Deliverable Report

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Deliverable Title	Determine most effective type and concentration of algae used in grey mullet larval rearing		
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Task Title:	Effect of algal type and concentration on larval performance		
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Lead Scientist preparing the Deliverable: Koven, B. (IOLR)

Other Scientists participating: Gisbert, E. (IRTA), Estevez, A. (IRTA), Rosenfeld, H. (IOLR), Meiri-Ashkenazi, I. (IOLR)

Objective: To determine the most effective type and concentration of algae to use in grey mullet larval rearing in terms of growth, survival, prey ingestion and digestive tract enzyme activity.

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1. Introduction

In the commercial rearing of marine fish larvae, the tanks are frequently "greened" with algae such as *Nannochloropsis oculata* or *Isochrysis galbana*. It is widely believed and demonstrated that the provision of these algae to the tanks significantly improves larval performance and has become an inseparable part of commercial rearing protocols in fish farms around the Mediterranean basin (Der Meeren et al., 2007). On the other hand, how algal supplementation contributes to larval growth and survival or if different algal species are equally effective remains unclear. The biochemical composition of algal species (e.g. fatty acids) varies and it is entirely possible that species-specific compounds secreted from the algal cell (e.g. polysaccharides) released during digestion might stimulate the immune system or enhance the digestive process (Hemaiswarya et al., 2011). Having said that, water turbidity from specific algal concentrations may provide optimal backlighting for larvae to facilitate prey identification (e.g. rotifers) and thereby enhance hunting success (Rocha et al., 2008). As algal cell size differs among species, achieving a specific tank turbidity

would require the addition of species-specific concentrations. Recent NCM studies (unpublished data) showed that gilthead sea bream (*Sparus aurata*) larvae consumed significantly (P<0.05) more rotifers when the *Nannochloropsis oculata* algal concentration in the tanks reached $500x10^3$ cells ml^{-1} compared to clear water or a concentration of $1x10^6$ cells ml^{-1}. On the other hand, grey mullet (*Mugil cephalus*) larvae have been reported to perform well when *Isochrysis galbana* was used "to green" the tanks (Harel et al., 1998). The question whether the benefits of algal addition to rearing tanks is due to back lighting and/or compounds secreted by the algae that promote larval growth and survival remains speculative and has not been addressed at all in the larval rearing of the grey mullet. In fact, if algal turbidity is the dominant factor influencing prey hunting success, a less costly method would be to just add potter's clay to the tank simulating the appropriate light scattering and back lighting. This approach was demonstrated to be a viable alternative in halibut larval rearing in Norway (Attramadal et al., 2012).

Consequently, the aims of the present study were to (1) compare the effect of microalgae type (*Nannochloropsis oculata* vs *Isochrysis galbana*) and concentration (cells ml^{-1}) on larval rotifer ingestion rate, biochemical composition, growth, survival and digestive tract enzyme ontogeny. (2) Determine if the benefit of live algal addition to the rearing tank is due to the effect of tank turbidity on efficient prey capture or to other factors.

2. Materials and methods

2.1 Experimental design

Grey mullet eggs (gastrula stage) were stocked in fifteen 1500 l tanks (100 eggs l^{-1}) in an open system where filtered (10 µm), UV-treated, 40 ‰ sea water (25 ^{0}C) entered from the tank bottom at a rate of two tank exchanges day^{-1}. Two turbidity levels (0.76 NTU, 1.20 NTU) and the no-algae control (0.26 NTU) were tested using two algal species (*Nannochloropsis oculata* and *Isochrysis galbana*) on 2-23 dph mullet larvae. Each of the 5 treatments was investigated with three replicate tanks.

The hatching and survival 24 h after hatching values of the stocked grey mullet eggs were calculated by placing a live egg in each of 12 (5 ml) wells in each of three plastic well plates. The plates were covered and placed in a temperature controlled incubator until hatching where the newly hatched viable larvae were counted. The next day, the larvae were recounted to calculate survival at end of 0 dph. After hatching, the salinity was incrementally lowered to 25 ‰ and the flow rate reduced to one tank water exchange day^{-1}. Lighting over the tanks provided 500 lux (14 h light day^{-1}) at the surface. After hatching, 225 µl of fish oil was added to the surface of each of the tanks three times day^{-1} where triangular skimmers simultaneously collected the oil layer. This reduced surface related mortality, which is quite high in mullet larvae due to their minute size (1.5 mm) and large yolk sac, which is frequently characterized by multiple oil globules. Once the yolk sac was depleted, the eyes pigmented and the mouth and anus opened (3 dph), fish oil addition to the water surface was stopped while essential fatty acid (Red Pepper, Bernaqua, Belgium) and taurine (600 mg l^{-1}) enriched preparations were fed to rotifers. These zooplankters were then offered as the main live food to the fish larvae from first feeding to 16 dph. After this age the larvae were co-fed rotifers and enriched *Artemia* until 24 dph (Red Pepper, Bernaqua, Belgium). From 25-57 dph, the fish were offered a 1:1 (w/w) mixture of Caviar (Bernaqua, Belgium) and *Ulva lactuca* produced at IOLR. After 57 dph, fish were fed only Ranaan Dry feed (RDF, Israel) until the end of the experiment at 79 dph (**Table 2**).

2.2 Treatments

All turbidity values were determined on triplicate water samples (including the no-algae control) that were first filtered (40 µm) before being read with a Turbidometer (Lovibond Turbi-check, Amesbury, England). The B turbidity value (1.20 NTU) was based on the current *Nannochloropsis oculata* concentration ($0.4x10^6$ cells ml^{-1}) used at IOLR for larval rearing. Previous studies (unpublished data) demonstrated that levels above this concentration significantly (P<0.05) reduced rotifer consumption in gilthead sea bream larvae.

Prior to the experiment, the necessary concentration of the larger *Isochrysis galbana* giving the B turbidity value of 1.18 was empirically determined. *Isochrysis galbana* has a cell size of ca 5 µm while *Nannochloropsis oculata* is ca 1.5 µm. The A turbidity values were the resulting NTU value when half of the B concentrations were used. The turbidity treatments were added twice daily, during the morning and afternoon. The afternoon turbidity reading was measured to calculate the amount of algae necessary to add to the tanks to compensate for algae washed out as a result of the water exchange rate in the rearing protocol. This algal amount was added in order to reach the designated control, A and B turbidity levels of 0.26, 0.76 and 1.19, respectively (**Table 1**) defined in the experiment.

Table 1. Turbidity levels of the no algal control treatment as well as the *Isochrysis galbana* and *Nannochloropsis oculata* concentrations added to the experimental tanks in order to give two higher turbidity levels (A, B). Turbidity values having different letters were significantly ($P<0.05$) different.

Treatments	Turbidity (NTU)
Control (no algae)	0.26[a]
Isochrysis galbana A ($0.0144x10^6$cells ml^{-1})	0.77[b]
Nannochloropsis oculata A ($0.2x10^6$ cells ml^{-1})	0.75[b]
Isochrysis galbana B ($0.0228x10^6$cells ml^{-1})	1.18[c]
Nannochloropsis oculata B ($0.4x10^6$cells ml^{-1})	1.20[c]

2.3 Rotifer consumption, growth and survival

To determine rotifer consumption, five larvae were sampled from each tank in the experimental system 90 min after feeding and were then fixed in 10% buffered formalin and stored at 4 ^{0}C until counting of the gut mastaxes. The mastax is the indigestible part of the rotifer feeding apparatus that remains in the larval gut for a period of time. They can be counted by quickly washing the fixed larvae in fresh sea water, placing a glass cover slip over them and then gently compressing the gut to see and count the mastaxes (**Fig. 1**). Fish growth was measured as dry weight (DW) at the end (23 dph) of the turbidity trial, while wet weight (WW) and length were measured individually on twenty-five 47 dph fish tank^{-1}. DW was determined by collecting ca 130 larvae, sacrificing them with an excess of MS-222 and then washing them with DDW. The samples were then dried at 70 ^{0}C for 24 h followed by weighing (A&D HD-120 analytical balance, Japan). Length and WW were determined by first lightly anesthetizing the fish in a small amount of MS-222. Length was then measured using a ruler and the fish were then weighed (BH BH-150 high precision balance, Casablanca, Morroco) in a tared Petri plate with sea water before being returned to the tank. Survival was expressed as a percentage of the surviving fish at the end of the trial over the number of eggs stocked (adjusted for hatching rate and survival after 24 h) in the experimental tank system.

