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Use of Ox-Aquaculture© for disinfection of live prey and meagre larvae, *Argyrosomus regius* (Asso, 1801)

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Abstract

Live prey used for marine larval fish (rotifers and *Artemia*) as well as intensive larval rearing conditions are susceptible to the proliferation of bacteria that are the cause for reduced growth and larval mortality. Hydrogen peroxide has been recently proved a good disinfectant in aquaculture, either for eggs, larvae or live prey. In this study the effects of a hydrogen peroxide-based product, Ox-Aquaculture©, on live prey (rotifers and *Artemia*) and meagre larvae bacterial load, composition and final status have been tested. A 34.6% reduction of total heterotrophic bacteria and 59.7% of *Vibrionaceae* were obtained when rotifers were exposed for 15 min to 40 mg L^{-1} of the product. A 34.3% reduction of total heterotrophic bacteria and 37.7% of *Vibrionaceae* were obtained when *Artemia* were exposed for 5 min to 8000 mg L^{-1} of the product. More than 95% reduction of total heterotrophic bacteria and 75% of *Vibrionaceae* were obtained when meagre larvae were exposed for 1 h to 20 mg L^{-1} of the product. Furthermore, disinfection of enriched live prey with the product did not change the fatty acid composition and survival of the live prey and improved final larval survival.

Keywords: hydrogen peroxide, bacterial load, live prey, larval rearing, *Argyrosomus regius*

Introduction

Intensive fish larvae production is highly susceptible to the proliferation of bacteria, which may cause poor growth or mass mortality of the larvae. In most cases, mortality cannot be attributed to specific pathogens, but to the proliferation of opportunistic bacteria (Olafsen 2001). The rearing environment, with high larval densities and high load of organic matter is highly susceptible to bacterial growth. Fish larvae at hatching are commonly colonized by very few bacteria, which may originate from the egg surface, or from the surrounding water. As soon as fish larvae start to capture and ingest live prey, the numbers of bacteria in the larvae increase exponentially. These bacteria are located mainly in the larval gut, being *Vibrio* species the main component of the gut microbiota mainly derived from bacteria associated with live prey (Skjermo & Vadstein 1993; Verschuere, Dhont, Sorgeloos & Verstraete 1997).

Live prey (rotifers and *Artemia*) carry a large diversity of associated microbiota that, although they are not pathogenic to live prey, can be transferred to their larval predators causing detrimental effects (Dhert, Rombaut, Suantika & Sorgeloos 2001; Giménez, Padrós, Roque, Estévez & Furones 2006). The dominant bacterial groups in rotifers are *Pseudomonas*, *Vibrio* and *Aeromonas* that account for 10^7 CFU mL^{-1} in the rearing water or 10^4–10^5 CFU $rotifer^{-1}$ (Nicolas, Robic & Ansquer 1989) with similar levels of bacteria in the case of *Artemia* (Austin & Allen 1981). Most of bacterial strains associated with rotifers are located on the external surface (Munro, Henderson, Barbour & Birkbeck 1999), thus several studies have been conducted to reduce the bacterial load in rotifers and *Artemia* prior to feeding the fish larvae, by the use of freshwater baths (Rodríguez, Planas & Otero 1991), antibiotics (Haamed & Balasubramanian 2000), chemical products (Gomez-Gil, Abreu-Grobois, Romero-Jarero & Herrera-Vega 1994; Martinez-Diaz, Álvarez-González, Moreno Legorreta,

Vázquez-Juárez & Barrios González 2003; Giménez *et al.* 2006), ultraviolet (UV) radiation (Munro *et al.* 1999) and ozone-treated seawater (Davis & Arnold 1997). However, the use of antibiotics to reduce the bacterial flora in live prey causes several secondary effects (interaction with gut microflora of the larvae, increase in strain resistance) and adverse environmental impacts (Minkoff & Broadhurst 1994) whereas the use of chemical products, such as formaldehyde, can cause the formation of by-products or toxic compounds (Haamed & Balasubramanian 2000).

