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Inflammatory reactions in rainbow trout fins and gills exposed to biocides

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ABSTRACT: Several biocides are widely used in rainbow trout aquaculture against various ectoparasites and ectobionts, but the inflammation induced in treated fish is less well described. Dose-response studies were conducted to elucidate the effects on rainbow trout (gills and fins) induced by a series of biocides including formalin, hydrogen peroxide (H_2O_2), peracetic acid (PAA) and the surfactant SPH6, which was isolated from the bacterium *Pseudomonas* H6. The compounds have documented antiparasitic effects, but the specific effects on fish needs further documentation. This study was performed over 24 h, and inflammatory reactions were evaluated in gills and fins. A dose-dependent effect was noted for expression of immune genes encoding for IL-1β, TNFα, IFNγ, IL-10, IL-8, lysozyme, serum amyloid A (SAA), hepcidin, precerebellin and complement factor C3. PAA induced the strongest upregulation of cytokine and acute phase reactant genes followed by H_2O_2 and formalin. SPH6 showed a lower effect, and in several cases the compound induced downregulation of several genes. Gills showed a stronger response compared to fins. The mucous cell density in fins showed a range of changes which varied by compound. PAA, and to a lesser degree H_2O_2 and formalin, initially induced mucous cell hyperplasia, whereas SPH6 immediately decreased the number of cells containing mucus.

KEY WORDS: Biocides · Inflammation · Cytokines · Gene expression

1. INTRODUCTION

Aquaculture production worldwide is challenged by a range of viral, bacterial and parasitic diseases (Woo et al. 2020), as systemic infections caused by microorganisms can elicit significant morbidity and mortality (Assefa & Abunna 2018). Even subacute infections may be associated with stress and thereby reduced growth and suppressed immunity in farmed fish (Tort 2011). In addition, freshwater fish production, in both classical ponds/raceways as well as modern recirculated aquaculture systems (RAS), can suffer from epibiont colonization of fish surfaces including amoebae, flagellates and ciliates (Buchmann & Bresciani 1997, Dyková et al. 2010, Jensen et al. 2020). Fish tank water in RAS may also show elevated levels of bacteria (Becke et al. 2020) and occurrence of harmful algae (Moestrup et al. 2014). Colonizing protozoans can reduce oxygen uptake by the gill and elicit inflammation in mucosal fish surfaces (Buchmann et al. 2004a, Jørgensen et al. 2009, Chettri et al. 2014). For example, the infective stages (theronts) of the intradermal parasitic protozoan *Ichthyophthirius multifiliis* can invade the fish surface and cause severe inflammation (Jørgensen et al. 2018). This is a highly pathogenic parasite, causing high mortality at high infection rates, which emphasizes the need for reduction of microorganisms in rearing water. Even non-viable and inert particles in the fish tank water elicit inflammatory reactions in

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fish gills (Lu et al. 2018). This is the basis for frequent use of biocides (formalin, hydrogen peroxide [H_2O_2] and peracetic acid [PAA]) at the farm level (Polinski et al. 2013, Lieke et al. 2020), aiming at reducing the concentration of infective stages in rearing water (Rintamäki-Kinnunen et al. 2005). The effect of some of the biocides on the microorganisms, such as ciliates (Buchmann et al. 2003, Meinelt et al. 2009, Bruzio & Buchmann 2010), oomycetes (Jussila et al. 2011, Straus et al. 2012), flagellates (Jaafar et al. 2013) and amoebae (Jensen et al. 2020) is well documented, but the use has been questioned due to environmental and occupational health concerns. The compounds are degraded in the environment (Pedersen et al. 2007, 2013), but depending on exposure time and concentration, usage may induce injuries in surfaces of fish or induce stress and thereby challenge the welfare of the fish (Buchmann et al. 2004b, Jørgensen & Buchmann 2007, Liu et al. 2017). PAA and H_2O_2 are considered to be more environmentally safe than formalin, since degradation of PAA results in acetic acid and H_2O_2, which eventually degrades to water, oxygen and carbon dioxide. The search for novel and less environmentally detrimental compounds has recently revealed that a novel lipopeptide biocide, isolated from a bacteria *Pseudomonas* H6 strain (SPH6), is effective against both *I. multifiliis* (Al-Jubury et al. 2018), amoebae (Jensen et al. 2020) and the oomycete *Saprolegnia* (Liu et al. 2015). This biologically derived compound may be applied as a control agent in farm settings, but before implementing large-scale usage, any effect on fish health, survival and fish welfare must be fully documented. The inflammation induced in fish surfaces exposed to biocides can be measured by determining the expression levels of genes encoding pro-inflammatory cytokines and acute phase reactants. The present study examined the effects of SPH6, PAA, H_2O_2 and formalin on the expression of these innate immune genes in rainbow trout gills and skin and the mucous cell density in fins over a 24 h exposure period.

2. MATERIALS AND METHODS

2.1. Ethics and legislation

The experiments were conducted under license 2019-15-0201-00388 issued by the Experimental Animal Inspectorate, Veterinary and Food Administration, Denmark. The general welfare laboratory guidelines at the University of Copenhagen were followed, which require that fish showing any abnormal clinical signs be taken out from the study and euthanized immediately.

2.2. Experimental fish

Rainbow trout *Oncorhynchus mykiss* were hatched (at 7°C) from iodophore disinfected eyed eggs originating from the Hallesø trout farm (Jutland, Denmark) and subsequently reared (at 12°C) at the disease-free recirculated facility (Bornholm Salmon Hatchery) (Xueqin et al. 2012). When the fish reached an average body weight of 1 g, they were transported to the experimental fish facility at the University of Copenhagen, Frederiksberg, and accommodated for 14 d acclimatization at 15°C in two 200 l tanks containing internal biofilters (20 l min^{-1}, EHEIM) with continuous aeration. Feeding was conducted with commercial pelleted feed, at a rate of 1% of the biomass per day (Inicio Biomar), up until experimentation. No feed was offered during the 24 h experimental period.