2.4 Biochemical analyses
Larval samples for fatty acid body composition were taken at hatching, 15, 18 and 25 dph while fish for digestive tract enzyme specific activity were sampled at hatching, 18, 25, 40, 61 and 79 dph.

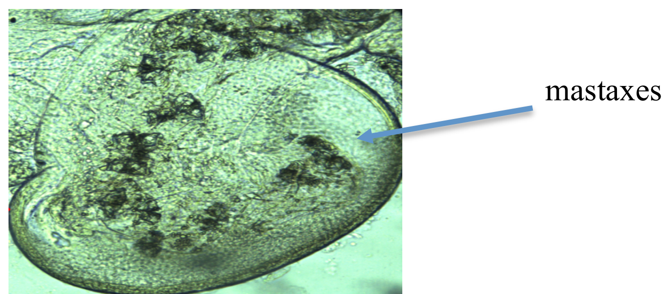

Figure 1. Rotifer mastaxes in the compressed larval gut 90 minutes after feeding.

2.4.1 Fatty acid analysis

For fatty acid analysis, the following procedure was carried out. The total lipid in freeze-dried tissue samples (20 mg) was chloroform-methanol (2:1) extracted (Folch et al., 1957). The lipid-containing chloroform upper phase was removed and evaporated to dryness under a stream of nitrogen and the remaining total lipid weighed (GH-120 analytical balance, A&D, San Jose, CA, USA). This was followed by the addition of the internal standard (17:0; heptadecanoic acid) to each of the samples. The samples were then transmethylated to their fatty acid methyl esters (FAME) by adding 1 ml mg^{-1} lipid of a 14% solution of boron trifluoride methanol (BF3) and sonicated for 1 h at 50 ^{0}C. 0.5 ml of double distilled water (DDW) and 1 ml mg^{-1} lipid of analytical grade hexane (99%), which was vortexed and then centrifuged (2300 rpm) for 2 minutes, were added to the samples. The top hexane layer containing FAME was transferred to a vial and stored at -20 ^{0}C until analysis in a Varian 450-220 GC/MS/MS (Agilent technologies, California, USA). Injected FAME samples (1 µl) were separated on a Varian WCOT fused silica column (50 M x 0.32 mm) at a flow rate of 1.5 ml min^{-1} and identified by known purified standards and quantified using a response factor to the internal standard (17:0; Sigma, St. Louis, MO, USA). The 30 min oven temperature program began at 70 ^{0} C for 4 min following injection and then increased to 300 ^{0}C at 10 ^{0}C min^{-1} for 3 min.

2.4.2 Digestive enzyme activities

Lyophilized larvae were shipped to IRTA-Sant Carles de la Ràpita (Spain) from the IOLR in Eilat, Israel where they were processed for determining the activity of digestive enzymes. Larvae younger than 60 dph were completely homogenized for analytical purposes, since they were too small for dissection, while older fish (60 and 79 dph) were dissected in order to separate the pancreatic and intestinal segments, as described by Cahu and Zambonino-Infante (1994).

Digestive enzyme activities were determined using the following numbers of fish per biological replicate at each sampling age: 350-400 fish at 18 dph, 92-172 fish at 25 dph, 12 fish at 41 dph, 3-6 fish at 61 dph, and 4-6 fish at 79 dph. Dissection was conducted under a dissecting microscope on a pre-chilled glass plate maintained at 0 °C.

For quantifying the activity of the pancreatic enzymes (trypsin, chymotrypsin, total alkaline proteases, α-amylase, and bile salt-activated lipase) and intestinal (alkaline phosphatase and leucine-alanine peptidase) enzymes, lyophilized samples were homogenized (Ultra-Turrax T25 basic, IKA$^{©}$-Werke, Germany) in 5 volumes (v/w) of mannitol (50 mM mannitol, 2 mM Tris-HCl buffer; pH 7.0), centrifuged at 3,300 x g for 3 min at 4 ºC and the supernatant removed for enzyme quantification and kept at -80 ºC until further analysis. After homogenization, 1 ml of the supernatant was pipetted and stored at -20 ºC for cytosolic enzyme (leucine–alanine peptidase) quantification. Then, the rest of the homogenate was used for brush border purification according to Crane et al. (1979).

Enzyme activities for pancreatic and intestinal enzymes were conducted as described in Gisbert et al. (2009). In brief, trypsin (E.C. 3.4.21.4) activity was assayed at 25 ºC using BAPNA (N-α-benzoyl-DL-arginine p-nitroanilide) as substrate. One unit of trypsin ml^{-1} (U) was defined as 1 µmol BAPNA hydrolyzed per min per ml of enzyme extract at 407 nm (Holm et al., 1988). Chymotrypsin (EC. 3.4.21.1) activity was quantified at 25 ºC using BTEE (benzoyl tyrosine ethyl ester) as substrate and its activity (U) corresponded to the µmol BTEE hydrolyzed per min per ml of enzyme extract at 256 nm (Worthington, 1991). Chymotrypsin activity was assayed only for 79 dph group whereas for younger groups the activity was weak (below the detection limit levels of the assay) or absent. Alpha-amylase (E.C. 3.2.1.1) activity was determined according to Métais and Bieth (1968) using 0.3% soluble starch. Amylase activity (U) was defined as the mg of starch hydrolyzed during 3 min per ml of tissue homogenate at 37 ºC at 580 nm. Bile salt-activated lipase (BALT, E.C. 3.1.1) activity was assayed for 30 min at 30 ºC using p-nitrophenyl myristate as substrate. The reaction was stopped with a mixture of acetone: n-heptane (5:2), the extract centrifuged for 2 min at 6,080 x g and at

4 ºC and the absorbance of the supernatant read at 405 nm. Bile salt-activated lipase activity (U ml^{-1}) was defined as the µmol of substrate hydrolyzed per min per ml of enzyme extract (Iijima et al., 1998). Regarding intestinal digestive enzymes, alkaline phosphatase (E.C. 3.1.3.1) was quantified at 37 ºC using 4-nitrophenyl phosphate (PNPP) as substrate. One unit (U) was defined as 1 µg BTEE released per min per ml of brush border homogenate at 407 nm (Bessey et al., 1946). All enzymatic activities were expressed as specific activity defined as units per milligram of protein (mU mg $protein^{-1}$). The assay of the cytosolic peptidase, leucine–alanine peptidase (E.C. 3.4.11) was performed on intestinal homogenates using the method described by Nicholson and Kim (1975), using leucine-alanine as substrate in 50 mM Tris-HCl buffer (pH 8.0). One unit of enzyme activity (U) was defined as 1 nmol of the hydrolyzed substrate per min per ml of tissue homogenate at 37 ºC and at 530 nm. Soluble protein of crude enzyme extracts was quantified by means of the Bradford's method (Bradford, 1976) using bovine serum albumin as standard. All the assays were made in triplicate (methodological replicates) from each pool of larvae (biological replicate) and absorbance read using a spectrophotometer (Tecan™ Infinite M200, Switzerland) and data presented in specific activity units (U mg $protein^{-1}$).