Hydrogen peroxide is a natural metabolite produced by the oxidative metabolism of the cells, being easily degraded by chemical reaction and enzymatic decomposition into water and oxygen in the presence of organic matter (algae, zooplankton and heterotrophic bacteria), light and turbulence. Hydrogen peroxide has been shown to be very effective in the disinfection of live prey (Gomez-Gil *et al.* 1994; Martinez-Diaz *et al.* 2003; Giménez *et al.* 2006) and fish (Lumsden, Ostland & Ferguson 1998; Rach, Gaikowski, Howe & Schreier 1998; Derksen, Ostland & Ferguson 1999; Gaikowski, Rach & Ramsay 1999; Thomas-Jinu & Goodwin 2004; Avedaño-Herrera, Magariños, Irgang & Toranzo 2006; Giménez, Padrós, Roque, Estévez & Furones 2009). It is considered a low risk product by US Food and Drug Administration and it is commonly used for the control of mortalities associated with external fungal infections in freshwater fish (Rach, Schreier, Schleis & Gaikowski 2005) and their eggs, for the control mortalities associated with bacterial gill disease on freshwater salmonids, and for the control of mortalities associated with external columnaris disease in freshwater-reared coldwater finfish and channel catfish (Schmidt, Gaikowski & Gingerich 2006). In the case of salmon it is commonly used for the treatment of sea lice in Canada, Scotland, Ireland, Norway and Chile (Bruno & Raynard 1994).

The aim of this study was to test the disinfection capacity of Ox-Aquaculture©, a commercial hydrogen peroxide-derived product (50% peroxide concentration), both in live prey, *Brachionus sp.* and *Artemia franciscana*, and in meagre larvae, to study its efficacy as a bacterial disinfectant and to evaluate its effects on live prey composition, and on larval growth and survival. Meagre was selected for the study not only because it is considered a new species for aquaculture diversification in Europe but also because it is prone to mortalities derived from stress conditions (inappropriate light and feeding as well as handling).

Material and methods

Experimental design

Fertilized eggs were obtained by GnRHa induced spawning from a wild broodstock of *Argyrosomus regius* (Asso, 1801) kept at the Institut de Recerca i Tecnologia Agroalimentària (IRTA) at Sant Carles de la Ràpita (Duncan, Estévez, Porta, Carazo, Norambuena, Aguilera, Gairin, Bucci, Vallés & Mylonas 2012). Eggs were incubated in 35 L PVC cylinders (baskets) with air-lifts to provide enough water removal and aeration, which were immersed in a 2000 L holding tank at 18°C until larvae hatched 48 h later. Larvae were counted volumetrically and transferred to 100 L tanks, at a density of 50 larvae L^{-1} and kept under recirculation at 18 ± 1°C with a photoperiod of 16 hL:8 hD. *Tetraselmis chuii* was added to the rearing water before larvae were stocked. From 2 to 15 days post hatching (dph) larvae were fed 10 individuals mL^{-1} of enriched *Brachionus sp.* (2 h, 26°C UV seawater, 250 ind mL^{-1}, 0.15 g L^{-1} Easy Selco, INVE, Belgium). Enriched (18 h, 28°C UV seawater, 300 ind mL^{-1}, 0.6 g L^{-1} Easy Selco) *Artemia* metanauplii were added from 14 to 25 dph (1 ind mL^{-1}).

Four treatments with four replicates each were used for the study. In treatment A (control) no disinfection of prey or larvae was used, in treatment B only larvae were disinfected, in treatment C only live preys were disinfected and in treatment D both larvae and live prey were disinfected.

Disinfection procedures

Meagre larvae were disinfected in the rearing tank at 1 and 13 dph using 20 mg L^{-1} of Ox-Aquaculture© for 1 h (Giménez *et al.* 2009). Rotifers were enriched, harvested, rinsed and disinfected using 40 mg L^{-1} Ox-Aquaculture© dissolved in UV treated seawater for 15 min. A similar procedure was used for *Artemia*, thus after enrichment, harvested and rinsed, they were disinfected using 8000 mg L^{-1} of Ox-Aquaculture© dissolved in UV treated seawater for 5 min. These conditions for live prey disinfection gave the best results in terms of live prey survival and bacterial reduction in a previous study by Giménez *et al.* (2006).

Data collection

Larvae were sampled ($N = 10$) at 1, 3, 6, 13, 16, 20 and 25 dph to measure standard length and dry weight. Larvae were anaesthetized with Tricaine methanesulfonate (MS-222) before measuring the standard length (SL) using a dissecting microscope (Nikon SMZ800, Nikon, Tokyo, Japan) connected to a digital camera (Olympus DP25) and an image analyser (Olympus analysis, Hamburg, Germany). The same larvae were collected in a 150 μm mesh sieve, rinsed with distilled water, dried in an oven at 60°C for 24 h and weighed to obtain the dry weight (DW). Survival was obtained at the end of experiment by counting the remaining larvae.

