REVIEWER #1

Reviewer wrote: This paper explores the implications of environmental stress (OA and hypoxia), as determined in lab experiments, on the growth and reproductive potential of mussels in two locations of the Mediterranean with simulations based on a dynamic energy budget model. The study capitalizes on the potential of DEB models to integrate the impacts of multiple environmental drivers on organismal level outcomes, including growth, reproduction, time to maturity, rates of feeding and respiration, and so on. This approach is powerful in potential and the application is new. However, there are some important shortcomings, especially in the way the model is parameterized. Also, I find the lack of some form of quality assessment problematic.

Author's reply: First, we thank the Reviewer #1 for the effort in providing his/her suggestions to the original version of our ms.

Author's changes: Apart from all possible not clear parts that we accordingly improved, we followed his/her suggestion in the hope to have increased the quality of this ms.

Reviewer wrote: It is very annoying that all sections consist of a single paragraph. Did something go wrong with the formatting of the manuscript?

Author's reply: We appreciated the referee's suggestion about the different paragraphs, but we believe that the current structure is already sufficiently sectioned; to increase the number of paragraphs or sub-paragraphs can only increase the text fragmentation which may limit the logical flow of the text.

Reviewer wrote: The author's use of respiration measurements as a proxy for DEB maintenance costs is problematic. In the DEB framework, respiration is emphatically not the same as maintenance, but also include energetic overheads, such that of growth. Respiration is a function of the commitment rate in DEB, of which maintenance could be a minor part, depending on size and nutritional status of the animal.

Author's reply: We acknowledge that respiration does not include only maintenance, but also include energetic overheads, such as growth. Nevertheless, there is no way - to our knowledge - to measure the different contribution of every energetic components apart from to experimentally measure oxygen consumption as a proxy for metabolism. Also, the proposed approaches measuring indirectly the $[\dot{p}_M]$ values (e.g. van der Veer et al., 2006; Ren and Schiel 2008), are not feasible in the context of the present experimental asset. While this approach is not experimentally feasible when assessing the effect of stressors on the energy budget, the only way to indirectly provide an estimation of the effect of disturbance is through the Jagger et al. (2016) approach which is based on the stress factor "s". Thus, after estimating the effect induced by a treatment on the oxygen consumption, that in the present case study was expressed as a percentage variation, we summed/subtracted the energetic amount due to the effect of a stressor to the species-specific $[\dot{p}_M]$ values of *M. galloprovincialis* then we run our models. However, we thank the referee for highlighting this point whose importance was addressed in the Discussion section of this ms. as we believe that is crucial to increase our understating on how we can mechanistically assess the effect of disturbance on individual performances through the DEB model. All these limitations show how much is important to date to increase the experimental and theoretical research effort in order to unravel this point, which is increasingly crucial to get realistic answers to management questions in a context of environmental change.

Reviewer wrote: In addition, oxygen deprived mussels, and possibly mussels enduring stress of hypercapnia, are able to use anaerobic metabolic pathways to fulfil their maintenance requirements. If stress increases maintenance requirements, one would expect respiration rates to increase with increasing stress intensity. However, we see the opposite happen (see Fig 2).

I think this is likely due to the fact that stressed mussels have their shells closed more often than unstressed conspecifics (see Fig. 1), and thus ingest less food. Less food leads to a lower energy reserve buffer and therefore a lower rate at which reserves are committed.
Author's reply: As reported in our results and Fig. 2, maintenance requirements in accordance with respiration rates, decreases with stress in agreement with what is reported in the current literature. We are sorry with Reviewer #1 and with all readers as we made a mistake in writing the text commenting the figure 1 (we wrote wrongly "opened" instead of "closed"). Actually, our mussels increased their openness with the increasing stressful conditions. At the present stage, we are not able to provide information about the amount of ingested food under different treatments and then we are not able to infer on the effect of openness degree on energetic performances.
Author's changes: Figure was fixed according to both referee's suggestion, and also the text in the paragraph has been rephrased accordingly.

Reviewer wrote: I suggest the authors change the maximum assimilation rate parameter of their model based on their behavioural observations and leave the maintenance rate parameter unchanged.
Author's reply: We appreciated the referee's suggestion but we prefer to focus on both the assimilation efficiency and the metabolic effect (through pM) as i) the main effect of acidification seem to be exerted on metabolism as widely reported in the current literature, and ii) also to show that our mechanistic DEB approach can be really effective in measuring the multiple stressor's effect on LHs.
Author's changes: Thus, we enlarged the discussion on these points to include possible shortcomings deriving from the fact that the stressor's effect on maintenance is not still well-experimentally measurable.

Reviewer wrote: The simulations suggest that unstressed mussels only grow to 3 cm in length and do not reproduce in Palermo. This seems implausible. How long do real mussels get in Palermo? Do they reproduce? How sensitive are the simulation results to the particular choices of parameter values? The authors do not reflect at all on the reliability of their assessments, which I find troublesome, especially given the politicized context of the subject matter.
Author's reply: Actually, to enlarge the discussion about the magnitude of effects at local level could be not influent for our purposes, although our results are in line with the environmental and trophic conditions reported in section 2.5: "Both sites were chosen as they represent two opposite temperature and food conditions for mussel growth in Italy… etc.". *M. galloprovincialis* in Sicily is observed to be limited by oligo-trophic conditions although it grows in highly trophic-enriched areas such as harbours or under Integrated Multi-Trophic Aquaculture (IMTA) conditions (Sarà et al 2012; 2013b, Giacoletti et al. 2018 in press JEMA) which supports what we gathered in the present ms. through the DEB simulations.

Reviewer specific comment n. 1
Reviewer wrote: Title. Functional spatial contextualization sounds impressive but I've no clue what it could mean. Also, the manuscript deals with only a single species; the title is too general.
Author's changes: We agreed with Reviewer's #1 point and changed the title.

Reviewer specific comment n. 2
Reviewer wrote: L27-33 Split up sentence.

Author's changes: Sentence was splitted up accordingly.

Reviewer specific comment n. 3
Reviewer wrote: L35 (and elsewhere) Put reference in the proper place of the sentence
Author's changes: All references were checked and put in proper spaces.

Reviewer specific comment n. 4
Reviewer wrote: L40 'lager'?
Author's changes: Changed with "larger".

Reviewer specific comment n. 5
Reviewer wrote: L68-70. This is a strong statement and should be substantiated with references. BTW, the only 2 papers using DEB in a OA context I'm aware of are 10.1111/gcb.12547 and 10.1016/j.jembe.2015.09.016
Author's changes: References regarding the effect of OA on functional traits such as feeding and assimilation, and on maintenance costs has been added accordingly.

Reviewer specific comment n. 6
Reviewer wrote: L72 the DEB [p_M] parameter does not relate to assimilation
Author's changes: The sentence was rephrased accordingly.

Reviewer specific comment n. 7
Reviewer wrote: L83 articulated ! consisted of
Author's changes: Changed accordingly.

Reviewer specific comment n. 8
Reviewer wrote: Section 2.4 contains material that should go in 2.3 (or combine the sections).
Author's reply: Section 2.4 refers to assimilation efficiency, while section 2.3 to oxygen consumption measures, so we consider not easy to combine both sections as we may incur in the risk to reduce the readability of this section.

Reviewer specific comment n. 9
Reviewer wrote: I didn't get how the authors calculate the assimilation efficiency.
Author's changes: A detailed explanation on how the assimilation efficiency was estimated, was added with supporting references.

Reviewer specific comment n. 10
Reviewer wrote: Section 3.1 belongs in the Materials and Methods Section. There is no need for a statistical analysis. Delete Table 2.
Author's changes: Table 2 was deleted according to both referee's suggestion and details were moved in the Materials and Methods Section.

Reviewer specific comment n. 11
Reviewer wrote: Combine Sections 3.2-4. There is no need to duplicate in the text what is already presented in the figures. The percentage of closed valves is simply 100 – percentage opened valves, so don't mention the former. I don't understand why the error measures differ so much, though.
Author's changes: We agree not to duplicate in text what is already presented in figures, and we worked to avoid this replication. Following Reviewer's #1 suggestion we also expressed

the percentage of closed valves 100 – percentage opened valve. Instead, merging the sections can increase the risk of confusion in the reader as section 3.2 is about behavioural observations, while the other two are about physiological measurements.

Reviewer specific comment n. 12
Reviewer wrote: Section 2.7 is incomprehensible for people without DEB modeling background. Include a figure and references to overview texts (e.g. Kooijman's book, Nisbet et al JAE, Sousa et al, and/or most recently Jusup et al Physics of Life Reviews 20:1-39).
Author's changes: We agree with Reviewer #1 that section 2.7 is difficult for someone without a DEB modelling background, and in order to made it more clear we added the suggested references and rephrased some parts.

Reviewer specific comment n. 13
Reviewer wrote: L263 addictive ! additive. The way the authors use 'additive' is confusing. Additive refers to impacts that can be summed, like 1+1=2, an unlikely situation with nonlinear models, such as DEB.
Author's changes: Rephrased following suggestions to "with a progressive contribution of hypoxia".

Reviewer specific comment n. 14
Reviewer wrote: What are the initial conditions of the simulation runs?
Author's reply: Results of simulation performed with unstressed organism were already reported in Table 5, while model parameters have been reported in Table 1.

Reviewer specific comment n. 15
Reviewer wrote: What is the rational for the choices for the frequency of events?
Author's reply: While we are aware that hypoxia events are more frequently during summertime, we decided to not apply any timing and frequency scheme to simulate hypoxia event's occurrence according to many papers published across the recent literature(Crain et al. 2008 Ecology Letters; Miller et al. 2009 PNAS).

