Determination of phytoplankton in water samples, algal biotoxins, microbiological parameters and microplastics in Mediterranean mussels (*Mytilus galloprovincialis* Lamarck, 1819) from an experimental pilot farm in the Calich Lagoon (Sardinia, Italy)

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Abstract

The aims of this paper were to collect and analyse preliminary data of phytoplankton in the water, biotoxins, *Escherichia coli*, *Salmonella* spp., *Vibrio* spp. and microplastic eventually present in farmed mussels, and to acquire information about the production capability from an experimental pilot farm of the Calich Lagoon. Two sampling sessions were carried out, in February and in May 2019, also monitoring the water condition (pH, temperature, salinity, dissolved oxygen, chlorophyll *a*). No potentially toxic algae were detected, and moreover no biotoxins (Paralytic Shellfish Poison, Diarrheic Shellfish Poison, Amnesic Shellfish Poison) were found in mussels. *E.coli* was present with the highest concentration in February (16000 MPN/100g e.p.). *Salmonella* and *Vibrio* spp. have not been detected. Almost a microplastic per grams was found, mainly fiber of different colours. Further studies, carried out for several months, will allow to better understand the possible problems related to the production of mussels in a lagoon not yet classified as a shellfish production area.

Introduction

Mediterranean wetland areas play an important economic part in the surrounding community, representing economic resources for fishing, agriculture, and touristic activities.

In Europe, the production of Mytilus species increased by 7%, the Italian production contributing in 2018 with 64×10^3 tonnes (EUMOFA, 2018). In Sardinia the mollusc farming has a long history and is an important sector for the regional economy. The increased development of various forms of aquaculture in the Mediterranean lagoons (Cataudella *et al.*, 2015), has raised the attention to multiple factors linked to climate change and variability that may affect food safety connected to shellfish consumption (Tirado *et al.*, 2010). Molluscs may be contaminated because of their nature as suspension feeders, by taking phytoplankton, zooplankton, bacteria, and inorganic matter from the surrounding water. Mussels can be a vehicle for the transmission of various diseases as a result of their consumption. In Europe the sanitary control of shellfish produced and sold for human consumption is based on the monitoring of *Escherichia coli,* considered a common indicator organism of faecal contamination in aquatic systems (Noble *et al.*, 2004), and *Salmonella* spp. (Reg. (EU) No 625/17 , Reg. (EU) No 627/19, Reg. (EU) No 2073/05).

As reported in EFSA and ECDC (2021), *Salmonella* spp. is the second most common cause of human gastroenteritis. In the aquatic environment, there are commonly find *Vibrio* spp. (Thompson *et al.*, 2004), some of which are involved in important infections, specifically *Vibrio parahaemolyticus, Vibrio cholerae*, and *Vibrio vulnificus* (Caburlotto *et al.*, 2011). Equally important is the possible presence of algal biotoxins produced by potentially toxic harmful algal species, such as Paralytic Shellfish Poison (PSP), Diarrheic Shellfish Poison (DSP) and Amnesic Shellfish Poison (ASP). Microplastics (MPs) in the marine environment have become a major environmental concern over the last years (Tsangaris *et al.*, 2021). Plastic are synthetic polymers, originated by the polymerization of organic and inorganic elements, such as carbon, silicon, hydrogen, oxygen and chloro (Shah *et al.*, 2006), petroleum based (Thompson *et al.*, 2009). A significant percentage of plastic produced in the world end up in the oceans (Thompson *et al.*, 2009). Increasing attention has been paid by the researcher towards the presence of plastic fragments, defined by Arthur *et al.* (2009) microplastics, as particles with size inferior to 5 mm. Bivalve molluscs are of particular interest because their feeding strategies expose them to particles present in the water column.