2.5 Statistics

Statistical analyses were carried out using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego California USA, www.graphpad.com). All data are presented as mean ± SEM. Outliers were identified by calculation of the Z value using the Grubbs test (Rousseeuw and Leroy 2003) and removed if calculated Z value was higher than tabulated value. Data values (percentage data were first arcsine transformed) analyzed by one-way ANOVA and Barlett's test for equal variances. If significance ($P<0.05$) was found after ANOVA analysis while Barlett's test was not significant ($P>0.05$), then testing differences between groups was carried out by Newman-Keuls Multiple Comparison test. In cases where ANOVA and Barlett's test were both significant ($P<0.05$), then the non-parametric Kruskal Wallis Test was applied followed by Dunn's multiple Comparison test to determine significant ($P<0.05$) differences among treatments. The effect of water turbidity on the overall functionality of the digestive system (activity of pancreatic and intestinal enzymes) was assessed by means of a Principal Component Analysis (PCA) in fish aged 79 dph using Statistica 7.0 (StatSoft, Inc.). PCA was only conducted in this group of samples, as it was the only age class with a full set of data for all the assayed digestive enzymes.

2.6 Ethics statement

All animal experimental procedures were conducted in compliance with the Guidelines of the European Union Council (86/609/EU) for the use of laboratory animals.

3. Results

3.1 Measured turbidity of the algal treatments.

Turbidity values of the control and the two treatment levels were significantly ($P<0.05$) different from each other each day after the morning algal addition (**Fig. 2**) and shortly before the morning rotifer feeding. The significant ($P<0.05$) differences between the A and B turbidity levels were maintained independently of the different *Nannochloropsis oculata* and *Isochrysis galbana* concentrations added to the tanks in each treatment. On the other hand, there were no turbidity differences among the treatments in the afternoon as a result of algal loss from the water exchange protocol. Nevertheless, the treatment turbidity levels returned to designated values, after calculation, with the afternoon algal addition which was shortly before the afternoon rotifer feeding.

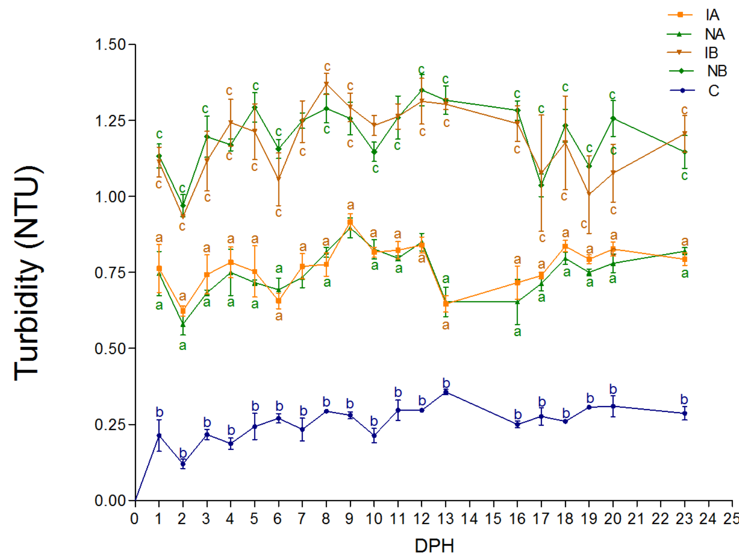

Figure 2. Turbidity levels (NTU) measured after morning algal addition to the tanks. The algal treatments no algae (C), *Isochrysis galbana* low turbidity (IA), *Isochrysis galbana* high turbidity (IB), *Nannochloropsis oculata* low turbidity (NA), *Nannochloropsis oculata* high turbidity (NB) were applied from first feeding at 1 to 23 dph. NTU values having different letters within each day were significantly (P<0.05) different.

3.2 Rotifer consumption, survival and growth as a function of turbidity treatments

Fig. 3a shows a significant (P<0.05) turbidity effect on rotifer consumption in 5 dph larvae that is independent of algal type. The larvae in the IB and NB treatments consumed significantly (P<0.05) more rotifers than the NA and C fish, although this was not significant (P>0.05) in the IA larvae. Interestingly, this pattern of rotifer consumption was similar to that of survival (**Fig. 3b**), where fish exposed to high algal turbidity levels from 2-23 dph survived significantly (P<0.05) better at 51 dph than fish feeding at the lower turbidity values or in clear water. However, despite the turbidity effect on prey consumption and survival, there was no significant (P>0.05) treatment effect on their growth (**Fig. 4**).

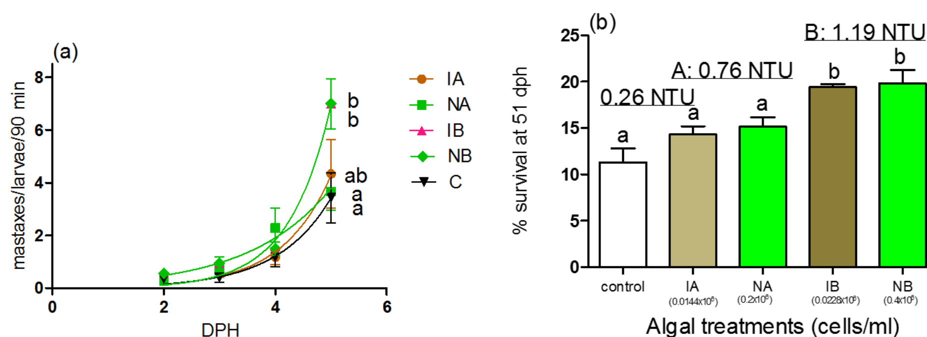

Figure 3. The effect of turbidity treatments; no algae (C), *Isochrysis galbana* low turbidity (IA), *Isochrysis galbana* high turbidity (IB), *Nannochloropsis oculata* low turbidity (NA), *Nannochloropsis oculata* high turbidity (NB) on (a) average rotifer (mastax count) consumption larva^{-1} found 90 min after feeding from 2-5 dph and (b) average percent (%) larval survival at 51 dph. Mastax number at 5 dph and percent survival values of the algal treatments on 51 dph having different letters were significantly (P<0.05) different.

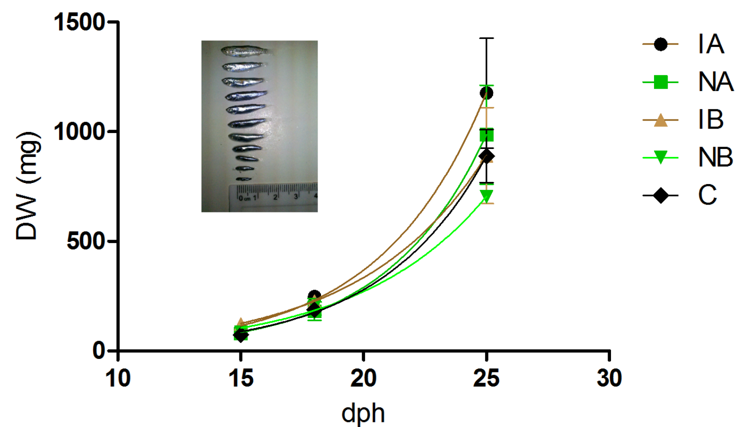

Figure 4. The effect of turbidity treatments; no algae (C), *Isochrysis galbana* low turbidity (IA), *Isochrysis galbana* high turbidity (IB), *Nannochloropsis oculata* low turbidity (NA), *Nannochloropsis oculata* high turbidity (NB) on growth at 15, 18 and 25 dph.

