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Review Article

Assessment of Fish-Farms Wastewaters Synergistic Impact on a Mediterranean Non-Tidal Lagoon

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Abstract

The impact of wastewater from two fish farms affecting a coastal lagoon has been assessed for water quality, sediments and macroalgae. Based on a previous study, three sampling areas of 135 hectares each were identified at increasing distance from the discharges and six monitoring surveys were carried out between May 2017 and November 2018. The results indicated macroalgal assemblages as the most suitable variables for assessing the impact of wastewater, since they showed a different species dominance in the three areas. No clear result emerged from sediments parameters due to the texture variability, while water quality was significantly different only for nitrates and orthophosphates in one of the three areas. Probably due to the specific lagoon morphology and eutrophication management methods adopted, only one of the surveyed areas was clearly affected by the wastewater impact.

Keywords: Eutrophication; Fish-farm impact; Labile organic matter; Lagoon water quality; Opportunistic macroalgae; Sediment

Introduction

In the last fifty years, global aquaculture production has greatly increased, reaching in 2016, 80 millions of tones, with an increasing number of products for direct human consumption, representing 47% of the total, and 53% if not human food uses are excluded (e.g. fishmeal and fish oil) [1]. 89% of this production takes place in Asia. In Europe and North America, aquaculture development was rapid between the 1980's and 1990's, but then slowed down, probably due to regulatory restrictions and market competition factors [2]. Although the percentage increases in annual production growth are lower than five years ago, in countries where production is high, these percentage increases mean a strong increase in terms of tones of product. Aquaculture is certainly destined to continue its growth and to largely replace fishing of the wild product, which can no longer meet the demands of the world population which greatly increased during the last fifty years.

During the period 1995 to 2015, production of farmed aquatic species reliant on feeds increased more than fourfold, from 12 to 51 million tonnes, and today, 66% of total global aquaculture production (excluding aquatic plants) is produced using exogenous feed, mostly commercially manufactured. In fact, in the same period, production of industrial aquaculture feeds increased sixfold, from 8 to 48 million tonnes. However, the proportion of fish from capture fisheries being reduced to fishmeal and fish oil has been declining in recent decades, and it is projected that a growing share of fishmeal and fish oil production will be obtained from fish processing co-products, such as fish carcasses. Furthermore, the dietary inclusion rates of fishmeal and fish oil in aquaculture feeds have also been falling, increasingly replaced by crops, especially oilseeds [1,3]. Much research is being directed into novel aquaculture feedstuffs, including seaweed and insect sources, but it is likely to be some years before these become widely available and affordable [4,5]. Despite the efforts to make aquaculture more sustainable, energetically more advantageous and environmentally friendly solutions will have to be studied and found necessary [6].

Moreover, the impact of aquaculture, in its different forms, on the host environment must be seriously considered. The coastal areas, also due to the contribution of aquaculture, have been subjected to a growing eutrophication with dramatic variations in the benthic communities, with the death of fish and enormous microalgal and macroalgal developments [7-10]. In marine finfish aquaculture, according to [11], for recently formulated feeds, 69 kg of Nitrogen (N) and 10 kg of Phosphorus (P) are released into the environment per tonne of fish produced. The same researchers also estimated that future improvements in feed production will lead in 2050 to a reduction in the Nitrogen (N) and Phosphorus (P) releases to 55 kg and 7 kg per ton of fish produced, respectively.

It is thus necessary to evaluate the impact of this practice on natural ecosystems and how the latter react to stress. A more in-depth knowledge of this issue would allow better intervention to mitigate of the consequences more effectively.

In this study, we examined the impact of wastewater from two sea bass and sea bream land-based farms, which are released into a non-tidal coastal lagoon.

Lagoon environments, despite being environments of shallow waters and poor water exchange compared to the nearby sea, have, in

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their community as a whole, a high resilience and a great capacity to integrate stress factors. However, since they are already perturbed environments, the stress produced by anthropic pressure, in our case the wastewater from the two fish-farms, in not easily distinguishable from the natural structural stress. This peculiarity is called estuarine quality paradox [12,13].

The aim of this study is to highlight a possible impact gradient of the wastewater of the two fish farms on the various compartments of the lagoon ecosystem, highlighting the best indicator variables of this type of impact. In the working hypothesis, the effects of this impact should decrease as the distance from the sources of waste release increases.

Materials and Methods

The study area and the fish-farms

The examined area was the eastern basin of the Orbetello lagoon (Figure 1). The Orbetello lagoon is a shallow, eutrophic coastal water body of about 25.25 km^2 in the southern Tuscan coast of Italy (42°25'-42°29' N, 11°10'-11°17' E). Three artificial canals, 0.5-3 km long and 10-15 m wide, two in the western and one in the eastern basin, connect the lagoon with the sea (Figure 1). Because they are small shallow canals, water turnover is poor and depends mainly on wind force and direction, as the Tyrrhenian tide range is narrow. The lagoon salinity ranges from 28 to 45 (practical salinity scale), depending on rainfall and evaporation.

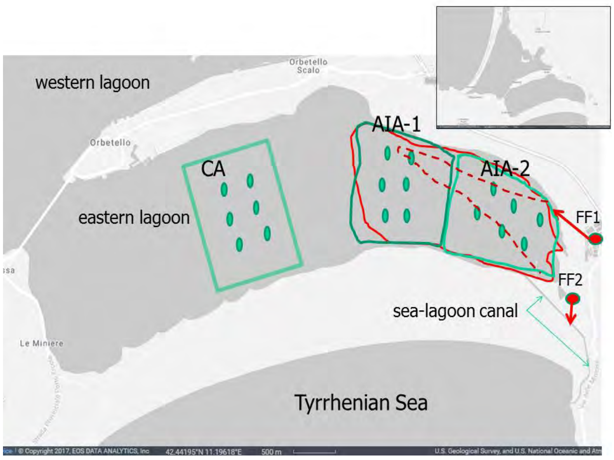

Figure 1: The Orbetello lagoon (Tuscany coast, Italy) and its eastern basin. Green squares, CA, AIA-1 and AIA-2 areas for sampling of water, sediment and macroalgal variables; green points, stations for water and sediment sampling. Red points and arrows, fish farms FF1 and FF2, and effluents of wastewaters; red line, area that has been hypothesized in this study to be affected by the wastewater; red dotted line, hydrodynamics wastewater propagation estimated by CORMIX model (Franchi et al., 2009).

