New species for EU aquaculture

Deliverable Report

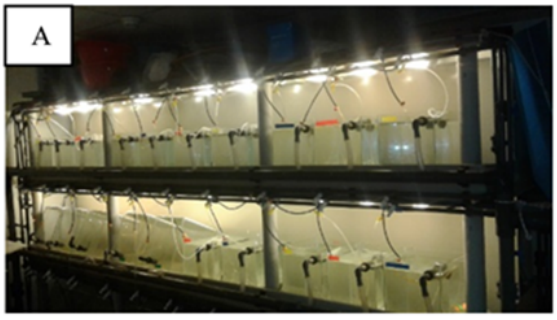
Deliverable No:	D19.4	**Delivery Month:**	56
Deliverable Title	Evaluate the effectiveness of replacing live algae with lyophilized algae during grey mullet larval rearing		
WP No:	19	**WP Lead beneficiary:**	P3
WP Title:	Larval Husbandry – grey mullet		
Task No:	19.2	**Task Lead beneficiary:**	P3
Task Title:	Comparing the selected microalgae type and protocol with lyophilized substitute		
Other beneficiaries:			
Status:	Delivered	**Expected month:**	48
…….			

Lead Scientist preparing the Deliverable: Enric Gisbert (P3. IRTA)

Other Scientists participating: Bill Koven (P4. IOLR), Alicia Estévez (P3. IRTA)

Objective: Comparing the selected microalgae type and protocol with lyophilized substitute

Table of Contents



1.0 Introduction

In the commercial rearing of marine fish larvae, tanks are frequently "greened" with microalgae 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, it remains speculative how algal supplementation contributes to larval growth and survival or if this benefit is species-specific. The biochemical composition of algal species (e.g. fatty acids) varies considerably and it is entirely possible that particular compounds secreted from the algal cell (e.g. polysaccharides) and/or are released during digestion might stimulate the immune system or enhance the digestive process (Hemaiswarya et al., 2011). In addition, water turbidity from specific algal concentrations may provide optimal backlighting for larvae to facilitate live prey identification (e.g. rotifers) and thereby, enhance hunting success (Rocha et al., 2008).

Sub-tasks 19.1.1 and 19.1.2 concluded that a turbidity of 1.2 NTU in the rearing tanks of 2-25 dph grey mullet (*Mugil cephalus*) larvae significantly improved rotifer consumption and survival of grey mullet fry independently of whether *Isochrysis galbana* or *Nannochloropsis oculata* was used in the rearing tanks. This further suggested that rotifer feeding continues to affect fish performance at much later stages of development, indicating that early nutritional condition has a direct impact on later ages. Although these results broadly hinted that the algal produced turbidity was a major factor influencing larval performance, there is an equally compelling argument that there are compounds common to these algal species that are promoting the growth and survival benefit. A follow up study verified that high *N. oculata* turbidity (1.2 NTU) improved larval performance over the lower *N. oculata* turbidity treatment (0.8 NTU). Importantly, larvae in the high *N. oculata* turbidity treatment significantly consumed more rotifers, as well as displaying better growth and survival than larvae exposed to the same turbidity obtained by means of red or white clay. This supports the argument that the live algae biochemical composition provides an advantage over its ability to produce turbidity in the larval rearing of grey mullet. Having said this, turbidity still contributes to larval performance, although to a lesser degree than previously thought in this species.

As microalgae production in hatcheries generally is considered to consume a large amount of resources (trained personnel, physical space and consumables), our group decided to explore potential alternatives to using live microalgae; consequently, we decided to test freeze-dried microalgae that can be easily found in the aquaculture market. Thus, in **Task 19.2** the use of live *N. oculata* and the best turbidity performing protocol (**Task 19.1**) was compared with lyophilized *N. oculata* to determine whether the benefit of this protocol would be conserved when using freeze-dried microalgae.

