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Effect of water treatment and aeration on the percentage hatch of demersal, adhesive eggs of the bullseye puffer (*Sphoeroides annulatus*)

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Abstract

The bullseye puffer (*Sphoeroides annulatus*) is a species with potential for aquaculture. However, preliminary studies on the biology and reproduction of the species in captivity have failed to produce consistent success at hatching. In this study, the effect of aeration and water treatment (filtration and ultraviolet (UV) sterilisation) on percentage hatch was assessed in a replicated experiment repeated on two occasions. Four treatments were used, filtered water (5 μm) treated with UV, in an aerated system (AT) and a static system (ST); water that had been coarse-filtered (20 μm) and not exposed to UV kept in aerated system (ANT) and a static system (SNT). The percentage hatch was ranked $87.5 \pm 5.0 > 80.6 \pm 9.4 > 77.2 \pm 12.8 > 62.8 \pm 10.4$ (mean of all replicates from both experiments $\pm$ S.D.) for the respective treatments AT>ST>ANT>SNT. However, percentage hatch for AT was only significantly ($P < 0.05$) higher than ANT and SNT, while hatch for ST was only significantly higher ($P < 0.05$) than SNT. A GLM analysis showed the use of treated water (filtered and UV treated) in the incubators and aeration had a significant positive effect on percentage hatch. These effects were additive with no significant positive or negative interaction. There was no significant differences in bacterial counts among treatments (AT, ANT, ST, SNT), but significant differences ($P < 0.05$) were observed among days. There was no significant interaction between days and treatments to affect bacterial counts. There was no correlation between bacterial counts and percentage hatch. The water treatments significantly improved percentage hatch, but did not affect bacterial counts. This paper reports an incubation system that consistently resulted in more than 80% successful hatching for bullseye puffer eggs. These results should help to improve the hatching success of this species and possibly others with demersal adhesive eggs.

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1. Introduction

The bullseye puffer *Sphoeroides annulatus* is an important commercial fisheries species found in coastal waters of the Pacific ocean. It is abundant in the Gulf of California and has a geographical range from San Diego (USA) to Peru (Thomson et al., 1987). In 2000, 147.6 tons of bullseye puffer were landed in the state of Sinaloa, Mexico with the value of US$226,600 (CONAPESCA 2000). High market value and overexploitation of natural populations during the reproductive season have stimulated interest in commercial cultivation of this species. The Mexican shrimp farming industry (production in 2000 of 33,478 tons of shrimp, FAO) is operating pilot-scale culture of the bullseye puffer using existing biotechnologies (Duncan and Abdo de la Parra, 2002). These include the induced spawning of wild-caught *S. annulatus* by injection of LHRHa (Duncan et al., 2003; Rodriguez and Duncan, 2001), description of egg and early larval development (Abdo de la Parra et al., 2001; Duncan et al., 2003) and weaning and juvenile production (Abdo de la Parra et al., 2001; García-Ortega et al., in press). The eggs are demersal, adhesive, 0.71 ± 0.01 mm in diameter, and contain a single large yolk cell and a group of lipid globules. Preliminary incubations of the eggs on glass plates in a rectangular 60-l tank with a 1 l/min flow of filtered and UV-treated water gave an inconsistent percentage hatch ranging from 18% to 70%. Low-percentage hatch appeared to be associated with the presence of bacteria and protozoa. No other studies have been published on the incubation and early development of the bullseye puffer. However, other Tetraodontidae have been studied in Australia (Gladstone, 1987; Gladstone and Westoby, 1988), USA (Sikkel, 1990) and in Asia where the puffer fish has the highest market value of any fish (Kanazawa, 1991; Yamahira, 1997).

The eggs of the puffer fish described in these and other studies were also demersal and adhesive (Arai and Fujita, 1988; Gladstone and Westoby, 1988; Sikkel, 1990; Fujita and Honma, 1991; Fujita and Abe, 1992; Yamahira, 1997). However, these studies either did not describe incubation conditions (Arai and Fujita, 1988; Fujita and Honma, 1991; Fujita and Abe, 1992) or described natural conditions (Yamahira, 1997; Gladstone and Westoby, 1988; Sikkel, 1990). In Japan, eggs of *Takifugu niphobles* are laid under pebbles of the upper intertidal zone (Yamahira, 1997). The sharpnose puffer *Canthigaster valentini*, native of Lizard Island in Australia, spawns on algae and after spawning, the eggs become hidden within the algae (Gladstone, 1987). In the US, the Atlantic puffer *Canthigaster rostrata* spawns in a nest made of a path of filamentous algae attached to a piece of coral rubble; the eggs adhere to the algal filaments and to one another (Sikkel, 1990). There is no information available regarding the spawning substrate of *S. annulatus*. The mature adults used in this and other studies (Duncan et al., 2003; Rodriguez and Duncan, 2001) were caught in the mouth of Teacapan estuarine lagoon in Mexico over a sandy bottom, but there is no evidence that this was the spawning area or what the spawning substrate may have been.

