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Effects of different hypoxia timing regimes on a transitional habitat's (Pialassa Baiona, Ravenna, Italy) benthic community

Tesi di laurea in Ecologia

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Alla mia famiglia.

ABSTRACT

Hypoxia is functionally defined as [DO] <2 mg DO L−1, it is a phenomenon that occurs when [DO] is sufficiently low to have negative (lethal or sub-lethal) effects on all organisms. Most past work has focused on identifying the duration of hypoxia that organisms can tolerate, while little attention has been paid to the tolerance to repeated hypoxia events. The aim of this thesis was to quantify experimentally in the field the effects of different timing regimes of hypoxia. On the structure of benthic communities in a transitional habitat.

The experiment was performed from 8 July to 29 July 2019 in a shallow subtidal area in Pialassa Baiona (Italy), a lagoon characterized by mixing regimes dominated by the tide. The benthic community was isolated using cylinders 15,5Cm x 20Cm size. Hypoxic conditions were imposed by covering the treated cylinders with a black plastic bag while control cylinders were left uncovered. We created 4 different timing regimes of hypoxia by manipulating both the duration of hypoxia (4 or 8 days) as well as the ratio between the duration of subsequent periods of hypoxia and the duration of a normoxic period of recovery between subsequent hypoxic events (D4R3/2, D8R3/2). At the end of each experimental trial, the benthic communities within each pot were retrieved, sieved in the field and subsequent analyzed in the laboratory where organisms were identified and counted.

Results showed that benthic organism were generally negatively affected by hypoxic stress events. As expected, longer hypoxic events caused a stronger decrease of benthic community abundance. When the hypoxic events were interrupted by the normoxic event there were two different results. If the hypoxic period was too long, the normoxic period didn't cause a positive recovery effect, and further decline of the benthic community was observed. Conversely normoxia had positive effects if the period of hypoxia was short enough not to compromise the benthic community. This resulted in a statistically significant interaction between the tested factores Duration and Ratio. Amphipods were the most sensitive organisms to hypoxia.

We conclude that the effects of hypoxia can be greatly relieved by short normoxic periods if they happen frequently enough.

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1 INTRODUCTION

1.1 HYPOXIA

1.1.1 WHAT IS HYPOXIA

Oxygen is necessary to sustain the life of organisms dependent on aerobic respiration. When the oxygen demand increases due to the request by animal life, the dissolved oxygen concentration ([DO]) declines (Diaz and Breitburg, 2009). In aquatic environments, oxygen dissolves in the water from the atmosphere and it fulfills to the respiration needs of all animals, including those that live near the sea bottom and those that have a sedentary life (Diaz, 2001). Diaz and Rosenberg (2008) reported that since the middle of the past century, the DO concentrations of many coastal ecosystems has declined. The ecosystem of the northern Adriatic Sea has been affected these phenomena at least since the 1950s. When the complete absence of oxygen occurs, we speak of Anoxia (Diaz, 2001). Anoxia is functionally defined by Porter et al. (2016) as [DO] of 0.2–0.0 mg DO L^{-1}. Water devoid of oxygen can contain lethal concentrations of metabolic products of microbial anaerobic organism (Diaz and Breitburgh, 2009). Furthermore, hypoxia can reduce habitat quality and quantity for macrofauna in the world's estuaries, coastal waters, and deep ocean. Hypoxia is the phenomenon that occurs when [DO] is sufficiently low to have negative (lethal or sub-lethal) effects on all organisms, in particular on animals that are directly exposed or indirectly influenced by food web interactions (Porter et al., 2016) and is functionally defined as [DO] < 2 mg DO L^{-1}. All this happens when the supply of oxygen in the bottom of the sea is below the point that sustains most animal life (Diaz, 2001). Indeed, decrease in DO and the following development of hypoxia was due to a combination of water stratification, stagnation and the increasing oxygen consumption at the bottom due to the eutrophication (Karlson et al., 2002). In a coastal system, hypoxia seems to partially follow a predictable pattern; the initial deposition of organic matter promotes the microbial growth, that increases the oxygen demand, but if the water column is stratified, the DO levels decreases rapidly. When these conditions appear, transient hypoxic events and the consecutive mass mortality of the benthic community occur. Over time, the organic matter and nutrients accumulate in the sediment, hypoxia becomes seasonal or periodic due to boom and bust cycles of animal populations. The hypoxic zone expands when the phenomena of hypoxia persist, and nutrients accumulate in the sediments. Therefore, while the DO continues to fall, hypoxia is established and H_2S is microbially generated (Diaz and Rosenberg, 2008).

Hydrogen sulphide is in general extremely toxic to aerobic organisms. The toxic effect consists in the inhibitory interaction with some enzymes such as cytochrome oxidase and blood pigments. Furthermore, Karlson et al. (2002) argued that the degree of tolerance to H_2S varies among species.

Keeling et al.(2009) named the loss of dissolved O_2 as "deoxygenation" and predicted that these events will manifest more frequently and intensely in the future, not just because O_2 is less soluble in warmer water but also because global warming may increase the stratification of the water column, inducing the decrease of DO in the deeper layers.

The zones with lower O_2 are called "dead zones", the thresholds that lead to the achievement of hypoxia typically depend, not just on O_2 levels, but also on levels of CO_2 and temperature. As it is known that O_2 plays a direct role in the biogeochemical cycling of carbon, nitrogen, and many other biogeochemically important elements (P, Fe, Mn, etc.), the deoxygenation of the ocean will have different consequences.

Once O_2 drops below a certain threshold, organisms suffer from a variety of stresses, leading ultimately to death if the concentrations stay too low for too long and such conditions are termed hypoxic. Tolerance to hypoxia varies greatly between marine taxa, crustaceans tending to be the most sensitive (Keeling et al.,2009).

The point beyond which changes in diversity composition and function occur, pushing the system into an alternative state that is called response threshold (Villnäs et al., 2012). Once the threshold is crossed, hypoxia tends to impair community contribution to ecosystem function and might lead to decrease ecosystem resilience and therefore stop the recovery of the system.

These effects are known to result in behavioural and compositional changes (e.g. in terms of abundance and biomass), which precede or accompany species loss, but what is not well known is how hypoxia affects the resilience of the ecosystem (Diaz and Rosenberg, 2008).

For all these situations descripted above, the phenomenon of hypoxia is particularly interesting because it is increasing in duration, intensity and frequency over time following various anthropogenic factors like climate change and eutrophication.

1.1.2 EUTROPHICATION

Coastal lagoons are enclosed water bodies, with limited connections to the sea. They are often very shallow, productive, bordered by human development, and have long residence times, which raise the concern over future eutrophication through climate change (Turner et al.,2015).Hypoxia is one of the common effects of eutrophication in coastal marine ecosystems and its increasingly caused by stressors (Conley et al.,2009).

Diaz and Rosenberg (2008) reported that since the middle of the past century, the DO concentrations of many coastal ecosystems have been adversely affected by eutrophication. The transitional habitats are threatened by local scale stressors, like the excessive nutrient input arising from intensive farming and inadequate wastewater treatment in the watershed, and relative sea level rise (RSLR) (Wong et al., 2015).

All these activities increase the nutrients input in the system, this nutrient enrichment produces excess organic matter that fuels the development of hypoxia and anoxia when combined with water column stratification (Diaz,2001).

Many coastal ecosystems experience due to the cumulative effects of local and global anthropogenic stressors, the loss of valuable ecosystem services (Wong et al., 2015). It is known that the response of coastal marine ecosystems is strongly modulated by physical processes like changes in temperature that can influence the phenomena of stratification and mixing, making organisms more susceptible to hypoxia (Conley et al.,2009). Indeed, the high-water temperatures induce the phytoplankton to die-off, and accumulate on the bottom layers accelerating bacterial aerobic degradation. The oxygen supply from the sea surface is restricted by stratification and stagnation, causing the sea floor to become hypoxic. Furthermore, Nakano et al. (2017) confirmed that the depleted DO on the sea floor, as a result of stratification, has affected the species richness and diversity of the benthic community.