2.0 Materials and methods

Grey mullet eggs were spawned from F1 broodstock at ARDAG Fish Farms Ltd., Eilat, Israel and stocked in a 6 m^3 V-tank (170 eggs/l). After 3 days, first feeding larvae were transferred and stocked (25 larvae/l) in eight 400 l V-tanks in a flow through system where 40 ‰ ambient, temperature controlled (24-25 ^oC), filtered (10 µm) and UV treated sea water enters at the bottom of the tank and exits at the top through a 150 µm filter. Sea water salinity was incrementally reduced over a few days to 25 ‰ (P4. IOLR grey mullet rearing protocol). This allowed the testing of live or lyophilized *Nannochloropsis oculata* derived turbidity (*ca* 1.22 NTU or concentration of 0.5 x 10^6 cells/ml) on 2-30 dph larval performance in replicates of 4 tanks per treatment. The study was carried out under a photoperiod of 14D/10L with a light intensity of 500 lux. Turbidity was measured (Turbicheck, Lovibond, Amesbury, England) after filtering (40 µm) during the morning and afternoon addition of algae. The larvae were fed rotifers enriched on taurine and the commercial enrichment "Red Pepper" (Bernaqua Ltd., Belgium) and then *Artemia* from 15 to 21 dph, which were enriched with the same protocol. From 22 to 30 dph, the fish were fed a 1:1 (DW) combination of dried ulva and the commercial weaning diet Caviar™ (Bernaqua, Belgium). On 30 dph, larval samples were also collected for pancreatic, brush border and

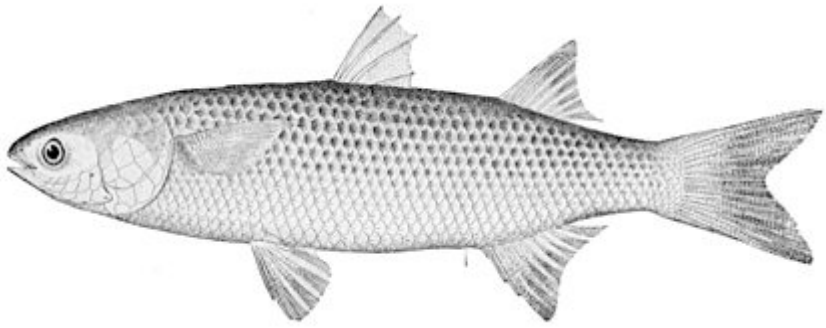
cytosolic enzyme analyses as described in Deliverable 19.1. Larval rearing was conducted at IOLR (Israel), whereas the analysis of the activity of digestive enzymes were conducted at IRTA (Spain).

2.1 Statistics

One way ANOVA, two way ANOVA and regression (linear and non-linear) analyses were carried out using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego California USA, www.graphpad.com). Regression data sets employed Akaike's Information Criteria (AIC) to compare linear, second order polynomial and other models to determine which most likely generated the data. ANOVA analyses and Barlett's test for equal variances were carried out simultaneously. If significance ($P < 0.05$) was found for ANOVA 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. One tailed T-tests were used to compare dry and live algae values in dry weight, survival and enzymes alkaline phosphatase and leucine alanine peptidase. All data are presented as mean ± standard error of the mean (SEM).

