Journal of Genetic Engineering and Biotechnology 23 (2025) 100541

Contents lists available at ScienceDirect

Journal of Genetic Engineering and Biotechnology

journal homepage: www.elsevier.com/locate/jgeb

A simplified, one step technique for disinfection of non-hardened rainbow trout eggs with tosylchloramide (Chloramine T) and peroxide (Wofasteril) compounds and the effects on bacterial load and microbiome composition in comparison to iodophore disinfection

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ARTICLE INFO

Keywords:
Egg
Salmonidae
Bacteria load
Bacteria diversity
Microbiome

ABSTRACT

Disinfection of the interior of non-hardened eggs with iodophors (Buffodine®) is an established hygienic practice in salmonid aquaculture to prevent pathogen transmission from the broodstock fish to their offspring. As iodophors inhibit sperm motility, fertilization is first performed in a 0.75 % NaCl solution, followed by egg disinfection in a second step after fertilization is complete. Although this two-step egg disinfection procedure is simple to perform under laboratory conditions, it presents challenges for fish farms using mass stripping. The process involves two highly time-sensitive steps, requiring precise execution, as any errors can lead to fertilization failure or ineffective disinfection. A more practical approach would be to simplify disinfection into a single-step procedure. The present study demonstrates that non-hardened rainbow trout eggs can be fertilized and disinfected simultaneously using a one-step method with tosylchloramide (100 mg/l Chloramine T®) or peroxide (100 µl Wofasteril®) compounds in 0.75 % NaCl for 40 min. This procedure is feasible as the applied concentrations of Chloramine T and Wofasteril have only low impact on sperm motility. The one-step methods do also not negatively impact embryo and early larval development. Non-hardened rainbow trout egg disinfection methods with Chloramine T and Wofasteril were as effective as the conventional Buffodine method in reducing total bacterial load of eggs at 3 h post-fertilization (hpf), the point at which water hardening is complete. Reanalysis of total bacterial load after 22 days of development (embryo stage at the onset of eye pigmentation) proved Chloramine T more effective than Wofasteril and Buffodine. Microbiome composition differed significantly across developmental stages and disinfection treatments. Notable variations were observed between non-disinfected controls and eggs treated with Buffodine, Chloramine T, or Wofasteril, in persistent bacterial communities, stage-specific bacteria, and bacteria colonizing the chorion during embryogenesis. Buffodine treatment increased bacterial diversity, while Chloramine T and Wofasteril led to reduced diversity compared to the control. These findings are discussed in the context of microbiome stability and resilience, key factors for long-term fish health. In summary, the simplified one-step disinfection protocol offers practical advantages in reducing time-sensitive handling steps and improving efficiency in large-scale aquaculture operations. Moreover, it is environmentally preferable as both Wofasteril and Chloramine T are used at lower concentrations than Buffodine and degrade rapidly in water, leaving no harmful residues.

1. Introduction

Salmonid fresh water aquaculture encompasses a variety of species, with rainbow trout (*Oncorhynchus mykiss*) being one of the most widely farmed fish species globally, with production spanning both freshwater and marine environments.[1] Its adaptability, rapid growth, and high market value have made it a main product in aquaculture across numerous countries.[1] In salmonid aquaculture, as in other branches,

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https://doi.org/10.1016/j.jgeb.2025.100541
Received 4 February 2025; Received in revised form 9 June 2025; Accepted 10 July 2025
Available online 28 July 2025

infectious diseases cause significant economic losses.[2] Disinfection of the interior of non-hardened eggs is an established hygienic practice in salmonid aquaculture to prevent pathogen transmission from the broodstock fish to their offspring.[3–4] Viruses and bacteria may be transferred into the oocytes from maternal fish during oogenesis, such as infectious pancreatic necrosis virus,[5–6] *Renibacterium salmoninarum*,[7] and *Flavobacterium psychrophilum*.[8] They can also be introduced via semen, ovarian fluid, or contaminants like mucus, blood, urine, or feces during stripping (e.g. *Flavobacterium psychrophilum* -.[9]

When the eggs of Salmonidae and other freshwater teleost fish come into contact with water, the process of egg water hardening is initiated. This process, lasting approximately two hours, involves water absorption into the perivitelline space—the space between the egg membrane and the chorion—while simultaneously hardening the chorion and making it highly impermeable.[10] As a result, disinfecting the interior of non-hardened eggs can only be performed within a narrow time window before the first contact with water. Once hardened, disinfectants can only affect microorganisms adhering to the egg surface [12].

Disinfection of non-hardened eggs is typically conducted using iodophors (polyvinylpyrrolidone-iodide-iodine complexes).[3–4] However, iodophors inhibit sperm motility and thereby their fertility,[13] necessitating a two-step procedure. First, the eggs are fertilized in a 0.75 % NaCl solution, which activates and stabilizes sperm motility while preventing water hardening.[3–4] After three minutes, when fertilization is complete, the fertilization solution is drained, and the eggs are immersed in a 1 % iodophor solution, which then diffuses into the eggs.[3–4] .

There are two key disadvantages in the described disinfection procedure, including disinfection efficacy and large-scale operation in fish farms. Disinfection efficacy depends on the diffusion of the disinfectant into the egg. However, the oolemma and egg yolk have low permeability,[10] limiting disinfectant penetration within the egg. Furthermore, the high protein content of the egg yolk may reduce disinfectant effectiveness by partially shielding microorganisms or binding to the disinfectant itself. Iodophor disinfection of non-hardened brown trout, *Salmo trutta*, eggs reduced bacterial and fungal loads by only approximately 50 %.[5] Similarly, infectious pancreatic necrosis virus,[14] *Renibacterium salmoninarum*,[7] *Cytophaga psychrophila*,[15] and the microsporid *Loma salmonella*[16] were only partially reduced in salmonid species following this treatment.

Although the two-step egg disinfection procedure is relatively simple under laboratory conditions, it presents challenges for fish farms using mass stripping. The process involves two highly time-sensitive steps, requiring precise execution, as any errors can lead to fertilization failure or ineffective disinfection. A more practical approach would be to simplify disinfection into a single-step procedure, but this is not feasible with iodophores due to their adverse effects on sperm motility. Recently, alternative disinfectants such as tosylchloramide (Chloramine T®) and peroxide (Wofasteril®) have been successfully tested for non-hardened egg disinfection.[17] These compounds may have less negative impact to spermatozoa than iodophor solutions, potentially allowing eggs to be fertilized directly in the disinfectant solution and left there for further disinfection. This method would offer several practical advantages, including reducing the risk of pathogen transfer during fertilization and improving disinfection efficiency. Additionally, using Chloramine T or Wofasteril might be more cost-effective and environmentally friendly, as their effective concentrations are approximately 100 times lower than those of iodophores[17] and they degrade rapidly in water, enhancing environmental protection standards. Moreover the further authorization of Buffodine as biocidal product is presently under discussion in the European community due to environmental concerns.

Beyond the feasibility of the proposed one-step disinfection procedure, its efficiency to reduce the load of microorganisms and the impact on the natural microbiome composition of the eggs are crucial factors for routine application. Disinfectants interact not only with pathogens but also with the naturally occurring microbiome, affecting viruses, bacteria, and fungi in a similar way.[18] Therefore, the total bacterial mass reduction rate serves as a critical efficiency metric since a high reduction rate of the total bacterial mass can be supposed to correlate with a high reduction rate of pathogens. Currently, data on this topic are limited. One preliminary study indicates that the total bacterial mass reduction achieved with Chloramine T and Wofasteril is comparable to that of iodophores.[17] However, disinfection conditions and procedures differed from the here proposed one-step method.