3.3 Fatty acid analyses of essential fatty acids and fatty acid classes of 15, 18 and 25 dph mullet larvae

In 15 dph larvae, there were significant (P<0.05) treatment effects on larval essential fatty acid profiles and fatty acid class content (**Fig. 5**). On the other hand, these results cannot be correlated to the high DHA and low EPA found in *Isochrysis galbana* or the low DHA and high EPA found in *Nannochloropsis oculata*. Moreover, there were no significant (P>0.05) treatment effects on these essential fatty acids or fatty acid classes in all other larval ages sampled (**Figs. 6, 7**).

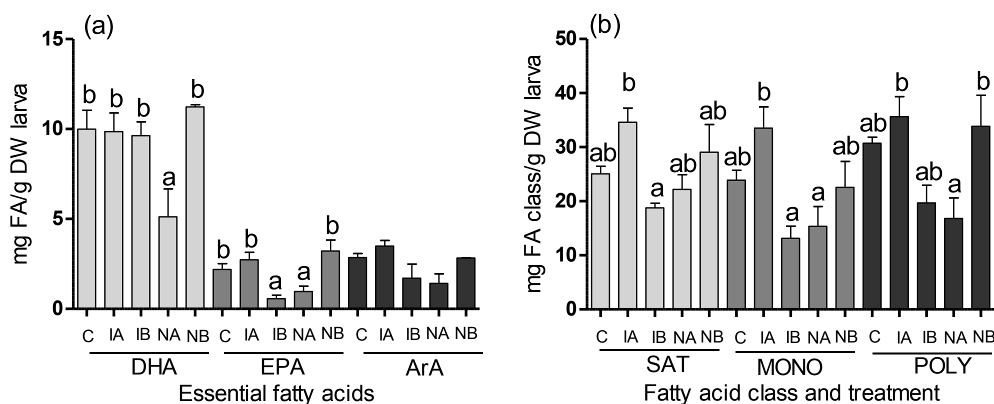

Figure 5. Essential fatty acids and fatty acid classes in 15 dph mullet larvae reared in the different turbidity treatments; no algae (C), *Isochrysis galbana* low turbidity (IA), *Isochrysis galbana* high turbidity (IB), *Nannochloropsis oculata* low turbidity (NA), *Nannochloropsis oculata* high turbidity (NB). Values within an essential fatty acid or fatty acid class having different letters were significantly (P<0.05) different.

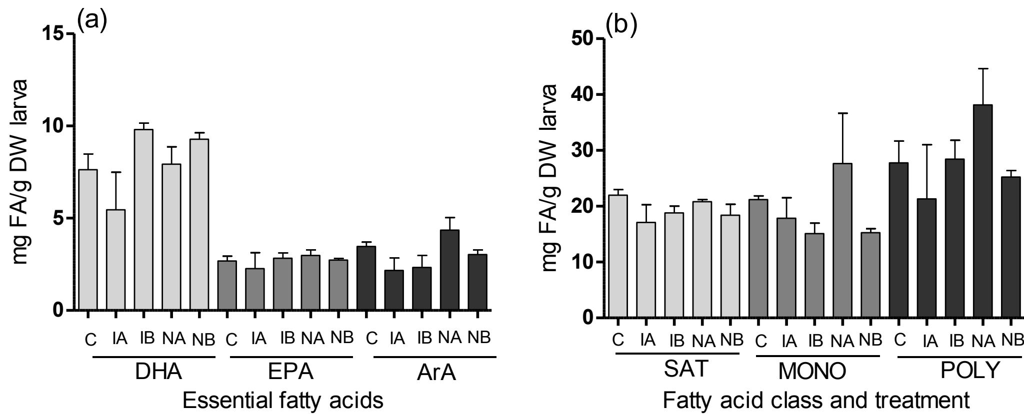

Figure 6. Essential fatty acids and fatty acid classes in 18 dph mullet larvae reared in the different turbidity treatments; no algae (C), *Isochrysis galbana* low turbidity (IA), *Isochrysis galbana* high turbidity (IB), *Nannochloropsis oculata* low turbidity (NA), *Nannochloropsis oculata* high turbidity (NB).

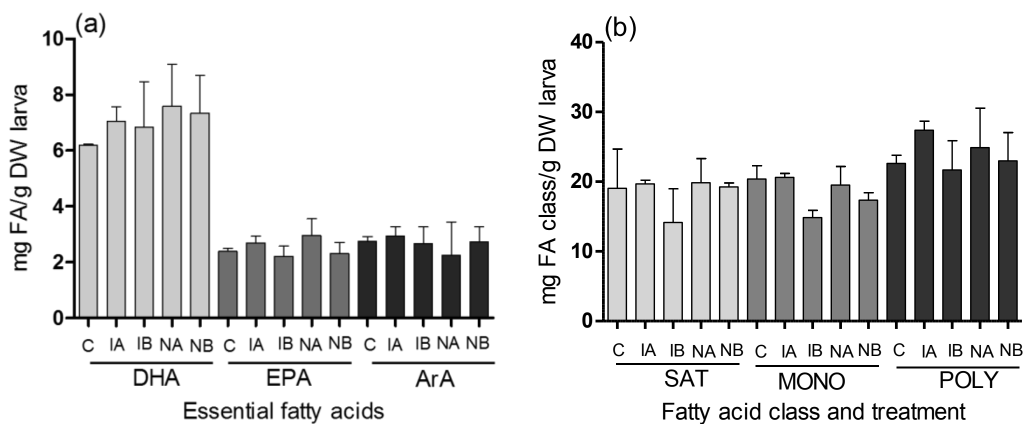

Figure 7. Essential fatty acids and fatty acid classes in 25 dph mullet larvae reared in the different turbidity treatments; no algae (C), *Isochrysis galbana* low turbidity (IA), *Isochrysis galbana* high turbidity (IB), *Nannochloropsis oculata* low turbidity (NA), *Nannochloropsis oculata* high turbidity (NB).

3.4 Digestive tract enzyme specific activity as a function of fish age and diet.

In general, it appears that the turbidity treatments tested on 3 to 23 dph larvae had no lasting effect on the specific activity of digestive tract enzymes when measured between 18 and 79 dph (**Figs 8-17**). Having said that, results from the PCA based on the matrix of co-variations calculated from the specific activity of all the studied (pancreatic and intestinal) enzymes, indicated that the digestive profile of grey mullet juveniles at 79 dph was affected by water turbidity provided during larval rearing (**Fig. 18**). In particular, regardless of the microalgae used, grey mullet larvae reared in low or high turbidity or clear water showed distinctive digestive enzyme profiles (centroid of each group) with the exception of Nanno A and Nanno B samples that showed intermediate values in comparison to the rest.

