Science of the Total Environment 782 (2021) 146888

Contents lists available at ScienceDirect

Science of the Total Environment

journal homepage: www.elsevier.com/locate/scitotenv

Combined effect of microplastics and global warming factors on early growth and development of the sea urchin (*Paracentrotus lividus*)

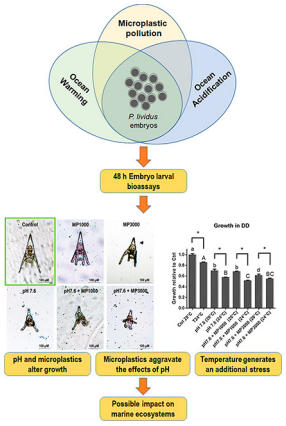
J.I. Bertucci *, J. Bellas

Centro Oceanográfico de Vigo, Instituto Español de Oceanografía, IEO, Subida a Radio Faro, 50, 36390 Vigo, Pontevedra, Spain

HIGHLIGHTS

- This work focusses on the effect of a multi-stressor environment in sea urchin.
- Embryo-larval bioassays were used to determine growth and morphometric parameters.
- A lower water pH (7.6) reduced larval growth and caused deformities.
- Microplastics aggravate the effect of water acidification in sea urchin larvae.
- High temperatures caused an additional stress and reduced larvae stomach volume.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 10 February 2021
Received in revised form 22 March 2021
Accepted 28 March 2021
Available online 1 April 2021

Editor: Henner Hollert

Keywords:
Bioassays
Climate change
Microplastics
Ocean acidification
Ocean warming
Sea urchin

ABSTRACT

The aim of this work was to estimate the potential risk of the combined effect of global change factors (acidification, temperature increase) and microplastic (MP) pollution on the growth and development of the sea urchin *P. lividus*. Embryo-larval bioassays were conducted to determine growth and morphology after 48 h of incubation with MP (1000 and 3000 particles/mL); with filtered sea water at pH = 7.6; and with their combinations. A second experiment was conducted to study the effect of pH and MP in combination with a temperature increase of 4 °C compared to control (20 °C). We found that the inhibition of growth in embryos reared at pH = 7.6 was around 75%. Larvae incubated at 3000 MP particles/mL showed a 20% decrease in growth compared to controls. The exposure to MP also induced an increase in the postoral arm separation or rounded vertices. The combined exposure to a pH 7.6 and MP caused a significant decrease of larval growth compared to control, to MP and to pH 7.6 treatments. Morphological alterations were observed in these treatments, including the development of only two arms. Increasing the temperature resulted in an increased growth in control, in pH 7.6 and pH 7.6 + MP3000 treatments, but the relative stomach volume decreased. However, when growth parameters were expressed per Degree-Days the lower growth provoked by the thermal stress was evidenced in all treatments. In this work we demonstrated that MP could aggravate the effect of a decreased pH and that an increase in water temperature generated an additional stress on *P. lividus* larvae, manifested in a lower growth and an altered development. Therefore, the combined stress caused by ocean warming, ocean acidification, and microplastic pollution, could threaten sea urchin populations leading to a potential impact on coastal ecosystems.

* Corresponding author.
E-mail address: jignacio.bertucci@ieo.es (J.I. Bertucci).

https://doi.org/10.1016/j.scitotenv.2021.146888
0048-9697/

1. Introduction

Anthropogenic activities are impacting the oceans, posing a serious threat not only to marine ecosystems, but also to human health. Among these activities, plastic pollution is currently one of the most important issue to address. Plastics make up ≈ 80% of marine litter and have become the fastest growing segment of the municipal waste stream since 1950. Its global production has raised from 1.7 to 359 million tons from the 1950s to 2018 (PlasticsEurope, 2015). This represents a high risk to marine ecosystems; in fact, lethal entanglement and plastic ingestion by sea animals have recently increased by 40% (Harding, 2016). Plastic particles smaller than 5 mm, so-called microplastics (MPs), can be specifically manufactured to be of small size (primary MPs) or formed from the breakdown of larger plastic items (secondary MPs) (Cole et al., 2011). MP are easily transported over long distance by wind and water-currents, being distributed widely across coastal areas (Ryan et al., 2009). Due to their ubiquity and persistence, MP pose a threat to marine wildlife as their small size makes them available to a wide range of organisms (Andrady, 2011; Beiras et al., 2018). Thus, ingestion of MP by marine organisms has been shown to cause stress, false feeling of satiation, reproductive complications, and reduced growth rate (Cole et al., 2015; Lee et al., 2013). In particular, adverse effects of MP in marine invertebrates such as sea urchins include a decreased fertilization rate, developmental alterations such as embryonic and larval abnormalities, and several cytogenetic and genotoxic effects (Trifuoggi et al., 2019).

