Materials and methods

1. The site

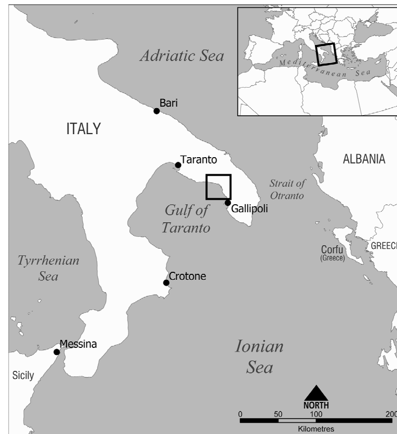


Fig 5.1 The South East of Italy where is located the study site .

The experiment was carried out for 12 months, starting in June 2007, at 6m depth, inside the no take zone of the Marine Protected Area of Porto Cesareo (SE Italy Fig 1.1). This area is a rocky-sand peninsula gently sloping from the surface to 4 – 6 m depth with a calcareous rocky plateau, extending for 30-60 meters until a vertical slop of about 5 meters. At this depth, rocky substrates turn into sandy substrates.

The MPA (40 ° 14 'N, 17 ° 54' E) (Fig 1.2) was established through the Ministry of Environment decree in the 1997. The 32 km of coast of the MPA falls in the municipalities of Porto Cesareo and Nardo, both in the province of Lecce. This MPA is on the Ionian Sea and it is featured by an heterogeneous coast including both rocky and sandy habitats.

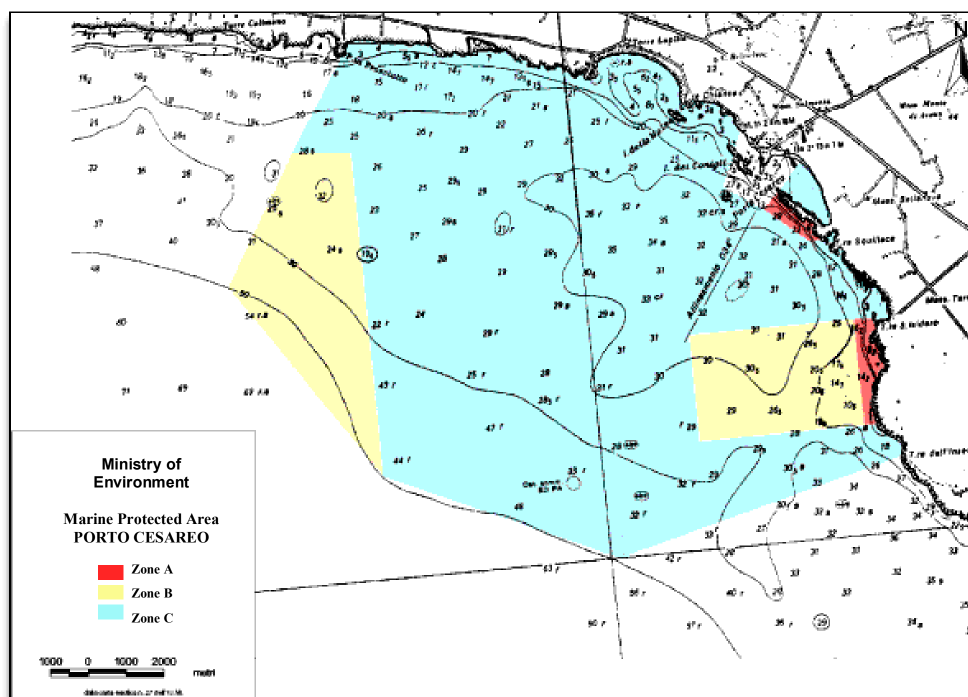


Fig 1.2 Chart of the Marine Protected Area of Porto Cesareo

The MPA covers an area of 16.654 hectares and is divided into 3 zones with different degree of protection:

Zone A – No take no man zone: it includes two areas, both extending up to 500 m from the coast. The first is located in front of the "Strea" Peninsula and the second is the stretch of coast going from Torre S. Isidoro to "Casa Giorgella";

Zone B – The buffer zone is also made up of two areas, one in the western boundary of the MPA and the other one delimiting one of the A zones;

Zone C – it includes the remaining area of the MPA.

The study area covers about 1000 m along the coast and it falls within one of two A zones, in front of a peninsula known as "La Strea" (Fig 1.3)

Fig 1.3 Study site: the rocky plateau included in the yellow rectangle.

