between dissolved organic carbon (DOC) and particulate organic carbon (POC) and both divalent cations (e.g. Ca^{2+} and Mg^{2+}) and metal ions such as Fe^{2+} Cu^{2+} and Al^{3+} have not been investigated with respect to the effects on *Paramoeba perurans*, efficacy of treatment and lesion resolution post-treatment and physiological effects in the fish. Additionally, interactions between the water chemistry, effects of organic load, fish biomass, temperature, salinity, oxygenation status and treatment chemicals such as H_2O_2 are poorly understood. Interactions between these parameters may account for the variation in efficacy of treatments and apparent toxicity often observed with oxidative disinfection. In this respect it is imperative that these factors be investigated with respect to each other so leading to improvements in bath efficacy, treatment safety, and fish welfare.

7. **Recommendations**

1. Water sources for freshwater bathing target optimal water chemistry and quality (summarized in Table 3). These parameters should include low Ca^{2+} and Na^{2+} content (characteristically soft waters) with a moderate pH optimally approximating 6.5. The water source would benefit from a high dissolved organic carbon content although it is recognized that the interactions of metal ions, divalent base metal ions (e.g. Ca^{2+} and Mg^{2+}) are unknown at pH values characteristic of bathing operations. Water sources with high alkalinity should be targeted although these often are buffered mostly by $CaCO_3$ and thus the risk is increases in the Ca^{2+} concentration. Alternatives may include the use of other buffering/neutralizing agents added to the bath water (e.g. NaOH, Na_2CO_3, $NaHCO_3$) although these have not been tested on a commercial scale. The use of carbonate based buffers would require the use of an active degassing process to facilitate the stripping of CO_2 produced in the buffering process.
2. It is recommended that all water sources used for commercial bathing of Atlantic salmon should be analysed as close to the time of use (for bathing) for water chemistry characteristics. Many of the historical analyses may be

changed over time depending upon catchment use, seasonal and annual fluctuations in rainfall etc.

3. Aversive action with fresh water treatments are rarely required based upon the experiences in Tasmania although large drops in pH and oxygen saturation would be clear indicators of failed handling of fish during the treatment. It should also be noted that the more severe the AGD score, the greater the risk of respiratory compromise in the fish and resultant treatment-related mortality.
4. The continued investigation of water quality and chemistry parameters and their interactions in freshwater treatment baths to optimize treatment efficacy and identify constraints for the control of AGD.
5. Use of existing information and the further investigation of water chemistry interactions in treatment efficacy to further develop an “industry best practice” for AGD treatments for the control of gill health on Norwegian sea farms.
6. The incorporation of farm site and environmental data into best practice AGD treatment including preceding environmental characteristics (e.g. algal blooms, jellyfish, freshwater runoff, DOC, temperature, salinity etc).
7. The up-skilling and further training of farm personnel in the diagnosis of AGD, scoring of gill lesions, handling of fish and monitoring of water quality and fish welfare during treatment baths.
8. The development of novel, alternatives to freshwater bathing (e.g. In-feed treatments) that could be used in conjunction with topical treatment of the sustainable control of AGD.

Conflicts of interest

There were no known conflicts of interest

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Figure legends

Figure 1. Range (± min/max) of Ca^{2+} (A), Na^{+} (B), concentrations pH (C) , alkalinity (D), total organic carbon (TOC) (E) and total Al concentrations (F) in Norwegian fresh waters. Adapted from Kristensen et al. (2009).

Figure 2. Gill bound aluminium concentrations (± 1 SD) with respect to salinity in Atlantic salmon. Adapted from Åtland et al. (2012).

Figure 3. Relationship between percentage of AGD lesioned gill filaments and plasma osmolality of Atlantic salmon over an 11 day AGD-challenge. (Pearson correlation coefficient 0.482, P value <0.01; y = 365.43 + x1.40, r^2 = 0.230)

Figure 4 Average number of chalimus, pre-adult, mature female stages and all counted stages of *L. salmonis* per fish recorded prior to treatment, immediately after pumping and after exposure to freshwater. Values represent means ± S.D. Mean values which do not share a letter were found to be significantly different by ANOVA and by Tukey`s multiple range test.

