No onset of the notochord flexion, giving the tail a heterocercal appearance, was observed. Larvae in the pre-flexion stage extended until at least 11 DAH, according to the sampling time period (Figures 4.4 E).

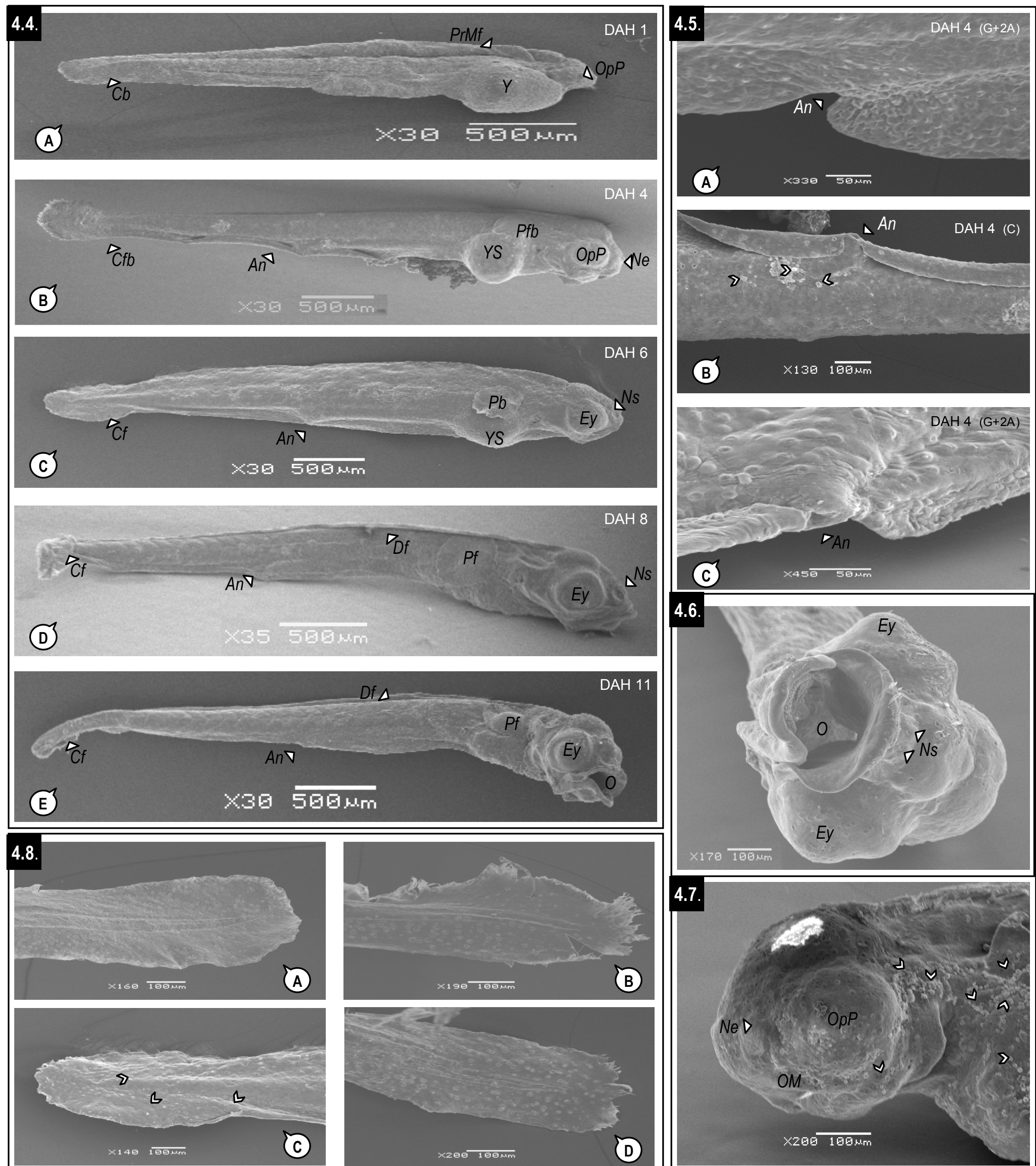


Figure 4.4. to 4.8. – SEM micrographs of *D.labrax* larvae, following egg exposure to different surface disinfection procedures.