Larvae were sampled at 1 and 13 dph whereas enriched rotifers and *Artemia* metanauplii were sampled at several times during larval rearing process for bacterial identification and counting. All the samples were collected before and after exposure to the product to estimate the effect of the hydrogen peroxide in the reduction of bacterial load in the live preys and larvae. For bacterial load determination 10 larvae, 50 000 rotifers and 10 000 *Artemia* metanauplii were harvested, filtered and homogenized in sterile manual homogenizer with 5 mL sterile saline solution. Sequential dilutions of 1 mL of homogenized sample in 10 mL of sterile saline solution were done, and 100 μL of each dilution tube were plated in tryptic soy agar (TSA) and thiosulphate citrate bile salt (TCBS) media in triplicates. After 24 h incubation at room temperature bacterial colonies were estimated by direct counting (colony forming units, CFU).

To check the effect of hydrogen peroxide on the fatty acid composition of the live prey, samples of rotifers before and after disinfection were taken and analysed in duplicates as in Giménez *et al.* (2006).

The survival of live preys (rotifer and *Artemia*) after disinfection was estimated by direct counting of three subsamples of 1 mL.

Statistical analysis

All the data were analysed using SigmaStat 3.1 statistical package (Systat Software Inc., San Jose, CA, USA). Statistical differences were analysed by one-way ANOVA ($P < 0.05$) followed by a pairwise comparison using Tukey's or Dunn's tests.

Results

Larval growth in standard length (SL) and dry weight (DW) is shown in table 1. Larvae from treatments A and D showed the highest and lowest growth in length and weight, respectively, with treatments B and C having intermediate results. At the end of the study, treatments D (live prey and larvae disinfected) and B (only larvae disinfected) had the highest survival rate (36.0 ± 10.6% and 28.0 ± 2.9% respectively) whereas treatment A (control) had the lowest (14.7 ± 9.0%).

The results in total heterotrophic bacteria and *Vibrionaceae* found in 1 and 13 dph meagre larvae before and after disinfection with hydrogen peroxide are presented in figure 1. Before disinfection, total heterotrophic bacteria in 1 dph larvae were $2.71 \pm 2.79 \times 10^2$ CFU ind^{-1} and in 13 dph larvae were $8.34 \pm 4.51 \times 10^5$ CFU ind^{-1} and $5.09 \pm 1.35 \times 10^5$ CFU ind^{-1} in treatments B and D respectively. After exposure to Ox-Aquaculture©, total heterotrophic bacteria in 1 dph were reduced to $1.90 \pm 1.13 \times 10^2$ CFU ind^{-1} and in 13 dph larvae were significantly reduced to $3.59 \pm 2.16 \times 10^4$ CFU ind^{-1} and $2.18 \pm 0.77 \times 10^4$ CFU ind^{-1} in treatments B and D respectively ($P < 0.05$). Before disinfection, *Vibrionaceae* accounted for $3.89 \pm 2.68 \times 10^1$ CFU ind^{-1} in 1 dph larvae and $3.25 \pm 0.12 \times 10^3$ CFU ind^{-1} and $6.25 \pm 1.77 \times 10^2$ CFU ind^{-1} in 13 dph larvae from treatments B and D. After disinfection, only in treatment B, *Vibrionaceae* were significantly reduced to $7.72 \pm 7.00 \times 10^2$ CFU ind^{-1} ($P = 0.018$), whereas in 1 dph larvae and 13 dph larvae of treatment D the reduction of *Vibrionaceae* (0 and $1.00 \pm 0.71 \times 10^2$ CFU ind^{-1} respectively) were not statistically significant ($P = 0.066$ and $P = 0.060$ respectively).