Today it is widely accepted that fish microbiota are involved in health, performance, and different physiological and metabolic functions, although a causal relationship is lacking in most cases.[19–20] A diverse and abundant microbiome can reduce the incidence of pathogenic infections.[21] Early stages of fish development are considered as very sensitive regarding to outbreak caused by opportunistic pathogens, and fish microbiota can act as a first barrier against opportunistic pathogens.[20,22–24] Beneficial bacteria can prevent the attachment and proliferation of opportunistic or pathogenic bacteria by competing for nutrients and space.[22–24] Notable beneficial bacteria that contribute to disease prevention and overall egg health include *Lactobacillus* improving survival rates in fish larvae by acting as a microbial barrier against pathogens, *Pseudomonas* spp. due to antimicrobial properties and ability to compete with pathogens, and *Shewanella* sp. and *Bacillus subtilis* by producing antimicrobial peptides and enzymes, contributing to pathogen inhibition. Therefore it is of importance to identify the fish microbiota of the eggs and their changes in relation to disinfection procedures. Understanding these effects is essential for establishing sustainable disinfection practices and selecting the most appropriate disinfection media for aquaculture. Research on the microbiome of salmonid eggs remains scarce and has been studied only in relation to embryogenesis[25] and ecological factors.[26–27] .

The present study addresses these questions using rainbow trout (Oncorhynchus mykiss) eggs as a model. First, it evaluated whether non-hardened egg disinfection could be performed as a one-step procedure with Chloramine T or Wofasteril by assessing their effects on sperm motility and embryo and larval viability at various concentrations and exposure times. Next, the bacterial load of eggs disinfected with the two types of disinfectants was compared to untreated controls and the classical iodophor disinfection method by quantifying bacterial mass cultivable on different types of culture media three hours (hpf) and 22 days after fertilization (dpf). After 3 hpf disinfection egg hardening is terminated and therefore this is the first time point to evaluate the disinfection efficiency. After 22 dpf the embryonic development is advanced as the embryos are at the start of eye pigmentation. This stage is useful to evaluate if disinfection has persistent effects. Finally, the microbiome composition of non-disinfected controls and eggs disinfected with iodophor, Chloramine T, or Wofasteril was analyzed at similar time intervals using next-generation high-throughput sequencing of the 16S rDNA bacterial gene.

2. Material and methods

2.1. Sample collection

All experiments were conducted in accordance with Austrian federal law for animal care. Gametes were collected from rainbow trout (*Oncorhynchus mykiss*) broodstock aged 4 + years (mean body weight: 1254 ± 98 g; mean total length: 56 ± 8 cm; n = 20) maintained at the Kreuzstein fish farm in Sankt Gilgen, Salzburg. Gametes were stripped by abdominal pressure and stored in appropriate containers at 9 °C for no longer than 30 min before the experiments began. The experimental desing is shown in Fig. 1.

2.2. Disinfection solutions

Chloramine T®, Wofasteril®, and Buffodine were sourced from a local supplier of aquaculture products. Chloramine T was tested at

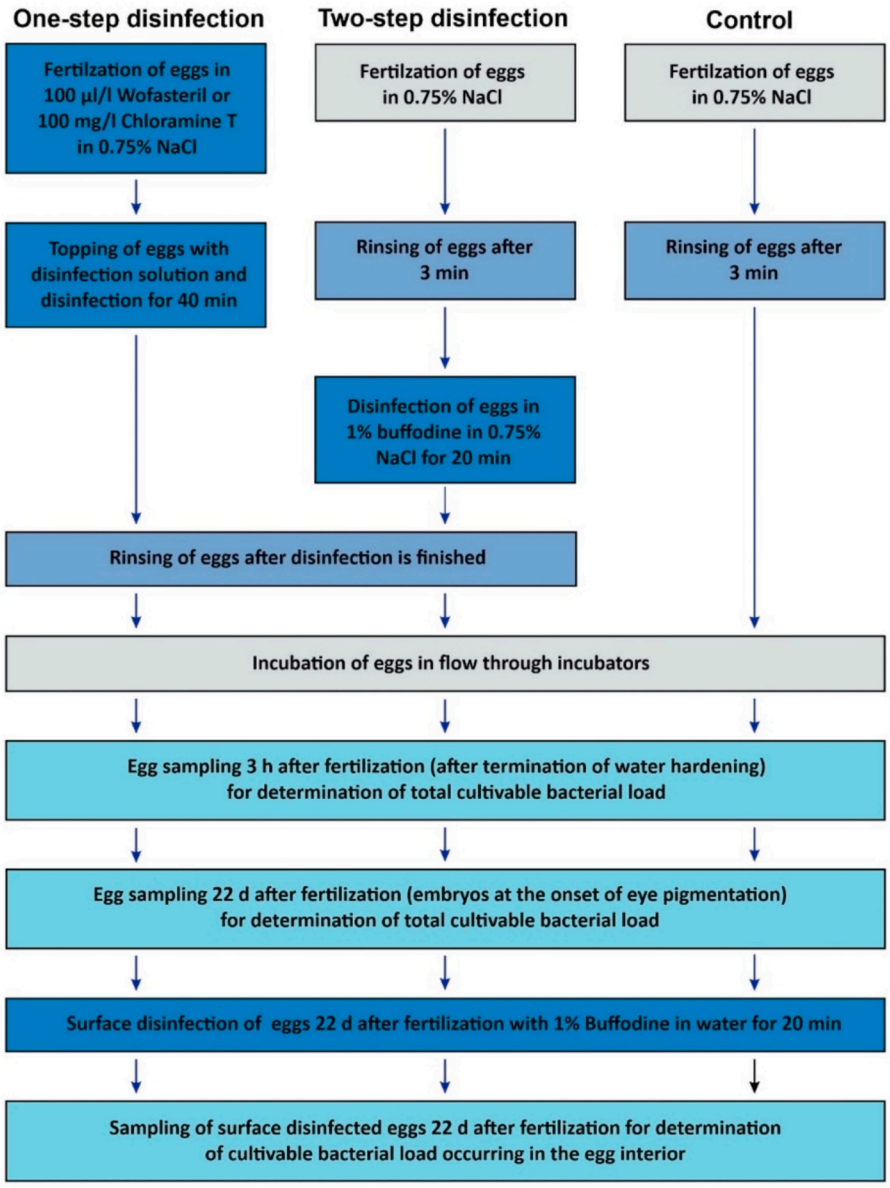

Fig. 1. Scheme of egg disinfection steps and of experimental and analytical procedures applied in the study.

concentrations of 25, 50, and 100 mg/L (active ingredient concentrations: 20, 40, and 80 mg/L sodium tosylchloramide), and Wofasteril at 25, 50, and 100 µL/L (active ingredients: 10, 20, and 40 µg/L of a mixture of acetylhydroperoxide, hydrogen peroxide, and acetic acid). Buffodine, serving as the positive control, was tested at a 1 % concentration (active ingredient: 100 mg/L iodine). All test concentrations were based on prior investigations.[13,17] Disinfectants were diluted in a 0.7 % NaCl solution prepared in well water.

Chloramine T and Buffodine did not affect the pH of the disinfection solutions, which remained at 7.78 ± 0.02 ($n = 3$). In contrast, Wofasteril decreased the pH in a concentration-dependent manner, with pH values of 7.42 ± 0.02, 7.30 ± 0.02, and 7.16 ± 0.02 for 25, 50, and 100 µL/L, respectively.

2.3. Feasibility of one-step disinfection with Chloramine T and Wofasteril

Effects on sperm motility: The effect of Chloramine T and Wofasteril on sperm motility was evaluated using eight semen samples. A 0.7 % NaCl solution served as the control. Sperm motility was assessed at 4 °C in a Makler investigation chamber. Two microliters of semen were activated with 100 µL of fertilization solution, and motility was recorded under an inverse phase-contrast microscope equipped with a video camera (200x magnification). Motility analysis was performed using the CellTrack Version 1.5 program (Motion Analysis Corporation).[28] For each sample, approximately 100 spermatozoa were analyzed 5 ± 1 s post-activation. Sperm velocity was calculated as average path velocity (µm/sec). Spermatozoa were classified as immotile (<5 µm/sec), locally motile (5–20 µm/sec), or motile (>20 µm/sec).

