5. Discussion

5.1. Axenically Grown Sea bass Larvae

The method which was developed, using surface disinfection of the European sea bass eggs, two days ± 12 hours after fertilisation, with 100 mg.L^{-1} glutaraldehyde and further antibiotic supplements, proved to be reliable and highly efficient, in obtaining larvae with no evidence of microbial contamination.

Furthermore, no adverse effects on larval hatching rate were found to result from such disinfection process. On the contrary, early larval survival was enhanced by the employment of this bactericidal treatment.

Moreover, the efficiency of this method appeared to be correlated with microbial load and egg quality.

In brief, the fish larvae were successfully maintained under axenic conditions for an additional period of 11 days, as was later on confirmed by classical microbiology platting and other efficacy tests.

5.1.1. Hatching Success and Larvae Survival after Disinfection Treatments

The mucosal surface of teleost fish eggs is a well-suited substratum for microorganism adhesion (Oppenheimer, 1955 *in* Mbaluka, 2002; Hempel, 1984 *in* Morehead & Hart, 2003; Verner-Jeffreys *et al.*, 2007). Microorganisms, such as epibiotic bacteria and viruses might persist and possibly proliferate at the egg surface, infecting offspring at hatching (Bergh *et al.*, 1990; Bergh & Jelmert, 1996; Mushiake, 1994 *in* Grotmol *et al.*, 2003). Release of exo-proteolotic enzymes from such adherent bacterial microflora, may also damage the chorion or destroy the zona radiata (Hansen & Olafsen, 1989), thus seriously hampering the development of the embryo and subsequently influence hatching success, as well as the larvae performance after hatching (Barker *et al.*, 1991 *in* Bergh & Jelmert, 1996; Olafsen, 2001; Verner-Jeffreys *et al.*, 2006).

Disinfection of eggs with suitable chemicals is a practical method to reduce bacterial loads before hatching (Harboe *et al.*, 1994; Salvesen *et al.*, 1997; Skjermo & Vadstein, 1999; Grotmol *et al.*, 2003), and it is common procedure in fish hatcheries.

The process of disinfection can be affected by a number of factors, each of which may nullify or limit its efficacy (Rutala, 1996). The most important factors for the success of an egg disinfection treatment are the concentration of the germicide, the duration of exposure and the stage of egg development when the treatment is applied (Douillet & Holt, 1994).

Although higher concentrated solutions were generally recommended; there are strong reasons to believe that the best chemicals were the ones that would have a high margin of safety, and which could be used at the lowest possible concentrations, to give the desired results (Wright & Snow, 1975).

Both the disinfectant concentration and the development stage at which the antimicrobial treatments were applied, seemed to have no adverse influence on hatchability of the exposed sea bass eggs, nor in the larvae performance, since there were no significant differences in the hatching success or the survival rates, between the treatment groups.

The 5 min exposure to 100 mg. L^{-1} glutaraldehyde, which was used, is supported by the results obtained in 2006 by Poletto (Poletto, 2006 – in cooperation with the main project). The antibiotic treatment was based on a standard protocol, historically used at the Artemia Reference Center (ARC) laboratory facilities. Broad spectrum antibiotics, applied at a dosage of 10 mg.L^{-1}, are effective in protecting the larvae during such sensitive period, and in enhancing survival figures to more than 70% (Dhert *et al.*, 1998). It is well known that some chemicals are more toxic to embryos than to juvenile fish (Cooper & MacGeorge, 1991) and Poletto's (2006) findings, also suggested a possible teratogenic reaction to kanamycin and tetracycline at embryonic stages.

Moreover, iodine in a manner similar to what was done with glutaraldehyde, was tested at levels below the established by the European Community, in numerous directives and regulations (in particular by Council Regulation (EEC) No. 2377/1990), and all standard procedures of the project, were conducted under circumstances that are relevant to the aquaculture industry sector.

Disinfection treatment at advanced embryonic stages has been recommended for other marine fish species (Bergh & Jelmert, 1996; Peck *et al.*, 2004), since decreased efficiency of the chemical compounds activity, during hardening of fish eggs, seems to be a common phenomenon (Lønning *et al.*, 1988 *in* Bergh & Jelmert, 1996).

Shortly after fertilization, soft chorions of teleost fishes are transformed into rigid, chemically resistant structures (Ohtsuka, 1960; Iuchi *et al.*, 1995). The sea bass chorion hardening is a relatively slow process, with maximum hardness reached after approximately 14 hours (Fausto *et al.*, 2004; Saka *et al.*, 2001), that being one of the reasons why the treatment was initiated two days after egg fertilization ± 12 hours.

Furthermore, to be sure of a surplus disinfection, throughout the entire procedure, the egg number was adjusted to the volume of disinfectant in use (following manufacture's indications).

Glutaraldehyde has long been known to improve hatching success, development and survival of larvae, of different marine fish species after egg disinfection (Skjermo & Vadstein, 1999). However, Harboe *et al.* (1994) reported that egg disinfection by this antimicrobial agent, although it did not have a very pronounced effect on hatching, or survival of halibut larvae, during the yolk sac stage, differences started occurring when the larvae started feeding, and survival became significantly higher on treated groups.

Furthermore, Salvesen & Vadstein (1995), when comparing the efficacy of glutaraldehyde with other chemicals, in the disinfection of eggs of plaice, cod and halibut, concluded that glutaraldehyde was the most promising agent, since no negative effects were observed, either on the hatching success, or on the survival rate. Other studies, using an identical antimicrobial agent (e.g. Morehead & Hart, 2003), reported similar findings.

