4. Results

4.1. Axenically Grown Sea bass Larvae

4.1.1. Hatching Success and Larvae Survival after Disinfection Treatments

In the first experiment, no hatching occurred. Four days after the arrival of eggs, none of the treatment groups exhibited a hatching percentage above 30%. At that point, the majority of the eggs were dead, having turned opaque and were sinking.

The remaining eggs were left in the bottles, to certify that they would hatch at a later stage, but in fact they didn't.

Regarding the second experiment, most of the larvae from the control and other treatment groups hatched in the course of 4 days of incubation. The morphological assessment done under the binocular magnifying glass did not reveal abnormal development or irregular behaviour of the newly hatched larvae.

For batch B, all treatments exhibited mean hatching percentages higher than 50%, C – 57.00±9.48%, I – 68.70±12.85%, G+2A – 53.93±16.01% (Figure 4.9).

The surface disinfection treatments did not have any adverse effect upon hatchability of the exposed sea bass eggs, given that Tukey's pair-wise comparisons after Bonferroni adjustments confirmed that there were no significant differences in the hatching success between the treatment groups (ANOVA one-way: $F(2,11)=1.68$, $p>0.05$).

Figure 4.1 illustrates the larvae survival rate, following egg exposed to different antimicrobial treatments approaches. Bars from each panel represent the survival mean values and standard deviation from treatment group replicates (n=6 per day, for each treatment group).

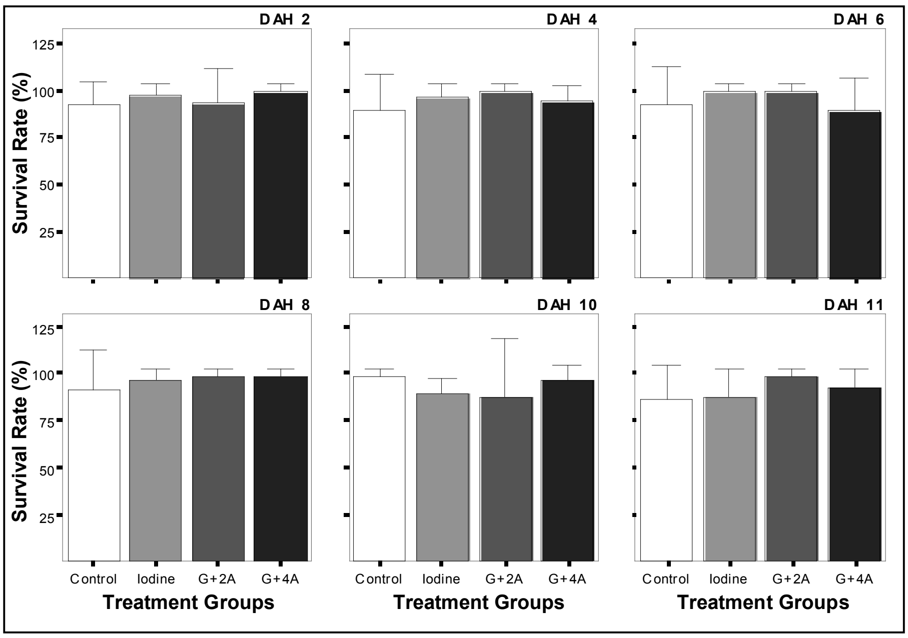


Figure 4.1. – Survival rate (%) of *D. labrax* larvae from batch B, during the eleven-day incubation period in multi-well test plates.

Throughout the eleven days following hatching, the larval survival for all treatment groups ranged from 86 to 98%, (Table I [Appendix B]). The glutaraldehyde treatment groups had no significant higher survival rates, compared to the other treatments (ANOVA two-way: $F(3,20)=1.302$, $p>0.05$).

At the end of the incubation time, most of the eggs from batch C had not yet hatched. More than 80% of the eggs were sinking or had already sunk, and in three out of six control treatment bottles not one single egg had hatched. Similar findings, were observed with 6 of the 12 bottles, from the G+2A treatment group.

4.1.2. Effect of Different Chemical Treatments on the Bacterial Load of Eggs and Larvae

Immediately after FASW rinsing procedures and disinfection with 100 $mg.L^{-1}$ glutaraldehyde, the bacterial density for both control and glutaraldehyde disinfection treatments was below the detection limit (< 30 colonies/plate) for batch A.

Despite the general initial increase of the bacterial densities, 24 hours after egg exposure to the different bactericidal treatments, the bacterial load levels were slightly lower for the glutaraldehyde treatment (Table 4.1), and was even below the detection limit for the glutaraldehyde disinfection with addition of 2 antibiotics treatment.

Regarding this last day data, the Kruskal-Wallis test yielded a highly significant difference among the treatment groups (H (2) = 9.26, $p < 0.05$). The average ranks from the actual statistical analysis test (C – 10.67, G – 8.33, G+2A – 3.50), confirm that the mean bacterial counts and colony-forming units recovered from the control and the glutaraldehyde surface disinfection treatments were fairly similar, whereas the mean average for the G+2A treatment group was significantly lower.