Reviewer specific comment n. 16
Reviewer wrote: From Table 5 remove data that are already presented in Figure 3. Round off # of eggs to 6.74e6. Units of frequency should be 1/time
Author's changes: Table 5 was corrected accordingly.

Reviewer specific comment n. 17
Reviewer wrote: Figure 3 label y axis 'Change relative to control'
Author's changes: Figure was corrected accordingly.

Reviewer specific comment n. 18
Reviewer wrote: L294 delete 'formally'
Author's changes: Deleted.

Reviewer specific comment n. 19
Reviewer wrote: L295 delete 'compensatory' and change contrast to compensate
Author's changes: Rephrased.

Reviewer specific comment n. 20
Reviewer wrote: L299 suppressed feeding activity

Author's changes: Changed.

Reviewer specific comment n. 21
Reviewer wrote: L304 what is crossing effect?
Author's changes: Sentence has been rephrased accordingly.

Reviewer specific comment n. 22
Reviewer wrote: L306 on! over. 'that' doesn't refer to anything
Author's changes: Rephrased.

Reviewer specific comment n. 23
Reviewer wrote: L333 sustainable and reliable ! practical
Author's changes: Changed.

Reviewer specific comment n. 24
Reviewer wrote: L337 write out TW, TRO and TM
Author's changes: Written out accordingly.

Reviewer specific comment n. 25
Reviewer wrote: The readability of the manuscript would improve if there were fewer references. Remove unnecessary repetitive references.
Author's changes:: All the references has been checked and unnecessary and repetitive ones have been deleted accordingly.

Predicting the multiple effects of acidification and hypoxia on *Mytilus galloprovincialis* (Bivalvia, Mollusca) life history traits

~~**Functional spatial contextualisation of the effects of multiple stressors in marine bivalves**~~

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Abstract. Many recent studies have revealed that the majority of environmental stressors experienced by marine organisms (ocean acidification, global warming, hypoxia etc.) occur at the same time and place, and that their interaction may complexly affect a number of ecological processes. Here, we experimentally investigated the effects of pH and hypoxia on the functional and behavioural traits of the mussel *Mytilus galloprovincialis*, we then simulated the potential effects on growth and reproduction dynamics trough a Dynamic Energy Budget (DEB) model under a multiple stressor scenario. Our simulations showed that hypercapnia had a remarkable effect by reducing the maximal habitat size and reproductive output differentially as a function of the trophic conditions, where modelling was spatially contextualized. This study showed the major threat represented by the hypercapnia and hypoxia phenomena for the growth, reproduction and fitness of mussels under the current climate change context, and that a mechanistic approach based on DEB modelling can illustrate complex and site-specific effects of environmental change, producing that kind of information useful for management purposes, at larger temporal and spatial scales.

Key-words: Acidification; Climate change; DEB Model; Hypoxia; *Mytilus galloprovincialis*; Multiple-Stressor; Mussel.

1 Introduction

Since the dawn of research investigating the possible effects of ocean acidification (OA) on aquatic organisms (e.g. Bamber, 1990), most studies have shown that elevated pCO_2 levels, as predicted for the next century, may affect ~~ ~~to some extent the functional traits (Schoener, 1986; Koehl, 1989) of marine organisms (Feely et al., 2004; Navarro et al., 2013). Referring to functional traits, we consider all those specific traits that define each species in terms of their ecological roles (Diaz & Cabido, 2001), and thereby the species' identity. In marine ectotherms such as bivalves, crabs, sea urchins and fish, these traits include tolerance and sensitivity to environmental conditions (e.g. physiological tolerance limits - Kearney & Porter, 2009) defining the ability of each species to support their own metabolic machinery (Sokolova et al., 2012; Sarà et al., 2014)~~,~~ They further include the ability to obtain energy from food, the so-called functional response (Holling, 1959) or those behavioural (e.g. swimming behaviour, habitat use, mating system) and morphological (e.g. shape, thickness) traits ~~(Schoener, 1986)~~ which led to optimise the energetic income (Krebs & Davies, 1992) and lastly to reach the ultimate fitness (Roff, 1992).

Research performed over the last decade and summarized in the recent IPCC (2014) report ~~(IPCC, 2014)~~ clearly shows that ocean acidification will affect marine organisms and ecosystems (Connell et al., 2017) in the coming decades, and such projections have stimulated new research that aims to understand the impact on calcifying marine organisms. Reductions in growth and calcification rates are just those kinds of the physiological impacts of ocean acidification (Thomsen et al., 2013; Byrne, 2012; Beniash et al., 2010). While much research showed that low pH may impair most functional traits (e.g. respiration), functions connected with energy uptake such as feeding and assimilation seem to be reduced at a larger extent in many species with expected implications for the amount of energy available for growth and reproduction (Kurihara et al., 2008; Appelhans, 2012; Navarro et al., 2013; Zhang et al., 2015). Such information has been obtained through both acute and chronic exposure to OA but no studies are yet available to assess the potential effects of OA on the magnitude of other Life History (LH) traits, such as maximum habitat body size, fecundity, time to reach maturation and the number of spawning events under future conditions of environmental change (*sensu* Kearney and Porter, 2009; Sarà et al., 2011; 2013b). ~~To obtain such LH traits, experiments should be long enough to assure a functional effect of lower pH for many weeks or months but probably no existing lab mesocosm could currently assure the stability of seawater acidification system for such a long time.~~ Thus, apart from long term experiments carried out in few field sites worldwide (e.g. Ischia [Hall-Spencer et al., 2008] and Vulcano [Duquette et al., 2015] islands) in the Southern Mediterranean Sea and in other Seas (Maug Island [Pala, 2009] or ~~CO2 vents in the~~ SW~~southwest~~

Pacific [Connell et al., 2017]) where lowered pH seawater is naturally available ~~through CO_2 natural emissions from vents~~, the recent introduction of mechanistic functional trait-based (FT) models based on the Dynamic Energy Budget theory (DEB; Kooijman, 2010) can offer a reliable opportunity for disentangling the effect of OA~~seawater acidification~~ on LH traits.

Functional trait-based DEB (FT-DEB) approach (Kearney and Porter 2009; Kearney et al., 2010; Kearney 2012; Sarà et al., 2011, 2012, 2013a, b, 2018) relies on the quantitative prediction of organismal functional traits and fecundity within the fundamental niche limits of one particular species (Hutchinson 1957). Such an approach aim to exploit mechanistic rules to connect environmental human-induced variability to functional traits (Schoener 1986; Diaz & Cabido 2001) and in turn functional traits to species LH (Stearns 1992) traits. The novelty of the FT-DEB approach relies on its intrinsic mechanistic nature deriving from the fact that it is based on flux of energy and mass through an organism which are traceable processes that are subject to conservation laws (according to the new posited concept of ecomechanics; Denny & Helmuth, 2009; Denny & Benedetti-Cecchi, 2012; Carrington et al., 2015). This provides an exceptionally powerful tool to predict organismal functional traits, capturing variation across species to solve a very wide range of problems in ecology and evolutionary biology (Lika et al., 2011; Kearney, 2012; Pouvreau et al., 2006; Pequerie et al., 2010; Sarà et al., 2011; 2012; 2013a; 2013b; 2014). FT-DEB could provide information about the effect of ~~seawater~~ acidification on the fecundity (as expressed by the number of gametes per life span, the so-called Darwinian fitness; Bozinovic et al., 2011) and the degree of reproductive failure of species providing theoretical predictions about LH traits having implications on population dynamics and community structure throughout the species range (Sarà et al., 2013a). Here, we specifically exploited the FT-DEB model spatially and explicitly contextualised along the Italian coasts under subtidal conditions ~~(Kearney et al., 2010; Sarà et al., 2011; 2012; 2013a; 2013b)~~, using four-year thermal series and satellite ~~Chlorophyll~~chlorophyll-a (CHL-a) concentrations, to test the multiple effect due to the combination of pH and hypoxia on the physiological and behavioural traits of our target species, the bivalve *Mytilus galloprovincialis* (Lamarck 1819).

Recent insights obtained by the experimental research have shown that OA mainly affects feeding rates (FR), assimilation efficiency (AE) and maintenance costs ~~rates~~ of marine organisms (Appelhans, 2012; Navarro et al. 2013; Kroeker et al. 2014; Zhang et al., 2015; Jager et al. 2016). Here, we translated the combined effects of hypoxia and hypercapnia on ~~AE~~ assimilation and oxygen consumption rates as measured under different treatments into effects on assimilation AE and somatic maintenance costs ~~as expressed by the DEB [$\dot{p}_M$] parameters~~. Somatic maintenance is a crucial suite ~~This latter is a crucial~~ of functional traits used in recent

bioenergetics based on the DEB theory that mechanistically can be used to investigate the role played by multiple stressors on LH traits of organisms by using first principles (Sarà et al., 2014). We further documented the effects of those stressors on *M. galloprovincialis* shells through the use of a scanning electron microscope (SEM), and compared the maximum shell breaking load of treated *vs.* control specimens. A behavioural analysis completed the frame concerning the individual's response to both single and combined stressors. Carried out in a context of OA, this exercise comprises a first step in linking the fields of ecomechanics and climate change ecology, which should yield a more mechanistic understanding of how biodiversity will respond to environmental change (*sensu* Buckley et al., 2012).

2 Materials and methods

This study articulated consisted of three steps: 1) laboratory investigation on the effects of pH and hypoxia on functional and (both behavioural and physiological) traits of *Mytilus galloprovincialis*; 2) collection of water temperature data, and Chlorophyll-a (CHL-a) data from two Mediterranean sites (Trieste and Palermo), as a further forcing variable in the DEB model and lastly 3) model running to simulate growth and fitness of *M. galloprovincialis* under stressful conditions by using estimated DEB parameters estimated arising from by the activities in the first step.