2.4. Effects on egg percentile mass increase during hardening and embryo and larvae development

The experiments were conducted using five different egg and semen samples. Each egg sample was drained of ovarian fluid and divided into 21 subsamples of 20 g (220–250 eggs). Three subsamples were fertilized in 15 mL of 0.7 % NaCl solution as controls, while the remaining subsamples were fertilized in 25–100 mg/L Chloramine T and 25–100 µL/L Wofasteril solutions by adding 20 µL of semen (sperm-to-egg ratio:

3000–4000:1). After three minutes, the solutions were topped up to 50 mL, and fertilized eggs remained in the disinfectants or NaCl solution for 20, 30, or 40 min.

For the determination of the percentile mass increase during hardening 10 eggs were taken from each subsample after 20, 30, and 40 min incubation in the disinfection solution or in the NaCl solution and weighted to the nearest 0.1 mg before and after a two-hour incubation in 9 °C well water.[29] .

The remaining eggs were rinsed three times in well water and incubated in horizontal flow-through incubators. Dead and fungus-infected eggs were not removed. The percentage of embryos reaching the eyed stage (eyes fully pigmented, mouth open) was recorded at 28 days post-fertilization (dpf) as the number of eyed stages embryos in relation to the total number of eggs. Subsequently, dead eggs were removed, and viable eggs were left to hatch. Hatched larvae were removed at 40 dpf (end of yolk sac phase), counted, and categorized as normally shaped or malformed in relation to the total number of incubated eggs. Total length of normal larvae was measured using ImageJ software to the nearest 0.1 mm from photographs taken with a Galaxy XCover 5 camera.

2.5. Bacterial load of disinfected eggs vs. untreated controls

Bacteria load was analysed as the mass of bacteria growing in different types of culture broths in comparison to the untreated control. Four egg and semen samples were used. Each egg sample was divided into four 20 g subsamples. Subsample 1 was fertilized in 0.7 % NaCl solution and served as a non-disinfected control. Subsamples 2 and 3 were fertilized and disinfected in 100 mg/L Chloramine T or 100 µL/L Wofasteril for 40 min as described above. Subsample 4 was disinfected with 1 % iodophore solution (Buffodine) and served as positive control. For iodophore disinfection eggs were fertilized in 0.7 % NaCl solution. After 3 min the NaCl solution was drained away and replaced by 10 ml/l Buffodine in 0.75 NaCl solution. Disinfection time was 20 min. After disinfection was finished all samples were washed 3 times in well water and incubated in flow through incubators.

Eggs were sampled at 3 h post fertilization (hpf) (when water hardening was terminated) and at 22 d post fertilization (dpf) (embryos in the early eyed stage). Samples at 22 dpf were split into untreated and surface-disinfected subsamples, the latter treated with a 1 % Buffodine solution for 20 min to remove bacteria colonizing the chorion.

3 eggs were taken from each sample, rinsed in sterile 0.7 % NaCl solution and placed in sterile tubes. 800 µl sterile 0.7 % NaCl solution was added and the eggs were homogenized. Tryptic soy broth, McConckey broth, and pepton water were prepared according to the manufacturer instructions. Tryptic soy broth and pepton water are general purpose culture media for bacteria, while McConckey broth is designed for the enrichment of Enterobacteriaceae and for detection of *Escherichia coli* which might occur in eggs due to contamination with faeces. 1600 µl of the culture broths was inoculated with 25 µl egg homogenate and incubated at 9 °C for 3d. 200 µl subsamples were taken in 24 h intervals under sterile conditions and sample turbidity was measured in a plate reader at 590 nm

2.6. Microbiome analysis

For microbiome analysis, it was assumed that bacterial DNA might only be partially degraded by disinfection procedures, potentially leading to biased results when sampling DNA directly from the eggs. To address this, bacterial DNA was sampled from the peptone water culture by preserving 300 µl of the culture broth in 700 µl of absolute ethanol. To minimize analysis efforts, only egg samples 1–3 were examined. Bacterial species present in the samples were identified using next-generation high-throughput sequencing of the 16S rDNA bacterial gene. This external analysis was performed by Vaiomer SAS, France, following the procedure described by.[30] Specifically, the V3-V4 variable regions of the 16S ribosomal RNA gene were amplified using universal 16S primers. For each sample, sequencing libraries were prepared by adding sequencing adapters. Sequencing was carried out using Illumina MiSeq technology with the 2 × 300 paired-end MiSeq kit V3. After demultiplexing the barcoded Illumina paired reads, individual read sequences were cleaned and paired for each sample to create longer fragments. Operational taxonomic units (OTUs) were generated through single-linkage clustering using the Swarm algorithm, followed by taxonomic assignment to profile the bacterial communities at the species level. Quality control measures included the removal of the last 10 bases from reads R1 and the last 30 bases from reads R2, as well as the exclusion of amplicons shorter than 350 nt or longer than 500 nt. Additionally, OTUs with abundances below 0.1 % of the total dataset were excluded to ensure data reliability and relevance.

2.7. Statistical analysis

The mass increase of eggs during hardening was calculated as percentile changes of 2 h water hardened eggs in relation to eggs incubated in the disinfection solution or in 0.7 % NaCl solution. Bacterial loads of Chloramine T, Wofasteril, and Buffodine disinfected eggs were calculated as percentile changes in comparison to the untreated control samples. Relative abundances of bacteria species were calculated as the number of specific OTUs in relation to the total number of OTUs. Data are presented as mean ± standard deviation. For statistical procedures percentage data were transformed by angular transformation ($\arcsin\sqrt{p}$) and metric data by log transformation to reach the assumptions of normal distribution. Data were analyzed by ANOVA using the treatment procedure as independent variable and the above mentioned analytical endpoints as dependent variable. Scheffés method was used as multiple comparison posthoc test at a significance level of $P \leq 0.05$. Microbiome α-diversity, the bacteria diversity within each sample, was determined using the Shannon index on the species level. Data were tested on significant differences by Kruskal-Wallis comparisons. β-diversity, the bacteria diversity between samples, was calculated on the species level, too, using the Bray-Curtis index. Data were analyzed on statistical significant differences at a significance level of $P \leq 0.05$ using Permanova.

3. Results

3.1. Feasibility of one-step disinfection with Chloramine Tand Wofasteril

Sperm Motility: Chloramine T solutions at concentrations of 25 and

Table 1
Motility rate and swimming velocity of rainbow trout spermatozoa in NaCl (0.7 %), Chloramine T (25 – 100 mg/l in 0.7 % NaCl), and Wofasteril (25 – 100 µl/l in 0.7 % NaCl) solutions. Sperm motility was measured 10 sec after activation. Sample number n = 8. Data superscripted by different letters are significantly different, $P < 0.05$).

	immotile, %	locally motile, %	motile, %	velocity, µm/sec
Control				
NaCl, 7.5 g/l	5.4 ± 9.0 [a]	12.0 ± 9.3 [a]	82.6 ± 7.5 [a]	98.1 ± 9.2 [a]
Chloramine T				
25 mg/l	8.2 ± 2.3 [a]	11.3 ± 7.8 [a]	80.5 ± 9.5 [a]	103.0 ± 17.1 [a]
50 mg/l	7.5 ± 3.5 [a]	13.7 ± 4.4 [a]	78.8 ± 6.1 [a]	100.9 ± 20.6 [a]
100 mg/l	15.3 ± 7.0[b]	24.6 ± 10.1[b]	60.1 ± 19.7[b]	88.2 ± 35.1[b]
Wofasteril				
25 µl/l	8.3 ± 3.9 [a]	11.7 ± 9.0 [a]	80.0 ± 11.0 [a]	105.3 ± 7.9 [a]
50 µl/l	13.3 ± 6.5 [a, b]	24.3 ± 14.3[b]	62.4 ± 11.9[b]	92.8 ± 11.7 [a,b]
100 µl/l	29.6 ± 5.5[c]	20.3 ± 12.3[b]	50.1 ± 7.9[c]	85.8 ± 11.6[b]

50 mg/L had no effect on sperm motility (Table 1). However, a concentration of 100 mg/L Chloramine T significantly reduced motility rates and swimming velocity, while increasing the proportion of locally motile and immotile spermatozoa (Table 1). Similarly, Wofasteril solutions at a concentration of 25 µL/L did not affect sperm motility parameters. In contrast, concentrations of 50–100 µL/L led to a dose-dependent decrease in the percentage of motile spermatozoa and sperm velocity, along with an increase in the proportion of locally motile and immotile spermatozoa (Table 1).