2. The experiment

2.1. Preliminary survey and experimental setup

We assessed the morpho-bathymetric features of the study area, the biological information about the algal community structure, sea urchins density and the trophic status in terms of nutrients and chlorophyll concentration. Sea urchin density was

estimated at the beginning, in the middle and at the end of the experiment counting individuals within 15 replicates of 1m2, randomly allocated in the study site.

Plots were enriched by a slow-release fertilizer (Osmocote-pro 18N:9P:10K, Scotts Company), contained in small mesh bags fixed on the rocky substrates (Fig 2.1.1).

Fig 2.1.1 The mesh bag with the fertilizer inside used to simulate the enrichment directly on the substrate

Properties of this fertilizer have been already tested in other experimental studies. It has particular dissolution features so that nitrogen and phosphorus levels can be maintained well above natural ambient concentrations during the entire period of experiment (Worm *et al.* 2000; Heck *et al.* 2000; Tracker *et al.* 2001; Littler *et al.* 2006). The concentration of nutrients and photosynthetic pigments in the water was measured collecting replicated samples in each plot.

Fig 2.1.2 The 12 points randomly choose where plots were been placed

Sampling was carried out in 25m^2 plots chosen randomly along 1 km of the rocky plateau, separated by approximately 20 m.

Spatial distribution of 12 experimental plots was decided through a specific tool associated with the GIS software package (Arcgis 9.1 ref). The random selection was done out of 25 geographical positions with similar features (Fig 2.1.2).

The presence of grazers was manipulated removing by hand all

sea urchins at the beginning of the experiment and every time was necessary through a twice-weekly monitoring activity. To better control the potential invasibility of the open plots to sea urchins, the invertebrates were also removed from a buffer zone around each plot of about $10m^2$. Four conditions were simulated: grazing × no enrichment (for a total of 4 plots: 2 control plots without experimental treatments and two plots to test potential artefacts due to the presence of mash bags), enrichment × no grazing, grazing × enrichment and no grazing × no enrichment.

2.2. Benthic percent cover estimation

Fig 2.2.1 Photographic sampling on one of the area with no treatments

Each time, 10 randomly photographic replicates of 16×23cm were sampled, for a total of 720 replicate units (Fig 2.2.1). All the slides were analysed in lab imposing a virtual grid of 24 equally sized squares (Fig 2.2.2) to help the quantification of taxa. Abundances of sessile benthic species were estimated by visual percentage cover estimation (Meese and Tomich 1992; Dethier *et al.* 1993): within each of the 24 squares defined by the grid, which has been superimposed on the sample unit, the abundance of each taxon is evaluated by assigning it a percentage value between 0% and 4% : nil value in the event of total absence of the taxon in question into one of the 24 squares in question, 1% where the taxon is present in 1/4 of the surface of the square, 2% if the covering layer affects 1/2 of surface of the square, 3% in case of presence of the taxon in 3/4 of the surface and, finally, 4% of the area where the coverage of the square by the taxon is total. In case of organisms that occur less than 1/4 of the surface of the square was still assigned a coverage rate of 1.

