# The effect of disinfection with ozone in restocking brown trout farm in North Italy

## *Effetto della disinfezione con ozono in un allevamento di trote fario da ripopolamento nel Nord Italia*

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SUMMARY - Since new EU aquaculture rules prevent the use of traditional chemical disinfectants because of their environmental impact, researchers are looking at alternative disinfection methods and ozone seems to be an interesting candidate. This experimentation was conducted at a brown trout (*Salmo trutta*) restocking farm. The experimental plan was bifactorial balanced with randomized groups and the tested experimental factors were: disinfection method and density of eggs, with 8 replicates per treatments. Two densities of eggs were utilised: 1.2 eggs per $cm^2$ (corresponding to a mono stratified layer of eggs) and 2.5 eggs per $cm^2$ (corresponding to a bi-stratified layer of eggs) and 4 disinfection treatments: 0.1 mg/L ozone (10 min/day), 0.4 mg/L ozone (10 min/day), Detarox (10 min/day) and a control group without disinfection were studied. The daily egg survival rate was measured 6 days per week in each tank. The hampseed trapping technique was utilised to collect *Saprolegnia* fungi directly from the aquatic habitat. Observing the results obtained from the final egg survival rate, it is possible to see that in this experimentation the higher concentration of ozone obtained the best results, but there is only a little more than 2% difference between the highest and lowest results.

*RIASSUNTO - Le regole imposte dalla Comunità Europea restringono notevolmente l'utilizzo dei disinfettanti tradizionali in acquacoltura in relazione al loro impatto ambientale. L'ozono è un disinfettante moderno, potenziale candidato alla disinfezione in acquacoltura. Il piano sperimentale, condotto in un impianto di riproduzione dove viene allevata la trota fario (*Salmo trutta*), è stato bifattoriale bilanciato a blocchi randomizzati e i fattori sperimentali testati sono stati il metodo di disinfezione e la densità delle uova, con 8 repliche per trattamento. Sono state utilizzate 2 densità di uova: 1,2 uova per $cm^2$ (che corrisponde a un monostrato) e 2,5 uova per $cm^2$ (che corrisponde a una condizione bistratificata di uova) e 4 trattamenti di disinfezione: 0,1 mg/l di ozono, 0,4 mg/l di ozono, Detarox (tutti utilizzati per 10 min/giorno) ed un gruppo di controllo senza disinfezione. La sopravvivenza giornaliera delle uova è stata misurata per 6 giorni alla settimana in tutte le vasche. Allo scopo di studiare la presenza dei miceti appartenenti al genere* Saprolegnia *direttamente nell'acqua è stata utilizzata una tecnica utilizzando semi di canapa. Dalle risposte ottenute nei confronti della sopravvivenza finale delle uova si può concludere che con la più alta concentrazione di ozono si sono ottenuti i migliori risultati di disinfezione, che corrispondono a un aumento della sopravvivenza finale del 2%.*

**Key words**: Trout farming, Disinfection, Ozone, *Saprolegnia* spp., Brown trout, *Salmo trutta*, Eggs.

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## INTRODUCTION

Aquaculture is one of the fastest expanding sectors of animal production. Trout farming is well developed in Italy and in all Europe, but the increase in the European freshwater aquaculture greatly depends on environmental sustainability. A drawback of trout hatchery is that it may contaminate recipient water bodies and can therefore become limited by environmental constraints. Ozone disinfection preserves an adequate quality of the output water. It was primarily used as a disinfectant for drinking water as a bactericide agent in different sectors of food production for many year (Da Silva *et al.*, 1998) and in wastewater treatment (Muela *et al.*, 1998; Freese *et al.*, 1999).

Ozonation is a disinfection technique that is often used in Asian shrimp farming, in fish hatcheries and less frequently in earthen ponds (Graslund & Bengtsson, 2001; Schuur, 2003) and in Taiwan finfish culture (Liao *et al.*, 2001). Ozone has been also used in abalone farming because of its antibacterial activity (Dixon *et al.*, 1991). Since new EU aquaculture rules prevent the use of traditional chemical disinfectants such as green malachite or formalin because of their environmental impact (Forneris *et al.*, 2003), researchers are now looking at new disinfection methods and ozone seems to be an interesting candidate because the only product of its degradation is oxygen (Bullock *et al.*, 1997; Krumins *et al.*, 2000; Suantika *et al.*, 2001; Grotmol *et al.*, 2002; Forneris *et al.*, 2003; Schuur, 2003; Summerfeld, 2003). Ozone is effective in fish pathogen control, but this treatment can reduce or delay hatching (Jorquera *et al.*, 2002). Moreover, comparing ozone with other disinfectants, like chlorine, Meunpol *et al.* (2003) showed that ozone removes some dangerous bacteria 600-3000 times faster than chlorine. For this reason and because ozone leaves only small amounts of residuals, it is a interesting candidate for water quality management in shrimp culture.