2.3. Biocide preparation

Aqueous solutions of different biocide concentrations for fish exposure were prepared based on municipal tap water (pH 7.6, $CaCO_3$ 450 mg l^{-1}, Frederiksberg municipality). Formaldehyde solutions were made by dilution of a 37% aqueous solution (cat. no. 10.005.000, Hounisen Laboratorieudstyr). PAA solutions were prepared from Aqua-Oxides Super 15% (cat. no. 241525, www.s-sorensen.dk) and H_2O_2 from 30% H1009 (cat. no. 16911-250ML-F, Sigma-Aldrich). The surfactant SPH6 was isolated by the company Sundew, Copenhagen, from *Pseudomonas* H6 bacteria as previously described by Liu et al. (2015). The freeze-dried compound (appearing as a white powder) was pre-dissolved into 100 ml water. Stock solutions at different concentrations of the different compounds were prepared in 100 ml beakers. The contents were then dripped into the experimental fish tank (water volume 15 l) with fish over 60 s. Continuous aeration ensured mixing and the near 100% oxygen content in the tank. Control tanks similarly received 100 ml water.

2.4. Experimental design and exposure

Experiments to evaluate the effect of biocides on rainbow trout, in comparison to untreated time point

controls, were performed in duplicate over 24 h. Five different concentrations were tested for PAA, formalin and H_2O_2 and 4 concentrations for SPH6 (see Fig. 1 key). The experimental procedure was performed over 2 d (2 × 24 h). At Day 1 fish were exposed for 24 h to different concentrations of PAA (2 × 5 tanks) and formalin (2 × 5 tanks) in parallel. At Day 2 the remaining groups were exposed similarly but to H_2O_2 (2 × 5 tanks) and SPH6 (2 × 4 tanks). Separate time point controls with fish exposed only to water were used for each of these experiments (2 × 2 tanks). One tank was kept for pre-experimental sampling. Samples of fish were taken at 3 time points following exposure: 2, 12 and 24 h post exposure (hpe) including 5 control fish in duplicate. At the start of the experiment a total of 645 fish with an average weight of 1.46 g and average total length of 5.23 cm were used. Fish were randomly allocated to 43 tanks (volume 15 l) each with 15 fish. Ten fish from 1 tank (pre-exposure control tank at Day 0) were taken as the basis before experimentation. Fish in 38 tanks were exposed to biocide, and fish in 4 tanks served as non-treated time point controls. All treatments and controls were run in duplicate. Water was aerated continuously, kept at 15°C (thermostat-controlled room), and water quality parameters were monitored daily for NH_3 mg l^{-1}, NO_2 mg l^{-1}, NO_3 50 mg l^{-1} and pH 7.6. The fish were kept in the exposure tanks 24 h prior to initiation of the experiment.

2.5. Sampling

Samples of 5 fish were taken from each tank at 3 timepoints following exposure: 2, 12 and 24 h hpe. At each time point the fish were captured by a hand-net and immediately euthanized by immersion into an overdose (300 mg l^{-1}) of tricaine methane sulphonate (MS-222) (cat. no. A5040, Sigma-Aldrich). For gene expression analyses all fins, except the caudal fin, and the gill arches, from one side of the fish, were sampled and placed in separate 2.5 ml cryotubes (cat. no. GR-122277, In Vitro) containing 0.5 ml RNAlater (cat. no. R0901, Sigma-Aldrich). The tubes were held at 5°C for 24 h before being stored at −20°C until processing according to the standard procedures. For recording mucous cell density, the caudal fin from the fish was excised and formalin fixed, stained and mounted on a microscope slide. All caudal fin samples from one tank were pooled in 4 ml Narrow-Mouth HDPE bottles (cat. no. 02-923-6A, Thermo Fisher Scientific), containing 4% neutral formaldehyde for fixation, and stored at room temperature until processing (staining and mounting).

2.6. Gene expression analysis

The gene expression analysis was performed as previously described by Jaafar et al. (2020). The gills and fins were homogenized using the Tissue-lyser II (cat. no. 85300, Qiagen). RNA from the gills were then extracted by GenEluteTM mammalian RNA kit (cat. no. RTN350, Sigma-Aldrich). The fins were pretreated with Proteinase K (cat. no. P4850-1ML, P4850, Sigma-Aldrich), due to the high collagen content, before being processed for RNA extraction. Samples were then treated with DNAse kit (AMPD1, Sigma-Aldrich) to remove genomic DNA contamination. The RNA concentration was determined by measuring optical density at 260/280 nm on a NanoDrop 2000 (cat. no. ND-2000, Saveen & Werner ApS) and the quality and integrity assessed visually by running 2 µl of each sample in a 1.5% agarose gel electrophoresis. The extracted RNA was then stored at −80°C. Subsequently, cDNA was synthesized in T100 thermocycler (Biorad) (10 min at 25°C, 60 min at 37°C and 5 min at 95°C) using TaqMan® Reverse Transcriptase Reagents (cat. no. N8080127, Thermo Fisher Scientific), Oligo d(T) primers and up to 1000 ng sample adding up to a total volume of 20 µl in each well. The cDNA was then diluted using 80 µl of RNA/DNA-free water (cat. no. 10977-035, Thermo Fisher Scientific) and stored at −20°C. To perform quantitative PCR (qPCR), we used an AriaMx Real-Time PCR machine (cat. no. G8830A-04R-010, AH diagnostics AS) with a set-up of 1 cycle at 94°C for 10 min followed by 40 cycles at 95°C for 10 s and 60°C for 15 s. A total volume of 12.5 µl reaction was added to each well. It consisted of 2.5 µl cDNA, 6.25 µl Brilliant III Ultra-Fast QPCR Master Mix (cat. no. 600881, AH Diagnostics AS), 1.0 µl primer-probe mixture (10 µM forward primer and reverse primer 5 µM TaqMan probe) and 2.75 µl RNA/DNA-free water. Reverse transcriptase and negative controls were used for each gene set up. The genes investigated in this study were related to inflammatory responses (cytokines IL-1β, TNFα, IFNγ, the regulatory cytokine IL-10, a chemokine IL-8) and other genes associated with the innate immune response (lysozyme, the acute phase proteins serum amyloid A [SAA], hepcidin, precerebellin and complement factor C3). For reference genes, ARP, β-actin and elongation factor α (ELF-1α) were applied. Sequence details for primer and probes were used according to Jaafar et al. (2020). By using the software NormFinder (Andersen et al. 2004), evaluating the applicability of reference genes and combinations of these, an average of the 3 genes were found to be the most suitable as reference.