It is common practice to, before incubation, remove the stickiness from demersal adhesive eggs spawned from freshwater fish such as carp (Rothbard, 1981), sturgeon (Kowtal et al., 1986) and white bass (Rottmann et al., 1988). However, it is unclear if the removal of adhesive properties of marine demersal eggs would lead to improved hatching success. Reports have described the successful incubation of eggs (from the rabbitfish,

Siganus randalli) stuck to plastic plates (Collins and Nelson, 1993) and eggs (from the wolffish, *Anarhichas lupus*) incubated in upwelling incubators after the stickiness had been removed (Pavlov and Moksness, 1995).

The hatching success of marine eggs has been shown to be associated with environmental influences such as aeration and bacterial loading of the water in which the eggs are incubated. Aeration of the water during incubation both provides dissolved oxygen (DO) to the eggs and physically moves them. Dissolved oxygen (DO) in the water is vital and significantly influences hatching rate. Low levels of dissolved oxygen can result in deformed fry and death of the fish eggs (Bromage, 1988). A desirable range of dissolved oxygen in water has been described for salmonid eggs as 7 mg l^{-1} and for catfish and tilapia eggs as above 2 mg l^{-1}. (Bromage, 1988). The minimum necessary DO apparently varies among species and it is important to ensure that the incubator provides adequate oxygen to allow normal development and hatching of the larvae. In Atlantic cod (*Gadus morhua*), it has been shown that the hatching rate decreased from around 30% at an oxygen level of 8 mg l^{-1} to less than 10% at 2 mg l^{-1} oxygen content (Waller et al., 1993). Aeration also produces movement in the water. In some species such as madtom *Noturus insignis*, higher hatching rates were achieved when eggs were constantly kept in motion until hatching (Stoeckel and Neves, 2000). On the contrary, salmonid eggs exposed to a mechanical shock before eyeing suffer very high egg mortality (Bromage et al., 1988).

Another parameter influencing the success of marine egg incubation is the microflora in the water. Microbiological problems may often arise in the early stages of larval rearing because cultivation of small and sensitive marine larvae includes a period with limited or no water exchange (Skjermo and Vadstein, 1999). Such conditions could result in bacterial overgrowth on the eggs. This can affect the commensal relationship between the indigenous microflora and opportunistic pathogens and subsequently hinder egg development, hatching rates and larval health (Hansen and Olafsen, 1989). Often, the mortality cannot be attributed to specific obligate bacterial pathogens but is associated with opportunistic bacteria (Munro et al., 1994). Relationships between bacterial loading and egg mortality have been shown in salmonid (Barker et al., 1989) and non-salmonid eggs (Subasinghe and Sommerville, 1985).

Various techniques are used in order to control the bacterial loading such as filtration, egg disinfection (Skjermo and Vadstein, 1999) or treatment of the water with ultraviolet light (UV) (Sommer et al., 1997). This study examined the effects of aeration and treatment of the water by combined filtration and exposure to UV on the percentage hatch of the eggs of *S. annulatus*. The total bacterial loads in the water were also analysed. The objective was to examine the relationship between water treatment and the conditions in the incubator, and to provide methods for the controlled incubation of eggs from *S. annulatus*.

2. Materials and methods

2.1. Broodstock and spawning induction

This study was conducted from 4 May to 18 May 2001 at CIAD, Mazatlan, Mexico (23°18′01″N, 106°28′48″W). Wild brood *S. annulatus*, recently caught (29 March) in the

