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Effects of stochastic hypoxia events on the benthic communities of transitional waters

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Abstract

Hypoxia is one of the most important and faster spreading threats to marine life and its occurrence has significantly increased in frequency, intensity and spatial extent in the last century. The effects of hypoxia on marine organisms and communities has mostly been studied in light of the intensity of the disturbance but not a lot of attention has been given to its interaction with other stressors and the timing of its appearance. In this thesis I started to explore these topics through laboratory and manipulative field experiments focusing my attention both on macrobenthos and on the microbial community that was studied through innovative meta barcoding techniques.

This thesis also aimed at furthering our knowledge about how to manage and protect anthropically controlled estuarine and coastal habitats where hypoxic events are expected to increase in the near future.

I studied the interactive effects of thermal stress and hypoxia on a European native bivalve species (*Cerastoderma edule*; Linnaeus, 1758) and a non native one (*Ruditapes philippinarum*; Adams & Reeve, 1850) through a laboratory experiment performed in the Netherlands. The non native species displayed a vastly greater tolerance to oxygen depletion than the native one both at winter and summer temperatures. This high tolerance may give the non native species a competitive advantage potentially increasing its invasive potential. Higher temperatures confirmed their important role in increasing the effects of hypoxia even when very low temperatures were compared.

The first field experiment was performed in an Italian brackish coastal lagoon (Pialassa Baiona) and aimed at testing the effects of different timing regimes of hypoxia on the natural benthic community. Hypoxic periods of different durations were imposed repeatedly and separated by different normoxic pauses and the effects of these treatments were assessed on the macrobenthic community. It emerged that the main factor affecting the community is the duration of the hypoxic period and that the normoxic pause resulted beneficial only when the disturbance was short enough.

The ability of the communities to recover after a set of repeated hypoxic periods is the topic that was explored in the second manipulative field experiment. We imposed three different timing regimes of hypoxia on sediment patches in Pialassa Baiona and we monitored the changes of both the benthic and the microbial communities after the disturbances.. Microbes were analyzed through metabarcoding techniques that allowed to compile a library of the whole community from sediment samples. We hypothesized that the communities resulting from the different disturbances would lead to variable trajectories, and potentially end results, of recovery. The preliminary analyses of the data from this last work suggest that the experimental manipulations caused limited detrimental effects on the communities and further and more refined analyses are needed to explore the recovery process.

Overall this thesis work suggests that the duration of hypoxic events, their repetitive nature and the associated thermal stress are key factors in determining their effects on the communities of coastal areas and that management measures should point towards a reduction of the duration of the single hypoxic periods more than their frequency.

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General Introduction

Hypoxia: a spreading threat.

Oxygen is a fundamental element in the functioning of marine and coastal ecosystems. Most metazoans in fact heavily rely on oxygen for their metabolism and dissolved oxygen concentration ([DO] from now on) is an important factor in shaping marine communities. Hypoxia is broadly defined as a condition where [DO] drops below 2 mg/l, a threshold below which most benthic organisms are expected to be negatively affected by the scarcity of oxygen (Pihl, Baden and Diaz, 1991). This definition, though, is limited and doesn't account for the wide range of tolerances to oxygen depletion displayed by marine organisms (Diaz & Rosenberg, 1995) that, based on different physiological, behavioral and ecological features, can either survive significantly lower [DO]s or be negatively affected at higher ones (Hagerman, 1998; Villnäs *et al.,* 2012). The existence of basins that present hypoxic or anoxic conditions is well documented in historical (Kamykowsky & Zentara, 1990) and geological (Robinson *et al.,* 2019) times but their number has drastically increased in the last century (Breitburg *et al*, 2018). According to Shmidtko, Stramma and Visbeck (2017) the global oceanic oxygen content has decreased by more than 2% since 1960 with a pronounced variability between different areas and depths. In the upper water column these changes are mostly linked to warming and consumption by organisms. Warming in fact decreases oxygen solubility in the water and increases stratification reducing vertical mixing (Helm, Bindoff & Church; 2011; Malone & Newton, 2020). Stratification linked to increased surface temperature and scarce ventilation has shown to be one of the factors that can single handedly trigger the occurrence of hypoxia (Rabalais*,* 2009; Ni *et al.*, 2016) or enhance its effects by preventing well oxygenated surface waters to reach the bottom. The combination of these factors are expected to extend the occurrence (Keeling *et al.*, 2010), both in time and space, of Oxygen Minimum Zones (OMZ) in the open ocean intermediate and deep waters (Levin *et al.*, 2009). Oxygen minimum zones are areas of the internal oceans where, mainly due to the reduced vertical mixing, [DO] is seasonally, or continuously, close to zero, making them unfit to host biodiversity and with strong effects on biogeochemical cycles in general (Keeling *et al.,* 2010).

Increasing temperature also increases the consumption of oxygen by marine organisms through respiration (Jones, 1977; Roman *et al.*, 2019) further enhancing both the intensity and the effects of hypoxia on communities (Vaquer-Sunyer & Duarte, 2011; Kim *et al.*, 2018).

Hypoxia is also increasing in intensity and extent in coastal and estuarine habitats (Breitburg *et al.*, 2018) with more than 500 sites experiencing low oxygen concentrations since 1950 mainly during the warm season due to increasing temperatures and anthropic pressures in terms of pollution and eutrophication (Malone & Newton, 2020; Diaz, 2001).

Hypoxia in coastal habitats

Coastal environments are complex and fragile: a high and increasing percentage of the world population inhabits coastal areas and their activities impact the habitats of those areas in multiple deep ways (Airoldi & Beck, 2007). These impacts, such as overexploitation, pollution and eutrophication, despite not necessarily catastrophic by themselves, cause habitat loss and fragmentation (Thrush *et al.*, 2008) that have been traditionally overlooked compared to those happening in terrestrial habitats.

Eutrophication represents one of the most important anthropic impacts affecting coastal habitats (Rabalais *et al.*, 2009) and is directly linked to the increase in extent and intensity of hypoxic events in coastal waters (Breitburg *et al.*, 2008; Malone & Newton, 2020). Nixon (1995) defined eutrophication as *'an increase in the rate of supply of organic matter to an ecosystem'* . The main causes of this increase in supply of organic matter and the consequent imbalance in the cycles of Nitrogen (N) and other elements are human activities: agriculture, industry and urban wastewater discharge deliver high quantities of nutrients in the surrounding waters (Diaz, 2001). Sources of eutrophication can either be diffused (nonpoint) or concentrated (point) (Prepas & Charette, 2003): the most pressing challenge for future control of eutrophication is the reduction of nonpoint sources of enrichment such as the intensive use of fertilizers in agriculture, animal waste and vehicle emissions while point sources such as sewage effluent can be controlled and limited with relative ease (Horne & Goldman, 1994). According to Pacés (1982), for example, N-NO_3 concentrations in the Elbe river reported a ten fold increase from 1892 to 1976 mostly due to anthropogenic contributions and similar trends have been observed in many other basins around the world (Turner & Rabalais, 1991; Davis & Koop, 2006; Fisher *et al.,* 2006).

Due to their proximity with urban and agricultural areas, their relatively shallow and often warm waters and the influence of riverine discharge (Kralj *et al.,* 2019), coastal and estuarine areas are particularly vulnerable to eutrophication and the subsequent hypoxic events.

High concentrations of nutrients cause an increase in primary production and the emergence of macroalgal or phytoplankton blooms (Miyamoto *et al.,* 2019; Burkholder *et al.*, 2007) that initially induce diel-cycling hypoxia: during the day they increase [DO] by performing photosynthesis while at night they reduce it drastically due to their high respiration rates (D'Avanzo & Kremer 1994, Tyler *et al.*, 2009). Macroalgae, when their concentration increases excessively tend to shade themselves, die and decompose releasing further organic matter into the water (Valiela *et al.,* 1997). This process stimulates excessive respiration by organisms causing sustained hypoxic periods and other detrimental phenomena such as acidification and the release of toxic compounds with major effects on fish and benthic communities.

Coastal waters are also often characterized by a strong water stratification enhanced by increasing temperatures (Orita *et al.*, 2015; Coma *et al.,* 2009) and this tends to aggravate the imbalances in nutrients cycles and to limit water mixing by stopping Oxygen from reaching bottom waters. The

Mediterranean sea, for example, is strongly stratified from May to October due to solar radiation during Spring (Coma *et al.,* 2009; Del Giudice *et al.*, 2018). As previously mentioned high water temperatures also reduce oxygen solubility and increases oxygen demand by organisms further contributing to the insurgence of hypoxic periods in the shallow and often stagnant waters of estuaries and coastal areas (Roman *et al.*, 2019; Wang *et al.*, 2017) and modulating the responses of marine ecosystems (Conley *et al.*, 2009).

Hypoxia in marine ecosystems causes severe detrimental effects for the communities reducing their abundance, biodiversity and resilience. Benthic and pelagic organisms are affected by hypoxia at different levels: despite presenting a wide range of tolerances, most of them experience some sort of lethal or sub lethal effects. The review by Gray and colleagues (2002) shows how some actively swimming fish start to be affected in their growth at a [DO] around 6 mg/l and and show metabolic responses at 4.5 mg/l, two concentrations significantly higher than that commonly accepted to define an hypoxic environment. Crustaceans also tend to be very sensitive to hypoxia with the larval stages of some species displaying a lethal concentration of 8.6 mg/l and with, in general, median lethal times significantly lower than those of other taxa (Vaquer-Sunyer & Duarte, 2008). Molluscs, cnidarians and priapulids, on the other hand, typically display higher tolerances (Levin *et al.*, 2009; Vaquer-Sunyer & Duarte, 2008) and better adaptations (Hagerman, 1998; De Zwaan *et al.*, 2002).

The 2008 review by Diaz and Rosenberg shows a number of case studies from different hypoxic areas from around the world highlighting how the structure of their benthic communities is deeply affected by oxygen depletion. Environments, after hypoxia, display communities characterized by low abundances and diversities with cases of complete defaunation in areas such as the Chesapeake Bay (Holland *et al.*, 1987) and parts of the Black Sea (Mee *et al.*, 2005).

Areas that historically experience periodic hypoxia may present communities composed by tolerant organisms capable to resist future hypoxic periods with relatively mild effects while oxygen depletion in previously unaffected habitats may have devastating consequences reducing the community to a point where recovery would take several years to take place (Diaz & Rosenberg, 2008). Intense hypoxic events often cause mass mortalities in costal habitats (Montagna & Ritter, 2006) leading to shifts to communities often composed by smaller, short lived and opportunistic organisms (Diaz & Rosenberg, 1995).

Physiological basis of tolerance and sensitivity to hypoxia of benthic organisms

Hypoxia causes physiological and tissue damages, reproductive impairment, and ultimately mortality on most marine organisms but a plethora of strategies have evolved to come with oxygen deprivation across all taxa.

Benthic organisms such as Nematodes, Annelids, Platyhelminths and Molluscs developed different behavioral strategies to face hypoxic or anoxic environments. Escaping hypoxic areas or unburrowing closer to the sediment-water interface (Sakurai *et al.*, 1996; Dominguez *et al.,* 2021, Verdelhos *et al.,* 2015) are basic behaviors adopted by organisms to tolerate dissolved oxygen depletion. However, important adaptations are also displayed at the physiological level: most benthic organisms in hypoxic conditions tend to improve the delivery of O_2 to compensate the reduced amount of oxygen provided by the environment through biochemical adaptations in their respiratory pigments. The main oxygen carriers among invertebrates are hemoglobins which are present in Annellids and some other phyla. In addition to them, nonheme iron proteins such as hemerythrin characterize sipunculids, brachiopods, priapulids and a few annelids while hemocyanins are typical of Arthropods and Molluscs. These pigments increase the efficiency of respiration allowing organisms to survive mild hypoxic conditions (Hochachka & Somero, 2002).

In oxygen deprived environments physiological mechanisms involving the respiratory pigments are aimed at regulating the provision of O_2 related to its availability, acting on the transportation of O_2 and on its delivery to gas exchange organs. However, when hypoxic conditions become too severe these systems do not manage to compensate the O_2 deficiency. So, at this point, other biochemical processes are put in place to survive extreme hypoxic episodes. Organisms for example can store energetic substrates such as glycogen to supply for the body needs during periods of oxygen limitation. In organisms with a long anoxic survival time metabolic suppression, namely a severe down-regulation of energy turnover, also occurs to limit the production of ATP and extend tissue's survival time. Further, many animals evolved anaerobic metabolic systems to enhance the production of ATP, increasing energetic efficiency of ATP-producing pathways (Hochachka & Somero, 1984; de Zwaan & Wijsman, 1976). However, the activation of fermentative pathways represents an extreme process put in place by organisms in completely anoxic conditions (Babarro & de Zwaan, 2008).

Concluding, in hypoxic conditions metabolic pathways maximizing the ATP produced per mole of oxygen are triggered while in anoxic conditions anaerobic pathways maximizing the production of ATP from fermentation are preferred. In hypoxic or anoxic environments the up-regulation of substrate coincides with the up-regulation of the metabolic potentials. However, it has be shown that the best strategies to face hypoxia is the suppression of energy turnover supplies.

Timing of insurgence

Due to the complex interactions between factors determining it, hypoxia may present with different timing regimes. Diel-cycling hypoxia is one of the most common: [DO] is high during the day due to the oxygen released with photosynthesis and drops drastically at night due to respiration (Miyamoto *et al.*, 2019) with lower levels at the end of the night. Diel-cycling hypoxia is common in shallow waters and is linked to a complex and scale-dependent hierarchy of factors (Tyler *et al.,* 2009; Porter & Breitburg, 2016) and seem to have a selective effect on benthic communities causing the loss of susceptible organisms or specific life stages (Gedan *et al.*, 2017).

Hypoxia also appears as a seasonal phenomenon. Some systems, such as the North Adriatic or nordic fjords, are characterized by persistent seasonal hypoxia (Diaz & Rosenberg, 2008). Seasonal hypoxia normally appears at the end of summer and persists until late fall due to summer stratification and increased oxygen consumption (Diaz & Rosenberg, 1995; Hagens *et al.*, 2014). Del Giudice and colleagues (2018) showed how the main factors triggering seasonal hypoxia are long-term nutrients loadings and spring temperatures.

Environments characterized by seasonal hypoxia often experience mass mortality events during summer (Diaz, 2001) with a partial annual recovery that, though, does not prevent drastic decrease in diversity, stock size and in the recruitment of fisheries (Baden *et al.,* 1990). The analysis of benthic communities from seasonally hypoxic habitats such as the Louisiana continental shelf (USA) or Lake Grevelingen (The Netherlands) show a pronounced reduction in the number of species and in total abundance (Sen Gupta *et al.*, 1996; Gaston, 1985; Capelle *et al.*, 2021) of organisms. Communities from seasonally hypoxic habitats are kept at early successional stages by the recurring disturbances and this allows them to recover relatively quickly while environments that experience aperiodic severe events present communities that are not shaped by previous stress and may take several years to recover (Boesch & Rabalais, 1991). For example the German Bight was interested by an intense hypoxic event between 1981 and 1983 (Niermann *et al.* 1990) that reduced species abundance of around 50%. A full recovery, in this case, was not observed until 1987.

These aperiodic catastrophic hypoxic events, often related to excessively warm seasons or sudden eutrophication phenomena, are bound to become more and more frequent in the future due to climate change and increasing anthropization (Gray, 2002) and, due to their unpredictable nature, will present important management challenges.

Not a lot has been studied about the effects of multiple stressful events and of their repetition (Benedetti-Cecchi al., 2006) and for phenomena such as hypoxia whose occurrence can both be cyclic and stochastic the relationships between frequency, duration, and temporal variance are aspects that need to be addressed (Nakano *et al.*, 2017; Dal Bello *et al.*, 2017). Nakano and colleagues (2017) used numerical modeling to show how a long and continuous hypoxic events have a stronger negative effect on the populations of the bivalve *Anadara kagoshimensis* than a series of short repeated events but only focused on 6 hours stressful periods simulating a diel-cycle. The capability of bivalves to tolerate diel-cycling hypoxia by quickly reacting to the re-introduction of oxygen has been also shown in laboratory experiments (Porter & Breitburg, 2016). These kinds of responses, though, are probably species-specific and have never been studied on whole communities. The other temporal aspect of the hypoxic stress that has been studied is the recovery: after an hypoxic period long enough to induce a partial or complete defaunation the time needed for the community to recover is key in determining the effects of a subsequent stressful event (Boesch & Rabalais, 1991). Van Colen and colleagues (2008) for example, monitored the recovery of experimentally defaunated areas and, despite observing a clear Pearson-Rosenberg succession sequence, they did not obtain, over several months, a community with high similarities with the starting (control). From later observations on the same experiment (Van Colen *et al.*, 2010) emerged a tendency for recovered patches to diverge from the ambient communities creating a potential situation of patchiness and instability that may characterize frequently hypoxic environments.

Other stressors have been studied because of their recurring occurrence and of their timing regimes. Temporal variance may in fact be an important factor in determining the effects of disturbances on the communities (Benedetti-Cecchi *et al.*, 2006) not necessarily in the same direction of the effects of intensity: a high temporal variance in the occurrence of stresses may determine situations where disturbances present at short time intervals preventing the recovery even of fast-growing organisms or even where the disturbances overlap with the recruiting seasons of some specific organisms with intense and point-like effects. Regularly distributed disturbances, on the other hand, if distanced enough in time, may allow for a stable adapted community to persist (Bertocci *et al.*, 2005; Van Colen *et al*; 2010; Nakano *et al.,* 2017). The increase in frequency and the changes in patterning of extreme climatic events are expected consequences of climate change, even more than the increase in intensity and their effects on the ecosystems need to be addressed.

Aims of the work

My PhD research aimed at investigating the effects of different aspects of the hypoxic disturbance on the benthic communities of coastal habitats. At first focusing on single species and the interactions between oxygen deprivation and temperature though a laboratory experiment.

Then I investigated the effects of different timing regimes of hypoxia on a natural benthic community to understand how repeated hypoxic events can shape the communities in different ways based on how they are clustered over time. At last we extended this focus on the timing regime of repeated hypoxic events monitoring the recovery of both the benthic and the microbial communities after different regimes of hypoxic disturbance.

The thesis is composed by three chapters intended as manuscripts for publication.

Chapter 1 I performed a laboratory experiment aimed at comparing the thermal tolerances to hypoxia of a native and a non native co-existing bivalve species, *Cerastoderma edule* and *Ruditapes philippinarum.* I tested their tolerance at summer and winter temperatures under different levels of oxygen deprivation in order to test whether the non-native invasive species could be favored over the native one when the two species are co-habiting habitats characterized by frequent or chronic hypoxia. This chapter is under review for publication on PeerJ.

Chapter 2: I focused on the evaluation of the effect of different temporal regimes of induced hypoxia on the benthic community in a coastal lagoon located in the Northern Adriatic Sea (Pialassa Baiona). Coastal habitats are key ecosystems that support a plethora of ecosystem services; notwithstanding, they are influenced by different anthropogenic pressures such as pollution and eutrophication that may induce hypoxia or anoxia. In transitional habitats and lagoons, hypoxia often appears in a stochastic and unpredictable way but currently, little information is available in terms of potential effects of different temporal regimes of hypoxia on communities. We performed two different experiment aimed at assessing the effect of single hypoxic events of different duration and the potential resilience of the community to hypoxic periods of different duration followed by different normoxic breaks on these transitional habitat's communities.

Chapter 3: I performed a manipulative field experiment imposing different timing regimes of hypoxia on the communities of an Italian coastal lagoon and monitored the recovery after the disturbance. The benthic community was visually analyzed while the microbial community was monitored through innovative meta barcoding techniques. Repeated hypoxic periods can in fact have different effects on communities and these effects can lead to variable recovery trajectories with potentially different end results. Hence, analyzing the recovery of communities after variable hypoxic disturbances is key to predict and manage the trends developing in habitats characterized by oxygen depletion.

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Chapter 1

Cold and heat tolerance in chronically hypoxic coastal habitat: an experimental comparison between native and non-indigenous bivalves.

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Abstract

The occurrence of thermal anomalies is more frequent under the ongoing climate change and likely to become a severe threat for communities inhabiting chronically hypoxic microhabitats. The combination of temperature stress and local hypoxia may facilitate the invasion of non-indigenous species that could likely outcompete the native ones. At the same time, non-indigenous species that originate from warm regions, may be negatively affected by cold stress especially in regions characterized by chronic hypoxia. We experimentally explored the interactive effects of different thermal stresses and chronic oxygen depletion on the native *Cerastoderma edule* and the non-indigenous *Ruditapes philippinarum,* in the Eastern Scheldt, Netherlands. In this region the two cohabiting bivalves are increasingly exposed to hypoxia and thermal stress and represent an excellent case study. *R. philippinarum* was found to have a much wider thermal tolerance even under severe hypoxic conditions compared to *C. edule* which was highly sensitive to heat exposure. The exposure to extremely low winter temperatures combined with anoxia caused only limited mortality in *R. philippinarum*. The occurrence of thermal stress in chronic hypoxic habitats could increasingly disadvantage the native species under future climatic scenarios. This type of information is essential to predict future changes in species distributions in areas where competition between native and non-indigenous species is emerging.

Key words

Chronic oxygen depletion – Non-indigenous species – Thermal stress – Thermal tolerance - *Cerastoderma edule* - *Ruditapes philippinarum* - Multiple stressors

1. Introduction

Coastal lagoons, estuaries and transitional habitats represent extremely valuable environments both from an ecological and socio-economical point of view in that they provide important goods and services and harbor unique biodiversity (Dolberth, 2011; Haines *et al.,* 2006). Yet, due to their intermediate position between land and sea, they are often threatened by a variety of natural and human stressors (Airoldi & Beck, 2007; Wong *et al.,* 2015, Lloret *et al.,* 2008).

Of all the abiotic variables driving biodiversity loss in transitional habitats, dissolved oxygen underwent, in the last years, a drastic change in the shortest period of time, due to the combination of anthropogenic impacts (e.g. nutrient inputs) and global climate change (Diaz, 2001; Santana *et al.,* 2018; Malone & Newton, 2020). Hypoxia is commonly defined as a dissolved oxygen concentration lower than 2 mg/l (i.e., $[DO] < 2$ mg/l) while anoxia is defined as the complete absence of dissolved oxygen in the water column. Hypoxia typically has deep effects on communities in that they often become dominated by organisms with physiological or behavioral adaptations to low oxygen concentrations (Bajer & Sorensen, 2009). The increased frequency of hypoxic events and the raising number of environments affected by chronic hypoxia have been shown to directly and indirectly affect benthic communities (Montagna & Ritter, 2006; Vaquer-Sunyer & Duarte, 2008), ranging from small changes in community structure to mass mortality events (Diaz *et al.,* 2004). The intensity and duration of hypoxic periods, as well as their effects on benthic communities, are expected to further increase with the increase of water temperatures (Vaquer-Sunyer & Duarte, 2011; Del Giudice *et al.,* 2018) and the hypoxic stress is known to interact with thermal stress in complex ways. Over time this could be a key driver of the loss of fisheries, biodiversity and alterations of food webs (Diaz *et al.,* 2001).