On the other hand, studies by Salvesen & Vadstein (1995), using plaice eggs, concluded that disinfection with 50 $mg.L^{-1}$ active iodine, did not ensure hatching enhancement. At higher doses the bactericidal effect was better, but with a reduced safety margin. Moreover, Khodabandeh & Abtahi (2006) stated that iodine (50-200 $mg.L^{-1}$) was not very effective in improving hatching success of common carp eggs.

However, judging from the present results, the extend of exposure of sea bass eggs to the different disinfection procedures, influenced both hatching success and survival rates throughout the experimental trials, varying with egg batches.

Larval viability in the early stages depends, to a great extent, on the initial quality of the eggs (Kamler, 1992 *in* Hansen & Falk-Petersen, 2001).

It is worth mentioning that the progeny of different females as well as males was tested, which makes it highly probable, that within any group, eggs of very high quality were to be found, mixed with eggs of poor quality, that being, possibly, the principal reason for the discrepancies observed in the results obtained within the same egg-batch (Dettlaff *et al*., 1993 *in* Yesaki *et al*., 2002).

Presently, one of the major constraints to evaluate the egg quality of different fish species, is the difficulty of establishing viable parameters of quality that would provide a reliable prediction of future performance (Bromage *et al*., 1994; Carrillo *et al*., 1995). Even so, simple and practical quality assessment procedures, based on physical characteristics of the egg, such as the appearance of the chorion, its shape, transparency and buoyancy, are still regarded as an important and reliable tool to distinguish good quality eggs from poor ones (Carrillo *et al*., 1989 *in* Bromage *et al*., 1994; Kjørsvik *et al*., 1990 *in* Hansen & Falk-Petersen, 2001; Linhart, 1991).

After the expected hatching time had elapsed, and in spite of the fact that when the experiments started, all the eggs were buoyant and transparent (an indication of expectable good quality), most of the eggs from batches A and C had not hatched, having instead, turned whitish and started sinking. Dead sea bass eggs are denser than seawater, and can be very easily distinguished from the live ones because they sink (Morales, 1991; Barnabé, 1991 *in* Olivar *et al*., 2000).

Although not one single larva hatched in most of the incubation bottles from egg-batch C, all the incubations bottles from batch A showed newly hatched larvae, though few in number.

Differences found among replicates within the same treatment group, do not seem to have been caused by disinfection procedures, since eggs underwent exactly the same disinfection treatment, before being stocked in separate bottles. These mortalities were, probably, due to undetected conditions, such as some form of bacterial contamination, which might have occurred. Although no attempt was made to confirm the contamination occurrence, and in spite of the fact that testing of all the material was normally processed, there is a possibility that bacteria may have been present on the system surfaces (e.g. bottles, caps, aeration tubes, etc.) and later on, after disinfection, rapidly colonized egg surfaces (Murchelano *et al.*, 1975 *in* Douillet & Langdon, 1994).

Treatments such as filtration, ultraviolet radiation and autoclave heating modify the generic composition of the present microflora, but are generally unable to cause permanent changes in bacterial concentrations (Murchelano *et al.*, 1975 *in* Douillet & Langdon, 1994), thus proliferation of opportunistic microorganisms (Andrew & Harris, 1986 *in* Olafsen, 2001), may have taken advantage of the ecological changes introduced, when such procedures were established (Skjermo & Vadstein, 1999).

Despite the absence of conclusive evidence of major infections, it is necessary to improve disinfection procedures, such as for instance cleaning all model system material with chromic acid (for glass) or sodium hypochlorite (for rubber and plastic), for two days, and afterwards rinsing it with tap water and distilled water. In addition to such strict hygienic control measures, it may be necessary to dismantle the barrier, for cleaning, disinfection and maintenance more regularly.

Microorganisms contamination will inevitably enter the barrier with time (Rutala, 1996), despite all preventive measures. Therefore, disinfection of the room in the interval between experiments, depending on the experimental routine, is advisable (Hem & Engh, 2001a) and should continued.

European sea bass is a batch spawner species (Tyler & Sumpter, 1996). Hirazawa *et al.* (1999 *in* Escaffre *et al.*, 2001) reported that the chorion thickness varies along seasonal spawnings, and that earlier egg batches tend to have thinner eggshells, compared to later ones. Thus earlier batches, such as egg-batch A, could have been more sensitive to surface chemical treatments. This might explain, at least partly, the mortality variance between different batches.

Bromage *et al.* (1994) stated that egg quality effects may be masked by a variety of environmental and husbandry factors. Furthermore, hatching failure from egg batches may have also been related and influenced by poor egg quality, which could be due to parental genetics or even to microflora adhering to fish eggs, during ripening.

Over and above the genetic of the broodstock, proliferation of large amounts of bacterial cells on the egg surface, not withstanding the possibility of contamination, may have caused other problems as well. Thus, reducing the exchange of gases and metabolic waste between the embryo and the surrounding environment, thereby causing hypoxia (Hansen & Olafsen, 1989; Barker *et al.*, 1991 *in* Bergh & Jelmert,

1996; Salvesen *et al.*, 1997).

Although hatched larvae survival performance observed during the experimental work, remained within a reasonable similar range when compared to customary values of 8 DAH obtained from sea bass commercial facilities (Panini *et al.*, 2001), further work should be carried out, in order to ensure that no long–term adverse effects, associated with the treatments, will result.

