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An IMTA in Greece: Co-Culture of Fish, Bivalves, and Holothurians

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Abstract: Integrated multitrophic aquaculture (IMTA) is an innovative mariculture methodology that reduces the environmental footprint and increases the profitability of the farm. It combines the cultivation of species belonging to different trophic levels, simulating a natural food web. In this study, five Mediterranean species were co-cultured in three operating fish farms in the Aegean (E. Mediterranean) Sea with different trophic conditions. The co-cultivated species were sea bream (*Sparus aurata*), European sea bass (*Dicentrarchus labrax*), Mediterranean mussel (*Mytilus galloprovincialis*), rayed pearl oyster (*Pinctada imbricata radiata*), and sea cucumber (*Holothuria polii*). Bream, bass, and mussels were cultivated according to the traditional on-growing methods (fish cages and longlines), whereas the pearl oysters and sea cucumbers were cultivated in baskets designed specifically for oyster farms. To estimate the growth of the co-cultivated species, growth indicators were calculated using length and weight measurements. Furthermore, the growth measurements from co-cultivated species were compared to the respective ones from natural populations. All the species showed high survival rates in the integrated multitrophic aquaculture (IMTA) conditions. Pearl oysters and Mediterranean mussels had positive growth in fish farms with high concentrations of nutrients. Mussel condition index (CI) was 42% in Aquaculture 1 (Aq1) and 33% in Aquaculture 2 (Aq2), compared to 35% in a typical Mediterranean mussel farm. Pearl oysters CI in Aq1 was 53%, in Aq2 56%, in Aquaculture 3 (Aq3) 19%, and in natural populations ranging from 30% to 45%. In contrast, holothurians did not gain weight under the fish cage regime despite the high survival rate. Their final total weight was 17.3 g in Ag1, 8.3 g in Aq2, and 18.3 g in Aq3, but in the natural population, the mean weight was 80 g.

Keywords: IMTA; finfish; sustainability; rayed pearl oysters; mussels; holothurians; Mediterranean species

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1. Introduction

During the past 50 years, mariculture has become one of the most productive industries worldwide, a fact that shows its importance for seafood supply as well as the dynamic profile of this industry and its ability to exploit resources and space. The organic enrichment of the environment near fish cages is related to the expansion of fish farming [1]. The most known effect of typical aquaculture is the releasing of dissolved nutrients and particulate organic matter in the water column as well as sediment enrichment, causing biological and geochemical changes [2,3].

One measure for mitigating the environmental impact of fish farming is the concept of IMTA. IMTA has been suggested as an innovative method of aquaculture development [4,5] that ensures sustainable development in agreement with the EU directions for Blue Growth and Blue Economy. It is defined as the cultivation of two or more aquatic species from different trophic levels in the same area in order to mimic the energy flow in natural ecosystems [6]. As a result, IMTA can maximize the productivity and cost-effectiveness of marine aquaculture through the exploitation of soluble and insoluble substances that have so far been lost in the framework of conventional monoculture [7–9]. The selection of co-cultivated species targets the greatest possible representation of natural ecosystem processes by balancing the chemical and biological interactions of cultivated organisms and the environment [10]. Species that can grow in multitrophic aquaculture depend largely on the environmental characteristics of the farming site, but the most common practice includes fish farming as a central crop, organisms that absorb dissolved inorganic nutrients (e.g., seaweeds), filter-feeding species exploiting suspended organic matter (e.g., bivalves), and deposit feeders, i.e., species using settled organic material (e.g., echinoderms) [4,11].

IMTA originated in China and has since been taken up in North and South America, Africa, and Europe [12]. IMTA farming has been tested mostly in selected fish farms with specific physicochemical water characteristics and trophic conditions [10]. European studies have demonstrated the efficiency of IMTA mostly in experimental conditions [13] and lately in open-water systems [14], but these trials had little further development has taken place.

IMTA organisms include bivalves and echinoderms that consume phytoplankton and particulate organic matter. In this study, the Mediterranean mussel (*Mytilus galloprovincialis*) and rayed pearl oyster (*Pictada imbricata radiata)* were co-cultured with fish. As a consumer of the settling particulate matter, the sea cucumber *Holothuria pollii* was used. Holothurians are deposit feeders and can recycle the nutrients in the organic material settled on marine sediments. Studies of sea cucumbers feeding on particle wastes deposited by fish cages have shown a reduction in total organic load [13]. As such, they may play an important role in the sustainable development of fish farming in the Mediterranean [13].

The bivalve *P. imbricata radiata* is an invasive but well-established species in the Mediterranean that lives mainly on hard and sandy substrates [15,16]. Soon after the settlement of natural populations, the pearl oyster became part of the diet of the locals, providing high-quality seafood [15,17,18].

Mediterranean mussels and pearl oysters are filter-feeding organisms that feed on phytoplankton and selected particles retained after filtering the water column [19]. Both have been shown to help reduce the organic load in the water without significantly burdening the benthic ecosystem with by-products of their metabolism [20,21]. As such, these organisms seem suitable for IMTA, and there have been trials to co-cultivate them with fish species [22].

The primary objective of this study was to develop an IMTA methodology suitable for use in the oligotrophic Eastern Mediterranean. The results can be used as a basis for setting up large-scale commercial IMTA systems in Greece. This was a pilot study designed to examine the survival, robustness, and growth of the species *Mytilus galloprovincialis*, *Pinctada imbricata radiata*, and *Holothuria polii* in IMTA systems compared with their growth in wild populations. The experimental IMTA systems were used in three Greek fish farms with different trophic statuses. This study is part of a wider study aiming to investigate the potential for using IMTA as a means of reducing the environmental impact of mariculture while increasing the revenues of mariculture farms.