Fig 1

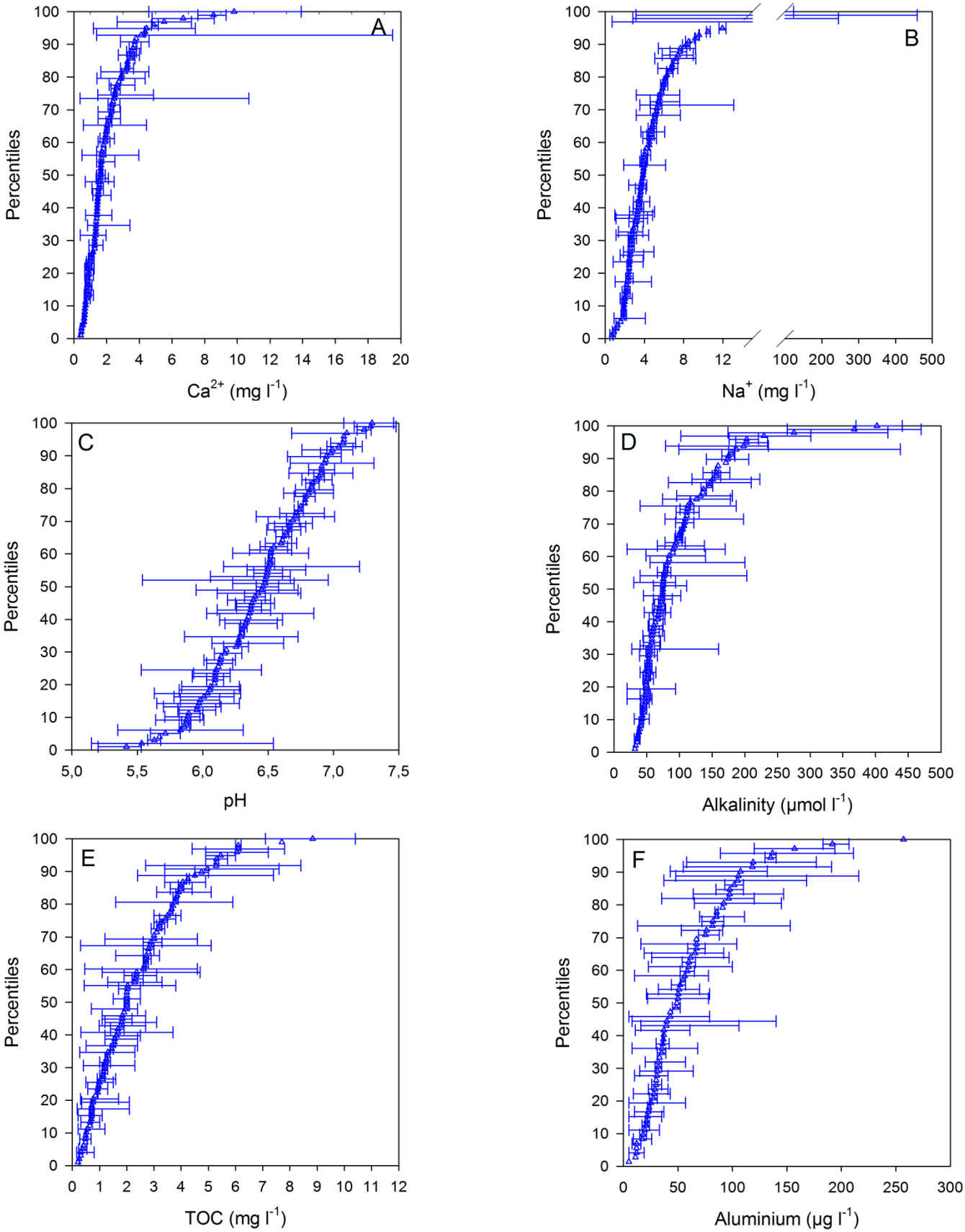
A
B
C
D
E
F
Percentiles
Ca2+ (mg l-1)
Na+ (mg l-1)
pH
Alkalinity (µmol l-1)
TOC (mg l-1)
Aluminium (µg l-1)

Fig 2.