Although generally associated with warmer waters, hypoxia and anoxia may also represent a threat in colder waters, in more northern temperate regions (Nordberg *et al.,* 2017; Magnuson *et al.,* 1985), particularly in small closed or semi-closed basins (Friedrich *et al.,* 2014). In these cold regions, harsh low winter temperatures pose serious challenges to many species (Houston *et al*., 2013), and the extra stress represented by hypoxia could potentially impair survival. Despite the greater frequency of anoxic events even in cold regions, the effects of extremely low temperatures on most aquatic ectotherms other than fish (Bajer *et al.*, 2009; Magnuson *et al.*, 1985) in chronically hypoxic habitats still deserve accurate investigations.

The increasing spread of non-indigenous species poses another important threat for marine communities (Hulme *et al.,* 2006; Booy *et al.,* 2017). Non-indigenous species may outcompete and displace native ones, causing serious ecological and economic impacts (Katsanevakis *et al.,* 2014). Furthermore, biological invasions can have large-scale effects on biodiversity (Walther *et al.,* 2009), ecosystem structure and functioning (Sgro *et al.,* 2005, Strayer *et al.,* 1999) and on the delivery of ecosystem services (Martinez *et al.,* 2007; Katsanevakis *et al.,* 2014). It has been suggested that higher tolerances to extreme or rapidly changing environmental conditions favor the spread and establishment of non-indigenous species, giving them a competitive advantage over native species (Kolar *et al.,* 2002, Diaz *et al*., 2012). At the same time, non-indigenous species often come from areas with different temperature regimes and may experience pronounced thermal stresses in the invaded environment. This means that species invading from warmer regions into temperate and cold systems could be facilitated if additional stress, such as that from hypoxia, occurs during the warm season, while the combined stress from low temperature and hypoxia might potentially limit, rather than favor, their spread during the cold season (Nie *et al.,* 2016). Understanding the combinations of factors that could facilitate or limit the success of species invasions is a pressing management need (Novoa *et al.,* 2020). To date, however, only few direct comparisons on hypoxia tolerance have been made between pairs of taxonomically or ecologically related non-indigenous and native species (Nati *et al.,* 2018; Sorensent *et al*., 2016; Stoffels *et al.,* 2017), and even less is known about the combined responses to hypoxia and thermal stress.

Here we compare the thermal tolerances of two bivalve species, the native *Cerastoderma edule* and the non-indigenous *Ruditapes philippinarum* that cohabit the Scheldt estuary, SW-Netherlands (Cozzoli *et al.,* 2013). In two laboratory experiments, we tested the tolerances of both species to thermal stress (extremely high and extremely low temperatures), simulating the occurrence of this stressful event under normoxic and hypoxic conditions. We hypothesized that, despite greater tolerance to hypoxia, *R. philippinarum* would display less tolerance to winter temperatures than *C. edule* and that this could potentially impair its invasion. The outcomes are discussed in the context of the interactive effects of temperature and hypoxia on the success of species invasions.

2 Materials & Methods

2.1 Model bivalve species

Cerastoderma edule is one of the most abundant native bivalve species in the Oosterschelde (Wijsman & Smaal, 2011), a delta area in the southwest of the Netherlands. *C. edule* is an active suspension feeder and is mostly found in the first 5 cm of sediment in intertidal habitats. It typically feeds on phytoplankton, zooplankton and organic particulate matter (Dabouineau & Ponsero, 2009; Tyler-Walters, 2007) and can survive between 0 to 32°C (Compton *et al.,* 2007), with its optimum temperature between 8 and 20°C (Brock & Kofoed, 1987). *R. philippinarum* is a non-indigenous species, native from the coasts of the Indian and Pacific Oceans, and known to have a high hypoxia tolerance (Li *et al.,* 2019; Vaquer-Sunyer & Duarte, 2008). In recent years it has become more abundant than the native common cockle (Troost *et al.,* 2018). *R. philippinarum* can survive in temperatures between 0 to 35°C (Laing & Child, 1996), with its optimum temperature estimated at 20°C (Nam *et al.,* 2008).

2.2 Collection and maintenance of bivalve species

Both species were collected in the Eastern Scheldt, which is an enclosed sea arm in SW Netherlands (Cozzoli *et al.,* 2014, Wijsman & Smaal, 2011), separated from the Sea by a storm surge barrier. The construction of the storm surge reduced significantly the tidal prism and flow velocities inside the basin (Louters *et al.,* 1998) and some of its waters, due to stagnation and the sources of pollution and nutrient overload surrounding the area may experience frequent, or even chronic hypoxic events amplified by the increasing temperatures (Capelle *et al.,* 2021; Van Colen *et al.,* 2012). Water temperature varies between 0 to 5 °C in winter and 18 to 22 °C in summer (Nienhuis & Smaal, 1994) and have shown an increasing trend in decent years (Capelle *et al.,* 2021). All organisms used were collected in the Oesterdam area of the Oosterschelde (51°28'11.0"N 4°13’12.5”E) during low tide, in May 2019 for the warm season experiment and in February 2020 for the cold season experiment.

After collection, bivalve specimens were directly transported to the NIOZ laboratories in Yerseke. They were acclimated for 10 days in a climate room (16 °C in May and 8.8 °C in February) in glass 100 l aquaria with a ≈ 5 cm thick layer of sandy sediment and filled with microfiltered water (both sediment and water were from the Oosterschelde). The bivalves were fed *ad libitum* with a monospecific microalgae culture. Water was continuously aerated and changed completely every 48 hours. After at least 10 days of acclimation, the experiments were started. No mortality was observed during acclimation in any of the two species.

2.3 Cold season experiment

After acclimation, 160 adult individuals of approximately the same size were selected for each of the two species of bivalves and divided in the 4 treatments per species resulting from the combinations of two temperatures (0.8 °C and 8.8 °C) and two levels of Dissolved Oxygen, hereafter DO, Anoxia ([DO] < 0.1 mg/l) and Normoxia ([DO] > 8 mg/l). 4 replicate bottles with 10 organisms each were set up for each treatment. The temperatures were chosen to represent a minimal, close-to-freezing winter temperature and an above average one. Two temperature-controlled baths were set up: one was maintained at the temperature of the climate room (8.8 °C), while in the second the temperature was lowered to 0.8 °C using a metal cooling spiral placed inside the water. Small aquarium pumps were used to continuously move the water to maintain a homogeneous temperature in both baths.

Anoxic water was prepared by bubbling N_2 into the microfiltered Oosterschelde water until oxygen level dropped to the desired concentration. Complete anoxia is a less frequent situation than moderate hypoxia but it is known to trigger significant species shifts in natural ecosystems (Jager *et al.,* 2018). The experiment was performed in air-tight glass bottles. The bottles for the anoxic treatment were filled with both the water and the organisms inside an anoxic tent to minimize the gas exchanges and manipulation was also simulated for the controls to check for the effects of handling. Bottles with the normoxic treatments were continuously gently bubbled with oxygen minimizing the water movement inside the bottles.

Mortality was noted twice per day by visual inspection. The transparent containers allowed a visual inspection of the organisms that were considered dead when the valves were completely opened. When the visual inspection was not decisive the responsiveness was tested by poking the organisms with a metal rod: individuals that did not respond to mechanical stimuli closing their valves were considered dead. Dead organisms were removed from the pots to avoid mortality due to the compounds released with decomposition. To remove dead organisms the containers were kept outside the water bath for the shortest possible time and the water was changed and readjusted to the desired [DO] level. Temperature and oxygen level were measured with the FireSting GO2(FSGO2)©. The experiment was suspended after 27 days.

2.4 Warm season experiment

For the summer temperature experiment a similar design was deployed, consisting of the combinations of two species (*R. philippinarum* and *C. edule*), two levels of Dissolved Oxygen (Hypoxia, [DO] < 2.2 mg/l, and Normoxia, [DO] > 7.1 mg/l) and two temperatures (16 °C and 23 °C). Temperatures were chosen to represent an average summer temperature and a substantially above average one. Two temperature-controlled baths were set up: one with water at 16 °C (room temperature) and one heated up to 23 °C. Each bath contained eight 5 l plastic jars with transparent caps. The caps had holes that allowed to bubble Nitrogen or Oxygen in the water in order to control the [DO] while minimizing gas exchanges with the water bath. Each water bath represented a level of temperature, while the pots represented 4 combinations of dissolved oxygen and species, each represented by 2 replicated pots. Each pot contained 5 specimens of one of the two species of approximately comparable sizes and where haphazardly chosen from the acclimation tanks in order to be representative of the population.

Hypoxia was obtained bubbling nitrogen directly into the pots until a [DO] <10% air saturation was reached. It was measured with a FireStingO2 optical oxygen and temperature meter 3 times a day, and adjusted to always remain below 25% air saturation. Normoxic treatments were also controlled 3 times a day and adjusted by bubbling oxygen in the pots if, due to respiration, [DO] dropped below 80% air saturation. Mortality was checked similarly to that in the cold season experiment.

The experiment was stopped after 15 days, when mortality had reached 100% for two treatments, while it was still at 0% in all the others.

2.4 Data analysis

Mortality curves and time when 50% mortality occurred (LT_{50}) were estimated and analyzed through a non-parametric Gehan-Breslow test with associated pair-wise comparisons with a

Bonferroni test for all the possible pairs of treatments. All tests were performed with SigmaPlot Version 14.5.

3 Results

3.1 Cold season experiment

Towards the end of this 27 days long experiment, some mortality appeared at both temperatures even in normoxic conditions. *C. edule* tolerated limited anoxia (Figure 1). Under hypoxic conditions the mortality of *C. edule* was significantly higher at 8.8 °C than at 0.8 °C (Tab. 1) and lower than that of *R. philippinarum* at both temperatures. The LT_{50} of *R. philippinarum* at 0.8 °C in anoxic conditions was surprisingly so long as to result similar to that measured in normoxic treatments. At 8.8 °C, the mortality of the invasive species under hypoxia was significantly higher compared to that observed at 0.8 °C, but it was still significantly lower compared to that of *C. edule* at the same temperature (Figure 1).

	C. e **anoxia - 0.8 °C**	***C. e*** **anoxia - 8.8 °C**	***C. e*** **normoxia - 0.8 °C**	***C. e normoxia - 8.8 °C***	***R. p*** **anoxia - 0.8° C**	***R. p*** **anoxia - 8.8 °C**	***R.p*** **normoxia - 0.8 °C**	***R. p normoxia - 8.8 °C***	***C. e*** **hypoxia – 16 °C**	***C. e hypoxia – 23 °C***
LT50 ± Standard error	214.922 ± 11.518	163.619 ± 8.058	475.336 ± 33.148	473.404 ± 34.773	460.227 ± 34.192	294.176 ± 14.784	408.753 ± 30.566	426.890 ± 25.382	92.412 ± 13.628	60.938 ± 7.149

Table 1: LT_{50} ± standard error (hours) for every treatment in the two experiments.

Among all anoxic treatments, *R. philippinarum* at 0.8 °C had the highest average LT_{50} (460 ± 34 hours; Fig. 1, Tab. 1), which was comparable to that measured in normoxic controls ($p > 0.99$, Tab.

2). However, at 0.8 °C in normoxia, *R. philippinarum* displayed a similar LT_{50} (408 ± 33 hours) compared to *C. edule* (475 ± 62.6 hours; Fig. 1).

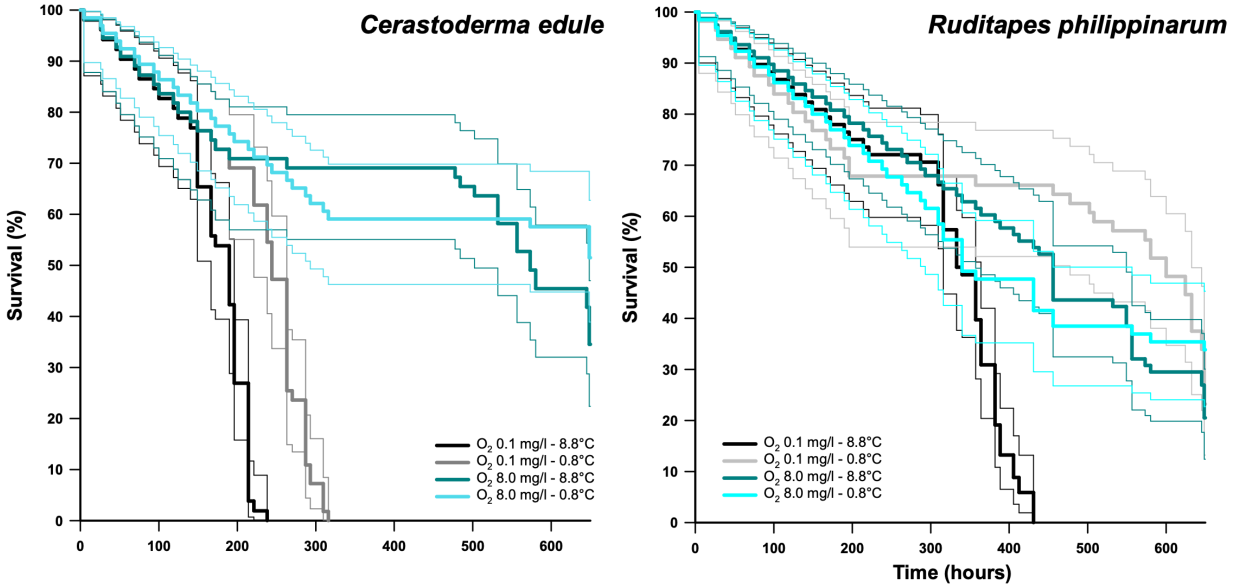

Figure 1: Survival of *C. edule* and *R. philippinarum* at winter temperatures (0.8°C and 8.8°C) under hypoxic and normoxic conditions. The highest recorded lethal time when 50% mortality occurred (LT_{50}) was observed for *C. edule* in normoxic conditions at 0.8 °C (475.336 ± 33.148), while the lowest was observed for *C. edule* in anoxic conditions at 8.8 °C (163.619 ± 8.058). The lowest LT_{50} for *R. philippinarum* was at 8.8 °C under hypoxia while at 0.8 °C and in normoxic conditions the LT_{50}s of the invasive species did not change significantly. Curves were performed on SigmaPlot Version 14.5, thin lines represent 95% confidence intervals.

C.e 0.1 O_2 – 8.8 °C	**C.e 0.1 O_2 – 0.8 °C**	**C.e 8.0 O_2 – 8.8 °C**	**C.e 8.0 O_2 – 0.8 °C**	**R.p 0.1 O_2 – 8.8 °C**	**R.p 0.1 O_2 – 0.8 °C**	**R.p 8.0 O_2 – 8.8 °C**	**R.p 8.0 O_2 – 0.8 °C**	
	21.812	**24.082**	**34.286**	**36.597**	**22.708**	**48.020**	**33.159**	**C.e 0.1 O_2 – 8.8 °C**
		21.101	**20.716**	**28.015**	**18.923**	**32.659**	**19.591**	**C.e 0.1 O_2 – 0.8 °C**
			1.240	**20.181**	0.257	2.135	0.820	**C.e 8.0 O_2 – 8.8 °C**
				11.674	3.345	3.595	1.659	**C.e 8.0 O_2 – 0.8 °C**
					17.361	**16.023**	4.314	**R.p 0.1 O_2 – 8.8 °C**
						0.862	0.154	**R.p 0.1 O_2 – 0.8 °C**
							0.038	**R.p 8.0 O_2 – 8.8 °C**
								R.p 8.0 O_2 – 0.8 °C

Table 2: F values of the Pair wise Bonferroni tests. In bold are shown significant differences.

3.2 Warm season experiment

Even under hypoxic conditions, all specimens of the non-indigenous *R. philippinarum* survived for 15 days at both temperatures. In contrast, the native *C. edule* showed high mortality under hypoxic conditions (Fig. 2). The mortality increased, albeit not significantly, at higher temperature, resulting in a LT_{50} of 60.938 ± 7.149 (Tab. 1) hours at 23 °C compared to 92.4 ± 13.6 hours at the ambient 16 °C. There were no differences in normoxic (control) conditions between the two species, which maintained 100% survival at both temperatures.

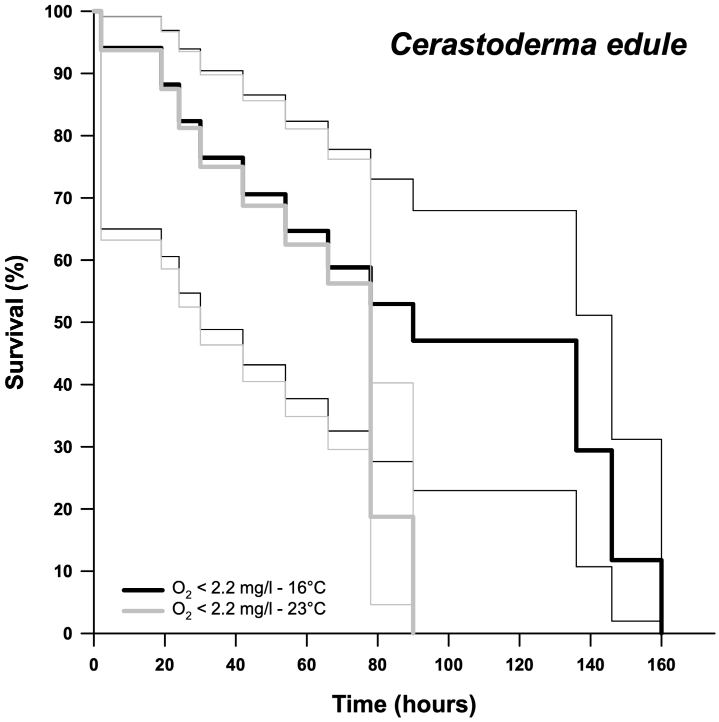

Figure 2: Survival at summer temperatures. Only the curve for *C. edule* under hypoxic conditions is shown, as *R. philipinarum* did not show any mortality during the experiment as well as *C. edule* under normoxic conditions, so those curves could not be estimated**.** The LT_{50} at 16 °C was 92.412 ± 13.628 hours while it dropped to 60.938 ± 7.149 hours with temperature increasing to 23 °C. Curves were performed on SigmaPlot Version 14.5, thin lines represent 95% confidence intervals.

4 Discussion

In our study the invasive *R. philippinarum* showed a significantly wider thermal tolerance under hypoxic conditions than the native *C. edule*. Conversely *C. edule* was very sensitive to both hypoxia and thermal stress, a condition which could increasingly disadvantage this native species under future climatic scenarios.

4.1 Cold tolerance

At 0.8 °C *R. philippinarum* showed a comparable mortality in the normoxic and the hypoxic treatments and, in hypoxic conditions, performed better at the lowest temperature. *C. edule* displayed a greater survival to cold stress than the non-indigenous species in normoxic conditions, while when subjected to hypoxia *R. philippinarum* always performed better than *C. edule*. Negative effects of hypoxia have been documented also previously for *R. philippinarum* (Kodama *et al.,* 2018, Kozuki *et al.,* 2013), but interestingly, in our study, the additional stress from cold conditions did not further impair the species. This could be related to advanced behavioral and metabolic adaptations (Li *et al.,* 2019; Velez *et al.,* 2017), even at extremely low temperatures (Nie *et al.,* 2018).

In *R. philippinarum* an increased expression of heat shock proteins under hypoxic conditions was reported by Nie and colleagues (2017; 2018), possibly explaining the comparable, if not greater, tolerance to extreme cold showed by the invasive species in the anoxic treatments. The surprising tolerance of *R. philippinarum* from the Oesterschelde to cold stress could also be explained through the *climate variability hypothesis* (Stevens *et al.,* 1989) which states that organisms acclimated to environments characterized by highly variable conditions, such as high latitude temperate areas, tend to have wider tolerance ranges and be adapted to more variable temperatures (Compton *et al.,* 2007). *C. edule* displayed a tolerance to extreme cold in normoxic conditions comparable to that of *R. philippinarum,* but its tolerance decreased steeply with anoxia. As reported by Beukema and Dekker (2020) and Compton *et al.,* (2007), native bivalves can be largely subjected to mass mortality during very cold winters. However, if low temperatures can mitigate some effects of hypoxia as suggested by our results, this could explain why in the winter mortality of both species significantly increased at higher temperatures only in the presence of hypoxia.

4.2 Heat tolerance

The simulated summer temperatures (16 °C and 23 °C), which lay within the thermal range of both species (Ansell *et al.,* 1981; Verdhelos *et al.,* 2015; Anacleto *et al.,* 2014), did not cause mortality in either species in normoxic conditions. However, when the hypoxic stress was introduced, mortality of *C. edule,* but not of *R. philippinarum,* significantly increased in treatments at the highest temperature (23 °C). The poor performance of *C. edule* observed in our experiments is consistent with the outcomes from previous experiments, where this species displayed poor anaerobic performances significantly worsened by high temperatures (Babarro & De Zwaan, 2008; De Zwaan *et al.,* 2001). This suggests that for the native species the temperature increase may be not stressful per se, but when associated to hypoxia it can seriously jeopardize the species' survival.

The invasive species, conversely, did not display any mortality at summer temperatures both in normoxic and hypoxic conditions, confirming its high adaptability to a wide range of temperatures and dissolved oxygen concentrations (Li *et al.,* 2019).

4.3 Implication for species invasiveness

Overall, these results suggest that chronic oxygen depletion would give competitive advantages to the invasive species independently of the time of the year when it occurs. Most interestingly, the disparity in tolerance to both thermal stresses between the invasive and the native species may have implications when it comes to the invasion of chronically hypoxic habitats. In an environment characterized by frequent or chronic hypoxia even a small increase in average temperature may cause mass mortality events in *C. edule* populations, which could advantage *R. philippinarum* eventually allowing it to take over. This competitive advantage is expected to persist under hypoxic conditions even during particularly rigid winters. Experiments performed on juveniles and larvae of the two species also showed that *C. edule* larvae have a moderate tolerance to hypoxic conditions in general (McArthur, 1998) while the early life stages of *R. philippinarum* display better performances even though significantly worsened by higher temperatures (Kodama *et al.,* 2018). This tendency could further facilitate the invasion of chronically hypoxic habitats by *R. philippinarum* enhancing its ability to spread and recruit. In addition to physiological and ontogenetic adaptations behavioral features could also be involved in determining the thermal and hypoxic tolerances of the species. Examining the burrowing activity of *R. philippinarum* Sakurai and colleagues (1996) concluded that its behavior is well adapted to the fluctuating conditions typical of high latitude environments. This could also potentially be due to their long siphons that allow the species to access the sediment-water interface while burrowed deeper in the sediment. Conversely the shallower average burrowing depth and the shorter siphon length typical of *C. edule* may decrease its tolerance to higher temperatures (Dominguez *et al.,* 2021, Verdelhos *et al.,* 2015). The tendency of bivalves to burrow deeper in the sediment in response to high temperatures also contrasts with the tendency to emerge towards the sediment-water interface in hypoxic conditions (Marsden & Bressington, 2009; Long *et al.,* 2008) which could further complicate the interaction between temperature and oxygen level on the two species.