2. Materials and Methods

The IMTA trials were implemented in three different fish farms in the Aegean, Mediterranean Sea. These fish farms were chosen because of their different eutrophic status and physical/chemical conditions. In all three fish farms, sea bream and sea bass are intensively farmed in cages and fed with commercial fish feeds (pellets). The same standard cultivation methodology was used on all farms.

2.1. Study Area

The first aquaculture farm (Aq1) is located in the wider area of Epidaurus in Saronikos Gulf (Figure 1). It includes three (3) cage sets in an area of 1×10^4 m^2 at a distance of

100 to 400 m from the nearest shore and at a depth of 45 m. The most frequent direction of the current in the area is from north to south along the coast. This farm has an annual production of 250 tons yr^{-1}, growing commercial Mediterranean fish species, i.e., gilthead sea bream (*Sparus aurata)* and European sea bass (*Dicentrarchus labrax*).

Figure 1. Locations of the three farms that are part of the IMTA trials. Aq1 is located in the area of Epidaurus in Saronikos Gulf, Aq2) is located in the Evoikos Gulf, and Aq3 is in the west of Rhodes.

The second aquaculture farm (Aq2) is located in Evoikos Gulf (Figure 1). It includes four sets of cages with a total area of 8×10^4 m^2 at a distance of 50 to 300 m from the nearest shore and at a maximum depth of 42 m. The direction of the current in the area changes periodically due to tidal effects. The farm has an annual production of 1600 tons of sea bream and sea bass.

The third farm (Aq3) is located on the west coast of Rhodes (Figure 1). It includes one set of cages in a farming area of 1×10^4 m^2 at an average distance of 100 m from the nearest shore and at a depth of 34 m. The most frequent direction of the current in the area is from north to south. This farm produces 300 tons of bream and bass annually.

2.2. Water Characterization

In every location, water samples were collected from three different depths (1 m, 3 m, 5 m) in the center of the fish farm near IMTA cages using Niskin bottles. Subsamples were collected for inorganic nutrient analysis and chlorophyll a (Chla) and stored in bottles for transportation. Dissolved phosphate (PO_4^{3-}), nitrite (NO_2^-), and nitrate (NO_3^-) concentrations were measured according to Strickland and Parsons [23]. Ammonium (NH_4^+) concentrations were measured after the method of Ivancic and Degobbis [24]. In order to measure Chl*a* concentration in the water column, 1 L of sample water was filtered through polycarbonate filters (2 μm and 0.2 μm) and stored in aluminum foil. Samples for the determination of Chl*a* concentration were analyzed according to Yentsch and Menzel (1963) [25], using a Turner fluorometer (model 112) following extraction with 90% acetone. Using the nutrient and Chla concentrations, the eutrophic index (EI) was calculated as described in Primpas et al. (2010):

$$EI : 0.279C_{PO_4} + 0.261C_{NO_3} + 0.296C_{NO_2} + 0.275C_{NH_3} + 0.214C_{Chla} \quad (1)$$

where C are the concentrations of each nutrient [26]. EI classifies the seawater body into five ecological status levels: (a) less than 0.04 (High), (b) 0.04–0.38 (Good), (c) 0.38–0.85 (Moderate), (d) 0.85–1.51 (Poor), and (e) more than 1.51 (Bad) [27]. Characterization of eutrophic status in the three fish farms was based on Chla concentration [28].

2.3. Cultivation of Mediterranean Mussels

For IMTA mussel cultivation, the traditional technique was used: The mussels were grown in long narrow nets placed in rows (longline culture) (Figure 2c). Initially, mussel seed (spat) was collected by hand either from ropes hanging near cages (spat-collectors) or from already existing anchoring ropes in the fish farm area. The mussel seeds were then put in elongated plastic cylindrical tubing nets (pergolaris) of 6 m in length and a net eye of 80 mm. The pergolaris were made using polyvinylchloride cylindrical tubes with a diameter ranging from 4 to 7 cm, which were then submerged around the middle cage in the center of each fish farm. The cultivation period was nine months, which is when they reached marketable size (5–6 cm) [29–31]. The longlines were placed on the ropes that tie the fish cages with the buoys at a distance of 2.6 m from the cages and at a distance of 80 cm from each other in each fish farm. In Aq1 farm, mussel seeds with a mean weight of 6.5 ± 4.6 g and length of 3.5 ± 1.0 cm were put in pergolaris with a density of 1307 ind/net. In Aq2, mussels with a total mean weight of 0.5 ± 0.4 g and length of 1.7 ± 0.5 cm had 3400 ind/net density. Mediterranean mussels were not placed in Aq3 as they were not locally available. After four months, it was necessary to harvest the mussels from the longlines and space them out in more nets to facilitate their growth. Mussel cultivation lasted from October (2020) until June (2021). To estimate the growth of the mussels, 30 individuals were randomly selected from different parts of the longline (10 mussels/2 m on pergolaris) and were measured in October (2020), February (2021), May (2021), and June (2021). Their total weight, as well as shell and flesh, were weighed separately using a two-decimal balance. After the shell of each bivalve was cleaned from any attached biofouling material before measuring, then the measurements were used to calculate the CI of the Mediterranean mussel [19,32]. Mussel morphometrics were also measured; more specifically, their shell length, width, and thickness. With these data, the weight to length ratio (W/L) was calculated.