2.7. Mucous cell density

In order to determine the mucous cell density, the excised caudal fins were fixed in neutral formalin and stained with Alcian Blue (Buchmann et al. 2004b). In brief, the fixed fins were rinsed using distilled water (dH_2O) and placed in filtered Alcian Blue: 1 g Alcian Blue (C74240, Gurr), 3 ml glacial acetic acid (ARK2183, Sigma-Aldrich), 97 ml dH_2O and pH adjusted to 2.6. After 20 min staining, the fins were rinsed twice in dH_2O to remove excess Alcian Blue, mounted (whole mounts) on microscope slides in AQUATEX® (cat. no. HC568794, Merck) and covered by a coverslip. Mucous cell densities (the number of stained goblet cells per unit area in 3 different locations on the caudal fin from each fish) were counted under a light microscope (magnification 200×) (Leica DM5000B). Pictures covering a fixed fin surface area were taken by photo software LAS V 4.12 (Leica Microsystems), and the number of cells was counted by use of the ImageJ programme (https://imagej.nih.gov). Positive superficial mucous cells were stained by Alcian Blue (indicating presence of mucus in the cell) within a total area of 0.4032 mm^2 per fish (3 different tail fin locations, each with an area of 0.1344 mm^2).

2.8. Data analysis

2.8.1. Testing for normality

The Shapiro-Wilk test was used to test for normality ($p > 0.05$). The Brown-Forsythe test ($p > 0.05$) was used to test for homogeneity of variances. Gene expression folds and levels were calculated by $2^{-\Delta\Delta Cq}$ and $2^{-\Delta Cq}$ (see Section 2.8.2). These are exponential data and by nature did not follow a normal distribution and did not pass tests for normality and homogeneity of variances. Log_2 transformation was therefore performed resulting in $-\Delta\Delta Cq$ and $-\Delta Cq$, respectively, which have equal distributions and SD. Transformed data (ΔCq-values) was then used for the tests.

2.8.2. Gene expression

Data were analyzed using the $2^{-\Delta\Delta Cq}$ method (Livak & Schmittgen 2001, Schmittgen & Livak 2008) as all qPCR assays had efficiencies within 100 ± 5%. This standard efficiency value provides a measure of variation and possible inhibition. Differences in gene expression between in fish exposed to various compounds and the corresponding time point controls was determined by an ordinary 1-way ANOVA with Dunnett's multiple comparisons test. Only gene expressions fulfilling both $p < 0.05$ and a minimum of 2-fold regulations were considered significant. All genes considered had enough valid Cq-values to be tested quantitatively. Please note that as folds are exponential data, they are presented as geometrical means with geometrical standard deviations (GSD). The histogram bars are to be divided/multiplied by GSD rather than added/subtracted.

2.8.3. Mucous cell density

The ordinary 1-way ANOVA with Dunnett's multiple comparisons test was used to compare mucous cell density in fish exposed to different biocide concentrations with a corresponding time point control.

2.8.4. Software

For all analyses data were analyzed using Microsoft Office Excel and GraphPad Prism 9. Differences were considered statistically significant at a probability level of 5% ($p < 0.05$).

3. RESULTS

3.1. Reactions of fish

No mortality occurred during the experiment. The biocide concentrations applied in the study did not induce significant adverse gross lesions or clinical signs in the fish. No balance disturbances or erratic swimming were observed during the 24 h observation period.

3.2. Gene expression analysis — general

A full summary with data of all gene expression analyses (with statistical details) are available in Table S1 in the Supplement at www.int-res.com/articles/suppl/d146p009_supp.xlsx. The regulation of immune genes in relation to exposure time and concentration (Fig. 1) indicated that the genes in the gills were mainly upregulated (except for those encoding for IL-10, IFNγ and TNFα), and to a higher

extent compared to the fins (Fig. 1B). SPH6 induced some increased expression at early time points (2 hpe) for genes encoding IL-10, IFNγ (Fig. 1B), lysozyme (Fig. 1C), and C3 (Fig. 1D). However, exposure to this compound resulted in most cases in a downregulation or no regulation at various time points.

3.3. Expression in gills

3.3.1. Exposure time

A significant upregulation associated with PAA exposure was recorded at 12 and 24 hpe for the genes encoding IL-1β, IL-8, IFNγ, TNFα, hepcidin, precere-