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

The disinfecting procedures concerning the bacterial load neutralization of eggs from several different fish species, have been studied by various authors (Table 5.1). Surface sterility, is a decisive criterion often used when evaluating the bactericidal effect of different antimicrobial treatments, which by definition, involve a process that is intended to eliminate or inactivate pathogenic organisms, but which does not necessary eradicate all types of microorganisms (Rutala, 1996; Gardner & Peel, 1986 *in* Salvesen *at al.*, 1997).

Eggs without bacterial growth are not necessarily surface sterile, because live epibacteria, although not able to grow on Marine bacteriological media, may still be present (Salvesen & Vadstein, 1995).

A state of viable but non-culturable (VBNC), has been recognized for a variety of marine bacterial species (Hansen & Olafsen, 1999). A survival strategy, adopted by bacteria when exposed to a number of adverse environmental conditions, by entering a phase whereby they may undergo morphological and biochemical changes resulting in dormancy (Poletto, 2006). Dormant cells are viable and in most instances probably also virulent, but unable to grow on conventional culture media (Hansen & Olafsen, 1999). As a consequence, the routine plating method could underestimate the real bacterial presence (Pianetti et al., 2004)

For convenience, in the present discussion, these eggs with “no bacterial load” associated, were termed surface sterile.

The efficacy of combining the 100 $mg.L^{-1}$ glutaraldehyde disinfection with antibiotic supplements, when compared with the control treatment group, reflected a rather highly significant bactericidal effect on both batches (A and B). With batch B, the initial bacterial load was strongly reduced, and remained under the detection limit, at no colony growth, throughout hatching day until further antibiotic supplements were applied. Eleven days after hatching, larvae bacterial load levels were still below detection limits for both glutaraldehyde groups (G+2A and G+4A), no significant statistical differences being observed in their antimicrobial activity.

A number of other studies describe glutaraldehyde as an efficient egg surface disinfectant (e.g. Douillet & Holt, 1994; Salvesen & Vadstein, 1995; Verner-Jeffreys *et al.*, 2007). This should naturally be expected from a compound which is a cheosterilizer, with a broad spectrum of activity, rapid anti-

microbial action and relatively high activity in the presence of organic matter (Borick, 1968 *in* Salvesen *et al.*, 1997; Gorman *et al.*, 1980 *in* Escaffre *et al.*, 2001).

Table 5.1. – Optimal conditions for egg surface disinfection, of various teleost species (ordered chronologically by chemical disinfectant).

Species	Amount	Exposure	Temperature	References
Glutaraldehyde				
Hippoglossus hippoglossus	400 mg.L^{-1}	10 min	no reference	Harboe *et al.* (1994)
Pleuronectes platessa	400-600 mg.L^{-1}	5-10 min	5-7ºC	Salvesen & Vadstein (1995)
Hippoglossus hippoglossus	400 mg.L^{-1}	10 min	5ºC	Salvesen *et al.* (1997)
Scopthalmus maximus	400-800 mg.L^{-1}	2.5 min		
Sparus aurata	200 mg.L^{-1}	4 min	18ºC	Escafre *et al.* (2001)
Anarhichas minor	150 mg.L^{-1}	5 min	8ºC	Hansen & Falk-Petersen (2002)
Latris lineata	400 mg.L^{-1}	10 min	14.5ºC	Morehead & Hart (2003)
Psetta maxima	100 mg.L^{-1} + 10 mg.L^{-1} Rifampicin	5 min	16 ºC	Poletto (2006)
Hydrogen peroxide				
Sciaenops ocellatus	3000 mg.L^{-1}	5 min	25ºC	Douillet & Holt (1994)
Polydactylus sexfilis and *Seriola rivoliana*	11 000 mg.L^{-1}	5 min	22-26ºC	Verney-Jeffreys *et al.* (2007)
Iodophor				
Esox masquinongy	100 mg.L^{-1}	10 min	12ºC	Schachte, (1979)
Micropterus salmonides	500-700 mg.L^{-1}	15 min	22.2ºC	Wright & Snow (1975)
Hippoglossus hippoglossus	20 mg.L^{-1}	10 min	5.5ºC	Bergh & Jelmert (1990)
Acipenser transmontanus	100 mg.L^{-1}	15 min	15.5ºC	Yesaki *et al.* (2002)
Gadus morhua and	1000 mg.L^{-1}	5 min	7-8ºC	Peck *et al.* (2004)
Melanogrammus aeglefinus	+5mg.L^{-1} (3Streptomycin:2Penicillin)	+24h		
Polydactylus sexfilis	100 mg.L^{-1}	10 min	22-26ºC	Verney-Jeffreys *et al.* (2007)
Ozone				
Hippoglossus hippoglossus, *Gadus morhua* and *Scophthalmus maximus*	2 mg.L^{-1}	2 min	6ºC	Grotmol *et al.* (2003)

The efficiency shown by the results also reflects the properties of the antibiotics used. Although most antibiotics are bacteriostatic, inhibiting only a limited bacterial spectrum, the combination of two wide-spectrum antibiotics, could offer better prospects (Provasoli, 1977), as proven herein.

The bactericidal activity from the other antimicrobial treatments seemed to be ineffective. This may be due to the concomitant discontinuation of disinfection, allowing bacteria to proliferate. Apparently in these instances sufficient bacteria survived the disinfection to affect the procedure efficiency.