On the other hand, diet composition during the period of enzyme sampling did affect specific activity in some enzymes. Lipase decreased (**Fig. 8**) from 18 dph, when larvae were feeding on high lipid containing rotifers and *Artemia* (**Table 2**), to the lower lipid level characterizing the co-feeding of Caviar: *Ulva lactaca* (1:1 w/w) from 25-57 dph (**Table 2**). However, after switching at 57 dph to feeding only on the inert diet RDF, the increased lipid of this inert diet (14%) resulted in an increase of the lipase specific activity ($P<0.05$). Similarly, dietary protein decreased from the high levels found in rotifers and *Artemia* (2 to 25 dph) (**Table 2**), which corresponded to high protease activity, to the reduced protein levels of the Caviar: *Ulva lactaca* (1:1) diet fed between 25 and 57 dph with the subsequent decrease in alkaline protease activity (**Fig. 9**). Nevertheless, the activity of this enzyme tended to increase when the fish were ingesting the higher protein of RDF from 58 to 79 dph (**Fig. 9**). On the other hand, trypsin specific activity was not markedly ($P>0.05$) affected by the diet throughout the entire sampling period (**Fig. 10**). The cytosolic enzyme leucine-alanine peptidase significantly ($P<0.05$) decreased in all algal treatments from 18 to 61 dph, but then increased ($P<0.05$) in 79 dph fish when the fish were fed the RDF diet (**Fig. 11**). Importantly, the digestive tract marker for brush border membrane (BBM) development, alkaline phosphatase (AP; **Fig. 12**), demonstrated significantly ($P<0.05$) increasing activity that was on average 9.6 times higher in fish from 61 to 79 dph (**Fig. 12**). Consequently, the AP/leu-ala ratio, an indicator of gut maturation, peaked at 61 dph, but then declined in 79 dph fish from all algal treatments (**Fig. 13**). During the period between 61 to 79 dph, amylase specific activity (**Fig. 14**) increased 2.3 times signaling a more herbivorous/detritivorous mode of feeding, as it has been described in juveniles (Gisbert et al., 1995, 2016). The alkaline protease/lipase ratio values showed higher protease activity over lipase activity at 18 dph when the larvae were feeding on highly digestible rotifer and *Artemia* protein (**Fig. 15**). However, protease activity dropped compared to lipase activity at 25 dph, when the fish began to feed on the Caviar: *Ulva lactaca* diet. On the other hand, the ratio increased significantly ($P<0.05$) from 25 to 61 dph, possibly signaling the fish's increasing ability to breakdown dietary carbohydrates (**Fig. 15**) and exposing more *Ulva lactaca* protein for protease digestion (**Fig. 15**). Moreover, the amylase/trypsin and amylase/protease ratios supported the increasing capability of amylase production with age (**Fig. 16 and 17**).

Table 2. Composition (%) of food used to feed the grey mullet at different stages of development.

Feed	Enriched Rotifers*	Unenriched *Artemia***	Enriched *Artemia****	Caviar (Bernaqua, Belgium) †	*Ulva lactaca* (IOLR, Israel)****	Ranaan Dry feed (RDF, Israel)†
Days fed (dph)	1-23	15	16-24	25-50	25-50	50-79
Protein	48.2	56.2	53.1	55	34	56
Lipid	14.0	17.0	28.7	15	7.4	14
Carbohydrate	18.5	3.6	3.5	8	56	1
Ash	9.3	7.6	2	12	2.6	14.8

*Demir and Diken 2011, **Garcia-Ortega et al. 1998, ***Koca et al 2015, ****analysis at IOLR, †according to manufacturer

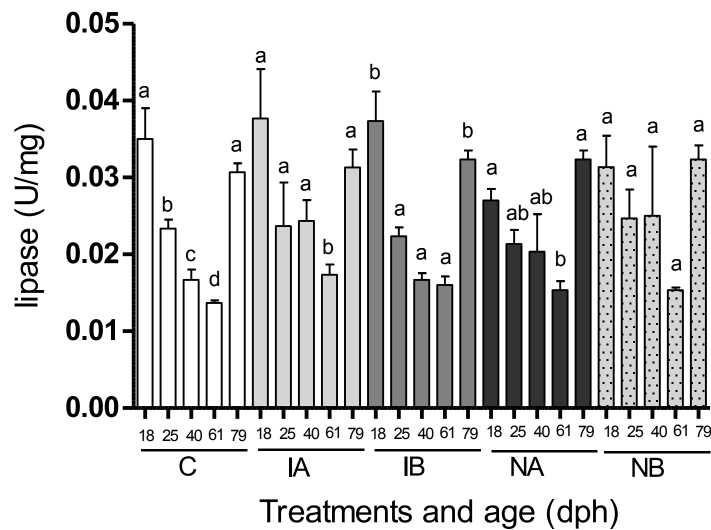

Figure 8. The effect of the turbidity treatments; no algae (C), low turbidity *Isochrysis galbana* (IA), high turbidity *Isochrysis galbana* (IB), low *Nannochloropsis oculata* (NA) and high *Nannochloropsis oculata* that were given to 3-23 dph grey mullet larvae on lipase activity sampled at different fish ages (18, 25, 40, 61, 79 dph). Specific activity values within a treatment having different letters were significantly (P<0.05) different.

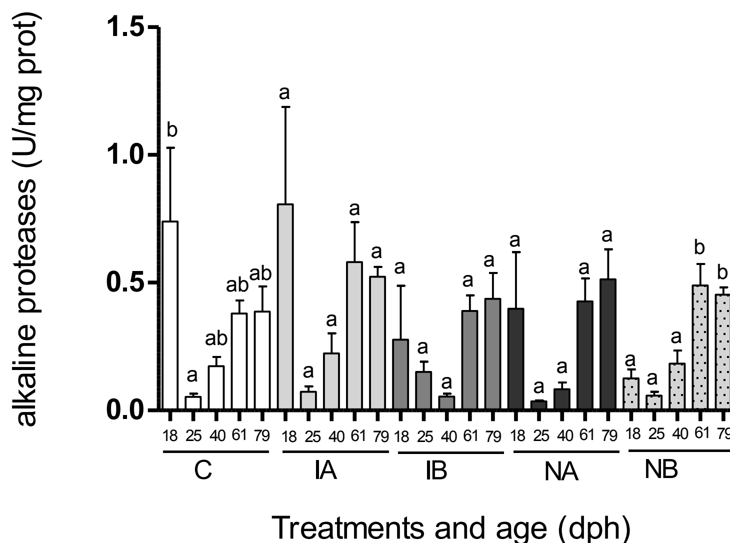

Figure 9. The effect of the turbidity treatments; no algae (C), low turbidity *Isochrysis galbana* (IA), high turbidity *Isochrysis galbana* (IB), low *Nannochloropsis oculata* (NA) and high *Nannochloropsis oculata* (NB) given to 3-23 dph grey mullet larvae on alkaline proteases activity sampled at different fish ages (18, 25, 40, 61, 79 dph). Specific activity values within a treatment having different letters were significantly (P<0.05) different.

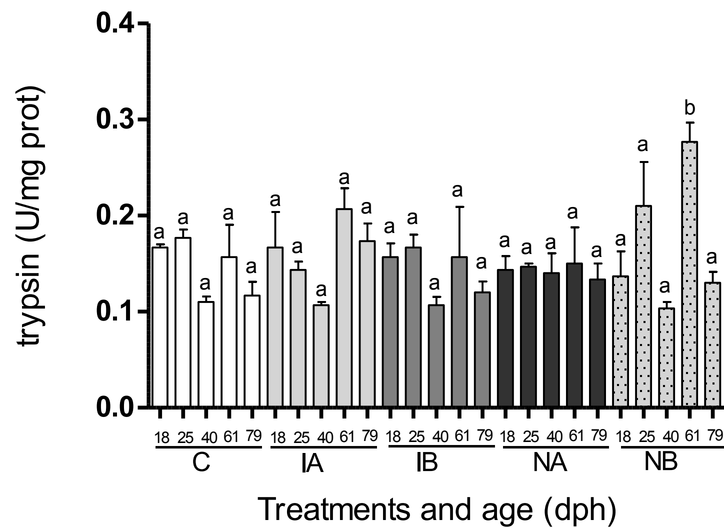

Figure 10. The effect of the turbidity treatments; no algae (C), low turbidity *Isochrysis galbana* (IA), high turbidity *Isochrysis galbana* (IB), low *Nannochloropsis oculata* (NA) and high *Nannochloropsis oculata* (NB) given to 3-23 dph grey mullet larvae on trypsin activity sampled at different fish ages (18, 25, 40, 61, 79 dph). Specific activity values within a treatment having different letters were significantly (P<0.05) different.