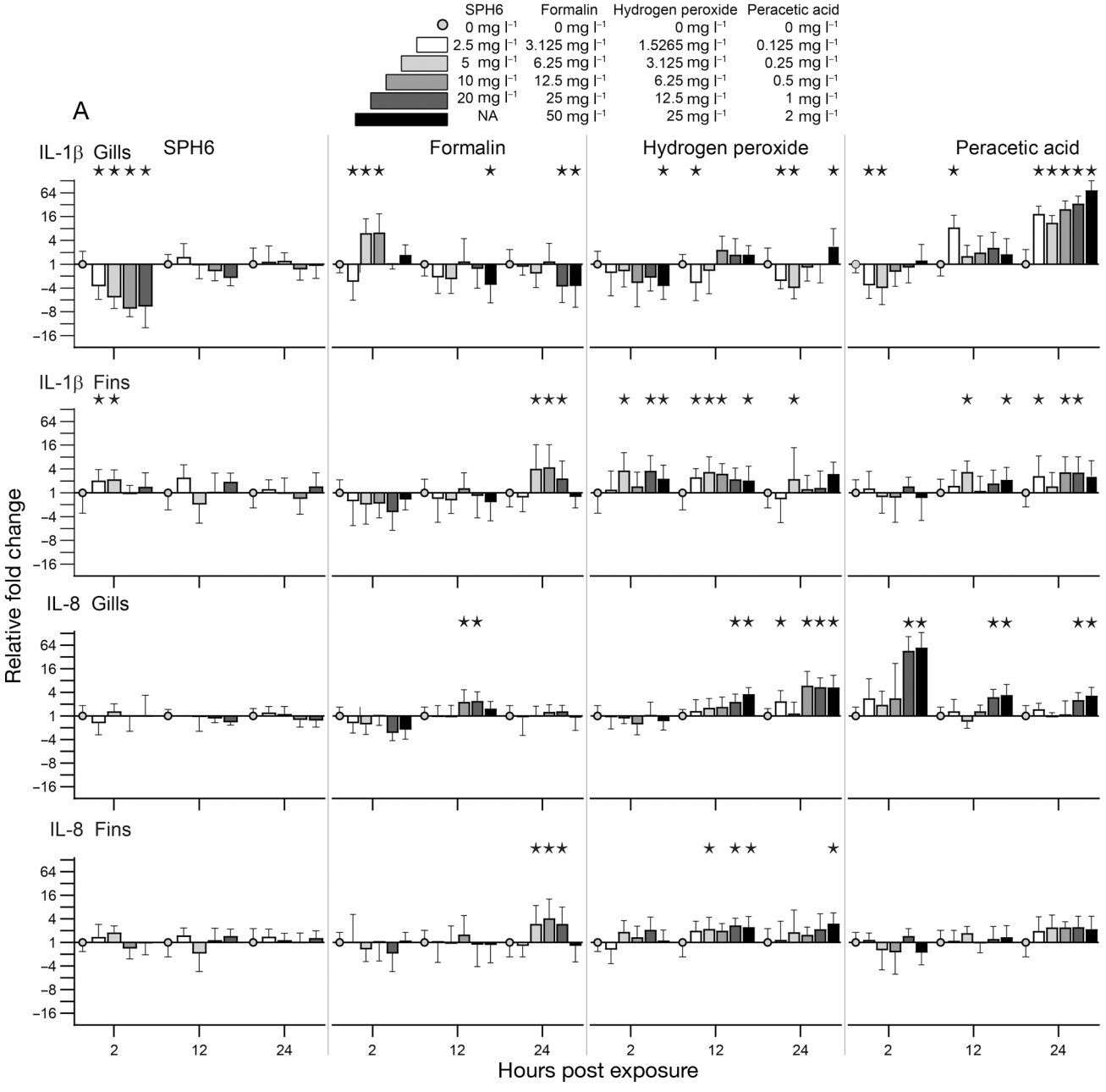
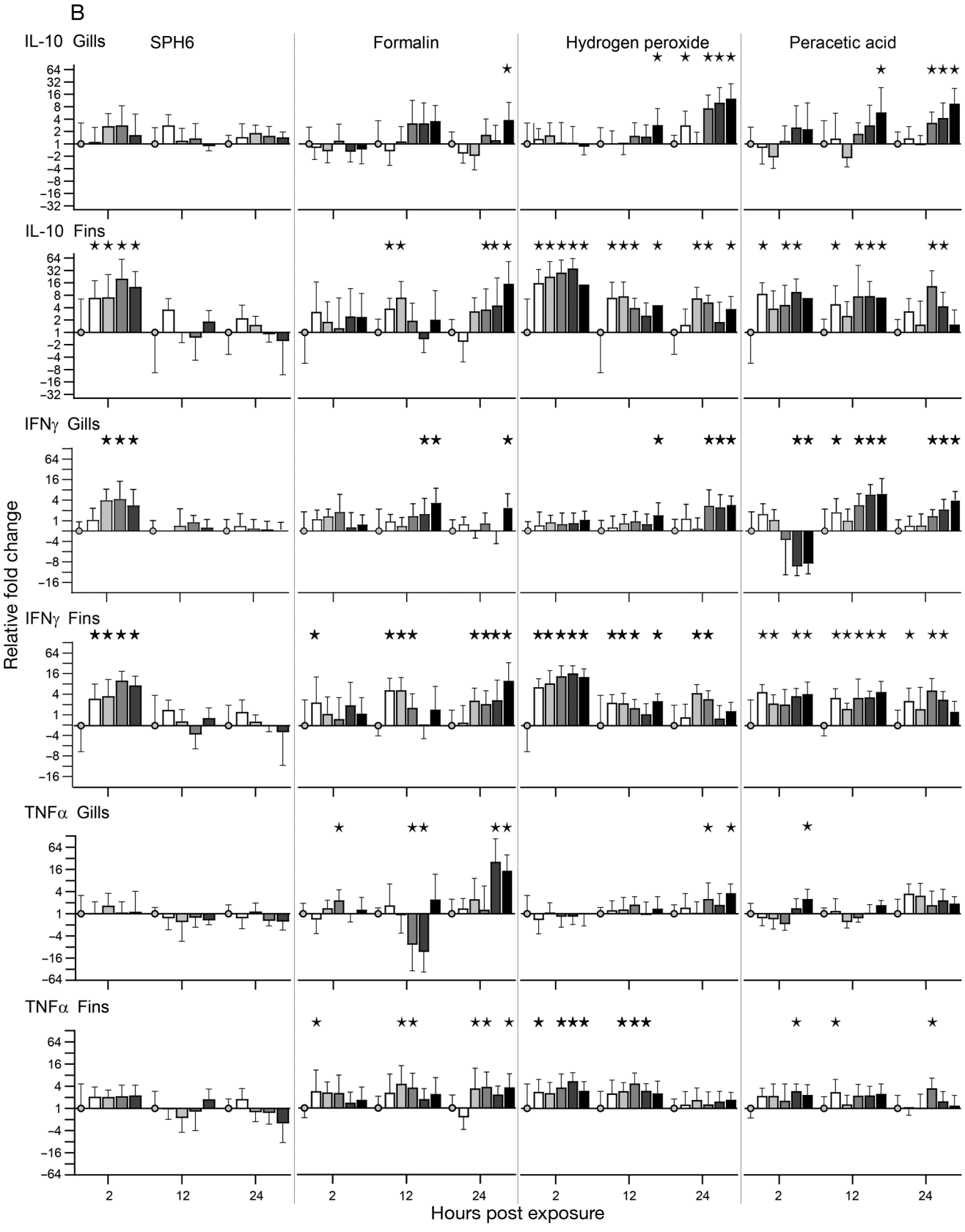