Iodophor solutions, on the other hand, are primarily effective against bacterial and viral pathogens (Wright & Snow, 1975), although, some sources have reported that iodine is also relatively effective as a fungicide (e.g. Vener-Jeffreys *et al.*, 2007).

Salvesen & Vadstein (1995), when comparing both disinfectants, found out that the range between iodine doses, which either have a direct lethal response or have no effect at all, was very narrow. In spite of such a slender safety margin (Wright & Snow, 1975), the use of iodine disinfectants may still be more attractive than many other alternatives, such as formalin and glutaraldehyde, when its relative safety and low-cost application are taken into consideration (Amend, 1974 *in* Salvesen & Vadstein,

1995; Peck *et al.*, 2004).

Withstanding iodine findings, were the results achieved in Verner-Jeffreys *et al.*, (2007) in their later studies, where high in vitro iodine sensitivity did not correlate with efficient disinfection of eggs, at lower iodine concentrations. Iodophor solutions have long been recognized as efficient egg surface disinfectants for many freshwater species (Schachte, 1979; Amend, 1974 *in* Bergh & Jelmert, 1996), but those buffered solutions may not necessarily be effective in seawater (Salvesen *et al.*, 1997), as the marine environment constitutes a different chemical and microbial environment from that of freshwater (Olafsen, 2001). In brief, the antimicrobial efficiency of a given compound in sea water, cannot be directly inferred from its successful application in freshwater.

However, in a number of marine species including the Atlantic cod (*Gadus morhua*), haddok (*Melanogramus aeglefinus*) and the Pacific threadfin (*Polydactylus sexfilis*), such efficiency correlation has been consistently observed. Iodine surface disinfection of such species has proved to be relatively useful (Peck *et al.*, 2004; Verner-Jeffreys *et al.*, 2007) minimizing the bacterial load.

Acceptable values presently adopted for optimal sea bass egg disinfection procedures, are notably lower than those previously recommended for other fish species.

Harboe *et al.* (1994) studies, found no bacterial growth on 400 $mg.L^{-1}$ glutaraldehyde disinfection for 10 min, of Atlantic halibut (*Hippoglossus hippoglossus*) eggs, after two days. Salvesen *et al.* (1995) found that 65-97% of plaice, *Pleuronectes platessa* eggs were surface sterile after 5-10 min disinfection with the same chemical compound (400-600 $mg.L^{-1}$).

Later, similar treatments with 400-800 $mg.L^{-1}$ glutaraldehyde for 10 min, had good antimicrobial effects at all concentrations tested, and gave 95-100% of surface sterile turbot, *Scophtalmus maximus* (Salvesen & Vadstein, 1997).

This discrepancy between the results may reflect either true interspecific variation in sensitivity, or may be due to differences in incubation temperatures (Table 5.1). The rate of glutaraldehyde's penetration depends on the type of tissue, exposure time and incubation temperature at which disinfection takes place (Hayat, 1970 *in* Escaffre *et al.,* 2001).

Physiological and structural differences among fish eggs are large, thus tolerance towards disinfectants should be highly variable (Davenport *et al.*, 1986 *in* Hansen & Falk-Petersen, 2001; Lønning *et al.*, 1988 *in* Hansen & Falk-Petersen, 2001; Bergh & Jelmert, 1990). Nonetheless, there is strong evidence that glutaraldehyde efficacy is temperature dependent (Sano *et al.,* 2005), with lower environmental temperatures partially mitigating effectiveness, and higher temperatures augmenting it (Salvesen & Vadstein, 1997; Gardner & Peel, 1986 *in* Sano *et al.*, 2005; Escaffre *et al.*, 2001).

The complexity increases further, due to the fact that glutaraldehyde is readily degraded by several microorganisms (Leung, 2001 *in* Sano *et al.*, 2005), and that differences in experimental design (flow-through *versus* static renewal) may diminish toxicity of the antimicrobial agents (Sano *et al.*, 2005).

Another constraint, which supports our results, is that the same bactericidal treatment may succeed or fail under ostensibly identical conditions (Provasoli, 1977; Norberg *et al.*, 1991 *in* Harboe *et al.*, 1994; Gatesoupe, 1995; Salvesen & Vadstein, 1995). This is not surprising, since the bacterial load varies widely in composition and density among different egg batches, with season, hatcheries and biocenosis (Sieburth, 1967 *in* Provasoli, 1977; Hansen & Olafsen, 1989).

Given that, identical amounts of microorganisms are inactivated in a disinfection reaction, per same unit of time (Chick, 1908 *in* Salvesen & Vadstein, 1995), the bactericidal effect of a standard dose can be expected to vary with the initial bacterial density (Bergh & Jelmert, 1996; Escaffre *et al.,* 2001).

Salvesen and Vadstein (1995) replicated experiments, under identical disinfection conditions, revealed a clear correlation between the degree of successful surface disinfection and the initial bacterial load of the egg batch. Treatments with recommended doses of disinfectants will thus not always ensure a total elimination of the epibiota microflora. The fraction of surface sterile eggs, after a standard treatment, will occasionally be higher, in groups with a low initial load of bacteria, than in groups having high numbers of associated bacteria.

The better bactericidal effect observed with batch B, can presumably be explained by a lower initial bacterial load, suggested by the fact that after 24h incubation, the fraction of CFU counts assessed for the control treatment group, was approximately 10^2 times lower than for the control group from batch A.