The environment is eutrophic due to fish-farm wastewaters, intermittent streams containing agricultural run-off and civil effluent, and historical input stored in sediment [14]. Due to high nutrient availability, morphology and low-water-turnover, this lagoon is subject to severe macroalgal proliferation, which can cause dystrophic crises with die-offs.

Owing to the low water renewal, sea water is pumped into the lagoon, between June and August, to promote water turnover, with input by two western pumping stations, at the mouth of the two western channels, and output by the eastern canal. This input creates a continuous one-way flow of about 13.000 L s^{-1}, with a weak speed of 1.0-1.5 cm s^{-1}. Although this water mass is relatively high, it follows short routes and does not allow sufficient water turnover in large part of the stagnating areas of the two basins, in shadow of this flow.

Therefore, in the summer months, in the eastern basin, the pumped waters coming from the western basin flow out towards the sea, with a very low flow velocity that can be hindered by a strong wind and rising of the marine front, which determine a consequent increase in the level of lagoon waters [15].

Two land based fish-farms discharge the wastewater in the easternmost part of the eastern basin, one (FF1) near the sea-lagoon channel, the other (FF2) in the middle of the same channel (Figure 1). Therefore during the pumping period it is probable that the FF1 wastewater is carried out by the outflow towards the sea and cannot extend much beyond the discharge area, while the FF2 wastewater flows mainly towards the sea, except for the periods in which a wind of contrast raise the sea front and let the waters enter from the sea, raising the lagoon level. In the remaining nine months, according to a study that used the CORMIX hydrodynamic model [16], the FF1 discharge mixes more and is removed towards the more central areas of the east basin, while the FF2 discharge follows the tidal flow, entering the lagoon at high tide. According to this study, the influx of wastewater extended towards the innermost areas of the east basin, along an extended brush about 1.8 km (Figure 1).

The two fish-farms breed sea-bass (*Dicentrarchus labrax* L.) and sea-bream (*Sparus aurata* L.), using water with salinity varying between 15 and 30, obtained by pumping from wells on brackish aquifers, which, due to a geothermal anomaly, are at constant temperature of 18-20°C.

FF1 consists of about 42 ground tanks covered in PVC of 400-600 m^3 for a total of 22.400 m^3 and a stream of outgoing waters of 560 L s^{-1}, FF2 consists of 45 ground tanks covered in PVC with dimensions and volumes similar to the previous ones, for an outgoing flow of 420 L s^{-1}.

Overall, fish production is 700-800 tonnes a^{-1}. Both fish farms are equipped with a system of small basins and sewage settling channels, for an extension of about 2 ha each, in which detritivorous fish (mugilids) are placed and where microalgae and macroalgae develop.

The experimental design

To identify the lagoon area subject to the impact of fish farm wastewater (area for impact assessment, AIA), the study by Franchi, et al., [16] was used. We hypothesized a possible extension of the influence of wastewater on the entire area between the two lagoon coasts that include the extension of the brush of influence established by the CORMIX model. Considering about 2 km this plume, the area of interest was about 270 hectares (Figure 1). Within AIA, two areas of 135 hectares each have been identified, AIA-1 and AIA-2, the second closer to the sources of impact. A third area of the same extension and more than 1 km away from the nearest margin of AIA-1, was selected as a control (CA) (Figure 1).

Three different compartments of the lagoon ecosystem have been considered: Water, sediment and macroalgae. The characterization of the three compartments took place in May 2017, in condition of incoming tide, aiming to highlight the wastewater dispersion towards the eastern lagoon centre. According to the results of May 2017, only macroalgae and sediment has been the subject of subsequent 5 campaigns: November 2017 and February, May, September, and November 2018.

Sampling and analytical determinations

Water column

In each of the 3 selected eastern lagoon areas (AIA-1, AIA-2 and CA), 6 sampling and measurement points were identified, arranged along two parallel transects (Figure 1). In May 2017, using a multi-parameter probe, Temperature (T, **°C**), pH, Salinity (S, psu), Dissolved Oxygen (DO, mg L^{-1}) and Nephelometric Turbidity (NTU) were measured in duplicate in each point. Water samples were then taken in duplicate in the same points and at the exit of the two fish farm wastewaters (FF1, FF2). Water samples were stored in a light-free and refrigerated environment, and then transported to the laboratory in a few hours for analytical determinations. Samples were filtered at 0.45 **µm** and the following analytical determinations were conducted: Ammonium nitrogen ($N\text{-}NH_4^+$), nitrous Nitrogen ($N\text{-}NO_2$), nitric Nitrogen ($N\text{-}NO_3$), Total Dissolved Nitrogen (TDN), Total Dissolved Phosphorus (TDP) and Soluble Reactive Phosphorus (SRP). The analyses were conducted according to APAT IRSA-CNR [17]. Dissolved Inorganic Nitrogen ($DIN=N\text{-}NH_4^+ + N\text{-}NO_2 + N\text{-}NO_3$), Dissolved Organic Nitrogen (DON=TDN-DIN), Dissolved Organic Phosphorus (DOP=TDP-SRP) and the atomic ratio DIN:SRP were then computed.