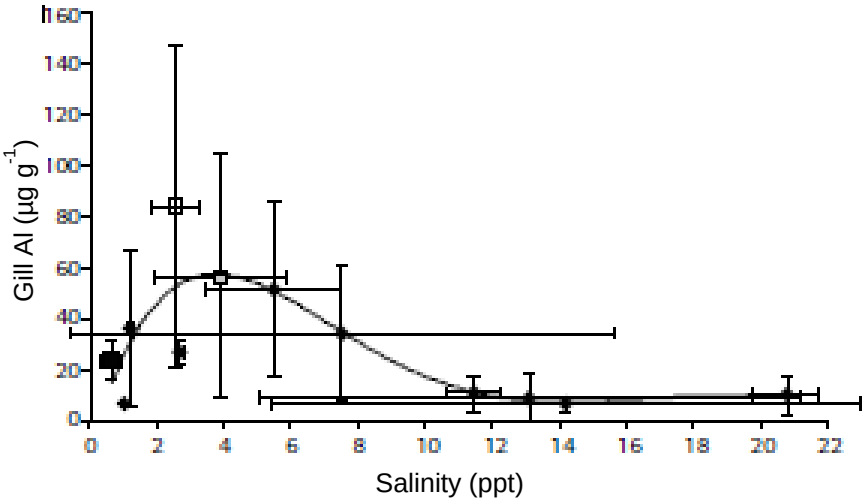
Gill Al (µg g-1)
160
140
120
100
80
60
40
20
0
0
2
4
6
8
10
12
14
16
18
20
22
Salinity (ppt)

Fig 3.

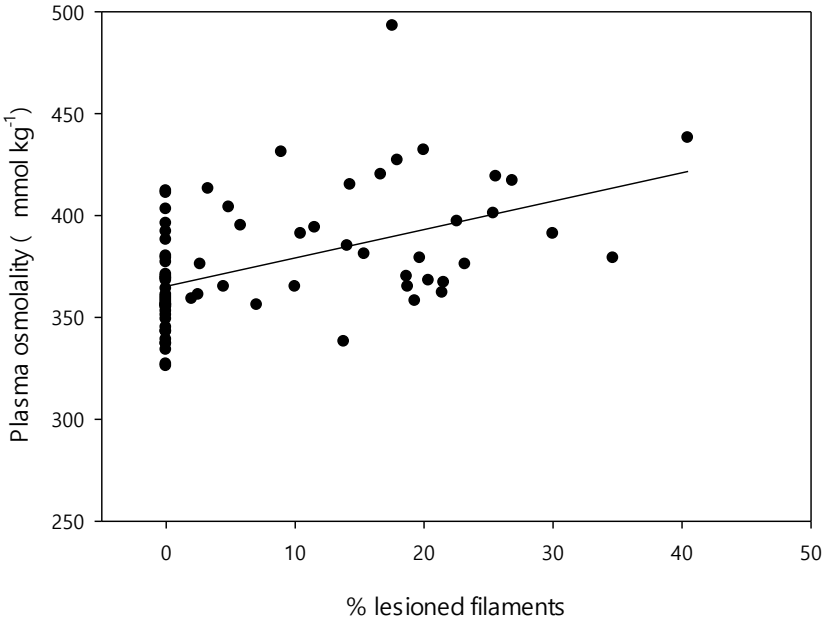
500
450
400
350
300
250
Plasma osmolality (mmol kg-1)
0
10
20
30
40
50
% lesioned filaments

Fig 4.