Embryo and larvae development: No significant differences were observed between control eggs and those disinfected with Chloramine T or Wofasteril for 20–40 min in terms of the percentile mass increase of water-hardened eggs (Fig. 2a), the percentage of embryos reaching the eyed stage (Fig. 2b), the proportion of normally shaped larvae at hatching (Fig. 2c), and the total length of larvae at the onset of exogenous feeding (Fig. 2d).

3.2. Bacterial load of disinfected eggs vs. untreated controls (Fig. 3)

When non-hardened eggs were disinfected with Buffodine, Chloramine T, or Wofasteril and analyzed at 3 h post-fertilization (hpf), the bacterial mass cultivable in tryptic soy broth and peptone water was significantly lower than in the control group. However, there were no significant differences in bacterial load among the three disinfectants. Notably, bacterial mass growing in McConkey broth was significantly reduced only by Buffodine disinfection.

At 22 dpf, the bacterial mass cultivable in tryptic soy broth was significantly lower in egg samples disinfected with Buffodine and Chloramine T compared to the control. The bacterial mass cultivable in peptone water was significantly reduced in eggs treated with Chloramine T and Wofasteril. For McConkey broth, a significant reduction in bacterial mass was observed only in eggs disinfected with Chloramine T.

Finally, surface disinfected 22 dpf eggs showed no significant differences in bacterial load across treatments Fig. 3.

3.3. Bacterial composition of disinfected eggs compared to untreated controls

The average number of raw read pairs was 61,745, with an average of 40,353 classified into operational taxonomic units. The proportion of discarded sequences between raw and classified read pairs was 34.6 %, which is within standard and acceptable limits.

Fig. 4 shows the relative frequencies of key bacterial species based on disinfection treatments and egg development stages. In control eggs at 3 hpf, *Acinetobacter*, *Sphingomonas*, and *Massilia* were the most prominent species, occurring at nearly equal frequencies. During embryogenesis, the bacterial composition shifted. At 22 dpf, *Pseudomonas*, *Enterobacter*, *Iodobacter*, *Janthinobacterium*, and *Acinetobacter* were most frequent. In surface-disinfected 22 dpf eggs, *Pseudomonas*, *Flavobacterium*, *Sphingomonas*, *Hydrogenophaga, Rhodoferax* and *Undibacterium* dominated. After disinfection of non-hardened eggs, the bacterial composition changed across all treatments, with *Pseudomonas* consistently among the most frequent species (Fig. 4).

To better understand bacterial composition, species were categorized into six groups based on their occurrence across development stages: (1) Bacteria persistent in the eggs were occurring in all development stages including surface disinfected 22 dpf eggs. Therefore, they were egg internal ones. (2) Bacteria developing in the eggs from 3 hpf to 22 dpf were absent at 3 hpf, but present at 22 dpf including the surface disinfection treatment. They were also classified as egg internal. (3) Bacteria of 3 hpf eggs were only detected in this development stage. (4) Bacteria colonizing the chorion of 3 hpf eggs were present in 3 hpf eggs and in non-surface disinfected 22 dpf eggs. (5) Bacteria colonizing the chorion from 3 hpf to 22 dpf occurred only in non-surface disinfected 22 dpf eggs. (6) Bacteria colonizing or recolonizing the surface disinfected 22 dpf eggs were detected after the surface disinfection treatment. A detailed list of bacteria species within these categories is provided in Table 2.

3.4. Bacteria frequencies across treatments

Based on the above defined categories bacteria frequencies across treatments could be described in the following way. In 3 hpf eggs the

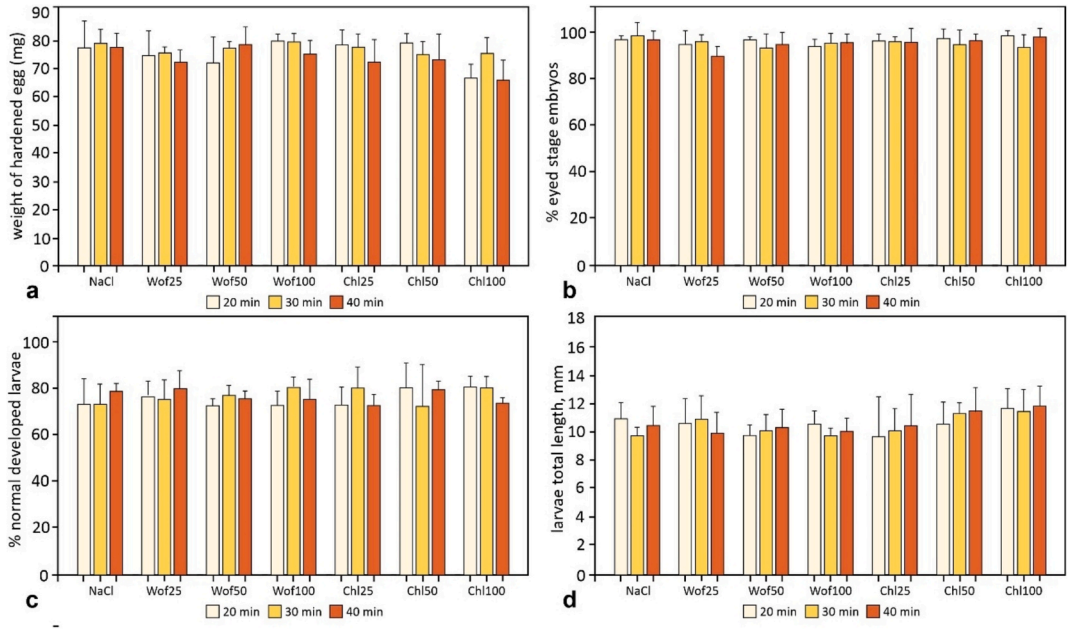

Fig. 2. Egg, embryo, and larvae viability characteristics of rainbow trout eggs disinfected with different concentrations of Chloramine T and Wofasteril for different time periods in comparison to untreated controls (0.7 % NaCl solution). Wof25, Wof50, Wof100: 25 – 100 µl/l Wofasteril; Chlo25, Chlo50, Chlo100: 25 – 100 mg/l Chloramine T. Sample number n = 5 for (a) and (b) and n = 50 for (c) and (d). Data are not significantly different, P ≥ 0.05.

