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Evaluation of Glutaraldehyde, Chloramine-T, Bronopol, Incimaxx Aquatic® and Hydrogen Peroxide as Biocides against *Flavobacterium psychrophilum* for Sanitization of Rainbow Trout Eyed Eggs

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Abstract

The effective conditions of glutaraldehyde, chloramine-T, bronopol, Incimaxx Aquatic® and hydrogen peroxide as some biocides commonly used by the aquaculture industry were investigated against *F. psychrophilum* in sanitization of rainbow trout eyed eggs. Bacteriostatic tests as well as bactericidal tests using ethidium monoazide bromide PCR assays were conducted *in vitro* on *Flavobacterium psychrophilum* while impacts of chemical treatments were studied *in vivo* on 240 [°C × days] rainbow trout eyed eggs. A 20-min contact time with bronopol (up to 2,000 ppm), chloramine-T (up to 1,200 ppm), glutaraldehyde (up to 1,500 ppm), hydrogen peroxide (up to 1,500 ppm) or with Incimaxx Aquatic® (up to 185 ppm, eq. peracetic acid) was effective against *F. psychrophilum* and did not affect the eyed eggs/fry viability. Collectively, the data obtained here clearly demonstrate that concentrations and duration of treatments commonly used to sanitize eyed eggs are widely overestimated in their effectiveness against *F. psychrophilum*. The new treatment conditions with the five studied biocides are bactericidal for *F. psychrophilum* and safe for rainbow trout eyed eggs. In this work, we developed an experimental approach to test some chemicals against fish pathogens to assist fish farmers in the effective and safe disinfection of eyed eggs.

Keywords: Aquaculture; Ethidium monoazide bromide; Disinfection susceptibility; *Flavobacterium psychrophilum*; Rainbow trout eggs; Viable qpcr

Introduction

Flavobacterium psychrophilum is the aetiological agent of 'rainbow trout fry syndrome' (RTFS) and 'bacterial coldwater disease' (BCWD), the two most significant systemic infections of primarily freshwater-reared salmonid fish [1] such as coho salmon (*Oncorhynchus kisutch*), rainbow trout (*Oncorhynchus mykiss*) and occasionally other fish species such as ayu (*Plecoglossus altivelis*) [2]. Several clinical manifestations have been described among which the most significant are mortality in juvenile fish (RTFS) and in adult, septicemia preceded by extensive necrotic lesions (BCWD) [3]. Consequently, considerable economic losses to fish aquaculture producers can occur (up to 90% in rainbow trout farmed in Norway [4]) and the erosion of tissue leading to a commercial downgrade of adult fish (for a review of *F. psychrophilum* biology, clinical signs and BCWD prevention and treatment, [5]). The control of *F. psychrophilum* infections is difficult and no effective vaccine is available yet despite numerous studies focused on the capability of some *F. psychrophilum* proteins to induce protection in fish. Potential targets identified for vaccine development include the OmpH-like surface antigen or the outer membrane glycoprotein OmpA [6,7] other immunogenic proteins such as trigger factor, ClpB, elongation factor G, gliding motility protein GldN and a conserved hypothetical protein [8]. Vaccination with FLAVO IPN and FLAVO AVM6, two mineral oil adjuvanted cocktails, induces responses that seemed capable of protecting rainbow trout against infections with *F. psychrophilum* [9]. However, recent study conducted with *F. psychrophilum* gliding motility N (GldN) protein underlines the importance of conducting multiple *in vivo* evaluations on potential vaccine(s) before any conclusions are drawn [10]. To date, the control of infections is yet achieved by antibiotic treatments using medicated feed (mainly florfenicol in 10 mg per kg of fish for 10 days) [11]. Some hatchery managers have expressed concerns about user safety and the impact on the environment of such molecules. Indeed, these pharmaceuticals or their metabolic residues (i) may be found inside the fish flesh, (ii) may lead to the emergence of resistant strain pathogens and/or (iii) may have side effects on aquatic organisms accidentally exposed to them. Due to environmental constraints, the aquaculture industry seeks to limit the use of antibiotics and emphasizes a preventive approach based on the implementation of effective hygiene measures.

Infections in fish (as for most other livestock) with bacterial pathogens involve either horizontal transmission by direct spread from contaminated animals or from their environment polluted by secretions/excretions of other infected animals, or vertical transmission from the spawners to their offspring through eggs. Such contamination may occur in two ways: the first is true vertical transfer where pathogens from parent broodstock invade the gonads and possibly infect gametes and the future embryos. The second is pseudo-vertical transfer where the surface of the eggs after spawning constitutes a matrix for environmental pathogens and the larvae are contaminated during the hatching of the contaminated eggs. Many molecules have been tested for the surface disinfection of fertilized fish eggs to prevent the pseudo-

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vertical transfer of pathogens [12]. They reduce the spread of pathogens from parent broodstock farms to hatchery farms and improve the survival to hatch. The list of disinfectants includes glutaraldehyde [13,14], hydrogen peroxide, iodine and tannic acid [15,16], ozone [17] and numerous others. For most of them, CT values have been defined as the concentrations of disinfectants (C; mg/L) multiplied by the exposition time (T; min) for which antibacterial effects have been observed with no significant side effect in hatching ability of the eggs. Thus, it has been shown that concentrations of copper sulfate needed to eliminate *F. psychrophilum* (above 300 mg/L) were toxic for rainbow trout eggs and thus are not recommended for control of RTFS or BCWD [18]. Therefore, the objective of this work was to specifically reassess both bacteriostatic and bactericidal effects of five biocides commonly used by the aquaculture industry against *F. psychrophilum* in sanitization of rainbow trout eyed eggs.