In general nitrogen and phosphorus are the principal elements that cause eutrophication, but nitrogen has received more attention because it often limits primary production in estuaries and because the nitrogen is globally used in synthetic fertilizers instead of phosphorus. (Glibert et al., 2005). It is known that increased phosphorus fluxes from sediments into overlying waters occur with hypoxia. In addition, reductions in the ability of ecosystems to remove nitrogen through anaerobic ammonium oxidation may be related to hypoxia and could lead to an acceleration in the rate of eutrophication (Conley et al.,2009).

Another phenomenon linked to estuaries susceptibility to eutrophication is the proliferation of opportunistic macroalgae. Macroalgae are a natural component of estuarine ecosystems, but algal blooms have increased over the past decades in many estuaries. These blooms now are more geographically spread and prolonged in time. Furthermore, they can negatively impact coastal recreation and tourism by fouling beaches and producing repulsive smell (Nakano et al., 2017). Indeed, eutrophication is now recognized to be one of the important factors contributing to habitat change and to the geographical and temporal expansion of algal blooms (Glibert et al.,2005).

Transitional habitats are recognized as important nutrient sinks, mediating the effects of excess nutrients, particularly nitrogen. Nitrogen-cycling is generally efficient in coastal lagoons, in fact these ecosystems tend to be nitrogen-limited. Excess nitrates are removed by assimilation into primary production, denitrification, and to a much lower extent by bacterially mediated anaerobic ammonia oxidation. On the other hand, concerns are being raised that increased nutrient availability in transition habitats could be facilitating their degradation (Wong et al., 2015).

Many studies have demonstrated a correlation through time between population growth, increased nutrient discharges, increased primary production in coastal areas, and increased occurrence of hypoxia and anoxia. The Northern Adriatic Sea is a good example in which this connection can be observed and studied (Diaz,2001). The system is heavily impacted by industrial pollution, infrastructural, agricultural activities and urbanization (Airoldi et al., 2016).

1.1.3 CLIMATE CHANGE

The IPCC (intergovernmental panel on climate change) is the United Nations body coordinating the science related to climate change. It prepares comprehensive assessment reports about the state of scientific technical on climate change.

These assessment reports highlight the impacts that the human activities have had on socio-ecological systems worldwide, including a global warming of about 1ºC range of 0.8 to 1.2°C compared to pre-industrial levels.

Future global warming is likely to further increase by 0.3°C to 4.8°C by 2081 and 2100. Furthermore, available data show significant increasing trends in the intensity and frequency of extreme climatic events. Climate change is modifying oceans in different ways, including ocean atmosphere circulation, water warming, and the effects of climate change will particularly be presented at the coast, where humans and oceans meet (Reguero et al., 2019).

Diaz and Breitburg et al. (2009) have shown that the stressors associate with climate change may make systems more susceptible to hypoxia. Indeed, when the water temperature increases due to warming, the oxygen solubility decreases, and metabolism of organism increases. Also, climatic instabilities with localized human perturbations are creating new disturbance regimes which are further accelerating the degradation and decline of coastal ecosystems (Wong et al., 2015).

Dissolved oxygen, one of the most important ecological variables has changed drastically over the past decade in the coastal waters. Vaquer-Sunyer and Duarte (2008) argued that the number of the coastal sites, where the hypoxia has been reported, has increased with an exponential growth that is not expected to stop. Benthic organisms are particularly vulnerable to coastal hypoxia because they live far from atmospheric oxygen and because coastal sediment tends to be low in oxygen due to intense decomposition processes.

Despite hypoxic and anoxic environments have existed trough geological time, their occurrence in shallow coastal and estuaries area appear to be increasing due to human activities (Diaz, 2001). It is known that nowadays the timing of occurrence and duration of the hypoxic events are changing due to the climate change and that this phenomenon can have profound effects on the shores (Benedetti-Cecchi et al., 2006).

Synthesis of literature of Diaz (2001) pertaining to benthic hypoxia and anoxia revealed that the oxygen budgets of many major coastal ecosystems have been adversely affected mainly through the process of eutrophication. Furthermore, many ecosystems that are now severely stressed by hypoxia may be near or at a threshold of change or collapse.

1.2 REPEATED EVENTS

The first studies about hypoxia showed a stress less frequent and more spatially restricted, but the past few decades have seen a drastic increase in the number of coastal areas characterized by episodic or seasonal hypoxia. Indeed, the increasing warming trends combined with eutrophication and strong longitudinal salinity gradients lead to the concern that hypoxic disturbance regimes will worsen the conditions of the ecosystems (Jager et al., 2018).

The periodic oxygen depletion may occur more often than seasonally but tends to be less severe. The problem is that smaller systems like shallow shores are vulnerable to periodic hypoxia because local weather events like tidal cycles influence stratification intensity (Diaz et al., 2008).

The magnitude of hypoxia is highly dynamic, and may vary in extent, severity and frequency. For example, the hypoxic period may last hours (diel cycles), days to weeks and even months to years. Therefore, projected increases in warming show an increase in the susceptibility of coastal marine ecosystems to hypoxia (Conley et al., 2009).

Models of climate change generally agree that extreme events such as storms and hypoxic events are becoming more frequent. Shifts in climate conditions can have profound ecological impacts, for example in patterns of distribution, abundance and diversity of the species (Benedetti-Cecchi et al.,2006). It is known that several climatic events occur over short intervals of time and they alternate with long periods in which no events occur (Benedetti-Cecchi, 2003).

Diel and seasonal hypoxia are the most common temporal pulses in which hypoxia manifests. In diel hypoxia, the [DO] decreases at night due to respiration and increases again during the day due to photosynthesis. In seasonal hypoxia, [DO] decreases during August and September, in summer months the minimum level of dissolved oxygen is reached (Porter et al., 2016).Other than these two main temporal pulses, hypoxic periods can have a stochastic nature: due to the complexity of the interactions and factors causing them they appear in an unpredictable way.

Often experimental studies have focused on the effects of a single hypoxic events on organisms, but in many coastal areas hypoxia does not present as a single event but as a series of repeated events that can change both in intensity and temporal distribution (Porter et al., 2016). The idea that these temporal regimes could be responsible of major changes in the benthic community has not been studied deeply (Benedetti-Cecchi al., 2006).

It is known that repeated hypoxic events lead to an increase in accelerated eutrophication and susceptibility to further hypoxic events. Whereby, once hypoxia occurs, there is high probability that this phenomenon will reoccur again and may be difficult to return to an original status. Indeed, Conley et al. (2009**)** said that it becomes important to know ecosystem thresholds of hypoxia and to link them to nutrient inputs for the management of coastal marine ecosystems.

Large-scale changes in benthic communities occur with hypoxia, indeed large and slowly growing animals show abundance decrease, while smaller, fast-growing animals, that can colonize surface sediments rapidly following hypoxia tend to show an abundance increase (Diaz & Rosenberg, 1995).

Repeated hypoxic events can increase the susceptibility of coastal marine habitats to further hypoxic events, causing changes in the community structure, and weakening the capability of the ecosystem to persist to the next hypoxic condition. If the natural ecosystem buffers that favour the maintenance of oxic conditions are lost, the resilience of the ecosystems against subsequent hypoxia events is eroded and new states appear that act in the opposite direction, causing the consolidation of hypoxic conditions (Conley et al., 2009).

The ability of coastal marine ecosystems to recover from hypoxia may be partly related to the ability of organisms to recolonize and the availability of nearby refuges. These observations suggest that the thresholds for hypoxia are dynamic, and that once trespassed, the changes in internal buffers discussed above making the ecosystems more prone to hypoxia (Conley et al.,2009).