5.1.3. Surface Disinfection Efficiency

Similar to most previous studies on this topic, axenic conditions were examined and confirmed, for the presence of bacteria using a combination of Marine Agar (MA) and Marine Broth (MB) culture based analyses. Even though, the bacteria present in the analyzed incubation culture medium, eggs or larvae samples appeared to have been MA and MB culturable it is not conclusive, that this would be generally the case with all sea bass egg-batches. Since it is well known that the majority of marine bacteria (>99%) are not recoverable using this approach (Amann *et al.*, 1995 *in* Verner-Jeffreys *et al.*, 2006).

Nevertheless, as mentioned by Salvesen & Vadstein (1995), the microbiological samples from the present experiments, displaying bacterial growth not only gave indications on the bactericidal effect from the different chemical approaches in use, but also gave an idea of the initial bacterial load on egg surface.

Regardless, analysis of the results obtained in most experiments, revealed that the validity of some conclusions concerning the efficiency of the bactericidal treatments pertaining to obtain axenic sea bass

larvae is limited, mainly by low reproducibility of the disinfection procedures. Earlier attempts (Poletto, 2006; Verner-Jeffreys, 2007) to develop such a system have also demonstrated that it is not easy to axenically produce fish larvae in a standard reproducible manner.

Even so, since highly reliable results were achieved, further evaluation of reproducibility should be undertaken as to consistently prove that certain treatments resort in a standardized successful effect.

As it was generally assumed that microbiological factors were responsible for the high variability within and between experiments, it can be anticipated that an agreement with the supplier of sea bass eggs on a standard operational routine procedure, would be beneficial.

Still, it is worth mentioning, that since several gram-negative bacterial strains are known to gain resistance to some disinfection procedures, because of a plasmidic resistance gene (Kummerle *et al.,* 1996 *in* Gatesoupe, 2002), it was of major importance to confirm whether the antimicrobial treatment in use, had caused resistance spread. Reason why, a combination of MA and MA+R+A medium was used. Fortunately no development of unwanted resistant bacteria, were confirmed.

However, VBNC forms may have been present, and since they cannot be detected by standard plating techniques (Pianetti *et al.*, 2004), other specific methodologies, such as molecular biology techniques (e.g. denaturing gradient gel electrophoresis analysis of PCR fragments generated by universal primers analysis) should be considered.

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

The larvae period represents a transitional and distinct ontogenic stage of simultaneous growth and development, which causes substantial changes in the structure, physiology, size and shape, all of which modify the performance (physiological and behavioral capabilities) and subsequently, the ability of the fish to deal with the challenges it will have to face for its survival (Koumoundouros *et al.*, 2001).

As regards to the action of certain disinfection agents, apart from the acute toxicity of numerous chemicals, that may result in direct mortality, fish larvae are very sensitive to sublethal concentrations of such toxicants. Several studies have shown, that hatching and larvae development may be delayed (Gormley & Teather, 2003 *in* Sánchez-Bayo & Goka, 2005), changes in temperature and/or salinity tolerances diminished (Heath *et al.,* 1997), and malformations (Hofer *et al.,* 1995; Birge *et al.*, 1985 *in* Luckenbach *et al.*, 2001; von Westernhagen *et al.*, 1988 *in* Abdel *et al.*, 2004) of several kinds occur.

Compared to adult fish, the detection of toxic effects is much easier in early ontogenetic stages, due to greater sensitivity and diverse well accessible end-points (Luckenbach *et al.,* 2001), thus making developmental events, which occur between hatching and the completion of metamorphosis, particularly interesting (Vegetti *et al.*, 1999).

Phase I, the time lasting out from hatching to the opening of the mouth, was a lecitotrophic period, in which the newly hatched larvae relied on the eosinophilic reserves contained in the large yolk sac (García Hernández *et al.,* 2001) for morphogenesis, growth and metabolic energy. Phase II, comprised the onset of exogenous feeding, from the opening of the mouth to the complete reabsorption of the yolk sac. According to the terminology usually employed, as convenient means of morphophysiological identification, phases I and II correspond to pre-larvae stages (e.g. Balon, 1975; Alemany, 2003).

Although amongst specimen collected on the same sampling day, there were some minor asynchronies, in the timing for certain morphological characters to develop, between the individuals reared in both the control and the G+2A treatments, no major differences were observed in the main development pattern. The timing at which the various anatomical features under evaluation developed, is in accordance with similar descriptions by well known authors (i.e. Tan-Tue, 1976; Deplano *et al.*, 1991; García Hernández *et al.,* 2001).

The allometric and morphological differences observed herein, reflect, most likely, variations in factors influencing the larval development, other than chemical exposure, namely: egg size, genetic background and broodstock aging (Blaxter 1988 *in* García Hernández *et al.,* 2001; Morrison *et al*., 2001; Kara *et al*., 1995 *in* Saillant *et al.*, 2001). As earlier mentioned, within the same assembled egg-batch, the progeny of different females as well as males were appraised, which makes it highly probable that within any treatment group, a wide range of egg sizes and even genetic background, were under evaluation.

A word of caution should be addressed to such an interpretation, since a very small number of larvae were used for evaluation, with some of the specimen being damaged throughout the process (and therefore omitted from subsequent measurements).

Furthermore, it is still under question, whether some morphometric differences between similar age-group larvae, were or not, associated in part, to the fixative in use. Existing literature supports that denaturation and dehydration, resulting from the commonly used fixatives (alcohol and buffered formaldehyde), may influence larval measurements (e.g. Takizawa *et al.,* 1994; Paulet & Kaisser, 2004).