The use of ozonated water is particularly interesting in rearing fishes during their embryonic development. During the first stages of fish development, bacterial disease has been assumed to be a major cause of both egg mortality and the occurrence of deformed larvae (Harboe *et al.*, 1994; Forneris *et al.*, 2003). Tipping (1988) instead observed that rainbow trout reared in ozone-treated water had a lower mortality rate and that they were larger than control fish. Ozonation is routinely used in some American trout farms (Zydlewski *et al.*, 2003), the presence of ozone in water during the day can help to maintain a stable population of nitratifying bacteria and it can be stated that the supplementation of ozone, mainly in recirculation systems, considerably improves water quality (Krumins *et al.*, 2000).

This experimentation was conducted at a brown trout (*Salmo trutta*) restocking farm. This farm is situated in a high tourist interest area in North West Italy (the Aosta Valley) where restocking aquaculture can improve the economic development for Salmonid farming. Brown trout fingerlings are diffused in the rivers in Aosta Valley. Ozone is more expensive than other disinfectants, but the absence of environmental impact could justify the high cost of ozone in this situation.

The aim of this study was to determine the effectiveness of ozone in relation to other disinfectants utilised in brown trout hatchery in a restocking oriented farm.

## MATERIALS AND METHODS

### Experimental plan

This research was conducted on an experimental farm in Morgex (the Aosta Valley - NW Italy) it lasted 2 months; 20 days for practical organisation of the experiment and 40 days for the disinfection trial (6 days per week), from January $18^{th}$ until February $28^{th}$ 2003. The daily egg survival rate was measured 6 days per week in each tank. The experimental plan was bifactorial balanced with randomized groups and the experimental factors were: disinfection method and density of eggs, with 8 replicates per treatment. Two density of eggs: 1.2 eggs per $cm^2$ (corresponding to a single stratified layer of eggs) and 2.5 eggs per $cm^2$ (corresponding to a double-stratified layer of eggs) and 4 disinfection treatments: 0.1 mg/l ozone (10 min/day), 0.4 mg/l ozone (10 min/day), Detarox (10 min/day) and a control group without disinfection, were utilised. Detarox is a mixture of hydrogen peroxide (20% by volume) and peracetic acid (4.5% by volume) which is normally used in northern Italian hatcheries. The resulting experimental design was 2 (egg density) x 4 (disinfection methods) x 8 replicates. During the entire period of the experiment, the water temperature was 9-10° C and the dissolved oxygen was about 10 mg/l. The experimental equipment that was utilised consisted of 8 concrete tanks with each tank holding 8 small metallic grid boxes containing trout eggs. The removal of eggs could have induced an artificial mortality that did not depend on our experimental conditions and, in order to avoid any human interference during trout embryonic development, dead eggs were therefore not eliminated from 4 of the boxes in any experimental treatment. In this way two groups of eggs were separately studied: not-cleaned and cleaned eggs. Ozone was produced using an ozone generator with pure gaseous oxygen, residual ozone concentration in water was measured using a specific ozone electrode. An ALPHAOMEGA® ozone generator was used to produce 1500 mg ozone/h. This generator was specifically modified for this experiment and it was supplied by Taurus T. s.a.s. (Turin, Italy). The apparatus was equipped with an instrument that indicates the concentration of ozone produced by a generator, while the monitoring of the ozone in the water was performed by a probe positioned in the basin directly near the incubating eggs. In this way the dose of supplied ozone was appropriately monitored. The sensitivity of the membrane cell probe allowed a ≥ 0.001 ppm measurement of the ozone concentrations to be made in the water.

Microbiological methods

According to Seymour method (1970), a small quantity of hempseeds was introduced into each experimental tank as a bioindicator of *Saprolegnia* in water, from January 23th to March 3th and the microbiological analysis of the water was subsequently conducted in the laboratory.