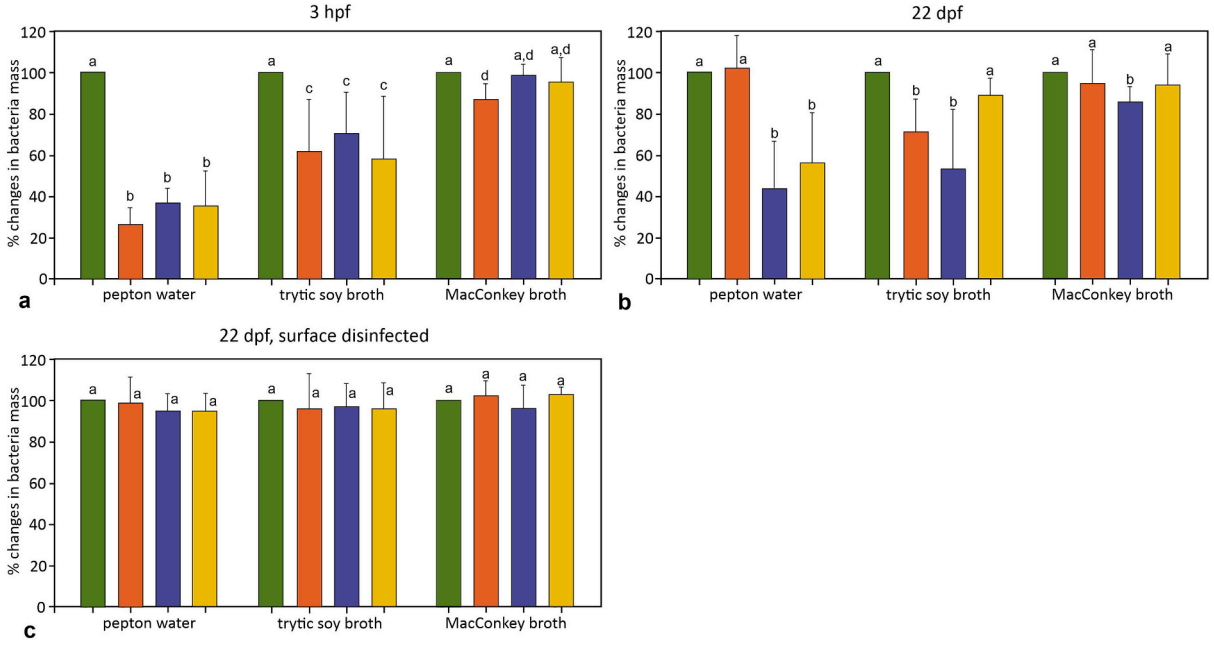

Fig. 3. Percentile changes in bacterial mass of rainbow trout eggs disinfected with 10 m/l buffodine for 20 min (orange bar), 100 mg/l Chloramine T for 40 min (blue bar), and 100 µl/l Wofasteril for 40 min (yellow bar) in comparison to untreated control eggs (green bar). Data are mean ± standard deviation (n = 5), those superscripted by different letters are significantly different, $P < 0.05$.

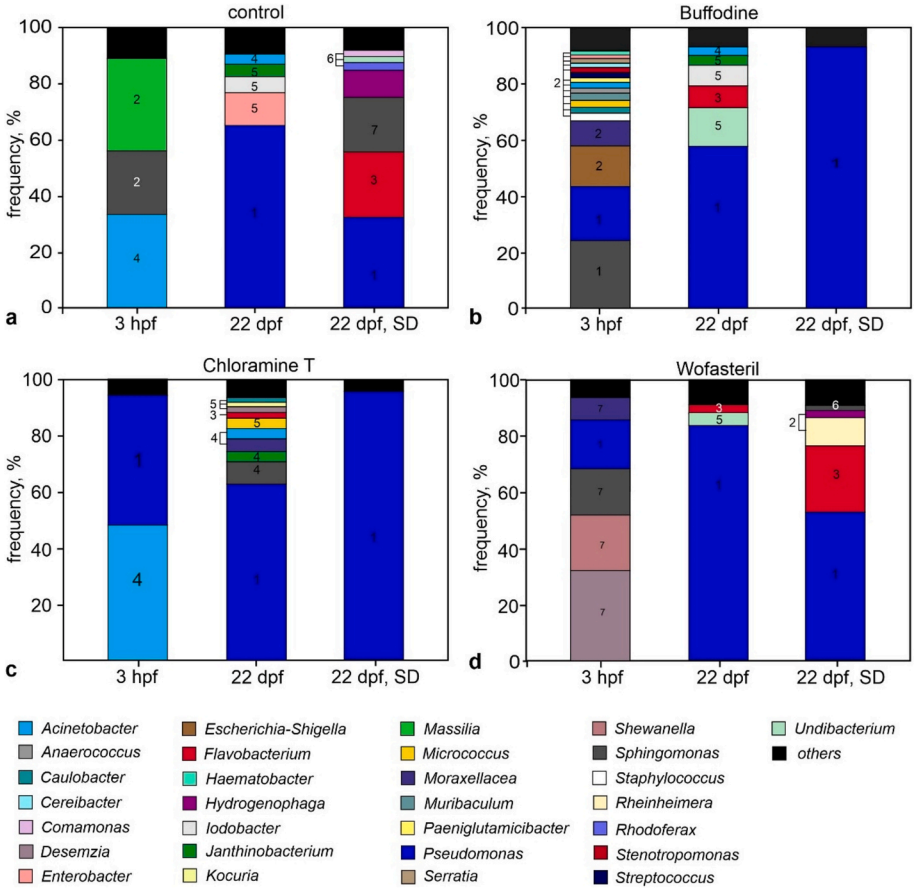

Fig. 4. Bacteria frequencies (specific OTUs in relation to the total number of OTUs) in untreated and disinfected rainbow trout eggs. (a) control, (b) 10 ml/l buffodine for 20 min, (c) 100 mg/l Chloramine T for 40 min, (d) 100 µl/l Wofasteril for 40 min. Data are mean of 3 individual egg samples. Numbers within the bars indicate the classification of the species in relation to the egg compartment and to the development stage. 1 – persistent bacteria, 2 – bacteria occurring only in 3 hpf eggs, 3 – bacteria colonizing the egg internal from 3 hpf to 22 dpf, 4 – bacteria colonizing the chorion of 3 hpf eggs, 5 – bacteria colonizing the chorion of 22 dpf eggs, 6 –bacteria colonizing or recolonizing the eggs after surface disinfection.

Table 2
Bacteria composition non-disinfected and disinfected rainbow trout eggs in relation to egg compartment and differentiation stage. Disinfection treatments: 10 ml/l Buffodine 20 min, 100 mg/l Chloramine T 40 min, 100 µl/l Wofasteril 40 min. No. = number of species.