The benthos of many coastal areas may be re-established by larval recruitment and succession; in this way the benthic community partially recovers, even if the species that establish during recovery may not be the same as those loss during DO deterioration (Diaz and Breitburg, 2008).

The key factors in determining the degrees of ecosystem degradation are the duration of the exposure of benthic organisms to the hypoxic event and the DO concentration. Diaz and Breitburg (2008) suggest that it may take years to benthic community to recover from hypoxic event. Opportunistic are the first colonizer, because they are more tolerant and can survive to strong stress event. Many polychaetes are opportunists and are favoured in unstable environments, for example, those where seasonal and irregular hypoxia occur (Karlson et al.,2002).

Phenomena like thermal stress due to aerial exposure, and disturbance by waves are typical events that occur in transitional habitats and they contribute to maintain spatial and temporal variability in the structure of these ecosystem. Global warming and the consequent increasing frequency and intensity of these stressors can amplify this spatial variability. For this reason, changes in the timing of occurrence and duration of all these events must be studied (Benedetti-Cecchi 2006).

1.3 AIMS OF THE STUDY

I performed a field experiment to test: if benthic communities, subjected to hypoxia of different duration vary significantly; if the benthic communities change in response to different ratios between hypoxic and normoxic periods; and if and how benthic organisms can absorb the disturbance from hypoxia.

We simulated hypoxic events of two, four and eight consecutive days acting on the benthic community and compared to the responses to normoxic treatments. We hypothesized that the benthic community would be more negatively affected by increasing the duration of hypoxia, as suggested by previous work (Jager et al., 2018).

We also simulated treatments where hypoxic periods were interrupted by normoxic periods of different duration. This allowed to test of the responses of the benthic community depends not only on the duration of the hypoxic events but also on the extent of the normoxic period, ultimately to compare different timing regimes of hypoxia.

2 MATERIAL AND METHODS

2.1 STUDY AREA

The experiment was conducted in Pialassa Baiona (Italy): a brackish lagoon located along the northern Adriatic Italian coasts between the Ravenna harbor and Lamone river. It is a eutrophic-microtidal lagoon with a surface of 11.8Km^2 (Ponti and Abbiati, 2004).

Pialassa Baiona is a transitional habitat characterized by mixing regimes; the external part is dominated by the tide, while the internal part is dominated by the river action. Tidal range can exceptionally exceed 1 m.

The lagoon has large areas of muddy bottoms with variable clay/silt proportion and organic matter contents. Sediments vary from sandy to muddy (sand range from 12.1% to 89.5% in weight) according to the occurrence of active sedimentation processes or relict sand dunes. (Ponti and Abbiati, 2005). It is affected by anthropogenic eutrophication, which causes extensive growth of seaweeds and phytoplankton blooms that are responsible for the events of hypoxia that occasionally occur in summer.

Nowadays the lagoon is divided by an artificial embankment into several shallow water ponds that are connected to each other and with the sea by channels. The total water volume is estimated in approximately 8.9 x 10^6 m^3, equally distributed between ponds and channels. In the channels the average depth varies from 0.5 m to 3 m, with a tidal range variable from 0.3 to 1 m, excluding extreme events. The tides cause large variations in the water levels and during low tides vast shallow areas emerge (Ponti and Abbiati, 2005). Overall water turnover in the lagoon has been estimated in 3 days (Ponti et al., 2009).

The small lake receives freshwater and nutrient inputs from five channels, some of them from urban and agricultural areas. Furthermore, the lagoon receives freshwater from treatment implants of urban and industrial wastewater and saltwater. The southern area of the lagoon receives wastewater from urban and industrial sewage treatment plants, and from two thermal power stations (Ponti et al., 2005).

Our experiment was conducted on a shallow, spatially homogeneous subtidal mudflat in the northern part of the lagoon, which is the further from the urban water discharges (Fig.1) and (Fig.2).

Figure 1: Pialassa Baiona (Google Maps)

Figure 2: Pialassa Baiona

2.2 EXPERIMENTAL DESIGN

In both experiments, we used normoxic reference controls, that allowed to understand if the variation in time of the benthic community was related to the hypoxic treatment or to temporal changes in environmental factors.

This set up was created to test two hypotheses: if the duration of hypoxia influences the survival of organisms and if the effect of repeated hypoxic events depend not only on the duration of the events but also on the extent of the normoxic period, ultimately to compare different timing regimes of hypoxia.

2.2.1 EFFECT OF DURATION OF HYPOXIA

Table 1: Combined effect of factor Time (three levels: D2, D4 and D8) and factor Treatment (hypoxic condition vs normoxic control).

	TIME			
TREATMENT		D2	D4	D8
	HYPOXIA (T)	D2T	D4T	D8T
	NORMOXIA (C)	D2C	D4C	D8C

This experimental design is composed by factor Time that represents the duration of the whole experiment with three levels (D2T=2 hypoxic days, D4T=4 hypoxic days and D8T=8 hypoxic days) and factor Treatment with two levels hypoxic condition(T) vs normoxic condition(C) (Tab.1).

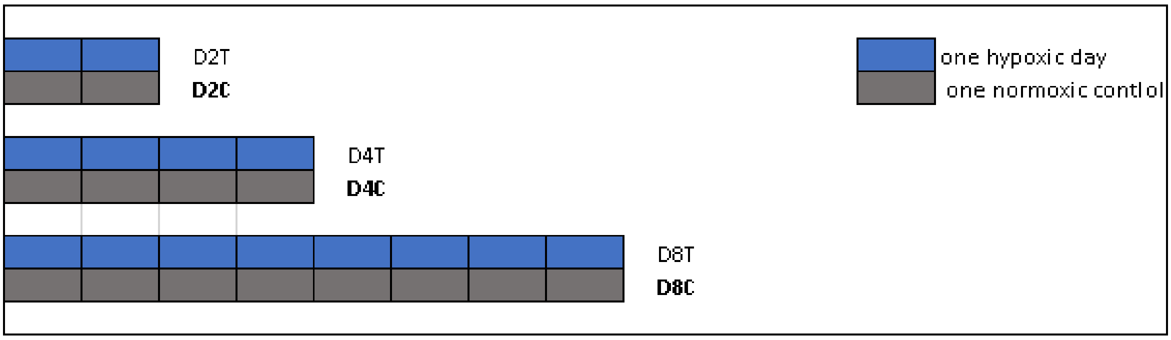

Figure 3: Experimental design testing the effects of different duration of hypoxia. Each box represents one day. Blue box represents the hypoxic days, grey boxes are the normoxic controls.

We experimentally manipulated hypoxic events of different "duration" (days): D2, D4 and D8 corresponding to two, four and eight total days of consecutive hypoxia, respectively. Each treatment was compared to a normoxic control (Fig.3).

2.2.2 COMBINED EFFECT OF DURATION AND RATIO

Table 2: Combined effect of duration of hypoxia(two levels: D4 and D8) and ratio among normoxic event and hypoxia regimes (two levels R0 and R3/2) .

		DURATION	
RATIO		**D4**	**D8**
	R0	D4RO	D8RO
	R3/2	D4R3/2	D8R3/2

Factor "Duration" was composed by two different levels, D4 and D8 total days of hypoxia, while "Ratio" indicated the ratio between the duration of the hypoxic period and the duration of alternating periods of normoxia. This included two different levels R0 (no normoxic period) and R3/2 (Tab.2), that included a midterm normoxic period with a duration equal to 3/2 of the total hypoxia (6 normoxic days vs 4 hypoxic days) and (12 normoxic days vs 8 hypoxic days).