Materials and Methods

Bacterial strains, media and growth conditions

F. psychrophilum strains used in this study were: the reference strain JIP02/86 (INRA) and some freshly isolated strains from rainbow trout showing clinical signs of the disease (Table 1). They were sampled in 2012-2013 from four French rainbow trout farms, where outbreaks of RTFS had been reported. Isolates were collected from organs of trout presenting clinical signs (brain, SESB02, MLEB15 and MTOB07; or kidney, PISK08, and ASOK05) and typed using qPCR [19,20] as well as pulsed-field gel electrophoresis [21]. The bacterial cells were cultivated in a modified FLP liquid medium [0.5% (w/v) tryptone, 0.05% (w/v) yeast extract, 0.02% (w/v) beef extract, 0.02% (w/v) sodium acetate (pH 7.2)] or in FLP solid medium (+ 15 g/L agar). Bacteria were incubated at 14°C under aerobic conditions (orbital stirring, 150 rpm). Purity of the bacterial suspensions was checked (i) by examination of Gram-stained smears and (ii) by qPCR using the universal primer or the *F. psychrophilum* specific set of 16S rDNA primers to calculate a specificity factor as indicated by Orieux et al. [20].

Trout hatchery

Egg samples were taken from a fish farm where recurring outbreaks of RTFS had occurred. The water sources were bore-hole water as well as surface water in a flow through system. Eggs were collected within an egg incubation tray stack 240 [°C × days] once every two days disinfected only with bronopol 50 ppm over a 1-hour period. The eggs were moved in a second farm for disinfection trials.

***F. psychrophilum* strains**		**JIP02/86**	**SESB02**	**PISK08**	**MLEB15**	**MTOB07**	**ASOK05**
Origin		Rainbow trout, Aquitaine (France)	Freshly isolated from rainbow trout, Aquitaine (France)				
MIC (ppm)	Bronopol	3.1	3.1	1.6	3.1	6.3	1.6
	Hydrogen peroxide	7.8	6.2	3.1	31.3	62.5	3.1
	Glutaraldehyde	300.0	160.0	800.0	300.0	300.0	160.0
	Incimaxx Aquatic® (eq. peracetic acid)	125.0	62.5	125.0	62.5	62.5	31.2
	Chloramine-T	313.0	313.0	313.0	313.0	313.0	156.0

Table 1: *F. psychrophilum* strains and related type strains used in this study and the corresponding MIC (minimal inhibitory concentration) observed in the presence of disinfectant.

Preparation of biocide solutions

F. psychrophilum isolates were tested for sensitivity to five biocides commonly used in the fish industry: (i) glutaric dialdehyde or glutaraldehyde (Across Organics, Illkirch, France); (ii) tosylchloramide or *N*-chloro tosylamide, named chloramine-T (Merk Chimie, Fontenay-sous-Bois, France); (iii) 2-bromo-2-nitropropane-1,3-diol, named bronopol (Sigma, Saint Quentin Fallavier, France); (iv) Incimaxx Aquatic® (i.e., a mix of peroctanoïc acid, peracetic acid and hydrogen peroxide, 7 g/L, 83 g/L and 55 g/L, respectively; ECOLAB Food and Beverage Division, Issy-les-Moulineaux, France); and (v) hydrogen peroxide (Merck). They were freshly prepared as stock solutions by dilution in water: (i) glutaraldehyde (50,000 ppm or 0.5 mole/L); (ii) chloramine-T (50,000 ppm or 0.22 mole/L); (iii) bronopol (50,000 ppm or 0.25 mole/L); (iv) Incimaxx Aquatic® (830 ppm or 11 mmole/L in equivalent peracetic acid); and (v) hydrogen peroxide (50,000 ppm or 1.47 mole/L). All tested concentrations of biocides are expressed in ppm.

Antimicrobial assays

Minimal inhibitory concentrations MICs were determined by the broth micro-dilution method in 96-well microtiter plates (Corning's Life Science, Costar N° 3370, Grosseron, France) with *F. psychrophilum* grown to early-exponential phase (optical density at 600 nm [OD_{600}] = 0.020). Aliquots of the cell suspension (10 µL; about 50 × 10^6 bacteria / mL) were cultured *in triplicate* in 200 µL of two-fold serial dilutions of disinfectant in FLP medium placed in wells of 96-well microtitration plates. Growth and sterility controls were included for each isolate. Microtiter plates were incubated at 14°C and the growth was spectrophotometrically monitored (OD_{600}) for four days in a Dynex MRX-II Microplate Reader (Dynex Technologies, France). The MIC was defined as the lowest concentration of disinfectant in which no absorbance change was recorded over a 3-days period. Alternatively, the minimum bactericidal concentration (MBC) was determined to evaluate the cell viability after disinfectant treatments. Culture aliquots (0.5 ml; OD_{600} = 0.05) of *F. psychrophilum* were exposed for 0 to 40 min at room temperature to one of the five disinfectants assayed. Chemical agents were removed by centrifugation (5,000 × *g*, 10 min, 4°C) and the cells were washed twice in PBS (50 mmol/L sodium phosphate buffer, 150 mmol/L NaCl, pH 7.4) and dispersed in PBS. Negative control (i.e. 100% of bacterial viability) was 0.5 mL of the working *F. psychrophilum* suspension untreated with any biocide while positive control (i.e. 100% of bacterial mortality) was 0.5 mL of the working *F. psychrophilum* suspension heat-treated at 95°C for 5 min using a standard laboratory heat block. No growth was observed after 5 days at 14°C when 50 µL of this suspension was spread on FLP solid medium. Then, the bacterial suspensions were subjected to EMA (ethidium monoazide bromide or phenanthridium, 3-amino-8-azido-5-ethyl-6-phenyl bromide, Sigma, Saint Quentin Fallavier, France) treatment to evaluate viable/dead cells according to Nocker and Camper [22]. Briefly, EMA dissolved in water (5 mg /mL) was added to *F. psychrophilum* suspensions to a final concentration of 2 µg/mL. A first 10-min incubation step in dark allowed to EMA to interact with DNA from permeabilized cells, only. The photoinduced cross linking EMA-DNA step was obtained by light exposition (2 cycles of 60 sec; 650 Watts halogen lamp) of samples on ice to avoid excessive heating. After EMA-treatment, cells were washed twice and dispersed in water for DNA analysis. The PCR experiment was performed with the *F. psychrophilum*-specific set of primers (Fp_16S1_fw and Fp_16Sint1_rev; [20]) and the amplified DNA was further analyzed by electrophoresis in a 2% agarose gel; the expected size of the amplicons was confirmed by comparison with DNA molecular weight markers (50 bp DNA step ladder, Promega, Charbonnières,