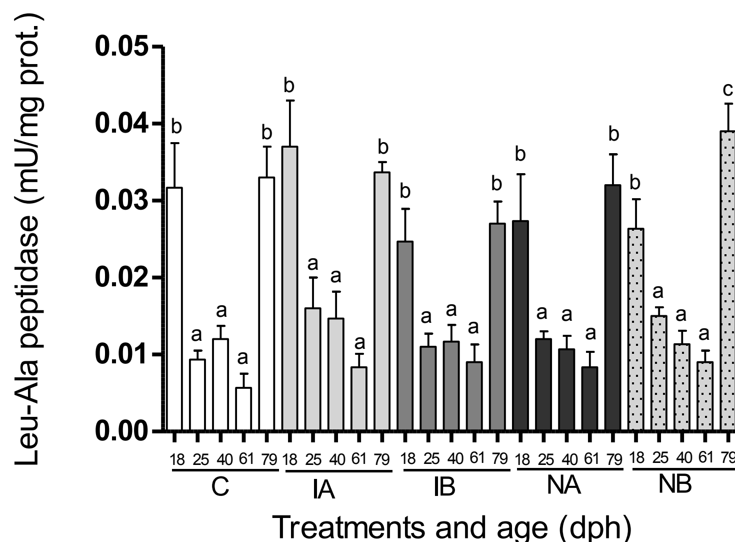

Figure 11. The effect of the turbidity treatments; no algae (C), low turbidity *Isochrysis galbana* (IA), high turbidity *Isochrysis galbana* (IB), low *Nannochloropsis oculata* (NA) and high *Nannochloropsis oculata* (NB) given to 3-23 dph grey mullet larvae on leu-ala peptidase activity sampled at different fish ages (18, 25, 40, 79 dph). Specific activity values within a treatment having different letters were significantly (P<0.05) different.

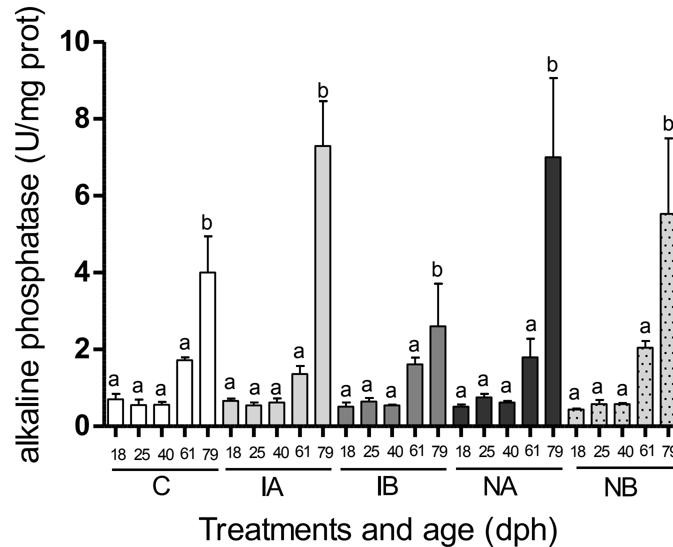

Figure 12. The effect of the turbidity treatments; no algae (C), low turbidity *Isochrysis galbana* (IA), high turbidity *Isochrysis galbana* (IB), low *Nannochloropsis oculata* (NA) and high *Nannochloropsis oculata* (NB) given to 3-23 dph grey mullet larvae on alkaline phosphatase activity sampled at different fish ages (18, 25, 40, 61, 79 dph). Specific activity values within a treatment having different letters were significantly (P<0.05) different.

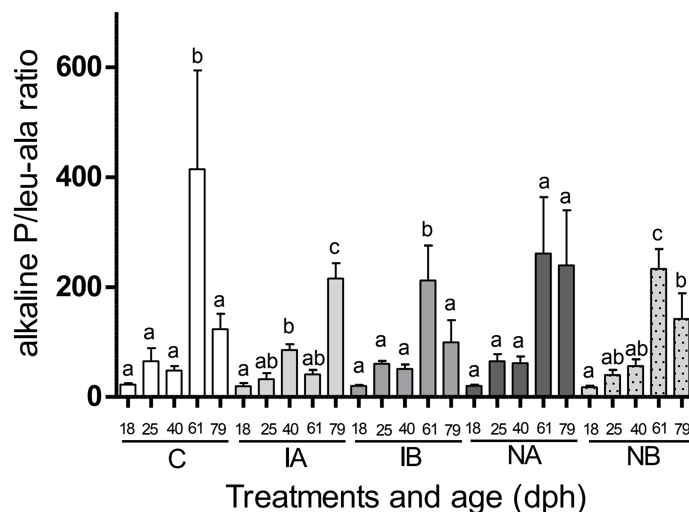

Figure 13. The effect of the turbidity treatments; no algae (C), low turbidity *Isochrysis galbana* (IA), high turbidity *Isochrysis galbana* (IB), low *Nannochloropsis oculata* (NA) and high *Nannochloropsis oculata* (NB) given to 3-23 dph grey mullet larvae on the alkaline phosphatase/leu-ala ratio sampled at different fish ages (18, 25, 40, 61, 79 dph). Specific activity values within a treatment having different letters were significantly (P<0.05) different.

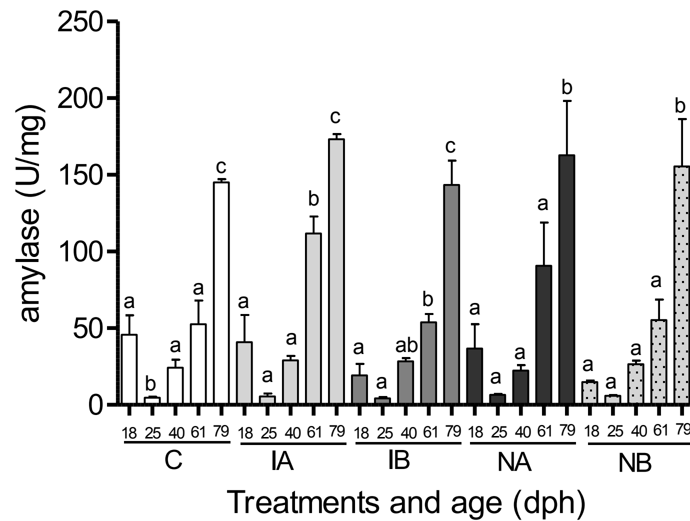

Figure 14. The effect of the turbidity treatments; no algae (C), low turbidity *Isochrysis galbana* (IA), high turbidity *Isochrysis galbana* (IB), low *Nannochloropsis oculata* (NA) and high *Nannochloropsis oculata* (NB) given to 3-23 dph grey mullet larvae on amylase activity sampled at different fish ages (18, 25, 40, 61, 79 dph). Specific activity values within a treatment having different letters were significantly ($P<0.05$) different.

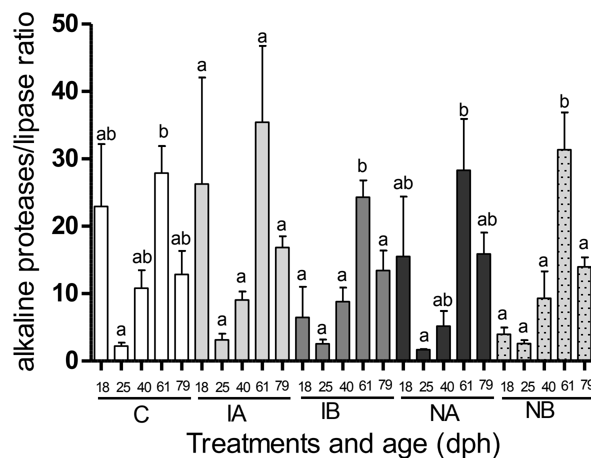

Figure 15. The effect of the turbidity treatments; no algae (C), low turbidity *Isochrysis galbana* (IA), high turbidity *Isochrysis galbana* (IB), low *Nannochloropsis oculata* (NA) and high *Nannochloropsis oculata* (NB) given to 3-25 dph grey mullet larvae on alkaline protease/lipase ratios at different fish ages (18, 25, 40, 61, 79 dph). Ratios within the same treatment having different letters were significantly ($P<0.05$) different.