[Figure 4.4] Longitudinal view, of the morphogenesis sequence of newly hatched sea bass larvae, over a period of 10 days. [Figure 4.5] Detail from the pre-anal region of 4-6 DAH sampled specimen. [Figure 4.6] Frontal-rostral view, of a 10 DAH larva specimen, with open mouth lacking maxilla teeth. A prominent eye-cup on either side of the head, and well-defined nostrils on the snout tip were quite evident. [Figure 4.7] Larva specimen, with no mouth development shows an indented region covered by an oropharyngeal membrane where the mouth will form. On the anterior part of the head, an optic primordium is present on both sides, and a well-developed nasal-olfactory present (3 DAH – G+2A). [Figure 4.8] Caudal end, showing normal and eroded fin tails. (A) Normal fin tail, 5 DAH – Control; (B) Eroded fin tail, 5 DAH – G+2A; (C) Normal fin tail, 6 DAH – G+2A; (D) Eroded fin tail, 7DAH – Control.

Legend: Anus aperture (*An*), Caudal fin (*Cf*), Caudal fin bud (*Cb*), Caudal fin base (*Cfb*), Dorsal fin (*Df*), Eye (*Ey*), Nasal-olfactory epithelium (*Ne*), Nostrils (*Ns*), Optic primordium (*OpP*), Oropharyngeal membrane (*OM*), Pectoral fin (*Pf*), Pectoral fin buds (*Pb*), Pectoral fin bases (*Pfb*), Primordial dorsal fin fold (*PrMf*), Yolk sac (*Ys*). *Note:* The white arrows, point out round-shaped “particles” dispersed over the skin surface.

At end of this period, most of the examined larvae showed eroded tails (Figures 4.8), although “degradation” in larvae specimen from the control treatment group was observed at earlier stages.

No maxilla teeth (Figure 4.6) or scales were visible (Figure 4.4 E).

Furthermore, large spheroid-shaped “elements”, appeared sparsely distributed on the surface of some larvae samples under evaluation (i.e. small arrows in Figures 4.3 G-H, 4.5 B, 4.7 and 4.8 C). No clear distinction was observed on the colonization pattern, neither along the sampling period, nor between different treatment groups. As a rough estimation, the average coverage of the epithelial tissues with such entities was bellow 2% total area of the larvae.

4.2. EVALUATION OF DIFFERENT HOLDING REGIMES

4.2.1. *D.labrax* Axenic Larvae

The eggs from batch D started eclosion 24 hours earlier than expected.

Glutaraldehyde surface disinfection treatment with further antibiotics supplement (G+2A) was not very effective in improving the hatching success of sea bass eggs, when compared with preceding experimental sets (ANOVA one-way: $F(3,21)=4.290$, $p<0.05$, Figure 4.9 and 4.10.), neither did it improve the hatching rate upon the control treatment group ($F(1,14)=4.909$, $p<0.05$). The hatching percentage for such treatment group was 28.13±14.73%, while for the untreated group it was 46.88±18.87%.

Both treatment groups exhibited different degrees of hatching success amongst replicates. This was also the case for the incubation bottles. One of the twelve glass bottles from the G+2A treatment group showed no hatching at all.

There was a strong initial effect of glutaraldehyde’s treatment on surface bacteria load counts. No colonies were visible after 48 hours incubation, except for one treatment bottle where a density of 91 CFU/egg was found.