The hampseed trapping technique is generally a successful method for collecting *Saprolegnia* fungi directly from an aquatic habitat. The submergence time for these traps varies according to the temperature (Seymour, 1970); one week was sufficient in our experimental conditions. The microbiological analysis was conducted on 100 ml of sample water after filtration, utilising cellulose nitrate filters with 0.2 µm pores (Sartorius, Germany). After water filtration, the filters were placed on Sabouraud Agar plates for fungi. The hempseed traps were also utilised for fungus reproduction useful for their classification. During the experimentation the number of non developed eggs was counted daily in each grid box (6 days per week) and the microbiological quality of the water and the microbiological quality of eggs was weekly controlled by mean the hemp seed traps. These analyses were conducted to investigate the relationship between the natural presence of *Saprolegnia* in the water and the occurrence of *Saprolegnia* on trout eggs.

Statistical elaboration

In the statistical elaboration of the data, only the survival rate of the cleaned eggs was considered and because of the low number of replicates (n = 4) the Kruskal Wallis test (non parametric ANOVA) was used for the statistical data elaboration (Venables & Ripley, 2002). Statistical analysis was performed using R, version 1.7.1 (R Development Core Team, 2003).

Bootstrap elaboration

Considering the slight differences that resulted from the median comparison, a computer intensive statistic was calculated to explore the sampling distribution of the medians. This statistical procedure is called bootstrap (Manly, 1991). Bootstrap is to take a number of random samples from the observed data with replacement and to calculate the median (bootstrap median) from these samples. The variability of sample median about the true unknown median can be investigated by difference between bootstrap median and sample median. Following Venables & Ripley (2002), 1000 random sample were utilised in resampling test. The bootstrap medians, a 95% confidence interval based on the percentile distribution, a bias of the sample median and the standard error of bootstrap median, were obtained. The bootstrap procedure was performed using R, version 1.7.1 (R Development Core Team, 2003).

## RESULTS

If the result obtained from the final egg survival rate is observed without considering the egg density, it is possible to see that the higher concentration of ozone led to the best results, but there was only approximately 2% difference between the highest and lowest results (Figure 1). The final survival rate in all the experimental treatments was higher in tanks 2, 3 and 1, which received ozone (Figure 2).

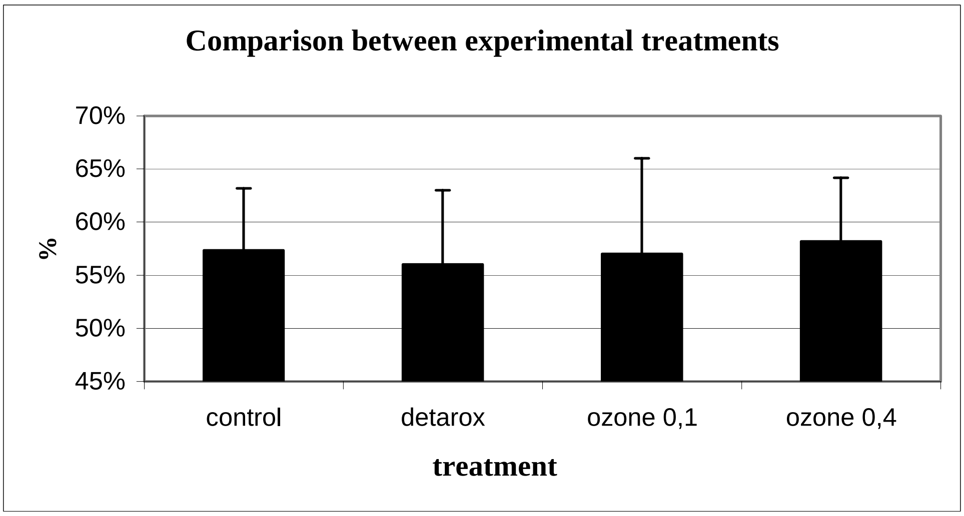


Figure 1 - Final survival rate in the experimental treatments.

*Figura 1 – Percentuale di sopravvivenza finale nei gruppi sperimentali con diverso trattamento.*