The degree of tissue shrinkage depends on the osmotic strength of the fixative, and water loss is amongst those factors responsible for shrinkage in fixatives. Thus, varying moisture content within the body of the fish larvae, can cause differential shrinkage rates (Takizawa *et al.,* 1994).

Percentage shrinkage has been shown to be specie-specific, and that it varied depending on the size of individuals. Tucker and Chester (1984 *in* Paulet & Kaisser, 2004) showed that shrinkage was greater when using small fish, because of their relatively higher water content.

Thus, for the given fixative, correction factors should be considered and determined before conducting other studies on the morphometric development of sea bass fish larvae (Kruse & Dalley, 1990 *in* Paulet & Kaisser, 2004; Takizawa *et al.,* 1994).

Foreign particle adhesion is a surface interaction phenomenon, and this makes it ideal for examination by scanning electron microscopy (SEM) (Knutton, 1995 *in* Ringø *et al.,* 2003). Likewise, some SEM micrographs from the present study easily revealed, areas of the larvae surface colonized by large spheroidal "particles", sparsely dispersed. Unfortunately, we were unable to identify those "elements".

It might be the presence of bacterial colonization remains, or even mucus produced by the goblet cells.

According to Varsamos *et al.* (2002), at hatching, the integument of the sea bass (*D.labrax*) pre-larvae is very thin (3–4 μm), and comprises two easily identifiable cell types: pavement cells with microridges and mucus cells. The later, might have been captured in some SEM micrographs from the present study. Figures 4.5c and 4.7, show the epithelial tissues with small, dense inclusions, which may have represented the mucus producing cells pores. Though higher SEM amplification should be used to confirm such assumption.

In a recent investigation, Ringø and Olsen (1999 *in* Ringø *et al.,* 2003) raised the question of whether or not there was a connection between SEM investigations and the dilution plate technique, when determining associated bacteria. The authors reported that SEM visualizations later confirmed the bacteria predominance, previously determined by classical microbiology platting.

In this particular study, (presuming the spheroidal "elements" were bacterial remains) discrepancies between quantitative bacterial determination in platting count (Table 4.2), and examination of the skin surface by means of a SEM, were observed. Surfaces from the control larvae specimen, often appeared devoid of bacteria, although cultured samples from the same treatment group, gave rise to high CFU numbers. It is likely that bacteria were washed off, along preparation for SEM or were internally present. On the other hand, the lack of bacterial growth in the culture platting from the disinfection treatment group, indicates that the G+2A egg surface disinfection was fairly effective, and the bacteria observed on larvae skin surface may have been dead, and probably fixed by the Karnovsky's fixative.

Bergh *et al.* (1989) and Pittman *et al.* (1990 *in* Morrison & MacDonald, 1995), have described larvae eroded tails, due to bacteria coverage, which could in some manner explain the present situation. Nevertheless, sea bass larvae in our study were evidently susceptible to tail abrasion, especially when confined to small multiwell plates. Though, it should be mentioned, this susceptibility doesn't seem to be an imperative factor, affecting larvae performance success.

Briefly, because of the ease in damaging the specimen samples during preparation for SEM, and for a meticulous overview comparison on the morphometric and morphological differences, arising from the different treatments in use, sampling a larger number of individuals is suggested. Higher SEM

amplification should also be considered in further studies.

Moreover, in combination with other former histological studies (Annamaria Rekecki *unpublished data*), these findings might provide a general overview on the effect that different disinfection and holding treatments, would have in the morphogenesis processes, and also provide an overall functional description of the developing gastrointestinal tract (GI) that arises during development of axenic *versus* conventional specimen, which in future, might be relevant to the host-microbial interaction perception when further probiotic strains are tested.

5.2. EVALUATION OF DIFFERENT HOLDING REGIMES

In order to study the effects of specific individual microorganism strains, more accurately, an acceptable and reliable model system was accomplished. It permitted an easy adjustment of the most important physical and chemical parameters, thus allowing successful axenic egg hatching, as well as further maintenance of sea bass larvae, under gnotobiotic conditions, for 13 days. The rotating devices used herein, although they did impose stress on the newly hatched larvae, still it may be the best feasible technical approach in order to maintain bacteria and yeast cells in suspension.

Moreover, the evaluation of the effect that specific microbial strains may have had on larvae survival performance, revealed that under such prevailing conditions, the selected strains acted as neutral microorganisms, thus not influencing the larvae performance during the first 11 days.

Although embryo survival, up to hatching day, did not improve significantly, by egg surface exposure to 100 $mg.L^{-1}$ glutaraldehyde for 5 min, with further antibiotic supplement (ampicillin and rifampicin, 10 $mg.L^{-1}$ each), present findings do indicate however, that such chemical treatment was effective at reducing microbial contamination and enhancing early larval survival for all set-up groups under evaluation.

Polystyrene multi-well plates proved to be very convenient for determining the hatching percentage of egg-batches. Data showed that there was high variation in the hatch rate, microbial colonization and potential sensitivity to chemicals, corroborating the high variation in the survival and larvae performance, amongst replicates.

5.2.1. *D.labrax* Axenic Larvae

Methods previously employed to monitor and assess the hatching success of egg-batches were often inaccurate, because the egg quantity stocked in each treatment bottle could be very easily under- or sub-estimated (Poletto, 2006), making it an inaccurate approach.