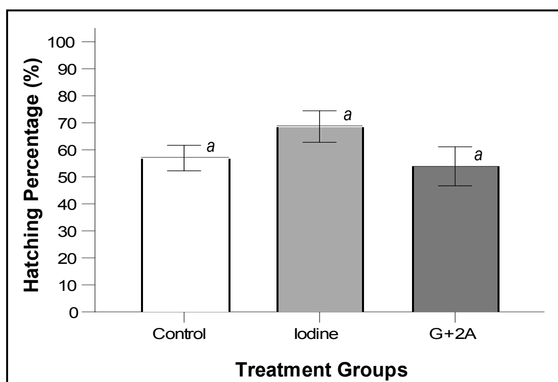


Figure 4.9. – Hatching percentage rate (%) of *D. labrax* eggs from batch B replicates (n=6 per treatment group), incubating for 4 days in 500 ml glass bottles, at 15ºC ± 1ºC.

Different superscripts within columns denote significant statistical differences (ANOVA one-way, $p<0.05$).

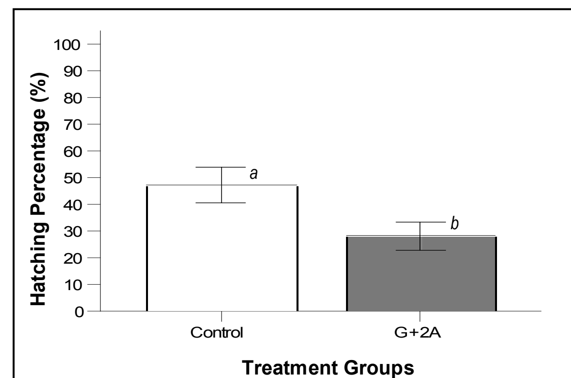


Figure 4.10. – Hatching percentage rate (%) of *D. labrax* eggs from batch D replicates (n=8 per treatment group), incubating for 3 days in multi-well plates, at 15ºC ± 1ºC.

Different superscripts within columns denote significant differences (ANOVA one-way, $p<0.05$).

Based on Cohen’s standard (η= 0.510), surface disinfection treatments had a moderate effect size.

Twenty six percent of the hatching's variance might be explained by the differences between treatment groups employed.

Overall, the effect of the disinfection treatments would seem to reflect the difference between the hatching rates from experiment 2 and 4, respectively (Figure 4.9 and 4.10). Yet, the *post-hoc* tests, so far, contradict this assumption (F=2.691, $p>0.05$).

The bactericidal effect, did, however vary considerably and seemed to be related to the initial bacterial load of the eggs from each batch.

4.2.2. Influence of Rotary Motion Devices on Larvae Performance

Survival rates obtained from the motionless experimental set (*Static*) were very low and cannot even be compared to the survival rates obtained with previous similar experimental sets (Table I and II [Appendix B]).

Mortality of larvae during the last days, from the latest set-up was high for all control groups (Figure 4.11), whilst on the previous experiment, survival rates reached 98%, ten days after hatching. When compared to the control rate, survival of the G+2A disinfectant treatment was, on the average, significantly higher (Pair-wise comparisons after Bonferroni adjustments: $p<0.001$) for all set-ups in evaluation. Hence reflecting the highly significant Cohen's standard effect (η= 0.917) that disinfection had on the surface bacterial load and subsequently on survival success.

Tukey *post-hoc* tests revealed significant differences between the three different holding groups (ANOVA two-way: $F(5,19)= 44.40$, $p<0.05$). The best results were obtained with the static multi-well setup when compared to the rotary motion devices. Moreover, it can be said that, the rotary motion devices