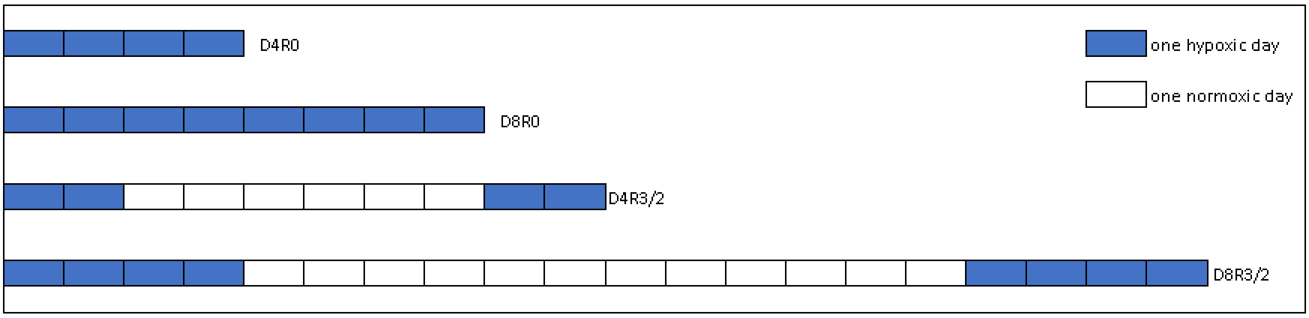

Figure 4: Experimental design testing the effects of different duration of hypoxia and ratio between hypoxia and normoxic intervals. Each box represents one day. Blue box represents the hypoxic days, white boxes are normoxic days applied to hypoxic treatments.

The treatments of the second experiment are detailed in Figure 4. Duration of hypoxia was four and eight days. In the first set of treatments there was no normoxic day (ratio between hypoxia and normoxia was 0) while in the second set of treatments the hypoxia was interrupted by a normoxic period of 6 and 12 days, with a ratio between normoxia and hypoxia of 3/2. These regimes were simulated by covering and uncovering the experimental mesocosms in correspondence of the hypoxic or normoxic periods respectively.

2.3 FIELD WORK

Sampling was performed from 8 to 29 July 2019. In the laboratory 15.5-centimeters diameter pots were cut and covered with a 0.5 mm mesh plastic net to eliminate the potential source of variability due to different predation or loss of organisms between covered or uncovered pots (Fig.5). The net indeed, allowed to maintain similar conditions between these two treatments, reducing potential confounding effects.

Figure 5:Experimental pots covered with a net sealed with a plastic cable tie to prevent access to predators and thereby removing any potential source of confounding from variable access of predators in hypoxic vs normoxic treatments

In the lagoon, pots were positioned at about five meters from the shore at low tide, completely submerged on two parallel rows. Tubes were buried 15 cm into the sediment around 50 cm apart and marked with sticks with colored labels (Fig.6) that allowed the operator to recognize them even with low visibility and poor weather condition.

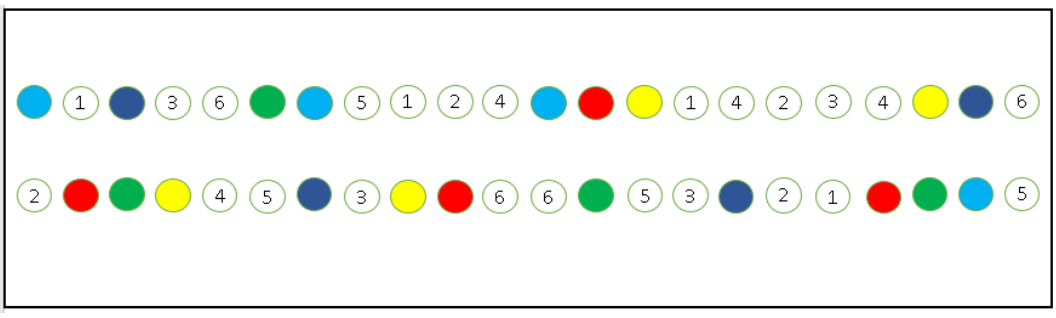

Figure 6: This picture shows pots haphazardly positioned; the controls coloured in white with the number that correspond to the time of sampling and treatments coloured in blue correspond to D2R0, light blue D4R0, red D8R0, yellow D4R3/2 and green D8R3/2.

The pots of the first and the second rows were placed and sampled avoiding disturbance and trampling as much as possible on the surrounding sediment in order not to affect the community when it was necessary to go to cover or uncover the pots or during the sampling of a specific treatment.

Hypoxic and normoxic conditions were imposed by covering the treated pots with a black plastic bag and uncovering the pots in set days. The plastic bag was fixed with two thick rubber bands that allowed the pot to be covered and uncovered easily even with high tide. The controls were left uncovered to see how the structure of the benthic community changed during time without any change caused by the treatments.

Every time at the end of the sampling the temperature, salinity and concentration of oxygen (% and mg/l) were measured from three different locations inside the experimental area.

When each treatment was ended, the mesocosm was removed with all its sediments, washed through a 0.5mm -sieve, to clean it from mud and clay, and put in a previously labelled plastic container with ethanol100%.

2.4 LABORATORY ANALYSES

All the samples were then moved into the university laboratories. Every sample was sieved through a 4000, a 2800 and a 500 µm mesh sieve stacked (Fig.7).

Figure 7: The picture shows a 4000, a 2800 and a 5 00 µm mesh sieve system.

The content of the pot sieved through the 4000 µm mesh sieve was analyzed with a magnifier while the sediment that was sieved through 2800 and 500 µm (Fig.7) were analyzed with a stereoscope (Fig.8).

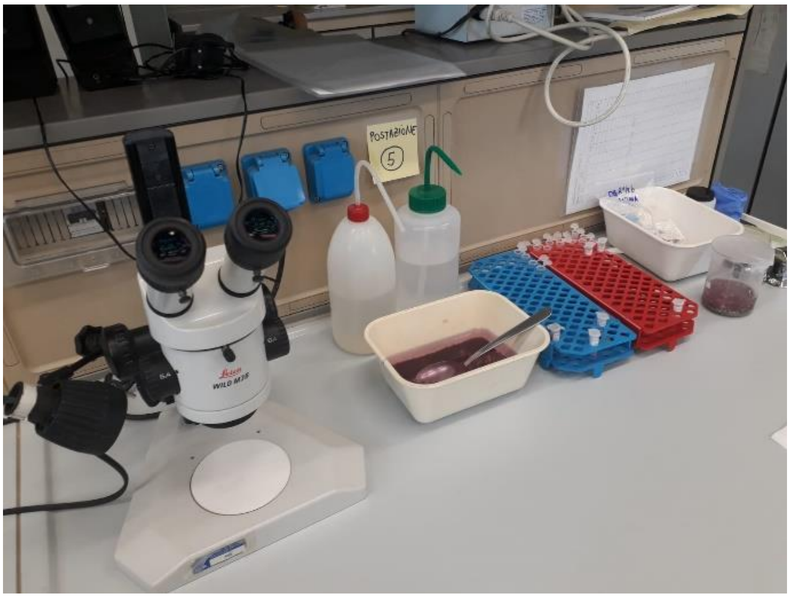

Figure 8: This picture shows the stereoscope, sample inside a Becker and some Eppendorf in which the organisms were put.

The smaller size class (500 µm) was stained with Rose Bengal, a stain that reacts with both live and dead cytoplasm and tissues (Bernhard et al.,2006).

Rose Bengal (4,5,6,7-tetrachloro-20 ,40 ,50 ,70 - tetraiodofluorescein disodium salt) is a stain that adheres to cytoplasmatic proteins, regardless of whether the cell/animal is dead or alive and colours different organisms differently and has the advantage of being easy to apply, and to require only a light microscope (Grego et al., 2013).

It was assumed that the organisms that were coloured and in a good condition in laboratory, were alive at the time of sampling in Pialassa Baiona.

The organisms were extracted from the sample and subsequently they were identified to the lowest taxonomic level and counted.

2.5 DATA ANALYSES

The benthic community was analyzed in terms of species abundance using R studio version 1.1.463. Before each analysis, Cochran's C test was performed to check for homogeneity of variances. If variances were heterogeneous (C> 0.05) the data set was Log10 transformed.