Treatment	No.	Species list
Persistent bacteria – Occurrence: 3 hpf: +; 22 dpf: +; 22 dpf SD: +		
Control	1	*Pseudomonas* sp.
Buffodine	3	*Pseudomonas* sp., *Acinetobacter* sp., *Sphingomonas* sp.
Chloramine T	1	*Pseudomonas* sp.
Wofasteril	1	*Pseudomonas* sp.
Bacteria occurring only in 3 hpf eggs – Occurrence: 3 hpf: +; 22 dpf: –; 22 dpf SD: –		
Control	3	*Massilia aurea, Massilia timonae, Serratia* sp.
Buffodine	31	*Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium, Anaerococcus* sp., *Caulobacter* sp., *Cereibacter* sp., *Corticibacterium populi, Corynebacterium* sp., *Delftia* sp., *Dickeya chrysanthemi, Dinghuibacter* sp., *Enterobacter* sp., *Escherichia-Shigella* sp., *Haematobacter* sp., *Klebsiella* sp., *Kocuria* sp., *Lachnospiraceae NK4A136 group, Methylobacterium-Methylorubrum, Micrococcus* sp., *Moraxella* sp., *Paramuribaculum* sp., *Nocardioides* sp., *Peptoniphilus coxii, Photobacterium* sp., *Prevotella oulorum, Pseudoglutamicibacter cumminsii, Roseomonas* sp., *Serratia* sp., *Stenotrophomonas maltophilia, Stenotrophomonas* sp., *Streptococcus* sp., *Vagococcus* sp., *Xanthomonas* sp.
Chloramine T	1	*Brevundimonas* sp.
Wofasteril	0	–
Bacteria colonzing the egg internal from 3 hpf to 22 dpf Occurrence: 3 hpf: –; 22 dpf: +; 22 dpf SD: +		
Control	3	*Chryseobacterium* sp., *Comamonas* sp., *Flavobacterium* sp.
Buffodine	2	*Flavobacterium* sp., *Hydrogenophaga* sp.
Chloramine	1	*Flavobacterium* sp.
Wofasteril	1	*Flavobacterium* sp.
Bacteria colonizing the chorion during water hardening Occurrence: 3 hpf: +; 22 dpf: +; 22 dpf SD: –		
Control	1	*Acinetobacter* sp.
Buffodine	1	*Chryseobacterium* sp.
Chloramine T	3	*Acinetobacter* sp., *Moraxella* sp., *Sphingomonas* sp.
Wofasteril	0	–
Bacteria colonizing the chorion from 3 hpf to 22 dpf Occurrence: 3 hpf: –; 22 dpf: +; 22 dpf SD: –		
Control	8	*Enterobacter* sp., *Flavobacterium hibernum, Iodobacter arcticus, Janthinobacterium* sp., *Oxalobacteraceae* sp., *Paeniglutamicibacter* sp., *Pedobacter* sp., *Sphingobacterium* sp.
Buffodine	10	*Arthrobacter* sp., *Rhodoferax* sp., *Comamonas jiangduensis, Flavobacterium hibernum, Iodobacter arcticus, Janthinobacterium* sp., *Undibacterium* sp., *Pantoea coffeiphila, Rheinheimera* sp., *Sphingobacterium* sp.
Chloramine T	18	*Caulobacter* sp., *Cetobacterium* sp., *Chryseobacterium* sp., *Comamonas jiangduensis, Corynebacterium* sp., *Desemzia* sp., *Enterobacter* sp., *Hydrogenophaga* sp., *Iodobacter arcticus, Janthinobacterium* sp., *Kocuria* sp., *Lactobacillus iners, Micrococcus* sp., *Moraxella* sp., *Undibacterium parvum, Roseomonas* sp., *Sphingomonas* sp., *Xanthobacter* sp.
Wofasteril	9	*Acinetobacter* sp., *Comamonas jiangduensis, Exiguobacterium* sp., *Flavobacterium hibernum, Gammaproteobacterium P1_1_1AA1, Iodobacter arcticus, Janthinobacterium* sp., *Undibacterium parvum, Sphingobacterium* sp.
Bacteria colonizing or recolonizing the eggs after surface disinfection Occurrence: 3 hpf: –; 22 dpf: –; 22 dpf SD: +		
Control	15	*Candidatus Fluviicola riflensis, Comamonas piscis, Corynebacterium* sp., *Escherichia-Shigella* sp., *Faecalibacterium* sp., *Fluviicola* sp., *Hydrogenophaga* sp., *Micrococcus* sp., *Moraxella* sp., *Phreatobacter* sp., *Rhodoferax* sp., *Sphingomonas* sp., *Stenotrophomonas maltophilia, Streptococcus* sp., *Undibacterium parvum*
Buffodine	4	*Cetobacterium* sp., *Gammaproteobacteria bacterium P1_1_1AA1, Rhodoferax* sp., *Staphylococcus* sp.
Chloramine T	1	*Gammaproteobacterium P1_1_1AA1*
Wofasteril	19	*Brevundimonas* sp., *Burkholderia* sp., *Caulobacter* sp., *Cloacibacterium* sp., *Corynebacterium* sp., *Escherichia-Shigella* sp., *Hydrogenophaga* sp., *Kocuria* sp., *Lepisosteus oculatus, Micrococcaus* sp., *Moraxella* sp., *Rheinheimera* sp., *Rhodoferax* sp., *Rothia mucilaginosa, Shewanella* sp., *Sphingomonas* sp., *Staphylococcus* sp., *Streptococcus* sp., *Undibacterium parvum*

frequency of persistent bacteria was significantly lowest in control eggs and eggs disinfected with Chloramine T. Buffodine and Wofasteril treatments significantly reduced the frequency of chorion-colonizing bacteria at 3 hpf (Fig. 5a).

In 22 dpf eggs the persistent bacteria were the dominant group across all treatments (Fig. 5b). Buffodine and Wofasteril treatments significantly reduced the frequency of chorion-colonizing bacteria and those colonizing the egg internal from 3 hpf to 22 dpf (Fig. 5b).

In surface-disinfected 22 dpf eggs the persistent bacteria were significantly less frequent in the control and Wofasteril treatments, while recolonizing bacteria were significantly more frequent (Fig. 5c). Buffodine and Wofasteril treatments significantly reduced the frequency of bacteria colonizing the egg internal from 3 hpf to 22 dpf (Fig. 5c).

4. Bacterial species diversity

The Shannon index, a measure of bacterial diversity, revealed a significantly higher diversity in 3 hpf eggs disinfected with Buffodine and a significantly lower diversity in those treated with Chloramine T (Table 3). At 22 dpf, the highest diversity was observed in control and Buffodine-treated eggs. For surface-disinfected 22 dpf eggs, diversity was highest in control and Wofasteril-treated eggs (Table 3). The Bray-Curtis index, which assesses diversity in species composition between treatments, showed significant differences across all treatments, with values ranging from 0.381 to 0.999 (data not shown).

5. Discussion

5.1. Disinfection technique

During water hardening of eggs of fresh water teleosts, the fluid within the perivitelline space absorbs water due to its high osmotic pressure[11]. As a result, the tension of the chorion increases due to the rising turgor pressure, and the chorion hardens. Simultaneously, the chorion and the oocyte membrane also becomes highly impermeable[11]. Water hardening begins as soon as the eggs come into contact with water. The primary function of this process is to create an elastic and protective barrier around the developing embryo.[11] Consequently, disinfection of the egg interior is only feasible during or immediately after fertilization, while the eggs remain immersed in a 0.75 % NaCl solution, which in Salmonidae suppresses the hardening process.[29] The present study demonstrates that non-hardened rainbow trout eggs can be fertilized in a 100 mg/l Chloramine T solution or a 100 µL/l Wofasteril solution prepared in 0.75 % NaCl. Following fertilization, the disinfection solution is either replenished or replaced, and the eggs undergo disinfection for a total of 40 min. Currently, the standard procedure for disinfecting non-hardened salmonid eggs follows a two-step method using an iodophore solution: fertilization in 0.7 % NaCl, followed by transfer to the disinfection medium.[17] The one-step disinfection procedure described in this study offers a practical alternative. The applied concentrations of Chloramine T and Wofasteril have minimal impact on sperm motility, unlike iodophore solutions, which inhibit the motility of salmonid sperm.[17] This simplified approach reduces time-