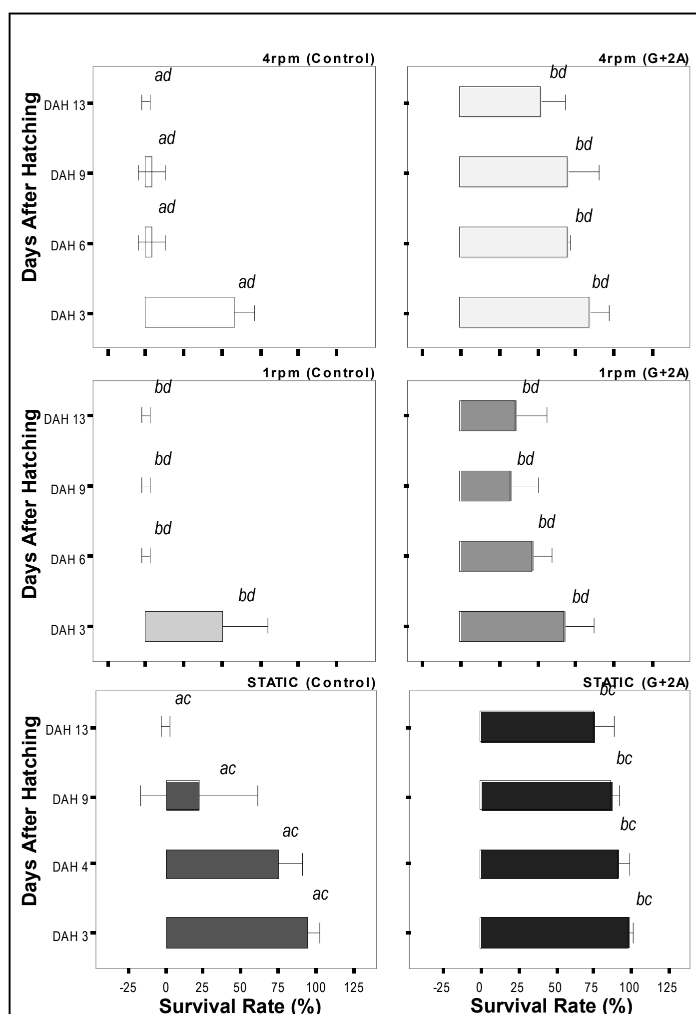


Figure 4.11. – Evaluation, along the thirteen-day period, of the outcome effect in the survival rate (%) of sea bass larvae, incubated in two different rotation devices (*Rotor A – 1rpm* and *Rotor B – 4rpm*), when compared with the results obtained in the motionless (*Static*) experimental setup.

Data represent survival mean values and standard deviation from batch D replicates (n≥4 per day, for each treatment group). Within each panel, bars with a common superscript letter are not significantly different (Tukey test, $p>0.05$).

seemed to have pronounced effect on larvae survival rate (Cohen's *size effect*, η= 0.913).

Remarkably, differences within survival started occurring immediately after the first sampling day. The survival rate was higher for the multi-well plates (*Static*: C – 94.00±8.94%, G+2A – 98.00±4.47%), than for the rotating devices (*Rotor A*: C – 50.00±29.44%, G+2A – 67.78±19.58%; *Rotor B:* C – 58.00±13.04%, G+2A – 84.44±13.24%).

By the end of the experimental period (DAH 13), differences between groups were quite substantial (Figure 4.11 and Table I [Appendix B]).

With the rotor turning at 4 rpm (*Rotor B*) in a parallel direction to the longitudinal axis of the vials, it resulted in better survival rates, than with the rotor turning at 1 rpm (*Rotor A*) in a perpendicular direction to the longitudinal axis of the vials, for both control and G+2A treatments groups (Figure 4.11). The sea bass larvae did manage to survive in both rotating devices. However these set-ups reduced in *circa* 30% of the survival rates, in a short 6-day period. It should be emphasized that no morphological abnormalities were visually detected, by means of a binocular magnifying glass.