The main aims of the present study were: a) to evaluate the occurrence of different microorganisms (*E.coli*, *Salmonella* spp., *Vibrio* spp.), and items in *Mitylus galloprovincialis* from an experimental pilot farm of the Calich Lagoon, a Sardinian lagoon (Italy); b) to analyse the phytoplanktonic population in the lagoon water; c) to assess the possible presence of biotoxins in *M. galloprovincialis.*

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Materials and methods

Sampling and analysis

Our study was carried out from February 2019 to May 2019, 1 sample of 2 liters (L) of lagoon water was sampled each month to analyse the present phytoplankton population. Water samples were taken using clean polyethylene bottles at a deep of 0.5 m. Two mussel samples (one in February 2019 and one another in May 2019) were collected and analysed to investigate the possible presence of different microorganisms (*E. Coli*, *Salmonella* spp.,*Vibrio* spp.), biotoxins, including domoic acid (DO, responsible for ASP), PSP group (responsible for PSP), LTs group (including Okadaic acid (OA) and its derivates), DTXs and associated esters (responsible for DSP) and besides: Pectenotoxins, Yessotoxin, Gimnotoxins, Spirolides, Pinnatoxins, Portimine and Azaspiracid (Wu *et al.,* 2019). Possible presence of MPs was also investigated.

Study area

The Calich Lagoon is situated in the Porto Conte Regional Natural Park, along the north-western coast (40°35′ 47.5″N; 8°17′59.9″ E) of Sardinia (Italy) (Figure 1). The lagoon extends for 92 ha with a depth varying between about 0.5 and 1.5 m. It is connected with the Alghero Gulf through a natural channel (60-m wide and 2-m deep) hosting a medium size shipyard and a tourist harbour. The lagoon receives freshwater from two natural fluvial tributaries and an artificial channel (Pulina *et al.*, 2017; Esposito *et al.*, 2021). The Calich Lagoon suffers of high eutrophication due to urban, agricultural, and industrial activities (Fenza *et al.*, 2014; Bazzoni *et al.*, 2018). Its catchment area extends for about 42,000 ha, receiving water from several municipalities. Water temperature in the Calich Lagoon follows a seasonal trend with the highest values between July and August (Baralla *et al.*, 2017). The Calich Lagoon is a highly productive environment (Bazzoni et al., 2019) and several studies (Chessa *et al.,* 2005; 2007; Pais *et al.,* 2006; 2007; Cannas *et al.,* 2011; Serra *et al.,* 2011) suggested that shellfish farming should be a sustainable exploitation strategy for this lagoon. Previous authors pointed out the occurrence of biological and chemical contaminants in native shellfish (Sedda *et al.*, 2016; Baralla *et al.*, 2017; Bazzoni *et al.*, 2019; Esposito *et al.*, 2018; 2021) and studied the ecology of different planktonic components (Ielmini *et al.*, 2014; Pulina *et al.*, 2017, 2018; Bazzoni *et al.*, 2018; Satta *et al.*, 2020).

Although the high abundance of natural beds of highly valuable commercial species of bivalves *e.g.,* grooved carpet shell (*Ruditapes decussatus*), olive green cockle (*Cerastoderma glaucum*), and Mediterranean mussel (*M. galloprovincialis*), the Calich Lagoon has not yet been classified as shellfish production area (Esposito *et al.*, 2018; 2021; Bazzoni *et al.*, 2019).

Experimental mussel pilot farm

The experimental mussel pilot farming was carried out in suspension, on a small "Trieste" type farm plant consisting of 2 single ropes supported by wooden poles fixed deeply in the muddy sediment of the lagoon and emerging from the water for about 1 meter. The mussels were inserted in n.60 droppers seeded with plastic stockings (80 cm length and 38 mm mesh) filled with tiny mussels (≈ 3.5 cm length and 1.9 cm width). The experiment began in December 2018 and continued until the achievement of the minimum commercial size (5 cm in length).

Water samples and phytoplankton analyses

A total of four monthly water samples were analysed from February to May 2019 to identify and quantify the phytoplankton taxa present in the mussel breeding site. Two liters (L) of water samples were taken using clean polyethylene bottles, of which, 1 L was preserved in situ with Lugol's iodine to be used in the microalgal species counts and 1 L for *in vivo* observation of phytoplankton. Samples were delivered to the laboratory under refrigerated conditions. Accordance to the EU reference method UNI EN ISO 15204:2006, the cell count was performed by means of Utermöhl's method (1958) on settling chambers (10 cm^3). An inverted microscope Olympus IX 73 (Olympus, Shinjuku, Tokyo, Japan) was utilized for the determination and enumeration of phytoplankton at magnifications of 200X and 400X.

Determination of physical and chemical parameters of water

Monthly temperature (°C), pH, salinity (psu), dissolved oxygen (mg L^{-1}) and chlorophyll *a* (µg L^{-1}) in Calich Lagoon water from February 2019 to May 2019 were recorded *in situ* with a multiparameter probe (Ocean Seven 316 Plus CTD, Idronaut, Brugherio, Italy).