2.2 Ethics statement

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



3.0 Results and Discussion

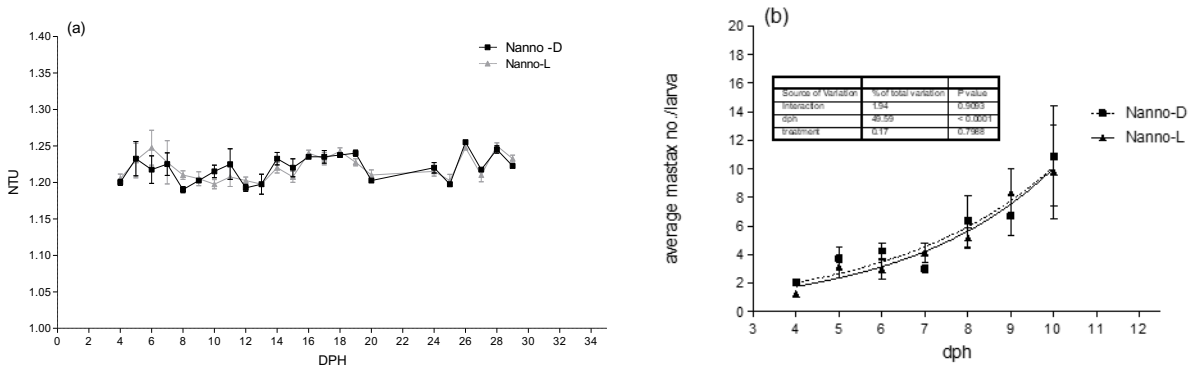


Figure 1 (a) Average NTU levels measured in the experimental tanks demonstrating that the lyophilized and live algae (Nanno-D, Nanno-L, respectively) provided very similar turbidity values from 4-30 dph. (b) The effect of lyophilized and live algae (Nanno-D, Nanno-L, respectively) turbidity on rotifer ingestion (mastax no.) in 4-10 dph mullet larvae. Regression and AIC analysis showed that the probability that the lyophilized (Nanno-D) and live (Nanno-L) *N. oculata* values were generated by an exponential growth equation were 75.5 and 67.1%, respectively. The curves were not significantly (P > 0.05) different from each other. The source of variation was a result of age (dph) and not algal treatment.

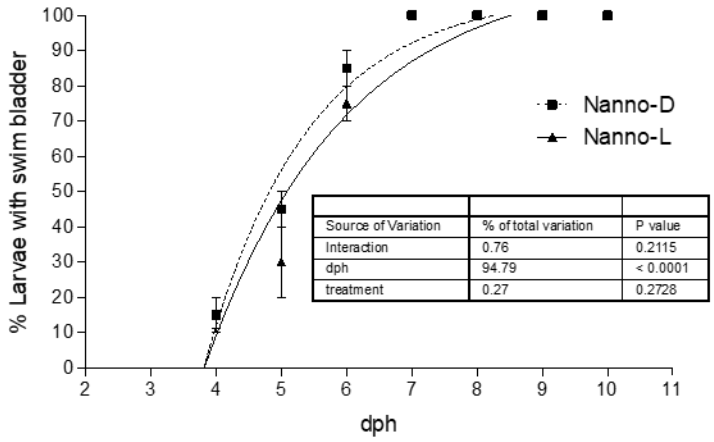


Figure 2 The effect of lyophilized and live algae (Nanno-D, Nanno-L, respectively) used for enhancing water turbidity on the percentage (%) of larvae with inflated swim bladder (n = 4). Regression and AIC analysis showed that the probability that the lyophilized (Nanno-D) and live (Nanno-L) *N. oculata* values were generated by a one phase association equation was 99.9% for both curves. The curves were not significantly (P > 0.05) different from each other. The source of variation was a result of age (dph) and not algal treatment.

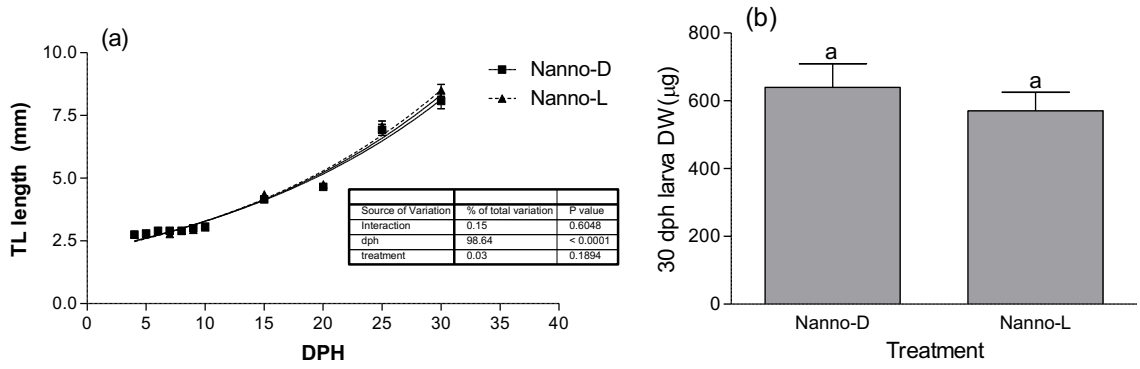


Figure 3 The effect of lyophilized and live algae (Nanno-D, Nanno-L, respectively) used for enhancing water turbidity on larval growth. (a) Changes in larval length between 3 and 30 dph (n = 4). Regression and AIC analysis showed that the probability that the lyophilized (Nanno-D) and live (Nanno-L) *N. oculata* values were generated by an exponential growth equation was 99.9% for both curves. The curves were not significantly ($P > 0.05$) different from each other. The source of variation was a result of age (dph) and not algal treatment. (b) Dry weight (DW) values in 30 dph larvae. One-way ANOVA for DW values was not significant ($P > 0.05$).