2.1 Sampling and eExperimental set-up. Specimens of *M. galloprovincialis* (45 - 55 mm) were provided by the Ittica Alimentare Soc. Coop. Arl. (Palermo) and transferred within 30 minutes to the laboratory. Mussels were then carefully cleaned and placed in a 300L tank filled with natural seawater at room temperature (18-20°C), field salinity (37-38 PSU), and fed *ad libitum* with cultured *Isochrysis galbana* (Sarà et al., 2011). According to common experimental procedures for studying the bioenergetics of bivalves (Sarà et al., 2008; Ezgeta-Balic et al., 2011), mMussels were acclimated for two weeks to reduce stress generated by manipulation and transport (Sarà et al., 2013a). and O once acclimated, 200 specimens were randomly divided in groups of 25 organisms, transferred to 8 independent rectangular glass tanks of 120L capacity (100 cm long, 30 cm deep, 40 cm wide) and kept in a conditioned room at 21°C for 4 weeks according to common protocol with bivalves (Braby & Somero 2006; Fields et al., 2012; Kittner and Riisgård 2005). Tanks 1 to 4 were filled with sea water and continuously with aerated through air pumpsand recirculating sea water, while Tanks 5 to 8 were not aerated and covered with a plastic film disposed on the water surface, in order to avoid gas-exchanges between air and water. Tanks 1-2 were used as a control (CTRL), while hypercapnia was imposed in Tanks 3-4 (Tr1), hypoxia (2

ppm) in Tanks 5-6 (Tr2), and both factor (pH 7.5 and hypoxia) in Tanks 7-8 (Tr3) (see Table 2). Mussels were acclimated to two different nominal pH treatments: (i) pH 8.0 in Tanks 1-2 (CTRL) and 5-6 (Tr2), corresponding to present average pH at the sampling site; and (ii) pH 7.5 in Tanks 2-3 (Tr1) and 7-8 (Tr3), deviating from present range of natural variability and relevant for 2100 ocean acidification scenarios. This last point is considered the critical dissolution threshold of calcium carbonate in shelled animals as reported in literature (Melzner et al., 2011; Gazeau et al., 2013). The carbonate system speciation ($pCO2$, HCO_3^-, CO_3^{2-}, ΩCa and ΩAr) was calculated from pH_{NBS}, temperature, salinity and alkalinity (T_A = 2.5 mM; Rivaro et al., 2010) using CO2SYS (see Table 2; Lewis and Wallace, 1998) with dissociation constants from Dickson & Millero (1987). The pH was manually controlled 8 times a day by an electronic pH-meter (Cyberscan 510, Eutech Instruments; accuracy = ± 0.01 pH) and gaseous CO_2 was injected directly into the aquarium through a commercial ceramic diffusor, when required. Oxygen concentration and temperature were monitored with the same frequency through the PiroScience FirestingO2 oxygen logger equipped with a dedicated temperature sensor. Water movement and recirculation were assured by water pumps. Tanks were siphoned at the end of each working day, removing all the faecal material in order to avoid the accumulation of waste products, and 20% of water was weekly changed with specific pre-conditioned sea water for each treatment.

2.2 Behavioural observations. The valve gape of mussels was recorded by means of the two simplest behavioural categories reported in Jørgensen et al. (1988): closed valves and opened valves. Each observation was carried out by an operator with the aim to record changes in the behavioural repertoire of bivalves in response to the exposure to a single stressor (pH or hypoxia) and to both pH and hypoxia, compared to individuals kept in normal environmental conditions. All experiments were conducted at environmental (37-38 PSU) salinity and with well-aerated sea water through a gentle flow (Ameyaw-Akumfi & Naylor, 1987), except for specimens of Tank 5-6 and 7-8, that were not aerated in order to maintain the hypoxia level set through the gaseous nitrogen. Behavioural observations were repeated six times a day at week 1 and 4 of exposure, on day 7, 14, 21, 28, and involved involving 5 random specimens for each treatment.

2.3 Oxygen consumption. The rate of oxygen consumption was determined twice (week 1 and week 4) in a respirometric glass chamber (0.3L) inside a temperature-controlled water bath, in order to compare investigate the effects of multiple stressors by converting rates intoon metabolic somatic maintenance costs the DEB parameter [$\dot{p}_M$] (expressed as J cm^{-3} h^{-1}) linked to the energetic cost of maintenance in orderand to integrate it in

the standard DEB model. Volume-specific somatic maintenance costs, as expressed by the [$\dot{p}_M$] parameter (J cm^{-3} h^{-1}), represent the amount of energy needed to fuel basal metabolism ($\dot{p}_M$) scaled with the organisms' volume, such as ([$\dot{p}_M$] = $\dot{p}_M$/V). All determinations were performed at 21°C using filtered seawater with the same pH and oxygen content as that of the respective treatment, stirred with a magnetic stirrer bar beneath a perforated glass plate supporting each individual (Sarà et al., 2008; Ezgeta-Balic et al., 2011). The decline in oxygen concentration was measured by a PiroScience FirestingO2 respirometer, capable of four sensor connections. We used a total of n = 64 mussels per week, 16 for each treatment (8 for each tank) acclimated as above, fed *ad libitum* until the day before the experiment. The decline was continuously recorded for at least 1 h, excluding an initial period (~ 10 min) when usually there is a more rapid decline in oxygen caused by a disturbance of the sensor's temperature equilibration. Respiration rate (RR, µmol O_2 h^{-1}) was calculated according to (Ezgeta-Balic et al., 2011; Sarà et al., 2008; 2013b): $RR = (C_{t0} - C_{t1}) x\, Vol_r x 60 (t_1 - t_0)^{-1}$, where C_{t0} is oxygen concentration at the beginning of the measurement, C_{t1} is the oxygen concentration at the end of the measurement, and Vol_r is the volume of water in the respirometric chamber. Volume-specific somatic maintenance costs were then calculated by converting oxygen consumption rates expressed in µmol h^{-1} in J h^{-1} through a conversion factor (Kooijman 2010) and then in J cm^{-3} (van der Veer et al., 2006; Ren and Schiel 2008) (for the calculation of dry weights refer to the end of section 2.4).

2.4 Assimilation efficiency. Assimilation is the final step of food processing and it represents the efficiency with which organic material is absorbed from the ingested food (Kooijman, 2010). The assimilation of food is assumed to be independent of the feeding rate *per se*, but proportional to the ingestion rate (Kooijman, 2010). Assimilation efficiency (AE) was measured through the Conover ratio (1966) $AE = (F - E)/[(1 - E)F]$, where F is the ratio between ash-free dry weight (AFDW) and dry weight (DW) for food, and E is the same ratio for the faeces; this represents the efficiency with which organic material is absorbed from the ingested food material. Here, after oxygen consumption measurement, the same 16 specimens of *M. galloprovincialis* per treatment were collected twice (week 1 and week 4) and placed into separate beakers containing 1L of filtered seawater (specific for each treatment) and a magnetic stirrer bar. In order to allow the mussels to open their valves and start their filtration activity, they were given 15 minutes before the introduction of food with an initial concentration of ~ 15,000 *Isochrysis galbana* cells ml^{-1}. After a period of 2 h mussels were moved to cleaned 1L glass beakers with filtered seawater for a period of 12 h, after that the water contained in each beaker was filtered on pre-ashed and weighted GF/C fibreglass filters. Once filtered, filters were washed with 0.5 M ammonium

formate (purest grade) to remove adventitious salts (~~Widdows & Staff, 2006~~Sarà et al., 2013a), dried in the oven (95°C for 24 h) and then incinerated in a muffle furnace (450°C for 4 h). After each step, the samples were weighted using a balance (Sartorius BL 120S ± 1µg). For the calculation of AE, together with the faeces collected from the mussels, filters containing algal food were dried and incinerated as above. After respirometric measurement and the collection of faeces each animal was killed by gentle freezing and dissected, and the shells were separated from the body tissue ~~in order~~ to calculate the condition index according to Davenport & Chen (1987) (CI = (body weight/shell weight) × 100), and their individual dry weights ~~and~~ to standardize respiration rates~~. to body weights.~~

2.5 Water temperature data. The main forcing driver of shellfish LH inside DEB models is represented by ~~mean~~ seawater temperature (Pouvreau et al., 2006; Kearney et al., 2010; Kooijman, 2010; Sarà et al., 2011; 2013). DEB simulations were run under subtidal conditions (body temperature was expressed by the mean seawater temperature; Montalto et al., 2014) with 4 years-hourly data (Jan 2006 - Dec 2009) of seawater temperature measured about 1 m below the surface at the closest meteo-oceanographic station held in Trieste (LAT 45° 38′ 57.81″; LONG 13° 45′ 28.58″) and Palermo (LAT 38° 07′ 17.08″; LONG 13° 22′ 16.79″). The period of 4 years is consistent with the normal life span of most Mediterranean shellfishes (Sarà et al., 2012; 2013b). Both sites were chosen as they represent two opposite temperature and food conditions for mussel growth in Italy, with Trieste as representative of lower temperature (average 16.98 ± 6.19 °C) and higher food levels (average 1.36 ± 0.37 CHL-a), and Palermo of higher temperatures (average 20.19 ± 4.64 °C) and lower food (average 0.19 ± 0.09 CHL-a). Data are available online from the Italian Institute of Environmental Research (ISPRA) web page (http://www.mareografico.it/).