Fig. 1. Relative fold change in expression of inflammatory immune cytokine genes encoding for (A) IL-1β and IL-8; (B) IL-10, IFNγ and TNFα; (C) innate effector molecules hepcidin, precerebellin and lysozyme; and (D) complement factor C3 and serum amyloid A (SAA) in rainbow trout over 24 h post exposure (hpe) to different biocides (at 4 or 5 different concentrations). Data are geometrical means with GSD (geometric standard deviation). NA: not applicable. ★Significantly different from the time point control (Dunnett's, $p < 0.05$) and fold change ≥2. Table S1 in the Supplement contains the gene expression results in a tabular form, which includes number of achieved quantification cycle (Cq)-values and the ANOVA *F*-statistics

(*Fig. 1. continued on next pages*)



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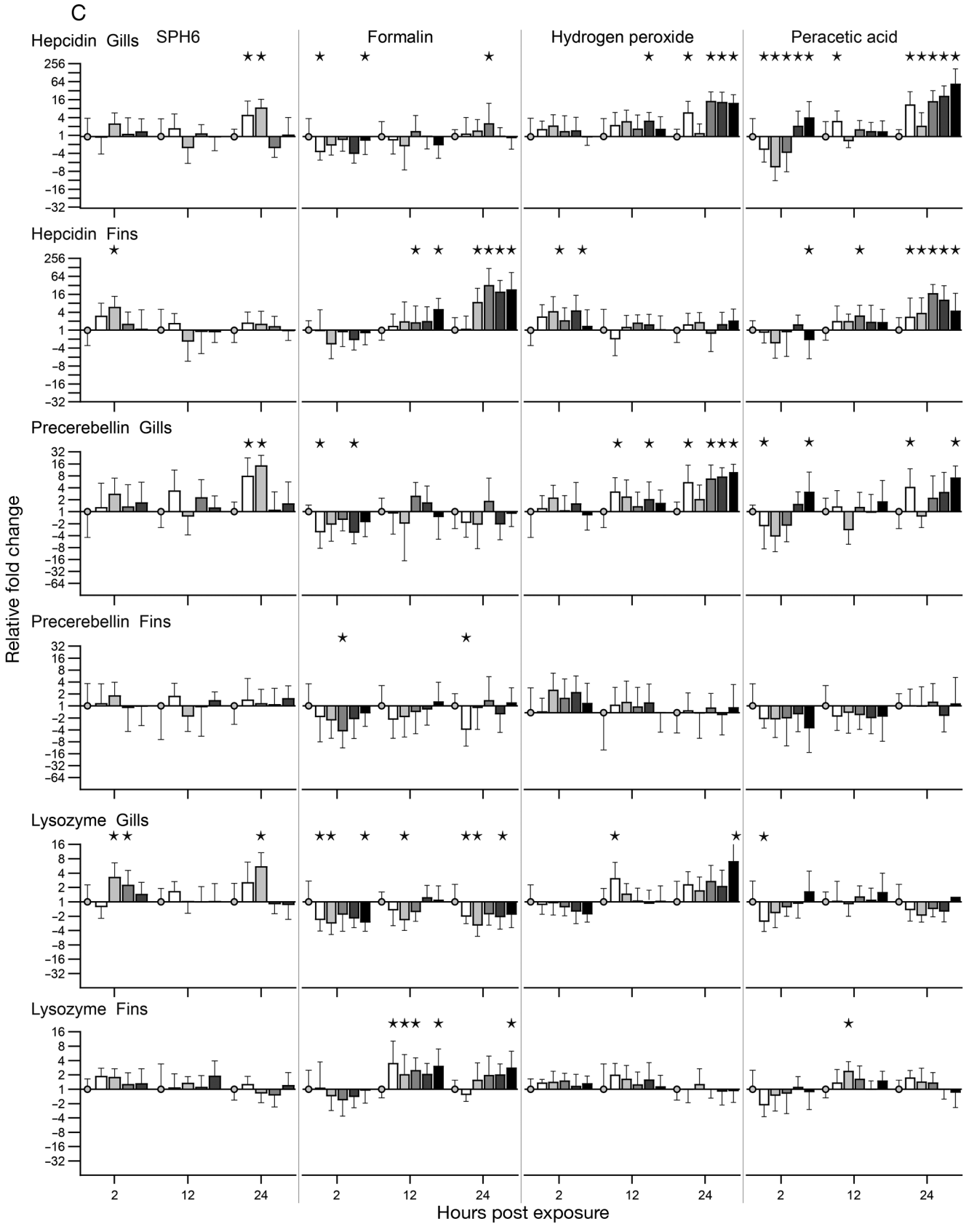