mouth of Teacapan estuarine lagoon, were held in a 2-m-diameter tank (2600 l) with flow-through (400% day^{-1}) seawater (35 ‰) filtered to 20 μm. The tank was maintained under ambient conditions, natural photoperiod (14 h light:10 h dark) and 23 ± 1 °C. The fish were fed twice a day with pellets (50:50 ratio of trout broodstock diet (El Pedregal Silver Cup, Toluca, Mexico) and a diet made in CIAD A.C. based on Kanazawa, 1991) at 2% body weight plus 10 g of squid per animal per day. Eggs of *S. annulatus* were obtained by induced spawning with synthetic analogue Des-Gly^{10}, [D-Ala^{6}]-luteinizing hormone-releasing hormone (LHRHa). Females for induced spawning were selected when their average oocyte diameter was greater than 500 μm (Duncan et al., 2003). The females were first injected with LHRHa at a dose of 20 μg kg^{-1} body weight, followed 48 h later by a second injection at a dose of 40 μg kg^{-1} body weight. The females were stripped by hand 12–24 h after the second injection. One male was used for each experiment; milt was checked and used only if it had 100% motility and remained activated for at least 1 min. Milt was then added to the eggs at the same time as the filtered and UV-treated seawater. After 2 min, eggs were rinsed and distributed for the incubation experiment.

2.2. Experimental system

Experimental units, static (S) and aerated (A) incubators consisted of 2-l translucent plastic incubators made from mineral water bottles (SierrAzul™). Each aerated incubator (Fig. 1) received 100 ml of 2-μm-filtered air per minute through an air stone (3 × 1.5 cm). The incubators were immersed in the same flow-through water bath at (24 ± 1 °C). Air temperature was maintained at 25 °C with an air-conditioning system (York™). Light was controlled by a timer to provide a photoperiod of 12 h light:12 h dark. Eggs from *S. annulatus* were stuck to glass microscope slides by gently pouring the eggs with UV-filtered water along the length of a wet slide. A total of 50 of eggs was stuck to each slide. Excess eggs were removed using a plastic spoon. Each incubator received a total of 200
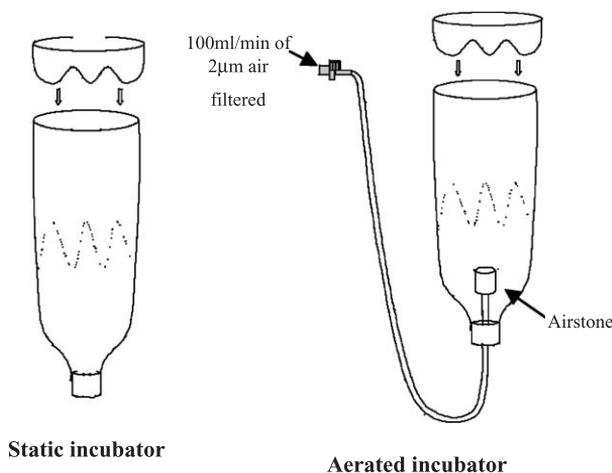

Fig. 1. The two different types of incubators, static and aerated.

eggs on four slides. The four glass slides in each incubator were held in plastic histology baskets, which were immersed in the incubator that contained 1.5 l of water. The nontreated water (NT) was filtered through a 20-μm filter. The treated water (T) was passed through a 5-μm filter followed by an exposure to two UV lights (model G37T6VH Light Sources™) at 5 l/min flow rate (Sommer et al., 1997).

To determine the combined effect of the type of incubator and water treatment on the percentage hatch, four different treatments were tested: static incubator filled up with nontreated water (SNT); static incubator with treated water (ST); aerated incubator with nontreated water (ANT); and aerated incubator with treated water (AT). There were five replicates for each treatment and the experiment was conducted twice. The system was tested in preliminary trials that were identical to the reported experiments; mean oxygen levels of 5.6 ± 0.3 mg l^{-1} ($n = 7$, range $5.2–6.2$ mg l^{-1}) were recorded in static incubators and 6.2 ± 0.2 mg l^{-1} ($n = 7$, range $5.9–6.5$ mg l^{-1}) in aerated incubators.

Percentage fertilisation was determined for each experiment by recording the number of developing and nondeveloping eggs in a sample of $50–81$ eggs taken at the $4–16$ cell stage (approximately 2 h after fertilisation). Four samples were taken from the batch of eggs used for experiment 1 and three for experiment 2. Percentage fertilisation was calculated as follows:

$$\text{Percentage fertilisation} = \frac{(\text{developing eggs}) \times 100}{(\text{developing eggs} + \text{nondeveloping eggs})}.$$

2.3. Determination of the percentage hatch

The percentage hatch was determined 6 days after fertilisation by counting the number of black larvae and dead eggs in each incubator. The contents of the incubator were emptied and rinsed into a white receptacle where the larvae were counted. Both the larvae that were not moving and the eggs still stuck to the slides were recorded as dead. The percentage hatch was calculated as follows:

$$\text{Percentage hatching} = \frac{(\text{swimming larvae} + \text{dead larvae}) \times 100}{(\text{live larvae} + \text{dead larvae} + \text{dead eggs})}.$$

2.4. Bacteriology

Every 2 days water samples were taken from each incubator for bacterial analysis. One-milliliter water samples were taken from each incubator 2 cm below the surface of the water using a sterile pipette (200–1000 μl, Labsystems™).