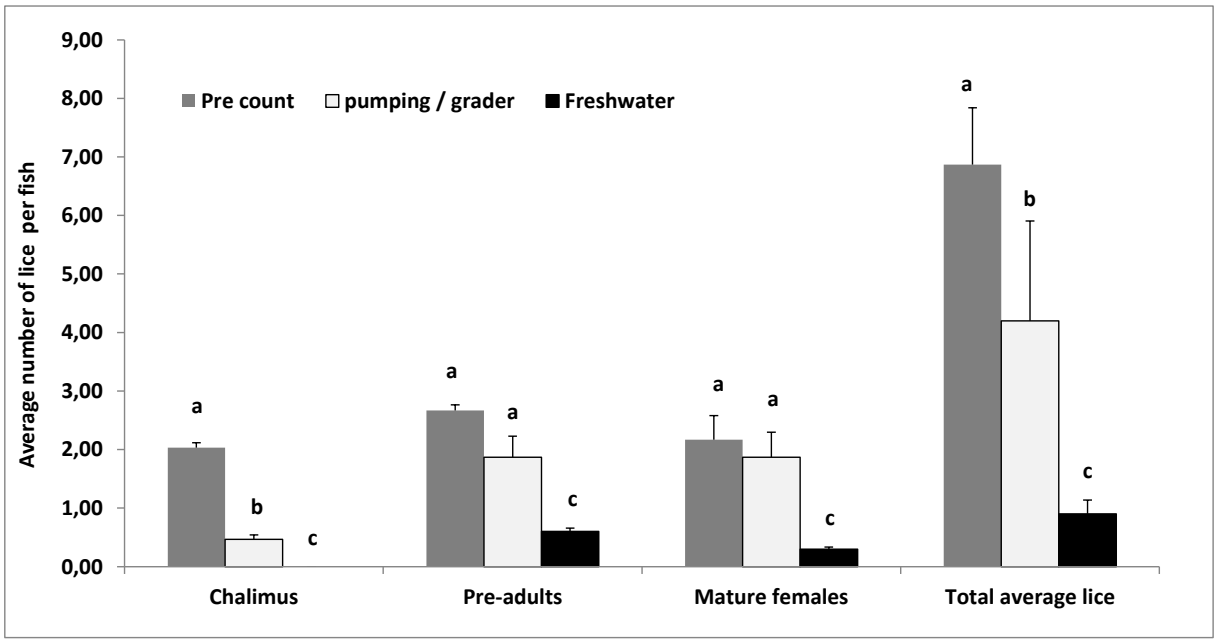
9,00
8,00
7,00
6,00
5,00
4,00
3,00
2,00
1,00
0,00
Average number of lice per fish
Pre count
pumping / grader
Freshwater
a
b
c
a
a
c
a
a
c
a
b
c
Chalimus
Pre-adults
Mature females
Total average lice

Table 1. Mean ± SEM with sample size (*n*) of caudal blood values for fork length (FL cm), mean gill score, blood Na^+, K^+, total CO_2 content, glucose and HCO_3^- (mM), hematocrit (%), pH and PCO_2 (kPa) corrected for temperature (15°C) of Atlantic salmon smolts acutely affect by AGD (days post-infection, dpi) at 34 ppt salinity. Superscripts indicate significant differences relative to controls.

	(*n*)	FL	Score[1]	Na^+	K^+	TCO_2	HCO_3^-	Glucose	Hct	pH	PCO_2
7 dpi	(5)	28.7	3.60^b	161.8^{ab}	4.7	5.00^a	3.75^a	6.8	25.5	6.821^a	0.769
		± 0.7	± 0.20	± 1.8	± 0.4	± 0.00	± 0.22	± 1.3	± 1.4	± 0.019	± 0.010
18 dpi	(8)	29.4	4.75^b	175.9^b	4.5	6.25^b	5.24^b	5.9	25.5	6.865^b	0.873
		± 0.7	± 0.25	± 2.4	± 0.1	±0.36	± 0.26	± 0.4	± 2.2	± 0.019	± 0.024
Control	(6)	29.5	0.00^a	155.0^a	4.4	5.17^{ab}	3.57^{ab}	5.4	26.7	6.742^c	0.825
		± 0.9	± 0.00	± 2.0	± 0.2	± 0.17	± 0.23	± 0.2	± 2.7	± 0.015	± 0.032

[1] based on Taylor et al. (2009)

Table 2. Mean (± SEM) blood parameters as measured by iSTAT demonstrating the effects of a single handling (1x) after 15 min and 1 h into a freshwater bath, or double handling (2x) where fish were transferred back to seawater after 15 min or 1 h in freshwater on adult marine *Lepeophtheirus salmonis* infested Atlantic salmon. Values with differing superscripts indicate significant differences from pre- treatment values.