2.6 CHL-a dataset. ~~Chlorophyll a (~~CHL-a~~)~~ was derived from satellite imageries (µg L^{-1}; http://emis.jrc.ec.europa.eu/)) ~~was~~ and adopted as a reliable food quantifier for suspension feeders (Kearney et al., 2010; Sarà et al., 2011; 2012)~~ and was downloaded from the EMIS website (http://emis.jrc.ec.europa.eu/).~~

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2.7 Model description. The Dynamic Energy Budget (DEB) Theory provides a general framework that allows to describe how physiological mechanisms are driven by temperature and food availability, and influences growth and the reproductive performances in marine organisms (Sousa et al., 2010; Monaco et al., 2014; Jusup et

al., 2017). Following the κ-rule (~~DEB theory;~~ Kooijman, 2010) a fixed energy fraction (κ) is allocated to growth and somatic maintenance, while the remaining fraction (1-κ) is allocated to maturity maintenance plus maturation or reproduction. If the general environmental conditions deviate~~s~~ from common natural patterns (i.e. changes in temperature, food availability etc.) reproduction and growth are consequently affected. According to the DEB theory, a reduction in growth can be caused either by reduced food assimilation ($\dot{p}_A$), enhanced maintenance costs $[\dot{p}_M]$~~($\dot{p}_M$)~~, or enhanced growth costs ($\dot{p}_G$). Using this approach, and through the DEB parameters ~~derived from Sarà et al. (2012)~~reported in Table 1, except for the variation in the maintenance costs $[\dot{p}_M]$~~($\dot{p}_M$)~~ and in the assimilation efficiency of food (AE) which were experimentally estimated throughout this study, we performed simulations using ~~the~~a s~~S~~tandard version of the DEB model (Nisbet et al., 2010) aimed at investigating the potential variations in growth and fecundity of our model species. To run the DEB simulations, local thermal series of selected sites were used together with satellite CHL-a concentrations, obtaining a first model with environmental conditions. A second model was run with the $[\dot{p}_M]$~~$\dot{p}_M$~~ calculated from the oxygen measurements on specimens of *M. galloprovincialis* from Tanks 3-4 (pH 7.5) simulating a chronic hypercapnia condition for the full cycle (4 years) and the relative estimated AE. Subsequently, further models were run by simulating one random hypoxia event (duration = 30 days) for each of the four years of the cycle, then simulating two yearly events, and so on up to six ~~monthly~~ hypoxia events. The starting month of each event was randomly chosen for every year with the use of a table of random digits. The $[\dot{p}_M]$~~$\dot{p}_M$~~ calculated from the oxygen consumption rate measurements on specimens from Tanks 7-8 (pH 7.5 and hypoxia) was used in substitution to $[\dot{p}_M]$~~$\dot{p}_M$~~ from pH 7.5 tanks 3-4, coupled with the relative estimated AE, when simulating both stressors. Simulations were performed using the R routine for Standard DEB model developed by M. Kearney (2012), and further modified (for use in bivalve modelling) by Sarà et al. (2013). Outputs of the DEB models ~~(Sarà et al., 2014)~~ were: the maximum theoretical total length of shellfish (TL), the maximum total weight (TW), the total number of eggs (TRO) produced during a life-span of 4 years, the total number of reproductive events (RE) and the time needed to reach gonadic maturity (TM) for each treatment.

2.7.1 Model limitation. DEB models are particularly useful to quantitatively assess the effects of multiple stressors on LH-traits in an integrated manner, leading to test the hypothesis on how OA may affect the maintenance costs of living organisms (Jager et al., 2016). Maintenance costs, as defined by Dynamic Energy Budget Theory (Kooijman, 2010), represent the energy requirement of an organism to survive, excluding investments in growth, reproduction and development. The volume-specific somatic maintenance costs

parameter [$\dot{p}_M$] within the standard DEB model has been up to date estimated only by indirect approaches through changes in energy content by starvation over time (van der Meer, 2006) or measurements of respiration rate of starved organisms (van der Veer et al., 2006; Ren and Schiel 2008). The idea of quantitatively assess the effect of a stressor including it as a modification of a specific parameter was first introduced by Jager et al. (2016) with the *stress factor* "s" applied to assimilation, maintenance and cost of growth. Thus, after estimating the effect induced by a treatment on the oxygen consumption, in our case expressed as percentage variation, we summed/subtracted the energetic amount due to the effect of a stressor to the species-specific [ṗM] parameter of *M. galloprovincialis* (Sarà et al. 2012) then we run our models. Previous proposed approaches, taking into account starvation for [$\dot{p}_M$] estimation, wouldn't be realistically applicable for testing and quantifying the effect of a stressor on the energy budget, without adding a further stressor. Jager et al. (2016) was therefore the first to adopt this concept, although using a simplified DEB model (DEBkiss; Jager et al. 2013) that did not involve the concepts of reserve and maturity that play a central role in DEB theory. Although this may not be considered a reliable measure of maintenance costs but a simpler proxy of metabolic effect, negligible costs for growth and gonadic development stand on the assumption of constant protein turnover throughout the experimental range (Hawkins et al. 1989).

2.8 Effects on shell: mechanical strength and SEM pictures. The functional impact of exposure to pH and to validate the pH effect on morphological structure of valves, was tested on mussels exposed to the two nominal pHs for 4 weeks. Twice (week 1 and week 4), 16 mussels for each treatment were collected and dissected, and both valves were cleaned and dried with absorbent paper. The left valve was then sliced transversely using a circular saw (Dremel® 300 series) to section the whole length of the shell. Age was estimated using the analysis of shell rings proposed by Peharda et al. (2011) by counting the number of rings with the use of a stereo microscope (Leica EZ4). The right valves were instead evaluated for their ~~mechanical~~breaking properties ~~at the Department of Mechanical Engineering. Experimental~~ through crushing tests~~, in order to estimate the shell's~~ (maximum breaking load ~~(~~in N)~~ as a further validation step, were realised with~~ as previously done in Martinez et al. (2018)~~a home-made press previously calibrated by an Instron 3367 machine controlled by the Bluehill 2.0 software~~. The effect~~s~~ of low pH exposure ~~were~~ was also documented by the use of a scanning electron microscope (SEM; Zeiss LEO 440) that led to a thorough investigation on the integrity of the mussels' external protein layer (*periostracum*) and on the underlying mineral layer, rich in calcite and aragonite.

2.9 Statistical analysis. The assumption of normal distribution has been tested through the Anderson–Darling test using Past® software. In order to test for significant differences in respiration rate and the assimilation efficiency, ANOVAs were performed using Treatment (CTRL, Tr1, Tr2, Tr3) and Time (Week 1 and Week 4) as fixed factors, with respectively four and two levels. In order to test for significant differences in behavioural categories, ANOVAs were performed using Treatment (CTRL, Tr1, Tr2, Tr3) as fixed factors, while Breaking load was tested with Treatment (CTRL, Tr1, Tr2, Tr3) and Time (Week 1 and Week 4) as fixed factors. When significant differences were detected, the Student-Newman-Keuls (SNK) post-hoc pair wise comparison of means was used (Underwood, 1997). Cochran's test was used prior to ANOVA to test the assumption of homogeneity of variance (Underwood, 1997). When no homogeneous variances were rendered with any type of transformation, the significance level was set at 0.01 instead of 0.05, as ANOVA can withstand variance heterogeneity, particularly in large balanced experiments, thereby reducing the possibility of a Type I error (Underwood, 1997).

3 Results

~~**3.1 Water chemistry.** Experimental target pH values were constantly maintained at significantly different levels in all tanks (normal pH and 7.5 pH treatments; Table 2; ANOVA, $p < 0.01$; SNK test: CTRL = Tr2, Tr1 = Tr3). Oxygen pO_2 was also maintained at significantly different levels between normoxia (7.3 ± 0.02 mg/l) and hypoxia (2.4 ± 0.02 mg/l) treatments (Table 2; ANOVA, $p < 0.01$; SNK test: CTRL = Tr1, Tr2 = Tr3). This translated into significant different pCO_2 levels in all treatments (Table 2; ANOVA, $p < 0.01$), and in different $CO_3^{=}$, ΩCa and ΩAr levels in all tanks (Table 2; ANOVA, $p < 0.01$) except between Tr1 and Tr3 (SNK test: Tr1 = Tr3).~~

3.~~2~~ 1 Valve gaping. During behavioural observations on *M. galloprovincialis*, specimens showed a significant difference in the behavioural categories, showing respectively ~~64.5 ± 5.6 % (CTRL), 57.3 ± 0.2 (Tr1), 24.5 ± 0.3 (Tr2) and 12.7 ± 0.2 % (Tr3) of opened valves (Fig. 1; Table 3, ANOVA, $p < 0.001$). The percentage of closed valveswas instead 35.5 ± 5.6 % (CTRL), 42.7 ± 4.8 (Tr1), 75.5 ± 5.7 (Tr2) and 87.3 ± 3.1 % (Tr3) (ANOVA, $p < 0.001$).~~ 33.3 ± 11.2 (CTRL), 50 ±4.5 (Tr1), 80 ± 8.9 (Tr2) and 83.3 ± 6.1 of opened valves (Fig. 1; Table 3, ANOVA, $p < 0.001$). The percentage of closed valves can be easily calculated as 100 – open valves. No significant differences resulted between week 1 and 4 (ANOVA, $p > 0.05$), between CTRL and Tr1 and between Tr2 and Tr3.