Sediment

To determine sedimentary content of organic Carbon (C), Nitrogen (N) and Phosphorus (P), in the same water sampling points (Figure 1), sediment samples were taken in May 2017, with a horizontal core drill, able to collect in the surface layer of the first 3-4 cm, using a 60 mL syringe. The sediment samples were transferred from the syringe into the polyethylene containers of the same volume and refrigerated and subsequently frozen pending for analysis. The samples were dried to constant weight at 75°C, and then subjected to analytical determinations. N and C were determined using an elementary analyser (CHN Thermoquest, model 1110), P according to Aspila, et al., [18]. Using the percentages of the three estimated macronutrients, the molar ratios C:N, C:P, N:P were subsequently calculated.

To determine the amount of organic matter present in the lagoon sediments as a labile fraction (LOM), sediment samples were collected in the same points, using the previously described method, in November 2017 and February, May, September and November 2018. The determination was carried out as combustion at 250°C in a muffle after reaching the constant dry weight (75°C) [19].

In February 2018, 3 samples were collected per area using the horizontal sampler, in order to define the texture of the first 3-4 cm of the sediments, in the sand, silt and clay components.

In November 2018, the detrital fraction >1 mm (cd) dried at 75°C, essentially consisting of shell debris, was considered for all sediment samples and was calculated as a percentage of the total of the sample according to the following equation: cd% = fraction>1mm*100: (fraction<1mm+fraction>1mm).

Macroalgae

In May and November 2017 and in February, May, September and November 2018, in each of the 3 areas, the number of macroalgal species present and the overall biomass were determined.

The Total Coverage (CT) of the substrate by the algal mats was estimated through Sentinel-2 satellite images obtained from the Land-Viewer site (EOS DATA ANALYTICS, USGS/NASA), and calculated through the Fiji-Image software. In the field, immediately following that of the available satellite image, the Biomass (b) was determined by collecting the plant material contained in a 60*60 cm panel lowered to 6 points per area, distributed according to the satellite images.

The material collected inside the box was drained for a few minutes and weighed in field with a portable electronic scale with a sensitivity of ±0.5 g. The data obtained were transformed to the surface unit of 1 m^2 (transformation factor 2.778) and expressed as kg wet weight m^{-2}. For the determination of Standing Crops (SC), the algal mass present in a given lagoon surface at the time of detection, the following equation was applied**:** $SC=b*CT*1000^{-1}$, where: SC is the standing crop expressed in Tonnes Wet Weight (Tww); b, the biomass expressed in kg_{ww} m^{-2}; CT, estimated total coverage with Fiji software in m^2; 1000^{-1}, the factor for bringing the final value to tones.

On the basis of described samplings, the specific Dominance (d) per point-station and the percentage of opportunistic species (% os) on the total species observed were determined.

In May 2017, samples of the most widespread species, common to the selected areas, were collected in each of the three areas, to determine C, N, P content. These samples were washed with sea water to remove debris and other impurities, transferred into plastic bags, stored in the dark, refrigerated and transported to the laboratory in few hours. The material was then quickly washed with fresh water, dried with tissue paper, dried at 40°C in a dryer with ventilation, and then further cleaned of impurities and small animals. The sample was stored in polyethylene containers in a dry place, up to the laboratory determinations. The analysis was carried out employing the same methods as described above for the sediment on samples dried to constant weight of 75°C. The molar ratios C:N:P and C:N were subsequently calculated.

Statistical analysis

Chemical-physical variables and nutrient components in the water and sediment sand, calcareous shell debris (cd) and nutrient contents were analyzed by one-way ANOVA with area (CA, AIA-1, AIA-2, 3 levels) as fixed factor. Nutrient content of macroalgal thalli was analyzed by the Student's t-test in order to detect significant differences between the source (AIA-2) and Control (CA) Areas.

LOM and biomass data were processed by two-way ANOVA to detect significant differences between the month (Nov17-Nov18, 5 levels for LOM; May17-Nov18, 6 levels for biomass) and area (CA, AIA-1, AIA-2; 3 levels) fixed and orthogonal factors.

Cochran's C-test was used before each analysis to check for homogeneity of variance [20], and datasets were transformed where necessary. The Student Newman Keuls (SNK) test was used for *a posteriori* multiple comparisons of means.

A regression analysis was performed in order to evaluate possible correlation between cd and LOM content in the sediment, with Oct-18 data-set (6 records per area). The degree of correlation between cd content of each studied area and the LOM one was calculated and reported as the squared correlation coefficient (determination coefficient, R^2).

All the statistical analysis was performed with the Statistica 10.0 software, and the critical value in all tests was P=0.05.

Results

Water column

In table 1, the means (± SD) of the chemical-physical variables (T, pH, S, DO, NTU) are reported for each area, for May 2017. In table 2, nutrients ($N\text{-}NH_4^+$, $N\text{-}NO_2$, $N\text{-}NO_3$, DIN, DON, TDN, SRP, DOP, TDP; expressed in µM) and atomic ratio DIN:SRP means (±SD) are reported for each lagoon area and for the two fish farm wastewaters as a reference for nutrient sources.

	CA	AIA-1	AIA-2
T	22.32±0.13	23.03±0.34	23,92±0.95
pH	9.15±0.22	9.19±0.05	9,06±0.17
S	37.00±0.82	34.75±0.99	26,17±4.14
DO	6,27±1.00	8.42±1.06	9,20±1.41
NTU	1.58±0.43	0.98±0.28	2,88±1.10

Table 1: Means±SD of temperature (T, °C), pH, Salinity (S, psu), Dissolved Oxygen, in mg L-1 (DO), redox (Eh; mV) and Nephelometric Turbidity Unit (NTU) conducted in the plots CA, AIA-1, AIA-2 (6 measures per plot), in May 2017.