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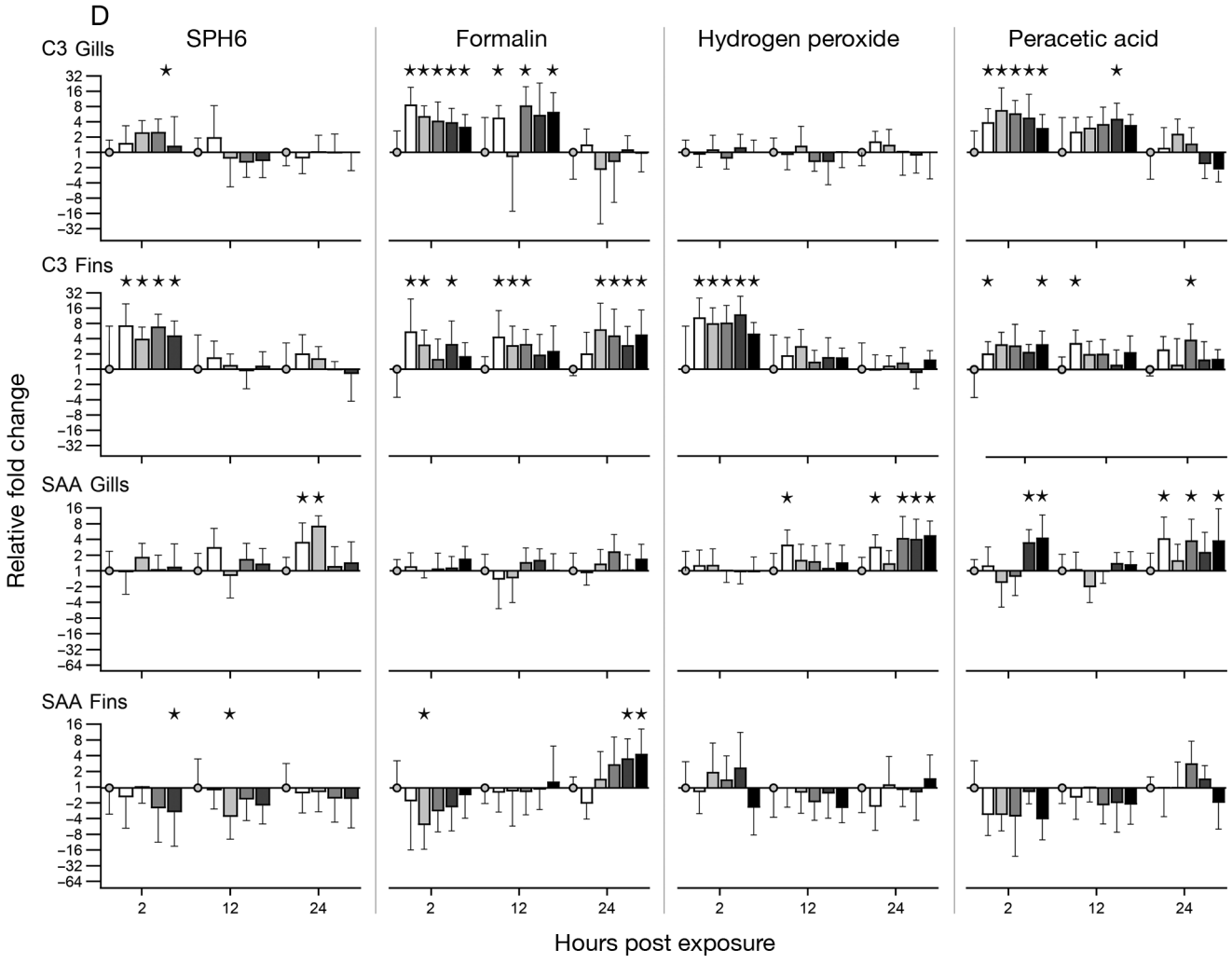

Fig. 1. (continued)

bellin and SAA. At 2 hpe the IL-8 and C3 genes were also up-regulated. For fish exposed to H_2O_2, time significant upregulations were measured for genes encoding IL-1β, IL-8, IL-10, IFNγ, TNFα, hepcidin, precerebellin, lysozyme and SAA. Formalin exposure resulted in an upregulation of genes encoding IL-8 (weakly at 12 hpe), IL-10, IFNγ, TNFα, hepcidin and C3. Some genes were downregulated at later time points (IL-1β, precerebellin, lysozyme). SPH6 exposure time was associated with an increased expression of hepcidin, precerebellin, lysozyme and SAA genes.

3.3.2. Concentration

A significant effect of increasing the PAA concentration was recorded for most of the genes investigated in this study, but the effect differed between time points. Several genes showed a positive correlation with the formalin concentration, but downregulation was seen for IL-1β, precerebellin and lysozyme genes. The H_2O_2 concentration had a lesser effect, but the expression of genes encoding IL-8, IL-10, IFNγ, TNFα, hepcidin, precerebellin, lysozyme and SAA were positively correlated with this parameter. SPH6 appeared to be the group with the lowest number of genes affected by concentration (IL-1β, IFNγ, C3, hepcidin, lysozyme, precerebellin and SAA).

3.4. Expression in fins

3.4.1. Exposure time

An effect of time on fish exposed to PAA was seen for genes encoding IL-8, IL-10, IFNγ, hepcidin and lysozyme. Prolonged H_2O_2 exposure merely affected the gene encoding IL-8, whereas IFNγ and TNFα

genes showed decreased expression over time. Extended formalin exposure induced a higher expression of genes encoding for IL-β, IL-8, IL-10, IFNγ, hepcidin, lysozyme and SAA. Increased SPH6 exposure time did not lead to elevated expression of any of the investigated genes in fins.

3.4.2. Concentration

An increasing PAA concentration affected genes encoding IL-1β and hepcidin. An elevated concentration of H_2O_2 influenced 4 genes positively (IL-1β, IL-10, IFNγ and lysozyme). An increased formalin concentration elevated expression of genes encoding IL-10, IFNγ, hepcidin and SAA. No effect of an increasing SPH6 concentration was reflected by an increasing expression of genes.

3.5. Specific actions of peracetic acid

PAA showed the overall strongest effect on most cytokine and acute phase reactant gene expression after short- or long-term exposure. In a few cases a downregulation was induced in fins for SAA and precerebellin.

3.6. Specific actions of hydrogen peroxide

Elevation of cytokine expression was evident (IL-10, IFNγ, TNFα) in fins at early time points, whereas the reaction occurred later in gills. The reaction to H_2O_2 in gills was evident (except for C3 reacting at 2 h) mainly after 24 h, where genes encoding acute phase reactants hepcidin, lysozyme, precerebellin and SAA were upregulated.