Climate anomalies and anthropogenic alterations are increasing globally, with particularly severe effects in coastal enclosed habitats (you could cite Malone and Newton 2020). Our results not only corroborates that increasing temperatures, together with more frequent hypoxic events, cause higher mortality rates among sessile benthic species, including economically important species, but further suggests that these changes may also bring coastal environments closer to optimal conditions for invasive species, potentially opening to long-term irreversible effects (Byers *et al.,* 2002; Sheffer *et al.,* 2001). The results further suggest the need to assess the different tolerances of species case by case and in relation to other multiple stressors, to be able to forecast future invasion scenarios in increasingly stressed environments.

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Chapter 2

Effects of different timing regimes of hypoxia on the benthic community of an Italian coastal lagoon (Pialassa Baiona, Ravenna)

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Abstract

The appearance of cyclic and aperiodic hypoxic events is already and important threat to coastal habitats and is expected to increase in the near future in a scenario of global warming and anthropic disturbances. Hypoxic events can appear in different timing regimes, both cyclic and aperiodic, and the effects of these changes in occurrence over time can have drastically different effects on the communities. We performed two manipulative field experiments in Pialassa Baiona, a hypertrophic coastal lagoon in the Northern Adriatic Sea, to test how different timing regimes of hypoxia affect the benthic community. In the first experiment we applied three hypoxic periods of increasing durations (2, 4 ,6 and 8 days). We observed how longer hypoxic periods caused more severe effects in terms of abundance and species richness but that the majority of the decline happened in the first two days of disturbance.

In the second experiment we applied two hypoxic periods of different durations separated by normoxic pauses of different durations. From the analysis of the benthic community emerged how a normoxic pause can be advantageous for the community only if the hypoxic events are short enough while, with longer hypoxic periods, the decline of the community is not significantly reduced by a pause between the two disturbances.

Key words

Chronic oxygen depletion - Coastal lagoons - Benthic community - Timing regime - Repeated events

1. Introduction

Coastal and estuarine lagoons are enclosed water bodies, with limited connection to the sea. They are often very shallow, highly productive, and extremely variable in terms of temperature, salinity, suspended organic matter and dissolved oxygen concentrations (from now on [DO]) (Sarà, 2009; Del Giudice *et al.*, 2018). Coastal lagoons constitute highly diversified habitats characterized by a great biodiversity and represent an important carbon sink (Duarte *et al.*, 2005; Sanders *et al.*, 2007; Donato *et al.*, 2011; McLeod *et al.*, 2011). Coastal habitats, lagoons and estuaries also provide a significant amount of ecosystem goods and services (Costanza *et al.*, 1997, Harley *et al.*, 2006) representing a valuable and large service-providing environment. Coastal areas, in fact, host a high (>60%) and increasing percentage of the global human population (EEA 1999a; Airoldi & Beck, 2007) and are consequently subjected to a variety of impacts such as pollution, habitat modification, over-exploitation and fragmentation (Mc Garigall & Mc Comb, 1999; Thrush *et al.*, 2008; Levin, 2009).

Transitional habitats such as lagoons and salt marshes, shaped by the blurring edges of land and sea and often surrounded by urban areas, are particularly affected by direct human impacts and are also vulnerable to the effects of climate change due to their physical and hydrological characteristics such as shallow and stagnant waters (Huges *et al.,* 2011). In these waters excessive nutrient inputs from surrounding urban or rural areas (Wong *et al.*, 2015), together with frequent heat waves and insufficient mixing can have deep effects on [DO] levels (Santana *et al.*, 2017; Rabalais *et al.*, 2009).

Dissolved oxygen, one of the most important ecological variables, has changed drastically over the past decade in coastal waters (Lafolley & Baxter, 2019). The number of the coastal sites where hypoxia has been reported, is progressively increasing with a consistent exponential growth (Vaquer-Sunyer and Duarte 2008). Hypoxia is often defined as a condition where [DO] drops below

2 mg/l but the susceptibility of species to this threshold is not general since the sensitivity to oxygen depletion greatly varies among taxa (Diaz & Rosenberg, 1995; Diaz, 2001; Breitburg *et al.*, 2019). Based on frequency, duration and intensity the effects of hypoxic events on communities can range from small community shifts to the emergence of dead zones (Diaz *et al.*, 2001; Vaquer-Sunyer & Duarte, 2008). In general, benthic communities in hypoxic environments present low biodiversity and are dominated by the few species presenting physiological or behavioral adaptations to oxygen deprivation (Bajer & Sorensen, 2009) and by opportunistic organisms (Kanaya *et al.*, 2015) with short life cycles that make them able to re-inhabit habitats after stressful periods.

The emergence of hypoxic events are multiple and complex and often linked to anthropogenic impacts such as the impact of local warming or excessive eutrophication. The increase of water temperatures, for example, provokes a decrease of oxygen solubility facilitating the insurgence of hypoxia (Diaz & Breitburg, 2009; Del Giudice *et al.,* 2018); higher temperatures also aggravate the effects of hypoxia on benthic organisms (Sturdivant *et al.*, 2015; Comandini *et al.,* 2022) making global warming one of the main phenomena related to the spread of hypoxic zones. Similarly, anthropogenic eutrophication contributes to the insurgence of environmental oxygen deprivation (Caballero-Alfonso *et al.*, 2015; Del Giudice *et al.*, 2018) since high levels of nutrients (mainly Phosphorus and Nitrogen) when released in the environment increase the primary production with a consequent cascade effect on decomposition and oxygen depletion (Correll, 1998; Diaz & Rosenberg, 2008). Hence, urban water treatment and discharge, the use of fertilizers in agriculture and other sources of pollution from heavily anthropized areas have deep impacts on the [DO] in many transitional and lagoon areas (Howarth; 1996; Diaz, 2001; Wong *et al.*, 2015; Breitburg *et al.*, 2019).

Due to this number of interacting factors and causes involved in its development, hypoxia is a complex phenomenon that can occur in multiple ways since its magnitude is highly dynamic and can vary in duration, intensity and frequency (Friedrich *et al.*, 2014). Hypoxia, from a temporal standpoint, can occur in a cyclic or stochastic ways. Diel hypoxia is a common form of cyclic hypoxia where [DO] decreases during the night reaching a minimum in early morning and increases again during the day due to the photosynthetic activity of plants and microorganisms (Tyler *et al.*, 2009). The second cyclic regime in which hypoxia occurs is seasonal: in this case [DO] is low during the warmer season, with a minimum in late summer or early autumn (Peterson *et al.*, 2013; Porter *et al.*, 2016). Other than these two main temporal pulses, hypoxic events can have a stochastic nature and due to the complexity of the interactions of the causing factors these events may occur in an unpredictable way. Often experimental studies on groups of organisms have focused on the effects of a single hypoxic event. The effects of the different temporal regimes of hypoxia on the benthic community, on the other hand, have not been studied exhaustively (Benedetti-Cecchi *al.*, 2006) even though the general consensus is that, after an impactful hypoxic event, the resilience of the ecosystems against subsequent hypoxic periods might be eroded (Conley *et al.*, 2009).

Other kinds of stressors have been studied in light of their repetitive nature and the results have shown a tendency of clustered events to have a less detrimental effect than randomly distributed ones (Benedetti-Cecchi *et al.*, 2006; Mumby *et al.*, 2011; Wolff *et al.,* 2016).

In light of this, duration, frequency and temporal distribution are factors that can tune the impact of hypoxia on ecosystems in different and complex directions. For example, the mass mortality of the ark clam *Anadara kagoshimensis* (Tokunaga, 1906) is caused by the duration of hypoxia but not by its frequency: 6 hour cycles of normoxic and hypoxic periods did not cause mortality as much as a continuous hypoxic period did (Nakano *et al.,* 2017).

In transitional environments and lagoons hypoxia is influenced by climate, nutrients discharge and freshwater inputs. These factors make the appearance of hypoxia often stochastic and unpredictable.

Therefore, understanding the effects of different temporal regimes of hypoxia represents a key factor to forecast the resilience of the benthic communities and plan management strategies. In the present work we aimed at studying the effects of different timing regimes of hypoxia on the benthic community of a coastal lagoon. Thus, we performed two parallel field experiments: the first to assess the effect of single hypoxic events of increasing durations and the second to quantify the impact of repeated disturbances separated by different normoxic pauses.

2 Materials and methods

2.1 Study site

The experiments were conducted in Pialassa Baiona (Italy): a brackish lagoon located along the northern Adriatic Italian coasts between the Ravenna harbor and Lamone river. It is an eutrophic microtidal lagoon with a surface of 11,8 km^2 (Ponti *et al.*, 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 *et al.*, 2005). It is affected by anthropogenic eutrophication, which causes extensive growth of seaweeds and phytoplankton blooms that are responsible for the events of anoxia that occasionally occur in summer (Ponti *et al.*, 2011).

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). Therefore, Pialassa Baiona is one of the most anthropogenically disturbed coastal lagoons in the Mediterranean Sea, due to its proximity to important urban, industrial and harbor areas. (Ponti *et al.*, 2009)

The coastline zone, where the experiment was performed, was chosen as homogeneous as possible. Our experiment was conducted on a shallow subtidal mudflat in the northern part of the lagoon, which is the further north from the urban water discharges.

2.2 Field work

The experiments were performed from 8 to 29 July 2019. Experimental units consisted of 15,5 cm diameter plastic tubes covered with a 0,5 mm mesh plastic net to eliminate the potential source of variability due to predation and organism's loss, both controls and treatments were continuously covered with the net to reduce any possible confounding effect as much as possible.

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 that allowed the operator to recognize them even with low visibility and poor weather condition._

All experimental units were placed and sampled avoiding disturbance and trampling as much as possible on the surrounding sediment in order not to affect the community.

Hypoxic conditions were imposed by covering the treated pots with a black plastic bag fixed with two thick rubber bands that allowed the pot to be covered and uncovered easily even with high tide. The controls were left covered by the plastic mesh in order to avoid the possible confounding factor due to migration in and out of the tubes. At the end of each treatment tubes were removed and their whole content was sieved through a 0,5 mm net and washed to remove as much sediment as possible before being fixated in alcohol on the field.

Temperature, salinity and concentration of oxygen (% and mg/l) were measured in three separate sites within the experimental area at the end of each sampling.

2.3 Experiment 1: Experimental design

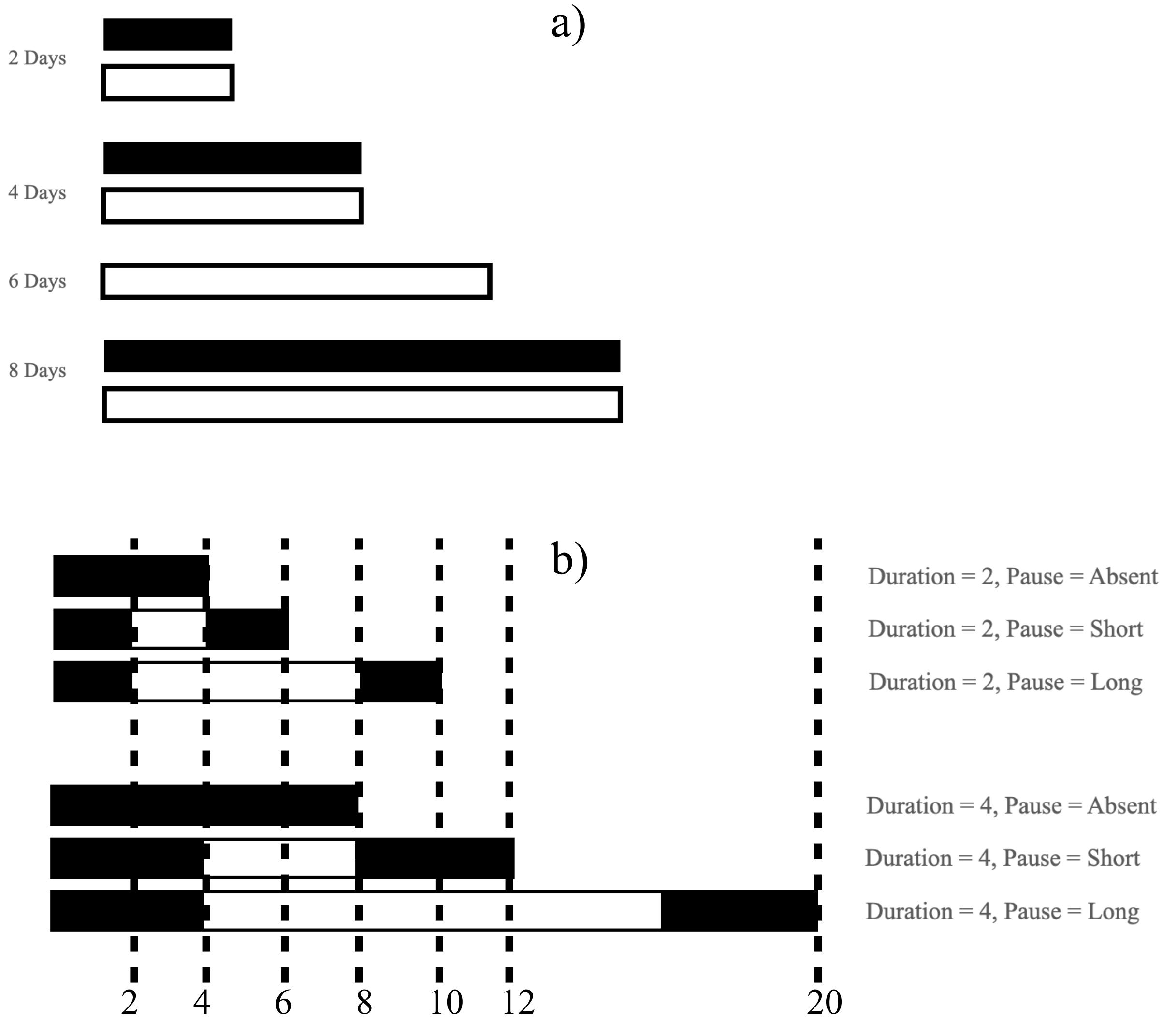

Fig. 1: Experiment Designs. Each bar represents a Treatment. Black portions represent Hypoxic periods and white ones represent Normoxic periods. Time is expressed in days. a) Represents the design of Experiment 1 and b) of experiment 2.

The first experiment focused on assessing the effect of increasing durations of hypoxia on the benthic community. Four replicate tubes were sampled before the beginning of the experiment

(Time 0) to have a base-line community and after 2, 4 and 8 days (Fig.1a). The four control replicates sampled after 6 days were also included into the experiment to increase the definition of our data. The factors that composed the design were Time (five factors: 0, 2, 4, 6 and 8 days) and Treatment (two factors: Hypoxic Treatment and Normoxic Control). The design is not balanced because levels 0 and 6 of factor Time do not present both levels of factor Treatment. We addressed this potential problem choosing statistical analyses suitable for unbalanced designs.

2.4 Experiment 2: Experimental design

The second experiment aimed at assessing the effects of two hypoxic periods separated by normoxic pauses of different durations (Fig.1b). The design included two factors: Duration of hypoxia (2 levels: 2 days and 4 days) and Duration of the normoxic Pause (3 levels: Absent, Long and Short). With an Absent pause the two hypoxic periods were consecutive, with a Short Pause they were separated by a normoxic period as long as the hypoxic one and with the Long Pause the normoxic period was three times as long as the normoxic one (Fig. 1b).

2.5 Laboratory analyses

The whole content of each experimental unit was analyzed as a sample. All the samples were moved into the Bologna University laboratories in Ravenna where they were sieved through a 4000, a 2800 and a 500 μm mesh sieve stacked.

The content of the pots sieved through the 4000 μm mesh was analyzed with a magnifier while the portions that were retained at 2800 and 500 μm were analyzed with a stereoscope (Fig.7).

The smaller size class (500 μm) was stained with Rose Bengal, a stain that reacts with cytoplasm and tissues (Bernhard et al, 2006), assuming that the organisms that were colored and in a intact in laboratory, were alive at the moment of the sampling in Pialassa Baiona.

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

2.6 Statistical analyses

Individual taxa abundances were standardized as the percentage of the highest abundance in which that taxon appeared in a sample. This transformation allowed to compare abundances reducing the impact of abundant species. Species considered too rare were excluded from the analysis. To show the trends in abundances we performed piece-wise regressions on the transformed data. This technique allowed to visualize eventual break-points and relate them with changes in oxygen conditions. All the regressions were performed on SigmaPlot Version 14.5.

Diversity indices were calculated and analyzed through one and two ways ANOVAs and visualized through box plots. Univariate analyses were performed on RStudio Version 1.2.5042.

The abundances of single taxa were then fourth root transformed, compared through a Bray-Curtis similarity matrix and visualized with a non-metric Multi Dimensional Scaling (nMDS). PERMANOVA analyses with pair-wise comparisons were performed on the transformed abundances to test the significance of the factors of the two experiments and their interactions.

The influences of the single taxa in determining the diversities between groups of treatments were analyzed through a SIMPER analysis. All multi-variate analyses were performed on PRIMER7&PERMANOVA (Anderson *et al*, 2008).

3. Results

3.1 Experiment 1

3.1.1 Abundances

Abundances tended to decrease over time both in controls and in hypoxic treatments (Fig.2). The treatments, however, showed a significantly faster decline in abundance (from 759±353 to 128±72) than the controls (from 759±353 to 392±147 individuals) over the 8 days as confirmed by the analysis of variances that showed a significant effect of both time (p=0,00641) and treatment (p=0,02228).

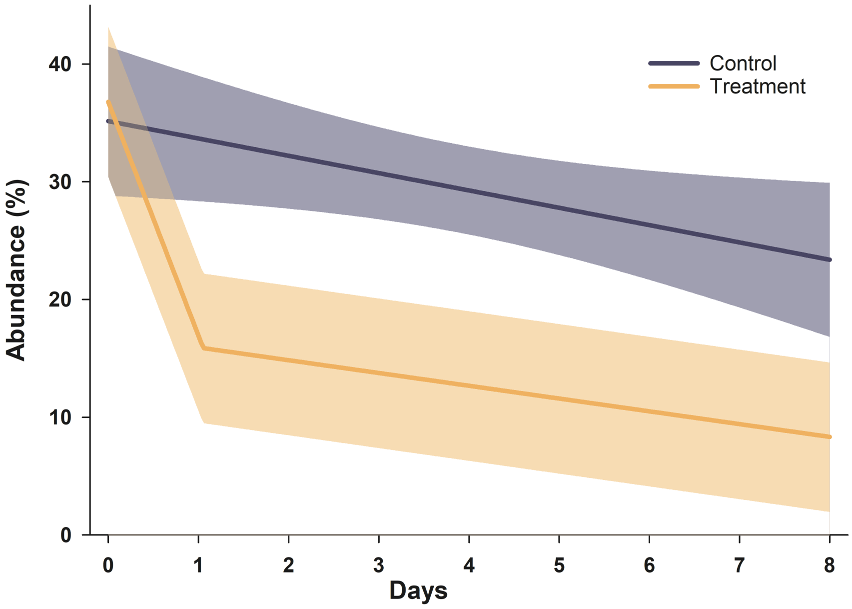

Fig. 2: Piecewise regression of abundances. Data were normalized as the 100% of the highest abundance of each individual taxon in a

Regressions show the trends over time and the normalization applied to the data allowed to treat all the species simultaneously. The piecewise regression (Fig.2) showed a steeper decline in the community abundance in the treatments during the first two days of hypoxia compared to the controls. After the first two days the curve of the treatments presents a clear breakpoint and assumes a slope comparable to that of the controls. Some of the species such as *Abra alba* (Wood, 1802) and *Cerastoderma edule,* when analyzed separately, display a tendency that reflects that of the whole community, with a significant difference between treatments and controls after the first two to four hypoxic days. Other species, such as the gastropod *Ecrobia* spp. and the polychaete *Capitella capitata* (Fabricius, 1780) did not display significant differences between treatments and controls (Fig. 3).

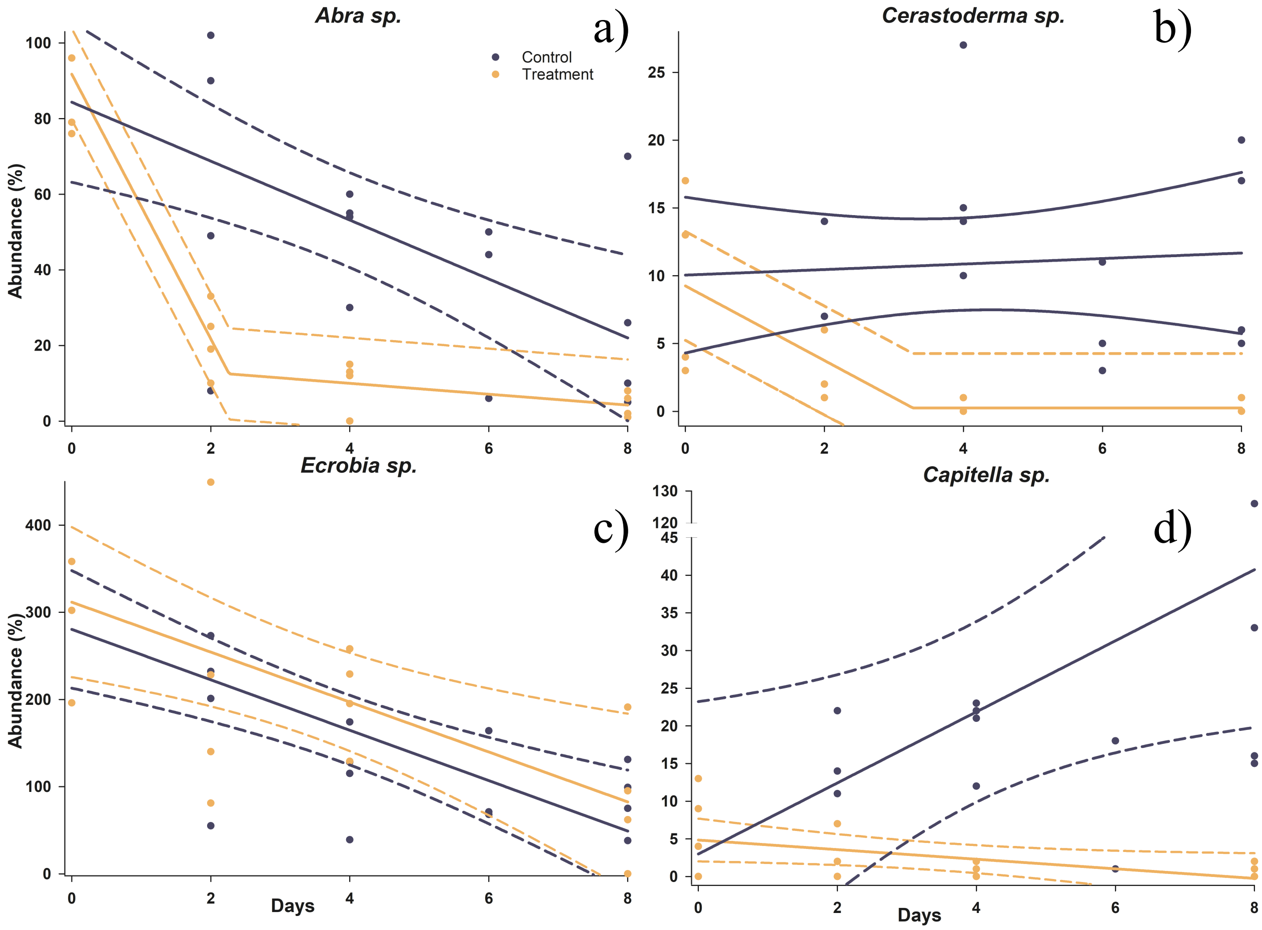

Fig. 3: Piece-wise regressions over Time (in Days) of the abundances of single species. The bivalves *Abra spp.* and *Cerastorderma spp.* (6a and 6b) show trends that reflect those of the overall community with a clear break point in the hypoxic treatments (in yellow) after 2 or 3 days of hypoxia. The gastropod *Ecrobia spp.* showed a clear decreasing trend (6c) with no differences between Treatments and Controls. The anellid *Capitella spp.* on the other hand showed an increase in abundance in the controls and a relatively stable situation in the hypoxic treatments.