France). The EMA-qPCR reactions were performed *in triplicate* with a MX3000p Stratagene thermocycler (Agilent Technologies, Massy, France) as previously described [20]. Data were expressed as quantities of viable bacterial cells (± SD).

Determination of D- and Z-values

For all chemical agents tested in this study, D-value (the decimal reduction time, min) was defined as the exposure time required causing 90% (= one decimal logarithm, i. e., one $\log_{10}$) reduction of the initial population of *F. psychrophilum* cells, under specified concentrations. Consequently, the initial population of bacterial (about 1-10 × 10^7 cells) exposed to the chemical compound at time zero was quantified by EMA-qPCR as well as the survivors at time 10, 20, 30 and 40 min. Residual living cells were expressed as percentage of the initial population. D-values were determined from the negative reciprocal of the slopes of the regression lines using $\log_{10}$-transformed percentage of survivors *vs* time of exposure to the biocide solution [i. e., $\log_{10} N_S/N_O \times 100 = f(\text{time})$, where N_S is surviving population and N_O is initial population]. Z value was defined as the increase in the concentration of a given biocide necessary to reduce the time of exposure to this biocide by a factor 10 (= one $\log_{10}$ reduction of the time). Practically, Z-value was determined from the negative reciprocal of the slope of the regression line using $\log_{10}$-transformed D-values *vs* the biocide concentrations [i.e., $\log_{10}$D-value = f([Disinfectant])].

Disinfection assays on eyed eggs

Triploid rainbow trout eggs were treated five days before hatching with one of the five disinfectants. Treatments were: (i) bronopol (50, 500 or 2,000 ppm), (ii) hydrogen peroxide (40, 1,000 and 2,500 ppm), (iii) glutaraldehyde (300, 1,500 and 2,000 ppm), (iv) Incimaxx Aquatic® (10, 150 and 185 ppm), and (v) choramine-T (50, 600 and 800 ppm). Untreated eggs were used as controls. Three replicate groups of 200 eggs were disinfected for each treatment in 500 mL beaker (400 mL of disinfectant solution). After a 20-min chemical treatment, eggs were rinsed in fresh hatchery water and placed on shelf (170 × 90 × 40 mm) as shown in Figure 1A and 1B, allowing the hatch and 5-7 days later, the fry development over a 5-weeks period (Figure 1C). Eggs and subsequent fry were daily observed after the disinfection step. Water parameters were the followings: (i) temperature: 12°C; (ii) pH 6.5; (iii) hardness below 3°fH. The cumulative percent mortality (CPM) was determined after 20 days, and the relative percent survival (RPS) was calculated using the following equation:

$$RPS = [1 - (\text{CPM of disinfected eggs/fry})/(\text{CPM of control eggs/fry})] \times 100$$

Values from experiments (n=3 per treatment) were expressed as mean ± SE.

Statistical analysis

GraphPad PRISM® (GraphPad Software, USA) was used to analyze data from disinfection assays on eyed eggs. The significance of cumulative percentage mortality of eyed eggs/fry/juvenile fish was analyzed using a 1-way ANOVA and comparisons of all chemical treatments *vs* control were performed by Bonferroni's Multiple Comparison Test. Differences were considered significant at $P < 0.05$.

Results

Effectiveness of disinfectants to prevent the *in vitro* growth of *F. psychrophilum*

Glutaraldehyde, chloramine-T, bronopol, Incimaxx Aquatic® and hydrogen peroxide were individually assayed to assess the capability of such products to inhibit the growth of five *F. psychrophilum* strains (Table 1). All of them were effective to control *F. psychrophilum* growth; among them, bronopol was the most effective with MICs less than 6.3 ppm. MICs recorded for the four other biocides ranged from 3.1-62.5 ppm for hydrogen peroxide, 160-800 ppm for glutaraldehyde, 31.5-1,000 ppm (eq. peracetic acid) for Incimaxx Aquatic® and 156-313 ppm for chloramine-T, respectively. Very slight differences in sensitivities to disinfectants were recorded with individual *F. psychrophilum* cultures suggesting possible different physiological states of the bacterial starting cells.