2.5.1 EFFECT OF DURATION OF HYPOXIA

We used a two-way fixed ANOVA to analyse the effect of hypoxic events of different durations on the abundance of the benthic community. The analysis included the factors Time (three levels: 2,4 and 8 hypoxic days) and treatment (2 levels: hypoxia and normoxic control). The variable considered in this analysis is the total organisms abundance.

Pair-wise test was performed to shows the difference between couples create with controls days (2,4 and 8 normoxic days) and treatments days (2, 4 and 8 hypoxic days).

Subsequently we performed multivariate analysis using the PRIMER 6 software to test the same effects on the whole community structure. Before each analysis the transformation "fourth root" of dataset was performed.

Starting from the transformed dataset, the Bray-Curtis similarity matrix has been created. This index is the most commonly statistical tool used to quantify the compositional similarity between two different samples.

We usually explored the data using multidimensional scaling (MDS), which shows levels of similarity between treatments into a Cartesian space (Anderson et al.,2008).

The effects of factor duration were assessed by permutational non-parametric multivariate analysis of variance (PERMANOVA) which allow to partition the variability and obtain F-statistics on matrices of Bray-Curtis calculated from the transformed data. P-values were calculated using 9999 random permutations of the appropriate exchangeable units and Type III sums of squares (Anderson et al.,2008).

2.5.2 COMBINED EFFECT OF DURATION AND RATIO

A two-way ANOVA was performed to analyze the effects of repeated hypoxic events on the benthic community, the factors included were Duration (4 vs 8 days) and Ratio (0 vs 3/2).
Subsequently we performed multivariate analysis using the PRIMER 6 software. Like in the first experiment the Bray-Curtis similarity matrix was created from the transformed dataset (fourth toot), and then multidimensional scaling (MDS) was performed.
The differences in benthic communities between factors Duration and Ratio were assessed by permutational non-parametric multivariate analysis of variance (PERMANOVA) followed by Pair-wise test if needed.
Furthermore, PRIMER software was used to perform similarity percentage analysis (SIMPER) between treatments and identify mostly contributed to the observed. SIMPER calculates the contribution of each species (%) to the dissimilarity between each two groups. It is calculated from the Bray-Curtis dissimilarity matrix. We obtained which couple of treatments had the higher dissimilarity percentages and which taxa represented the majority of that differences. We expected that these taxa correspond to those animals that were more susceptible to hypoxia.

3 RESULTS

During the experiment the seawater temperature ranged between 27.6ºC and 31.1ºC. We found a total of 29355 organisms Fig.9 shows the average abundance in the different experimental normoxic controls.

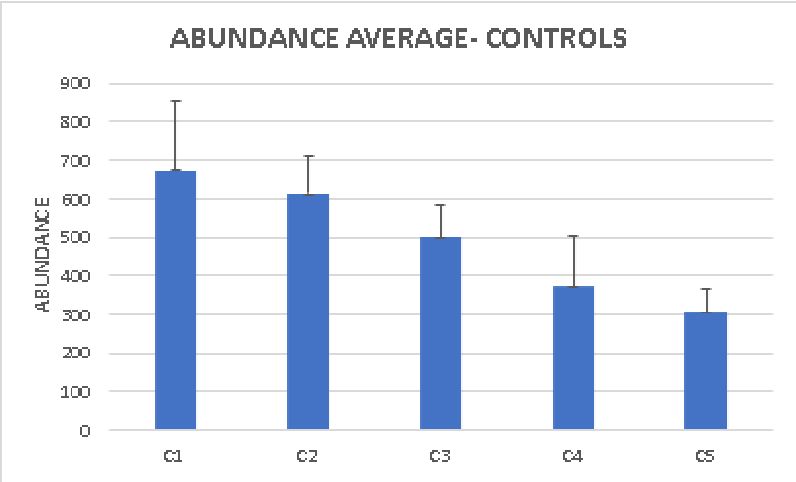

Figure 9: Average abundance (total number of organisms) in every control C1, C2,C3,C4 and C5,each control has four replicates (n=4) (+E. S).

The plotted averages show a gradual decrease in abundance in controls over time probably due to the isolation provided by the pots or to changes in the environmental conditions during the experiment.

3.1 EFFECT OF DURATION OF HYPOXIA

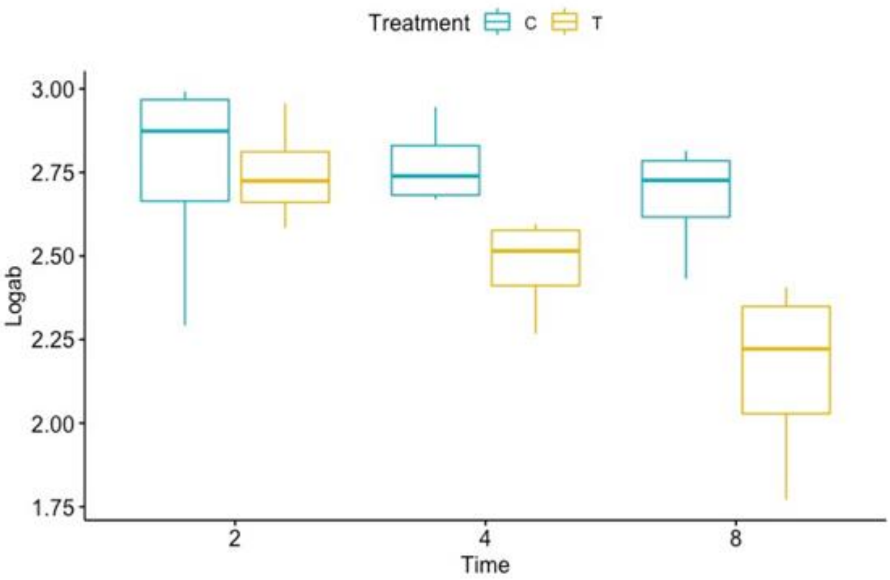

Figure 10:On the x-axis Time before sampling, on the y-axis total organisms abundance (log-transformed).

When the consecutive days of hypoxia increase (2-4-8 consecutive hypoxic days), the abundance of the benthic fauna decreased (Fig.10). There was a significant interaction between Time and the Treatment (p=0.027) (Tab.3).

Table 3:The table shows the results of one two-way ANOVA performed between two factors, Treatment(2 levels) and Time(3 levels).Asterisks indicate significant p-values.

	df	Sum Sq	Mean Sq	F value	Pr(>F)
Time	1	0,4626	0,4626	10,82	0,0037*
Treatment	1	0,4594	0,4594	10,75	0,0038*
Time:Treatment	1	0,242	0,242	5,66	0,027*
Residuals	20	0,855	0,043		

Table 4: Pair-wise test shows the difference between couples create with controls days D2C=2 normoxic days, D4C=4 normoxic days,D8C=8 normoxic days and treatments days D2T=2 hypoxic days, D4T=4 hypoxic days, D8T=8 hypoxic days.

	diff	lwr	upr	p adj
D8C-D2C	-0,083	0,57	0,4	0,99
D8T-D8C	-0,52	-1	-0,04	0,03*
D2T-D2C	-0,01	-0,5	0,47	0,99
D8T-D2T	-0,59	-1,08	-0,11	0,01*
D4T-D4C	-0,3	-0,78	0,18	0,39
D4T-D2T	-0,27	-0,75	0,21	0,49
D8T-D4T	-0,32	-0,8	0,1	0,33
D4C-D2C	0,02	-0,5	0,5	0,99
D8C-D4C	-0,09	-0,58	0,39	0,99

The Pair-wise test shows a significant difference between the 8 and 2 days of hypoxia (D8T and D2T) Furthermore, we can see a significant difference between eight consecutive hypoxic day and his control (D8T-D8C) as Table 4 shows.

Finally, this test doesn't show a significant difference between other pairs.