3.1.2 Diversity indices

Both species richness (S) and the diversity index N1 declined over time in the anoxic treatments but not in the controls displaying a significant interaction between Time and Treatment (p=0.027). The linear regressions (fig. 4) displayed a significant decreasing trend over time in the hypoxic treatments (p=0.0127) for species richness but no significant changes in the controls (p = 0.583). The diversity, on the contrary, showed a significant increase in the controls (p=0,00071) but no changes in the treatments.

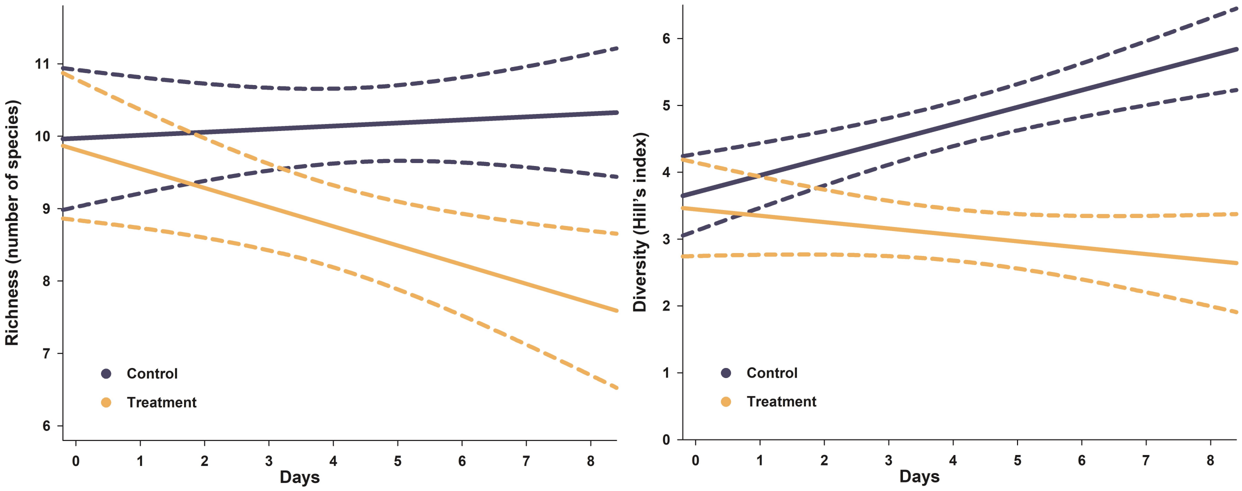

Fig. 4: Linear regressions of the Diversity indices. a) Shows the regression over Time (in days) of the Species richness while b) shows the trend of Hill's index of diversity. In black are represented the normoxic controls while in yellow the Hypoxic treatments.

3.1.3 Multivariate analyses

The PCO (Fig. 5) performed to explore the structure of the whole community showed a clear separation between the hypoxic treatments (shades of blue in the plot) and the controls (shades of red in the plot) and different trajectories over time. These differences are confirmed by the PERMANOVA analysis that showed a significant effect of both Time and Treatment on the structure of the community.

Pairwise comparisons showed significant differences between control and treatments at 4 and 8 days. Within the hypoxic treatments the community after 4 and 8 days differed significantly from the initial one and that after 2 days days resulted significantly different from the one after 4. Globally the community became increasingly poor, less diverse and its structure changed deeply over time. The Control and the Treatment after 4 days showed a 29,44% dissimilarity constituted for the 42,73% by Amphipods, the bivalve *C. glaucum* and the annelid *Capitella capitata* cumulatively.

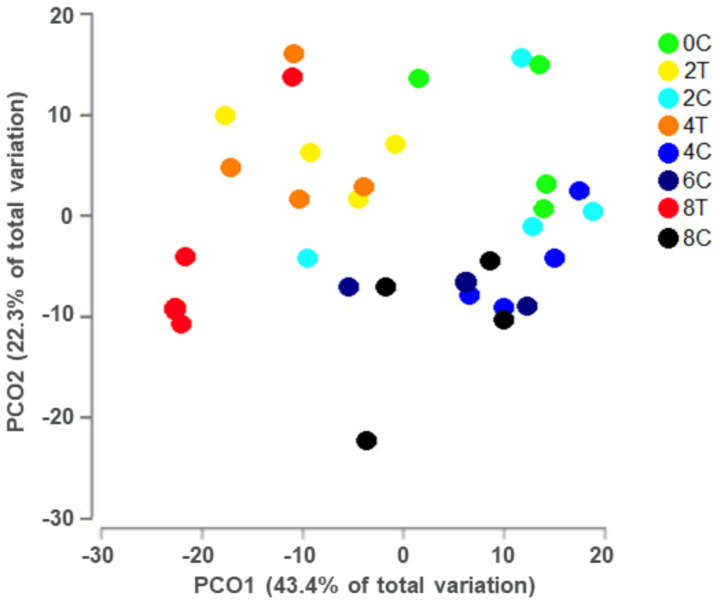

Fig. 5: PCO representing the community structure over time.
Controls are in shades of blue and hypoxic treatments in shades of red.

After 8 days the Treatment and the Control displayed a 33.29% dissimilarity to which *Capitella capitata, C. edule* and Naididae sp. contributed cumulatively for the 44.70%.

Hypoxic treatments after 4 days differed from the initial community for the 28.49% constituted for the 44.88% by Amphipods, the bivalves *Abra alba* and *Cerastoderma edule.*

After 8 days of hypoxia the dissimilarity with the initial community was the 39.71% constituted for the 39.94% by Amphipods, the gastropod *Ecrobia* and *Abra alba.*

Hypoxic treatments after 2 and 4 days displayed the 19.86% dissimilarity and *Cerastoderma, Abra alba* and the annelid *Capitella capitata* contributed to it for the 40.00% cumulatively.

3.2 Experiment 2

3.2.1 Abundances

Linear regressions indicate that in the treatments with 2 days hypoxic periods (Fig. 6a) the presence of a longer pause resulted beneficial for the benthic community while with 4 days hypoxic periods (Fig. 6b) there was no difference in the slope of the regressions from the treatments with a Long and a Short pause.

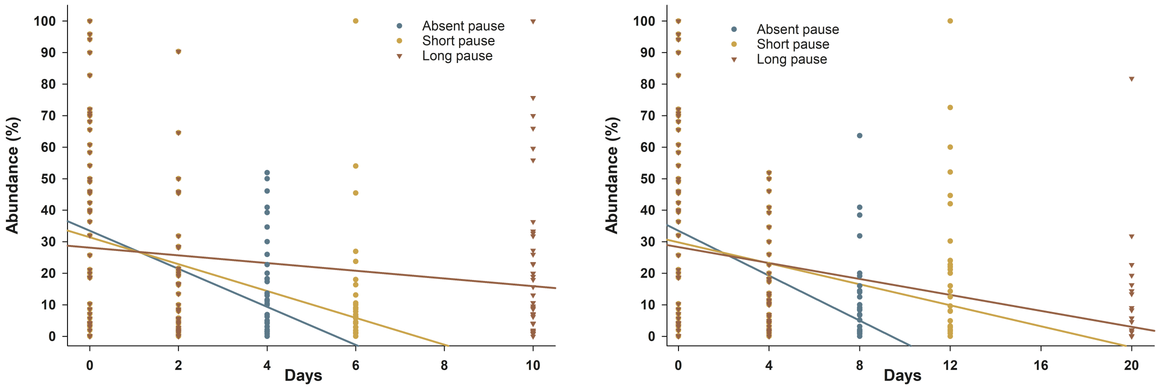

Fig. 6. Linear regressions of normalized abundances over time. On the left abundances from the 2 days Duration treatments and on the right those from the 4 days Duration Treatments. In blue treatments with Absent Pause, in yellow Short Pause and in red Long Pause. In the 2 days Treatment the slope of the regression with the Long pause resulted significantly different from the other two while in the 4 days Treatment all the three slopes differed significantly from each others.

With a two days hypoxic period the only significant difference in the slope of the regression resulted with the Long Pause, where the decrease in overall abundance was slower than in the other two treatments, while with a 4 days hypoxia both the Long and the Short Pause treatments displayed a significant difference in slope: the Absent Pause treatment displayed a steeper decline than that with a Short Pause (ANCOVA p<0,001) and a Long Pause (ANCOVA p<0,001); the Short Pause and The Long Pause treatments also displayed a significant difference in slope (ANCOVA p=0,019).

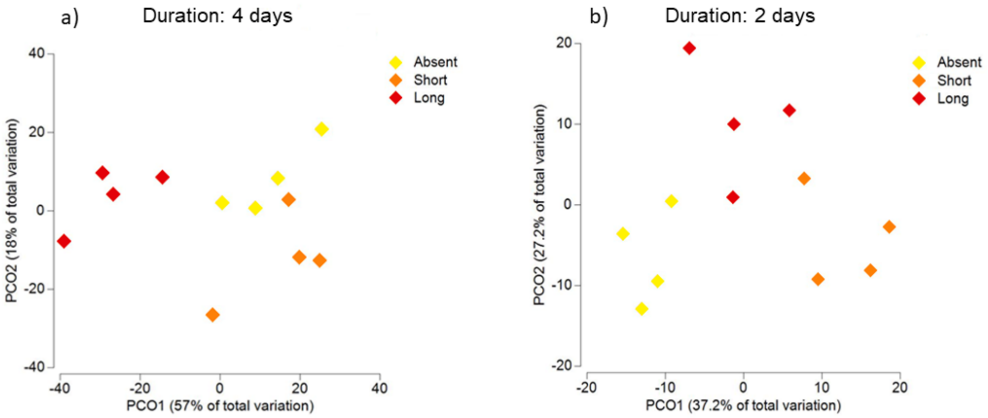

Fig. 7. PCOs from experiment 2. Duration 2 and Duration 4 Treatments were separated for readability. In a) are shown samples with 4 days hypoxic periods. Absent Pause in yellow, Short Pause in orange and Long Pause in Red. In b) samples with 2 days hypoxic periods. Absent Pause in light blue, Short Pause in blue and Long Pause in black.

3.2.2 Multivariate analyses

PCOs (Fig. 7) also show different trends between the 2 days treatment and the 4 days treatment: with 2 days of hypoxia the treatment with the Short Pause separated clearly from the others while with 4 days of hypoxia the samples from the treatment with a Short Pause were closer to those with Absent Pause while those with a Long Pause separated clearly.

PERMANOVAS (Table S1, Supplementary Materials) confirmed the significant interactions of Duration of Hypoxia and Duration of the Pause. Pairwise comparisons show a significant difference between all the treatments with 2 days of hypoxia while within the treatments with 4 days of hypoxia the treatment with the Absent Pause and that with the Short Pause did not differ significantly. The difference between treatments with a Long Pause with 2 and 4 days of hypoxia resulted significant.

The SIMPER analysis allowed to explore how the single taxa contributed to the dissimilarity between the pairs of treatments. Similarly to what happened in the first experiment the majority of the dissimilarity between the treatments were constituted by different taxa. Among those, Amphipods, Isopods, bivalve *Cerastoderma edule* were recurring as the most influential ones (Table S2, S3 and S4. Supplementary Materials).

4 Discussion

These experiments aimed at assessing the effect of different timing regimes of hypoxia on the benthic communities of a brackish lagoon. From our first experiment emerged a clear negative effect of hypoxia on the benthic community. The experimental units were almost completely defaunated after eight hypoxic days but the majority of the decrease appeared to happen during the first two days as shown by the piece-wise regression. Both species richness and diversity displayed different trends in Treatments and Controls with the hypoxic treatments. Stressed communities appear less diverse and poorer than the unstressed ones. Long hypoxic periods have shown to cause such effects on benthic communities (Rabalais *et al.,* 2002) together with a strong biomass and abundance decrease. The taxa that appeared to be the most affected by the stressing period were Amphipods and the bivalves *Cerastoderma glaucum* and *Abra alba.* Multiple laboratory experiments confirmed the low tolerance of amphipods to hypoxia (Hoback & Barnhart, 1996; Hervant, Mathieu & Culvert, 1999; Sandberg-Kilpi, Vismann & Hagerman, 2013) and crustaceans in general, have shown to be one of the least tolerant taxa (Vaquer-Sunyer & Duarte, 2008). Also, bivalves of the genus *Cerastoderma* displayed poor performances under hypoxic conditions in experiments despite their physiological adaptations to oxygen depletion (De Zwaan *et al.,* 2001; Babarro & De Zwaan, 2008; Comandini *et al,* 2022). Our experimental units were designed to prevent movement of organisms in and out simulating an environment completely interested by oxygen depletion but it's interesting to note how in areas that are only locally hypoxic mobile organisms may be favored by their ability to escape stressful conditions (Rabalais IUCN). Unburrowing closer to the sediment-water interface or migrating away from hypoxic waters are in fact all behavioral reactions that burrowing, mobile benthos and fish developed in hypoxic environments (Long *et al*., 2008; Magnuson *et al.,* 1985, Verdelhos *et al*., 2015) increasing their tolerance.

In the second experiment two hypoxic periods were imposed and separated by normoxic periods of different durations. It emerged that a normoxic pause separating two hypoxic periods is beneficial for the community only if it is long enough and if the stressing event isn't too long. With a short hypoxic event the presence of a longer normoxic pause appeared to favor organisms: the community, after two consecutive 2 days hypoxic periods, resulted similar to that where the 2 days hypoxic events were separated by 2 days of normoxia. Organisms' abundances, on the other hand, were significantly higher and the community resulted richer and more diverse if the hypoxic periods were separated by a 6 days normoxic pause. The situation is exacerbated with hypoxic periods of 4 days: in terms of abundance the community decreased slightly faster in the treatment with no Pause while with a Short or Long Pause the normoxic periods appeared to be beneficial for the community in terms of overall abundance, diversity and structure. Analyzing the communities in detail emerged that the taxa representing the highest amount of diversity between the treatments with 2 and 4 days hypoxic periods were Amphipods, the gastropod *Ecrobia* spp and Isopods. Within the treatments with 4 days hypoxia also *Cerastoderma edule* emerged as a relevant species in determining the dissimilarities between communities. Previous experiments support this idea that the effects of intermittent hypoxia increase with the increase in duration of the single hypoxic periods (Kim *et al.* 2013). Organisms such as Molluscs and Arthropods can increase the efficiency of their respiration through alternative oxygen carrying proteins (Hochachka & Somero, 2002) or move away from deprived oxygen areas (Sakurai *et al.*, 1996; Verdelhos *et al.,* 2015; Dominguez *et al.,* 2021) but these strategies are often only effective in situations of mild or spatially limited hypoxia. When oxygen deprivation is extended and intense some extreme adaptations may arise, such as the activation of fermentative patterns, but they cannot sustain organisms of long times (de Zwaan & Wijsman, 1976; Hochachka & Somero, 1984; Babarro & de Zwaan, 2008).

In general, we can expect that at least a baseline portion of the community in the Pialassa Baiona was shaped by its ecological history characterized by previous, naturally occurring hypoxic periods (Ponti *et al.,* 2011). This history of periodic oxygen deprivation may have shaped a community with a relatively high overall tolerance to hypoxia potentially mitigating the magnitude of the effects of these experiments: further research should analyze whether in previously unaffected environments the effects of the same timing regimes of hypoxia may change both in magnitude and in nature. As stated by Rabalais (IUCN Report), environments strongly affected by hypoxia tend to present communities characterized by low diversities and abundances and dominated by smaller non deeply-burrowing species. These trends appeared clearly in areas with seasonally severe hypoxia such as the Northern Gulf of Mexico (Rabalais *et al*., 2001; Kim *et al.*, 2013) but the effects were less severe in areas with intermitted hypoxia. Environments characterized by hypoxic conditions are in fact expected to increase in the near future (Lafolley & Baxter, 2019) and understanding how emerging, repeated, hypoxia will affect them will be an important challenge for ecology. The significant changes in communities emerging from various hypoxic regimes may also lead to different recoveries at the end of the hypoxic occurrence. The analysis of recovery is another step forward needed to formulate better predictions and management plans of coastal environments susceptible to hypoxia.

Acknowledgments

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Supplementary Materials

Comparison 2 days	**Diff. of Means**	**t**	**P**
Long vs. Absent	2.227	6.126	<0.001
Long vs. Short	1.518	4.204	<0.001
Short vs. Absent	0.709	1.949	0.052

Comparison 4 days	**Diff. of Means**	**t**	**P**
Long vs. Absent	4.219	6.021	<0.001
Long vs. Short	2.569	3.691	<0.001
Short vs. Absent	1.65	2.354	0.019

Table S1: pairwise comparisons from the PERMANOVA analysis. Comparisons are made within levels of factor Duration (2 days on top, 4 Days on bottom).

	Group 4Short	Group 4Long				
Species	**Av.Abund**	**Av.Abund**	**Av.Diss**	**Diss/SD**	**Contrib %**	**Cum.%**
Isopoda	1.88	0.00	8.33	4.63	15.94	15.94
Anfipoda	2.04	0.25	7.65	3.96	14.65	30.59
Hydrobia sp.	3.65	2.32	5.85	1.81	11.20	41.79
Polyplacophora	0.37	1.59	5.68	1.67	10.88	52.67

Table S2: Results of the SIMPER analysis for groups 4 Short and 4 Long. Dissimilarity was 52.24%. In the table are shown the taxa that contribute cumulatively to the 50% of that dissimilarity.

	Group 2Long	Group 4Long				
Species	**Av.Abund**	**Av.Abund**	**Av.Diss**	**Diss/SD**	**Contrib%**	**Cum.%**
Anfipoda	3.44	0.25	11.57	6.33	22.19	22.19
Cerastoderma	1.88	0.00	6.80	3.48	13.04	35.22
Isopoda	1.81	0.00	6.54	5.29	12.55	47.77
Abra alba	1.56	0.30	4.66	1.89	8.94	56.71

Table S3: Results of the SIMPER analysis for groups 2Long and 4Long. Dissimilarity was 52.14%. In the table are shown the taxa that contribute cumulatively to the 50% of that dissimilarity.

	Group 4Absent	Group 4Long				
Species	**Av.Abund**	**Av.Abund**	**Av.Diss**	**Diss/SD**	**Contrib %**	**Cum.%**
Anfipoda	1.81	0.25	7.60	2.67	15.93	15.93
Isopoda	1.45	0.00	7.18	4.23	15.07	31.00
Hydrobia sp.	2.41	2.32	6.35	1.61	13.31	44.31
Abra alba	1.36	0.30	5.45	1.90	11.44	55.75

Table S4: Results of the SIMPER analysis for groups 4Long and 4Long. Dissimilarity was 47,68%. In the table are shown the taxa that contribute cumulatively to the 50% of that dissimilarity.

Chapter 3

Study on the recovery patterns of the microbial and benthic communities of a coastal lagoon following different timing regimes of hypoxia.

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Abstract

Global warming and increasing anthropic impacts on coastal areas are expected to increase the frequency, intensity and severity of hypoxic events in the near future. Different timing regimes in the occurrence of hypoxic events may have radically different effects on the interested communities and the study of these effects is a key for understanding, managing and protecting coastal habitats in the future. In this work we performed a manipulative field experiment in a North Adriatic coastal lagoon (Pialassa Baiona). We imposed three different timing regimes of hypoxic disturbance and monitored the recovery of both the benthic and the microbial communities over time. We hypothesized that the different hypoxic regimes would create different effects on the communities leading to variable recovery trajectories and final results. From the first exploratory analyses of both benthos and the microbial community a significant difference between the hypoxic treatments and the controls did not emerge neither in the in the final result of the treatment nor in the recovery trajectory. This may suggest that the experimental procedures did not induce a sufficient oxygen depletion to deeply affect the communities. Further and deeper analyses should investigate more taxonomic levels and functional groups to give a batter view on the changes we observed.

Key words

Repeated hypoxic events - Hypoxia - Recovery - Microbial Community - Benthic community - Pulse stressors

1 Introduction

The complex interactions between global climate change and other anthropogenic pressures on coastal and marine environments is increasing the number and extent of episodes of oxygen depletion (Levin *et al.*, 2009). These events are defined, based on their intensity, as hypoxic (Dissolved Oxygen Concentration [DO] < 2 mg/l) or anoxic ([DO]<0,1mg/l) (Vaquer-Sunyer & Duarte, 2008). They can cause a variety of consequences on the communities affected, including slight losses of diversity, drastic community shifts up to mass mortalities (Levin *et al.*, 2009; Diaz & Rosenberg, 2008). Environments characterized by periodic or even continuous hypoxia have existed in historical and geological times (Robinson *et al.,* 2019) but in the last 50 years their occurrence has increased by more than 50% (Shmidtko, Stramma and Visbeck, 2017; Breitburg *et al*, 2018). In chronically hypoxic areas, hypoxic events often occur cyclically. The most common cycles in which hypoxia can manifest are diel-cycling hypoxia, where [DO] decreases at night due to respiration and increases during the day due to photosynthesis (Miyamoto, 2019), and seasonal cycles, where normoxia alternates with hypoxic periods of varying duration and severity from late summer to late autumn (Diaz & Rosenberg, 2008; Del Giudice *et al.*, 2018). Hypoxia, however can also appear aperiodically due to multiple factors such as, for example, sudden and unpredictable increases in nutrient loading in the water column. These different timing regimes of hypoxia can have different long term effects on the communities and determine differences in the recovery process. Long lasting hypoxic events can for example lead to mass mortalities and almost complete defaunation to the point that a complete recovery and convergence to the initial state is impossible (Van Colen *et al.*, 2010). Short repeated disturbances can, on the other hand, lead to milder detrimental effects but, at the same time, t heir periodic reappearance, can hinder and interrupt the recovery process (Nakano *et al,* 2017; Boesch & Rabalais, 1991). Numerical models (Nakano *et al.* 2017) as well as experimental studies (Diaz & Rosenberg, 2008) show how the duration of hypoxic periods is more important than its frequency in determining its effects on the community while other studies show the importance of temporal variance (Bertocci *et al.*, 2016) and of clustering regimes (Dal Bello *et al.*, 2017) in shaping the communities after stresses and their potential reactions to future disturbance.