Optimization of EMA-qPCR to determine anti-*F. psychrophilum* susceptibility

The viable qPCR was used to quantify the susceptibility of *F. psychrophilum* to disinfectants. The effectiveness of 20 min-hydrogen peroxide exposition time was first tested by classical PCR with the reference strain JIP02/86 and five freshly isolates (Figure 2). The PCR amplification of DNAs using Fp_16S1_fw and Fp_16Sint1_rev primers generated an expected 146 bp-product from all untreated *F. psychrophilum* strains in the absence of EMA. Unlike this, no PCR product was observed from 95°C-heated cells in the presence of EMA indicating that most if not all of the bacterial cells were permeabilized in the course of the heat-treatment. Hydrogen peroxide displayed contrasted efficiencies when tested against the six strains

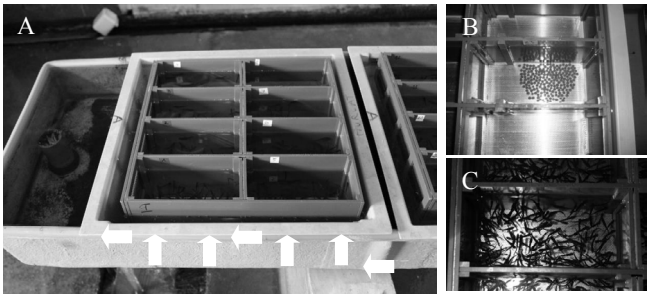

Figure 1: Pictures of the experimental device used to test the impact of different disinfectants on different batches of rainbow trout eggs.

(**A**) White arrows underline the water circulation inside all batches.
(**B**) A set of 200 eggs deposed onto shelf just after one biocide treatment.
(**C**) A set of survival fry 5-weeks post-treatment.

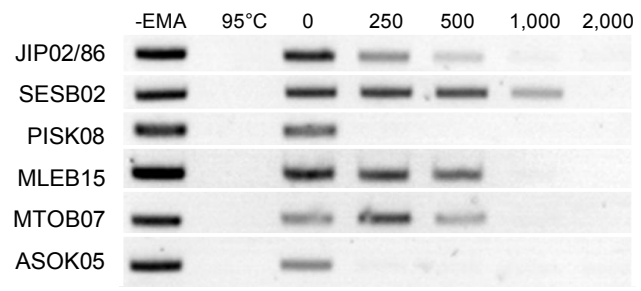

Figure 2: PCR products obtained by PCR using *F. psychrophilum*-specific primer set from EMA treated *F.psychrophilum* cells.

The cells had been previously exposed 20 min to hydrogen peroxide 250, 500, 1,000 or 2,000 ppm (250-2,000, respectively) or not-exposed (0). Positive and negative controls were EMA-untreated cells (-EMA) and 95°C-heated cells (95°C), respectively. *F. psychrophilum* strains were the type-strain JIP02/86 and five other strains freshly isolated from five French farms.

of *F. psychrophilum*. The strains PISK08 and ASOK05 were shown to be highly sensitive to the action of hydrogen peroxide because no amplification occurred for the weakest hydrogen peroxide concentration assayed here (250 ppm). On the other hand, the strain SESB02 exhibited a high resistance to hydrogen peroxide (1,000 ppm) while the three other strains, including the reference strain JIP02/86, were sensitive to concentrations above 500 ppm. Taking into account these results, *F. psychrophilum* JIP02/86 as moderately susceptible strain and reference strain was used to assess *F. psychrophilum* viability by EMA-qPCR after exposure to these biocides.

The next step was to evaluate the potential use of viable qPCR to investigate the hydrogen peroxide capability to kill *F. psychrophilum*. Stress gradients were tested in a preliminary screening over an assay period of 40 min with hydrogen peroxide concentrations ranging from 500 to 2,500 ppm (Figure 3). Increasing stress resulted in an increasing loss in *F. psychrophilum* viability during the first 20-minutes with a maximal three $\log_{10}$ unit reduction in the presence of 2,500 ppm hydrogen peroxide. This observation indicates clearly that not only the membrane integrity of *F. psychrophilum* was compromise by hydrogen peroxide treatment but so the effects observed were stress-dependant (i.e., the tested concentrations and the exposure times). Cell viability recorded after an incubation time above 20 minutes were not included in the exponential portions of the survivor curves for each of the hydrogen peroxide concentrations assayed and consequently not consistent with those observed at 10 and 20 minutes. A possible EMA oxidation with hydrogen peroxide could not be ruled-out; such a chemical alteration may have consequences in the EMA-capability to correctly-interact with DNA and therefore to inhibit further DNA amplifications. Based on this observation, an exposure time of 20 minutes only was selected in subsequent assays with other studied biocides considering that periods of 30 or 40 minutes were too long.

Bactericidal susceptibility of disinfectants against *F. psychrophilum*

The effectiveness of five antibacterial compounds was evaluated by of EMA-qPCR (Table 2). For each of the biocide concentrations tested, D-values were determined in order to calculate the Z-values. Data obtained with bronopol are shown for illustration in Figure 4. A reduction concentration-dependent in the *F. psychrophilum* population was recorded with bronopol treatments; about 0.8 to 3.0 $\log_{10}$ reduction units were observed in the course of 20-min treatments with bronopol 500 ppm to 2,500 ppm, respectively (Figure 4A). D-values derived from slopes of regression lines were found to be included between 25.7 min (bronopol 500 ppm) to 7.2 min only (bronopol 2,500 ppm) (Table 2). Z-value corresponding to bronopol was determined as 3,533 ppm (Figure 4B) indicating that theoretically, one treatment of *F. psychrophilum* with bronopol about 4,000 ppm (i. e., 500 + 3,533 ppm) is required to reduce the exposure time from 25.7 min to 2.6 min with same efficiencies against this pathogen. Alternatively, treatment durations corresponding to 5-$\log_{10}$ reduction in viability were included in a range of about two hours for bronopol 500 ppm to 36 min for bronopol 2,500 ppm. Similarly, D-values and Z-values were calculated for the four other chemical compounds as described above for bronopol (Table 2 and Figure 5). A 14-min exposition to hydrogen peroxide 500 ppm was needed to kill 90% of one suspension of *F. psychrophilum* while it was reduced to about 6 min with hydrogen peroxide 2,500 ppm. The theoretical Z-value was calculated to less than 5,900 ppm and 5-$\log_{10}$ reductions in bacterial viability were observed for treatment expositions between 1 h 30 min to 33 min for hydrogen peroxide 500 ppm and 2,500 ppm, respectively.