	CA	AIA-1	AIA-2	FF1	FF2
$N\text{-}NH_4$	35.63±18,01	27.52±22.53	11.86±13.34	142.61±14.54	103.68±3.46
$N\text{-}NO_2$	0.27±0.45	0.07±0.00	1.15±1.55	20.61±0.11	8.46±0.12
$N\text{-}NO_3$	40.38±1.32	45.45±3.57	48.06±4.28	127.60±0.96	50.18±0.18
DIN	76.29±19.25	73.05±21.06	61.07±15.93	290.82±15.61	162.32±3.39
TDN	848.40±186.97	766.25±399.08	685.88±175.86	344.36±12.79	654.39±2.75
DON	772.12±190.91	693.20±407.22	624.81±172.02	53.54±2.82	492.07±0.64
TDP	0.35±0.26	0.60±0.53	0.80±0.34	8.58±1.11	6.08±0.37
SRP	0.07±0.08	0.15±0.13	0.42±0.27	7.45±0.77	5.04±0.02
DOP	0.23±0.20	0.45±0.57	0.38±0.10	1.12±0.34	1.04±0.39
DIN/SRP	2103±1067	1399±1272	285±302	39±2	32±1

Table 2: Determination in µM of ammonium Nitrogen ($N\text{-}NH_4^+$), nitrous Nitrogen ($N\text{-}NO_2$), nitric Nitrogen ($N\text{-}NO_3$), Dissolved Inorganic Nitrogen (DIN), Total Dissolved Nitrogen (TDN), Dissolved Organic Nitrogen (DON), Total Dissolved Phosphorus (TDP), Soluble Reactive Phosphorus (SRP), Dissolved Organic Phosphorus (DOP), and DIN:SRP atomic ratio estimated in the water samples collected in the CA, AIA-1, AIA-2 areas and in the FF1 and FF2 fish-farms wastewater, in May 2017.

The ANOVA analysis showed a significant effect of the area factor on the variables T, S, DO and NTU (P=0.0023, P<0.0001, P=0.0030, P=0.0012, respectively). The post hoc SNK showed significantly higher T values towards the sources (AIA-2), compared to the other two areas (CA=AIA-1), while the S values were significantly lower in AIA-2 (*P*<0.01). DO was significantly lower in CA than the other two areas (P<0.01) (AIA-1=AIA-2); for NTU, each area was significantly different from the other, with higher values in AIA-2 (P<0.01) and lower in AIA-1 (P<0.05).

For nutrients, ANOVA showed a significant effect of the area only for $N\text{-}NO_3$, SRP and DIN:SRP (P=0.0072, P=0.0122, P=0.0289, respectively). With the post hoc SNK, $N\text{-}NO_3$ values resulted significantly lower in CA than in AIA-1 (P<0.05) and AIA-2 (P<0.01), while the two AIA areas were similar. The SRP values in CA and AIA-1 were significantly lower than in AIA-2 (P<0.01, P<0.05, respectively), while the values of CA and those of AIA-1 were similar. The values of DIN:SRP were significantly higher in CA than in AIA-2 (P<0.05).

Sediment

Table 3 shows the means (±SD) of the percentages of sand, silt and clay on sediment samples dried at constant weight at 75°C collected in February 2018, and the percentages of carbonate concretions (cd) obtained from samples collected in November 2018. The amount of sand in sediment was significantly different in all three areas examined (P<0.01), with the highest values in AIA-2 and lower values in AIA-1. The quantities of cd were significantly higher in the two AIA areas than in the control area (P<0.05), with the highest values in AIA-2.

	sand	silt	clay	cd
	%	%	%	%
CA	79.80±0.50	14.60±0.10	5.60±0.40	10.57±5.26
AdI-1	78.65±0.45	14.30±0.20	7.05±0.25	17.44±11.19
AdI-2	84.40±0.50	9.45±0.85	6.15±0.35	30.75±15.81

Table 3: Percentages of sand (>63 µm), silt (63-4 µm) and clay (<4 µm) present in the sediments (on driedmatter to constant weight) collected in February 2018 in the control area, CA, and in the two areas of interest, AdI-1 e AdI-2. Percentage of shell debris (cd) present in the sediments of the three areas collected in November 2018.

Table 4 shows the means (±SD) of C, N, P content and the relative molar ratios C:N, C:P and N:P for the May-17 sampling. ANOVA showed a significant effect of the area factor only on the variables C:P and N:P (P=0.0003 e P<0.0001, respectively). With the post hoc SNK, the estimated values in AIA-2 for these two variables were significantly lower (P<0.01) than those found in the other two areas (CA=AIA-1).

	CA	AIA-1	AIA-2
%C	4.40±2.57	6.48±0.28	5.10±2.69
%N	0.52±0.32	0.73±0.04	0.52±0.32
%P	0.04±0.02	0.06±0.01	0,09±0.05
C:N	10.87±2.90	10.34±0.65	14.60±3.22
C:P	287.18±62.53	280.22±23.14	163.48±31.82
N:P	27.15±2.99	27.14±1.94	12.23±3.62

Table 4: Carbon (C), Nitrogen (N) and Phosphorus (P) content, in percentage (%), on sedimentary matter of the three study areas (CA, AIA-1, AIA-2), collected in May 2017 and dried to constant weight at 75°C, and related C:N, C:P, N:P atomic ratios.

The percentages (±SD) of Labile Organic Matter (LOM) in sediment are shown in table 5, for the period from November 2017 to November 2018. ANOVA showed a significant effect of the area factor (P<0.0001), and the post hoc SNK showed lower values in AIA-2 (P<0.01) compared to the other two areas.

		17-Nov	18-Feb	18-Jun	18-Sep	18-Nov
LOM	CA	9.36±3.19	11.63±3.08	11.57±1.36	9.50±1.21	10.50±3.46
	AIA-1	11.94±3.93	10.31±1.95	12.80±4.72	12.70±5.15	10.64±4.17
	AIA-2	7.56±0.73	8.21±0.53	8.82±1.87	7.72±0.51	7.26±2.23

Table 5: Labile Organic Matter (LOM) % content on sedimentary matter, dried to constant weight at 75°C, of the three study areas (CA, AIA-1, AIA-2), collected during the 6 sampling trials, between November 2017 and November 2018.