The water was plated out directly and in two dilutions (10^{-2} and 10^{-3}). Each of the three concentrations was inoculated onto a separate Petri dish by placing six 20-μl drops onto TSA + 2.5% NaCl. The plates were then incubated at 30 °C for 24 h; after which the colonies formed in association with each drop were counted.

$$\text{CFU ml}^{-1} = \frac{(\text{average number of CFU/20 μl drop}) \times 50}{\text{dilution of the sample}}.$$

2.5. Statistical analysis

Statistical analyses were conducted using Statistica 6.0 (StatSoft, 2001). GLM models that included a full factor nested ANOVA were used to examine percentage hatch. Normality was determined with the Shapiro–Wilk W test. Distributions of bacterial counts were skewed towards high counts several magnitudes greater than the mean and could not be transformed to a normal distribution. Bacterial counts were compared with the two-way Scheirer–Ray–Hare extension of the Kruskal–Wallis test (Sokal and Rohlf, 1995), with a multiple comparison between bacterial counts per day made with the Dunn test (Zar, 1984). Relationships between bacterial counts and hatch rates were examined using the Spearman rank correlation (Zar, 1984). All means are expressed as $\pm$ 1S.D. Details of the specific GLM models applied are contained in Results.

3. Results

3.1. Percentage fertilisation

Percentage fertilisation was $93.8 \pm 3.1\%$ for the first experiment and $91.3 \pm 1.6\%$ for the second.

3.2. Percentage hatch

Percentage hatch was normalised by arcsine square root transformation and compared as the dependent variable, with treated water and aeration as independent variables nested within the two experiments in a GLM. Both water treatment (Sum of Squares = 0.292, $df = 1$, $F = 20.51$, $P = 0.000078$) and aeration (Sum of Squares = 0.0.16119, $df = 1$,
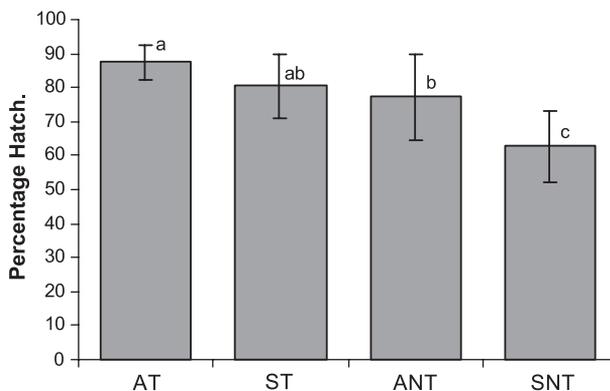

Fig. 2. Mean ($\pm$ S.D.) percentage hatch (combined data from the two experiments) for each treatment. The same letter above columns indicates no significant difference (Student–Newman–Kuels, $P < 0.05$). SNT = static incubator with nontreated water. ANT = aeration, nontreated water. ST = static, treated water. AT = aeration, treated water.

Table 1
Mean percentage fertilisation ± S.D. (*n*—number of replica counts of 50–81 eggs) and mean percentage hatch ± S.D. (*n*—number of replicas) for each treatment, aeration and treated water (AT), static and treated water (ST), aerated and nontreated water (ANT), static and nontreated water in experiments 1 and 2 and the combined results

	Fertilisation	Treatments			
		AT	ST	ANT	SNT
Experiment 1	93.4 ± 3.1 (4)	87.9 ± 3.4 (5)	83.0 ± 9.1 (5)	74.8 ± 18.7 (5)	65.6 ± 14.0 (5)
Experiment 2	91.3 ± 1.6 (3)	87.0 ± 6.6 (5)	78.2 ± 10.1 (5)	79.6 ± 2.4 (5)	60.0 ± 5.2 (5)
Combined results, experiments 1 and 2		87.5 ± 5.0 (10)	80.6 ± 9.4 (10)	77.2 ± 12.8 (10)	62.8 ± 10.4 (10)