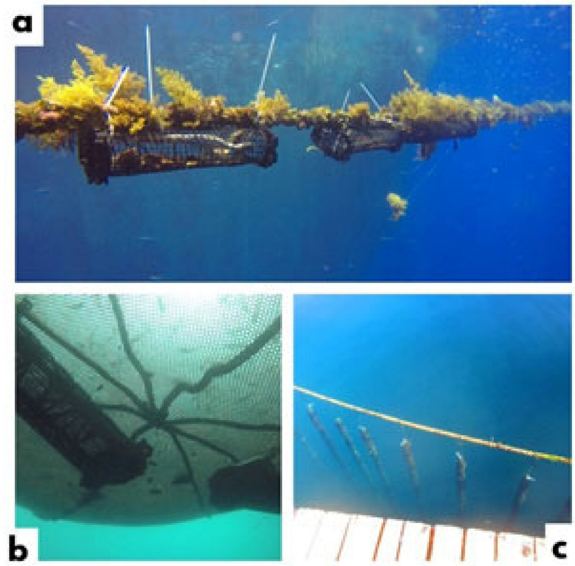

Figure 2. IMTA: Co-culture of sea bream, European sea bass, Mediterranean mussel, pearl oyster, and sea cucumber: (**a**) figure of pearl oysters cultivated in SEAPA© baskets, submerged next to the fish cages at 3 m depth; (**b**) figure of SEAPA baskets with sea cucumbers submerged under a fish cage; (**c**) figure of mussels in elongated plastic cylindrical tubing nets (pergolaris), a traditional mussel cultivating method.

The specific growth rate was calculated for all cultivated species as follows:

$$\mathrm{SGR} = 100 \times \frac{\ln(\mathrm{W_f}) - \ln(\mathrm{W_i})}{\mathrm{t}} \quad (2)$$

where W_f and W_i are the final and initial wet weight of Mediterranean mussels in each station (g), respectively, and t = the duration of cultivation (days) [14,33].

The CI and meat yield (MY) were calculated for Mediterranean mussels as follows:

$$CI = \left(\frac{W_F}{W_s}\right) \times 100\% \quad (3)$$

$$MY = \left(\frac{W_F}{W_t}\right) \times 100\% \quad (4)$$

where W_F and W_S are the flesh weight, and shell weight of Mediterranean mussels, respectively, and W_t is the total wet weight.

To determine Mediterranean mussel growth, our data from IMTA mussels were compared with data from Keskin and Ekici, 2021 [34], and Hatzonikolakis et al., 2017 [35], which had calculated CI and MY for a typical mussel farm in the Northern Aegean Sea [34].

2.4. Cultivation of Pearl Oysters

Pearl oysters were cultivated in baskets fabricated by SEAPA© (1998, Seapa, Edwardstown, SA, Australia) for oyster growers (Figure 2a). Although these baskets are designed for longline oyster farming, they can be adapted to suit a range of alternative farming systems and methods.

For the pearl oyster cultivation, spat was collected from natural populations in the vicinity of each fish farm. Before putting them in the IMTA system, the pearl oysters were divided into three different size classes and placed in separate baskets with holes of increasing diameter. The smallest oyster size class (mean length: 3.8 ± 0.8 cm) was grown in baskets with 3 mm-sized holes, the medium (mean length: 5.3 ± 0.5 cm) in 6 mm, and the large ones (mean length: 7.4 ± 0.6 cm) in 12 mm-sized baskets. All SEAPA baskets had 15 L volume, and there were 40 pearl oysters per basket. The density of pearl oysters inside the cage is a factor that affects both survival and growth; following the observations of Macedo et al. (2021), low-density pearl oyster cultivation was selected in order to avoid losses due to lack of feeding [36]. Oyster baskets were deployed around the middle fish cage in the center of each fish farm at a depth of 3 m for 9 months in total on Aq1 and Aq2. In Ag3, oyster cultivation lasted 12 months. The baskets were periodically retrieved from the water from July 2020 until July 2021 in order to estimate their growth and place them in new baskets with larger diameter holes if needed. The growth of the pearl oysters was recorded by measuring the total weight and the weight of shell and flesh of ten individuals at the beginning and at the end of the cultivation in each fish farm.

The shell of ten bivalves was cleaned from attached biofouling organisms in every sampling, and then the total weight was measured using a two-decimal scale. In addition, morphometrics (shell length, width, and thickness) were measured using vernier calipers in order to calculate the specific growth rate of the bivalves.

The following indices were used to monitor the growth and robustness of the bivalves, as in Bayne 2002 [19]: Specific growth rate was calculated for all cultivated pearl oysters using Equation (1) above. The CI and MY were calculated for pearl oysters with Equations (3) and (4), respectively.

To determine the pearl oyster's growth, data from IMTA baskets were compared with data from the natural population in the Evoikos and Saronikos gulfs [15]. In both studies, the same method was used to weigh and measure the morphometric characteristics of pearl oysters and then calculate the CI and MY [15]. It should be noted that these comparisons refer to eutrophic and mesotrophic areas as there are no data available regarding pearl oyster growth in natural populations in highly oligotrophic environments.

2.5. Holothurian Cultivation

SEAPA baskets were also used for the *Holothuria polii* cultivation (Figure 2b). Initially, 40 individuals were collected from natural populations with a mean weight of 59 ± 10.7 g and then transferred to aquaculture sites in a tank with continuous ventilation. At each aquaculture site, 13 individuals of *H. polii* were placed in a SEAPA basket and submerged beneath the bottom of the fish net at the central cage of each aquaculture farm. Each SEAPA basket had a 15 L capacity and 3 mm-sized holes. It is important to mention that after some trials, the minimum available hole size of the baskets was chosen because holothurians have the ability to change the shape of their body [37] and escape from the larger holes.