Subsequently multivariate analysis was performed with fourth root transform data.

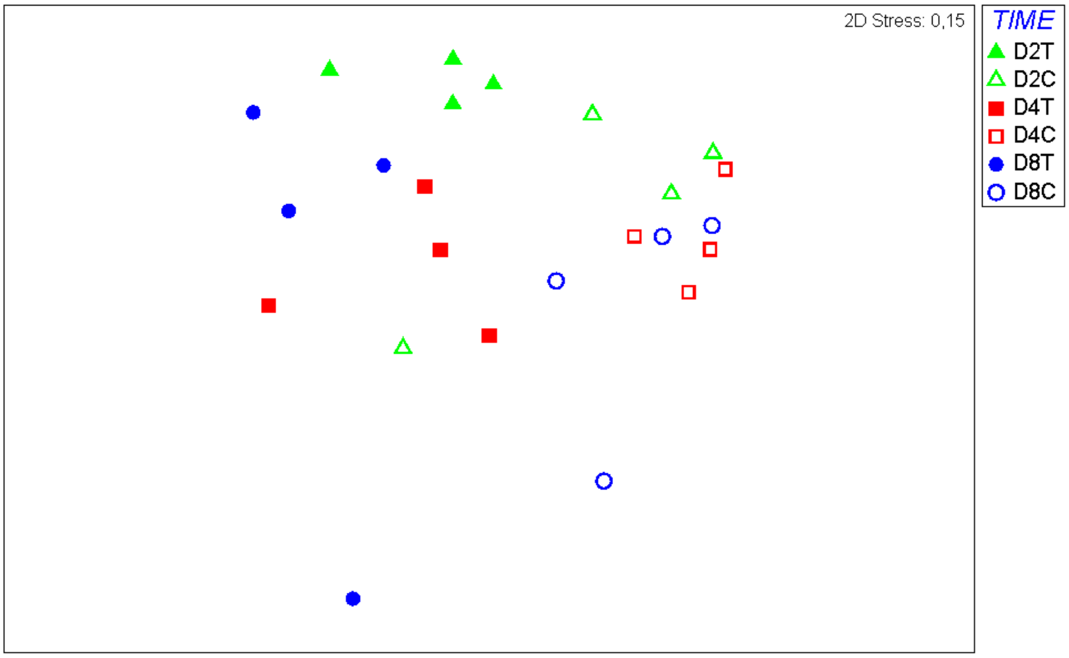

Figure 11: MDS analysis showing the differences among three levels of factor Time (D2T=2 days of hypoxia,D4T=4 days of hypoxia ,D8T=8 days of hypoxia and normoxic controls D2C=2 normoxia days D4C=4 normoxia days and D8C=8 normoxia days). There were 4 replicated plots for each treatment.

MDS analysis (Fig.11), showed that the controls are well separated from the treatments, and despite the high variability, some reasonably clear separation emerges between the treatments too as confirmed by the PERMANOVA test.

Table 5:PERMANOVA test performed between Treatment (2 levels: Treatment and control) and Time (3 levels: 2,4 and 8 hypoxic days). Both factors are significant, while the interaction among two is not.

	PERMANOVA TABLE RESULTS					
Source	df	SS	MS	Pseudo-f	P(perms)	unique perms
Treatment	1	4332,4	4332,4	10,04	0,001*	998
Time	2	2256,7	1128,4	2,62	0,009*	996
Treatmentxtime	2	1058	529,01	1,23	0,273	998
Res	18	7763,7	431,32			
Tot	23	15411				

PERMANOVA test showed a significant effect of both duration and hypoxic treatment (Tab.5).

3.2 COMBINED EFFECT OF DURATION AND RATIO

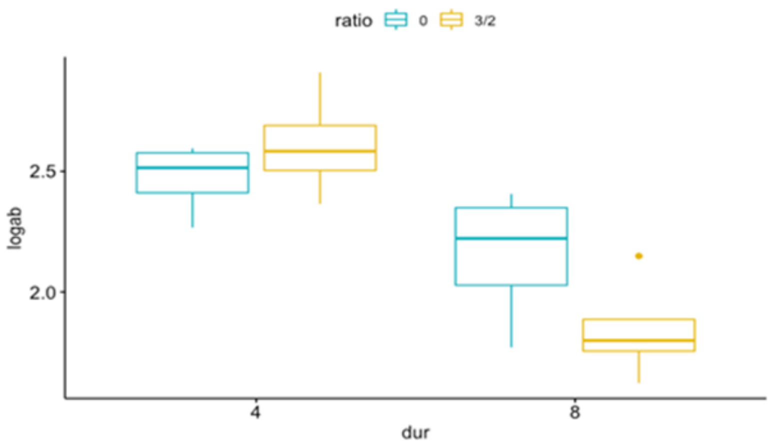

Figure 12: Faunal abundance in treatments of different duration of hypoxia (4 days vs 8 days) and different ratios (0 normoxic days vs a normoxic period equal to 3/2 of the duration of hypoxia).

The boxplot of faunal abundances (Fig.12) shows that the normoxic period have a slight positive effect when hypoxia was only 4 days long, while with a more prolonged hypoxic period the effect of normoxia was negative. The ANOVA showed a significant effect only of the factor Duration (Tab.6).

Table 6: Results of one two-way ANOVA performed between two factors with own levels , Duration (4 hypoxic days vs 8 hypoxic days) and Ratio (R0 and R3/2).Asterisks indicate significant effects.

	df	Sum Sq	Mean Sq	F value	Pr(>F)
Duration	1	1,18	1,18	23,19	0,0004*
Ratio	1	0,03	0,03	0,6	0,45
Duration:Ratio	1	0,2	0,2	3,98	0,069
Residuals	12	0,61	0,05		

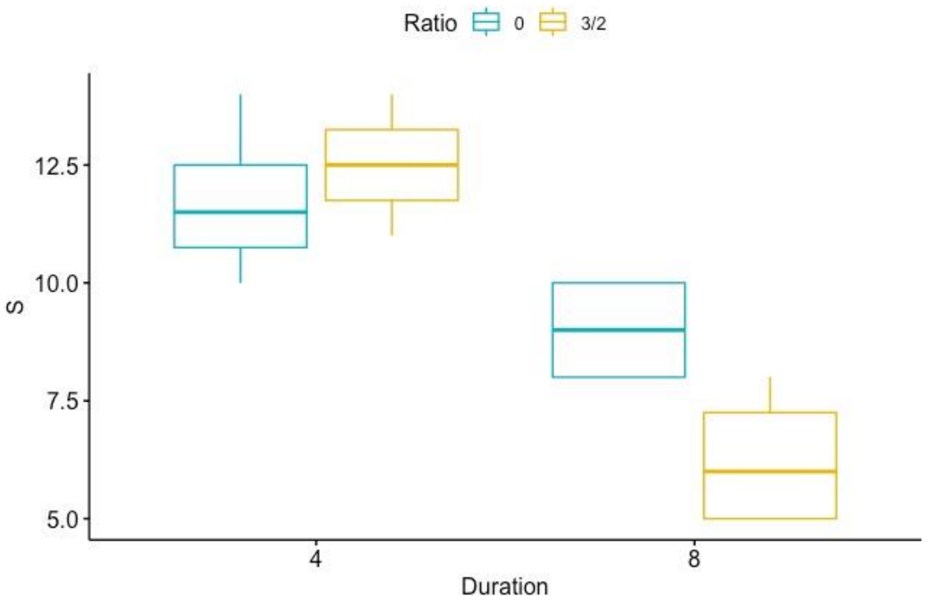

Figure 13: The species richness (S) was analysed with a two-way ANOVA that was performed with factor Duration D4R0 and D8R0 light blue and factor Ratio D4R3/2 and D8R3/2 yellow.