As shown in figure 2, an inverse correlation between LOM and Carbonate Debris (cd) content was found. Although the degree of correlation was R^2=0.3437, the regression was significant (F=10.34; P=0.0054), indicating a significant loss of LOM at the increasing cd content in the sediment.

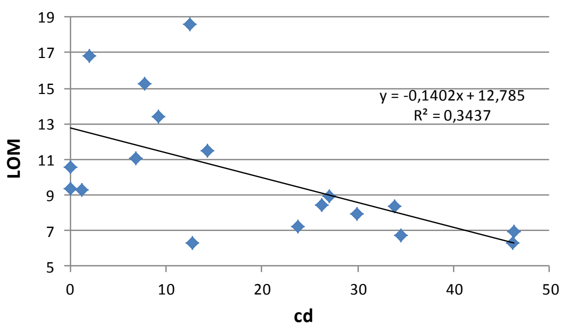

Figure 2: Regression analysis performed in order to evaluate possible correlation between calcareous shell debris (cd%) and Labile Organic Matter (LOM%) content in the sediment, with November 2018 data-set (6 records per area, 18 in total).

Macroalgae

Table 6 shows the floristic list of the observed species and the number of opportunistic species present in each area, during the whole survey. The total number of macroalgal species increases from CA (n=8) to AIA-2 (n=12) as well as the number of opportunistic species. The latter were 50% and 45% of the species present in CA and in AIA-1, respectively, and 67% of those in AIA-2.

In table 7, the average (±SD) of the biomass (b, kg wet weight m^{-2}), the corresponding Standing Crop (SC, in Tww), the total coverage in hectares compared to the overall surface of each area (CT, estimated using Fiji software) and the dominant species (d) are reported for each area and for each survey. The time course of SC for the three areas is shown in figure 3.

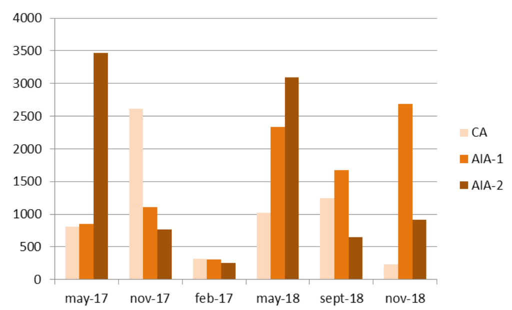

Figure 3: Seaweed standing crop in Tonnes Wet Weight (T_{WW}), between May 2017 and November 2018, in CA, AIA-1 and AIA-2 areas, 135 hectares each.

		CA	AIA-1	AIA-2
Spyridia filamentosa (Wulfen) Harvey	R	**X**	**X**	**X**
Gracilaria gracilis (Stackhouse) Steentoft	R	**X**	**X**	**X**
Gracilariopsis longissima (S.G. Gmelin) Steentoft, L.M. Irvine & Farnham	R			**X**
Polysiphonia sp.	R	X	X	X
Ceramium sp.	R	X		X
Alsidium corallinum C. Agardh	R	X	X	
Sphaerococcus coronopifolius Stackhouse	R		X	X
Dasia ocellata (Grateloup) Harvey	R		X	
Ulva rigida C. Agardh	C			**X**
Ulva prolifera O.F. Müller	C		**X**	**X**
Valonia aegagropila C. Agardh	C	**X**	**X**	**X**
Chaetomorpha linum (O.F.Müller) Kützing	C	**X**	**X**	**X**
Cladophora vagabunda (L.) Hoek	C			**X**
Cystoseira barbata (Stackhouse) C. Agardh	O	X	X	
Dictyota dichotoma (Hudson) J.V.Lamour.	O		X	X
n		8	11	12
% s		53.3	73.3	80
% os		50	45.5	66.7

Table 6: Floristic list of macroalgae observed in the 3 areas (CA, AIA-1, AIA-2) of the eastern basin of the Orbetello lagoon, between May 2017 and November 2018. R, Rhodophyceae; C, Chlorophyceae; O, Ocrophyceae; n, number of species; X, presence of species; X in bold, opportunistic species; % s, percentage of species observed; % os, percentage of opportunistic species in each group.

		CA	AIA-1	AIA-2
17-May	b	1,04±0,86	0,93±0.62	3,15±1,85
	CT	0.58	0.68	0.82
	SC	808	848	3468
	d	s, G>V	S, G, E	G>E, CH
17-Nov	b	2,25±1,56	1,44±1.61	0.86±0.62
	CT	0.68	0.57	0.66
	SC	2617	1104	768
	d	a, V	a, G, S	S, a, CH
18-Feb	b	0,60±0,88	0,41±0,32	0,37±0,39
	CT	0.4	0.55	0.51
	SC	322	306	257
	d	a, V	G, S	G
18-May	b	1.89±0.69	3,14±1,02	4,51±2,53
	CT	0.49	0.51	0.77
	SC	1021	2331	3098
	d	a>S	S>G, Cb	G>>S
18-Sep	b	2.31±1.23	2.25±1.61	0.94±0.36
	CT	0.4	0.55	0.51
	SC	1245	1672	647
	d	A	S	S, G
18-Nov	b	0.67±0.25	3.06±1.99	0.88±0.16
	CT	0.26	0.65	0.77
	SC	234	2688	913
	d	a>CH>V	S>a>G	G, CH

Table 7: Macroalgal Biomass (b) in kg m-2, Standing Crop (SC) in tonnes wet weight and dominant macroalgae (d) in the three areas of about 135 ha (CA, AIA-1, AIA-2), estimated in May and November 2017, and in February, May, September and November 2018. CH, *Chaetomorpha linum*; E, *Ulva prolifera*; S, *Sphaerococcus coronopifolius*; G, Gracilariaceae; s, *Spyridia filamentosa*; V, *Valonia aegagropila*; a, *Alsidium corallinum*.