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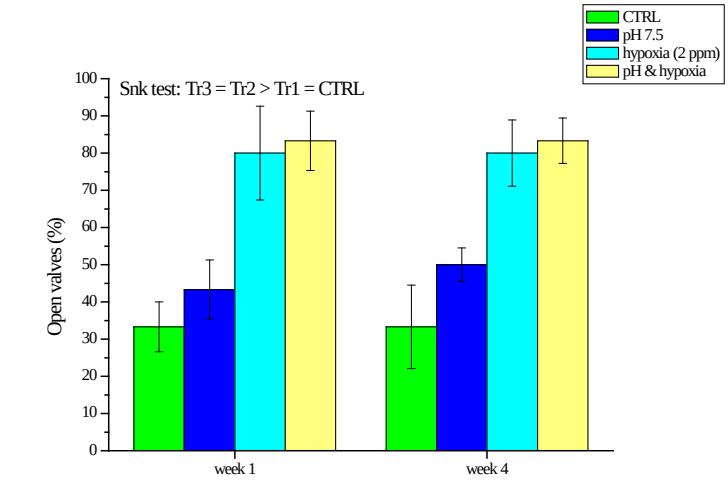

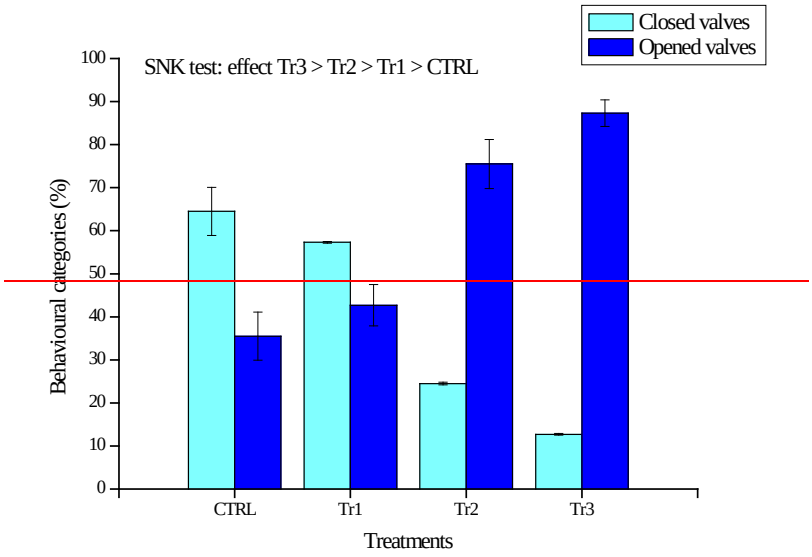

Fig. 1 Behavioural observations (± se) of *Mytilus galloprovincialis* under different treatments of oxygen (normoxia – hypoxia 2ppm) and pH (7.5 – 8.0). ~~The two behavioural categories represented were: closed and opened valves.~~

3.~~3~~ 2 Oxygen consumption. Results showed a significant reduction in the oxygen consumption rate by specimens of *M. galloprovincialis* exposed to treatments (Table 4, ANOVA, $p < 0.01$), although the SNK test revealed no significant differences among the various groups (Fig. 2a). No significant effects were highlighted

for the time factor (Table 4, ANOVA, $p > 0.05$), so in Fig. 2a we reported only results for week 4. The rate of oxygen consumption was reduced by up to 42% in Tr1, to 35% in Tr2, and to 41% in Tr3, causing a decrease in the $[\dot{p}_M]\dot{p}_M$ by up to 29% in Tr1, to 47% in Tr2, and to 49% in Tr3 across the four weeks of exposure.

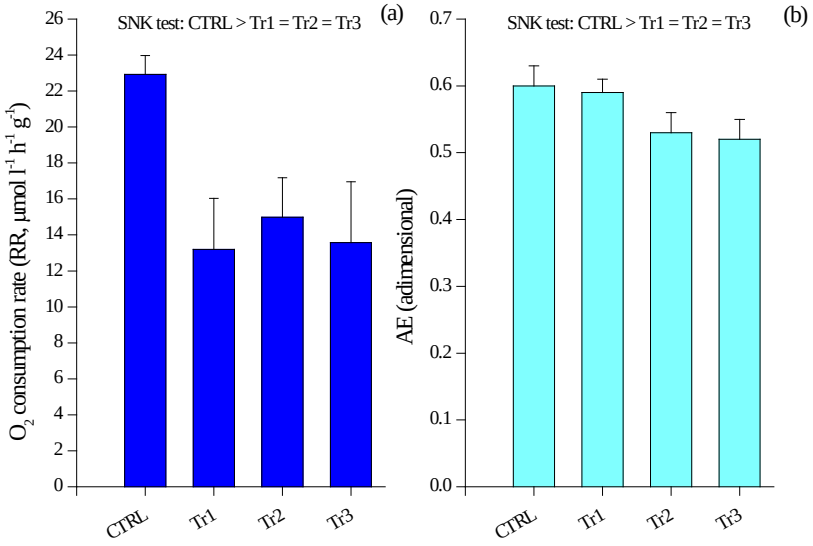

Fig. 2 (a) Oxygen consumption rates (RR) and (b) Assimilation efficiency (AE) ± se of *Mytilus galloprovincialis* under different treatments of oxygen (normoxia – hypoxia 2ppm) and pH (7.5 – 8.0) at week 4. CTRL = control; Tr1= pH 7.5; Tr2 = hypoxia 2ppm; Tr3 = pH 7.5 & hypoxia 2ppm.

3.4 3 Assimilation efficiency. Assimilation efficiency of food (AE) resulted was in significantly affected by treatments (Table 4, ANOVA, $p < 0.001$) after four weeks of exposure. No significant effects were highlighted for the time factor (Table 4, ANOVA, $p > 0.05$), so in Fig. 2b were reported only results for week 4. In particular, AE decreased of 2.4% in Tr1, of 12.4% in Tr2, and of 14.4% in Tr3, although the SNK test revealed no significant differences among the various groupstreatments (Fig. 2b). At the end of the 4 weeks exposure, the BCI resulted comparable (35.16 ± 1.12 %) to the initial values (39 ± 1.9 %).

3.5 4 DEB simulation results. Once $[\dot{p}_M]\dot{p}_M$ and AE were experimentally estimated under each treatment, we introduced obtained those parameters were used values under the different treatments to run DEB models and to obtain the derivedin order to predict the effects In on in terms of LH traits. Thus, we performed DEB simulations

were performed under local thermal conditions (as expressed by the thermal series recorded in Trieste and Palermo; see ~~M&M for details~~Section 2.5) and using satellite CHL-a concentrations (2006-2009) as a proxy of food. Results showed a remarkable effect exerted by hypercapnia and an increasing ~~additive effect~~ hypoxia contribution ~~of hypoxia~~ related to the intensity of disturbance (*i.e.* number ~~of yearly~~ hypoxic events *per* year) on LH traits of *M. galloprovincialis* by the end of 4th year (Table 5). Total length (TL) and total weight (TW) in Trieste and in Palermo were similarly reduced by hypercapnia (Fig. 3), with a progressive addictive effect of hypoxia (Table 5). Total length (TL) resulted unaffected by hypoxia up to a frequency of 2 hypoxia events (f = 0.17; 1/Time), then the Trieste site (representative of eutrophic conditions) reported a~~n~~ larger~~higher~~ effect. On the opposite the total weight (TW) highlighted a larger~~an higher~~ effect of hypoxia on the oligotrophic site (Palermo) (Fig. 4). The total number of eggs produced (TRO) and the total number of reproductive events (RE) in Trieste were strongly reduced by hypercapnia (Fig. 3), with the same progressive ~~addictive effect from~~ hypoxia contribution (Table 5). Maturation time (TM) was affected both in Trieste and Palermo by hypercapnia, with the same hypoxia contribution previously shown. Palermo showed no reproductive events in the DEB simulations.

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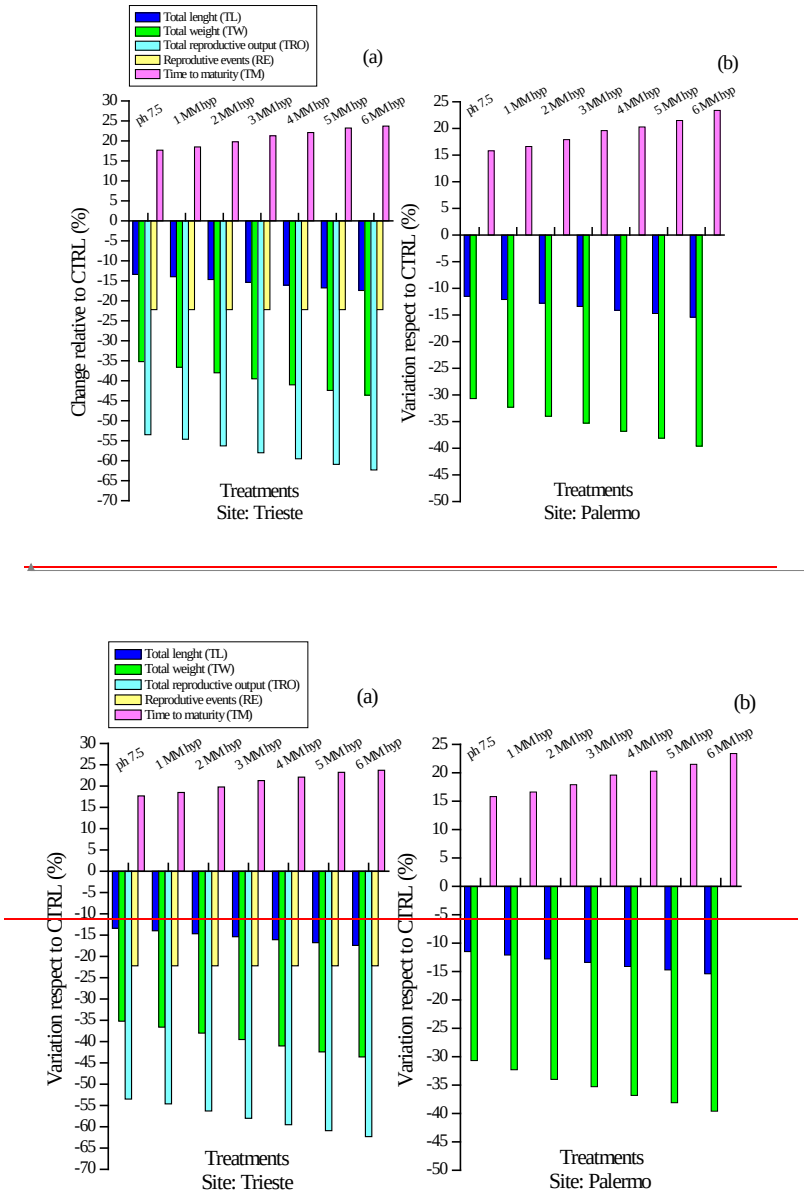

Fig. 3 Results from DEB simulation for (a) Trieste and (b) Palermo sites, percentage variation of DEB outputs respect to CTRL. TL and TW were reduced by 13.4% and 35.2% in Trieste, and by 11.5% and 30.7% in Palermo by hypercapnia, with a progressive addictive hypoxia effect up to 8.9%. TRO and RE were reduced by 53.4% and 66.7% in Trieste by hypercapnia, with a progressive addictive hypoxia effect up to 8.8%. TM increased by 17.8% in Trieste and by 15.7% in Palermo with a similar hypoxia effect (up to 7.6%).