Shellfish samples

Two mussel samples composed of 60 *M. galloprovincialis* specimens each, were collected in February 2019 and in May 2019 from one sampling point of the experimental pilot farm (Figure 1). All samples were stored in refrigerated bags and immediately brought to the laboratories to be analyzed within 24 hours.

Toxin analyses

Starting from shellfish tissue, according to the AOAC 2005 Official Method 2005.06, the determinations of PSP toxins were performed. The LTs toxins were analysed according to the Regulation (EC) 15/2011 by means the liquid chromatography-tandem mass spectrometry approach (LC-MS/MS). The extraction procedure is reported in AESAN EU-RL-MB Lipophilic toxins Version 5: 2015.

Finally, the DO acid was detected

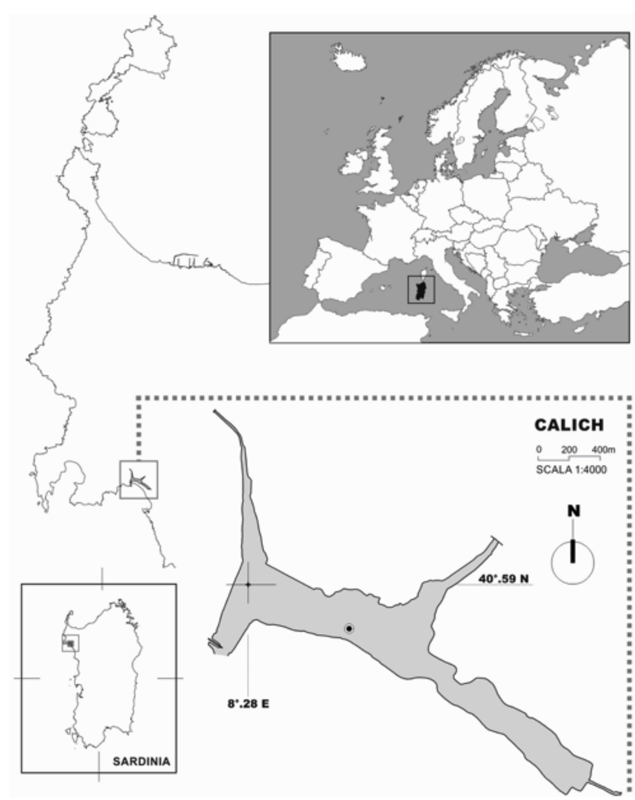

Figure 1. Study area.

according to the Standard Operating Procedure AESAN, 2008. The description of the methods has been previously described in Mudadu *et al.*, 2021.

E. coli

The enumeration of *E. coli* was conducted on 100 g of sample using the Most Probable Number (MPN) method (ISO 16649-3) according to Bazzoni *et al.,* 2019.

Salmonella spp.

The analyses were performed according to the ISO 6579-1:2017. The description of the method has been previously described in Bazzoni *et al.,* 2019.

Vibrio spp.

The analyses for the determination of *Vibrio* spp. were performed according to the ISO/TSS 21872-1-2:2017 method with two pre-enrichment steps. The method has been previously reported in Lorenzoni *et al*., 2021.

Microplastics

The determination of MPs in mussels was performed according to an internal protocol based on Phuang *et al*. (2018). The digestion efficiency and the recovery rate have been determined according to Karami *et al.,* 2017. These values have been calculated as follows:

$$\text{Digestion efficiency (\%)} = \frac{Wi - (Wa - Wb)}{Wi} \times 100$$

Wi= Initial weight of biological materials; Wa=Weight of dry filter membrane after filtration.

Wb = Weight of dry filter membrane before filtration.

$$\text{Recovery rate (\%)} = \frac{Wa - Wb}{Wi} \times 100$$

Wa=Weight of dry filter membrane after filtration, and Wb =Weight of dry filter membrane before filtration, and Wi=Initial weight of spiked MPs.