Treatment	**Na^+ mM**	**K^+ mM**	**TCO_2 mM**	**Glucose mg/L**	**Hct %**	**pH**	**PCO_2 kPa**	**HCO_3^- mM**	**Hb g/100mL**
Pre	155.1[a]	4.06	9.6	78.9[a]	26.9[a]	7.353[a]	1.094[a]	9.11	9.14
	(± 0.7)	(± 0.24)	(± 0.4)	(± 2.2)	(± 1.0)	(± 0.033)	(± 0.002)	(± 0.37)	(0.34)
1h 1x handling	152.6[a]	3.56	10.6	97.4[b]	27.4[a]	7.213[b]	1.086[b]	9.72	9.30
	(0.6)	(0.18)	(0.9)	(3.8)	(0.9)	(0.045)	(0.003)	(0.81)	(0.30)
1h 2xhandling	162.4[b]	4.16	10.8	96.6[b]	27.6[a]	7.202$_b$	1.085[b]	9.94	9.42
	(2.5)	(0.30)	(0.8)	(4.4)	(1.9)	(0.018)	(0.001)	(0.70)	(0.63)
15 min 1x handling	158.6[a]	3.02	10.2	91.4[b]	31.6[b]	7.119[b]	1.080[b]	9.18	10.76
	(0.7)	(0.37)	(0.5)	(3.0)	(0.8)	(0.019)	(0.001)	(0.38)	(0.28)
15 min 2x handling	166.4[b]	4.24	9.0	89.0[a]	31.0[a]	7.145[b]	1.082[b]	8.14	10.54
	(1.3)	(0.72)	(0.7)	(3.5)	(0.8)	(0.038)	(0.002)	(0.61)	(0.27)
ANOVA	F 19.267	H 7.452	H 4.44	F 6.777	F 3.617	F 8.798	F 8.775	F 1.335	F 3.600
df	4,29	4	4	4,29	4,29	4,29	4,29	4,29	4,29
P value	<0.001	0.114	0.350	<0.001	0.019	<0.001	<0.001	0.284	0.019

Table 3. Recommended values and limits for water chemistry of freshwater baths for the treatment of AGD affected Atlantic salmon with gill scores < 3[1] and the percentile of Norwegian waters with the appropriate characteristics. Blank boxes mean no action can be expected to be taken.

Parameters	**Prior to bathing**	**%ile of waters**	**During bathing**	**Aversive action options**
Conductivity	< 500 µS cm^{-1}	0-100	< 1000 µS cm^{-1}	Add low conductivity water to treatment Continue treatment monitoring closely
pH	6.0-6.7	19-80	6.0-6.8	Increase buffer capacity if possible Continue treatment monitoring closely Terminate treatment
ORP[2]	FW[3] 40-100 mV SW[3] 140-170 mV		< 350 mV[4]	Terminate treatment immediately
TOC/DOC	< 3 mg L^{-1}	0-70	If possible sample for later analysis	
Ca^{2+} concentration	< 10 mg L^{-1}	0-100	If possible sample for later analysis	
Na^{2+} concentration	< 10 mg L^{-1}	0-90		
O_2 saturation	90-110%		90-110%	Increase oxygen input and solubilisation
CO_2 concentration	< 5 mg L_{-1}		< 25 mg L^{-1}	Actively de-gas using compressed air Continue treatment monitoring closely Terminate treatment
Water characteristics	Freshwater < 5ppt salinity		Freshwater < 5ppt salinity	

[1] The gill score is likely to have a direct effect upon the resultant mortality associated with a treatment bath. With a higher gill score, the risks of respiratory compromise and cardiovascular collapse is increased.

[2] Recommended monitoring continuously when using H_2O_2 treatments as an indicator of oxidative toxicity. This can be used in combination with Total Residual Oxidation measurements using spectrophotometric analysis.

[3] Values will vary between different water sources and decision s and actions should be dependent upon and relative to baseline values

[4] Critical value based upon data from Harris et al. (2004).