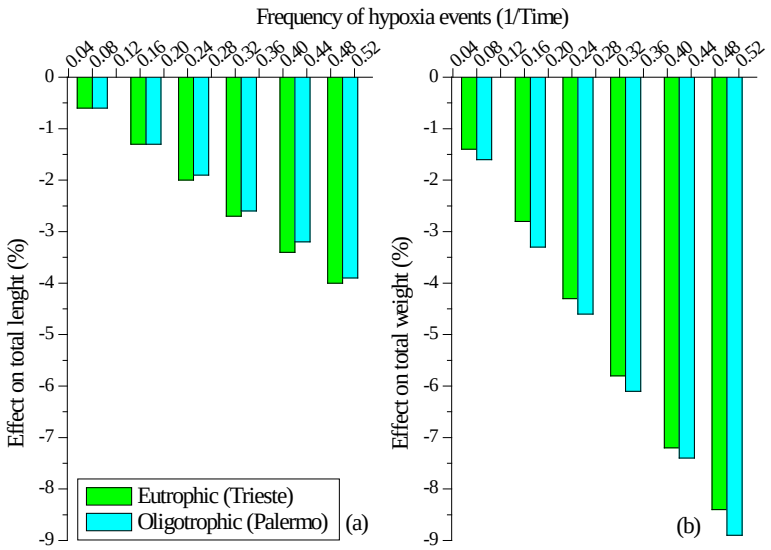

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Fig. 4 Percentage effect of Hypoxia from DEB simulations on TL and TW considering the two different trophic conditions represented by Trieste (a) and Palermo (b).

3.6 Effects on shell. ~~Specimens of *M. galloprovincialis* collected ranged in age from 1 to 3 years with a mean age of 1.8 ± 0.04 years (n = 128). Overall, 97% of individuals were > 2 years old.~~ Results from the breaking load experiment revealed a significant effect of pH (58.8 ± 5 N) and of combined stressors on the breaking load (50 ± 2.7 N), compared to hypoxic (64.4 ± 3.7 N) and CTRL specimens (77.2 ± 2.2 N) (Fig. ~~4~~5) (Table 3, ANOVA, $p < 0.001$). In addition, the effect was stronger at week 4 than after one week of exposure (Table 3, ANOVA, $p < 0.01$). Deeper investigations through scanning electron microscopy validated an effect by showing an increasing erosion of the shell after exposure to CO_2-induced acidification. The external dissolution pattern usually started from the umbonal region and progressed toward the margin of the shell, usually associated with some degree of damage to the *periostracum*. The damage was present at differing extensions in all specimens exposed to treatments, except in the control mussels (Fig. ~~5~~ 6 b, c, d). The alteration of the underlying carbonate layer was instead visible only in Tr1 and Tr3, with details in Fig. ~~6~~ 7 (b, d). This kind of alteration was never ~~recorded~~ observed under control pH (Fig. 4a).

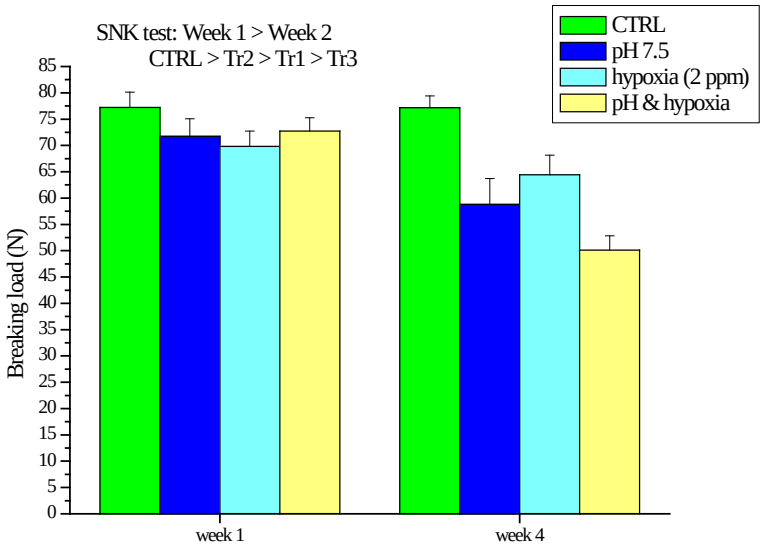

Fig. ~~4~~ 5 Breaking load of valves (in Newton, N ± se) exposed to different treatments of oxygen (normoxia – hypoxia 2ppm) and pH (7.5 – 8.0) at week 1 and 4.

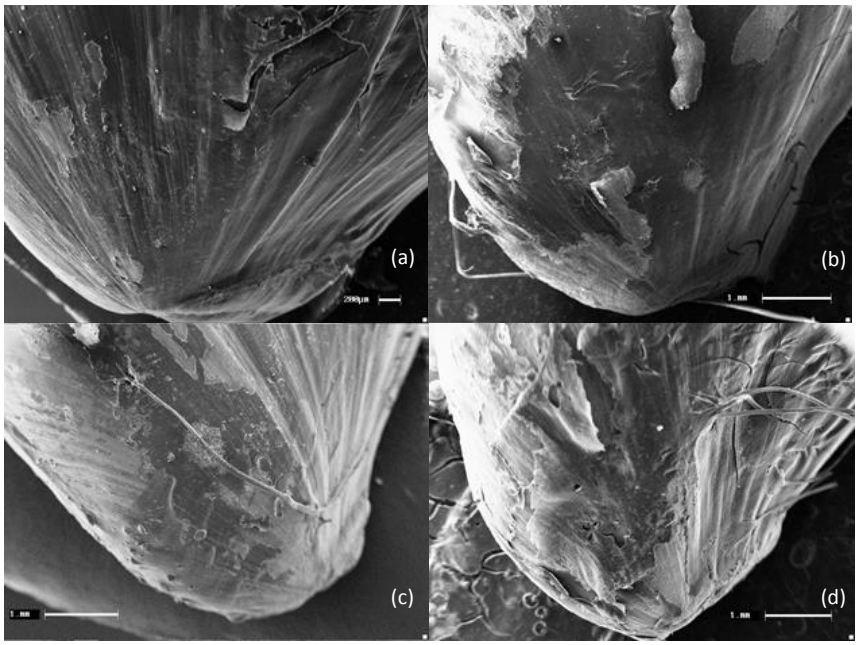

Fig. ~~5~~ 6 SEM pictures of different shells exposed to (a) control condition (CTRL); (b) pH 7.5 and normoxia condition (Tr1); (c) normal pH and hypoxia condition (Tr2); (d) both pH 7.5 and hypoxia conditions (Tr3).

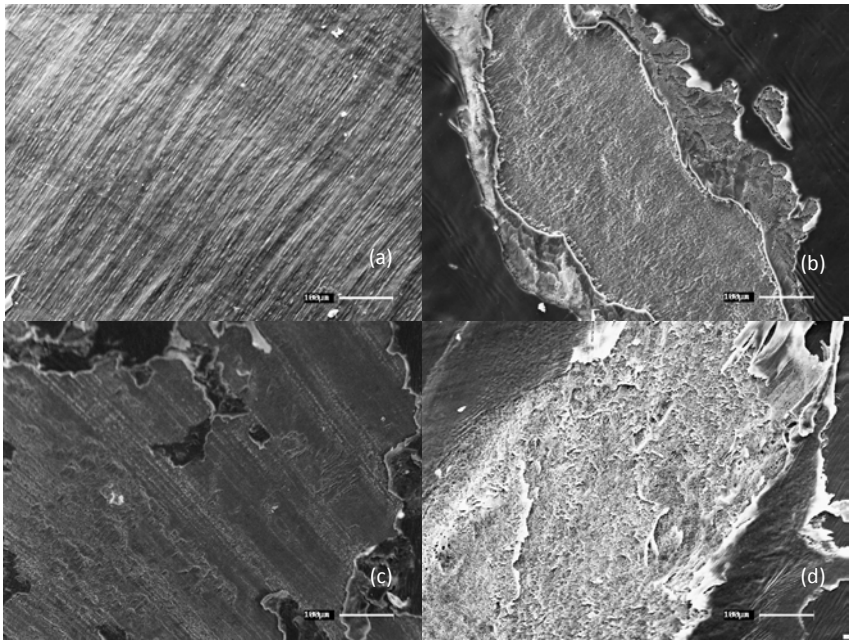

Fig. ~~6~~ 7 Details of different shells exposed to (a) control condition (CTRL); (b) pH 7.5 and normoxia condition (Tr1); (c) normal pH and hypoxia condition (Tr2); (d) both pH 7.5 and hypoxia conditions (Tr3).