While searching for a more reliable system, the use of sterile multi-well plates for egg incubation seemed sufficiently adequate. Not only did it not require extensive facilities, a simple controlled-temperature chamber being adequate, so as to maintain environmental temperatures within the required

limits, making this technical-model very cost effective (Panini *et al.*, 2001). Another important aspect is the ease with which the monitoring of individual eggs and larvae from several different egg-batches can be performed.

Under such a laboratory-scale model, each well was an independent experimental unit (Grotmol *et al.*, 2003), making it an accurate tool for hatching percentage assessment.

Similar studies have demonstrated how polystyrene multi-well dishes can be successfully used as culture chambers for individual eggs/larvae of European sea bass, during the first days of development (e.g. Panini *et al.,* 2001). The hatching percentages were not affected by this culturing method, indicating that the environmental conditions in the wells of the multi-well plates were adequate for the proper development of the embryos (Panini *et al.*, 2001).

The glutaraldehyde surface disinfection treatment with further antibiotics supplement (G+2A), used as surface egg disinfection in the present study, has been selected, based on previous successful achievements, and due to its potential compatibility with probiotics (Gatesoupe, 2002). In the present studies, such chemical treatment, was neither very effective in improving the hatching success of sea bass eggs, when compared with preceding experimental sets, nor did it improve the hatching rate relative to the control treatment group.

Although biocenosis influences played a significant part, variations in the chorion thickness (Helvick, 1988 *in* Bergh *et al.*, 1990) and in the egg quality, within the same egg-batch (Kamler, 1992 *in* Hansen & Falk-Petersen, 2001) along the seasonal spawnings, may again explain some of the quoted differences amongst replicates.

In contrast to what was observed in the previous experiments, the eggs from batch D started hatching 24 hours earlier than expected. The first idea that came to mind was that those particular sea bass eggs, were somehow, more developed than was normally expected. However, the explanation for the premature hatching of the eggs may lie eventually, on the damage to the egg's membrane, caused by protozoan activity and bacterial degradation, or even by hypoxia, as a result of substantial bacterial colonization of the chorion by obligate aerobes, which is known to stimulate the hatching procedures (Hansen & Olafsen, 1989; Haya & Waiwood, 1981 *in* Hansen & Falk-Petresen, 2001; Barker *et al*., 1989 *in* Olafsen, 2001).

Even so, glutaraldehyde's treatment showed to be highly efficient in inactivating microbial larvae load, as no colony growth was found after 48 hours incubation.

5.2.2. Influence of Rotary Motion Devices on Larvae Performance

Whilst several studies (e.g. Bergh & Jelmert, 1990; Jelmert & Naas, 1990; Morrison & MacDonald, 1995; Forniés, 2000 *in* Panini *et al.*, 2001), support that rearing yolk sac larvae in multi-well plates, is an

adequate way of obtaining healthy fish larvae for experimental purposes, on traditional static set-ups such as those, bacteria and yeast cells tend to sink very fast.

Microbial aggregation is observed in virtually all microbial taxa (i.e. bacteria, yeast, cellular slime molds, filamentous fungi, algae, and protozoa), leading to sedimentation (Calleja, 1984). Genetic, physiological, nutritional, environmental, and physical factors all come into play in determining whether a cell suspension will aggregate and to what degree they will start sinking to the bottom (Hjortso & Ross, 1994). This fact not only compromises ingestion by larvae, but also promotes a breeding-ground for microbial growth and to water quality deterioration.

Rotating devices seem to offer convenient advantages over long-established static set-ups, since, by re-suspending the strains and making them once more available to the larvae, considerably reduces the water confinement risks.

Most fish larvae species are very sensitive to disturbances caused by shaking or other mechanical means (Piper *et al.*, 1982 *in* Escaffre *et al.*, 2001), particularly during their most critical early stages. As an example, a sudden strong flow of water may very easily affect the health performance of larvae (Woynarovich & Horváth, 1980).

Evidence provided by the present study, suggests that *D.labrax* larvae managed to survive the physical stress imposed by water movement, generated by the rotating devices (Rotors *A* and *B*). However 6 days following larvae stocking, both control treatments for the rotation devices, showed a mortality rate of nearly 96-100%.

At the end of the experimental period (DAH 13), differences in the survival rates between the two rotating groups were quite substantial. Contrary to what would be expected, the rotor turning at 4 rpm (*Rotor B*), inspite of its higher rotation speed, produced better survival rates, than were obtained with *Rotor A*, turning at 1 rpm. This apparent discrepancy may be explained by the different water-mass and air translocation dynamics, induced by each rotor on their respective vials, those effects being more pronounced in the case of *Rotor A*, due to the axis of the rotating vials being perpendicular to the axis of the rotor. In the case of Rotor B, with the axis of the vials parallel to the axis of the rotor, the water-mass and air translocation dynamic effects were considerably less, in spite of the higher speed of that device, thus resulting in better survival rates than obtained with *Rotor A.*

It has been demonstrated by several authors that the mortality increases when larvae are exposed to physical stresses, such as light and water movement (Bolla & Holmefjord, 1988 *in* Harboe *et al.*, 1994; Pittman *et al.*, 1990 *in* Bergh & Jelmert, 1996).

In the early stages of rearing, morphologic and physiological problems often arise, as cultivation of such small and sensitive marine larvae requires a period with no or low water exchange (Skjermo & Vadstein, 1999). Visual observations by means of a binocular magnifying glass, did not reveal any inconsistent behaviour or anomalous morphological development though treatments without any

observable negative effects, may still have caused damages that could negatively affect the viability of the larvae at a later development stage. Further tests should be performed to investigate such possibility.