The glutaraldehyde treatments assayed (500 to 3,000 ppm) required exposition durations comprised between 1 h to 7 min to kill 90% of a *F. psychrophilum* population while they were between 5 h to less than 40 min to observe a 5-$\log_{10}$ reduction in viability. Z-value was estimated to about 3,000 ppm for glutaraldehyde. Similar efficient exposition times were observed for treatments with the peroctanoïc acid, peracetic acid, hydrogen peroxide based product (i.e., Incimaxx Aquatic®) as well as with chloramine-T. However, the Incimaxx Aquatic® concentrations assayed here were 100 to 250 ppm eq. peracetic acid, only. The theoretical Z-value was calculated to about 200 ppm eq. peracetic acid. The efficient concentrations of chloramine-T were between 400 to 1,200 ppm and the Z-value was evaluated to 775 ppm.

Viability of rainbow trout eggs/fry after immersion disinfections

Triplicate groups of 200 rainbow trout eyed eggs were used in the study to evaluate the egg survival to hatch as well as the fry development after one 20-min period of egg chemical treatments (five disinfectants; three concentrations each). Data of viability studies collected at 5 weeks post-disinfection are summarized in Table 3. The viability of eggs/fry was not significantly impacted by disinfection treatments with four over the five chemical products used in this study. Indeed, no excess mortality was recorded when the eggs were treated with bronopol, hydrogen peroxide, Incimaxx Aquatic® as well as chloramine-T at the concentrations tested. Positive RPS values observed in the presence of bronopol and hydrogen peroxide suggest a protective effect of the two biocides relative to the negative control (e.g., untreated eggs) avoiding any multiplication of pathogens on the egg surface. On the other hand, negative RPS values were observed for eggs treated with Incimaxx Aquatic® at all concentrations tested. It could be that Incimaxx Aquatic® has a side effect on the eggs, very low when the disinfectant concentration is low and slightly stronger in the presence of higher concentrations. Similarly, a negative value RPS was obtained with chloramine-T 50 ppm while positive values were recorded for concentrations widely above 50 ppm suggesting the benefit of the disinfection. I could be that a treatment with chloramine-T 50 ppm was not included within the range needed for an efficient immersion disinfection of eggs. Unlike the previous observations, the viability of eggs/fry was clearly impacted by disinfection treatments with glutaraldehyde. Indeed, egg mortality was about 10% in glutaraldehyde 300 ppm, i.e. not different

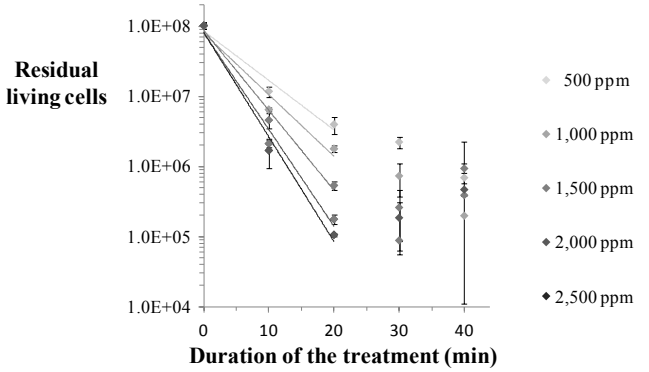

Figure 3: Impact of the hydrogen peroxide exposition on the *F. psychrophilum* JIP02/86 viability.

The bacterial cells were exposed to hydrogen peroxide 500, 1,000, 1,500, 2,000 or 2,500 ppm, treated with EMA
and the residual living cells quantified by EMA-qPCR. Error bars represent standard deviation.