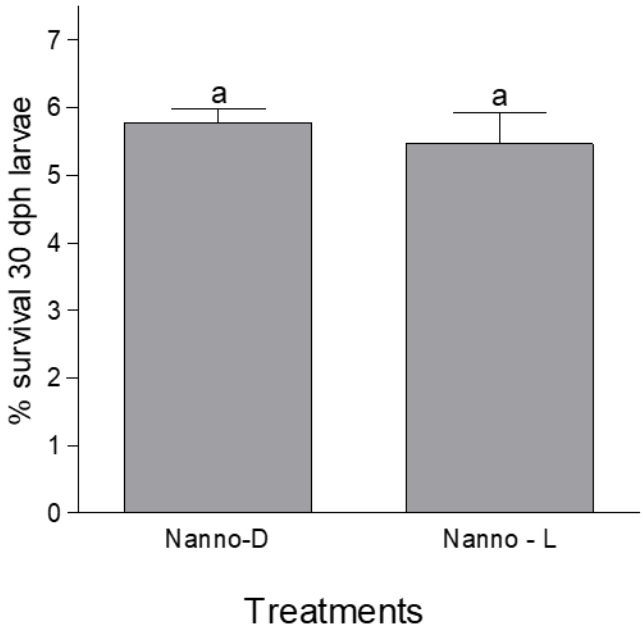


Figure 4 The effect of lyophilized and live algae (Nanno-D, Nanno-L, respectively) used for enhancing water turbidity on on the percent survival (%) in grey mullet fry at 30 dph (n = 4). One way ANOVA of survival values (after arsine transformation) were not significant ($P>0.05$).

Figure 1a demonstrated that the lyophilized (Nanno-D) and live (Nanno-L) algae treatments provided almost identical daily turbidity values during the 3-30 dph experiment. This resulted in very similar larval performances in the two treatments in terms of rotifer ingestion rate (**Figure 1b)**, swim bladder inflation (**Figure 2**), growth (TL and DW) (**Figure 3a,b**) and survival (**Figure 4**). In support of this, Navarro and Sarasquete (1998) found no differences between the use of live and freeze-dried *N. oculata* during the first 15

days of larval rearing in gilthead sea bream (*Sparus aurata*). Similarly, Cañavate and Fernández-Díaz, (2001) concluded that the positive aspects of using algae in marine fish larviculture were not affected if *Nannochloropsis gaditana* was live or freeze dried.

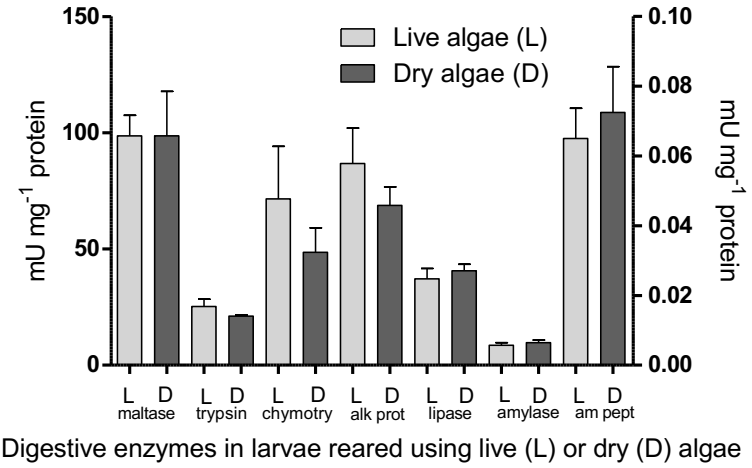


Figure 5 The effect of using live or dry algae (*Nannochloropsis oculata*) on the specific activity of pancreatic (trypsin, chymotrypsin, total alkaline proteases, bile-salt activated lipase and α-amylase) and brush border (maltase and aminopeptidase N) enzymes in grey mullet fry. Aminopeptidase N is read from right Y axis.