$F = 11.330$, $P = 0.001996$) had significant effects on percentage hatch (model $R^2 = 0.255$); the two experiments had no effect on percentage hatch ($P = 0.58$) and there were no significant interactions ($P = 0.367, 0.281, 0.782, 0.7171$). Post hoc Student–Neuman–Keuls tests demonstrated that the static system with no water treatment (SNT) had the worst hatch rate, 62.8 ± 10.4%; the aerated system with no water treatment (ANT) had a significantly ($P < 0.05$) higher hatch rate, 77.2 ± 12.8%, which was not significantly different from static, treated water system (ST), 80.6 ± 9.4%. The aerated, treated water system (AT) had the highest hatch rate, 87.5 ± 5.0%, which was significantly ($P < 0.05$) higher than the aerated system with no water treatment (ANT), but was not significantly different from static, treated water system (Fig. 2 and Table 1).

3.3. Bacteriology

The trend in number of colony-forming units per milliliter (CFU ml^{-1}) was similar in all treatments and both experiments; counts were similar on each day with an increase from day 0 to day 2, followed by a decrease to day 4 and a second increase to day 6 (Fig. 3). There were no significant differences between bacterial counts from the different treatments, SNT, ANT, ST and AT, in each experiment or when the data for the two
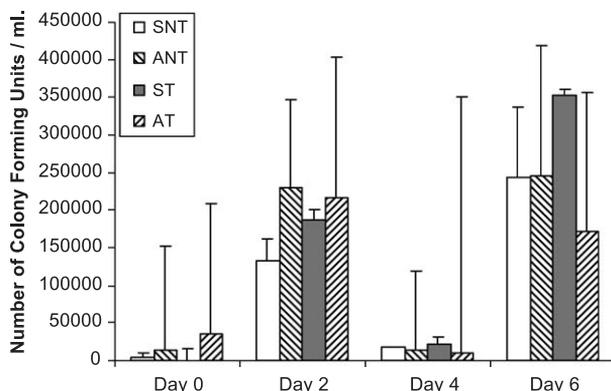

Fig. 3. The mean bacterial count (combined data from experiment 1 and 2) of colony forming units (CFU) per milliliter for all treatments on each day: 0, 2, 4 and 6 of the experiments.

experiments were combined. There were significant differences ($P < 0.05$) between bacterial counts for each day; the combined counts increased significantly ($P < 0.05$) from $13,431 \pm 7762$ to $190,875 \pm 21,618$ CFU ml^{-1}, then decreased significantly ($P < 0.05$) to $15,077 \pm 1999$ CFU ml^{-1} before again increasing significantly ($P < 0.05$) to $252,946 \pm 36,611$ CFU ml^{-1}; there was no significant difference between counts on days 0 and 2 or between days 4 and 6. There were no significant interactions between treatments and days to affect CFU ml^{-1}. There was no correlation on any day between number of CFU ml^{-1} and percentage hatch (r values were: day $0 = 0.12$, day $2 = 0.05$, day $4 = 0.03$, day $6 = -0.24$).

4. Discussion

In the present study, both the use of treated water (filtered and UV-treated) and aeration had a significant positive effect on percentage hatch. These effects were additive with no significant positive or negative interaction. This suggests that reducing the numbers of organisms entering the system with the water and maintaining a more homogenous environment while adding oxygen and removing some waste products (aeration mixing the system) increased egg survival.

Water treatment had the biggest effect on hatching percentage. The alternative water treatments consisted of water similar to that in the fish holding tanks (coarse filtered through 20 μm) compared with water filtered through 5 μm and exposed to UV light. Fine filtration and UV treatment have been shown to reduce the bacterial loading of water (Sommer et al., 1997), and in many commercial systems the water used to fill the incubators is UV-treated to reduce the risk of pathogenic organisms entering the system.