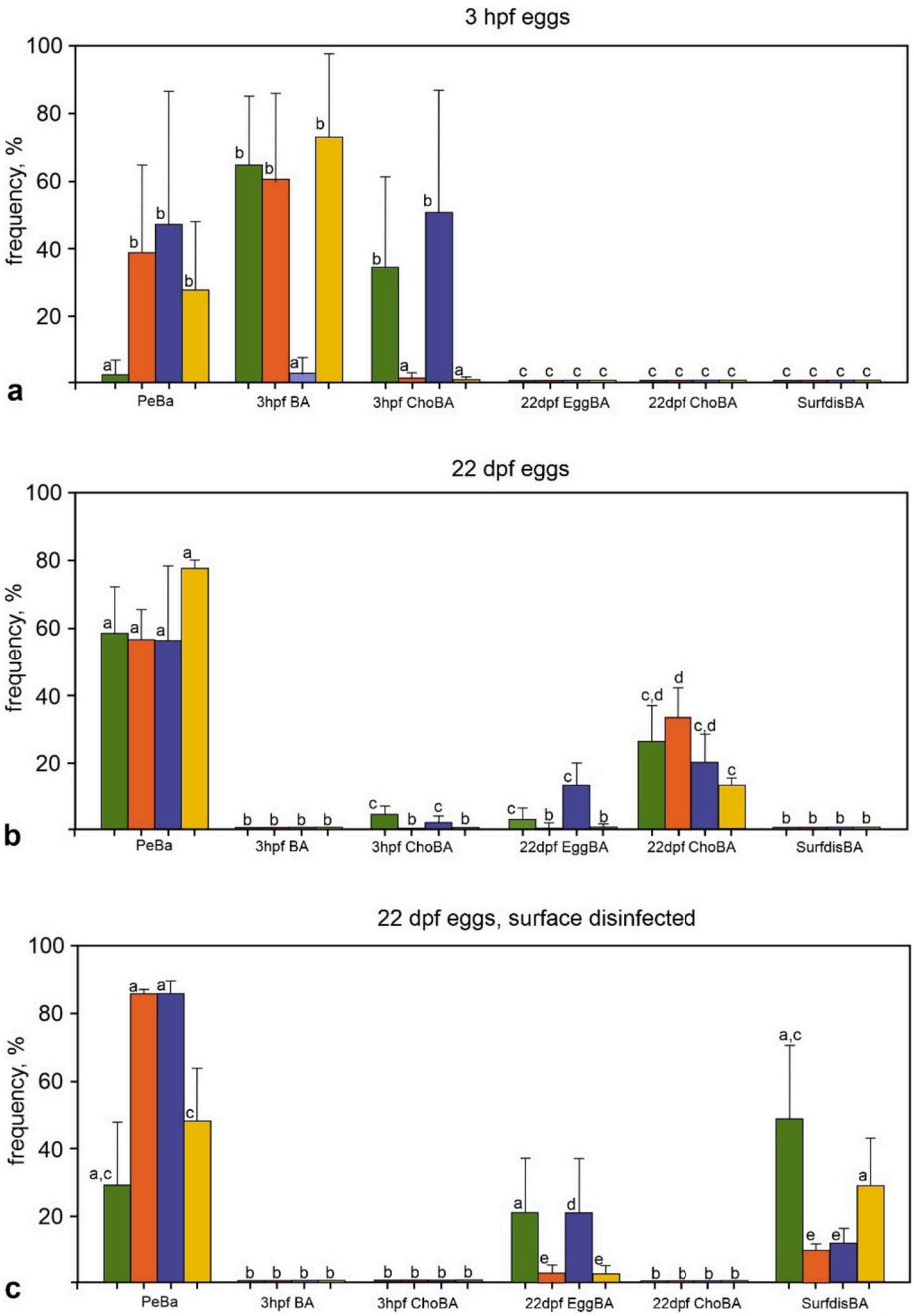

Fig. 5. Frequency (percentage of specific OTUs in relation to the total number of OTUs) of bacteria categories in relation to differentiation stages. Green bar – control eggs, orange bar – 10 ml/l Buffodine 20 min, blue bar – 100 mg/l Chloramine T 40 min, yellow bar 100 µl/l Wofasteril 40 min. PeBa – persistent bacteria, 3hpf BA – bacteria occurring only in 3 hpf eggs, 3hpf ChoBA – bacteria colonizing the chorion of 3 hpf eggs, 22 hpf EggBa bacteria colonizing the egg internal from 3 hpf to 22 dpf, 22dpf ChoBA – bacteria colonizing the chorion of 22 dpf eggs, SurfdisBa – bacteria colonizing or recolonizing the eggs after surface disinfection. Data are mean ± standard deviation of 3 individual egg samples per treatment, those superscripted by different letters are significantly different, $P < 0.05$.

sensitive handling steps, making it more efficient for large-scale fish farm operations where numerous fish are stripped simultaneously. Additionally, iodophore products are currently under review in the European Community due to their potential environmental impact. The method presented here offers an environmentally compatible alternative, as Wofasteril and Chloramine T are used in lower concentrations and degrade rapidly in water without leaving harmful residues. Therefore, this method serves as a viable option should iodophores be banned as biocides in aquaculture due to legislative decisions. Since salmonid gametes exhibit similar tolerance levels to disinfectants,[13] this approach is applicable to other salmonid species.

5.2. Effects of Wofasteril and Chloramine T on gamete viability and embryo development

Wofasteril concentrations exceeding 50 µl/l and Chloramine T concentrations above 100 mg/l significantly reduced sperm motility and swimming velocity, indicating that Wofasteril has a more detrimental effect on spermatozoa than Chloramine T. The reported concentrations therefore represent the upper tolerance threshold for spermatozoa. As oxidizing agents, both disinfectants may induce oxidative damage to spermatozoal proteins and lipids, potentially disrupting metabolic pathways and changing membrane potentials essential for motility.[31] In teleost fish, sperm motility plays a crucial role in fertilization[32–33].

Table 3
Shannon index of rainbow trout eggs disinfected with 10 ml/l Buffodine for 20 min, 100 mg/l Chloramine T for 40 min, and 100 µl/l Wofasteril for 40 min in comparison to untreated control eggs. Data are mean ± standard deviation (n = 3), those superscripted by different letters are significantly different, $P < 0.05$.

Shannon	3 hpf	22 hpf	22 dpf, surface disinfected
control	0.592 ± 0.332 [a]	1.234 ± 0.155[b]	1.369 ± 0.485[b]
Buffodine, 10 ml/l for 20 min	2.382 ± 0.379[c]	1.650 ± 0.274[b,c]	0.645 ± 0.401 [a]
Chloramine T, 100 mg for 40 min	0.351 ± 0.129 [d]	0.892 ± 0.257 [a]	0.583 ± 0.308 [a]
Wofasteril, 100 µl for 40 min	0.730 ± 0.550 [a]	0.995 ± 0.164 [a,b]	1.148 ± 0.304[b]

However, reduced motility can be partially compensated by increasing the sperm-to-egg ratio.[32–33] Since fish farms typically use excess semen, a 20–30 % reduction in sperm motility is considered negligible in practical applications.

Beyond sperm motility, disinfection media could also impact egg hardening, an enzyme-mediated process of protein linking[11], as well as embryonic and larval development, which relies on complex cellular mechanisms such as differentiation, proliferation, migration, transformation, and apoptosis.[34] However, no adverse effects were observed on egg mass increase during water-hardening, embryo development to the eyed stage, hatching success of normally shaped larvae, or larval total length. Therefore, this disinfection method does not negatively affect development.

5.3. Effects of disinfectants on bacterial load

The reduction in total bacterial mass serves as a key metric for evaluating disinfectant efficiency. In this study, disinfection treatments with Chloramine T, Wofasteril, and Buffodine significantly reduced bacterial mass in 3 hpf eggs, achieving similar levels of effectiveness. Bacterial reduction rates were 60–70 % for peptone water cultures, 20–45 % for tryptic soy broth, and 10 % for McConkey broth. Even after a 22-day development period, bacterial load differences remained between control and disinfected eggs. Chloramine T, Wofasteril, and Buffodine continued to reduce bacterial mass by 30–55 % for peptone water cultures, 20–40 % for tryptic soy broth cultures, and 25 % for McConkey broth cultures. Chloramine T proved the most effective, with reductions comparable to those observed in 3 hpf eggs.

During embryogenesis, the chorion serves as a primary target for bacteria from water, equipment, and human handling. To assess the long-term impact of disinfection on bacteria occurring in the egg interior, the bacterial load of surface-disinfected 22 dpf eggs was compared to that of 22 dpf non-disinfected eggs. Post-surface disinfection, no significant differences were found between eggs disinfected at the non-hardened stage and those left untreated at the non-hardened stage. This suggests that on long term non-hardened egg disinfection primarily affects surface-associated bacteria with minor impact on internal bacteria.

The findings of this study indicate that the two-step disinfection process with Buffodine can be replaced also under aspects of disinfection efficiency by a single-step procedure using Wofasteril or Chloramine T, with Chloramine T offering superior efficacy. Reducing bacterial load and microbial presence may support the immune system of developing eggs and embryos. In salmonids, immune-related genes are weakly expressed during embryogenesis but become highly upregulated after hatching[35]. Lysozyme plays an essential early role in egg protection before embryonic cells begin expressing immune genes[36]. For a disinfection procedure to be considered highly effective, it must achieve a bacterial reduction of at least 6 log (>99 %).[18] The lower success of disinfection in salmonid eggs may be attributed to the low permeability of the yolk[10], which restricts disinfectant diffusion. Additionally, the protein-rich yolk may shield bacteria from disinfectants or reduce disinfectant efficacy by binding to proteins—a phenomenon known as protein failure. This has been documented for peroxide and chlorine compounds.[18] Variability in bacterial reduction across different culture media likely results from bacteria residing in different egg compartments, where varying degrees of protection influence disinfection effectiveness.

5.4. Bacterial composition of non-disinfected and disinfected eggs

According to their occurrence across different treatment and developmental stages, several types of bacteria could be distinguished and classified. These were persistent bacteria, bacteria unique to 3 hpf eggs, bacteria colonizing the eggs and chorion during various stages of embryogenesis, and bacteria recolonizing the eggs after surface disinfection at 22 dpf. Notably, bacteria colonizing the chorion of 3 hpf eggs may also include those present in the perivitelline space, as the analytical methods used were not capable of distinguishing between these compartments. The Bray-Curtis index, alongside the data presented in Table 2 and Figs. 3 and 4, revealed distinct qualitative and quantitative microbiome compositions across developmental stages and treatment conditions.