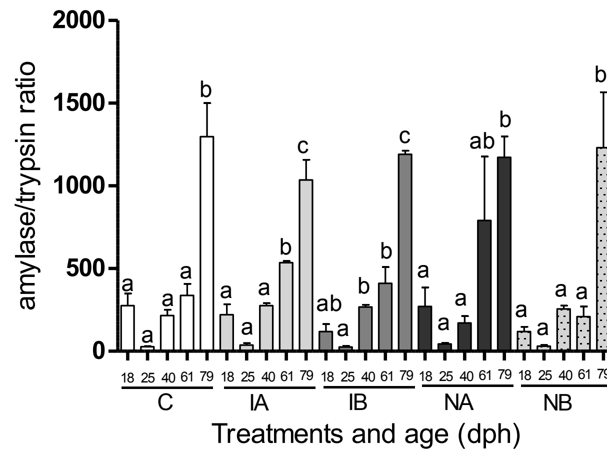

Figure 16. The effect of the turbidity treatments; no algae (C), low turbidity *Isochrysis galbana* (IA), high turbidity *Isochrysis galbana* (IB), low *Nannochloropsis oculata* (NA) and high *Nannochloropsis oculata* (NB) given to 3-25 dph grey mullet larvae on amylase/trypsin ratios at different fish ages (18, 25, 40, 61, 79 dph). Amylase/trypsin ratios within the same treatment having different letters were significantly ($P<0.05$) different.

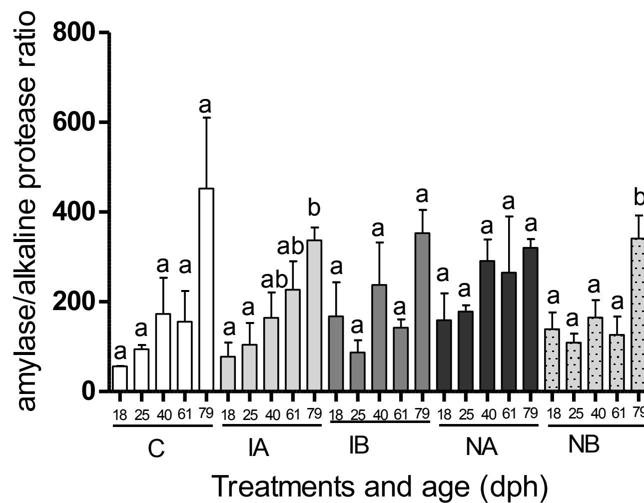

Figure 17. The effect of the turbidity treatments; no algae (C), low turbidity *Isochrysis galbana* (IA), high turbidity *Isochrysis galbana* (IB), low *Nannochloropsis oculata* (NA) and high *Nannochloropsis oculata* (NB) given to 3-25 dph grey mullet larvae on amylase/proteases ratio at different fish ages (18, 25, 40, 61, 79 dph).

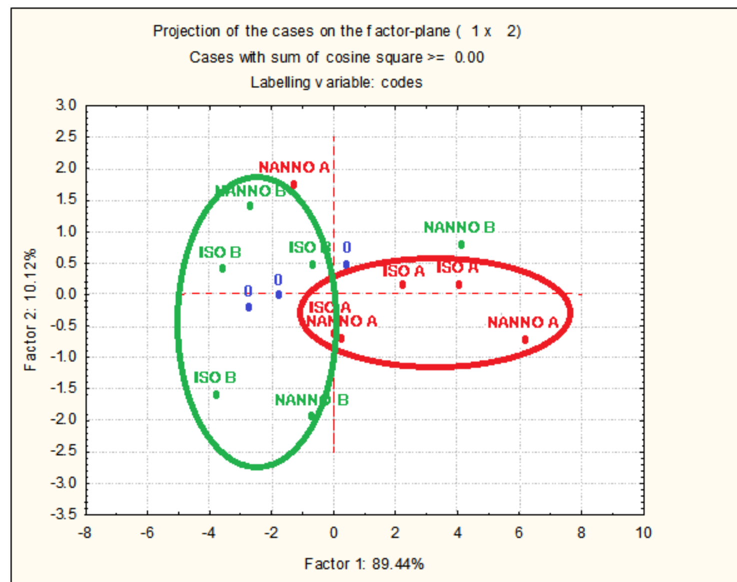

Figure 18. The effect of the turbidity treatments; no algae (0), low turbidity *Isochrysis galbana* (ISO A), high turbidity *Isochrysis galbana* spp. (ISO B), low *Nannochloropsis oculata* (NANNO A) and high *Nannochloropsis oculata* (NANNO B) given to 79 dph grey mullet larvae. PCA is based on the matrix of covariations that, in turn, was calculated on the specific activity of all the studied (pancreatic and intestinal) digestive enzymes in 79 dph specimens.

4. Discussion

The beneficial effects of the presence of microalgae in the rearing tanks for the larvae of many farmed species have long been recognized (Naas et al., 1992; Reitan et al., 1997; Cahu et al., 1998; Lazo et al., 2000; Skiftesvik et al., 2003; Faulk and Holt, 2005). Tamaru et al. (1994) similarly concluded that algal addition was necessary for the rearing of striped mullet larvae. Various hypotheses have been put forth explaining how the algae might benefit the larvae that include (1) providing a direct supply of micronutrients (Van Der Meeren, 1991) that trigger key physiological processes (Hjelmeland et al., 1988), (2) releasing of appetite stimulating components (Stottrup et al., 1995) and (3) influencing the bacterial composition of the rearing water and consequently the larval gut microbial flora (Skjermo and Vadstein, 1993). Apart from these potential advantages, microalgae might be indirectly contributing to larval welfare by affecting the diffusion of light in the water column and thereby, creating a backlighting effect. A number of authors have suggested that this would contrast the zooplankton prey against its background facilitating detection by the larvae, which would contribute to hunting success (Boehlert and Morgan, 1985; van der Meeren, 1991; Utne-Palm, 2002). In the present study, the increased rotifer consumption at the higher turbidity, independent of algal type, suggests that the developing fish could detect and hunt the prey more effectively under these conditions. Moreover, turbidity appears to be a more dominant factor affecting prey consumption than the algal biochemical content. Although the biochemical composition considerably differs between *Nannochloropsis galbana* and *Isochrysis oculata* microalgae, particularly in essential fatty acid content, these phytoplankton species similarly affected prey consumption at the same turbidity level. In addition, the results do not suggest any evidence of algal essential fatty acid or fatty acid class affecting larval fatty acid composition from 18 to 79 dph. In support of this, Cahu et al. (1998) found, at similar *Isochrysis galbana* concentrations used in our study, that algal nutrients, energy and fatty acids had a minor impact on larval European sea bass (*Dicentrarchus labrax*) performance. In contrast to the present study, Harel et al. (1998) found that the "greening" of grey mullet larval rearing tanks with *Isochrysis galbana* contributed more to larval survival than when adding *Nannochloropsis oculata.* On the other hand, these authors used considerably higher and

variable concentrations of *Nannochloropsis oculata* and *Isochrysis galbana*, resulting in generally higher turbidity NTU readings, which possibly contributed to the lower survival (<1%).