The results in total heterotrophic bacteria and *Vibrionaceae* counts (CFU ind^{-1}) in rotifer before and after the treatment with the product were found in figure 2. Before disinfection, total heterotrophic

Table 1 Standard length (SL, mean ± SD), dry weight (DW, mean ± SD) and survival (mean ± SD) rate of 30 dph meagre larvae. Superscripts indicate significant differences ($P \leq 0.05$) among the treatments

Treatment	DW (mg)	SL (mm)	Survival (%)
A	0.53 ± 0.25^{a}	5.83 ± 0.47^{a}	14.67 ± 9.02^{a}
B	0.39 ± 0.09^{ab}	5.45 ± 0.20^{ab}	28.00 ± 2.94^{b}
C	0.40 ± 0.05^{ab}	5.60 ± 0.13^{ab}	25.67 ± 4.99^{ab}
D	0.32 ± 0.05^{b}	5.35 ± 0.23^{b}	36.00 ± 10.61^{b}

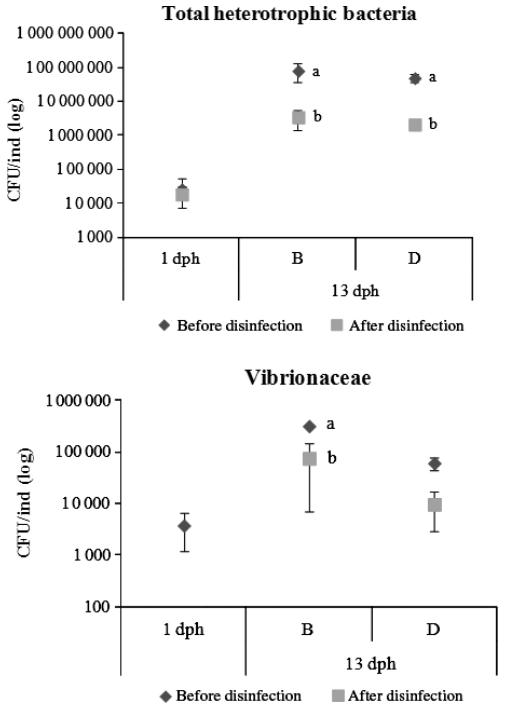

Figure 1 Bacterial load (CFU ind^{-1}) in meagre larvae (1 and 13 dph) before and after disinfection with H_2O_2 in treatment B and D. Results are given as mean ± SD. Superscripts indicate significant differences ($P < 0.05$) between before and after disinfection.

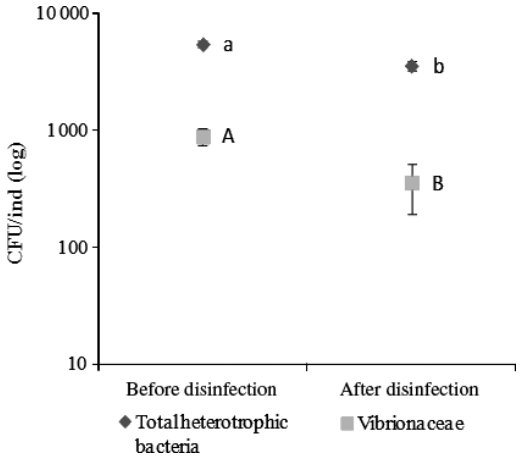

Figure 2 Bacterial load (CFU ind^{-1}) in rotifer before and after disinfection with H_2O_2. Results are given as mean ± SD of three replicates. a and b superscripts indicate significant differences ($P < 0.05$) found in total heterotrophic bacteria whereas A and B superscripts are used for *Vibrionaceae*.

Table 2 Total lipid content (mg g^{-1} DW) and fatty acid composition (%) of rotifer enriched disinfected and non-disinfected with hydrogen peroxide

	Disinfected	Non disinfected
Total lipids (mg g^{-1} DW)	72.7	80.1
Total fatty acid (mg g^{-1} lipid)	487.5	422.3
16:0	9.34	10.74
18:0	4.33	4.98
Total saturated	17.64	19.62
16:1n-7	14.04	14.81
18:1n-9	22.99	24.06
Total monounsaturated	46.95	48.22
18:2n-6	9.42	9.80
20:4n-6	1.30	1.49
Total n-6 PUFA	13.27	14.13
18:3n-3	3.44	3.02
20:5n-3	5.68	5.84
22:5n-3	2.12	1.67
22:6n-3	4.70	4.82
Total n-3 PUFA	20.65	16.65

bacteria in rotifers were $5.47 \pm 0.38 \times 10^3$ CFU ind^{-1} being significantly reduced to $3.58 \pm 0.32 \times 10^3$ CFU ind^{-1} after exposure to Ox-Aquaculture© ($P = 0.003$). *Vibrionaceae* were also significantly reduced after disinfection with Ox-Aquaculture, from $8.91 \pm 1.47 \times 10^2$ CFU ind^{-1} to $3.59 \pm 1.63 \times 10^2$ CFU ind^{-1} ($P = 0.014$).