4 Discussion

Marine organisms, and in particular intertidal species (Montecinos et al., 2009), have been ~~formally~~ recognized as being equipped with well-developed and conserved ~~compensatory~~ mechanisms to ~~contrast~~ compensate ocean acidification such as (i) passive buffering of intra- and extracellular fluids; (ii) transport and exchange of relevant ions; (iii) transport of CO_2 in the blood in those species that have respiratory pigments; (iv) metabolic suppression to wait out periods of elevated CO_2 (e.g. Lindinger et al., 1984; Cameron, 1989; Walsh and Milligan, 1989; Hand, 1991; Heisler, 1993; Guppy and Withers, 1999; Pörtner et al., 2004). Several authors recorded suppressed~~ion~~ of feeding activity and growth, depressed metabolism, increased N excretion and loss of tissue weight for marine bivalves exposed to reduced seawater pH (Bamber, 1990; Michaelidis et al., 2005; Berge et al., 2006; Gazeau et al., 2010). Bivalves are in fact capable of maintaining a constant internal pH by decreasing their metabolic rates and/or dissolving their shell; the shell acting then as a source of CO_3^{2-} (Bamber, 1990; Michaelidis et al., 2005; Berge et al., 2006) counterbalancing the ~~crossing~~ effect ~~due to lowering~~of dissolved CO_2 crossing~~through~~ biological membranes ~~(Fabry et al., 2008)~~. Compensation of low pH, associated with anthropogenic increases in seawater pCO_2 (Fabry et al., 2008), through adjustments in ionic composition appears to be a trade-off that is not likely sustainable ~~on~~ over longer time-scales~~, such as that associated with anthropogenic increases in seawater~~

~~pCO_2 (Fabry et al., 2008)~~ From our behavioural observations mussels exposed to low pH resulted in a higher, even if not significant, percentage of opened valves respect to CTRL individuals, with the highest significant difference relative to hypoxia exposition. The effect of low pH on the adductor muscle of bivalves has been already documented by Pynnönen & Huebner (1995) and the same effect has been reported after exposition to hypoxia (Sheldon & Walker 1989). In agreement with current literature showing deleterious effects of CO_2-induced acidification on a wide range of invertebrates (Barnhart & McMahon, 1988; Barnhart, 1989; Rees & Hand, 1990)~~,~~ and similarly to other studies by *M. galloprovincialis* (Gestoso et al., 2016; Michaelidis et al., 2005), our results showed how hypercapnia (pH reduced by 0.6 units, relative to the natural pH of the lower Tyrrhenian waters) was able to induce a decline in metabolic rates of mussels. This kind of decline has already been noticed by other authors as an adaptive strategy for survival under transiently stressful conditions (Michaelidis et al., 2005). According to Pörtner et al. (2004), metabolic reduction due to hypercapnia could be a result of acid-base disturbances and therefore be similar to the response of intertidal individuals to anaerobic conditions. Direct effects of hypoxemia have been further proven to cause fatal decrements in an organism's performance in growth, reproduction, feeding, immunity and behaviour (*sensu* Pörtner & Farrell, 2008). ~~Synergistic~~ Combined effects by ~~s~~stressors ~~like~~ such as ocean acidification and hypoxia narrow ~~are capable of narrowing~~ the thermal window of functioning according to species-specific sensitivities, modulating biogeographical distributions~~es~~, coexistence ranges, community shifts and in general ecological~~other~~ interactions (Pörtner & Farrell, 2008). The mussel *Mytilus edulis* has been proven able to compensate both short- and long-term exposure to hypercapnia by dissolution of ~~its~~ shells (Lindinger et al., 1984; Michaelidis et al., 2005), resulting in reduced growth and metabolism. A similar mechanism of release of inorganic molecules into the pallial cavity (as $CaCO_3$ from valves) has been documented during periods of anaerobic metabolism, to maintain the acid-base balance (Chaparro et al., 2009), determining further physiological and energetic cost such as decreased growth, respiration rate and protein synthesis (Pörtner et al., 2005). During periods of environmental oxygen limitation, many organisms are able to suppress ATP demand, shut down expensive processes, such as protein synthesis (Hand, 1991), but at the same time limiting growth and the reproductive potential. Although suppression of metabolism under short-term experimental conditions is a ~~"~~sub-lethal~~"~~ reversible process, reductions in growth and reproductive output will effectively diminish the survival of the species on longer time-scales (Fabry et al., 2008). The contemporary occurrence in our simulations, of monthly hypoxia events, revealed a growing ~~additive~~ contribution to what was already elicited by hypercapnia alone on growth and reproduction. Current literature has not currently explored the combined effects of multiple stressors on long-term experiments by modulating the intensity and duration of disturbance. This would probably

translate as a very complex experimental set-up which would be hardly practicable, especially on long-term scales. On the other hand, mechanistic models offer a more ~~sustainable and reliable~~practical alternative to long-term, in-field research when studying the effects of multiple-stressors~~-~~, with the advantage of testing, at the same time, the magnitude and the duration of disturbance on LH ~~-~~traits of a model species. Our results highlighted the general hypoxia growing effect following the increasing duration of disturbance, with a particular focus in Trieste on TW and TRO, while in Palermo on TW and TM (~~Table 5~~Fig. 3). A further important peculiarity of the ~~DEB~~ mechanistic modelling ~~simulations~~ deals with the possibility to spatially contextualise the effects of single and multiple stressors on selected outputs by integrating local thermal conditions and food concentrations (Sarà et al. 2018c). Comparing the effect of hypoxia across frequencies (Fig. 4), total length (TL) resulted unaffected up to a frequency of 2 hypoxia events in both sites, then the highest effect was recorded in the eutrophic site (Trieste). Trieste, between the two chosen sites, had ~~represented also the one with~~ the lowest temperature. On the contrary a ~~lower~~ smaller effect of hypoxia was detected ~~resulted considering~~ on ~~the total weight (~~TW~~)~~ in ~~the eutrophic site~~Trieste, suggesting ~~some~~ a sort of food compensation capacity on the effect of environmental stressor (Mackenzie et al., 2014). ~~In particular~~Also the DEB model easily allowed the estimation of the fecundity potential of ~~cultivated and natural~~ organisms, that is often omitted in other ecological studies, but that represents a crucial quantity for resource (e.g. aquaculture) (Sarà et al. 2018c) and conservation purposes. To verify impacts on shellfish fecundity, we contextualised our simulation by introducing Trieste hourly temperature series after those of Palermo, with the respective local actual CHL-a concentrations, as long as in the first site no reproductive events came out from our simulations, probably due to food limitations and temperature threshold. This is reflected by natural populations in Palermo colonising ~~being represented only in~~ only substrates in ~~highly~~ trophic-enriched ~~areas~~sites. A combined effect of the simultaneous stressors, such as those considered across this study, has proven in the present study~~, through our experimental and mechanistic integrated approach,~~ to affect the organism's performance in growth, reproduction and behaviour. Our results highlighted an effect of pH alone and when combined with hypoxia on the breaking load of shells of our experimental mussels. Through a similar approach, Martinez et al. (2018) showed that temperature was a primary factor driving shell's fragility along a latitudinal gradient. Present findings corroborates that idea that fragility can be affected by both stressors through a combined effect. Multiple stressors ~~Those specific and synergic effects of each stressor seem capable~~can narrow, especially ~~at~~ when organisms are on the edge of their thermal tolerance range~~extreme temperatures~~, ~~of narrowing~~ the thermal window~~s~~ and this has a potential for generating repercussions on ~~, modulating~~ biogeographical distribution, coexistence ranges, community shifts, food webs and species interactions (*sensu* Pörtner & Farrell,

2008). Moreover, an appropriate knowledge of species' biological traits~~,~~ and a mechanistic understanding of the effect of each stressor, reached through an FT-based approach, will allow the translation of the effects of environmental change into realistic management measures taking into account the optimisation of the species' biological traits (Sarà et al. 2018a,b).

~~6 Conclusions~~

Additional research is still required to improve our knowledge of organismal response to multiple stressors, in particular, of many marine ectotherms with indeterminate growth amongst invertebrates (e.g. crustaceans, molluscs). Nevertheless, modelling the growth and reproductive potential (and failure) of species vulnerable to those stressors with predictive tools, such as bioenergetic models is a useful approach for management and protection purposes, but also for shellfish culture in general.

Authors' contributions

Both authors contributed to all phases of this ms.~~AG and GS conceived the idea and led the writing. AG carried out all experiments in mesocosms, performed modelling work and analysed data. GS provided lab facilities and research funds. All authors contributed critically to the drafts and gave final approval for publication.~~

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Tables

Table 1. DEB parameters for *Mytilus galloprovincialis* (1 = Kooijman, 2010; 2= van der Veer et al., 2006; 3 = Sarà et al., 2011; 4 = Thomas et al., 2006; 5 = Schneider, 2008); Lb, Lp, Ls = length at birth, puberty and seeding respectively; dVw = specific density to convert weight into volume; *f* = functional response type II f = X/(XK + X); µx = chemical potential to convert moles into food energy; SMI is the somatic mass index of both starved and well-fed animals, expressed as somatic ash free dry mass (AFDM, mg); X_K = saturation coefficient expressed as a concentration of chlorophyll a (µg CHL-a l^{-1}), where the ingestion rate is half of the maximum.