As shown in table 7 and figure 3, in spring macroalgal biomass progressively increased from the CA control area to the AIA-2 area, where the biomass values were clearly higher than the other two areas. Two-way ANOVA showed a significant effect of the interaction between the factors month and area. In fact, the post-hoc SNK showed significant changes in biomass in May 2017 (CA=AIA-1≠AIA-2, $P<0.05$) and in May 2018 (CA≠AIA-1≠AIA-2, $P<0.01$) due to the important algal developments in AIA-2, and in November 2018 for higher biomass values in CA and AIA-1, compared to AIA-2 ($P<0.05$), while for the other 3 months the differences in biomass were not significant.

The pattern of biomass observed in the 3 areas, in the various sampling months, indicates that there were no significant biomass variations for CA, while AIA-1 showed significant changes between February 2018 and May 2018 and November 2018 ($P<0.05$); AIA-2, on the other hand, confirmed a significant variation in the biomass between the two spring months and the remaining months (May-17≠Nov-17, Feb-18, Set-18, Nov-18, $P<0.05$; May-18≠Nov-17, Feb-18, Set-18, Nov-18, $P<0.01$; the other months did not show significant differences between them).

The dominant vegetation was constituted by *Gracilariopsis longissima*>>*Chaetomorpha linum*>*Ulva prolifera* in AIA-2. Elsewhere, a winter carpet of low thickness prevailed, consisting mainly of a mixture of *Gracilaria gracilis* and *Spyridia filamentosa*, alternating, in the other seasons, a higher biomass with high dominance of *Alsidium corallinum* in CA, and *Sphaerococcus coronopifolius* in AIA-1 (Table 7).

In table 8, number of analysed samples (n), means (±SD) of C, N, P content in dried matter of *C. linum*, *U. prolifera* and *Gracilaria* spp., and the relative molar ratios C:N:P are reported, for each area in the May 2017 survey.

The sample numbers allowed only for *C. linum* a macronutrients content comparison by Student T-test, between the areas CA and AIA-2. There were no significant differences for the carbon ($P=0.6329$) and nitrogen ($P=0.0917$) content, while a significantly higher phosphorus content was found in AIA-2, compared to CA ($P=0.0108$).

C. linum and *Gracilaria* sp. showed similar C:N:P ratios in CA and in AIA-1, while lower values were found in AIA-2. The same result was found, though less markedly, for *U. prolifera*. Lowest C:N ratio were found in AIA-2 for *C. linum* and *U. prolifera*. Values of the N:P ratio around 30, indicating P-limitation [21,22], were observed in CA for *C. linum*, more markedly in CA and AIA-1 for *Gracilaria* sp. and slightly in CA for *U. prolifera*.

Carbon content remained substantially stable for the various species with the variation of the area, and *Gracilaria* sp. had the highest content. N values were discordant, higher in AIA-2 for *C. linum* and *U. prolifera*, but lower for the nitrophilous *Gracilaria* sp., while for the first two the lowest values were estimated in AIA-1. P was markedly higher in all samples collected in AIA-2.

Discussion

Water

The results of the water analyses showed a wide variability not only between the three areas, but also between the various sampling points within the same area. This suggests the not homogeneity of water masses, subjected to local phenomena. On the other hand, heterogeneous data sets are typical of transitional waters and the assessment of environmental quality is often carried out considering wide ranges of variation [23].

Some significant variations, however, are highlighted for salinity and temperature. The two fish farm wastewaters lower the salinity (significant changes in AIA-2 compared to the other two areas), despite the proximity of a marine mouth, thereby contributing to countering summer evaporation and mitigating the temperature, counteracting excessive lowering in winter and raising in summer. This result, on the one hand highlights the poor marine turnover in the 9 months in which there is the natural flow of the tides, on the other it suggests that the mitigation of salinity conditions could affect macro and microalgal growth, favoring it even in extreme seasons.

The fact that DO was found to be significantly lower in CA than in AIA areas in May 2017, could be the consequence of the significant increase in the presence of plant biomass in AIA-2, but, as in the case of NTU, which resulted significantly among all areas, many possible factors might have affected this result.

A gradient was detected for N-NO_3 and SRP, with significant increase towards AIA-2, especially for the last variable. The increase in P in AIA-2, closer to the fish farms discharge, is the cause of the significant reduction in the DIN:SRP molar ratio in that area compared to the other two.

		n	C	N	P	C:N	C:P	N:P
CH	CA	6	25.73±2.34	1.06±0.39	0.050±0.020	33.11±13.99	1402±488	44±8
	AIA-1	2	22.83±2.27	0.67±0.03	0.048±0.011	39.77±5.70	1260±169	32±9
	AIA-2	6	24.87±3.14	1.44±0.26	0.140±0.060	21.03±5.97	546±221	26±6
G	CA	2	39.81±3.59	2.65±0.53	0.110±0.013	17.72±1.95	933±22	53±5
	AIA-1	2	35.83±0.91	1.86±0.18	0.076±0.003	22.57±2.70	1214±83	54±3
	AIA-2	7	31.09±4.74	1.76±0.35	0.121±0.045	21.37±6.15	712±180	34±9
U	CA	1	19.19	1.02	0.06	22.06	828	38
	AIA-1	3	22.77±1.94	0.96±0.43	0.062±0.012	30.53±9.92	969±115	34±8
	AIA-2	4	22.96±3.84	1.60±0.62	0.094±0.027	17.98±4.41	616±143	31±3

Table 8: Carbon (C), Nitrogen (N), Phosphorus (P) percentage content on macroalgal thalli dried to constant weight at 75°C, and related atomic ratio (C:N:P; C:N), of *Chaetomorpha linum* (CH), Gracilariaceae not determined (G) and *Ulva prolifera* (U), collected in CA, AIA-1, AIA-2 areas, in May 2017. n, number of records.