The same analyses performed on species richness (Fig.13) shows the same trend confirmed by the ANOVA test where the interaction between Ratio and Duration is significant (p=0.031), as shown in Table 7. The normoxic period had a negative effect on species richness with a longer hypoxic period.

Table 7: Two-way ANOVA test was performed with factor Duration (4 and 8 consecutive hypoxic event) and Ratio (R0 and R3/2). Asterisks indicate significant effects.

	df	Sum Sq	Mean Sq	F value	Pr(>F)
Duration	1	81	81	39,67	3,96e-05*
Ratio	1	4	4	1,96	0,19
Duration:Ratio	1	12,25	12,25	6	0,031*
Residuals	12	24,5	2,04		

This MDS shows separation between the four treatments despite the variability expectedly shown by the community (Fig.14).

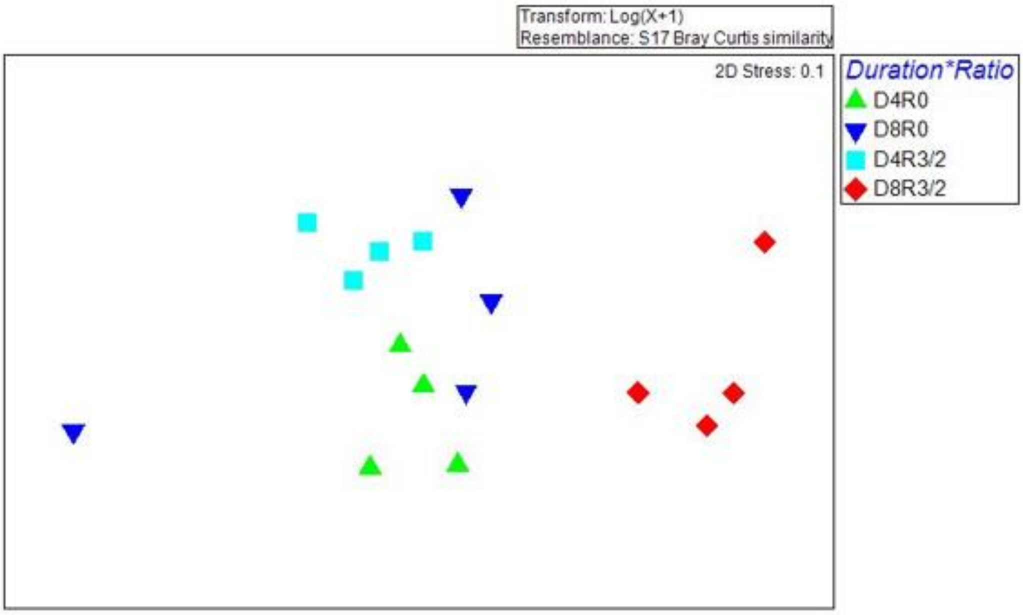

Figure 14: MDS plot showing the differences between plots of different duration of hypoxia (4 days and 8 days) and different ratios (no normoxia vs normoxia with a length of 3/2 compared to hypoxia)

Table 8: PERMANOVA test with factor duration (2 levels) and factor Ratio (2 levels). Asterisks indicate significant effects.

	PERMANOVA TABLE RESULTS						
Source	df	SS	MS	Pseudo-f	P(perms)	unique perms	P(MC)
Duration	2	3617,5	1808,7	4,03	0,0002	9925	0,0012*
Ratio	1	1854,9	1854,9	4,13	0,0023	9938	0,0069*
DurationxRatio	1	2710,4	2710,4	6,03	0,0001	9956	0,0003*
Res	15	6737,5	449,17				
Tot	19	15333					

The PERMANOVA test (Tab.8) resulted significative for each factor and interaction between the two, which probably indicated that the magnitude of the differences was more pronounced with a duration of hypoxia of 8 days compared to 4 days, as pairwise test always showed significant differences among treatment levels.

Table 9: Pair-wise test among levels of factor Duration (D4 and D8). Asterisks indicate significant effects.

Groups	t	P(perm)	Unique perms
D4R0-D8R0	2,28	0,0009*	9944

Table 10: Pair-wise test among level of factor Ratio (R0 and R3/2). Asterisks indicate significant effects.

Groups	t	P(perm)	Unique perms
R0-R1,5	2,01	0,0014*	9920

Table 11:Pair-wise test within level D4, among levels of factor Ratio (R0 and R3/2). Asterisks indicate significant effects.

Groups	t	P(perm)	Unique perms
R0-R1,5	2,014	0,03*	35

Table 12: Pair-wise test within level D8, among levels of factor Ratio (R0 and R3/2). Asterisks indicate significant effects.

Groups	t	P(perm)	Unique perms
R0-R1,5	2,34	0,029*	35

Table 13: Pair-wise test within level R0, among levels of factor Duration (D4 and D8).

Groups	t	P(perm)	Unique perms
D4R0-D8R0	1,09	0,36	35

Table 14:Pair-wise test within level R3/2, among levels of factor Duration (D4 and D8). Asterisks indicate significant effects.

Groups	t	P(perm)	Unique perms
D4R0-D8R0	3,44	0,03*	35

From Pair-wise test results that within factor Duration and within factor Ration the difference among the levels of every factor results significant (Tab.9 and Tab.10).

The Pair-wise test performed in Table13 within level R0 between levels of Factor Duration, results not significantly.

The other Pair-wise tests show significantly differences (Tab.11), (Tab.12), (Tab.14).

Table 15: SIMPER analysis

Groups	AVERAGE DISSIMILARITY
D8R0-D8R3/2	46,81
D4R0-D8R3/2	44,18

The results of the SYMPER analysis (Tab.15) were that the pairs of treatments with more differences were D8R0-D8R3/2 and D4R0-D8R3/2 furthermore, both couples had amphipods as a more differentiated group. To visualize this, result a box plot was performed (Fig.15) with the abundance of amphipods.

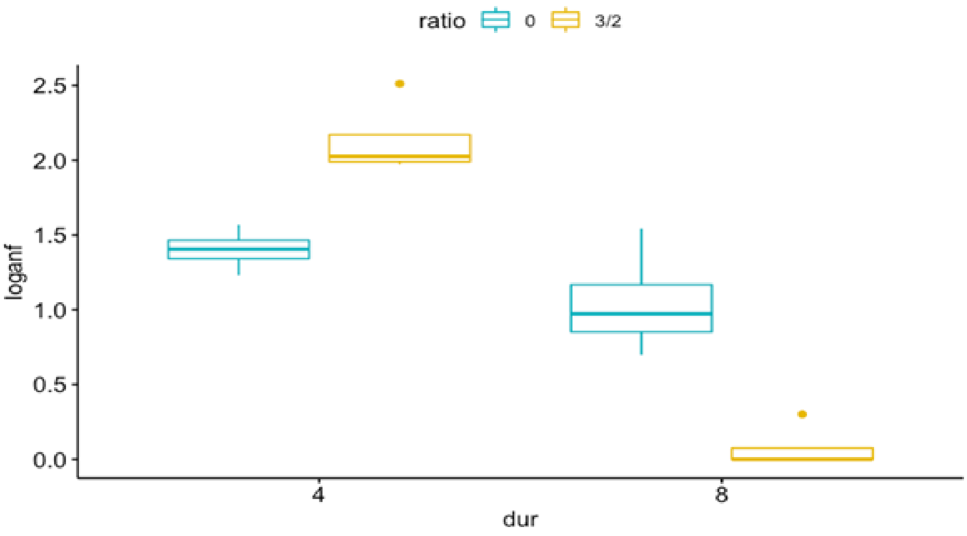

Figure 15: Abundance of Amphipoda in treatments of different duration of hypoxia (4 days vs 8 days) and different ratios (no recovery vs recovery with a length of 3/2 compared to hypoxia. Treatments D4R0 and D8R0 light blue while D4R3/2 and D8R3/2 yellow

This representation clearly shows that the effect of the normoxic period is opposite depending on the duration of hypoxia.