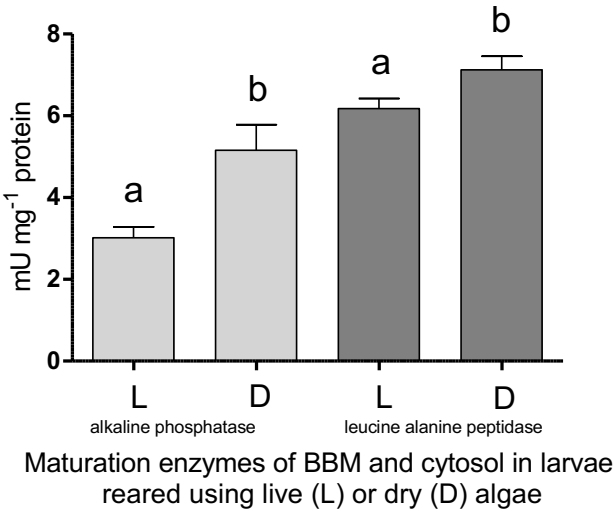


Figure 6 The effect of using live or dry algae (*Nannochloropsis oculata*) on the specific activity of the brush border alkaline phosphatase and the cytosolic leucine alanine peptidase intestinal enzymes in grey mullet fry. L and D values of an enzyme having different letters were significantly ($P<0.05$) different.

In this study, digestive enzyme activities were very similar regardless of the use of lyophilized or live *Nannochloropsis oculata* algae treatments for enhancing water turbidity (**Fig. 5**). On the other hand, the dry or lyophilized microalgae markedly ($P < 0.05$) affected the activity of alkaline phosphatase and leucine alanine

peptidase (**Fig. 6**). In particular, the use of freeze-dried microalgae enhanced the maturation of the intestine in grey mullet fry as the ratio of alkaline phosphatase (AP) / leucine alanine peptidase (LAP) indicated (**Fig. 7**; $P < 0.05$).

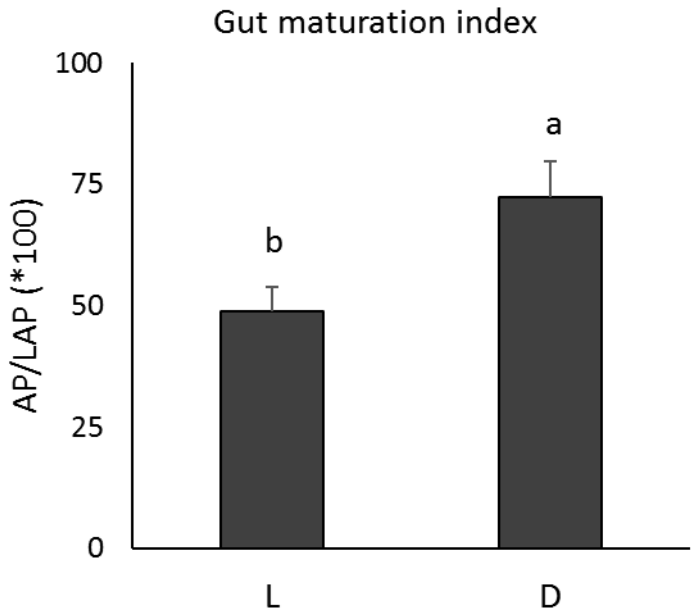


Figure 6 Intestinal maturation index, defined as the ratio between alkaline phosphatase (AP) and leucine alanine peptidase (LAP) reared using live or dry microalgae (*Nannochloropsis oculata*) in order to enhance water turbidity in rearing tanks. Different letters denote statistically significant ($P<0.05$) differences between groups.