Communities interested by hypoxic events tend in general to follow a Pearson-Rosenberg succession during recovery (Pearson & Rosenberg, 1978; van Colen *et al.*, 2008). However, their recovery process is often slow and can have a delayed start from the end of the disturbance (Lim *et al.*, 2006) and may be interrupted by further hypoxic events before a complete return to the original state (Diaz, 2001). Environments that experience oxygen depletion frequently and regularly often present benthic communities that are tolerant to and shaped by the stressful conditions (Sen Gupta *et al.*, 1996; Garegston, 1985; Seitaj *et al.*, 2017; Capelle *et al.*, 2021) and are often kept at an early successional stage with opportunistic and fast growing organisms (Boesch & Rabalais, 1991; Diaz, 2001). On the other hand, in environments where hypoxic conditions appear for the first time or in an occasional and irregular way, they may cause extremely drastic effects on communities that are not dominated by hypoxia tolerant organisms (Boesch & Rabalais, 1991).

Microbes are major contributors of ecosystem functions in marine ecosystems, playing an essential role in organic matter mineralization, nutrient regeneration, and energy flow. Microbial communities are also deeply linked with oxygen depletion in the sediment and the water column. On one hand microbial activity is one of the factors determining the occurrence of hypoxia (Keeling *et al.,* 2010; Wilhelm *et al.*, 2013). On the other the dynamics and structures of microbial communities are deeply shaped by oxygen levels (Shang *et al.* 2022). Mahmoudi *et al.* (2015) showed how, in the Caspian sea, hypoxic waters do not strongly affect the total microbial biomass but they shape its composition and functional role. Bacterial taxa responsible for anaerobic degradation of organic matter such as *Desulfubacteriales* are common in oxygen depleted waters and sediments (Orphan *et al.*, 2001) together with other groups involved for example in Nitrogen cycling.

Microbial and benthic dynamics in response and after hypoxic periods are also tightly linked. Microbes are responsible for the degradation of the organic material derived from the accumulation of dead benthic organisms. Anaerobic degradation of organic matter performed by microbes is slower than that occurring at higher oxygen levels, significantly enhancing the preservation of organic matter and further changing the biogeochemical characteristics of the environment (Canfield *et al.,* 1993) . The microbial community, on the other hand, also has a key role in determining the recovery of benthos and the overall environments after prolonged and severe hypoxic events: sulphide oxidizing microbes, for example, are important in detoxifying strongly reduced sediments and making them suitable for colonization by further steps in the recovery succession (Pearson & Rosenberg, 1978; Middelburg & Levin, 2009).

In this work we performed a field experiment imposing different timing regimes of hypoxia in a coastal lagoon in the Northern Adriatic Sea and monitored the recovery of both the benthic and the microbial communities to understand the succession after a stressful period and whether different histories may lead to different outcomes in the recovery. We hypothesized that different timing regimes of hypoxia would have led to different community structures (Comandini *et al.,* 2022) and consequently to potentially different recovery trajectories. We expected longer hypoxic events to have increasing detrimental effects on the community and lead to a slower and more delayed recovery than shorter ones.

2 Materials and methods

2.1 Study site

The experiment was conducted in Pialassa Baiona, a coastal lagoon located in the northern Adriatic

Sea and linked to the Ravenna Harbor (Airoldi *et al.*, 2016). Piallassa Baiona is an eutrophic microtidal lagoon where with a tidal range normally smaller than 1 m. The small lagoon (11,8 km2) receives water from multiple sources: five channels provide freshwater and nutrients from urban and agricultural areas, wastewater treatments and two thermal power stations also bring water to the lagoon and saltwater from the Ravenna harbor enters the pond with the tides (Ponti *et al.*, 2005).

Piallassa Baiona is characterized by mixing regimes with the external areas dominated by the effects of the tides and the internal part dominated by the river action. The sediments in the lagoon are variable in organic content, grain size and clay/silt proportion with sand ranging from 12.1% to 89.5% in weight based on the differences in the sedimentation process or the presence of sand dunes (Ponti *et al.*, 2005).

Due to the wastewater input from the town and adjacent industrial areas that introduce high concentration of nutrients into the lagoon periodic phytoplankton and seaweeds blooms occur causing occasional hypoxic events (Ponti *et al.*, 2010). Piallassa Baiona is a severely impacted area due to the aforementioned proximity with highly anthropized zones and has been historically interested by multiple sources of pollution, organic and inorganic. Our experiment was performed in a shallow mudflat located in the northernmost part of Piallassa Baiona, far from direct sources of anthropic disturbance.

2.2 Field experimental set up and design

To test and monitor the different recovery patterns three timing regimes of hypoxia were imposed: a Single Short (SS) Hypoxic period (5 days), a Single Long (SL) Hypoxic period (10 days) and Two Short Hypoxic (TS) periods (5 days) separated by a Normoxic period (5 days). Control (C) experimental units were also set up (Fig. 1).

Microbial samples were taken at the end of the treatment period (T1) and after 1 (T2), 2 (T3), 4 (T4) and 7 days (T5). The short recovery times were chosen for the microbial community in order to intercept as much change as possible in such a fast evolving community (Katherine Dafforn, personal communication). Benthic samples were taken at the end of the treatments (T1, November 27), after 48 days (T2, in January) and after 180 days (T3, in July).

Fig. 1: Design of the four treatments. Hypoxic periods are represented in black and normoxic periods are in white. Treatment 'Two Short' (TS) consisted of two hypoxic periods of five days separated by a normoxic period of 5 days. Treatments 'Single Long' consisted of one hypoxic period of 10 days. Treatment Single Short consists of one single 5 days hypoxic period and the Control is represented by a 15 days normoxic period.

To impose hypoxia 4mx4m sediment patches were thoroughly covered with polypropylene plastic sheets carefully fixed to the bottom through bricks. Three different timing regimes of hypoxia, representing the three treatments of the experiment, were imposed by covering and uncovering the patches at different times (as shown in Fig. 1). Experimental units were signaled with colored wooden sticks that allowed to distinguish the different treatments even in poor visibility conditions and determined the margins of the patch even after the plastic sheets were removed.

2.3 Benthic community

2.3.1 Sampling

Benthos was sampled from each experimental unit at at least 0.5 m from the edge of the patch to avoid any border effect. Sampling was performed with a 12.5 cm diameter steel corer that was used to sample the top 15 cm of sediment. From each replicate patch one core was taken at each sampling time and its whole content was sieved through a 0.5 mm mesh net to remove as much sediment as possible before fixating the whole content of the net in ethanol on the field and moving the samples to the lab. All the sampling was performed minimizing trampling and disturbance inside the experimental units.

2.3.2 Laboratory work

All the samples were moved into the Bologna University laboratories in Ravenna where they were sieved through a 4000, a 2800 and a 500 µm mesh sieve stacked. The macroscopic portion of each sample was visually inspected while the portion between 4000 and 500 µm was analyzed through a stereoscope. The smaller size class was stained with Rose Bengal and organisms that were intact at the time of the analysis were assumed to be alive at the moment of the sampling. The organisms were extracted from the sample, identified to the lowest taxonomic level and counted.

2.3.3 Statistical analyses

Total abundances, species richness and diversity indices were calculated and analyzed through two-way ANOVA analyses with Time (3 levels) and Treatments (4 levels: Control, Single Short, Two Short, Single Long) as factors. Linear regressions of Abundance and species richness over time were also performed to visualize their trend over time and differences in trend between treatments. Univariate analyses were performed on RStudio version 2021.09.2

The structure of the community was examined through multivariate analyses all performed on Primer7 & Permanova. The community was visualized through non metric Multi Dimensional

Scaling (MDS) and analyzed through PERMANOVA analyses with Time (3 levels) and Treatments (4 levels: Control, Single Short, Two Short, Single Long) as factors. A SIMPER analysis later allowed to explore which taxa contributed the most to the dissimilarities between groups. To compare the trends of each Treatment over Time a Second Stage analysis (Clarke et al., 2006) was performed, visualized through a nmMDS and analyzed through an analysis of similarities (ANOSIM).

2.4 Microbial community

2.4.1 Sampling

Microbial samples were taken at the end of the treatment period (T1) and after 1 (T2), 2 (T3), 4 (T4) and 7 days (T5). The microbial samples were taken using 150 ml syringes. For each replicate patch three sub-samples of sediment were taken and merged together to homogenize spatial variability that may occur inside each patch. Each sub sample consisted of the amount of sediment obtained by pushing the syringe about 2 cm into the sediment. Sub samples were merged in a Falcon tube, labeled and stored in dry ice on the field and immediately transported to the laboratories at the University of Padua to be stored at -80 °C. Samples were taken at the beginning of the experiment then at the beginning of the recovery and after 1, 2, 4 and 7 days.

2.4.2 DNA extraction

Total DNA was extracted from 0.25 g of each sample using the DNeasy PowerLyzer PowerSoil Kit (Qiagen GmbH, Hilden, Germany) following the manufacturer's protocol. The DNA obtained was purified using magnetic beads. The concentration and quality of the purified DNA was measured by Qubit 1.0 fluorometer and NanoDrop Lite spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA), respectively.

2.4.3 DNA amplification and preparation of libraries

The genetic analyses targeted the bacterial 16s rRNA gene that was amplified using 515F-Y (5′-GTGYCAGCMGCCGCGGTAA-3′) and 926R (5′ CCGYCAATTYMTTTRAGTTT-3′) primers to encompass the V4 and V5 hypervariable loops of 16S rRNA genes (Parada *et al.*, 2016). PCR reactions were performed in 25 µl final volume reactions containing 5 ng of template DNA, 0.5 U of Phusion High-Fidelity DNA polymerase (Thermo Fisher Scientific, Waltham, MA, USA), 1X Phusion HF buffer, 200 µM of each dNTP, and 0.5 µM of each primer. PCR conditions consisted of an initial denaturation of 4 min at 98°C followed by 25 cycles of 20 s at 98°, 30s at 57°C, 30 s at 72°C and a final extension step of 5 min at 72°C. PCR amplifications were carried out in triplicate to reduce possible intra-sample variance and all PCR products were visualized on 1.5% agarose gels. The amplicon triplicates were merged and purified using 0.65X volumes of AMPure XP beads (Beckman Coulter Life Sciences, Brea, CA, USA). Then, all the purified products were indexed following the "16S Metagenomic Sequencing Library Preparation" protocol provided by Illumina (Illumina, San Diego, CA, USA) and the PCR amplicons were normalized using a SequalPrep Normalization Plate (Thermo Fisher Scientific, Waltham, MA, USA). Finally, amplicon libraries were sequenced on the Illumina MiSeq system using the MiSeq reagent kit v3 (600 cycles).

2.4.4 Sequence analysis

The sequences obtained were trimmed of primers, Illumina adapter sequences and distal bases using the Trimmomatic software v 0.36 (Bolger *et al.*, 2014). Low quality reads were removed if the quality was lower than Q score 20 together with all the reads shorter than 100bps and orphan reads. Maximum error rates were calculated based on the whole sequencing run and combined with the DADA2 denoising algorithm (Callahan *et al.,* 2016) to determine exact amplicon sequence variants (ASV). All resulting paired end sequences were merged and remaining chimeric sequences removed (consensus method; Callahan *et al.*, 2016). To assign taxonomy of bacteria and archaea all sequences were classified using an RDP naive Bayesian classifier method (Wang *et al.*, 2007) against the SILVA SSU Ref NR 99 138.1 Database (Quast *et al.*, 2013).

We obtained a total of more than 17000 ASVs on which a series of filters were applied. Analyses were performed using phyloseq package v 1.36 (McMurdie and Holmes, 2013) in R v 4.1.0 and following protocols introduced in Callahan et al., 2016. All the ASVs not assigned to any Phyla were removed and only ASVs present in at least four samples and with a total count above 10 reads in all samples were retained in order to remove very low abundance features and potential sequencing errors. A final of 3057 ASVs was analyzed further.

2.4.5 Statistical analyses

The community was mainly analyzed both at the phylum and at the class level. First the relative abundances of all phyla were visualized through bar plots. Total abundances, richness and diversity indices were then calculated and analyzed through a two way ANOVA with Time (5 levels) and Treatments (4 levels: Control, Single Short, Two Short, Single Long) as factors. Linear regressions were also used to visualize trends over time of the interesting metrics.

Community structure was explored though multivariate analyses: first it was visualized through a Principal Component Analysis then the effects of factors Time (5 levels) and Treatment and their interaction (4 levels: Control, Single Short, Two Short, Single Long) were examined through PERMANOVA analyses. The contribution of each taxa to the dissimilarities between groups were investigated with a SIMPER analysis. To account for the non independence of the replicates a Second Stage Analysis was performed to compare the trajectories over time of the treatments and was visualized through an MDS and analyzed through an ANOSIM. All multivariate analyses were performed on PRIMER7+PERMANOVA.

3 Results

3.1 Benthic community

After removing the rarest groups 29 taxa remained. Species richness in samples varied from 22 to 3 and organism abundance from 477 to 40. Relative abundances of benthic taxa are shown in Figure 2.

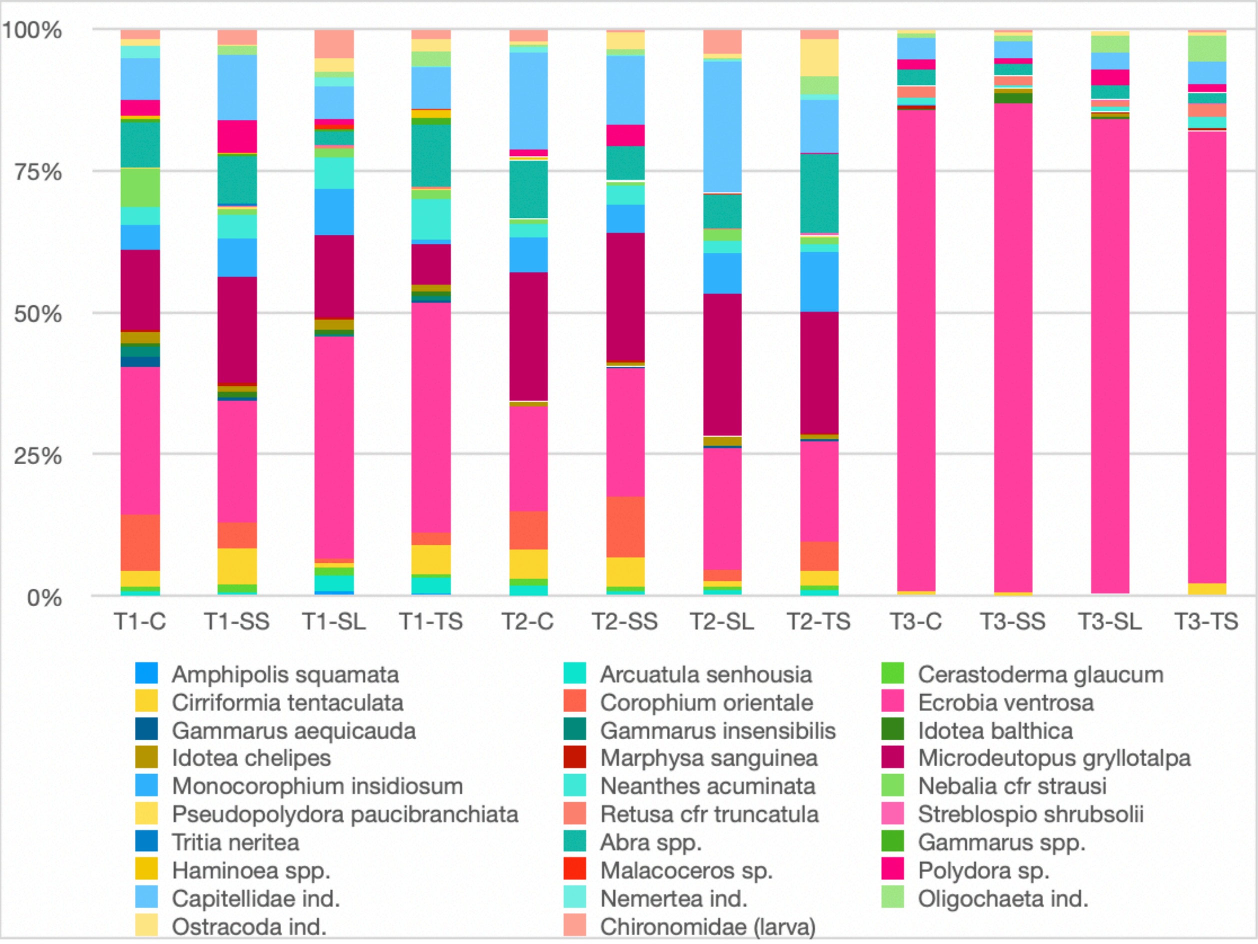

Fig. 2: Barplot with the average relative abundances

Abundance increased overall through the experiment in all the treatments apart from the normoxic control (Fig.3). *Ecrobia ventrosa* (Montagu, 1803) was the most abundant species in all samples and its relative abundance increased significantly in the last sampling time. The amphipod *Microdeutopus gryllotalpa* (Costa, 1853) was the second most abundant species in the first three sampling times and almost disappeared in the last one. Organism abundance (N) significantly increased over time (p = 0,00745) while the number of Species (S, in Fig.3) and the diversity (H) decreased steeply. The community in July was abundant but dominated by the gastropod *Ecrobia ventrosa* and presented very low diversity and evenness. No effect of the treatments was detected with the univariate analyses (Table 1).

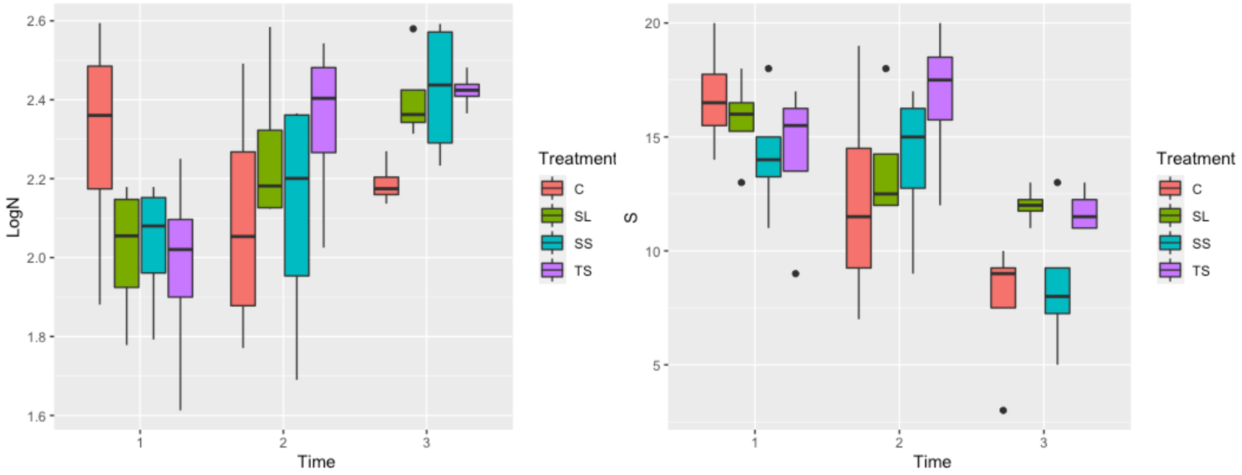

Fig. 3: Boxplots of abundance (N, log transformed) and Species richness (S) of the benthic community at the three sampling times.

A)	Df	Sum square	Mean square	F value	P value
Time	2	0.6273	0.31367	6.433	0.00409
Treatment	3	0.0283	0.00943	193	0.90015
Time:Treatment	6	0.5664	0.09440	1.936	0.10140
Residuals	36	1.7553	0.04876		
B)	Df	Sum square	Mean square	F value	P value
Time	2	246.5	123.27	13.062	5.43e-05
Treatment	3	39.6	13.19	1.397	0.2590
Time:Treatment	6	78.1	13.02	1.380	0.2490
Residuals	36	339.8	9.44		

Tab. 1: ANOVA results on the total abundances (A) and the Species Richness (B) of the benthic community. Abundance data were log transformed.

The significant variation of the community over Time was confirmed by the PERMANOVA analysis (p=0,001) and is shown in the MDS (Fig.4) where, in all the treatments, the samples of the last sampling time (July) clearly separate from the others. The pairwise tests following the PERMANOVA analysis Showed a significant difference between all the three times (Table S1) and the SIMPER (Tables S2, S3) confirms the observation that most of the dissimilarity between groups was due to the increase of *Ecrobia ventrosa* and the almost complete disappearance of amphipods (*Microdeutopus gryllotalpa*, *Monocorophium insidiusum* Crawford, 1937 and *Corophium orientale* Schellenberg, 1928). The Second Stage Analysis showed no differences between the trends of the different treatments over Time (Fig. 8). The associated ANOSIM suggested that the similarity within groups was comparable to that between groups (R= 0.03).

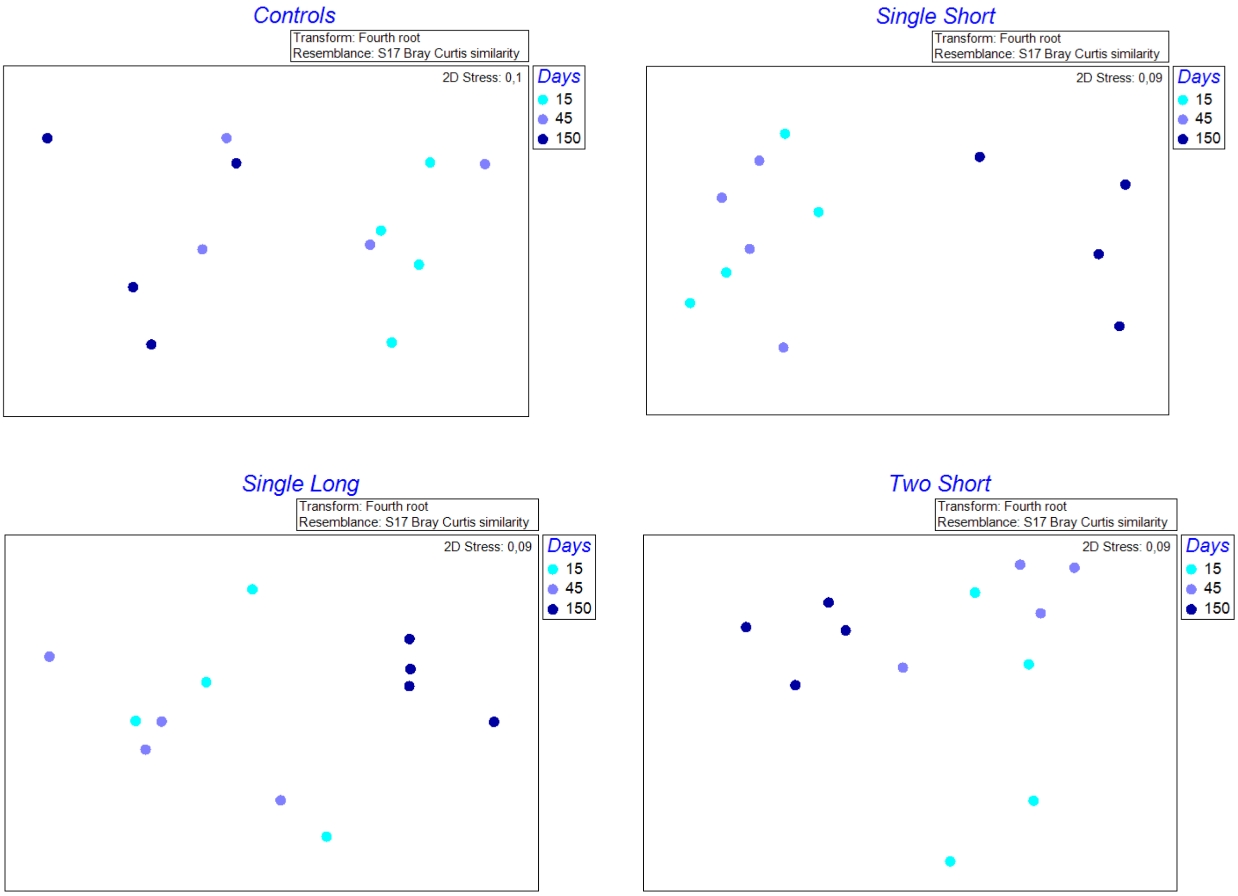

Fig. 4: MDS representing the trends of the benthic community over Time (in days) divided by Treatment. For all the treatments the community after 180 days separates clearly from those at the other sampling Times.