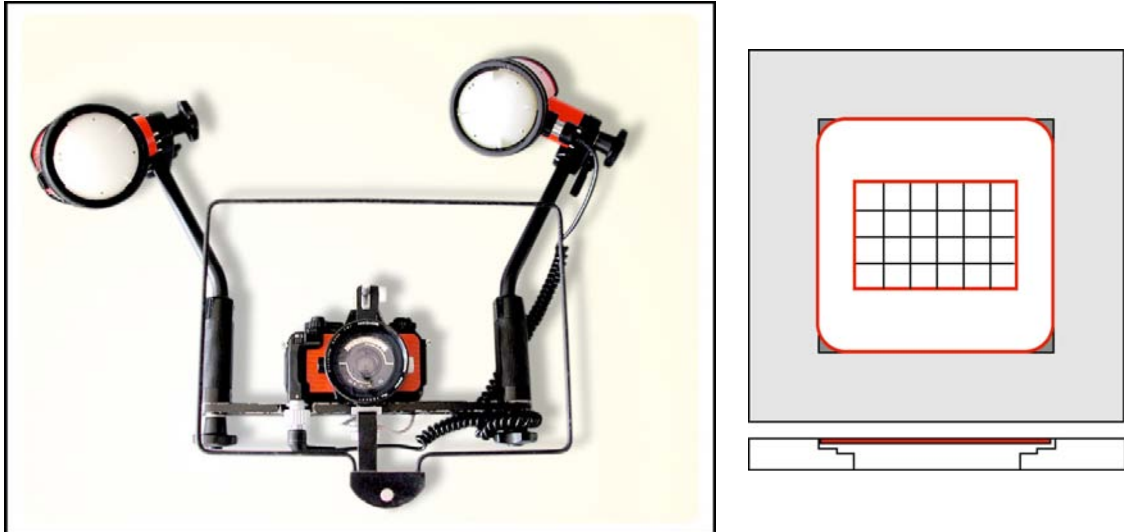


Figura 2.2.2 Photographic equipment and the 24 equally sized squares grid used to estimate the covere of benthic organisms and the percentage of bared rock.

Summing the estimation in each of 24 squares for each taxon, we obtained the total coverage of each taxon for each separate sampling unit

Motile animals (e.g. gastropods, ophiuroids and amphipods) were not counted.

When the identification at the species level was not possible, taxa were identified to the genus, or family level, and in some cases in morphological group. In a second time all taxa have been also grouped for functional characteristics according to Steneck and Dethier (1994) reviewed by MARBEF Team of the BIOFUSE project (MARBEF network, 2006).

2.3. Nutrient enrichment

2.3.1. Water sampling

Water samples were collected every three month in order to estimate photosynthetic pigments distribution and monitor the concentration of nutrients in each plot. Chemical analyses were conducted in the laboratory of Ecology of the University of Salento to measure the concentration in water of phosphate (Deniges 1920), nitrite (Bendschneider and Robinson 1952), nitrate (Morris e Riley 1963), ammonium (Aminot and Chassepied 1983).

A Niskin bottle was used for water samples, allowing collecting a volume of about 5 litres at specific depths.

2.3.2. Analytical methods

The analytical methods used to measure the concentrations of nutrients (particularly phosphates, nitrites, nitrates and ammonium) and the phytoplankton are based on the analytical protocols proposed by the Istituto Centrale per la Ricerca Scientifica e Tecnologica Applicata al Mare (ICRAM) and by the Institute of Water Research (IRS).

Phosphates

The analysis model is based on the formation of a blue phosphomolybdic complex whose concentration is determined by spectrophotometer (Deniges, 1920). Given the remarkably low concentrations of phosphate and the relative sensitivity of the analytical methods, at least two determinations for each sample to be analyzed should be made. Once the procedure followed for sample preparation is done on a spectrophotometric assay to measure the absorbance at 882 nm of the cuvettes in which it was spilled the solution. Then, we proceed to calculate the concentration of phosphate following the next report:

$[PO_4] = (ABS - bl - blc, i) \cdot f$

where

$[PO_4]$ = concentration of phosphate (expressed in μmol / L)

ABS = absorbance of the sample

bl = blank reagent

blc, i = i-th white cell used

f = colorimetric factor

Nitrite

The method is based on a series of reactions that lead to the formation of a diazo compound which is determined by colorimetry. This procedure is specific for nitrites and has no change in efficiency in relation to the ionic strength of the solution and has been applied to the analysis of seawater from Bendschneider and Robinson (1952). After performing the analytical procedure for the preparation of test samples, the spectrophotometric assay has been performed for measuring the absorbance at 543 nm of the solutions inside the cells. Then, the concentration was calculated according to the report:

$[NO_2^-] = (ABS - bl - blc, i) \cdot f$

where

$[NO_2^{-}]$ = concentration of nitrite (expressed in μmol / L)

ABS = absorbance of the sample

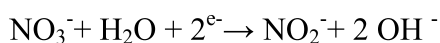
bl = blank reagent

blc, i = i-th white cell used

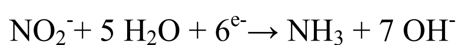
f = factor colorimetric

Nitrates

The method used for the analysis of nitrate is based on their reduction in heterogeneous phase, nitrite and its subsequent determination of total nitrite using a colorimetric method. The method was introduced by Morris and Riley (1963), but later was deepened by Nydhal (1976), Grasshoff (1983). The main reactions in alkaline environment, are



$NO_3^{-} + H_2O + 2^{e-} \rightarrow NO_2^{-} + 2\ OH^{-}$



$NO_2^{-} + 5\ H_2O + 6^{e-} \rightarrow NH_3 + 7\ OH^{-}$

Once the solutions is prepared, we proceed to analyze the spectrophotometric assay, using the same technique above descrbed for nitrates, but taking into account the following relationship for calculating the concentration of nitrites:

$[NO_3^{-}] = (ABS - bl - bl\ c, i - [NO_2^{-}] / f2) \cdot f1$

where

$[NO_3^{-}]$ = concentration of nitrate (expressed in μmol / L)

$[NO_2^{-}]$ = concentration of nitrite in the sample (obtained independently and expressed in μmol / L)

ABS = absorbance of the sample

bl = blank reagent

blc, i = i-th white cell used

f1 = factor colorimetric nitrate

f2 = factor for nitrite colorimetric

Ammonia

The proposed method is based on a series of reactions catalyzed photo-chemically, leading to the formation of blue indophenol. This methodology was first used by Berthelot (1859). Even here, after having prepared the solutions for analysis by the

appropriate analytical procedure, we proceed to determine the concentration of ammonia through the spectrophotometric assay, measuring the absorbance of the samples at 640 nm. The formula to calculate the concentration of reference is the following:

$$[NH_4^+] = (ABS - bl - blc, i)\ f$$

where

$[NH_4^+]$ = concentration of ammonia (expressed in µmol / L)

ABS = absorbance of the sample

bl = blank reagent

bl c, i = i-th white cell used

f = factor colorimetric

Chlorophyll

"Chlorophyll ***a***" is the most common photosynthetic pigment, since widespread among plants. This pigment is present in all algal groups, while other chlorophylls (b, c and d) are specific of some groups. The cellular content of chlorophyll in algal cells varies between 0.3 and 2.0% of dry weight, depending on the physiological state of cells or their adaptation to particular conditions of light radiation. When the algal cells die and are decomposed, the chlorophyll is degraded and forms other molecules called feo-pigments. Often the relationship between chlorophyll and these pigments provides valuable information on the state of health of a population of phytoplankton.

The analytical model used to determine the concentration of chlorophyll a is based on the fluorimetric method based on the measurement of fluorescence acetone extract of pigments before and after acidification with hydrochloric acid. In this way, is possible to measure photosynthetically active fraction (chlorophyll *a*) and inactive (feo-pigments) of pigments present in samples. The primary method used was the Holm-Hansen and Riemann (1978) which offers significant advantages over the spectrophotometric methods because it is more rapid, precise and sensitive.

2.4. Statistical analyses

2.4.1. Univariate analyses

The experimental design included four factors: Time (T, nine levels, fixed), nutrient Enrichment (E, two levels, fixed, crossed with Time), Grazing (G, two levels, fixed, crossed with Time and Enrichment) and Plot (P two levels, random, nested in the three factors) with ten replicate units for combination of factors.

The analysis of variance (ANOVA) was conducted on the number of taxa and the total cover, to test differences between treatments in time. The ANOVA was also carried on the percentage cover of the functional groups. Prior to analyses, the assumption of homogeneity of variances was checked using Cochran's *C*-test and data were appropriately transformed, if required. The analyses were performed using GMAV v5 software (University of Sydney, Australia).

2.4.2. Multivariate analyses

The experimental design was the same described for the univariate analyses. There were 46 variables (taxa) included in multivariate analyses. In order to test the effects of treatments on assemblage structure, a distance-based permutational multivariate analyses of variance (PERMANOVA, Anderson 2001a; McArdle and Anderson 2001) was performed on the two full data set. The tests were conducted with 4999 random permutations of appropriate units (Anderson 2001b; Anderson and ter Braak 2003) on Bray-Curtis dissimilarities (Bray and Curtis, 1957) of untransformed data. The same analysis was run on functional groups. In this case there were 9 variables included in the multivariate analyses. The PRIMER v6 and PERMANOVA + software (Plymouth Marine Laboratory, Plymouth, UK) was used.

Terms significant in the full model, Enrichment × Grazing on the whole dataset and Time × Enrichment × Grazing on the functional groups, were examined individually using appropriate pairwise comparisons. Non-metric Multi-Dimensional Scaling ordination (nMDS) (Kruskal and Wish 1978) of the interaction terms Enrichment× Grazing and Time×Enrichment×Grazing in time 1, 2, 4, 5, 7 and 8 were visualized. SIMPER analysis (Clarke 1993) was used to identify the taxa/functional groups mostly contributing to differences highlighted by the pair wise tests. For each

comparison, taxa and functional groups that cumulatively contributed for more than 10% of dissimilarities were shown.