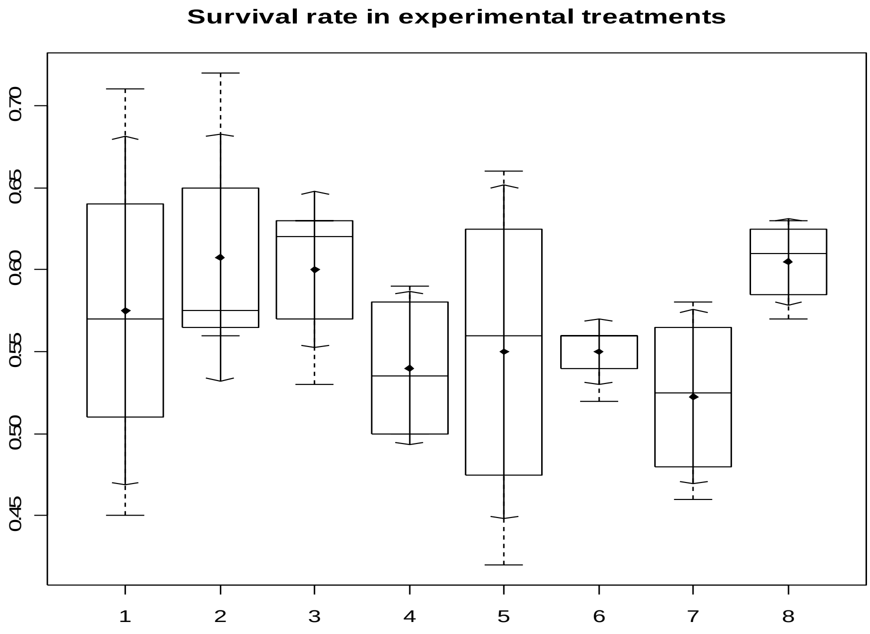


Figure 2 - Final survival rate in the 8 disinfection treatments that were considered.
Treatments: 1 = LD Ozone 0.1 ppm; 2 = HD Ozone 0.1 ppm; 3 = LD Ozone 0.4 ppm; 4 = HD Ozone 0.4 ppm; 5 = LD Detarox; 6 = HD Detarox; 7 = LD control; 8 = HD control.
(LD: low density of eggs; HD: high density of eggs).
*Figura 2 – Percentuale di sopravvivenza finale negli 8 gruppi considerati con diversi trattamenti disinfettanti. Trattamenti: 1 = BD Ozono 0,1 ppm; 2 = AD Ozono 0,1 ppm; 3 = BD Ozono 0,4 ppm; 4 = AD Ozono 0,4 ppm; 5 = BD Detarox; 6 = AD Detarox; 7 = BD controllo negativo; 8 = AD controllo negativo.*
*(BD: bassa densità di uova; AD: alta densità di uova).*

If we observe the variability of data, it can be seen that the survival rate in the control treatments (tanks 7 and 8) is regular while it is more variable in the low density tanks (1, 3 and 5). This suggests that a high density causes a greater homogeneity of the hatching conditions.

Figure 3 shows the differences in the survival rate between the cleaned and not-cleaned eggs at the end of the experiment. It is evident that the dead eggs caused a rapid mortality in the surrounding eggs. Figure 4 shows the daily egg mortality in all the considered tanks; regardless of the treatment, an initial mortality peak was observed in the first days of hatching and a second major peak about 14-15 days after enclosure of the eggs. This fact could be due to a critical embryo development phase which normally occurs after two weeks of incubation.

During the experiment, a hempseed sample was analysed weekly in order to detect the presence of *Saprolegnia* and at the end, the cumulative percentage of the *Saprolegnia* presence was calculated. *Saprolegnia* was never found in tanks 2, 3, 5, 6 and 7 (Table 1). The relationship between the presence of *Saprolegnia* in the water and the infected eggs was not clear, tanks 1, 4 and 8, where *Saprolegnia* was found, in fact showed different final egg mortality rates.