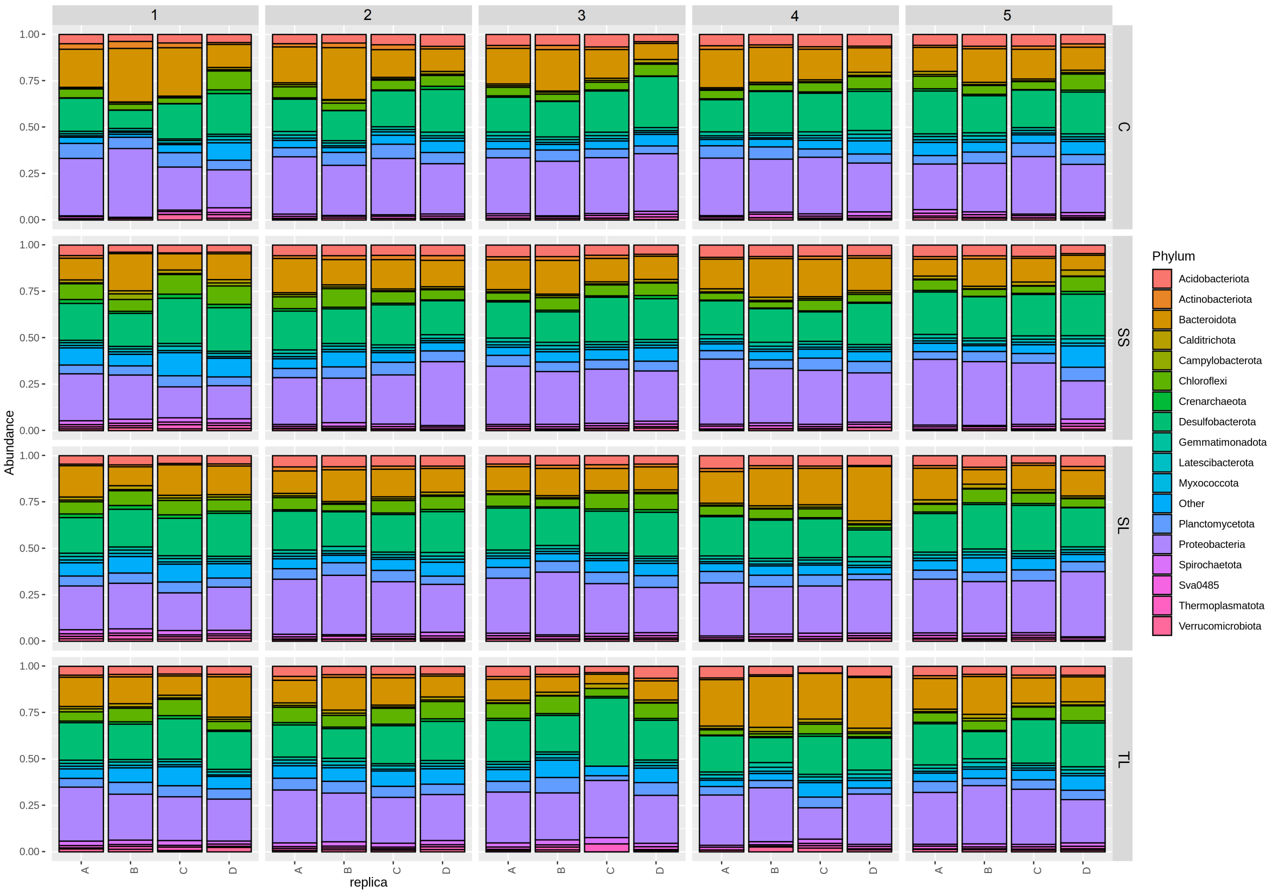

Fig. 5: Barplot of the relative abundances of the dominant microbial phyla. In each square a represented the 4 replicates of a single Treatment (Control, Single Short, Single Long, Two Long) at a single Sampling Time (1, 2, 3, 4, 5).

3.2 Microbial community

From the data retrieved 943 ASVs were not assigned at the phylum level and were removed from the analyses. ASVs that were below 10 total counts or were not shared in at least 4 samples were also removed from the dataset. This filtering resulted in 3057 total ASVs. The prevalence plot was performed at the phylum level. Figure 5 shows the relative abundance of phyla in the samples. The extremely low values appeared in one of the replicates from Time 2 of Treatment SL led to think to a sampling mistake so the sample was removed.

Bacteroidota, Proteobacteria and Desulfobacterota were the dominant phyla in all the samples. Each of 18 phyla represented more than 1% of the abundance in each sample and are represented in the figure. From the end of the treatment period microbial abundance increased consistently for the week in all of the experimental units, including the controls (Fig.6).

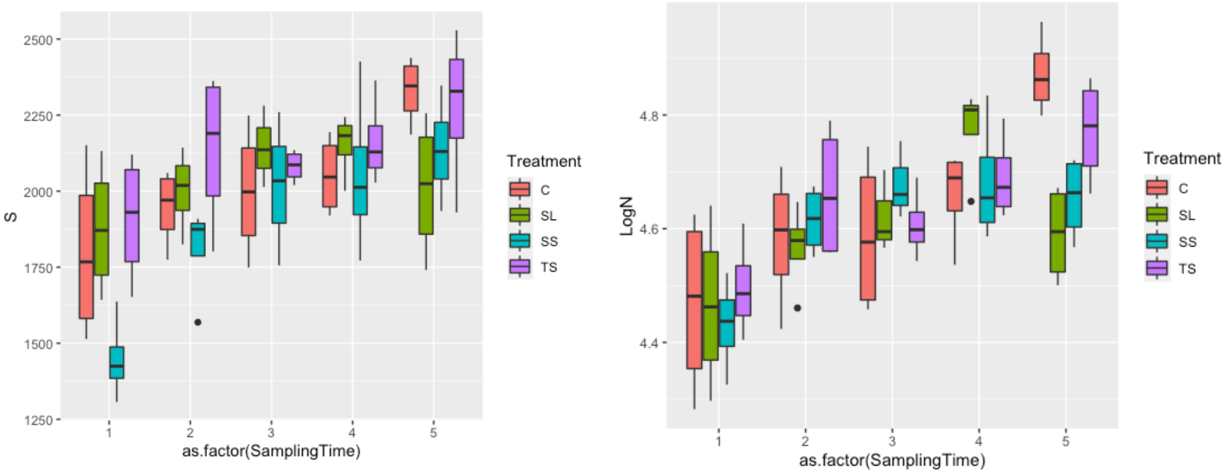

Fig. 6: Boxplots of Species richness (S) and abundance (N, log transformed) of the microbial community at the five sampling times. Abundance data was log transformed.

The ANOVA displayed a significant interaction between Time and Treatment was detected (Table 2) but the pair wise tests did not highlight consistent differences between treatments and controls and all the detectable differences were to be attributed to the treatment with a Single Short hypoxic period. At the beginning of the recovery period abundances were similar between the treatments and remained comparable within sampling times until the last one (T4) where slightly more marked differences seemed to appear. Species richness showed a similar increasing trend over time but a significant effect of the Treatment also appeared. The SS Treatment displayed significantly lower species abundances compared to the TS one.

A)	Df	Sum square	Mean square	F value	P value
Time	4	0.6585	0.16463	16.431	4.71e-09
Treatment	3	0.0251	0.00837	836	0.4798
Time:Treatment	12	0.2382	0.01985	1.981	0.0427
Residuals	58	0.5811	0.01002		

B)	Df	Sum square	Mean square	F value	P value
Time	4	1718379	429595	11.357	7.12e-07
Treatment	3	508871	169624	4.484	0.00673
Time:Treatment	12	589663	49139	1.299	0.24408
Residuals	58	2193845	37825		

Tab. 2: ANOVA results on the Species Richness (A) and abundance (B) of the microbial community.

At the Class level the PERMANOVA analysis highlighted a significant effect of factors Time (p=0,001) and Treatment (p=0,002) on community composition but no interactive effect of the two factors. Treatment SS displayed a significant difference with all the other three treatments but no other differences appeared for the factor Treatment. Within factor Time, Time1 was significantly different from all the other times (p values in a table) but no other significant differences were detected. The SIMPER analysis allowed to understand which taxa contributed the most to the variability between samples. Comparing the levels of factor Time the level that displayed the highest dissimilarity with the others is level 1, the one at the end of the Treatment period.

Various taxa appeared responsible for the dissimilarities with the decrease of *Dehalococcoidia*, *Nanoarchaeia* and *Thermoplasmata* important for the differences between Time 1 and 2 (27,44%dissimilarity) and 1 and 3 (26,91% dissimilarity). A relevant amount of the dissimilarity between times 1 and 3 was also represented by the increase in the abundance of *Desulfovibrionia* and *Mixococcia*. Times 1 and 4 presented a 28.02% dissimilarity and a relevant part of that dissimilarity constituted by the increase of *Bacteroidia*, *Gammaproteobacteria*, *Desulfovibrionia.*

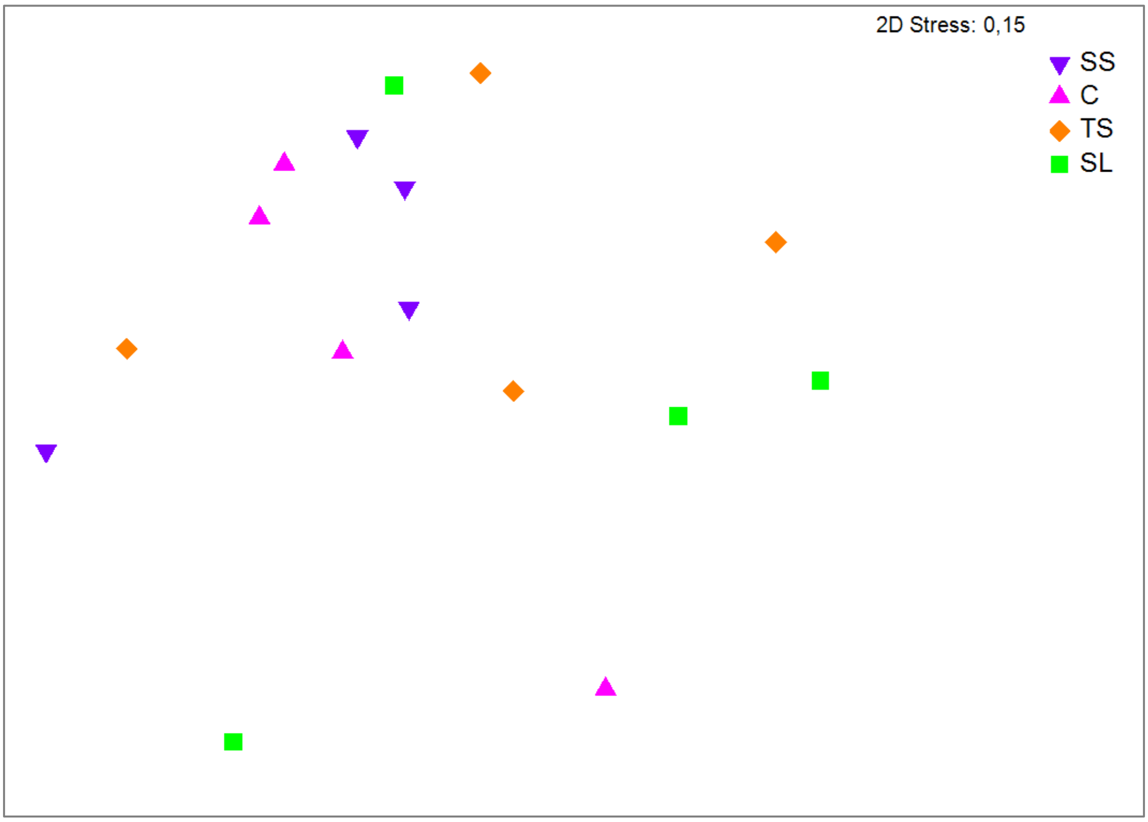

Fig. 7: MDS resulting from the Second Stage Analysis. Analyses are performed on Bray-Curtis similarly matrices of fourth root transformed abundance data. Each dot represents the pattern in the recovery of the microbial community from one single experimental unit over time. Different colors represent the different treatments. As confirmed by the ANOSIM analysis the similarity within Treatments are comparable to those between Treatments.

The three trends determining the highest dissimilarities between levels 1 and 5 of factor Time (27,67%) were the decreases of *Gammaproteobacteria*, *Desulfobulbia* and *Alphaproteobacteria.* Within factor Treatment the highest dissimilarity appeared between levels Single Short and Single Long (26,77%). The three classes that contributed the most to this dissimilarity were *Dehalococcoidia*, *Nanoarchaeia* and *Thermoplasmata*. All these three taxa resulted less abundant in the treatment Single Short. Within level 1 of factor Time, treatment SS displayed a high dissimilarity (>33%) with the others. The taxa that contributed the most to that dissimilarity were the Archea Nanoarchaeia, Thermoplasmata and the bacteria Dehalococcoidia and Fibrobacteria.

All those taxa appeared at particularly low abundances in treatment SS. Treatment SS at Time 1 also displayed a high dissimilarity with the same Treatment at Time 2 and 3 with the increase of Nanoarchaeia and Thermoplasmata being the tendencies contributing the most to the dissimilarity. The second stage analysis showed no significant differences in the trends over Time of the four Treatments (Fig. 7, Tables S4, S5 and S6.) and the ANOSIM confirmed the comparable Similarity within and between treatments (R=0.074).

4 Discussion

4.1 Benthic community

The dominant species in the community, the gastropod *Ecrobia ventrosa* is a species with a high tolerance to hypoxia (Vaquer-Sunyer & Duarte, 2008) and thanks to its mobility can escape and recolonize hypoxic areas quickly. Other abundant groups, such as amphipods and other crustaceans presented high abundances during winter and spring and decreased steeply in summer probably due to their lower tolerance to high temperature and lower dissolved oxygen (Vaquer-Sunyer & Duarte, 2008; Hoback & Barnhart, 1996; Hervant, Mathieu & Culvert, 1999; Sandberg-Kilpi, Vismann & Hagerman, 2013). The benthic community displayed a significant variation over time but no significant differences were detected between the treatments. The preliminary explorative analyses presented here suggest a negligible effect of the experimental procedure on the communities. Probably the intensity of the imposed stress was not enough to cause drastic damage on a community already adapted to frequent and short stressing periods. The positioning of the plastic sheets may have allowed oxygenated water to still enter the experimental units or photosynthetic organisms trapped under the plastic sheets may have, through their activity, increased oxygen levels during the day limiting the severity and duration of hypoxia.

Indeed, at the end of the treatments the abundance in the controls was in fact only slightly higher than that in the treatments but the difference was not significantly higher than in the treatments and the community composition was similar as well. The limited size of the experimental units may also have enhanced the changes in the community when the experimental units were uncovered facilitating the migration of mobile organisms in and out of the patches (Gamenick *et al,* 1996). The changes observed in the community over time, then, cannot be considered an actual recovery and should only be viewed as the natural seasonal variation of the community in that area of the lagoon in response to the multiple changing factors.

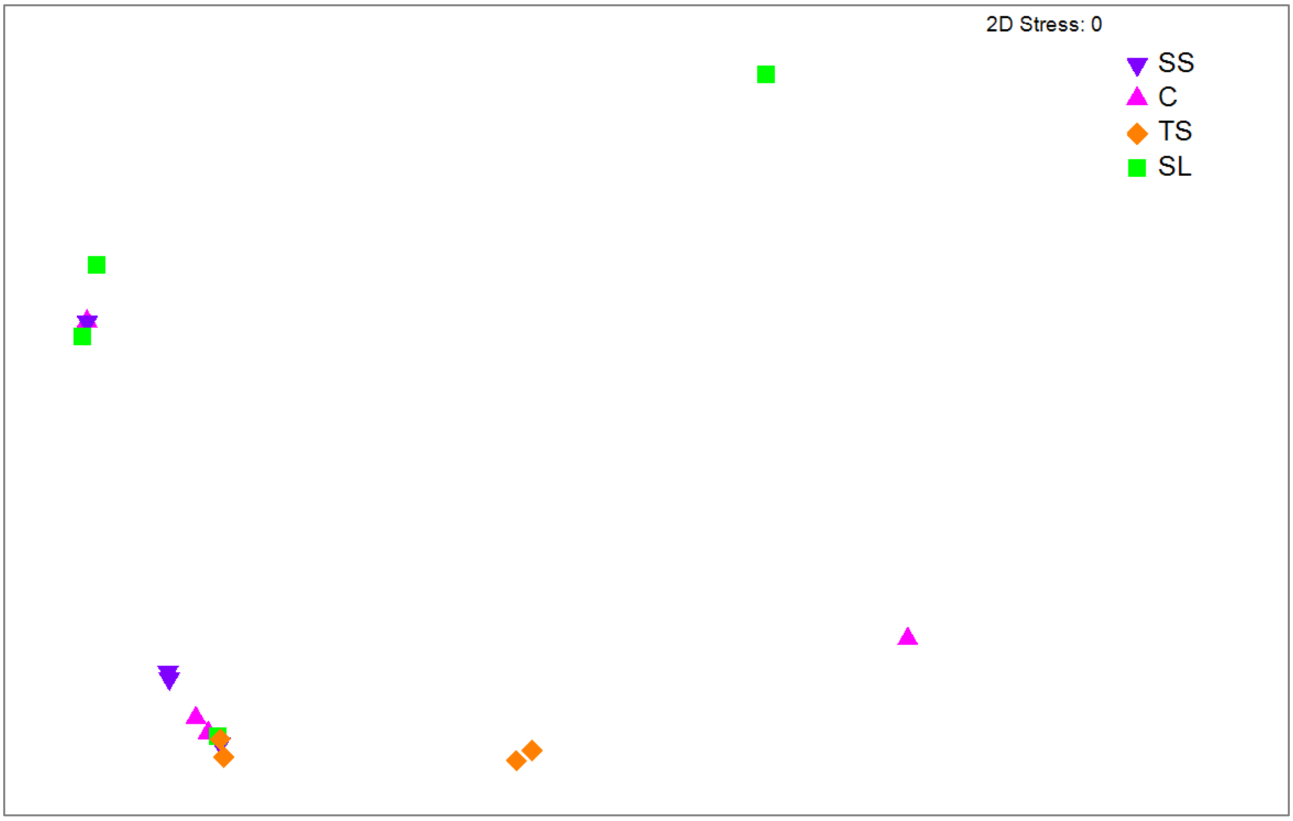

Fig. 8: MDS resulting from the Second Stage Analysis. Each dot represents the trajectory in the recovery of the community from one single experimental unit over time. Different colors represent the different treatments. As confirmed by the ANOSIM analysis the similarity within Treatments are comparable to those between Treatments.

4.2 Microbial community

The absence of a consistent difference between the normoxic controls and the hypoxic treatments tend to confirm that the experimental units did not undergo an oxygen depletion strong enough to significantly affect the communities. Other differences can probably be attributed to other, non controlled, factors. After the end of the Treatment period, the whole community abundance increased quickly already within the first 24 hours both in the control and in the treatment groups. The taxa responsible for most of the increase are Archaea and Bacteria typical of hypoxic and highly impacted areas. Thermoplasmata perform sulfide or organosulfonate reduction (Lin et al., 2015), Dehalococcoidia (Cupples, 2008) are anaerobe dechlorinating bacteria that use halogenated compounds, generally from highly polluted areas, as electron acceptors. Various undetermined classes of the phylum Latescibacterota were very abundant in all the samples and gave an important contribution to the dissimilarities between groups. Latescibacterota are bacteria typical of anoxic ponds and hydrocarbon impacted environments important in the turnover of algal detritus from anoxic and microoxic zones (Farag et al., 2017). Bacteroidia and Gammaproteobacteria are two other very abundant groups of anaerobic bacteria (Podosokorskaya et al., 2020) whose abundance also decreased over time (most important taxa in determining the dissimilarities between Times 1 and 4). In general the community seemed to be strongly shaped by oxygen levels and relatively adapted to hypoxic conditions but our experiment failed at applying a meaningful disturbance.

5 Conclusion

Oxygen depletion clearly is one of the factors shaping both the benthic and the microbial communities in Piallassa Baiona. Macrobenthos in the lagoon presents a high spatial and temporal variability due to the occurrence of point-like or seasonal stressors and a strong environmental heterogeneity (Ponti *et al.*, 2005). The prevalence of specific taxa and periods of low diversity may be the direct consequence of the multiple stressors that emerge in Piallassa Baiona and in other estuarine and coastal environments. Time of recovery and the successional stage reached depend on the frequency of the stressors and we can expect that in environments with frequent point-like disturbances will not present simple stress/recovery cycles but different communities every time based on the temporal and spatial extent of the stress. Further research is needed on the recovery of frequently stressed habitats to clarify how different timing regimes of disturbance may influence the stability of the communities over time.

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Supplementary Materials

Source	df	SS	MS	Pseudo-F	P(perm)	Unique perms
Time	2	16995	8497.6	12.313	0.001	999
Treatment	3	2525.8	841.94	1.22	0.228	998
TimexTreatment	6	5082.9	847.15	1.2275	0.179	997
Res	36	24845	690.13			
Total	47	49449				

Table S1: results of the PERMANOVA of the benthic community. The only significant effect is that of factor Time.