Chemical product	Concentration (ppm)	Duration of treatment[1]			D-value (min)	$t = n \times D$ $n = 5\text{-}\log_{10}$ (h:mm)	Z-value (ppm)
		0 min	10 min	20 min			
Bronopol	500	$2.92 \pm 0.79 \times 10^7$	$1.99 \pm 0.36 \times 10^7$	$4.86 \pm 1.45 \times 10^6$	25.7	2:08	3,571
	1,000		$4.39 \pm 2.45 \times 10^6$	$1.88 \pm 1.12 \times 10^6$	16.8	1:24	
	1,500		$2.23 \pm 1.33 \times 10^6$	$4.68 \pm 0.43 \times 10^5$	11.1	0:56	
	2,000		$1.28 \pm 0.57 \times 10^6$	$1.06 \pm 0.81 \times 10^5$	8.2	0:41	
	2,500		$4.32 \pm 3.00 \times 10^5$	$4.72 \pm 0.84 \times 10^4$	7.2	0:36	
Hydrogen peroxide	500	$10.2 \pm 0.90 \times 10^7$	$1.18 \pm 0.20 \times 10^7$	$3.97 \pm 1.10 \times 10^6$	14.2	1:11	5,882
	1,000		$6.45 \pm 0.42 \times 10^6$	$1.78 \pm 0.18 \times 10^6$	11.4	0:57	
	1,500		$4.57 \pm 1.12 \times 10^6$	$5.36 \pm 0.82 \times 10^5$	8.8	0:44	
	2,000		$2.11 \pm 0.26 \times 10^6$	$1.77 \pm 0.27 \times 10^5$	7.2	0:36	
	2,500		$1.69 \pm 0.76 \times 10^6$	$1.06 \pm 0.04 \times 10^5$	6.7	0:33	
Glutaraldehyde	500	$3.36 \pm 0.89 \times 10^7$	$2.23 \pm 1.98 \times 10^7$	$1.57 \pm 0.68 \times 10^7$	60.6	5:03	2,941
	1,000		$7.36 \pm 1.62 \times 10^6$	$3.32 \pm 1.10 \times 10^6$	19.9	1:40	
	2,000		$8.27 \pm 3.03 \times 10^5$	$1.71 \pm 0.68 \times 10^5$	8.7	0:44	
	3,000		$9.79 \pm 4.46 \times 10^4$	$8.25 \pm 4.74 \times 10^4$	7.7	0:38	
Incimaxx Aquatic® (eq. peracetic acid)	100	$1.19 \pm 0.34 \times 10^7$	$8.29 \pm 3.52 \times 10^6$	$4.93 \pm 0.43 \times 10^6$	52.4	4:22	194
	150		$7.62 \pm 0.78 \times 10^6$	$3.29 \pm 1.46 \times 10^5$	12.8	1:04	
	200		$1.66 \pm 0.70 \times 10^5$	$5.91 \pm 3.94 \times 10^4$	8.7	0:43	
	250		$1.39 \pm 1.07 \times 10^5$	$4.48 \pm 2.94 \times 10^4$	8.2	0:41	
Chloramine-T	400	$7.91 \pm 2.85 \times 10^7$	$4.90 \pm 1.22 \times 10^7$	$3.62 \pm 0.96 \times 10^7$	58.8	4:54	775
	600		$2.08 \pm 0.51 \times 10^7$	$1.08 \pm 0.04 \times 10^7$	23.1	1:56	
	800	$8.27 \pm 4.81 \times 10^7$	$3.41 \pm 0.19 \times 10^6$	$4.39 \pm 1.07 \times 10^6$	15.7	1:18	
	1,000		$3.42 \pm 0.52 \times 10^5$	$1.83 \pm 1.50 \times 10^5$	7.5	0:38	
	1,200		$1.09 \pm 1.32 \times 10^5$	$1.38 \pm 0.76 \times 10^4$	5.3	0:26	

[1]*Flavobacterium psychrophilum* suspensions were treated with the indicated chemical compound and the viability of the bacterial cells was evaluated by EMA-qPCR as described in the Material and Method section.

Table 2: Impact of chemical treatments on *F. psychrophilum* JIP02/86 viability.

Chemical products	Concentration (ppm)	Viability[1]	
		CPM[2] % (mean ± SE)	RPS[3] (%)
Control (PBS)		$11.36^a \pm 2.64$	0.00
Bronopol	50	$11.10^a \pm 3.60$	2.3
	500	$10.50^a \pm 2.82$	7.5
	2,000	$8.50^a \pm 3.56$	25.2
Hydrogen peroxide	40	$9.60^a \pm 2.77$	15.5
	1,000	$8.30^a \pm 2.14$	26.9
	2,500	$10.10^a \pm 3.12$	11.1
Glutaraldehyde	300	$10.30^a \pm 4.46$	9.3
	1,500	$27.30^a \pm 13.83$	-140.4
	2,000	$44.50^b \pm 15.83$	-291.8
Incimaxx Aquatic®	10	$11.60^a \pm 5.15$	-2.1
	150	$12.60^a \pm 4.98$	-10.9
	185	$14.30^a \pm 4.04$	-25.9
Chloramine-T	50	$14.10^a \pm 5.85$	-24.2
	600	$9.40^a \pm 3.75$	17.2
	800	$8.50^a \pm 2.95$	25.2

[1]Rainbow trout eggs (5 days before hatching) were treated 20 minutes with the corresponding chemical product and viability of eggs/fry was recorded over a 5-weeks period.
[2]CPM, the cumulative percentage mortality (n=5 per treatment).
[3]RPS, the relative percentage survival; it was determined relative to PBS treatment.
Mean CPM values with different superscripts indicate significant difference at $p<0.05$.

Table 3: Impact of chemical treatments on rainbow trout eggs/fry viability.

to that observed for untreated eggs, while it was about 30% and 45% in glutaraldehyde 1,500 ppm and 2,000 ppm, respectively. However, the only significant difference was found between the treatment with glutaraldehyde 2,000 ppm and negative control and all almost other chemical treatments assayed in this work.

Discussion

Bronopol has been shown to be effective in protection against parasites infecting rainbow trout such as *Saprolegnia parasitica*, when administered as a daily bath/flush treatment at concentrations of 15 ppm and greater [23] or by *Ichthyophthirius multifiliis*, when exposed as long, low doses (24 h; 1 ppm) as well as short, high doses (30 min;