Table 4.1. – Bacteria density of *D. labrax* eggs (CFU/egg) from batch A, assessed on 10% MA medium replicate plates from egg arrival and the following day.

Treatment Group	*Bacteria Density (CFU/egg)*	
	Egg Arrival	1Day Incubation
Control	BDL*	$3.54x10^4±4.02x10^4$ *a*
Disinf. Glutaraldehyde	BDL	$3.54x10^3±3.28x10^3$ *a*
Disinf. Glutaraldehyde+2Antib.	NP§	BDL

Different superscript letters within the same column indicate significant statistical differences among treatments (n≥12 per treatment group), following Kruskal-Wallis test ($p<0.05$).
* Colony Forming Units (CFU) below the detection limit of 30 colonies/plate.
§ Assay not performed.

When compared to the untreated group, surface disinfection with G+2A generated a considerable reduction of the bacterial loads (Mann-Whitney test after Bonferroni adjustments: U = 0.00, r = - 0.81, $p<0.025$), while between both control and glutaraldehyde treatments no statistical significant difference in the antimicrobial activity was observed (Mann-Whitney test after Bonferroni adjustments: U = 1.00, $p > 0.025$).

Cohen's value[1] (η =0.670) clearly reflects an effect from the different treatment groups employed, thus indicating, that such effect accounts for 44.9% of the bacteria load variability.

19 Cohen's statistical power analysis, according Cohen (1988,1992) and Field (2002).

In batch B, the average bacterial load of the eggs upon arrival was below detection limit for all treatment groups, after applying the same bacteriological procedures employed in the first egg-batch. Twenty-four hours later, the bacterial density for the untreated control group was still below detection limit (Table 4.2).

During the following days, bacterial load levels were not significantly affected by the surface disinfection with iodine (Mann-Whitney test after Bonferroni adjustments, p>0.0125).

Overall effects resulting from egg surface disinfection, were more pronounced for the glutaraldehyde treatment groups (Table 4.2), which have shown to be highly efficient in reducing the microbial load.

The combination of glutaraldehyde disinfection with antibiotic supplements had a highly significant bactericidal effect compared to the control treatment group ($H(3) = 11.32$, $p < 0.05$, $\eta = 0.628$), since no colony growth was observed throughout hatching day until further antibiotic supplements were applied.

Table 4.2. – Bacteria density of *D. labrax* eggs (CFU/egg) and larvae (CFU/larvae) from batch B, assessed on 10% MA and MA+R+A medium replicate plates, during the eleven-day incubation period.

Treatment Group	*Bacteria Density (CFU/egg)*		*Bacteria Density (CFU/larvae)*	
	Egg Arrival	1Day Incubation	DAH 0	DAH 11
Control	BDL* ^{a}	BDL ^{a}	$2.23x10^{4}±1.86x10^{4\,a}$	$3.25x10^{4}±2.30x10^{4\,a}$
Disinf. Iodine	BDL ^{a}	$4.96x10^{3}±8.56x10^{3\,a}$	$3.06x10^{4}±6.43x10^{3\,a}$	$3.23x10^{4}±5.01x10^{4\,a}$
Disinf. Glutaraldehyde	BDL ^{a}	NP	NP	NP
Disinf. Glutaraldehyde+2Antib.	NP§	0†	0	BDL ^{b}
Disinf. Glutaraldehyde+4Antib.	NP	NP	NP	BDL ^{b}

Different superscript letters within the same column indicate significant statistical differences among treatments (n≥6 per day, for each treatment group), following Kruskal-Wallis test (p<0.05).
* Colony Forming Units (CFU) below the detection limit of 30 colonies/plate.
† Zero represents, no colonies growth detectable.
§ Assay not performed.

Nonetheless bacterial densities increased over time, still to negligible levels, when compared to the control treatment group. Eleven days after hatching, larvae bacterial load were still below detection limit.

Between both G+2A and G+4A treatments, no statistically significant differences, in the antimicrobial activity, were observed (Mann-Whitney test after Bonferroni adjustments: $U = 11.50$, $r = -0.08$, $p > 0.025$).

For egg-batch C, twenty-four hours after surface disinfection, egg bacterial loads from all treatment groups, were still above the detection limit (> 30 colonies/plate). Every single one, of the replicate incubated plates, shown growth of several small opalescent whitish bacteria colonies, suggesting the disinfection procedure was unsuccessful. As a result, from this moment on, the third experiment was aborted.

4.1.3. Surface Disinfection Efficiency

Due to the fact that there was a rather anomalous result in the hatching rate success from all treatment groups of the first experiment, four days after the egg arrival, samples from the incubation culture medium of each incubation bottle of the G+2A treatment group were tested for microbiology assessment.

No colony forming units were detected in the incubation medium, except for one 10^2 fold dilution plated on 10% MA, where a density of $2.00x10^4$ $CFU.ml^{-1}$ was assessed.

Similarly, for each one of the twelve filters used to aseptically filter the incubation culture medium no bacterial colony growth was detected, indicating that axenic conditions were provided throughout all the experimental procedures.