	Group 3	Group 9				
Species	**Av.Abund**	**Av.Abund**	**Av.Diss**	**Diss/SD**	**Contrib%**	**Cum.%**
Microdeutopus gryllotalpa	1.68	0.00	4.37	1.98	8.38	8.38
Ecrobia ventrosa	2.40	3.72	3.63	1.93	6.95	15.33
Corophium orientale	1.16	0.00	2.93	1.48	5.61	20.94
Retusa cfr truncatula	0.14	1.15	2.84	1.62	5.43	26.37
Chironomidae (larva)	1.33	0.42	2.78	1.65	5.33	31.70
Monocorophium insidiosum	0.98	0.26	2.46	1.13	4.71	36.41
Arcuatula senhousia	0.93	0.00	2.45	1.53	4.70	41.11
Polydora sp.	0.75	1.03	2.34	1.21	4.47	45.58
Oligochaeta ind.	0.69	0.97	2.33	1.29	4.45	50.04

Table S2: Results of the SIMPER analysis comparing benthic communities at times 3 and 9. Dissimilarity was 52.22%. In the table are shown the taxa that contribute cumulatively to the 50% of that dissimilarity.

	Group 2	Group 3				
Species	**Av.Abund**	**Av.Abund**	**Av.Diss**	**Diss/SD**	**Contrib%**	**Cum.%**
Microdeutopus gryllotalpa	1.91	0.00	4.73	1.56	9.11	9.11
Ecrobia ventrosa	2.39	3.72	3.63	2.36	6.99	16.09
Monocorophium insidiosum	1.57	0.26	3.57	1.77	6.87	22.96
Corophium orientale	1.31	0.00	3.27	1.46	6.30	29.26
Retusa cfr truncatula	0.19	1.15	2.80	1.53	5.38	34.64
Arcuatula senhousia	1.04	0.00	2.75	2.12	5.30	39.94
Capitellidae ind.	2.06	1.40	2.58	1.18	4.97	44.91
Oligochaeta ind.	0.82	0.97	2.40	1.25	4.62	49.53
Polydora sp.	0.58	1.03	2.35	1.24	4.53	54.06

Table S3: Results of the SIMPER analysis comparing benthic communities at times 2 and 3. Dissimilarity was 51.95%. In the table are shown the taxa that contribute cumulatively to the 50% of that dissimilarity.

	Group 1	Group 5				
Species	**Av.Abund**	**Av.Abund**	**Av.Diss**	**Diss/SD**	**Contrib%**	**Cum.%**
Gammaproteobacteria	8.77	10.59	0.18	1.83	0.66	0.66
Desulfobulbia	6.32	7.88	0.16	1.71	0.58	1.24
Alphaproteobacteria	5.71	7.22	0.15	1.70	0.54	1.78
Desulfobacteria	7.54	8.86	0.15	1.46	0.53	2.32
Desulfovibrionia	0.78	1.59	0.14	1.37	0.51	2.83
Dehalococcoidia	4.42	4.50	0.14	1.32	0.50	3.33
Nanoarchaeia	3.20	3.30	0.13	1.09	0.48	3.81
Thermoplasmata	3.88	3.88	0.13	1.27	0.47	4.28
Planctomycetes	5.33	6.51	0.13	1.70	0.46	4.75
Thermoanaerobaculia	5.23	6.47	0.12	1.86	0.45	5.19
Bacilli	0.78	1.52	0.12	1.37	0.45	5.64
Latescibacterota_Undefined12	1.04	1.12	0.12	1.22	0.45	6.09
Latescibacterota_Undefined27	0.55	1.62	0.12	1.54	0.44	6.53
Actinobacteria	1.92	2.89	0.12	1.29	0.44	6.96

JS1	2.75	2.73	0.12	1.30	0.43	7.40
Myxococcia	0.82	1.41	0.12	1.20	0.43	7.83
Calditrichia	4.34	5.42	0.12	1.30	0.43	8.25
Sva0485_Undefined23	0.44	1.52	0.12	1.63	0.43	8.68
Zixibacteria_Undefined17	0.40	1.39	0.12	1.52	0.42	9.10
Latescibacterota_Undefined37	0.28	1.29	0.12	1.46	0.42	9.53
Latescibacterota_Undefined7	0.99	1.95	0.12	1.25	0.42	9.95
Sva0485_Undefined13	0.83	1.71	0.12	1.35	0.42	10.36
Latescibacterota_Undefined43	0.33	1.31	0.11	1.46	0.41	10.77
NB1-j_Undefined3	1.39	1.56	0.11	1.25	0.41	11.18
Schekmanbacteria_Undefined1	1.86	1.83	0.11	1.16	0.41	11.59
Latescibacterota_Undefined21	0.70	1.58	0.11	1.35	0.40	11.99
Zixibacteria_Undefined12	0.61	1.27	0.11	1.54	0.40	12.39
Syntrophobacteria	4.87	5.61	0.11	1.32	0.40	12.80
Bacteroidia	8.16	9.04	0.11	1.40	0.40	13.19
DG-56	1.78	1.71	0.11	1.18	0.40	13.59
Zixibacteria_Undefined16	0.46	1.45	0.11	1.66	0.40	13.99
Zixibacteria_Undefined3	1.42	1.44	0.11	1.14	0.39	14.38
Latescibacterota_Undefined19	0.56	1.21	0.11	1.16	0.39	14.77
Desulfobacterota_Undefined1	0.52	1.23	0.11	1.20	0.39	15.16
Phycisphaerae	4.75	5.01	0.11	1.15	0.39	15.55
Micrarchaeia	1.17	1.06	0.11	1.24	0.39	15.94
Latescibacterota_Undefined32	0.76	1.58	0.11	1.24	0.39	16.33
Latescibacterota_Undefined34	0.61	1.46	0.11	1.37	0.39	16.72
Desulfitobacteriia	0.20	1.13	0.11	1.21	0.39	17.10
Zixibacteria_Undefined6	0.71	1.56	0.11	1.35	0.38	17.49
Zixibacteria_Undefined5	1.02	1.57	0.11	1.17	0.38	17.87
Latescibacterota_Undefined9	1.27	1.77	0.11	1.11	0.38	18.26
Schekmanbacteria_Undefined6	1.37	0.87	0.11	1.28	0.38	18.64
TA06_Undefined3	1.64	1.50	0.11	1.25	0.38	19.02
BD7-11	1.27	0.89	0.11	1.27	0.38	19.41
Fibrobacteria	2.56	2.79	0.11	1.02	0.38	19.79
NB1-j_Undefined4	1.05	1.54	0.11	1.23	0.38	20.17
CK-2C2-2_Undefined2	0.62	1.31	0.10	1.27	0.38	20.54

Sumerlaeia	3.12	3.42	0.10	1.20	0.38	20.92
Sva0485_Undefined19	0.63	1.10	0.10	1.21	0.37	21.30
Latescibacterota_Undefined28	0.92	1.49	0.10	1.20	0.37	21.67
Chlamydiae	1.31	1.23	0.10	1.32	0.37	22.04
NB1-j_Undefined14	0.41	1.16	0.10	1.43	0.37	22.41
Sva0485_Undefined25	0.62	1.13	0.10	1.20	0.37	22.78
Latescibacterota_Undefined5	1.89	1.99	0.10	1.09	0.37	23.15
Latescibacterota_Undefined11	1.05	1.73	0.10	1.23	0.37	23.52
Latescibacterota_Undefined67	0.00	1.02	0.10	1.24	0.37	23.89
Sva0485_Undefined20	0.48	0.98	0.10	1.04	0.37	24.26
Latescibacterota_Undefined49	0.55	1.29	0.10	1.36	0.37	24.63
Desulfobacterota_Undefined2	0.64	1.37	0.10	1.35	0.37	24.99
Sva0485_Undefined24	0.75	1.00	0.10	1.16	0.37	25.36
NB1-j_Undefined9	0.67	1.30	0.10	1.26	0.37	25.73
Anaerolineae	6.08	6.91	0.10	1.41	0.37	26.09
LCP-89_Undefined4	1.14	0.78	0.10	1.26	0.36	26.45
Methanosarcinia	1.48	1.43	0.10	1.10	0.36	26.82
Latescibacterota_Undefined18	0.90	1.80	0.10	1.16	0.36	27.18
Schekmanbacteria_Undefined5	1.19	0.91	0.10	1.22	0.36	27.54
NKB15_Undefined1	1.26	1.57	0.10	1.10	0.36	27.91
Babeliae	2.82	3.21	0.10	1.03	0.36	28.27
Zixibacteria_Undefined18	0.66	1.18	0.10	1.20	0.36	28.63
Bathyarchaeia	4.09	4.20	0.10	1.30	0.36	28.99
Schekmanbacteria_Undefined7	1.32	0.99	0.10	1.24	0.36	29.35
Sva0485_Undefined26	0.47	0.94	0.10	1.08	0.36	29.71
NB1-j_Undefined13	0.57	1.30	0.10	1.32	0.36	30.07
KD4-96	2.79	3.69	0.10	2.07	0.36	30.43
Latescibacterota_Undefined10	0.97	1.83	0.10	1.30	0.36	30.78
Latescibacterota_Undefined25	1.10	1.47	0.10	1.17	0.36	31.14
Latescibacterota_Undefined29	1.01	1.03	0.10	1.22	0.36	31.49
Latescibacterota_Undefined16	0.84	1.65	0.10	1.25	0.36	31.85
Dissulfuribacteria	1.73	1.69	0.10	1.08	0.36	32.21
Zixibacteria_Undefined4	1.37	1.54	0.10	1.13	0.36	32.56
Latescibacterota_Undefined33	0.68	1.36	0.10	1.23	0.35	32.92

Zixibacteria_Undefined9	0.92	1.38	0.10	1.17	0.35	33.27
Schekmanbacteria_Undefined4	1.28	1.28	0.10	1.11	0.35	33.62
Zixibacteria_Undefined21	0.53	1.29	0.10	1.33	0.35	33.98
Schekmanbacteria_Undefined8	0.55	1.11	0.10	1.20	0.35	34.33
Elusimicrobia	1.48	1.34	0.10	1.15	0.35	34.68
Gracilibacteria	1.03	1.27	0.10	1.17	0.35	35.03
Zixibacteria_Undefined14	0.70	1.28	0.10	1.30	0.35	35.38
Latescibacterota_Undefined8	1.16	1.79	0.10	1.17	0.35	35.73
Schekmanbacteria_Undefined2	1.39	1.40	0.10	1.15	0.35	36.08
NKB15_Undefined4	1.02	0.55	0.10	1.22	0.35	36.43
NKB15_Undefined2	1.25	1.48	0.10	1.08	0.35	36.77
RBG-16-55-12	1.38	1.80	0.10	1.17	0.35	37.12
Latescibacterota_Undefined35	0.94	0.81	0.10	1.15	0.35	37.47
NB1-j_Undefined12	0.68	0.95	0.10	1.16	0.35	37.81
LCP-89_Undefined5	0.88	0.90	0.10	1.12	0.35	38.16
LCP-89_Undefined6	0.95	1.27	0.10	1.22	0.35	38.51
Lokiarchaeia	3.44	3.35	0.10	1.37	0.35	38.85
Latescibacterota_Undefined41	0.82	1.26	0.10	1.22	0.35	39.20
Thermococci	0.98	0.84	0.10	1.20	0.35	39.54
Omnitrophia	2.41	2.55	0.10	1.02	0.35	39.89
Latescibacterota_Undefined31	0.79	1.41	0.10	1.27	0.34	40.23
Zixibacteria_Undefined10	0.99	1.24	0.09	1.08	0.34	40.57
Zixibacteria_Undefined11	0.25	1.00	0.09	1.17	0.34	40.92
P9X2b3D02	0.89	1.69	0.09	1.20	0.34	41.26
Latescibacterota_Undefined39	0.93	1.12	0.09	1.15	0.34	41.59
Sva0485_Undefined15	1.01	1.69	0.09	1.25	0.34	41.93
LCP-89_Undefined2	0.90	1.32	0.09	1.18	0.34	42.27
Subgroup 22	3.60	4.49	0.09	1.53	0.34	42.61
Zixibacteria_Undefined1	1.53	1.98	0.09	1.05	0.34	42.95
Latescibacterota_Undefined51	0.59	1.01	0.09	1.12	0.34	43.28
Latescibacterota_Undefined26	0.67	0.73	0.09	0.96	0.34	43.62
Kapabacteria	0.77	1.22	0.09	1.24	0.34	43.96
Latescibacterota_Undefined50	0.53	1.22	0.09	1.34	0.34	44.29
Sva0485_Undefined12	1.46	1.70	0.09	1.03	0.34	44.63

Desulfomonilia	1.43	2.16	0.09	1.22	0.34	44.96
Latescibacterota_Undefined54	0.86	0.59	0.09	1.08	0.33	45.30
MBNT15_Undefined5	0.26	1.01	0.09	1.22	0.33	45.63
Desulfobacterota_Undefined3	0.88	1.01	0.09	1.17	0.33	45.97
Subgroup 26	0.59	1.36	0.09	1.33	0.33	46.30
Zixibacteria_Undefined7	0.85	1.58	0.09	1.15	0.33	46.63
WWE3	1.54	1.41	0.09	1.20	0.33	46.97
WS2_Undefined2	0.62	0.95	0.09	1.26	0.33	47.30
Latescibacterota_Undefined47	0.39	1.00	0.09	1.17	0.33	47.63
Zixibacteria_Undefined24	0.71	1.10	0.09	1.35	0.33	47.97
Latescibacterota_Undefined40	0.70	1.31	0.09	1.25	0.33	48.30
Sva0485_Undefined17	1.29	1.60	0.09	1.19	0.33	48.63
LCP-89_Undefined3	0.68	1.29	0.09	1.21	0.33	48.96
Halanaerobiia	0.52	1.26	0.09	1.46	0.33	49.29
Latescibacterota_Undefined38	0.62	0.93	0.09	1.11	0.33	49.61
NB1-j_Undefined5	1.33	1.86	0.09	1.12	0.33	49.94
Latescibacterota_Undefined53	0.96	0.94	0.09	1.21	0.33	50.27

Table S4: Results of the SIMPER analysis comparing microbial communities at times 1 and 5. Dissimilarity was 27.57%. In the table are shown the taxa (Class level) that contribute cumulatively to the 50% of that dissimilarity.

	Group 1	Group 2				
Species	**Av.Abund**	**Av.Abund**	**Av.Diss**	**Diss/SD**	**Contrib%**	**Cum.%**
Dehalococcoidia	4.42	4.12	0.15	1.29	0.55	0.55
Nanoarchaeia	3.20	3.00	0.15	1.21	0.54	1.09
Thermoplasmata	3.88	3.52	0.14	1.33	0.52	1.62
JS1	2.75	2.55	0.14	1.24	0.50	2.12
Bacilli	0.78	1.49	0.13	1.38	0.48	2.60
Planctomycetes	5.33	6.39	0.13	1.67	0.47	3.07
NB1-j_Undefined3	1.39	0.91	0.13	1.28	0.47	3.54
Latescibacterota_Undefined5	1.89	1.36	0.13	1.16	0.47	4.01
Latescibacterota_Undefined12	1.04	0.96	0.13	1.15	0.46	4.47

Sva0485_Undefined23	0.44	1.52	0.13	1.68	0.46	4.93
Schekmanbacteria_Undefined1	1.86	1.30	0.13	1.23	0.46	5.39
Actinobacteria	1.92	2.79	0.13	1.24	0.46	5.85
Gammaproteobacteria	8.77	9.82	0.12	1.54	0.45	6.30
Latescibacterota_Undefined19	0.56	1.31	0.12	1.22	0.44	6.75
Zixibacteria_Undefined3	1.42	1.18	0.12	1.14	0.44	7.19
Latescibacterota_Undefined7	0.99	1.55	0.12	1.23	0.44	7.63
Latescibacterota_Undefined9	1.27	1.35	0.12	1.17	0.43	8.06
Myxococcia	0.82	1.09	0.12	1.13	0.43	8.49
DG-56	1.78	1.60	0.12	1.14	0.43	8.92
Desulfobulbia	6.32	7.24	0.12	1.50	0.43	9.35
NKB15_Undefined1	1.26	1.31	0.12	1.17	0.43	9.78
Dissulfuribacteria	1.73	1.43	0.12	1.14	0.43	10.21
Zixibacteria_Undefined6	0.71	1.58	0.12	1.36	0.42	10.63
Schekmanbacteria_Undefined7	1.32	0.58	0.12	1.38	0.42	11.05
Fibrobacteria	2.56	2.36	0.11	1.20	0.42	11.47
Schekmanbacteria_Undefined6	1.37	0.69	0.11	1.29	0.41	11.88
NB1-j_Undefined4	1.05	1.86	0.11	1.20	0.41	12.29
Alphaproteobacteria	5.71	6.66	0.11	1.63	0.41	12.70
WWE3	1.54	1.07	0.11	1.29	0.41	13.11
Methanosarcinia	1.48	1.43	0.11	1.12	0.41	13.52
LCP-89_Undefined1	1.79	1.62	0.11	1.05	0.41	13.92
Desulfovibrionia	0.78	0.72	0.11	0.95	0.41	14.33
Sumerlaeia	3.12	3.01	0.11	1.05	0.41	14.73
BD7-11	1.27	0.79	0.11	1.28	0.40	15.14
Bacteroidia	8.16	8.78	0.11	1.28	0.40	15.54
Latescibacterota_Undefined43	0.33	1.10	0.11	1.28	0.40	15.94
LCP-89_Undefined4	1.14	0.68	0.11	1.25	0.40	16.33
Omnitrophia	2.41	2.22	0.11	1.05	0.40	16.73
Zixibacteria_Undefined4	1.37	1.30	0.11	1.11	0.39	17.12
Chlamydiae	1.31	1.39	0.11	1.27	0.39	17.52
Phycisphaerae	4.75	4.70	0.11	1.20	0.39	17.91
Latescibacterota_Undefined11	1.05	1.82	0.11	1.21	0.39	18.30
Micrarchaeia	1.17	0.83	0.11	1.29	0.39	18.69

Latescibacterota_Undefined18	0.90	1.55	0.11	1.17	0.39	19.07
Zixibacteria_Undefined5	1.02	1.36	0.11	1.22	0.38	19.46
Sva0485_Undefined13	0.83	1.26	0.11	1.23	0.38	19.84
Zixibacteria_Undefined10	0.99	0.81	0.11	1.12	0.38	20.22
RBG-16-55-12	1.38	1.50	0.10	1.16	0.38	20.60
Latescibacterota_Undefined10	0.97	1.79	0.10	1.27	0.38	20.98
Schekmanbacteria_Undefined4	1.28	0.94	0.10	1.25	0.38	21.36
Elusimicrobia	1.48	1.15	0.10	1.16	0.38	21.74
Latescibacterota_Undefined26	0.67	0.79	0.10	1.01	0.38	22.11
Sva0485_Undefined19	0.63	0.90	0.10	1.13	0.38	22.49
Babeliae	2.82	2.69	0.10	1.19	0.38	22.86
WCHB1-81	0.98	0.92	0.10	1.21	0.37	23.24
Latescibacterota_Undefined28	0.92	0.90	0.10	1.16	0.37	23.61
Latescibacterota_Undefined38	0.62	1.06	0.10	1.13	0.37	23.98
Gracilibacteria	1.03	0.97	0.10	1.23	0.37	24.35
Latescibacterota_Undefined32	0.76	1.11	0.10	1.18	0.37	24.73
Planctomycetota_Undefined6	0.25	1.01	0.10	1.32	0.37	25.10
Latescibacterota_Undefined16	0.84	1.31	0.10	1.16	0.37	25.46
Chloroflexi_Undefined1	0.66	0.87	0.10	1.11	0.37	25.83
Latescibacterota_Undefined37	0.28	0.95	0.10	1.10	0.37	26.20
Oligoflexia	0.58	1.17	0.10	1.24	0.37	26.56
TA06_Undefined3	1.64	1.45	0.10	1.27	0.36	26.93
CK-2C2-2_Undefined2	0.62	1.04	0.10	1.17	0.36	27.29
Latescibacterota_Undefined30	1.01	0.74	0.10	1.22	0.36	27.66
Latescibacterota_Undefined21	0.70	1.18	0.10	1.23	0.36	28.02
Zixibacteria_Undefined7	0.85	1.32	0.10	1.13	0.36	28.38
Lokiarchaeia	3.44	3.27	0.10	1.32	0.36	28.75
Sva0485_Undefined28	0.81	0.95	0.10	1.15	0.36	29.11
Zixibacteria_Undefined8	1.06	1.04	0.10	1.15	0.36	29.47
P9X2b3D02	0.89	1.12	0.10	1.16	0.36	29.84
Acidimicrobiia	4.02	4.83	0.10	1.65	0.36	30.20
NKB15_Undefined4	1.02	0.91	0.10	1.21	0.36	30.56
Latescibacterota_Undefined35	0.94	0.63	0.10	1.14	0.36	30.92
Sva0485_Undefined7	1.37	1.55	0.10	1.03	0.36	31.28

Planctomycetota_Undefined4	0.56	1.02	0.10	1.14	0.36	31.63
LCP-89_Undefined7	0.97	0.40	0.10	1.24	0.36	31.99
TA06_Undefined5	1.25	0.89	0.10	1.18	0.36	32.35
Latescibacterota_Undefined27	0.55	1.03	0.10	1.19	0.36	32.70
Bathyarchaeia	4.09	4.06	0.10	1.24	0.35	33.06
MBNT15_Undefined1	1.01	1.30	0.10	1.13	0.35	33.41
Thermococci	0.98	0.64	0.10	1.19	0.35	33.76
Latescibacterota_Undefined45	0.22	0.88	0.10	1.10	0.35	34.12
Thermoanaerobaculia	5.23	6.08	0.10	1.73	0.35	34.47
NB1-j_Undefined9	0.67	1.07	0.10	1.20	0.35	34.82
Latescibacterota_Undefined8	1.16	1.99	0.10	1.10	0.35	35.17
Kapabacteria	0.77	1.27	0.10	1.23	0.35	35.52
Chloroflexia	0.25	0.87	0.10	1.12	0.35	35.87
Sva0485_Undefined17	1.29	1.27	0.10	1.19	0.35	36.23
LCP-89_Undefined6	0.95	0.67	0.10	1.18	0.35	36.58
Latescibacterota_Undefined42	0.61	1.08	0.10	1.20	0.35	36.93
Odinarchaeia	0.90	0.83	0.10	1.15	0.35	37.28
KD4-96	2.79	3.51	0.10	1.65	0.35	37.63
Latescibacterota_Undefined29	1.01	1.26	0.10	1.25	0.35	37.98
Desulfobacterota_Undefined2	0.64	1.20	0.10	1.34	0.35	38.33
Latescibacterota_Undefined25	1.10	1.28	0.10	1.17	0.35	38.68
Latescibacterota_Undefined39	0.93	0.98	0.10	1.17	0.35	39.02
LCP-89_Undefined5	0.88	0.67	0.10	1.12	0.35	39.37
Sva0485_Undefined30	0.92	0.21	0.10	1.22	0.35	39.72
Latescibacterota_Undefined20	1.21	0.96	0.10	1.14	0.35	40.07
Latescibacterota_Undefined54	0.86	0.52	0.10	1.07	0.35	40.42
Planctomycetota_Undefined5	0.95	0.27	0.10	1.23	0.35	40.76
Sva0485_Undefined24	0.75	0.62	0.10	1.02	0.35	41.11
Latescibacterota_Undefined34	0.61	0.92	0.10	1.12	0.35	41.46
Sva0485_Undefined15	1.01	1.33	0.09	1.19	0.35	41.80
Zixibacteria_Undefined1	1.53	1.92	0.09	1.00	0.35	42.15
Latescibacterota_Undefined33	0.68	1.08	0.09	1.14	0.34	42.49
NKB15_Undefined2	1.25	1.40	0.09	1.09	0.34	42.83
Desulfobacteria	7.54	8.00	0.09	1.25	0.34	43.18

Zixibacteria_Undefined11	0.25	0.90	0.09	1.11	0.34	43.52
Bacteroidota_Undefined1	1.60	1.13	0.09	1.14	0.34	43.86
Zixibacteria_Undefined9	0.92	1.09	0.09	1.14	0.34	44.20
Latescibacterota_Undefined48	0.68	0.91	0.09	1.11	0.34	44.55
Subgroup 26	0.59	1.08	0.09	1.23	0.34	44.89
NB1-j_Undefined5	1.33	1.66	0.09	1.07	0.34	45.23
Schekmanbacteria_Undefined2	1.39	1.25	0.09	1.09	0.34	45.57
Latescibacterota_Undefined53	0.96	0.85	0.09	1.21	0.34	45.91
NKB15_Undefined3	1.16	1.29	0.09	1.11	0.34	46.25
MBNT15_Undefined6	0.93	0.56	0.09	1.14	0.34	46.59
NB1-j_Undefined14	0.41	0.83	0.09	1.04	0.34	46.93
Schekmanbacteria_Undefined5	1.19	1.17	0.09	1.05	0.34	47.27
Zixibacteria_Undefined18	0.66	0.74	0.09	1.02	0.34	47.60
Sva0485_Undefined27	1.01	0.85	0.09	1.26	0.34	47.94
NB1-j_Undefined17	0.76	1.13	0.09	1.22	0.34	48.28
NB1-j_Undefined10	0.89	1.07	0.09	1.17	0.34	48.61
Zixibacteria_Undefined13	0.35	0.89	0.09	1.12	0.34	48.95
Anaerolineae	6.08	6.73	0.09	1.32	0.34	49.29
Latescibacterota_Undefined46	0.91	0.69	0.09	1.18	0.34	49.62
NB1-j_Undefined18	0.56	0.93	0.09	1.27	0.34	49.96
MBNT15_Undefined2	0.99	0.93	0.09	1.10	0.34	50.29

Table S5: Results of the SIMPER analysis comparing microbial communities at times 1 and 2. Dissimilarity was 27.44%. In the table are shown the taxa (Class level) that contribute cumulatively to the 50% of that dissimilarity.