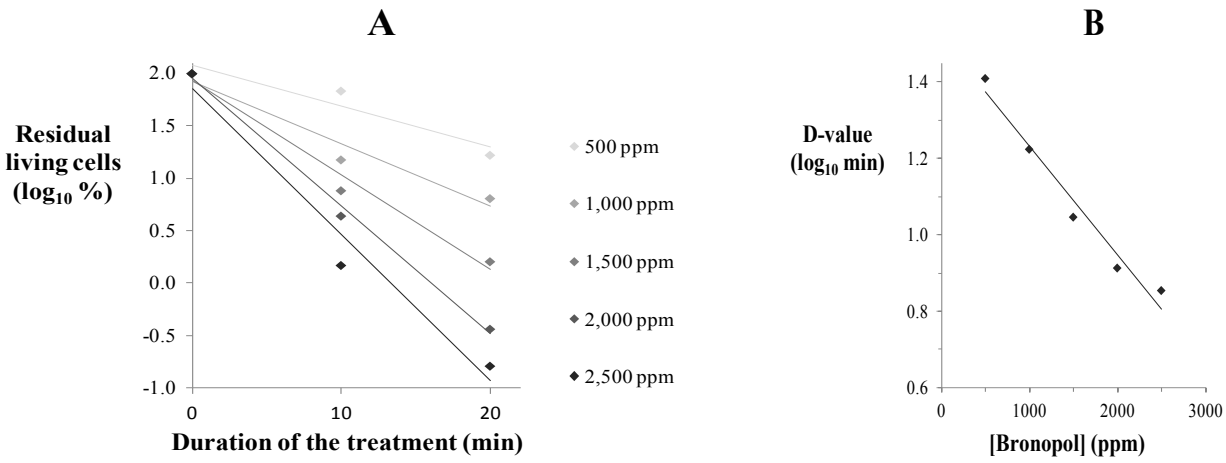

Figure 4: (A) Impact of the bronopol exposition on the *F. psychrophilum* JIP02/86 viability. The bacterial cells were exposed to different concentrations of bronopol, treated with EMA and the residual living cells were evaluated by qPCR. **(B)** D-values (i.e., the exposition time needed to destroy 90% of the initial cells) were determined for each of the bronopol concentrations assayed and were log_{10}–transformed. A correspondence curve was drawn where each data point represents log_{10}D-value determined for the corresponding bronopol concentration.

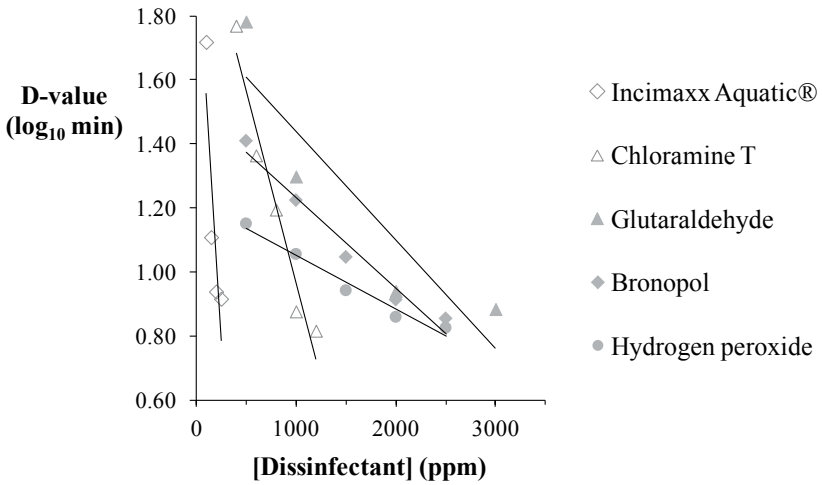

Figure 5: D-values and Z-values were calculated for the four other chemical compounds as described above for bronopol.

100 ppm). Accordingly with Birkbeck et al. [24] who determined MIC of bronopol for 13 bacterial pathogens isolated from marine fish, bronopol MIC ranging from 1.6 to 6.3 ppm were observed for the *F. psychrophilum*. However, none of them were bactericidal because *F. psychrophilum* growth was observed when samples of each mixture were taken two days post-treatment and spread on FLP-agar plate (Data not-shown). Consequently, bactericidal conditions [(i) concentration and (ii) treatment time] for bronopol were determined using the EMA-qPCR approach to identify the effective biocide conditions leading to the permeabilization of *F. psychrophilum* cells. Much higher bronopol concentrations than previously described were needed to observe an impact on the flavobacterial viability. Indeed, one decimal reduction of the viability was observed for treatments ranging from 500 to 2,500 ppm, for 26 to 7 min (D values), respectively. Such high concentrations or bronopol have been already recorded as effective for disinfection. Indeed, a bronopol concentration of 500 ppm was required for surface disinfection of Haddock eggs to achieve a significant reduction in bacterial numbers [25]. Such bronopol doses did not show any side effects on the rainbow trout egg survival: (i) the eggs viability was not affected by 20 min-long baths in bronopol up to 2,000 ppm; and (ii) the hatching time was not modified significantly compared with controls. Similar observations have been recently made on the insensitivity of crustacean eggs at high bronopol concentrations (until 3,000 ppm administered for 15 min every second day on crayfish eggs) [26].

Chloramine-T is a biocide used worldwide as a disinfectant and antiseptic. Although, it is used in aquaculture against bacterial and protozoal infections eliciting little or no response of oxidative stress biomarkers from *Oncorhynchus mykiss* when exposed to chloramine-T 10 ppm for 20 min (3 days; 3 expositions per day; [27]), it is not yet approved by the U.S Food and Drug Administration (FDA) for use on fish [28]. Previously, chloramine-T 15 ppm was found to be effective within 10 min against some *Aeromonas* spp. bacteria (*A. hydrophila* and *A. salmonicida* subsp. *salmonicida*) while 60 min of contact time was required to be effective against *A. salmonicida* subsp. *achromogenes* [29]. In this work, *F. psychrophilum* is much more resistant to chloramine-T since 300 ppm were needed to inhibit the bacterial growth and only severe chloramine-T treatments ranging from 400 to

1,200 ppm (for about 1 hr to 5 min, respectively) were bactericidal. Experiments performed to evaluated chloramine-T as possible candidate for approval for use to control mortality in freshwater-reared salmonids caused by bacterial gill disease have been shown that the fry and fingerlings viability was unaffected by exposure to concentrations less than 100 and 60 ppm, respectively [30]. Clearly, rainbow trout eggs are much more resistant to chloramine-T since none of the 20-min treatments of chloramine-T up to 1,200 ppm did reduce significantly the egg viability.