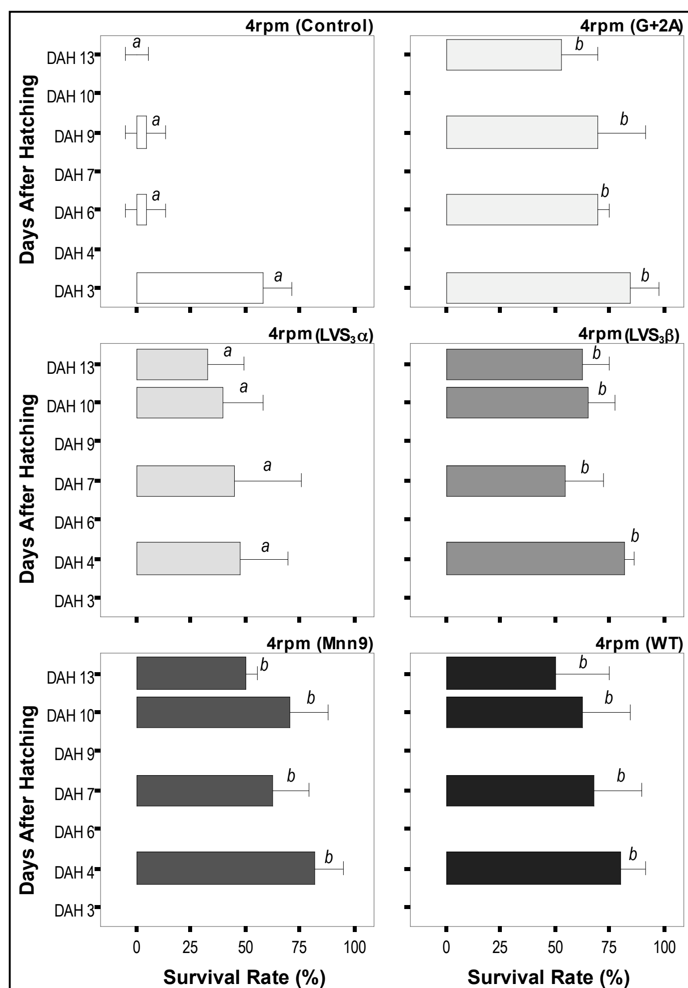
Furthermore, there was no statistical evidence that a two-way interaction would exist between the surface disinfection and the rotation motion, on the survival successes of sea bass larvae ($F(1,21)=2.99$, $p>0.05$).

Finally, to ensure that the larvae remained under axenic conditions throughout the experiment, after survival assessment, 1ml of 30% MB was added to each well of the multi-well plates and to the polystyrene vials regarding the G+2A treatment groups. No pots became turbid, indicating that they remained axenic during such period, as a result of the efficient combination of glutaraldehyde surface disinfection with further antibiotic supplement.

4.2.3. Effect of Different Individual Microbial Strains on Gnotobiotically Grown Larvae Performance

The combination of selected individual strains with previous surface disinfection of the eggs, further increased the larvae survival rate, in comparison with the untreated group ($F(3,20)=18.169$, $p<0.01$, η= 0.909, Figure 4.12). Despite the lower survival rates observed with the controls, survival was significantly higher for all treated groups and 11 days after hatching it averaged 65-70% success rate (Table III [Appendix B]).

When normalized to the G+2A treatment group, none of the strains tested appeared to affect the survival rate of the sea bass larvae throughout the experiment (Tukey test: $p>0.05$, Figure 4.12). However the numbers of surviving larvae exposed to *Aeromonas hydrophila* LVS_3, with a final concentration of approximately 10^4 cells.ml^{-1}, were significantly lower ($p<0.05$).



It is important to remember, that larvae from this treatment group were the last to be stocked[2], remaining in the incubation culture medium, within which eclosion occurred.

By means of the diet composed of yeast, the larvae group fed on Wild Type baker's yeast, showed relatively lower survival rates than the group fed on it's *mnn9* isogenic mutant. However none of these differences were found to be significant ($p>0.05$).

4.12. – Evaluation of different treatment holding regimes concerning the effect, that different individual microorganism strains have on survival rate (%) of grown gnotobiotically incubated *D. labrax* larvae.

Data represent survival mean values and standard deviation from batch D replicates (n=4 per day, for each treatment group). Within each panel, bars with different superscript letters denote significant differences (Tukey test, $p<0.05$).

[20] Stocking of larvae into the polystyrene sterilised vials, happened according to the replicates, not according to the treatment groups, in order to avoid loss of complete sampling points in case some incubation bottles would prove to be not axenic (i.e. replicate N.° 1 of all treatments were stocked first, then replicate N.º 2 of all treatments, ... , until all were done).