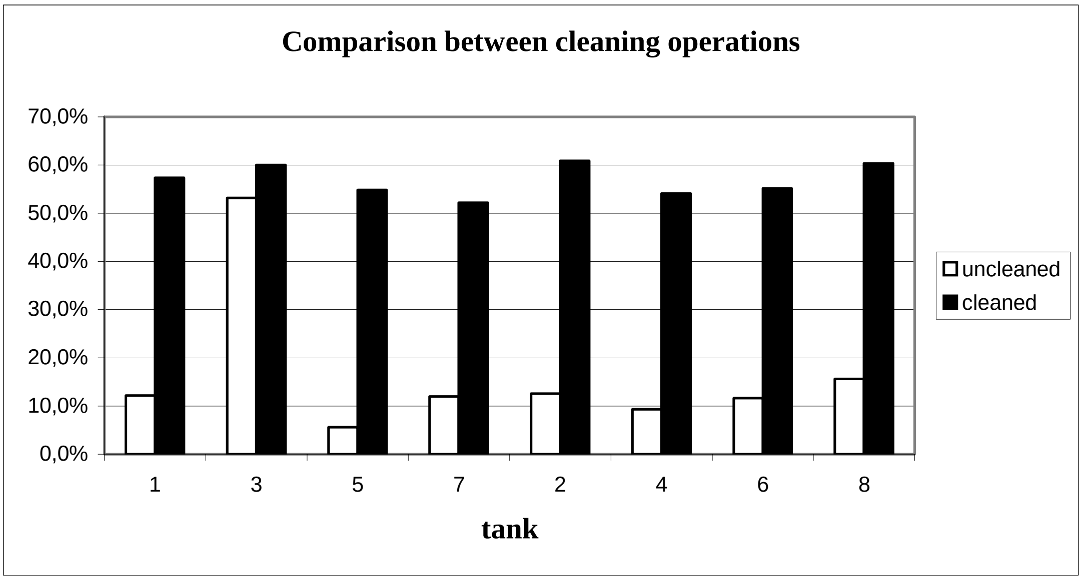


Figure 3 - Comparison of the final survival rate between the cleaned and not-cleaned eggs.
*Figura 3 – Confronto della percentuale finale di sopravvivenza tra gruppi di uova trattati e non trattati.*

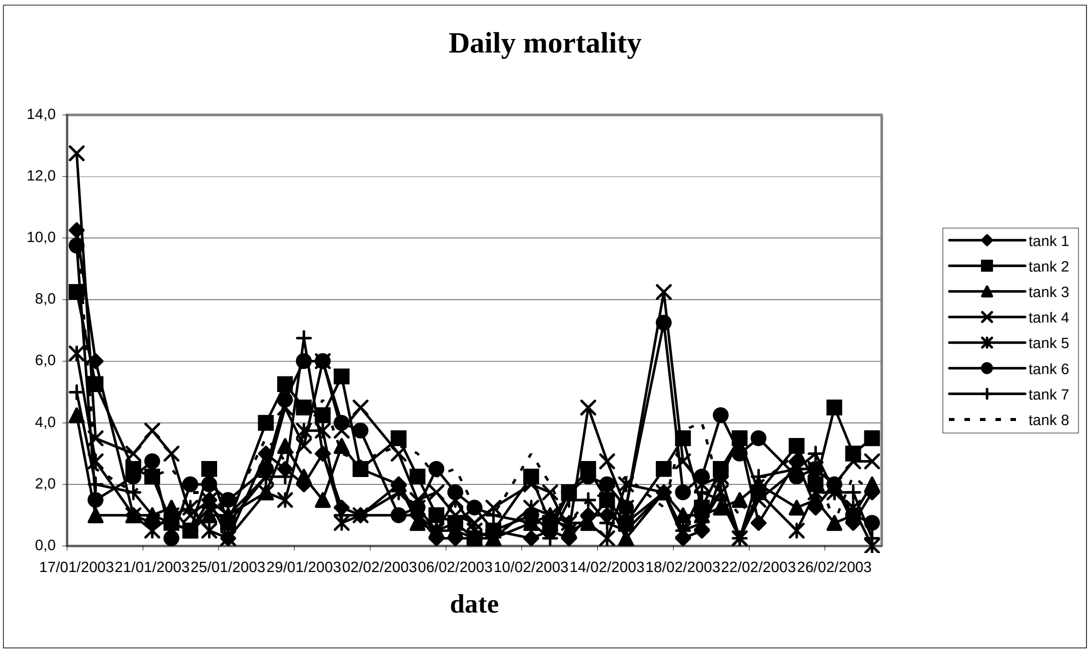


Figure 4 - Daily mortality during the experiment.
*Figura 4 - Mortalità giornaliera durante la sperimentazione.*

| Tank | % |
|---|---|
| 1 | 40% |
| 2 | 0% |
| 3 | 0% |
| 4 | 80% |
| 5 | 0% |
| 6 | 0% |
| 7 | 0% |
| 8 | 80% |

Table 1 - *Saprolegnia* incidence obtained from the hempseeds traps.
Treatments: 1 = LD Ozone 0,1 ppm; 2 = HD Ozone 0,1 ppm; 3 = LD Ozone 0,4 ppm; 4 = HD Ozone 0,4 ppm; 5 = LD Detarox; 6 = HD Detarox; 7 = LD control; 8 = HD control.
(LD: low density of eggs; HD: high density of eggs).
*Tabella 1 – Incidenza di* Saprolegnia *ottenuta mediante l'uso di semi di canapa.*
*Trattamenti: 1 = BD Ozono 0,1 ppm; 2 = AD Ozono 0,1 ppm; 3 = BD Ozono 0,4 ppm; 4 = AD Ozono 0,4 ppm; 5 = BD Detarox; 6 = AD Detarox; 7 = BD controllo negativo; 8 = AD controllo negativo.*
*(BD: bassa densità di uova; AD: alta densità di uova).*