There are various interpretations of the influence of microbiological conditions on hatching of fish eggs. Hayes (1930) stated that embryos did not die from external infection, and Sauter (1987) found no particular organism was consistently associated with high egg mortality during incubation of chinook salmon (*Oncorhynchus tshawytscha*) eggs. Barker et al. (1990) found that when bacterial levels on incubating rainbow trout eggs were reduced chemically for short periods of time, no improvement in survival was observed. However, conversely, studies have shown that microbial fauna does affect percentage hatch. Hameed (1997) found a highly significant correlation between the total number of bacteria and the hatching rate of shrimp (*Fenneropenaeus indicus*). Keskin et al. (1994) found bacterial loading to be more important in artificial incubation than in the wild. In turbot (*Scophthalmus maximus*) farms, at the end of an incubation period, the number of colony-forming units (CFU) in incubation jars was up to four orders of magnitude higher than in seawater. Large numbers of eggs and bacteria could reduce percentage hatch by oxygen deprivation (Trust, 1972). It would appear that bacteria can play some role in egg mortality (Sauter, 1987), but should be considered among a whole range of factors (e.g. flow rate, dissolved oxygen, pH or temperature), which can influence egg mortality (Barker et al., 1989). A study on *Takifugu rubripes* found that hatching rate in UV-treated incubators was 10% higher than in non-UV-treated incubators at $15-17$ °C (Miyaki et al., 1992).

In the present study, variations in the bacterial counts in the different treatments were similar and there were no significant differences in counts among treatments. Therefore,

the water treatment did not have a significant effect on bacterial counts, but did have a significant effect on percentage hatch. It is probable that the water treatment reduced the number of bacteria that entered from one source, the water. Other sources were the air (which was filtered), the incubator (which was disinfected before the experiment) and the eggs (washed with water treated with UV, but not disinfected). It would appear that altering the source of bacteria did not affect bacterial counts. Each treatment appeared to provide a similar potential for bacterial growth that resulted in similar counts, irrelevant of the source of the bacteria. It is possible that the species composition of the microbial population that developed from the different sources was different and this in turn may have affected the percentage hatch.

The bacterial CFU counts did exhibit a pattern, increasing significantly from day 0 to day 2, then decreasing significantly to day 4 before finally increasing significantly to day 6. From the available data it is impossible to expand on this pattern; however, it is highly probable that initially the bacteria present in the system bloomed using the available free nutrients supplied with the eggs and water; the bloom exhausted these nutrients and crashed; more nutrients were then introduced to the system by the hatching eggs and a second bloom was observed.

Aeration also significantly affected percentage hatch. The adverse effects of reduced dissolved oxygen (DO) on embryos have been documented for a wide range of fish species, such as steelhead trout, *Oncorhynchus mykiss* type vernacular (Coble, 1961); coho salmon, *Oncorhynchus kisutch* (Shumway et al., 1964), channel catfish, *Ictalurus punctatus* (Carlson et al., 1974); smallmouth, *Micropterus dolomierii* and white bass, *Morone chrysops* (Siefert et al., 1974); goldenshiner, *Notemigonus crysoleucas* and goldfish, *Carassius auratus auratus* (Stone and Park, 1999); sneep, *Chondrostoma nasus* (Kecheis et al., 1996).

However, DO in both aerated and nonaerated incubators was adequate (6.2 ± 0.2 and 5.6 ± 0.3 mg l^{-1}) (Piper et al., 1982) and it would appear that the relatively small number of eggs (200) and microbial population had an almost negligible effect on DO. Aeration also provides motion and turbulence, which can break up any microenvironment close to the eggs, thus avoiding localised low DO levels or accumulation of waste products. It is possible that the motion over the eggs either reduced microenvironment effects or gave some physical motion that aided incubation. Giorgi (1982) states that adequate ventilation of adhesive marine fish eggs is necessary to ensure normal embryo development. Bakerville-Bridges and Kling (1996) have suggested that Atlantic cod (*G. morhua*) eggs should be vigorously moved during incubation and larval rearing; however, too much movement can have a detrimental effect. Yamahira (1996) reported that physical stress (wave action and exposure during low tide) significantly decreased egg survival of the puffer, *T. niphobles*. In other species, levels of turbulence produced by different diffused aeration levels did not affect the hatching rate, for example, the grouper, *Epinephelus striatus* (Ellis et al., 1997).

5. Conclusion

The present study showed that water treatment (filtration and UV) and aeration had positive effects on percentage hatch. Bacterial counts were unaffected by treatments,

suggesting that treatments affected the source of bacteria but not the count. It is possible that hatcheries should consider that the techniques used in the present study enable the control of the source of bacteria but not the number of bacteria present in an incubation system. The environment created by these treatments repeatedly gave a percentage hatch higher than 80%. This reliable incubation will allow comparison of egg quality for this species and possibly for other marine species with demersal adhesive eggs. It is also important for the result of the culture of this species and in subsequent studies that the systems used here should be scaled up to commercial production to examine the effects of scale on the relationships.

6. Uncited references

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Acknowledgements

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