As can be seen from table 2, N-NH_4^+, N-NO_x and DON conveyed with fish-farms wastewater were 42%, 37% and 16% for FF1, and 16%, 9% and 75% for FF2, respectively. These decidedly different percentages between the two fish-farms, although they breed the same species at the same densities, could be due to the different conformation of the settling basins and canals and to their dynamics, so it is possible that uptake or release processes and decomposition of plant masses take place at different times and quantities. Among the inorganic chemical species, N-NH_4^+ is normally dominant in fish-farm wastewater, since it is the major excretion product of nitrogen from fish [24], however, in the three areas it showed large fluctuations in data sets and the averages are paradoxically decreasing towards the source. On the contrary, the oxidized form prevailed in the area closest to the wastewater. This could be due to an intense nitrification favored by the high DO values produced by macroalgae, moreover, the same macroalgae that in AIA-2 are present with *Gracilaria-Gracilariopsis* dominance, could have subtracted particularly ammonium at spring temperatures [25].

Giordani, et al. [26], have hypothesized that the environmental quality value for the variable DIN is inversely proportional to the value of the variable itself. DIN values greater than 100 µM, correspond to the lowest quality (score=0), while values of DIN=0 µM to the highest quality (100). The same Authors suggested the best quality (100) is found at SRP=0 µM and the worst (0) at values >6 µM. Viaroli, et al. [23] suggested that, for transition environments, the optimal condition for good productivity is in the range from 0 to 20 µM of DIN, although other Authors considered 20 µM the critical threshold for the coastal lagoons for this variable [27,28]. Therefore, the concentrations of DIN in water found in the present study indicate scarce environmental quality in all three areas examined, including the control area. In fact, the average values of DIN ranged from 61 to 76 µM, while SRP values were lower than 1 µM.

DIN abundance in all the examined areas resulted in very high values of the DIN:SRP atomic ratio, which progressively decreases moving closer to the source. This indicates P-limitation in all the three areas [21,22], including AIA-2, the nearest to the nutrient input of fish farm wastewaters, while only for the wastewater of the two fish farms DIN:SRP reached the lowest values (39 for FF1 and 32 for FF2). However, it is clear that high values of the DIN:SRP ratio in AIA-1 and AIA-2, do not express a real limitation of P, but a relative deficiency of P, an imbalance of abundance, since N concentrations were decidedly greater than P, which is not lacking.

DON, in all the areas, and DOP, to a lesser extent, were the most abundant components of dissolved nitrogen and phosphorus in the water column, varying between 87% and 91% of TDN and between 46% and 79% of TDP. High values of DON and DOP have been reported in other studies of this lagoon [29-31], and are probably a characteristic of eutrophic environments with algal blooms, due to the release of cellular exudates and the presence of macromolecules that come from cell lysis, from the decomposition of plant masses and from bacterial extracellular enzymatic activities, in an environment that has a relatively modest water mass and abundant primary production. Different DON content in fish farms wastewater could be due to processes occurring in the settling basins.

Sediment

Nutrient concentrations in sediments showed a wide variability between the various sampling points within the same area, and between the three areas, as in the case of the water column. The wide variability does not allow to detect any nutrient gradient, not even for P, although C:P and N:P were significantly lower in AIA-2.

A significant difference was found for LOM between all the three areas, with unexpected higher values in CA and AIA-1 than in AIA-2. Higher values of LOM in areas more distant from the nutrient source, and the variability of nutrient data are probably due to the granulometric differences of the sediments of the sampling areas (Table 3). In fact, there is a significant inverse correlation between organic matter and Calcareous Detritus (cd) produced by shells (Figure 2), whose relative deficiency was probably the cause of the highest LOM values in CA and AIA-1. Sediments with sandy dominance and high quantities of coarse shelled debris retain the organic components to a lesser extent and favor interstitial oxygen penetration enhancing nitrification/denitrification processes [32]. This was the case of AIA-2, the area with the lowest LOM content, while the highest LOM values in AIA-1 and in CA were probably due to a relative lower content of sand. Sand was lower in AIA-1 than in CA, but the first had higher values of shell debris. These results did not allow identifying the fish farms input as a source of LOM that seems mainly due to the lagoon intrinsic dynamics.

Phosphorus in sediments is present as insoluble orthophosphate adsorbed by other mineral components [33,34]. It is probable the high variability in P accumulation, that was found within a same area and among the three areas, could be also attributable to the variable granulometry of the sediment.

N:P molar ratio in CA and AIA-1 sediments was similar to the average value of 30 estimated by Atkinson and Smith [21] in thalli of different macroalgal species, which would suggest that it derives directly from the decay of masses macroalgal, as substantially confirm the molar ratios N:P in the algal thalli, a little more P-limited (Table 8). In AIA-2, the values were decidedly lower than 30, indicating, although without statistical support, a lower relative presence of N, which reflects the N:P values of the thalli, but where this value is due to a greater accumulation of P.

Macroalgae

Macroalgae have provided clearer information than water and sediment variables. The algal biomass, in fact, showed an evident significant spring increase in AIA-2 of at least 4 times higher than in CA and AIA-1 (Table 7). Occasional high developments were also estimated in these latter areas, in CA in Nov-17, in AIA-1 in Nov-18. The high-density spring masses in AIA-2 have caused the summer decay of the macroalgae and, therefore, have determined the presence of a significant biomass reduction in the other seasons, with a slow recovery due also to increase of turbidity of the waters due to consequent microphitic developments. The development of biomass in AIA-2 was local and not produced by accumulations of vegetation carried by the wind, since the nitrophilous rodophycea *Gracilariopsis longissima*, the dominant species in AIA-2, has been observed only in this area; also the nitrophilous chlorophycea *Ulva prolifera*, that, even if present in AIA-1 and observed in an CA, was abundant only in AIA-2. That these macroalgal developments are to be considered endogenous, and therefore to exclude wind transports of vegetable masses, is also confirmed by Lenzi, et al. [35], according to which the 270 hectares of AIA were substantially sheltered from strong winds able to affect the bottom layer and transport the masses.