The same representation was performed with the abundance of polychaetes, one of the taxa that consistently represented the least variability.

Table 16:ANOVA with data of Amphipoda results with factor Duration (2 levels) and factor Ratio (2 levels)

	df	Sum Sq	Mean Sq	F value	Pr(>F)
Duration	1	5,831	5,831	98,69	3,85e-07*
Ratio	1	0,057	0,057	0,973	0,34
Duration:Ratio	1	2,902	2,902	49,11	1,42e-05*
Residuals	12	0,709	0,059		

The effects of duration and the interaction between duration and ratio resulted significative as show table 16.

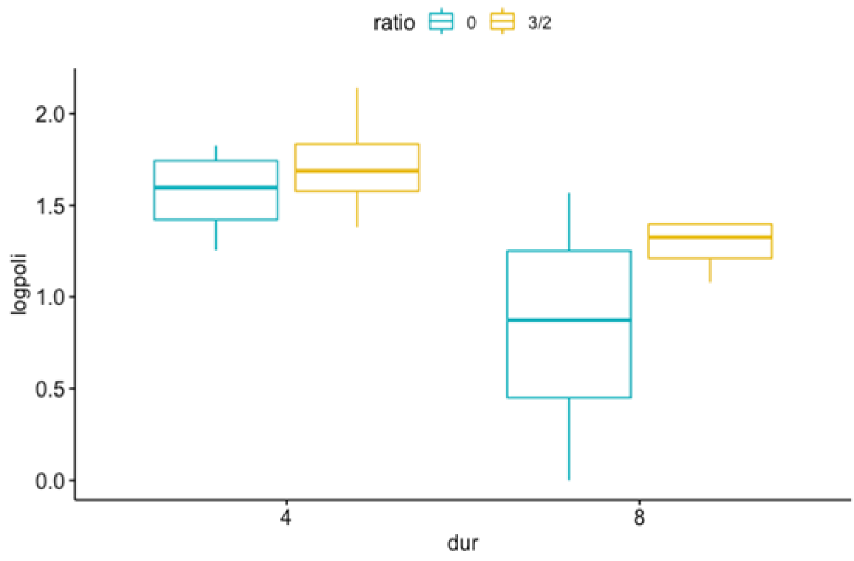

Figure 16: Two-way ANOVA test was performed with polychaetes abundance (log transformed) in the treatments of different duration of hypoxia (4 days vs 8 days) and different ratios (no normoxia vs normoxia with a length of 3/2) . Treatments D4R0 and D8R0 light blue while D4R3/2 and D8R3/2 yellow.

In this case the abundance of polychaetes resulted higher in the treatments with the normoxic period that stopped hypoxia showing a different trend from the overall community and amphipods.

The ANOVA shows that Duration was the only factor that was resulted significant with a P-value=0.012 as seen in (Tab.17).

Table 17:ANOVA with data of Polychaeta results, the only significant effect is the one of hypoxia Duration.

	df	Sum Sq	Mean Sq	F value	Pr(>F)
Duration	1	1,39	1,39	8,59	0,012*
Ratio	1	0,37	0,37	2,28	0,15
Duration:Ratio	1	0,089	0,089	0,55	0,47
Residuals	12	1,95	0,16		

4 DISCUSSION

In transitional habitats, like the Pialassa Baiona study area, hypoxic events occur as series of events repeated over time that can change both in intensity and frequency (Porter et al., 2016). Results showed that the averages faunal abundance decrease rapidly with duration of hypoxia when there were single continuous hypoxic events.

This confirms the results from most literature and the common-sense expectation that the population density decreases with increasingly long periods of hypoxia. Indeed, Nakano et al. (2017) suggested that mass mortality is caused by the duration rather than the frequency of hypoxia. For example, the variation in the rate of decrease in the ark shell population density showed that the duration of hypoxia is a more important determinant of mass mortality than the frequency of hypoxia.

On the other hand, Jager et al. (2018) suggested that repeated shorts events of hypoxia, even if individually not so severe, can lead to pass a threshold beyond which a new regime occurs. Once the new regimes occur it is very difficult to return to the original ecosystem. This threshold could be linked to the big differences in tolerance to hypoxia displayed by benthic organisms (Vaquer-Sunier et al., 2008). This hypothesis seems supported by the results of the experiments presented in this thesis: when a hypoxic event is short enough, like in the treatment D4R3/2 the decrease of the benthic community was significantly smaller than in a long hypoxic event stopped from a period of normoxia. This seems counterintuitive but can be explained by the idea that, over a certain threshold of hypoxia mortality carries on also when the oxygen conditions go back to normal.

Our results suggest that the effect of normoxia is positive only when the period of hypoxia is short enough not to compromise the community too much, while when the period of hypoxia is longer the positive effects of normoxia do not show up. Moreover, with longer hypoxic periods the decline of the community seemed to continue even during the normoxic period. Therefore, even if D8R3/2 has 8 hypoxic day stopped by 12 normoxic days, this relief did not allow to maintain the community in good condition. Indeed, at the end of the D8R3/2 treatment the community was drastically reduced, even more than after 8 consecutive hypoxic days.

It is known that benthic community of many coastal areas may be re-established by larval recruitment and succession; in this way the benthic community can recovered to a partially community composition and density that occurred before the hypoxic event occurred (Diaz and Breitburg, 2008). Since the experiment was performed with isolated pots, this limited the possibilities of recovery from immigration and recruitment, but about a positive effect of the normoxic period on the benthic community and its ability to persist in a stressed habitat, probably linked to physiological traits.

Johasson et al. (1997) said that the ability of the individual species to persist in a stressed microcosm it depends on the tolerance to oxygen deficiency. In response to decreasing oxygen concentrations, the abundance and species richness decreases.

Amphipods were the taxon associated with most of the variability observed in the SIMPER analysis. Indeed, crustaceans and amphipods are particularly sensitive to hypoxia as reported by Vaquer-Sunyer et al. (2008).

Crustaceans are particularly sensitive to hypoxia, as also confirmed by Johasson et al. (1997). In their experiment three species of crustaceans were studied (2 amphipods and 1 isopods) in the Baltic sea, amphipods were more sensitive than isopods with over 50% mortality recorded after 24 h exposure to nearly anoxic water. Isopod can survive anoxia for up to 11±12 day.

In our results, the tendencies in amphipod abundance were consistent (and even more pronounced) with the ones of the whole community while the abundances of polychaetes, a more tolerant taxon (Vaquer-Sunyer et al., 2008) were different.

The presence of a normoxic period was beneficial for polychaetes even after a longer hypoxic period: Polychaete abundance was slightly higher in D4R3/2 than in D4R0 but, notably, also in D8R3/2 than in D8R0.

Karson et al. (2002) argued that many polychaetes are opportunists and are favoured in unstable environments, for example, where seasonal and irregular hypoxia and/or anoxia occur.

Our result about changes in the abundances of different taxa due to the treatments leads to think that the duration of hypoxia, necessary to allow a normoxic period to have a positive effect, is directly linked to the tolerance of the single species.

Indeed, a laboratory studio descripted by Karlson et al. (2002) show that a strategy of amphidos allow them to cope with reduced oxygen condition.

The abundance of amphipods, in the treatment with hypoxia stopped by normoxic period ,is higher probably due to the capacity to minimise the costs associated with obtaining oxygen, as well as the risk of predation by moving little on or above the sediment when critical oxygen concentrations prevail.

In general, few species can dominate in a habitat's oxygen deficiency. While at higher oxygen concentrations the benthic community is composed by a higher diversity of organisms (Johasson et al., 1997).

In our experiment we considered only the structure of the benthic invertebrate community, it should be interesting to also analyse the responses of the phytobenthic and bacteria community because it allows you to have a wider view of how an ecosystem responds to this type of stress.

In conclusion my work shows that benthic invertebrate reacts to different duration and timing regimes of hypoxic stress.

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