3.7. Specific actions of formalin

Formalin induced a strong reaction (for genes encoding IL-8, IL-10, IFNγ, TNFα, hepcidin, lysozyme, C3, SAA), but gills and fins differed in their response. In fins mainly TNFα and IFNγ genes showed an early upregulation. With regard to the acute phase reactants the expression of the C3 gene was high at 2 and 12 hpe in gills. In fins the reaction was also seen at 24 hpe, at which time point hepcidin and SAA genes were clearly regulated as well.

3.8. Specific actions of SPH6

The surfactant exposure showed no or minimal effect at early time points. The IL-1β gene was downregulated to some extent at 2 hpe, whereas IL-10, IFN and C3 genes were upregulated. At 24 hpe the acute phase reactant genes encoding hepcidin, lysozyme, precerebellin and SAA were upregulated in gills.

3.9. Mucous cell density

3.9.1. Peracetic acid

A significant increase in mucous cell density (Fig. 2) was observed at 2 hpe in all concentrations

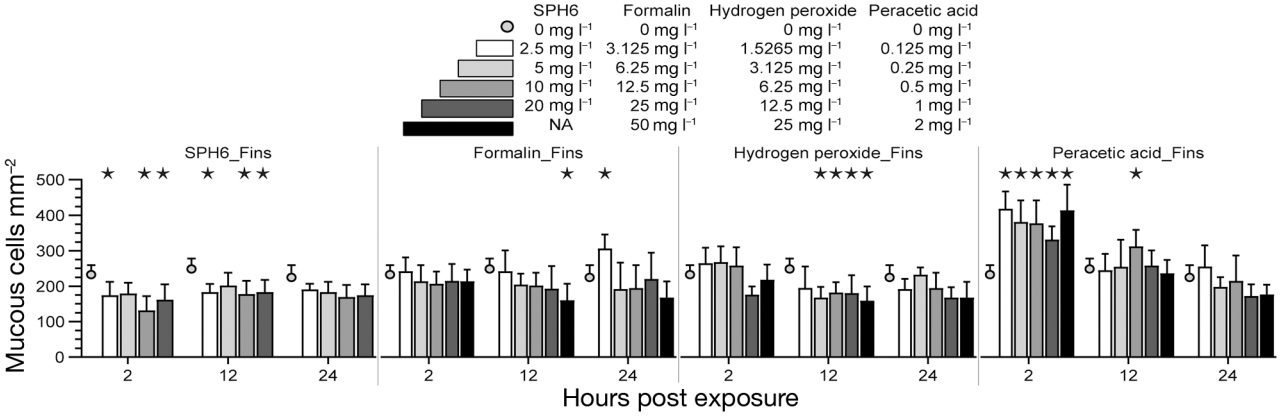

Fig. 2. Mucous cell densities in fins of rainbow trout exposed to different biocides (different concentrations) over 24 h. Each column represents the number (mean and SD) of mucous cells mm^{-2} (caudal fin) in 10 fish. NA: not applicable. *x*-axis shows hours post exposure (hpe). Filled circles: time point control; histogram bars with increasing level of shading: increasing concentrations. Five concentrations were tested for each compound except for SPH6 (4 concentrations) using the ordinary 1-way ANOVA. *Significantly different from the time point control ($p < 0.05$)

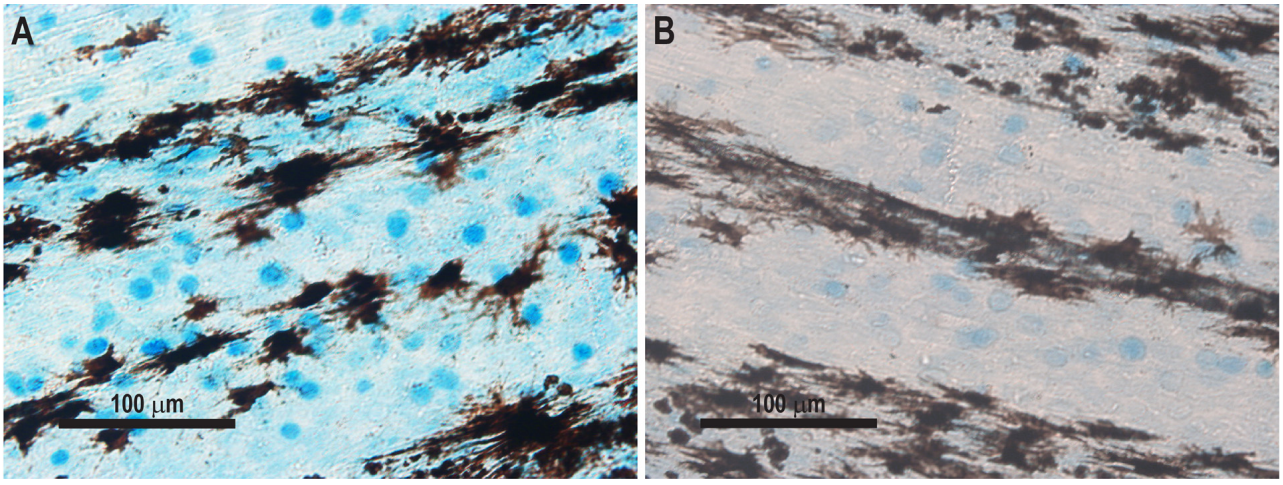

Fig. 3. Superficial mucous cells in rainbow trout caudal fins stained by Alcian Blue. (A) Non-stimulated mucus-filled mucous cells; (B) stimulated cells with partial exhaustion of mucus

of PAA (Fig. 3A). At 12 hpe, this response was seen only for 0.5 mg l^{-1}, where cell densities in fish fins exposed to the other concentrations had decreased to control levels. At 24 hpe, significantly lower mucous cell densities (Fig. 3B) were observed for the 2 highest concentrations of PAA (2 and 1 mg l^{-1}).

3.9.2. Hydrogen peroxide

At 2 hpe a significantly higher mucous cell density was observed in the groups receiving 3.125 mg l^{-1} of H_2O_2 whereas the groups receiving 12.5 mg l^{-1} showed a significantly lower density. At 12 hpe a significant decrease in cell density was recorded in all concentrations. At 24 hpe lower cell densities were observed in the groups receiving 1.56, 12.5 and 25 mg l^{-1} of H_2O_2.