In this lagoon basin, two marine species are commonly found, *Alsidium corallinum* e *Sphaerococcus coronopifolius*, which seem to well tolerate the eutrophic conditions, degenerating to a large extent during the hot season, but developing shortly afterwards. Although thalli may occasionally be found everywhere, they have two distinct areas in which development is dominant: *A. corallinum*, in CA and widens a little towards the westernmost part of the eastern basin; *S. coronopifolius*, essentially in AIA-1. Therefore, the three areas of this study are characterized by dominance of different three algal species. However, the presence of the sea-lagoon communication channel in AIA-2 further could confuse the picture because typically marine species can be found in AIA-2 and AIA-1 occasionally conveyed with the waters of the incoming tide, as is undoubtedly the case of the Rhodophyta *Dasia ocellata* observed in AIA-1 in May 2018.

This horizontal distribution certainly cannot be random, also because it is stable over time, as we have observed over the years (unpublished data). Many factors may have affected this distribution: nutrients, salinity, sediment grain size, LOM accumulation, light radiation, hydrodynamism. Certainly, the nutrient intakes of fish farms may have contributed, as in part the N and P accumulations in thalli suggest, however the high nutritional availability can constitute a limit for many marine algal species, for which the optimal conditions are normally oligotrophy or mesotrophy, and they cannot tolerate lagoon eutrophy or hypertrophy due to continuous external nutrient inputs. Therefore, a relative nutritional abundance could be one of the factors that prevent the development of *A. corallinum* and *S. coronopifolius* in AIA-2. However, these two species could be further confined to their respective areas of dominance by salinity values. In fact, in CA there is salinity values similar to those of the near sea and quite stable, and in AIA-1 the values are a little lower and equally stable; it is in AIA-2 that the salinity values are the lowest, with greater variability than the other two areas, which is also accompanied by a greater thermal rise (Table 1). This combination of factors, to which the fish-farm wastewaters contribute to a great extent, could favor the *G. longissima* competition in AIA-2 but cause its summer decay, while the other two species find more favorable conditions in the other areas and result to survive to summer criticality.

C. linum physiologically tends to P-limitation, so as soon as phosphorus becomes available it accumulates this nutrient and then uses it when needed [36], therefore this behavior would explain its relative abundance in AIA-2, compared to the other two areas, although less competitive than *G. longissima*. Although the records were relatively few, both *G. longissima* and *U. prolifera* showed the highest value of P among all the samples analysed in AIA-2, highlighting a predisposition to eutrophication of these species.

Conclusion

In a non-tidal lagoon, water column and sediment can present large variations of environmental variables, both chemical-physical and nutritional, in a very complex dynamic, influenced by many factors: The wind thrust on the lagoon water masses; the tides, however weak; the sea front (sea level that lowers or rises following the wind direction, favoring the outflow of the lagoon waters towards the sea, or preventing it); the stratification of water masses with different thermal and salt characteristics; the sediment resuspension by wind and human activities; the natural predisposition to eutrophication; sedimentary texture; bioturbation by the infauna and fish schools. All these factors produce a complex picture and can hinder the source of impact whose environmental impact is to be established.

In the case under examination, the heterogeneous data sets did not allow, for all the considered variables, a clear assessment of the environmental impact of the two fish-farm wastewaters in the area for impact assessment. For the dissolved nutrients in the water column, only N-NO_3 and SRP were significantly higher, and DIN:SRP atomic ratio significantly lower, in the area closest to wastewaters discharges, compared to control area. For the sediments, among all the variables, only C:P and N:P showed significant values in AIA-2 to support a nutrient impact. By contrast, LOM had significantly lower values in AIA-2, contrary to expectations. It was clear that the presence of detrital organic matter is strongly influenced by sedimentary texture, which sees in AIA-2 greater presence of sand and limestone detritus and shell >1 mm, which favor both oxidation and removal.

Macroalgae, on the other hand, provided clearer information than water and sediments, both in terms of biomass, higher in the area closest to wastewaters discharges than in the other two areas, and in terms of the number of opportunistic species, more present in AIA-2. A different horizontal stratification of macroalgal species was highlighted in this study, with the dominance of *G. longissima* in AIA-2, *S. coronopifolius* in AIA-1 and *A. corallinum* in CA, although for these last two species it was not possible to establish if and to what extent their location depends on the influence of fish-farm wastewater. In a previous study concerning the influence of wastewater from urban treatment plant in the western basin of the same lagoon, a clearer horizontal stratification of nitrophilous and P demanding species was observed near the nutrient input.

Also for the tissue content of the *C. linum* thalli, it was possible to highlight a significant difference due to the higher levels of P in the thalli collected in AIA-2 compared to those collected in CA, and also the few records of the other two algal species have shown the same behavior; this suggests macroalgae can better highlight the contributions of P, compared to sediment variables, due to the differences in texture. Eventually, the incidence of fish farm wastewaters on macroalgae seemed to occur predominantly in AIA-2 area.

However, even for submerged vegetation there can be numerous factors that can confuse, mask or amplify the extent of the impact. For example, even in other areas of the same lagoon, the wind can bring the pleustophytic algal masses away from the area of influence and accumulate in different areas; therefore it is necessary to take these factors into account.

This study suggests that the variables that can better and more quickly highlight a eutrophic anthropogenic impact in an already eutrophic, low-turnover and shallow water environment, such as lagoons or estuaries, are those related to macroalgal vegetation. The water and sediment variables require many sampling points, relatively long times and a complex study plan, with a strong uncertainty on the final result. On the contrary, the picture provided by the qualitative-quantitative distribution of macroalgae and their content in tissue macronutrients can quickly provide a reliable assessment of the extent of anthropogenic impact.

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