Because high survival and growth rate have been reported for *Apostichopus japonicus* in cultivation conditions when the density was low [13,38], in this study, the density in SEAPA baskets was also kept low, with 13 individuals per basket. Then, at time intervals of one to three months (at the same dates as above), the cages with holothurians were taken out of the water in order to assess the growth and the robustness of the organisms. The cultivation period for holothurians lasted 12 months, i.e., from July 2020 until July 2021. Throughout the deployment period, farm divers cleaned the cages regularly from biofouling in order to prevent hypoxic conditions. In the Aq3 farm, the holothurian cage was lost after a storm before the last sampling in June 2021. Missing and sick holothurians from cages were considered dead (not counted in the final population) in the equation of survival:

$$\frac{\mathrm{N_f}}{\mathrm{N_i}} \tag{5}$$

where $\mathrm{N_f}$ is the final number of holothurians in cages, and $\mathrm{N_i}$ is the initial number of holothurians from all cages.

No apparently sick individuals were found in any of the sampling events. Sick holothurians would exhibit body wall perforations [39]. The growth of holothurians was recorded by measuring total weight and their total length. Specific growth rate (SGR) was calculated using Equation (1) above.

To determine robustness, data (weight and length) from holothurians in IMTA baskets were compared with data from the natural populations sampled with the same methods and in the same week as those in the farm sites.

2.6. Statistical Analysis

Statistical analysis was carried out using the Statistical Package for the Social Sciences (SPSS), (University of Crete, Biology Department, Heraklion, Greece) program. The CI of mussels in Aq1 and Aq2 for different months was compared using two-way ANOVA (University of Crete, Biology Department, Greece) at $p < 0.05$ statistical significance after variables were checked for assumptions of normality (Shapiro–Wilk test) and homogeneity of variance (Levene’s test). One-way ANOVA was also conducted for W/L data after variables were checked for assumptions of normality (Shapiro–Wilk test) and homogeneity of variance (Levene’s test). To compare the CI of Aq1 and Aq2, a one-way ANOVA was conducted for each month. In addition, for comparison of wild and cultivated holothurians, one-way ANOVA was conducted in SPSS after variables were checked for assumptions of normality (Shapiro–Wilk test) and homogeneity of variance (Levene’s test).

3. Results

3.1. Water Characterization

In Aq1, Chla was 0.33 ± 0.20 $\mu\mathrm{gL}^{-1}$ and indicated higher mesotrophic conditions in the water column (Table 1).

The Aq2 farm is situated in a mesotrophic area with EI 0.86 ± 0.40 and Chla concentration 0.58 ± 0.40 $\mu\mathrm{gL}^{-1}$; the characterization of the ecological status of the water column is poor/moderate (Table 1). The farm Aq3 is in an oligotrophic area with EI = 0.54 ± 0.50 and Chla = 0.05 ± 0.0002 $\mu\mathrm{gL}^{-1}$; dissolved inorganic nutrients and Chla of the area characterized the ecological status as “Good”/“High” (Table 1).

Table 1. Average concentrations of nutrient and chlorophyll a (Chla) for the three different aquaculture sites. Calculation of EI and characterization of each fish farm. Data are expressed as the mean ± standard deviation.

Site	PO4 (μM)	NO2 (μM)	NO3 (μM)	NH4 (μM)	Chla (μgL^{-1})	EI	Categories Of Trophic Levels	Ecological Status
Aq1	0.24 ± 0.09	0.10 ± 0.1	0.67 ± 0.4	4.02 ± 1.5	0.33 ± 0.2	1.42 ± 0.5	Higher Mesotrophic	Bad/Poor
Aq2	0.16 ± 0.1	0.10 ± 0.1	0.96 ± 1	1.58 ± 0.5	0.58 ± 0.4	0.86 ± 0.4	Lower Mesotrophic	Poor/Moderate
Aq3	0.15 ± 0.1	0.02 ± 0.02	0.47 ± 0.2	1.95 ± 1.4	0.05 ± 0.002	0.54 ± 0.5	Oligotrophic	Good/High

3.2. Growth of Mytilus Galloprovincialis

In Aq1, which had the highest mesotrophic conditions among the three sites, Mediterranean mussels were able to survive for months, and their SGR was 0.4%/day (Table 2). In addition, in the Aq2 mesotrophic waters, the SGR was 1.3%/day. Mediterranean mussels with an initial total mean weight of 6.5 g grew to 21.2 g in 9 months in Aq1 and 0.5 g to 16.0 g in 9 months in Aq2.

Table 2. Culture duration, initial total weight, final total weight, final total length, specific growth rate (SGR), condition index (CI), and meat yield (MY) at three IMTA farms of the Mediterranean mussel (Aq1: Epidauros, Aq2: Evoikos, Aq3: Rhodes) and CI and MY of the Mediterranean mussel under natural conditions in the North Aegean Sea [34].

Site	Culture Duration (Months)	Initial Mean Weight (g)	Final Mean Weight (g)	Final Total Length (cm)	SGR (%/days)	(CI)	(MY)
Aq1	9	6.5 ± 4.6	21.2 ± 5.2	5.8 ± 0.8	0.4	42%	19%
Aq2	9	0.5 ± 0.4	16.0 ± 5.5	5.7 ± 0.5	1.3	33%	28%
Aq3	-		-	-	-	-	-
North Aegean Sea	-		-	-	-	35%	10%

Shell length had a logarithmic relationship with the total body weight of IMTA mussels in both Aq1 and Aq2 (See Appendix A, Figure A1). In Aq1 and Aq2, mussels had the same relationship between their length and weight.

In Aq1, Mediterranean mussels had a CI of 42% and an MY of 19% in Aq1 (Table 2). In Aq2, values were 33% and 35%, respectively. However, in a typical Mediterranean mussel farm in the North Aegean Sea, the CI of the marketable mussels is 35%, and the meat yield is 10% (Table 2 and [34]).