The mortality of larvae observed during the last days, in all the experimental set-ups, was unexpectedly high for each and every control group, whilst on previous experiments, survival rates reached 98%, ten days after hatching.

The reason for such a generalised mortality, probably lies on the accumulation of certain harmful material that larvae excrete during their development, which if allowed to accumulate, may contribute to the surrounding environmental deterioration, and even favour microorganisms proliferation (Kaway *et al.*, 1964 *in* Douillet & Holt, 1994; Panini *et al.*, 2001). It has long been known that the combination of high nutrient concentrations and water confinement, is of a great stimulus to microbial growth (Douillet & Langdon, 1994), and that microorganisms play an extremely important role in the mortality of many fish larvae (Austin & Austin, 1999; Hansen & Olafsen, 1999; Bricknell & Dalmo, 2005).

Even so, survival of the G+2A disinfectant treatment group when compared with the control rate, was significantly higher for all set-ups under evaluation. This is most probably a reflection of the highly significant bactericidal effect that disinfection had on the surface bacterial load hampering larvae survival.

Despite the fact that the design of the rotating systems employed cannot be considered as optimal, axenic newly hatched larvae reared in UV-sterilized vials, were successfully maintained under gnotobiotic conditions for an additional 13 day period, as was later confirmed, after incubation in sterile test-broth failed to yield bacterial growth.

The method presently used to check the efficiency of treatments does not allow assessment of a complete absence of microflora. Not only it does not acquire for the presence of VBNC bacteria, but also, it merely evaluates bacteria from the incubation culture medium and liberated from larvae (Escaffre *et al.*, 2001). Nevertheless, it is indicative of the efficient combination of glutaraldehyde surface disinfection, with further antibiotic supplement, in obtaining “sterile” surface sea bass larvae.

5.2.3. Effect of Different Microbial Strains on Gnotobiotically Grown Larvae Performance

The use of new preventive approaches such as immunostimulants and probiotic strains to reduce stress and mortality, maintain cultured organisms in good health and stimulate the non-specific defence mechanisms, is becoming increasingly important in aquaculture (Marques *et al.,* 2006b). Immersion of marine larvae in immunostimulants enriched with probiotic strains, at an early pre-feeding stage (before starting with the exogenous feeding), is believed to be a suitable technique for immunostimulation, preferably during periods of prevailing stagnant or low water exchange (Gatesoupe, 2002), such as those prevailing.

Recently, claims have been made of the beneficial effects of some promising dietary live yeasts (Pérez *et al.*, 1997; Tovar *et al.*, 2002; Marques *et al.*, 2004; Tovar-Ramirez *et al.,* 2004; Gatesoupe, 2007) and *Aeromonas hydrophila* LVS_3 bacteria strains (Harris, 1993; Verschuere *et al.*, 1999; Irianto & Austin, 2003), said to have performance-enhancing characteristics, thus significantly improving the survival rates of various aquatic organisms. Yet the results obtained in the present study, during the first thirteen days of the sea bass larvae, have shown that the different selected strains acted as neutral microorganisms (Marques *et al.,* 2005), thus not influencing sea bass larvae performance. This most probably happened because of time being insufficient for the microorganisms strains to settle in the culture environment, and to adhere to the mucus layer of the gut section and to the external surfaces of the host organisms, making it impossible for any potential beneficial effect to occur. It should be mentioned that *Saccharomyces cerevisiae* X2180 was referred by Tovar *et al.* (2002), to adhere to the digestive tract of sea bass, sensibly at 41 DAH.

Another important condition for the strains efficiency has to do with their viability state (Verschuere *et al.*, 1999; Lagat, 2001). Several investigations have demonstrated that various non-differentiating strains can respond to a number of environmental stresses, by hampering their metabolic activity (e.g. Pianetti *et al.*, 2004).

Furthermore, according to Verschuere *et al*. (2000a), when cultures are performing under optimal confinement conditions, in the absence of opportunistic pathogens, potential strains applications may not produce any notable benefits.

The number of surviving larvae exposed to *Aeromonas hydrophila* LVS_3, with a final concentration of approximately 10^4 cells.ml^{-1}, was significantly low, most probably, because they were the last ones to be stocked, and the ones to spend more time in the incubation culture medium, within which hatching occurred. Hatching normally results in the release of high amounts of organic matter into the incubation water, leading to significant enhancement of microbial growth and to a decrease in survival (Douillet & Holt, 1994; Poletto, 2006).

The molecules of β-glucans and of chitin, are known to be involved in the optimal function of the immune system of aquatic animals and to enhance overall disease resistance, thus improving their performance (Marques *et al.,* 2005). Since mnn9 yeast cells contain more β-glucans and chitin, than "Wild Type" yeast cells (Marques *et al.,* 2004), one would be tempted to speculate that the mnn9 isogenic mutant offered much better conditions to improve larvae performance, than the Wild Type" yeast cells. However, in the present findings, that expectation did not hold totally true. Although the larvae group fed on “Wild type” baker’s yeast showed a lower survival rate than the group fed on this mnn9 isogenic mutant, the difference was not significantly relevant.

Independently of the results attained for the first thirteen days, such strains have already proven to be highly appropriate as performance-enhancing, thus they should be considered as candidates for further tests, using the present gnotobiotic system.