If the results obtained from the bootstrap resampling are considered (Table 2), the low values of bias show that there is only a slight difference (nearly 0) between the sample median and the bootstrap median. Furthermore, the bootstrap standard errors are very low in all the considered treatments. This means that the calculated medians are statistically correct and the differences not revealed in this case are true.

| | **Ozone 0.1 ppm** | **Ozone 0.4 ppm** | **Detarox** | **Control** |
|---|---|---|---|---|
| Bootstrap median | 57 % | 58 % | 56 % | 57.5 % |
| Bias | 0.0089 | - 0.0054 | - 0.0061 | - 0.0028 |
| E.S. of median | 0.029 | 0.032 | 0.018 | 0.026 |
| 95% lower limit | 56 % | 50 % | 52 % | 50 % |
| 95% upper limit | 71% | 63 % | 59 % | 62 % |

Table 2 – Bootstrap statistics (n = 1000) in the experimental treatments.
*Tabella 2 – Statistica Bootstrap (n = 1000) dei trattamenti sperimentali.*

Bootstrap permutation techniques confirmed the statistical results obtained from the Kruskal Wallis test. Bootstrap techniques could become very common in the future for the elaboration of this kind of data, where there is no clear information on the data distribution. Bootstrap and classical non parametric statistics were compared in this research.

## DISCUSSION AND CONCLUSIONS

The numerous uses of ozone for disinfection, water aeration, the removal of metabolic by-products and the improvements in suspended solids removal that have been showed in other researches, make it especially appropriate treatment process for fish hatcheries (Rueter & Johnson, 1995). In this research the use of ozone led to a 2% increase in the egg survival rate. It is interesting to consider that this farm annually produces about 5 million eggs and a 2% difference could consequently lead to an additional 100,000 trout eggs in the hatchery. The effect of disinfection was monitored during all of embryonic development phases and the temporal trend of the egg incubation was similar in all the experimental treatments; no differences were observed for the different disinfectants. As the principal cause of mortality in Italian hatcheries is *Saprolegnia* (Forneris *et al.,* 2003), the occurrence of *Saprolegnia* in the water before contact with the eggs was investigated in this study. *Saprolegnia* is a very common pathogen, but little attention has been paid to the relationship between water occurrence and egg infection of this fungus. According to Seymour method (1970), an unusual method (hempseed trap) was utilised to study the presence of *Saprolegnia* in farm water, but no clear results were obtained. Since the water temperature in trout hatcheries is very low, it is probably necessary to increase the submergence time, from 2 to about 10 days, but this very simple method is promising as a preventive technique in trout farms.

At the moment, ozone is more expensive than other disinfectants, except for particular situations. Suantika *et al.* (2001) and Summerfeld (2003) for example, stated that ozone is much more economically sustainable in recirculation systems than traditional aquacultural systems. Schuur (2003) stated that ozone could be economically interesting in intensive shrimp farming. This experimentation was conducted in a public restocking hatchery. In these conditions a high disinfection cost could be tolerable to minimize the trout farm environmental impact. Disinfection with ozone may help to reduce the transfer of pathogens from broodstock to their offspring and, above all, this method may reduce the spatial diffusion of pathogens from farms to the wild. A negative aspect of ozone utilisation is that fish exposed to ozone may result in oxidative damage of some physiological functions (Ritola *et al.*, 2002). Moreover, from the practical point of view, stable concentrations of ozone are difficult to maintain and dissolved ozone is rapidly lost from water due to several processes such as reactions with particulate, decomposition to oxygen and the loss of ozone into the atmosphere. These factors make ozone determination in water problematic. During ozonation, the ozonator reached is maximum value, stabilized and declined; this caused some difficulties in determining the effective dosage of ozone. In a research regarding shrimp farming (Schuur, 2003), shrimp larvae were exposed to 6 h exposure at 0.5 mg/l ozone without any loss of shrimps. Presently, ozone disinfection is used in two rainbow trout farms in Northern Italy, in the Trentino and Lombardy regions and the ozone generators are prototypes produced by the French AIR LIQUID company. Despite ozone's potential benefits, few Italian fish farm have adopted its use because of its high costs, probably because the operational details are often poor and because of weak results from research.

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