Nevertheless, green water may be still contributing to larval welfare in other ways. The significant ($P<0.05$) effect of turbidity level on rotifer consumption in 5 dph larvae was markedly similar to the treatment effect on survival in 51 dph fish. This means that 2-23 dph larvae, which were exposed to the high turbidity treatments, survived significantly better at 51 dph than the lower turbidity and control treatments. This occurred despite the fact that more than three weeks had elapsed since the fish were exposed to the algal treatments and that during this period the fish were all fed an identical diet. It is conceivable that the high algal turbidity larvae were already surviving better than larvae from the no algae control and low turbidity treatments after 23 dph, but this parameter was not measured in our study until 51 dph. The addition of microalgae to the larval rearing tanks has been shown to improve survival in a number of species such as Atlantic halibut (*Hippoglossus hippoglossus*; Naas et al., 1992), Atlantic cod (*Gadus morhua*; van der Meeren et al., 2007), European sea bass (Cahu et al., 1998) and cobia (*Rachycentron canadum*; Faulk and Holt, 2005). The influence of green water on enhanced pancreatic enzyme production and accelerated brush border membrane development and its subsequent enzyme activity improved survival in European sea bass larvae (Cahu et al., 1998; Cahu and Zambonino Infante, 1994). However, the long term turbidity effect on juvenile survival may also be at play here as the benefits from the exposure to suitable levels of “green water” during larval rearing (2-23 dph) may have given a protracted survival advantage over cohorts not exposed to the algal addition, which was still expressed in juvenile fish (51 dph). In our study, we found no difference in enzyme activity among larvae from different treatments at 18 dph. However, this might have been past the point where green water improves pancreatic enzyme activity and BBM development. Cahu et al. (1998) reported that the effect of algal addition (5-20 dph) on larval pancreatic and intestinal digestive function was only observed until 16 dph. Nevertheless, survival in European sea bass at 32 dph was significantly improved. In addition, such differences between both species might be also explained by different rates in maturation of digestive organs, as well as different reproductive guilds between both species (Gisbert et al., 2013).

The composition of the diets the grey mullet were consuming during development appears to have influenced the specific activities of lipase and total alkaline proteases, while there did not appear to be an age or treatment effect on trypsin despite reports claiming that this enzyme may be considered as a growth indicator in fish larvae (Rungruangsak-Torrissen et al., 2006). The level of lipase and total alkaline protease activities increased when ingesting the relatively lipid and protein rich rotifers and *Artemia* (18 dph), while decreased when co-fed the lower lipid and protein levels in the Caviar: *U. lactaca* diet (25-57 dph) and increased again when ingesting the moderate lipid and protein levels of RDF (> 57 dph). Protease activity dropped compared to lipase activity at 25 dph, when the fish commenced to feed on the Caviar: *U. lactaca* diet. The considerable increase in total alkaline protease activity may also be due to the improved ability to breakdown carbohydrates. It should be noted that no acid protease activity was found (data not shown); thus, protein digestion in grey mullet larvae is mainly accomplished by alkaline proteases and not acid proteases as in other marine fish larvae and early juveniles (Gisbert et al., 2013). *Ulva* spp. are a relatively rich source of starch (Korzen et al., 2016) where amylase can hydrolyze the α-1, 4 glycoside bonds in glycogen and starch. This suggests that the increased amylase activity from 25-61 dph, which was likely genetically based, resulted in an increasing ability to digest *Ulva lactaca* carbohydrate and potentially exposing more *Ulva* protein for protease digestion. Similar high amylase activities were found in grey mullet fry weaned onto diets rich in fishmeal or with a high level of fish meal substitution by plant proteins (Gisbert et al., 2016).

In fact, the ontogeny of digestive tract enzyme activity in the grey mullet larvae and juveniles appears to be more a function of age and genetic programming than dietary modulation. A case in point are the two enzymes indicative of enterocyte development; alkaline phosphatase (AP), which is a marker of nutrient absorption (Segner et al. 1995) found in the enterocyte brush border membrane (BBM) and the cytosol based leucine-alanine peptidase (leu-ala), which is involved in protein intracellular digestion in the cytosol of enterocytes. As the BBM develops together with increasing enzyme activity, there is a parallel decrease of intracellular digestion activity, resulting in a rising AP/leu-ala ratio (Ma et al. 2005) until reaching the adult mode of enterocyte digestion. In the carnivorous European sea bass (Cahu and Zambonino Infante, 1994),

Senegalese sole (*Solea senegalensis*, Ribeiro et al., 1999) and red drum (*Scianops ocellatus*, Buchet et al., 1997), the BBM rapidly increases around the 3rd week after hatching, signaling gut maturation at this time. On the other hand, the AP/leu-ala peptidase ratio abruptly increased in the thick-lipped grey mullet (*Chelon labrosus*) at 8 dph and then significantly ($P<0.05$) decreased to 36 dph. This prompted the authors to conclude that gut maturation takes place rapidly. In contrast, the present study on grey mullet showed that AP dramatically increased over 9 fold between 61 to 79 dph while the peak AP/leu-ala peptidase ratio was at 61 dph. Conceivably, this indicated late maturation of the BBM and/or the transition from a carnivorous to an omnivorous/herbivorous mode of feeding. This may be also due to the lack of acid proteases in grey mullet requiring the combination of extracellular (intestinal lumen) and intracellular (enterocyte) digestion in this species for this specific stage of development. The carnivorous-omnivorous transition would be expected to occur in grey mullet juveniles as they swim to less saline estuaries (Oren, 2011). Although the AP/leu-ala ratio peaked at 61 dph, there was a marked drop at 79 dph as leu-ala peptidase levels increased greatly. Zouiten et al. (2008) studying thick-lipped mullet found a similar, but much earlier AP/leu-ala peptidase ratio pattern. The rational explaining the abrupt increase in leu-ala peptidase after maturation remains unclear, although larval rearing conditions may be one of the main factors explaining such differences between both mullet species.

Nevertheless, the suggested late age of the carnivorous-herbivorous shift in grey mullet is supported by the steadily increasing activity of amylase from 25-79 dph (particularly between 61 to 79 dph). Amylase activity is much higher in herbivorous and omnivorous fish compared to carnivores (Hidalgo et al. 1999). Overall, the results suggest that 61-79 grey mullet juveniles, which approximate the size of juvenile mullet moving to estuaries (Gisbert et al., 2016), have the capacity to digest protein and starch allowing for the exploitation of the relatively starch rich microalgae (Zemke-White and Clements, 1999) and macroalgae (Horn et al., 1989), as well as benthic organisms characterizing these lower salinity estuarine waters (Oren, 1981). Moreover, the results broadly suggest that aquaculture feeds at this developmental stage should include high levels of starch or other low cost amylolytic energetic compounds.

In conclusion, the beneficial effect of “green water” in the rearing tanks for larval grey mullet was derived predominantly from the resultant turbidity on prey ingestion rate (within the turbidity levels measured in this study) and less so to the algal type or biochemical content (*i.e.,* fatty acid profile). Nevertheless, ingested algae by the larvae may have stimulated and improved gut maturation in early developing larvae resulting in markedly improved survival during the juvenile stage. The algal treatments given to 2-23 dph larvae did not have a significant effect on older larvae and juveniles in terms of pancreatic and digestive tract enzyme activities. On the other hand, diet composition may have influenced the lipase and total alkaline protease specific activities. Nevertheless, overall the ontogeny and activity of the pancreatic and digestive tract enzymes measured appeared to be genetically programmed. The enzymatic activity of AP and leu-ala peptidase individually, and in ratio indicated gut maturation around 61 dph and an increasing amylase capacity to at least 79 dph. This suggests (1) the capacity to feed on micro- and macroalgae as well as benthic organisms when the fish move to the lower saline and shallower waters of estuaries at this age and (2) when to include significant levels of low cost starch in prepared feeds in order to efficiently grow grey mullet following gut maturation.

Deviations: There were no deviations from the DOW, except the delay in submitting the deliverable, which was due to technical problems with the analyses.

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