No differences in total lipid and fatty acid content as well as in the fatty acid relative composition (Table 2) were observed between before and after disinfection of rotifers. The survival of rotifers after disinfection was 77.5 ± 8.1% along the experiment.

Figure 3 shows total heterotrophic bacteria and *Vibrionaceae* counts (CFU ind^{-1}) in *Artemia* metanauplii before and after the treatment with the product. A significant reduction in total heterotrophic bacteria from $1.38 \pm 0.20 \times 10^3$ to $9.07 \pm 1.02 \times 10^2$ CFU ind^{-1} was observed ($P = 0.022$). The same can be said in the case of *Vibrionaceae* that were significantly reduced from $8.40 \pm 0.30 \times 10^2$ to $5.23 \pm 0.41 \times 10^2$ CFU ind^{-1} ($P = 0.000$). The survival of *Artemia* after disinfection was 95.6 ± 6.7% along the experiment.

Discussion

Treatments B and D had higher survival rates than those from control group (A) indicating the positive effect of Ox-Aquaculture© added to the rearing water at precise periods of larval development.

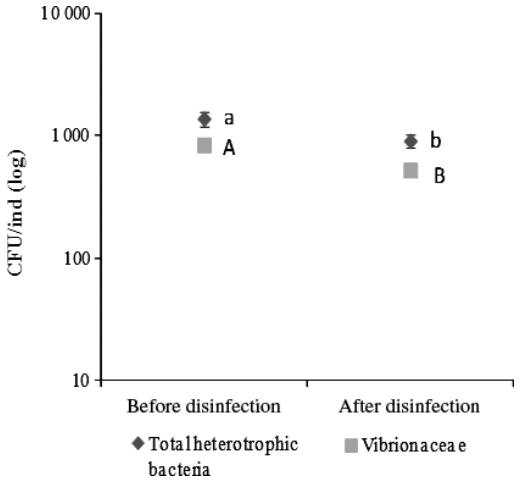

Figure 3 Bacterial load (CFU ind^{-1}) in *Artemia* before and after disinfection with H_2O_2. Results are given as mean ± SD of three replicates. a and b superscripts indicate significant differences ($P < 0.05$) found in total heterotrophic bacteria whereas A and B superscripts are used for *Vibrionaceae*.

Higher survival was also obtained by Giménez *et al.* (2009) in a previous experiment using this same product for disinfection of sea bream and common dentex although at different concentrations. A reduction in larval bacterial load (total heterotrophic and *Vibrionaceae*) was recorded in both groups of larvae after treatment with the product. Not only the bacterial load in the water (Giménez *et al.* 2009) but also in the larval body (this experiment) showed a clear reduction in number after the treatment. In the case of group D both live prey and larvae were disinfected contributing to these better values of survival. Disinfecting only the live prey had no significant effects on larval survival being the results of group C not significantly different to those from control group (A). Nicolas *et al.* (1989) observed that larval feeding behaviour changes depending on the bacterial concentration of the live prey, as turbot larvae refused to ingest prey which contained a high number of bacteria. Munro, Barbour and Birkbeck (1994) also observed that only the slow colonization rate of the larval gut by bacteria correlates well with larval survival. Thus, rapid bacterial colonization of the gut in early larvae is deleterious due to the presence of fast-growing colonizers (Vadstein, Oie, Olsen, Salvesen & Skjermo 1993) and the only way to reduce colonization rate is rotifer disinfection, especially considering that most of the bacteria associated to rotifers are found in the body surface. This might explain why meagre larvae of group D treated, as well as their live food, with hydrogen peroxide experienced a higher survival rate than the rest of the groups.