The MPs determination was performed only in May, because the mussels from the previous sampling in February 2019 were used to test the protocol. Samples were collected, immediately posed in cooled bags and transported to the laboratory of the Istituto Zooprofilattico Sperimentale della Sardegna within two hours of collection. The samples were frozen at -20°C until analysis. Our method was based on the use of a stereomicroscope as a first step for a preliminary evaluation of items that potentially could have been made of plastics. All mussels were firstly rinsed with distilled water. Each sample was composed by 3 mussels randomly selected. During the experiment were considered 2 groups of mussels, called group A and group B. Length, width and weight of each mussel was determined. KOH 10% (Potassium Hydroxide Pellets, Carlo Erba, Milano, Italy) was added in each flask, the KOH quantity was determined on the basis of the weight difference of the flask with muscle tissue minus the weight from empty flask and the value obtained was multiplied by 3. For each group was prepared a procedural blank (WH), which was a sample with KOH 10% without mussels. In addition, to assess the possible presence of items in the laminar flow hood, a filter was placed in a glass Petri dish located near the workstation. The flasks were stored to 48 h at 60°C. Afterwards the samples were filtered using 5 µm nitrocellulose filters and 47-50 mm in diameter (Merck TM SSWP04700), with the aid of a vacuum pump (Thermo Savant VLP200 Valu Pump, Thermo Fischer Scientific, USA). Consequently, filters were placed in glass petri dishes and allowed to dry at room temperature for 24 hours. As a final step, filters were observed by means a stereomicroscope (LEICA M205 C, Microsystems Gmbh, Germany). MPs were classified according to a morphologic classification, by shape (sphere, fiber, fragment, etc.) and color (white, black, red, blue, green, other colors, transparent, opaque, etc.), and counted. Several characteristics should be indicative of a non-biologic origin. Major plastic features are the absence of repetitive structures indicative of biological origin, homogeneous colouring unless due to transparency, and in case of fibrous forms equal thickness and three-dimensional bending (Enders *et al.,* 2015).

Results

Determination of physical-chemical parameters of water

Table 1 shows the values of temperature, salinity, dissolved oxygen, concentration of chlorophyll *a* and pH of the water, recorded by means of a multiparametric probe at the experimental pilot plant. The values of salinity, dissolved oxygen and pH were in the desirable ranges for lagoon shellfish areas and appeared almost always within the survival limits of *M. galloprovincialis* (Specchiulli *et al.,* 2008; Serra *et al.*, 2011).

Phytoplankton community and biotoxins

Between February to May 2019, a total of 24 taxa were observed (Table 2). The “Ultraplankton”, a category with organisms too difficult to classify because of their small size (<5µm; Murphy and Hagen, 1985), is also considered. The mostly represented class was Bacillariophyceae, with 17 taxa. Other classes were Dinophyceae (3 taxa), Cryptophyceae (1), Dictyophyceae (1), Chlophyceae (1) and Pyramimonadophyceae (1). The highest abundances were observed in March, with in a peak of *Cyclotella* sp. (57×10^6 cells/L) and *Skeletonema costatum* (41×10^6 cells/L). Ultraplankton was always present with abundances relevant throughout the period considered, with a peak in May (95×10^6 cells/L). No potentially toxic algae were found, moreover no biotoxins were found in mussels.

Bacteria

E. coli was found with a concentration of 16000 MPN/100 g in February and 1300 MPN/100 g in May. *Salmonella* spp. and *Vibrio* spp. were never detected.

Microplastics

The digestion efficiency was 96% and the recovery rate was 95%. In all groups of mussels were detected fibers, while fragments were found more rarely. Items were found in both May sampling groups (18 items in group A and 21 items in group B) and the results are summarized in Table 3. As shown in Table 4, an average of one item per grams of muscle has been pointed out.

Table 1. Determination of physical-chemical parameters and concentration of chlorophyll *a* in Calich Lagoon’s water in 2019.

Month	Depth (cm)	Water temperature (°C)	Salinity (psu)	Oxigen (mg L^{-1})	Chlorophyll a (g L^{-1})	pH
February	60	10.7	1.8	4.4	1.3	7.9
March	60	14.8	18.3	7.1	1.95	8.2
April	60	17	32.3	6.4	1.15	8.6
May	60	14	23.5	7	0.71	9

Discussion and conclusions

This paper reports the results on the determination of phytoplankton in water samples, algal and their biotoxin, microbiological parameters and MPs in Mediterranean mussels from an experimental pilot farm in the Calich Lagoon (Sardinia, Italy), a brackish environment located in an area of strong anthropogenic impact.

No potentially harmful algae, able to determine PSP, DSP or ASP events, has been detected. Several classes have been found, in particular *Bacillariophyceae*. Microalgae are a food source for mussel population and *Bacillariophyceae* represent a well know source of food for bivalve molluscs (Muniz *et al.*, 2017).