3.9.3. Formalin

Slight but non-significant decreases of mucous cell densities were seen after treatment with formalin at 2 hpe. At 12 hpe a significantly lower cell density was observed in all concentrations except 3.125 mg l^{-1}. A significantly higher mucous cell density was seen for the lowest concentration (3.125 mg l^{-1}) at 24 hpe.

3.9.4. Surfactant

A significantly lower mucous cell density was observed in all groups treated with SPH6 at 2, 12 and 24 hpe.

4. DISCUSSION

The need for auxiliary compounds and biocides is positively correlated with the content of organic matter in fish culture systems. Recirculated production facilities reuse the water containing dissolved organic matter and particles released from biofilters and fish (Becke et al. 2020, Schumann & Brinker 2020), which is associated with a risk of propagation of several types of microorganisms (bacteria, virus, amoebae, flagellates, ciliates) (Jørgensen et al. 2009, Moestrup et al. 2014). Even if some of these microorganisms are relatively benign epibionts, their abundance and overgrowth in gills may create respiratory problems for the fish. Others are primary pathogens (*Ichthyobodo, Ichthyophthirius*) and may cause severe problems even at lower intensities. The organisms may also aggregate to particles with direct effects on fish gills (Lu et al. 2018). Future development of filtration techniques may be a solution to some of these problems, but at present elimination of these organisms is usually achieved by use of certain biocides. Among these, PAA, H_2O_2 and formalin are commonly used (Straus & Meinelt 2009, Straus et al. 2012, Jaafar et al. 2013). The present study has documented that these biocides, when used for bath exposure of rainbow trout, induce strong inflammatory reactions especially in gills and to some extent in fins. This may explain why farmers and researchers have recognized adverse reactions after exposure to auxiliary compounds such as PAA (Liu et al. 2017, Straus et al. 2018, Soleng et al. 2019), H_2O_2 (Jia et al. 2021) and formalin (Buchmann et al. 2004b). We measured expression of genes

encoding inflammatory cytokines and acute phase reactants in gills and fins of rainbow trout. Some differences between compounds were noted, but PAA exhibited a marked effect at almost all levels. Bath treatments of rainbow trout, using solutions of these compounds, may therefore induce some irritation or pain in the skin of the fish. This question should be further elucidated. In some cases, the compounds (e.g. formalin and PAA) downregulated expression of some cytokine and acute phase reactant genes, which calls for a study on the implications for fish health. We also investigated effects on fish of a novel biocide SPH6, which is a lipopeptide with a surfactant effect (Liu et al. 2015) able to eliminate pathogenic ciliates (Al-Jubury et al. 2018) and amoebae (Jensen et al. 2020). Although the compound has promising effects on parasites *in vitro*, it is necessary to determine its effects on fish gills and fins if it is to be applied at the farm level. Other auxiliary compounds hitherto used were shown to affect fish adversely. Tissue injuries and physiological disturbances occur following exposure to similar concentrations of formalin (Buchmann et al. 2004b, Jørgensen & Buchmann 2007), PAA (Straus et al. 2018) and H_2O_2 (Polinski et al. 2013, Henriksen et al. 2015, Chalmers et al. 2018, Jia et al. 2021). It was therefore noteworthy that the surfactant SPH6 had a relatively low stimulatory effect on expression of inflammatory genes in rainbow trout, and in some cases it downregulated some genes. We also recorded the presence and mucus content of mucous cells in the fin epidermis. The density of superficial mucous cells in the fish epidermis is flexible and sensitive to environmental disturbances. The cells are recruited from the lower epidermal cell layers and increase in numbers a few hours after formalin stimulation, but extended exposure may stimulate mucus expulsion from cells (Buchmann et al. 2004b). The process is highly temperature dependent (Quiniou et al. 1998), but the present study was performed at a stable temperature. We documented that especially PAA, and to a lesser extent H_2O_2 and formalin, induced an increase in superficial mucous cells in the fin, but extended stimulation resulted in an elevated release of mucus. This was shown as a reduction or absence of Alcian Blue-stained mucus in the cells. Mucous cell densities in caudal fins following SPH6 exposure decreased within 2 h. This suggests that this biocide stimulates mucous cells to release their content of mucus, but no evidence of elevated recruitment of new cells to the fin surface was found. The implications of this for practical use in farms should be further investigated. We cannot rule out the possibility that a strong expulsion of mucus, from superficial mucous cells in the fish epidermis, may assist the elimination of epibionts on fish surfaces and act as a biological cleaning process. With a relatively benign effect on the fish host, and a significant lethal effect on various parasites, the novel biocide SPH6 may show promise for future use in aquaculture. It is not expected that biocides of any kind will have no side effects on fish, but the SPH6 surfactant may challenge the welfare of the fish to a lesser degree than other commonly used biocides (formalin, H_2O_2, PAA). It is unknown if this compound will be commonly applied in the future, but history has shown that all microorganisms exposed to various antimicrobials may achieve some resistance due to selection of resistant organisms (White et al. 2002, Miller & Harbottle 2018). It is therefore recommended that this issue is also elucidated in any future study or use of the compound.

5. CONCLUSIONS

A range of parasites and epibionts which are severely challenging freshwater aquaculture can be controlled by water treatments using various biocides or auxiliary substances. We have shown that many of the compounds applied induce an inflammatory reaction in the surfaces (skin and gills) of the fish present in the fish tank. Gene expression studies showed that genes encoding inflammatory cytokines and acute phase reactants become upregulated to various degrees by use of formalin, H_2O_2 and PAA. A novel biological biocide, a surfactant lipopeptide isolated from *Pseudomonas* H6, was shown to affect fish surfaces to a considerably lower degree. No regulation or downregulation of the genes was demonstrated in several cases. The compound stimulated immediate release of mucus from superficial mucous cells. This action may together with its direct antimicrobial effect call for further studies on its application in aquaculture enterprises.

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