Symbol	Description	Formulation	Units	*Mytilus galloprovincialis*	
				Value	Ref
V_b	Structural volume at birth	$V_b = (L_b \times \delta_m)^3$	cm^3	0.0000013	1
V_s	Structural volume at seeding	$V_s = (L_s \times \delta_m)^3$	cm^3	-	-
V_p	Structural volume at puberty	$V_p = (L_p \times \delta_m)^3$	cm^3	0.06	2
δ_m	Shape coefficient	$\delta_m = (Ww \times d_{Vw}^{-1}) \times L^{-1}$	-	0.2254	3
$\{J_{Xm}\}$	Maximum surface area-specific ingestion rate	$\{J_{Xm}\} = J_X/(f \times V^{\frac{2}{3}})$	$J\ cm^{-2}h^{-1}$	8.2	4
ae	Assimilation efficiency	$ae = (\mu_x \times J_X)/p_A$	-	0.88	3
X_K	Saturation coefficient	-	$\mu g\ l^{-1}$	2.1	3
$[E_G]$	Volume-specific cost of growth	$[E_G] = SMI_{starved} \times 23 \times (\delta^3_m)^{-1}$	$J\ cm^3$	5,993	5
$[E_m]$	Maximum storage density	$[E_m] = (SMI_{fed} - SMI_{starved}) \times 23 \times (\delta^3_m)^{-1}$	$J\ cm^3$	2,190	2
$[p_M]$	Volume-specific maintenance cost	$[p_M] = p_M/V$	$J\ cm^{-3}\ h^{-1}$	1	2
κ	Fraction of utilized energy spent on maintenance and growth	-	-	0.7	2
K_R	Fraction of reproductive energy	-	-	0.8	3
T_A	Arrhenius temperature	$T_A = \ln\lvert K_{(T_0)}/K_{(T_1)}\rvert x \frac{(T_1 x T_0)}{(T_0 - T_1)}$	°K	7,022	2
T_L	Lower boundary of tolerance range		°K	275	2
T_H	Upper boundary of tolerance range		°K	296	2
T_{AL}	Rate of decrease at lower boundary		°K	45,430	2
T_{AH}	Rate of decrease at upper Boundary		°K	31,376	2

Table ~~1~~2. Seawater carbonate chemistry parameters (mean ± se). Seawater pH on the NBS scale (pH_{NBS}), temperature (T; °C), and salinity were used to calculate CO_2 partial pressure (pCO_2; µatm) as well as aragonite and calcite saturation states (respectively Ωar and Ωca), for a total alkalinity of 2500 mmol kg^{-1}.

	Measured	Calculated

	Temperature (°C)	pH_{NBS}	O_2 mg/l	Salinity (PSU)	pCO_2 (µatm)	CO_3^-	Ωca	Ωar
CTRL	20.77 ± 0.01	8.01±0.001	7.29±0.02	37.18±0.11	624.31±4.9	167.93±0.95	3.95±0.02	2.58±0.01
Tr1	20.77 ± 0.01	7.53±0.002	7.30±0.02	37.12±0.05	2151.17±22.02	62.05±0.73	1.46±0.02	0.95±0.01
Tr2	20.77 ± 0.01	8.01±0.001	2.44±0.02	37.07±0.04	729.88±18.24	152.53±1.51	3.59±0.04	2.34±0.02
Tr3	20.77 ± 0.01	7.53±0.002	2.44±0.02	37.21±0.17	2238.83±20.72	59.59±0.42	1.40±0.01	0.91±0.01

Table 2. ANOVA on seawater chemistry parameters. Comparison between CTRL (normal pH) and TREAT (low pH and hypoxia) (* = p < 0.05; ** = p < 0.01; *** = p < 0.001; ns = not significant).

		pH_{NBS}			pO_2		
	df	MS	F	p	MS	F	p
TREAT	3	10.73	41450.84	**	1083.21	18798.36	**
Residuals	548	0.0003			0.06		
Cochran's Snk				*			*

		pCO_2			CO_3^-		
	df	MS	F	p	MS	F	p
TREAT	3	1.06e08	2426.84	**	460157.7	3433.17	**
Residuals	548	43851.09			134.03		
Cochran's Snk				*			*

		Ωca			Ωar		
	df	MS	F	p	MS	F	p
TREAT	3	254.09	3432.44	**	108.26	3426.14	**
Residuals	548	0.07			0.03		
Cochran's Snk				*			*

Table 3 ANOVA table of results. Effect on valve gape and breaking load of *Mytilus galloprovincialis* (* = p < 0.05; ** = p < 0.01; *** = p < 0.001; ns = not significant).

Source	df	Valve gape			Source	df	Breaking load		
		MS	F	P			MS	F	P

Treatment (Tr)	3	~~34.53~~17.41	~~26.03~~15.60	***	Treatment (Tr)	3	3838.12	15.18	***
Time (Ti)~~Residuals~~	84	~~1.33~~2.08	1.87	ns	Time (Ti)	1	777.19	9.22	**
Tr x Ti~~Cochran's C~~		0.3056	0.27	ns	Tr x Ti	3	132.92	1.58	~~Ns~~ns
Residuals	40				Residuals	56			
Cochran's C				ns	Cochran's C				ns

Table 4 ANOVA table of results. Respiration rate (RR) and assimilation efficiency (AE) of *Mytilus galloprovincialis* (* = p < 0.05; ** = p < 0.01; *** = p < 0.001; ns = not significant).

Source	df	RR st			AE		
		MS	F	P	MS	F	P
Treatment (Tr)	3	312.9183	6.95	***	0.2783	12.21	***
Time (Ti)	1	205.1325	4.56	*	0.0424	1.86	ns
Tr x Ti	3	40.7752	0.91	ns	0.0198	0.87	ns
Residuals	120	45.0271			0.0228		
Cochran's C				*			ns

Table 5 DEB simulation outputs. Percentage variation of treatments from CTRL: Total length (TL), Total weight (WW), Total reproductive output (TRO), Total reproductive events (RE), Time to maturity (TM).

DEB outputs (CTRL) after 4 years								
Site	Stressor	Hypoxia events (days)	Frequency (1/Time)	TL (cm)	WW (g)	TRO (n° egg)	RE	TM (days)
Trieste	CTRL	0	0	9.55	11.19	6.74e637889	9	232
Palermo	CTRL	0	0	3.08	0.31	0	0	739

~~DEB outputs: percentage variation respect to CTRL after 4 years~~							
~~Site~~	~~Stressor~~	~~Frequency~~	~~TL (%)~~	~~WW (%)~~	~~TRO (%)~~	~~RE (%)~~	~~TM (%)~~
~~Trieste~~	~~pH 7.5~~	~~baseline~~	~~-13.44~~	~~-35.20~~	~~-53.49~~	~~-22.22~~	~~18~~
~~Trieste~~	~~pH+hypoxia~~	~~1 month~~	~~-14.04~~	~~-36.58~~	~~-54.61~~	~~-22.22~~	~~18~~
~~Trieste~~	~~pH+hypoxia~~	~~2 month~~	~~-14.71~~	~~-38.04~~	~~-56.34~~	~~-22.22~~	~~20~~
~~Trieste~~	~~pH+hypoxia~~	~~3 month~~	~~-15.40~~	~~-39.52~~	~~-58.01~~	~~-22.22~~	~~21~~
~~Trieste~~	~~pH+hypoxia~~	~~4 month~~	~~-16.09~~	~~-40.97~~	~~-59.52~~	~~-22.22~~	~~22~~
~~Trieste~~	~~pH+hypoxia~~	~~5 month~~	~~-16.77~~	~~-42.37~~	~~-60.95~~	~~-22.22~~	~~23~~
~~Trieste~~	~~pH+hypoxia~~	~~6 month~~	~~-17.38~~	~~-43.62~~	~~-62.26~~	~~-22.22~~	~~24~~
~~Palermo~~	~~pH 7.5~~	~~baseline~~	~~-11.47~~	~~-30.69~~	~~0~~	~~0~~	~~16~~
~~Palermo~~	~~pH+hypoxia~~	~~1 month~~	~~-12.09~~	~~-32.26~~	~~0~~	~~0~~	~~17~~
~~Palermo~~	~~pH+hypoxia~~	~~2 month~~	~~-12.84~~	~~-33.97~~	~~0~~	~~0~~	~~18~~
~~Palermo~~	~~pH+hypoxia~~	~~3 month~~	~~-13.42~~	~~-35.26~~	~~0~~	~~0~~	~~20~~
~~Palermo~~	~~pH+hypoxia~~	~~4 month~~	~~-14.10~~	~~-36.78~~	~~0~~	~~0~~	~~20~~
~~Palermo~~	~~pH+hypoxia~~	~~5 month~~	~~-14.70~~	~~-38.08~~	~~0~~	~~0~~	~~22~~
~~Palermo~~	~~pH+hypoxia~~	~~6 month~~	~~-15.42~~	~~-39.63~~	~~0~~	~~0~~	~~23~~

Percentage ~~additive~~ contributing effect of Hypoxia							
Trieste	pH+hypoxia	30	0.081~~ month~~	-0.6	-1.4	-1.1	0	0.8
Trieste	pH+hypoxia	60	0.172~~ month~~	-1.3	-2.8	-2.8	0	2.1
Trieste	pH+hypoxia	90	0.253~~ month~~	-2	-4.3	-4.5	0	3.6
Trieste	pH+hypoxia	120	0.334~~ month~~	-2.7	-5.8	-6	0	4.4
Trieste	pH+hypoxia	150	0.425~~ month~~	-3.4	-7.2	-7.4	0	5.5
Trieste	pH+hypoxia	180	0.506~~ month~~	-4	-8.4	-8.8	0	6
Palermo	pH+hypoxia	30	0.081~~ month~~	-0.6	-1.6	0	0	0.8
Palermo	pH+hypoxia	60	0.172~~ month~~	-1.3	-3.3	0	0	2.1
Palermo	pH+hypoxia	90	0.253~~ month~~	-1.9	-4.6	0	0	3.8
Palermo	pH+hypoxia	120	0.334~~ month~~	-2.6	-6.1	0	0	4.5
Palermo	pH+hypoxia	150	0.425~~	-3.2	-7.4	0	0	5.7

			~~month~~					
Palermo	pH+hypoxia	180	0.506 ~~month~~	-3.9	-8.9	0	0	7.6