The CI of cultivated mussels varied significantly between the two aquaculture sites (Aq1, Aq2) and between months ($F_{stat} = 38.408$, $p < 0.05$) (Figure 3), and this is expected since the mussels were of different sizes during the onset of the experiment. In October, the CI did not vary statistically between the two fish farms ($F_{stat} = 1.296$, $p > 0.05$). In February, the CI of the mussels was statistically different in two aquaculture sites ($F_{stat} = 56.491$, $p < 0.05$) and also in May ($F_{stat} = 70.301$, $p < 0.05$). However, in June, when the average length was similar (and therefore, comparisons between the two populations are valid), the mussels in Aq1 had significantly higher CI than in Aq2 ($F_{stat} = 28.062$, $p < 0.05$) (Figure 3).

The ratio W/L of cultivated mussels varied significantly between the two aquaculture sites (Aq1, Aq2) and between months ($F_{stat} = 28.65$, $p < 0.05$) (Figure 3). In October, the W/L did not vary statistically between the two fish farms ($F_{stat} = 2.44$, $p > 0.05$). In February, the W/L of the mussels was statistically different in two aquaculture sites ($F_{stat} = 182.43$, $p < 0.05$) and also in May ($F_{stat} = 42.16$, $p < 0.05$). Finally, in June, the mussels in Aq1 had a significantly lower W/L ratio than in Aq2 ($F_{stat} = 492.73$, $p < 0.05$) (Figure 4).

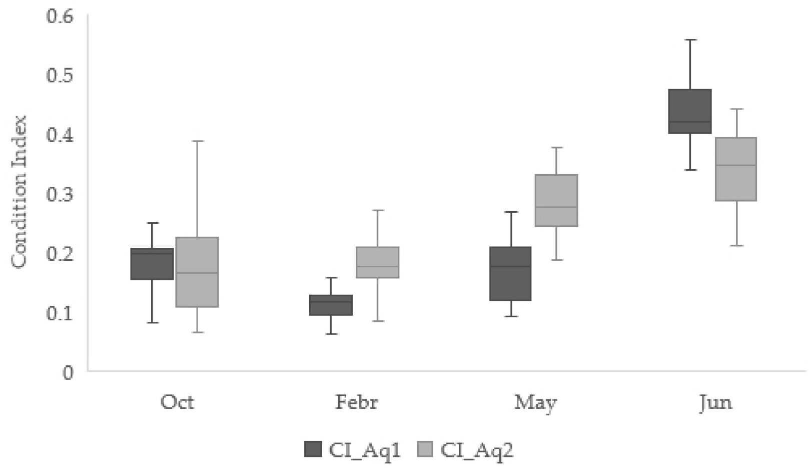

Figure 3. Median condition index (CI) of cultivated mussels (*Mytilus galloprovincialis*) in four seasons for the two fish farms (Aq1: Epidauros and Aq2: Evoikos) from October 2020 until June 2021. Data are expressed as the mean ± standard deviation (n = 30 individuals).

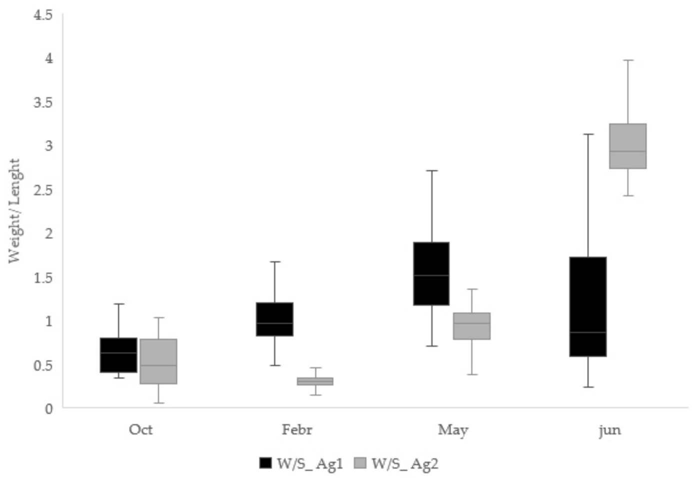

Figure 4. Median weight/length (W/L) ratio of cultivated mussels (*Mytilus galloprovincialis*) in four seasons for the two fish farms (Aq1: Epidauros and Aq2: Evoikos), from October 2020 until June 2021. Data are expressed as the mean ± standard deviation (n = 30 individuals).

3.3. Growth of Pinctada Imbricata Radiata

Pearl oysters showed positive growth rates (SGR) in all the study sites. In high mesotrophic waters (Aq1), the SGR was 0.7%/day, in Aq2, it reached 0.9%/day, and in the most oligotrophic Aq3, it was found to be 0.4%/day (Table 3). Within 9 months, in Aq1, pearl oysters grew from 6.6 g to 39.5 g, and in Aq2 from 4.4 g to 48.9 g, and within 12 months, in Aq3 from 7.2 g to 25.5 g (Table 3).

Table 3. Culture duration, initial total weight, final total weight, final total length, specific growth rate (SGR), condition index (CI), and meat yield (MY) at three IMTA farms of the pearl oyster Pinctada imbricata radiata (Aq1: Epidauros, Aq2: Evoikos, Aq3: Rhodes) and CI and MY of P. imbricata radiata under natural conditions the Evoikos and Saronikos Gulfs [13].