The samples were incubated separately in six sterile tubes containing 10% MB. At the end of the incubation period, only two of those tubes were contaminated (turbid).

Unlike data from the last holding day of the first experiment, all the 1 ml samples from the G+2A treatment group of the second experiment failed to yield bacterial growth, and did not become turbid after 72 hours incubation. In other words, indicating the existence of axenic conditions.

On the last day (DAH 11), the incubation culture medium from the same treatment group was aseptically filtered, and the filters set to incubate in growth medium plates. All sample replicates were negative, with the exception of a 10% MA medium plate where very low CFU numbers were encountered, after a 24 h period of incubation at 25ºC.

In brief, bacteria density results were in general lower for the second experimental set (Table 4.1 and 4.2), although not significantly different from the first experimental set (Mann-Whitney tests, $p>0.05$).

Larvae with no evidence of microbial contamination were obtained eleven days after hatching by glutaraldehyde surface disinfection treatment with further antibiotics supplement. Control cultures were all contaminated. The success rate for obtaining such larvae was roughly 54%, for the second experimental set. Survival rate for those axenic larvae, after 11 days incubation in multi-well plates, was around 92% and 98% (Table I [Appendix B]).

4.1.4. Different Chemical Treatments and their Effect on the Morphological Ontogeny of Sea Bass Larvae

Two distinct phases have been established, in the ontogeny of the sea bass specimen replicates under evaluation. The onset of this phases, have by reference some of the most important events in the sea bass development. Thus, hatching, opening of the mouth (meaning appetence to start with the exogenous feeding) and the complete absorption of the yolk sac, sets out phase I and II, respectively.

In general, no major differences were observed in the morphological development pattern, between the individuals reared in both the control and the G+2A treatments.

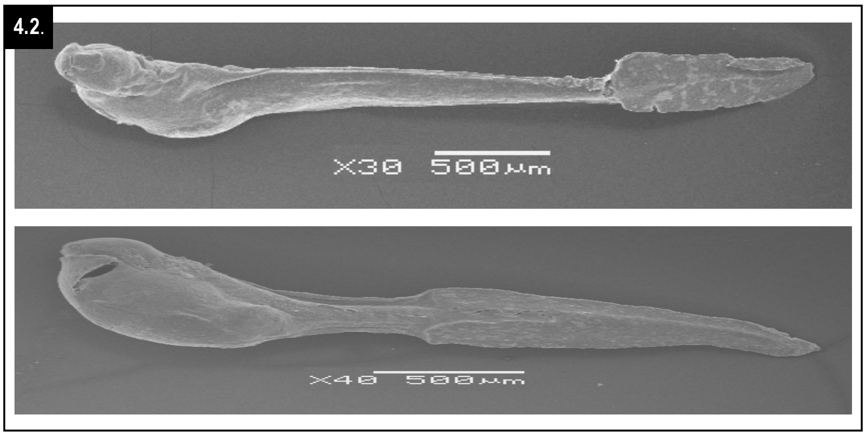
It is worth mentioning, that some of the specimens were damaged throughout the process, and were thus omitted from subsequent measurements. Nevertheless an average of two to three larvae per day, were assessed.

Phase I (from hatching to mouth opening – yolk sac pre-larva stage)

At hatching, despite their undifferentiated aspect and the inter-individual variation among fish larvae from the same treatment group, larvae from both treatment groups already showed some ultra-structural variations in the rostral, trunk and caudal zones (Figure 4.2).

Newly hatched larvae showing a moderately elongated, laterally compressed and bilaterally symmetrical body, ranging from 2.74-3.26 mm of the total body length (TL), had considerable large yolk sacs, which extended along approximately one quarter of their standard body lengths (SL). At hatching, the yolk sac volume, assuming its spheroid form, oscillated between 0.35 and 0.55 mm^3 (Figure 4.2).

At this stage, the larvae mouth were still usually closed and covered by an oropharyngeal membrane. In the central part of the head, the eyecups were becoming prominent (Figure 4.2).



Figures 4.2 – Longitudinal SEM micrographs, showing *D.labrax* newly hatched larvae from batch B replicates, following egg exposure to different surface disinfection approaches.

While the primordial marginal fin-fold was the only formed fin at hatching, about midway through this period (DAH 2-3), the pectoral fin buds began to develop (Figure 4.3 A). By the end of this phase, the already well-established pectoral fin bases started rotating, so that their blades were now facing back instead of upwards (Figure 4.3 A-E).

The anus and the mouth opened during (DAH 4-5) and at the end of phase II respectively (Figures 4.5 and 4.3 C, F and G respectively). During this phase, relative pre-anal length (prAnl) and overall body depth decreased, as the yolk was progressively diminished. Head depth (HD) and respective length (HL) increased along time. The relative eye diameter (ED), following the same pattern of development, increased until DAH6, but later kept constant (Figures 4.4 A-B).

During the yolk sac larval stage, TL rapidly increased by approximately 10-15% (Figures 4.4 A-B).