	Group 1	Group 3				
Species	**Av.Abund**	**Av.Abund**	**Av.Diss**	**Diss/SD**	**Contrib%**	**Cum.%**
Desulfovibrionia	0.78	1.56	0.14	1.42	0.54	0.54
Myxococcia	0.82	1.92	0.14	1.40	0.53	1.07
Sva0485_Undefined20	0.48	1.60	0.14	1.63	0.52	1.59
Thermoplasmata	3.88	3.49	0.14	1.35	0.51	2.10
Nanoarchaeia	3.20	2.95	0.14	1.28	0.51	2.61
Latescibacterota_Undefined19	0.56	1.56	0.13	1.37	0.50	3.11
Gammaproteobacteria	8.77	9.97	0.13	1.59	0.50	3.61
Desulfobulbia	6.32	7.49	0.13	1.64	0.49	4.10
Dehalococcoidia	4.42	4.27	0.13	1.27	0.48	4.59
Latescibacterota_Undefined12	1.04	1.21	0.13	1.31	0.48	5.06
Latescibacterota_Undefined7	0.99	2.00	0.13	1.29	0.47	5.54
Bacilli	0.78	1.65	0.12	1.57	0.46	6.00
Schekmanbacteria_Undefined1	1.86	1.32	0.12	1.27	0.46	6.46
Planctomycetes	5.33	6.37	0.12	1.83	0.45	6.90
Fibrobacteria	2.56	2.35	0.12	1.13	0.44	7.35
Actinobacteria	1.92	2.68	0.12	1.24	0.43	7.78
BD7-11	1.27	0.48	0.11	1.31	0.43	8.20
Chlamydiae	1.31	1.20	0.11	1.26	0.42	8.62
JS1	2.75	2.56	0.11	1.33	0.42	9.04
Alphaproteobacteria	5.71	6.72	0.11	1.75	0.42	9.46
Zixibacteria_Undefined3	1.42	1.11	0.11	1.11	0.42	9.88
NB1-j_Undefined4	1.05	2.01	0.11	1.23	0.41	10.29
Zixibacteria_Undefined13	0.35	1.19	0.11	1.36	0.41	10.70
Methanosarcinia	1.48	1.14	0.11	1.13	0.41	11.11
Zixibacteria_Undefined11	0.25	1.14	0.11	1.38	0.41	11.53
Latescibacterota_Undefined45	0.22	1.10	0.11	1.25	0.41	11.94
Sva0485_Undefined13	0.83	1.53	0.11	1.29	0.41	12.34
Bacteroidota_Undefined1	1.60	0.78	0.11	1.37	0.41	12.75
TA06_Undefined3	1.64	1.14	0.11	1.29	0.41	13.16
Schekmanbacteria_Undefined6	1.37	0.77	0.11	1.31	0.41	13.57
NB1-j_Undefined3	1.39	1.49	0.11	1.22	0.40	13.97
Sva0485_Undefined19	0.63	1.04	0.11	1.16	0.40	14.37

Sva0485_Undefined25	0.62	1.24	0.11	1.28	0.40	14.78
NB1-j_Undefined9	0.67	1.48	0.11	1.29	0.40	15.18
Zixibacteria_Undefined5	1.02	1.58	0.11	1.16	0.40	15.58
Dissulfuribacteria	1.73	1.56	0.11	1.11	0.40	15.98
Schekmanbacteria_Undefined5	1.19	0.79	0.11	1.21	0.40	16.37
Zixibacteria_Undefined6	0.71	1.39	0.11	1.27	0.40	16.77
Phycisphaerae	4.75	4.75	0.11	1.18	0.39	17.16
Zixibacteria_Undefined16	0.46	1.17	0.11	1.30	0.39	17.56
WWE3	1.54	1.11	0.11	1.23	0.39	17.95
Micrarchaeia	1.17	0.87	0.11	1.29	0.39	18.34
Latescibacterota_Undefined9	1.27	1.90	0.11	1.11	0.39	18.73
Latescibacterota_Undefined49	0.55	1.24	0.10	1.33	0.39	19.12
Bacteroidia	8.16	8.63	0.10	1.35	0.39	19.51
Zixibacteria_Undefined10	0.99	0.70	0.10	1.11	0.39	19.89
Schekmanbacteria_Undefined10	0.19	1.00	0.10	1.12	0.39	20.28
LCP-89_Undefined4	1.14	0.60	0.10	1.24	0.38	20.66
DG-56	1.78	1.57	0.10	1.17	0.38	21.04
Latescibacterota_Undefined16	0.84	1.66	0.10	1.22	0.38	21.43
Thermoanaerobaculia	5.23	6.14	0.10	1.54	0.38	21.81
Kapabacteria	0.77	1.47	0.10	1.25	0.38	22.19
NKB15_Undefined1	1.26	1.27	0.10	1.09	0.38	22.57
Latescibacterota_Undefined5	1.89	1.79	0.10	1.09	0.38	22.95
Sva0485_Undefined24	0.75	0.83	0.10	1.12	0.38	23.33
Sva0485_Undefined23	0.44	1.09	0.10	1.24	0.37	23.70
Latescibacterota_Undefined11	1.05	1.48	0.10	1.21	0.37	24.07
Desulfobacterota_Undefined2	0.64	1.26	0.10	1.31	0.37	24.45
Schekmanbacteria_Undefined4	1.28	1.03	0.10	1.19	0.37	24.82
Gracilibacteria	1.03	0.70	0.10	1.14	0.37	25.19
Latescibacterota_Undefined28	0.92	1.20	0.10	1.30	0.37	25.56
Latescibacterota_Undefined8	1.16	1.72	0.10	1.16	0.37	25.93
Schekmanbacteria_Undefined7	1.32	0.88	0.10	1.25	0.37	26.31
Elusimicrobia	1.48	1.26	0.10	1.13	0.37	26.67
NKB15_Undefined2	1.25	1.33	0.10	1.11	0.37	27.04
CK-2C2-2_Undefined2	0.62	1.07	0.10	1.16	0.37	27.41

Chloroflexi_Undefined1	0.66	0.92	0.10	1.19	0.37	27.78
Zixibacteria_Undefined7	0.85	1.26	0.10	1.17	0.37	28.15
Sumerlaeia	3.12	3.13	0.10	1.18	0.37	28.51
Desulfobacteria	7.54	8.13	0.10	1.28	0.37	28.88
Latescibacterota_Undefined27	0.55	1.09	0.10	1.23	0.37	29.25
Chloroflexia	0.25	0.93	0.10	1.17	0.37	29.62
Latescibacterota_Undefined31	0.79	1.32	0.10	1.27	0.37	29.98
Latescibacterota_Undefined18	0.90	1.72	0.10	1.12	0.37	30.35
Latescibacterota_Undefined47	0.39	1.02	0.10	1.18	0.37	30.71
Latescibacterota_Undefined32	0.76	1.09	0.10	1.11	0.36	31.08
Latescibacterota_Undefined21	0.70	1.14	0.10	1.21	0.36	31.44
P9X2b3D02	0.89	1.66	0.10	1.19	0.36	31.80
Latescibacterota_Undefined50	0.53	1.12	0.10	1.24	0.36	32.17
Latescibacterota_Undefined25	1.10	0.87	0.10	1.22	0.36	32.53
Latescibacterota_Undefined10	0.97	1.58	0.10	1.21	0.36	32.89
Thermococci	0.98	0.71	0.10	1.14	0.36	33.25
Zixibacteria_Undefined14	0.70	0.97	0.10	1.13	0.36	33.61
NB1-j_Undefined12	0.68	0.91	0.10	1.16	0.36	33.97
Sva0485_Undefined15	1.01	1.65	0.10	1.23	0.36	34.32
Omnitrophia	2.41	2.36	0.10	1.08	0.36	34.68
Sva0485_Undefined26	0.47	0.92	0.10	1.17	0.36	35.04
NKB15_Undefined4	1.02	0.36	0.10	1.26	0.36	35.39
Latescibacterota_Undefined41	0.82	1.16	0.10	1.15	0.36	35.75
Latescibacterota_Undefined30	1.01	1.23	0.10	1.21	0.35	36.10
Zixibacteria_Undefined9	0.92	0.92	0.10	1.18	0.35	36.46
Sva0485_Undefined17	1.29	1.19	0.10	1.22	0.35	36.81
NB1-j_Undefined10	0.89	1.23	0.10	1.14	0.35	37.17
Latescibacterota_Undefined34	0.61	1.03	0.10	1.16	0.35	37.52
Zixibacteria_Undefined8	1.06	1.13	0.10	1.08	0.35	37.87
Subgroup 26	0.59	0.90	0.09	1.21	0.35	38.22
LCP-89_Undefined3	0.68	1.23	0.09	1.20	0.35	38.57
Latescibacterota_Undefined35	0.94	0.92	0.09	1.14	0.35	38.92
WCHB1-81	0.98	0.56	0.09	1.18	0.35	39.27
Latescibacterota_Undefined29	1.01	1.03	0.09	1.20	0.35	39.62

LCP-89_Undefined5	0.88	0.66	0.09	1.10	0.35	39.97
Zixibacteria_Undefined12	0.61	0.78	0.09	1.10	0.35	40.32
Latescibacterota_Undefined33	0.68	1.13	0.09	1.16	0.35	40.67
Zixibacteria_Undefined15	0.68	1.14	0.09	1.17	0.35	41.01
Bathyarchaeia	4.09	4.08	0.09	1.25	0.35	41.36
LCP-89_Undefined6	0.95	0.48	0.09	1.21	0.35	41.71
Lokiarchaeia	3.44	3.24	0.09	1.35	0.34	42.05
Latescibacterota_Undefined22	0.97	0.99	0.09	1.17	0.34	42.39
MBNT15_Undefined1	1.01	1.21	0.09	1.11	0.34	42.74
Sva0485_Undefined27	1.01	0.78	0.09	1.27	0.34	43.08
Sva0485_Undefined30	0.92	0.60	0.09	1.21	0.34	43.42
LCP-89_Undefined1	1.79	1.87	0.09	0.89	0.34	43.77
Babeliae	2.82	2.78	0.09	1.04	0.34	44.11
MBNT15_Undefined6	0.93	0.24	0.09	1.10	0.34	44.45
Desulfobacterota_Undefined3	0.88	1.06	0.09	1.13	0.34	44.79
Latescibacterota_Undefined55	0.28	0.91	0.09	1.11	0.34	45.13
RBG-16-55-12	1.38	1.82	0.09	1.06	0.34	45.47
Latescibacterota_Undefined48	0.68	0.92	0.09	1.10	0.34	45.81
NB1-j_Undefined13	0.57	1.00	0.09	1.16	0.34	46.15
Latescibacterota_Undefined44	0.59	0.85	0.09	1.16	0.34	46.49
Odinarchaeia	0.90	0.84	0.09	1.18	0.34	46.83
Desulfobacterota_Undefined1	0.52	0.80	0.09	0.98	0.34	47.16
Latescibacterota_Undefined26	0.67	0.58	0.09	0.96	0.34	47.50
Latescibacterota_Undefined54	0.86	0.26	0.09	1.01	0.34	47.84
LCP-89_Undefined2	0.90	1.25	0.09	1.14	0.33	48.17
Zixibacteria_Undefined4	1.37	1.75	0.09	1.10	0.33	48.51
Latescibacterota_Undefined39	0.93	1.48	0.09	1.08	0.33	48.84
Latescibacterota_Undefined40	0.70	1.21	0.09	1.21	0.33	49.17
Planctomycetota_Undefined5	0.95	0.52	0.09	1.19	0.33	49.51
Syntrophobacteria	4.87	5.28	0.09	1.13	0.33	49.84
Sva0485_Undefined28	0.81	0.57	0.09	1.07	0.33	50.17

Table S6: Results of the SIMPER analysis comparing microbial communities at times 1 and 3. Dissimilarity was 26.99%. In the table are shown the taxa (Class level) that contribute cumulatively to the 50% of that dissimilarity.

Main findings, conclusions and future perspectives

The aim of the thesis was to explore the potential effects of hypoxic events, and in particular of their repetitive nature and the timing regimes of their occurrence, on the communities of estuarine and transitional habitats. The initial focus was on single species and their tolerance different temperatures combined with different intensities of hypoxia. It clearly appeared that increasing temperatures strongly increased the mortality of both species under conditions of oxygen deprivation. This result confirms the tendency of high temperatures to increase the effects of hypoxia on organisms suppressing their strategies to cope with oxygen deprivation and increasing their oxygen demand (Babarro & De Zwaan, 2008; De Zwaan *et al.,* 2002).

Coastal lagoons and estuarine habitats tend to present stagnant and shallow waters that can be especially susceptible to hypoxia (Diaz & Rosenberg, 1995; Miyamoto, 2009) and, in our warming world, both the occurrence and the effects of hypoxic periods can be enhanced causing mass mortalities for a variety of species (Nakano *et al.*, 2017; Coma *et al.*, 2009; Lim *et al.,* 2006) with limited adaptations to drastic changes in abiotic factors (Conley *et al.*, 2009; Wu, 2002). These tendencies suggest the need for a special attention and focused management actions aimed at limiting the occurrence of hypoxic events in warming basins whose communities may not be well adpated to extreme abiotic conditions.

Chapter 1: The two species tested in the experiment were the European native *Cerastoderma edule* and the non native *Ruditapes philippinarum.* The sub-tropical species *R. philippinarum* displayed a significantly higher thermal tolerance than *C.edule* that results even more apparent under hypoxic conditions. Behavioral and physiological adaptations of bivalves to hypoxia and temperature are well known (Ansell *et* al., 1981; Kodama *et al.*, 2018; Beukema & Dekker, 2020) but, despite those, *C. edule* displayed a very high mortality that could determine the ability by *R. philippinarum* to outcompete the native species in increasingly stressful environments. This tendency by non native invasive species to present higher tolerances to hypoxia is not universal (Sorensen & Branstrator, 2017; Nati *et al.*, 2018) but widespread. Stoffels and colleagues (2017) for example show how increasing hypoxia and higher temperatures may give to an invasive fish species a competitive advantage over a native one and similar situations present for other species (Lagos *et al.,* 2017; Kim *et al.*, 2018). This aspect of the hypoxic stress is important to address in a world where species migration and invasion success is already increased by global warming (Walther *et al.*, 2009).

Chapter 2: Another aspect that is expected to increase in the future about hypoxia is the frequency and stochastic nature of its appearance: increasing temperatures, nutrient loading, organic pollution and extreme climatic events can enhance the appearance of aperiodic and repeated hypoxic periods (Del Giudice *et al.,* 2018; Levin *et al*., 2009) whose effects may be especially detrimental for communities. Repeated events have different effect on organisms based on their duration and frequency (Nakano *et al.*, 2017; Dal Bello, 2017): the duration of a stressful event is critical in determining its effect, the effect of subsequent disturbances and also the possibility for the communities to take advantage of non stressful periods to increase their performances and their resilience. A normoxic period following an hypoxic one can in fact help the community and increase its tolerance to subsequent events only if the initial hypoxic period is short enough. Our results back up those by Nakano *et al.* (2017) suggesting that whenever in an environment it is possible to manage, at least partially, the occurrence of stressful events, the priority should be to minimize the duration of each single event preferring, on a short time scale, a series of short events to a single long one.

Chapter 3: The timing of occurrence of hypoxic periods is also key in determining the recovery ability of communities. In habitats where hypoxic events appear seasonally, for example, the full recovery is interrupted before a complete return to the pre-stress structure and it tends to stabilize at and early successional stage (Boesch & Rabalais, 1991; Diaz, 2001) with short-lived, smaller and opportunistic organisms (Pearson & Rosenberg, 1978; van Colen *et al.*, 2010). An hypoxic period particularly close to the previous one could, on the other hand, be almost irrelevant if it happens before the recovery process from the previous one has even started (Lim *et al.*, 2006; Mumby *et al.*, 2011) and only delay, more then directly impact, the recovery. The ability and the time required for a community to return to a state comparable to that before the disturbance is linked to multiple factors such as the extent (spatial and temporal) of the disturbance itself, the initial structure of the of the community, its tolerance and adaptations and eventual overlapping stressors. We can expect communities from historically hypoxic habitats to resist hypoxic periods well and to recover quickly but, in a world where the amount of basins interested by hypoxia is expected to widely increase in the next years, the emergence of such a devastating pressure in new habitats may lead to severe and potentially irreversible damages.

These results may be an important step forward in understanding, predicting and planning the effects of repeated hypoxic events and open up a plethora of additional questions and future research perspectives: the study of the interaction between multiple stressors is already one of the hot topics in ecology and adding to it the temporal component appears to be a pressing issue for the future. A deeper understanding of the long term effects of repeated hypoxic events in shaping the tolerance and resilience of communities for the future is also a question that sparks from the findings of this work together with a more thorough knowledge about the short and mid-term recovery dynamics after repeated and aperiodic disturbances.

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Appendix

List of activities

1 Papers published or in preparation

- Piarulli, S., Vanhove, B., Comandini, P., Scapinello, S., Moens, T., Vrielinck, H., Sciutto, G., Prati, S., Mazzeo, R., Booth, A. M., Van Colen, C., & Airoldi, L. (2020). Do different habits affect microplastics contents in organisms? A trait-based analysis on salt marsh species. Mar Pollut Bull, 153(January), 110983. https://doi.org/10.1016/j.marpolbul.2020.110983

- Piarulli, S., Scapinello, S., Comandini, P., Magnusson, K., Granberg, M., Wong, J.X.W., Sciutto, G., Prati, S., Mazzeo, R., Booth, A.M., Airoldi, L., 2019. Microplastic in wild populations of the omnivorous crab Carcinus aestuarii: A review and a regional-scale test of extraction methods , including micro fibres. Environ. Pollut 251, 117–127. https://doi.org/10.1016/j.envpol.2019.04.092

- Paolo Comandini, Folco Giomi, Valentijn Elsman, Laura Airoldi, Tjeerd Bouma. Cold and heat tolerance in chronically hypoxic coastal habitat: an experimental comparison between native and non-indigenous bivalves. (Under Review on PeerJ)

- Paolo Comandini, Folco Giomi, Simona Rovera, Tjeerd Bouma, Laura Airoldi. Effects of different timing regimes of hypoxia on the benthic community of an Italian coastal lagoon (Pialassa Baiona, Ravenna). Manuscript in preparation

2 Other activities

- Project Report for Spelen met Stroom(ing) (SmS) project: Paolo Comandini, Jeroen Van Dalen, Valentijn Elsman, Folco Giomi, Tjeerd Bouma and Laura Airoldi, 2021. Title: Understanding the importance of timing of anoxia events for benthic tolerances

- "Census of Marine Mediterranean Benthic biodiversity: an integrative metabarcoding approach (CoMBoMed)"- a conference I attended in Ravenna about the integration of different taxonomic and phylogenetic techniques for the census of marine biodiversity (10 hours)

- Presentation at the SdS project final meeting

- Attended the seminar ‘Funzioni Cause e Meccanismi’ about Philosophy of Science

- Course: Introduction to Systematic Review and Meta-Analysis. Coursera Inc.

- Supervision of high school students during their school/work alternation

- Periods Abroad: Visits at the Royal Netherlands Institute for Sea Research for the execution of laboratory experiments

- Various NIOZ seminars and talks about a variety of Marine Biology topics

- Collaboration to the experiment led by NIOZ about the degradation of artificial structures

- “Through the Water art show” (20 October 2018, Grinder coffee lab, Ravenna, Italy)

- Co-founder and scientific curator for the scientific divulgation project “Through the Water”

- DIGITA: Educational event for primary school students with various activities linked to the marine environment

3 Supervised Theses

- Co-supervision of the Master Thesis by Martina Bernardi. ‘Does Hypoxia facilitate the invasions in aquatic environment? A systematic review’

- Co-supervision of the Bachelor Thesis by Valentijn Elsman. ‘The effect of anoxia and low temperatures on the survival of R. philippinarum and C. edule’

- Co-supervision of the Master Thesis by Simona Rovera. ‘Effects of different hypoxia timing regimes on a transitional habitat’s (Pialassa Baiona, Ravenna, Italy) benthic community’

- Co-Supervision of the Bachelor Thesis by Federica Iezzi. ‘Behavioral reactions of a native (Cerastoderma glaucum) and an invasive bivalve (Ruditapes philippinarum) to hypoxic conditions.