Site	Culture Duration (Months)	Initial Total Weight (g)	Final Total Weight (g)	Final Total Length (cm)	SGR (%/days)	CI	MY
Aq1	9	6.6 ± 3	39.5 ± 6.1	6.4 ± 0.6	0.7	53%	26%
Aq2	9	4.4 ± 0.8	48.9 ± 5.2	7.8 ± 0.2	0.9	56%	33%
Aq3	12	7.2 ± 1.2	25.5 ± 1.8	5.6 ± 0.3	0.4	19%	27%
Evoikos Gulf	-	-	42.6 ± 14.6	6.7 ± 0.7	-	30%	20%
Saronikos Gulf	-	-	34.9 ± 10.5	6.8 ± 0.7	-	45%	31%

A comparison of CI and MY from natural populations of pearl oysters and those grown in multitrophic aquaculture (Aq1 and Aq2) (Table 3) showed that: (i) both categories had increased values, i.e., conspicuous growth, (ii) the cultivated pearl oysters had higher values of CI (53–56%) than the natural populations in two central Aegean coastal areas (30–45%), and (iii) the differences in MY values between IMTA-farmed and natural pearl oysters were less visible. In the oligotrophic waters of Aq3, oyster CI was 19% and MY 27%.

The shell length and total weight relationship was used as an index of their growth (Figure A2) [40]. Pearl oyster shell length and weight increased logarithmically in the three fish farms (Aq1, Aq2, Aq3).

3.4. Growth of Holothuria Polii

Survival of holothurians was rather successful, with a rate of 80% for Aq1, 62% for Aq2, and 40% for Aq3. In Aq1, sea cucumbers survived for 12 months in cages, with their weight changing from 59.4 g to 17.3 g and an SGR of −35%/day (Table 4). In addition, in Aq2, holothurian weight decreased from 58.7 g to 8.3 g, and the SGR was calculated as −56%/day. In Aq3, sea cucumbers survived for nine months, and their weight decreased from 70.0 g to 18.3 g; s, the specific growth rate, was calculated as −50%/day. In the last sampling event (July 2021), no holothurians were found in the cages beneath Aq3 (Table 4).

Table 4. Culture duration, initial total weight, final total weight, final total length, specific growth rate (SGR), and survival of sea cucumber (Holothuria polii) at three IMTA farms (Aq1: Epidauros, Ag2: Evoikos, Aq3: Rhodos).

Site	Culture Duration (Months)	Initial Total Weight (g)	Final Total Weight (g)	Final Total Length (cm)	SGR (%/day)	Survival (%)
Aq1	12	59.4 ± 11	17.3 ± 3.5	5.7 ± 0.6	−35	80
Aq2	12	58.7 ± 12	8.3 ± 3.0	5.3 ± 0.4	−56	62
Aq3	9	70.0 ± 16	18.3 ± 6.9	5.7 ± 1.4	−50	40

The initial average weight of the holothurians did not differ between individuals placed in aquaculture cages and natural populations (Figure 5). In the following months, the average weight of cultivated holothurians was significantly different from that of the natural population.

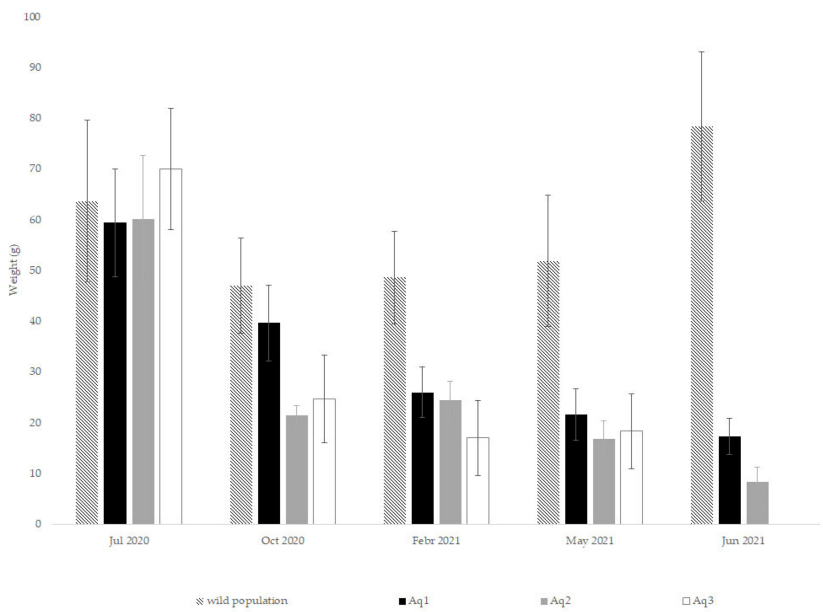

Figure 5. Weight of holothurians in IMTA farms (Aq1: Epidauros, Ag2: Evoikos, Aq3: Rhodos) and in natural populations for four seasons, from July 2020 to July 2021. Data are expressed as the mean ± standard deviation (n = 15 individuals).

In October, the average weight of holothurians in Aq1 was 40 g, not statistically different from the average weight of holothurians in the natural population, which was 47 g. In contrast, in aquaculture sites with mesotrophic (Aq2) and oligotrophic waters (Aq3), the average weights of holothurians were 21 g and 25 g, respectively, which varied statistically ($F = 44.32$, $p < 0.05$) from the natural population. In January and April, the average weight of cultivated holothurians ranged from 26 g in Aq1 to 17 g in the oligotrophic site, while in natural populations, it was 49 g. Finally, in July 2021, as shown in Figure 1, the weight of cultivated holothurians had decreased to 17 g in Aq1 and 8 g in Aq2, while in the natural population, it had increased to 78 g.