In 3 hpf control eggs, a significant portion of the bacteria were unique to this stage (e.g. Sph*ingomonas* sp. and *Massilia* sp.), while *Acinetobacter* sp. was found colonizing the chorion. Persistent bacteria were present only in low abundance, suggesting that the microbial composition at this stage remains immature or is still undergoing dynamic changes. The identified bacterial species are commonly found in aquatic environments and within the skin and digestive tracts of Salmonidae species.[37–38] Previous studies have also reported that the initial microbial composition of fish eggs often reflects that of the surrounding water and includes also bacteria vertically transmitted from parent fish.[39] Consequently, both the bacterial composition of the fertilization solution and potential contamination introduced during the stripping process may play crucial roles in shaping the future egg microbiome.

By 22 dpf, the persistent bacterium *Pseudomonas* had become the most abundant species in control eggs (>60 %), followed by species colonizing the chorion at 3 hpf (*Acinetobacter* sp.) and others colonizing the chorion from 3 hpf to 22 dpf (*Enterobacter, Iodobacter, Janthinobacterium*). *Pseudomonas* species are ubiquitous in aquatic environments and are also found in animal hosts, including fish.[40] In a previous study, *Pseudomonas* sp. has been described as a vertically transmitted pioneering species as it is dominant in mature fish but absent from water and feed.[19] *Iodobacter* and *Janthinobacterium* are of particular interest as they are able to inhibit the growth of *Saprolegnia* hyphae,[41–42] a common fungal pathogen in hatcheries known to infect non-fertilized and dead eggs. *Enterobacter*, while typically associated with the fish digestive tract, is also widespread in natural freshwater environments.[43] .

Surface disinfection of 22 dpf eggs significantly altered the bacterial composition. Although *Pseudomonas* remained dominant (30 %), there was a marked increase in *Flavobacterium* and *Sphingomonas* (20 % each), with minor increases in *Hydrogenophaga* and *Comamonas* (5 % each), and *Rhodoferax* and *Undibacterium* (2.5 % each). Since *Pseudomonas* and *Flavobacterium* were identified as internal egg bacteria, these findings suggest that surface disinfection affects not only chorion-associated bacteria but the total egg microbiome. These dynamics contradict the theory of chorion impermeability, raising the possibility that disinfectants may diffuse into the egg interior or that specific bacterial species may migrate between different egg compartments. Such findings have important implications for the future design of surface disinfection procedures. *Flavobacterium*, in particular, is of concern due to its known association with fish diseases. The genus includes pathogens such as *F. psychrophilum*, which causes cold-water disease and rainbow trout fry syndrome, and *F. columnare*, the causative agent of cotton-wool disease.[42] In contrast, *Hydrogenophaga, Comamonas, Rhodoferax*, and

Undibacterium are freshwater bacteria,[43] with *Hydrogenophaga* also present in the digestive tract of rainbow trout.[44] .

Disinfection of non-hardened eggs with Buffodine, Chloramine T, or Wofasteril increased the frequency of persistent bacteria in 3 hpf eggs compared to controls, suggesting the potential establishment of a more stable microbiome. Notably, Chloramine T reduced bacteria unique to 3 hpf eggs, while Buffodine and Wofasteril reduced bacteria colonizing the chorion. This indicates that the different disinfectants act via distinct mechanisms, as also evidenced by data on reductions in bacterial load. Buffodine treatment was associated with increased bacterial diversity (as measured by the Shannon index), whereas Chloramine T reduced it. These effects also extended to 22 dpf, where Buffodine-treated eggs maintained greater microbial diversity, while Wofasteril- and Chloramine T-treated eggs exhibited reduced diversity. Taken together, the observed reductions in both bacterial load and number again indicate that Chloramine T is the most effective disinfectant for non-hardened rainbow trout eggs. Across all treatments, *Pseudomonas* remained dominant (>50 %), followed by internal bacteria like *Flavobacterium*, and chorion-associated species such as *Janthinobacterium*. Surface disinfection at 22 dpf reduced the Shannon index in Buffodine- and Chloramine T-treated eggs, but not in Wofasteril-treated or control eggs. These findings indicate that disinfection creates opportunities for opportunistic bacteria to colonize or recolonize eggs.

The differing actions and efficiencies of the disinfectants are likely related to their distinct mechanisms of action. Wofasteril, as a strong oxidizing agent, damages biomolecules by electron removal[45]. Chloramines also oxidize biomolecules and disrupts cell metabolism, though their precise mode of action is unclear[46–47]. Iodine destabilizes membranes by oxidizing unsaturated fatty acids, disrupting protein synthesis, enzyme activity, and DNA structure, culminating in cell death.[47] These mechanistic differences likely influence bacterial survival and colonization patterns on and within the egg.

Despite the significant differences in microbiome composition between treatments, no effects on egg development, development of early larval stages and on larvae malformation rate were observed. The fish microbiome remains poorly understood, especially regarding the functional relationships between its composition and functionality. Therefore the present data on microbiome composition remain at a descriptive level at present. The microbiota of fish is considered a critical barrier against opportunistic pathogens.[48] Therefore, disinfectants should not only be evaluated based on their efficiency in reducing the bacterial numbers and the total bacterial load (which is beneficial to reduce pathogen transfer from parents to offspring) but also in terms of their impact on the natural microbial community. Establishing a stable, diverse, and resilient microbiome may have long-term benefits for fish health, particularly as many fish pathogens are opportunistic.[48] The present study provides an initial scientific indication of these relationships. Future research should evaluate disinfectants not only from a sanitary perspective but also in terms of their ability to support a diverse and resilient microbiome. Moreover, artificial manipulation of the bacterial community immediately after disinfection of non-hardened eggs may offer a way toward establishing more robust microbial profiles. In aquaculture, various bacterial species have shown potential for promoting fish health. *Lactobacillus* species can improve larval survival by forming a protective microbial barrier. *Pseudomonas* spp., *Pediococcus* spp., and *Enterococcus* spp. are known for their antimicrobial properties and ability to compete with pathogens, while *Shewanella* sp. and *Bacillus subtilis* produce antimicrobial compounds that inhibit harmful bacteria.[22–24] These species represent promising candidates for egg microbiome enhancement strategies in aquaculture.

6. Conclusions

Non-hardened rainbow trout eggs can be simultaneously fertilized and disinfected using a one-step method. This involves treatment with 100 mg/l Chloramine T or 100 µl/l Wofasteril in a 0.75 % NaCl solution for 40 min. This approach does not negatively affect embryo or early larval development. Wofasteril demonstrated similar disinfection efficacy in reducing bacterial load when compared to the traditional two-step disinfection process using iodophore solutions, while Chloramine T proved to be even more effective. Reducing bacterial load in non-hardened eggs lowers the risk of pathogen transfer from parent fish and supports the immune system of developing embryos. Moreover, the microbiome composition of the eggs underwent significant changes across developmental stages in response to different disinfection treatments. This highlights the importance of evaluating future disinfection methods not only for their efficacy but also in terms of their impact on microbiome stability and resilience which are critical factors for long-term fish health. Finally, the one-step disinfection procedure offers practical advantages, particularly for large-scale fish farming operations where numerous fish are stripped simultaneously. By reducing time-sensitive handling steps, this method enhances efficiency. Additionally, it provides an environmentally friendly alternative, as Wofasteril and Chloramine T are applied at much lower concentrations than iodophore solutions, degrade rapidly in water, and leave minimal harmful residues.

CRediT authorship contribution statement

Franz Lahnsteiner: Writing – original draft, Conceptualization, Validation. **Anna Dünser:** Methodology, Supervision, Conceptualization.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgement

The authors are grateful to the team of the fish farm Kreuzstein for care of experimental fish and for supervision of experiments.

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