These results indicate a higher gut maturation level in fry reared in tanks where freeze-dried microalgae were used to enhance water turbidity, which was consistent with the findings in older fish of other deliverables linked to this topic (**D19.1**, Determine most effective type and concentration of algae used in grey mullet larval rearing). According to Zambonino-Infante and Cahu (2001), the normal maturation of the enterocytes in developing fish larvae, and also in other species including mammals, is characterized by a decrease in the activity of LAP and a progressive increase in AP activity, which represents the establishment of an efficient brush border membrane digestion that is indicative of the adult mode of digestion by enterocytes. In the present study, freezing the *N. oculata* cells before lyophilization, particularly if it was not a rapid process, can cause large ice crystals and rupturing of the cell walls. This may have allowed more microalgae cell content to be available to the larvae once ingested through drinking or these compounds may have accumulated in the rotifers. On the other hand, the live, intact *N. oculata* cells may have passed through larvae with only partial or no release of their contents, as they have limited capacity to digest their complex cellular walls.

There are many compounds in phytoplankton that could modulate digestive enzyme activity in fish larvae. Algal polyamines such as spermidine and spermine, that has been associated with gut maturation in birds and mammals (Sousadias and Smith, 1995; Peulen et al., 1998) as well as fish larvae (Peres et al. 1997; Tovar-Ramirez et al., 2004), may have been more available from the lyophilized microalgae. However, the more rapid gut maturation of larvae exposed to lyophilized *N. oculata* did not result in improved fish performance. This may have been due to the relatively early age (30 dph) that larvae were sampled in this study. In D19.1, we demonstrated that gut maturation in grey mullet fry peaks at 61 dph and that both BBM extracellular and

cytosol intracellular are being utilized. This suggests that older than 30 dph juveniles may have expressed a growth or survival advantage if exposed earlier to lyophilized microalgae.

Taken together, the results of this study clearly show that lyophilized *N. oculata algae* can be used to replace live algae which would be a significant saving in time, labour and infrastructure and may have expressed a growth advantage in older fish. Consequently, the results of the present study recommend the use of lyophilized *Nannochloropsis oculata* in the larva rearing of grey mullet. Nevertheless, more work is needed to determine the algal compounds that contribute to gut maturation and provide an improvement to grey mullet larvae and juvenile performance.

4.0 References

Cañavate, J.P., Fernández-Díaz, C., 2001. Pilot evaluation of freeze-dried microalgae in the mass rearing of gilthead seabream (Sparus aurata) larvae. Aquaculture 193, 257–269.

Hemaiswarya S., Raja R., Kumar R.R., Ganesan V., Anbazhagan C. 2011. Microalgae: a sustainable feed source for aquaculture. World Journal of Microbiology and Biotechnology 27, 1737–1746.

Navarro, N., Sarasquete, M.C., 1998. Use of freeze-dried microalgae for rearing gilthead seabream, Sparus aurata, larvae: Part I. Growth, histology and water quality. Aquaculture 167, 179–193.

Pères, A., Cahu, C.L., Zambonino Infante, J.L., 1997. Dietary spermine supplementation induces intestinal maturation in sea bass (Dicentrarchus labrax) larvae. Fish Physiology and Biochemistry 16, 479–485.

Peulen, O., Pirlet, C., Klimek, M., Goffinet, G., Dandrifosse, G.1998. Comparison Between the Natural Postnatal Maturation and the Spermine-Induced Maturation of the Rat Intestine, Archives of Physiology and Biochemistry, 106:1, 46-55

Rocha, R.J., Ribeiro, L., Costa, R., Dinis, M.T., 2008. Does the presence of microalgae influence fish larvae prey capture? Aquaculture Research 39, 362–369.

Sousadias, M.G., Smith, T.K., 1995. Toxicity and growth-promoting potential of spermine when fed to chicks. Journal of animal science 73, 2375–2381.

van der Meeren, T., Mangor-Jensen, A., Pickova, J., 2007. The effect of green water and light intensity on survival, growth and lipid composition in Atlantic cod (*Gadus morhua*) during intensive larval rearing. Aquaculture 265, 206–217.

Zambonino-Infante, J.L., Cahu, C., 2001. Ontogeny of the gastrointestinal tract of marine fish larvae. Comparative Biochemistry and Physiology 130C, 477-487

Deviation from DOW

This deliverable was completed on month 55 and not on month 48 due to technical problems in the performing of the experiment and analyses.

Co-funded by the Seventh Framework Programme of the European Union