4. Discussion

Bivalves and holothurians are potential candidates for culture in commercial IMTA systems. The Mediterranean mussel was able to successfully grow in IMTA conditions, but not in all waters; the Mediterranean mussels have not colonized oligotrophic waters being unable to find adequate food resources to ensure the survival of their population. The pearl oysters, on the other hand, are a species highly suitable for IMTA cultivation because they can grow successfully in environments with very different trophic statuses. Finally, holothurians did not gain weight as expected despite their relatively high survival rate.

The Mediterranean mussel can survive and grow normally in IMTA conditions using traditional methods such as longlines. Comparing the values of CI and MY, the IMTA mussels had higher CI and MY than reported values for traditionally farmed mussels from the North Aegean. These results indicate that when Mediterranean mussels grow in proximity to multiculture farms, they have more flesh in their shell in comparison to those growing on a typical mussel farm. Although the Mediterranean Sea is mostly oligotrophic, the areas near fish farms may be an exception due to the significant amount of released nutrients from fish cages [3]. Therefore, fish farms are probably a favorable environment for Mediterranean mussels as they provide plenty of nutrients for the growth of phytoplankton and, subsequently, mussels. However, in very oligotrophic aquaculture sites such as Aq3, the fish farm effluents were obviously not enough to counteract the nutrient-starving system of the water column, so these areas have not been colonized by the Mediterranean mussel.

Mussel CI in Aq1 increased faster from May to June in comparison with mussels in Aq2 (Figure 3). Although the average weight did not show differences between the two fish farms (Table 2), the shells of the mussels growing in Aq1 were thinner and lighter than the shells of the mussels in Aq2 (Figure 4). The difference in the morphology of the shell between the two fish farms may be attributed to the colonization by an endemic sea vase (*Ciona intestinalis)*, which was found growing between and on mussels in Aq1.

As opposed to Mediterranean mussels, oysters were successfully cultivated in IMTA conditions using traditional methods, such as oyster baskets; they grew properly at sites with all types of trophic conditions despite their high requirements for phytoplankton biomass. A comparative study of different bivalves regarding their clearance rate (CR) and particle retention efficiency [41] showed that members of the genus *Pinctada* had a CR of 2–3 times higher than that of the species of *Mytilus* although the mytilid species were more efficient in retaining small particles (i.e., 1–4 μm). The *Pinctada* species (in contrast to the mytilids) lack the eu-latero-frontal cirri used to retain small particles. Pouvreau et al. (1999) found this lack surprising since the relative abundance of picoplankton in the natural habitat of *P. margaritifera* in Polynesia is as high as 60% of the total OM in suspension, and this is also the case in the oligotrophic waters in the Eastern Mediterranean [42,43]. *Pinctada* and other tropical bivalves counterbalance this low efficiency in retaining picoplankton by feeding on higher trophic levels, including protists micro-algae and protozoa [41].

The "protozoan trophic link" [44] has been recognized as an alternative feeding strategy for oysters, which cannot efficiently filter very small picoplankters but can do so with their grazers, i.e., the ciliates. Various studies in the vicinity of fish farms in the Mediterranean have shown that dissolved nutrients released from farmed fish are readily absorbed by small-size plankton and rapidly transferred to higher trophic levels through ciliate grazing [3,45]. Besides ciliates, oysters have been found to feed on aggregates, assemblages of mucus, detritus, bacteria, etc. [41]. In the vicinity of fish farms, there is abundant particulate material of non-phytoplanktonic origin, mainly dust or crumbs of fish feed and small fragments of fish feces, immediately available to filter feeder organisms; on the other hand, phytoplankton needs time to capitalize on the released nutrients and therefore it is found to increase in abundance at an intermediate distance from fish farms [45]. All the above may explain the differences between *Mytilus galloprovincialis* and the pearl oyster *P. radiata* regarding their performance in the vicinity of Mediterranean fish farms. The mussels need mostly fresh phytoplankton biomass, which may be found in eutrophic or mesotrophic conditions but not in highly oligotrophic ones and, as a rule, not at small distances from the farms. On the other hand, pearl oysters can use a variety of feeding sources, including detritus, which is present in large quantities, but also ciliates that have "bio-packaged" pico- and nanoplankton produced in situ, consuming released dissolved nutrients. Due to their feeding strategy and the increased CR, pearl oysters are able to thrive in a wide spectrum of trophic statuses, including the highly oligotrophic ones.

Furthermore, although pearl oysters were able to survive and grow to commercial size in all examined trophic conditions, they exhibited a different growth rate. They reached commercial size (5–6 cm) within a few months in Aq1 and Aq2, but in the oligotrophic aquaculture (Aq3), they needed more than a year.

Comparing the cultivated pearl oysters with natural populations in terms of weight and length measurements, no significant differences were observed. This suggests that pearl oysters can grow in multitrophic conditions successfully. To our knowledge, there are no published studies on the use of pearl oysters in IMTA. In conclusion, both species of bivalves show high growth and robustness compared to natural populations, showing that IMTA is a cultivation method that improves their growth efficiency. Furthermore, their increased growth performance in the close vicinity of fish farms indicates that these organisms are able to consume at least part of the wastes released by the fish farm, and, therefore, they may be used as a mitigation measure for decreasing the ecological footprint of aquaculture.

Study results showed that holothurians can survive in SEAPA baskets for a yearlong period despite the negative growth. The high survival rate of holothurians was perhaps related to cage position under the fish cages. It has been shown that when holothurians were placed directly on the sediment, they died because of anoxic conditions [8,13,46]. Thus, tying holothurians under the fish cages may have protected them from hypoxia. In addition, this method may be used in offshore fish farms where the water depth favors dispersion of the precipitating organic material over a wide area of the seabed and therefore becomes available only in small quantities per seabed unit area.