Hydrogen peroxide as a strong oxidizing agent is widely used by aquaculture industry to treat fungal infections of fish with recommended concentration for bath treatments (500 ppm for 20 min; [31]) as well as to sanitize fish eggs for concentrations ranging from 500 until to 30,000 ppm for few minutes to 60 min [32,33]. While hydrogen peroxide is a useful and environmental friendly biocide, it can promote or boost, in some cases, fish infections (e.g., *Tenacibaculum maritimum* in turbot [34]). This has been smartly used to pre-stress rainbow trout fry with peroxide hydrogen (until 200 ppm; 60 min) to obtain a reproducible immersion model of *F. psychrophilum* infection [35]. Less than hydrogen peroxide 65 ppm was needed to specifically inhibit the growth of the six *F. psychrophilum* strains assayed here, while bactericidal effects on *F. psychrophilum* JIP02/86 were observed from hydrogen peroxide treatments ranging from 50 to 2,500 ppm for less than 15 min to 7 min, respectively. None of these hydrogen peroxide treatments (from 40 to 2,500 ppm; 20 min) on rainbow trout eyed eggs did modify significantly neither the hatching time nor the fry viability or deformity rates. Our data are in agreement with those reported by the Wagner's group. Indeed, short expositions of eyed eggs to hydrogen peroxide 30,000 ppm (1 min) or 6,000 rpm (5 min) reduced well the bacterial load on eggs but did not affect the subsequent development of eggs [15]. Because high hydrogen peroxide concentrations used, attention was focused on the need to maintain hydrogen peroxide solutions to pH values close to neutrality with additions of $NaHCO_3$. More recently, experiments with different hydrogen peroxide treated groups of trout eggs (from 10,000 ppm for 2 min to 500 ppm for 35 min) confirmed that mortalities did not significantly differ to that in untreated eggs; they pointed-out also that the bacterial abundance on control eggs was higher than treated eggs, with a prevalence of yellow colonies, possibly *F. psychrophilum* [33]. Taken into the whole, these results underline the effectiveness of peroxide against *F. psychrophilum* and its safety in disinfection process of rainbow trout eggs.

Incimaxx Aquatic® has been shown as promising formulation in aquacultural systems since at weaker doses than 8 ppm eq. peracetic acid, it is effective to control the free-living stages of the parasitic protozoae *Ichthyophthirius multifiliis* [36]. Due to the strong bactericidal, virucidal, fungicidal, and sporicidal activities, peracetic acid has been used as an effective therapeutic treatment against some fish pathogens including *Flavobacterium columnare* [37]. Reduction of the *in vitro* growth was observed for *F. columnare* with increasing peracetic acid concentration ranging from 1 to 10 ppm and concentrations higher than 15 ppm have been shown to be toxic for channel catfish eggs [38]. Unlike this, our findings underline the high resistance of *F. psychrophilum* to Incimaxx Aquatic® since no growth inhibition was observed from 31.2-125 ppm (eq. peracetic acid) and reduction of viability was observed for Incimaxx Aquatic® incubations 100-250 rpm for about one hour to 8 min, respectively. No toxicity for rainbow trout eggs was recorded by 20 min-long Incimaxx Aquatic® treatments up to 185 ppm. Due to the acidic character of Incimaxx Aquatic®, it should be stressed that the water hardness has to be monitored and adjusted close to the neutrality to avoid deleterious effect of acidosis [39].

Glutaraldehyde is routinely considered as egg surface disinfectant for aquaculture. Doses of glutaraldehyde between 400–800 ppm for 5-10 min have been shown to improve the hatchability and larval survival in egg batches of Atlantic halibut when used for disinfection of egg surface [40]. However, these authors have cautioned that concentrations and contact times should be evaluated if disinfection with glutaraldehyde is to be applied to other fish species. Here, we found that such doses were bacteriostatic concentrations for *F. psychrophilum* and they were shown bactericidal *in vitro* (i.e., one $\log_{10}$ reduction of bioburden, only) for contact times ranging from 20 to 60 min. Less than 10 min were needed to observe similar effectiveness for glutaraldehyde 2,000-3,000 ppm. However, a 20-min treatment with glutaraldehyde 2,000 ppm did reduce significantly the survival of the rainbow trout eggs. Glutaraldehyde failed to reduce the survival of *F. psychrophilum* at concentrations that were safe for the rainbow trout eggs and some concerns about user safety (i.e. the possible long-term exposure for handlers causing irritations of the eyes, nose, throat, and skin and its potential adverse effects on the aquatic environment, [41]). Therefore there is a need for alternative methods using other chemicals for egg disinfection leading to the elimination of *F. psychrophilum*.

In conclusion, successful aquaculture requires knowledge of toxicity of applied chemicals, and acute toxicity tests are considered essential in selecting appropriate parameters of therapeutic baths. Here, we developed a new toolkit for assistance to fish farmers for the choice of effective chemicals against *F. psychrophilum* used to treat eyed eggs. However, treatments proposed in field condition have been employed in a particular situation of water quality (i.e., pH, temperature, hardness, organic matter.) and consequently they need to be adapted to different water qualities.

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