According to the Regulation EU 625/2017 and the implementing regulation 627/2019 regarding the microbiological quality for the production and placing on the market of bivalve molluscs, the overall results of the present study suggested a possible classification of the Calich Lagoon as Zone B. However, the seasonal fluctuations of *E.coli* counts in mussels should be continuously monitored, *Salmonella* spp. and *Vibrio* spp. were not found in shellfish samples collected in spring. On the contrary, Bazzoni *et al.* (2019) detected these bacterial pathogens in spring sampling sessions.

Table 2. List of Phytoplankton taxa and species detected with relative cellular abundances (cells/L) from February to May 2019 in the Calich Lagoon (Sardinia).

Algal Class	Algal species	February	March	April	May
Bacillariophyceae	*Achnanthes* sp.	<300	<300	400	<300
	Amphora sp.	3893	<300	3958	<300
	Chaetoceros subtilis	<300	756146	117054	642576
	Chaetoceros tenuissimus	<300	<300	3958	<300
	Cocconeis sp.	<300	<300	32458	491570
	Cyclotella sp.	<300	55898550	198294	19773
	Fragilaria sp.	3142	<300	<300	<300
	Licmophora sp.	<300	<300	<300	9500
	Melosira spp.	11458	2049	600	<300
	Melosira varians	2083	<300	<300	<300
	Navicula spp.	7663	7197	23373	4750
	Nitzschia sp.	<300	7661	<300	15797
	Skeletonema costatum	<300	4121077	949057	<300
	Synedra sp.	<300	992	100	<300
	Thalassiosira sp.	7663	3074	<300	116320
	Tryblionella sp.	61430	<300	<300	<300
	Entomoneis sp.	<300	<300	<300	<300
Dinophyceae	*Gymnodinium* sp.	<300	<300	<300	5523
	Kryptoperidinium foliaceum	<300	11075	<300	<300
	Minuscola bipes	<300	7197	<300	<300
Chlorophyceae	*Tetraselmis* sp.	<300	<300	3958	23750
Cryptophyceae	*undetermined Cryptophyceae*	11433	965555	484500	141396
Dictyophyceae	*Apedinella spinifera*	<300	<300	15323	<300
Pyramimonadophyceae	*Pyramimonas* sp.	<300	183174	<300	<300
ultraplankton		17367188	13364022	51471000	95285000

<300 cells/L = detection limited

Table 3. Type and quantity of microplastics in Calich Lagoon's mussels in May 2019.

Mussels group A	Quantity	Mussels group B	Quantity
Light blue fiber	6	Light blue fiber	14
Dark blue fiber	6	Dark blue fiber	4
Red fiber	1	Red fiber	2
Dark fiber	3	Dark fiber	n.d
Transparent white	2	Transparent white	n.d
Fragments	n.d	Fragments	1

n.d: not detected

Table 4. Ratio of MPs found to the weight of the sample analysed.

Sampling data	Weight (g) Group A	Length (cm) Group A	Width (g) Group A	Weight (g) Group B	Length (cm) Group B	Width (g) Group B	Total Weight group A (g)	MPs/grams of muscle group A	Total Weight group B (g)	MPs/grams of muscle group B
6/5/2019	7.9	49.6	26.40	4.8	44.04	21.63	17.5	1.02	14.4	1.45
	4.9	45.02	25.26	5.1	45.47	23.66				
	5.3	45.23	23.35	4.5	46.00	22.26				

In recent years, MPs have aroused interest in the scientific community, in particular for their presence in the digestive system and in the tissues of marine species. Our results showed that both concentration and type of the detected items (mainly fibers) were similar in the two batches. According to previous authors (Avio *et al.,* 2015, Corami *et al*.,2020) they confirmed the capacity of MPs accumulation in the mussels. Mussels may be utilized to assess the abundance of MPs in the marine environment. At present, no specific national or European legislation indicated the maximum levels of MPs that can be found in the tissues of bivalves intended for human consumption. The role of mussels as source of MPs for humans is still scanty and a real evaluation of the human risk due to the MPs ingestion in mussels is still under investigation (Catarino *et a*l., 2018, Barboza *et al.,* 2018). The airborne MPs contamination should be considered as a major risk for the human health (Prata, 2018).

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