However, factors that negatively affect their development despite their high survival are still under question. The growth of holothurians may be affected by physicochemical characteristics of the water or the lack of available nutrition. The negative growth of IMTA holothurians was probably due to underfeeding, as well as the fact that for the cultivation, adult individuals, not offspring, were used since the latter was not available. In addition, antibiotics and metals [47] may negatively affect the growth of holothurians. Sea cucumbers had, as shown in Figure 4, lower values of weight and length compared to natural populations. Their weight seemed to decrease during the winter months, even in the natural populations, due to the degeneration of their gonads in their annual sexual cycle [48]. So, one explanation can be that IMTA holothurians did not create gonads at all in the summer months, and that is why their weight did not increase.

In an aquaculture farm, in addition to organic material, other substances such as antibiotics and metals are released into the environment [42]. Thus, it is reasonable to expect that organisms that filter water may also retain these substances [47]. Studies on mussel co-culture with salmon showed no difference in metals or antibiotics compared to traditional monoculture [11]. In addition, food safety studies for holothurians showed no difference in metal concentrations in comparison to natural populations [49]. In order to find out if all the above-mentioned co-cultivated organisms are appropriate for consumption, samples were collected for analysis of biotoxins, metals, and antibiotics in the context of an ongoing follow-up research project.

In conclusion, IMTA is a dynamic system that can be adapted to the conditions prevailing in each region. Depending on the water variables, IMTA may involve cultivating different types of organisms near the fish cages. This is an additional advantage of this method since fish farmers can adapt to different conditions using their own strategy depending on the market needs and their management abilities.

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Appendix A

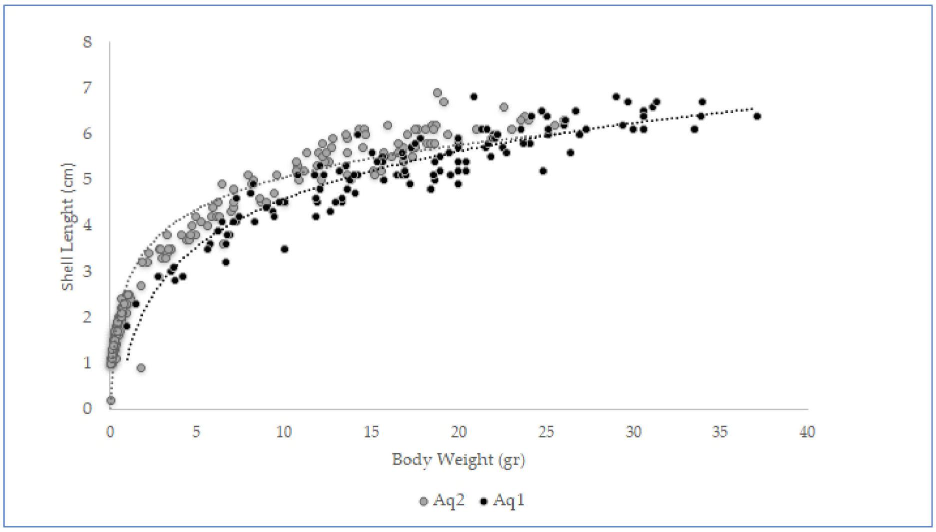

Figure A1. Shell length (cm) and body weight (g) measurements of mussel (*Mytilus galloprovincialis)* in Aq1 (Epidavros) and Aq2 (Evoikos). Each dashed line represents the length-weight relationships (W = aln(L) + b) for mussels under IMTA conditions in Aq1 and Aq2.

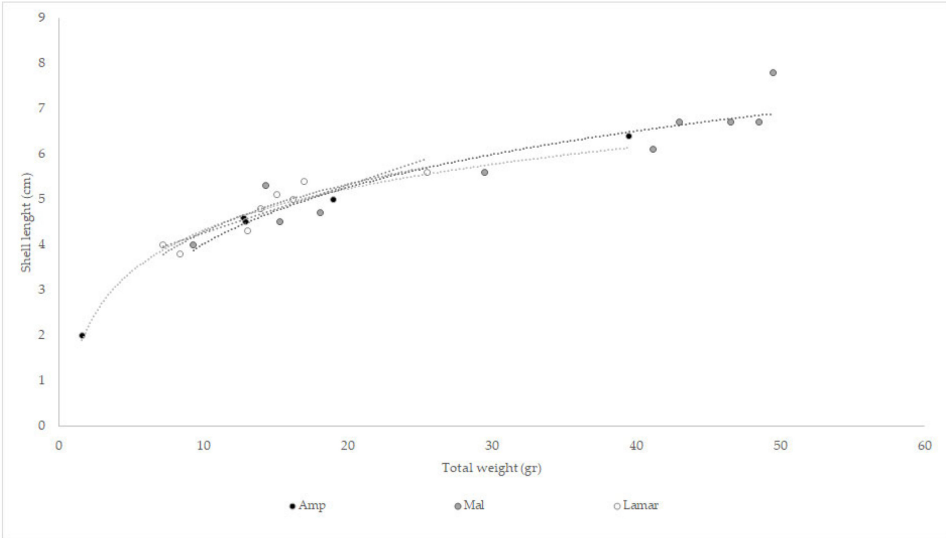

Figure A2. Shell length (cm) and body weight (g) measurements of pearl oyster (*Pinctada imbricata radiata*) at 3 IMTA farms (Aq1: Epidauros, Aq2: Evoikos, Aq3: Rhodos). Each dashed line represents the length-weight relationships (W = aln(L) + b) for pearl oysters under IMTA conditions in Aq1 Aq2 and Aq3.

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