When meagre larvae were disinfected with Ox-Aquaculture© at 13 dph total heterotrophic bacteria were significantly reduced. *Vibrionaceae* were present in low amounts in the larvae, and after disinfection a reduction was observed although only in treatment B was statistically significant. Although hydrogen peroxide is a natural metabolite, Gaikowski *et al.* (1999) observed a reduction in the survival rate depending on the lifestage of different cold-, cool- and warmwater fish. The optimum concentration of hydrogen peroxide depends on the species, thus, the survival of *Sparus aurata* and *Dentex dentex* larvae decreased if concentrations higher than 50 mg L^{-1} and 20 mg L^{-1} were used respectively (Giménez *et al.* 2009). In the case of *Ictalurus punctatus,* the ongrowing fish cannot tolerate concentrations greater than 75 mg L^{-1} (Thomas-Jinu & Goodwin 2004), whereas *Scophthalmus maximus* had respiratory distress when 8–10 g fish were treated with 30 mg L^{-1} of H_2O_2 (Avedaño-Herrera *et al.* 2006). Finally, rainbow trout *Oncorhynchus mykiss* exposed to H_2O_2 concentrations greater than 100 mg L^{-1} had an increased ventilatory rate and appeared agitated (Derksen *et al.* 1999).

Total heterotrophic bacteria and *Vibrionaceae* were significantly reduced in rotifers treated with Ox-Aquaculture©. Giménez *et al.* (2006) and Munro *et al.* (1999) obtained a reduction in total bacterial counts in rotifer treated with this same product (hydrogen peroxide) or after 2 min exposure to UV radiation respectively. Other strategies such as the use of disinfected rotifer eggs for the production of axenic cultures (Dhert *et al.* 2001) have also been applied, although the easiest solution to solve the problem of bacterial colonization of live prey is the use of antibiotics (Perez Benavente & Gatesoupe 1988). However, the use of antibiotics induces the development of resistances (Dhert *et al.* 2001), which can be transferred to other bacteria, including pathogenic bacteria to fish, or adverse environmental impacts (Minkoff & Broadhurst 1994). Therefore, the use of antibiotics should be kept to a minimum.

The reduction in the bacterial load in *Artemia* metanauplii obtained in this experiment was

similar to the results found by Haamed and Balasubramanian (2000) and Giménez *et al.* (2006). Different techniques have been used to reduce numbers of associated bacteria in *Artemia*, i.e. incubation with *Tetraselmis* (Olsen, Olsen, Attramadal, Christie, Birkbeck, Skjermo & Vadstein 2000) a microalgae well known for producing antibacterial substances, the use of freshwater baths after harvesting the nauplii (Rodríguez *et al.* 1991), UV treatment of incubation water (Munro *et al.* 1999) and the use of antibiotics or chemical disinfectants (Gomez-Gil *et al.* 1994; Haamed & Balasubramanian 2000; Giménez *et al.* 2006). Gomez-Gil *et al.* (1994) preferred sodium hypochlorite as an agent of disinfection while Haamed and Balasubramanian (2000) recommended the use of formaldehyde. None of them recommended the use of antibiotics as Haamed and Balasubramanian (2000) observed that more than 60% of bacterial isolates were resistant to antibiotics. The use of these two chemical products can cause the formation of by-products or toxic compounds with adverse consequences on the environment. On the contrary hydrogen peroxide is a natural metabolite that is naturally degraded into oxygen and water by chemical reduction or enzymatic decomposition, by many organisms and by the direct action of sunlight on water, without producing any undesired pollutant. Treatment with any of these products might have effects on the fatty acid composition of live prey, especially when applied after enrichment. One of the advantages of hydrogen peroxide is that it is not inducing any change in the fatty acid profile of enriched *Artemia* (Giménez *et al.* 2006) or rotifers (this study, see Table 2) being the nutritional quality of the live prey used for larval rearing assured even after disinfection. Another advantage of this disinfectant is that the survival of live preys is not affected by the addition of hydrogen peroxide as Giménez *et al.* (2006).

Conclusions

The results of this experiment indicate that the use of the product Ox-Aquaculture© as a disinfectant of live prey and meagre larvae at early rearing stages contributes to a better survival although not affecting larval growth. In addition, disinfection of live prey (rotifers and *Artemia* metanauplii) prior to the introduction into larval rearing tanks reduces the number of total heterotrophic bacteria and *Vibrionaceae* preventing and/or reducing larval diseases caused by opportunistic bacteria. Thus, Ox-Aquaculture© can be considered a good disinfectant for aquaculture purposes.

Acknowledgments

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