Much less studied has been the interaction between MP and other environmental stressors. MP pollution can be aggravated by the effects derived from anthropogenic climate change, such as the increase in water ocean temperature (ocean warming) and their acidification (ocean acidification). Current models (FAO and IPCC) predict a temperature rise in the ocean surface and in coastal areas of 1.8–4 °C by the end of this century, and a decrease in the ocean pH due to the acidification caused by the increase in atmospheric CO_2 of 0.2–0.5 units in the next 60 years (FAO, 2019; Krinner et al., 2013). Climate change-derived phenomena have a substantial impact on ecosystem functioning and food-web dynamics (Perry et al., 2019; Gao et al., 2020), provoking additional stress to sea life besides that caused by other stressors such as MPs. Moreover, changes in water pH and/or temperature can alter the physicochemical properties of contaminants potentially increasing their toxicity (Alava et al., 2017). Water acidification and water temperature rise have been proved to cause calcification-, reproductive-, and food intake-related alterations in marine invertebrates (Dworjanyn and Byrne, 2018; Poloczanska et al., 2016). In general, acidification has a stunting effect on sea urchin growth, manifested as smaller larval and adult skeletons. For instance, larvae of the sea urchin *Arbacia lixula* grown at low pH exhibited reduced arm length (Foo et al., 2020). Although it was demonstrated that under near- and far-future ocean warming and acidification scenarios individuals of *Paracentrotus lividus* fully balanced their acid-base status, their skeletal growth was halved after 60 days (Rengifo et al., 2019). Thus, it is evident that the study of the effects of multi-stressor scenarios is crucial to have a realistic scheme of the impact that human activities are causing to aquatic life.

As it is impossible to test and study the impact of a stressor in every component of a particular ecosystem, a good approach is to study its effects on an ecologically relevant model species. In this regard, sea urchins are ecologically important grazers that have shown to mediate transitions in temperate kelp (Castro et al., 2020; Ling et al., 2018) and tropical coral ecosystems (Castro et al., 2020). Moreover, sea urchins have calcareous structures and calcify in their planktonic and benthic life stages (larvae and adult stages, respectively) and therefore alterations in water pH and pCO_2 can result in lower calcification and therefore development problems (Byrne and Hernández, 2020; Sheppard Brennand et al., 2010; Stumpp et al., 2012; Wolfe et al., 2013; Zhan et al., 2016). Thus, sea urchins represent an excellent group to assess the impact of climate change in the laboratory, being a recurrent model for this type of studies in the last few years (Foo et al., 2020). Particularly, the edible sea urchin *P. lividus* has gained interest as a model due to the several advantages they have as bioindicators and in their manipulation in laboratory conditions (Parra-Luna et al., 2020). Also, standard bioassays have been developed to test the impact of any stressor in the first 48 h of life of sea urchins, covering its development from the fertilized egg to the 4-arm pluteus larvae (Beiras et al., 2012), making them ideal indicators to study the effects of environmental stressors on the ecosystems using quick assays in the laboratory.

With this background, the goal of this work is to study the combined effect of global warming factors (acidification, temperature increase) and MP concentration on the growth and development of *P. lividus* larvae, aiming to estimate the potential risk of these stressors in a future climate change scenario.

2. Materials and methods

2.1. Reagents

The MP employed in this study was high density polystyrene. The product used, polystyrene microspheres (PSMS) with a size range of 9.5–11.5 μm (90%), a sphericity greater than 95% and a density of 1.07 g/cc, was purchased from Cospheric (California, USA). MP suspensions were prepared by addition of 1 mg of powder in 1 L of 1 μm-filtered seawater, sterilized with UV light and ozone (FSW, 30 psμ salinity, pH = 8.1). This suspension contains approximately 9000 MP particles per mL (particles/mL) that were counted with a portable particle counter (PAMAS Partikelmess- und Analyse systeme GmbH - S4031GO, Rutesheim, Germany) and constituted the stock suspension of MP that was used to prepare the experimental MP treatments. To avoid sinking and agglomeration of MP particles, 5 μL/L of Tween ® 20 were added to each MP solution and Tween ® 20 controls were included.

Experimental treatments (FSW or MP solutions) were acidified by adding 10% HCl (PanReac, Castellar del Vallés, Barcelona) in a dropwise manner under continuous stirring until pH stabilization (Bellas et al., 2003; da Silva Souza et al., 2019; Kurihara and Shirayama, 2004; Saco-Álvarez et al., 2010; Yamada and Ikeda, 1999). The pH was checked before starting the incubation and right after ending exposure. No major variations were detected in this parameter.

2.2. Biological material

Mature individuals of *P. lividus* were collected from a natural population inhabiting the outer part of the Ría of Vigo (Galicia, NW Iberian Peninsula) in the months from May to August. Individuals (around 50 males and 50 females) were maintained in 150 L tanks, with a natural photoperiod and natural running seawater (temperature 14 ± 2 °C, pH = 8.1 ± 0.1), until its utilization. Sea urchins were fed daily with green and brown algae (*Ulva lactuca*, *Laminaria* sp.).

2.3. Experimental procedures

The embryo growth bioassays were based on the methods described by Saco-Álvarez et al. (2010). Gametes were obtained by dissecting a single pair of mature *P. lividus* individuals per experiment to avoid genetic variation. A dense suspension of oocytes was fertilized with 10 μL of undiluted sperm in a measuring cylinder containing 40 mL of FSW. Subsequently, three aliquots of 25 μL were observed under the microscope to determine the fertilization success (indicated by the presence of a fertilization membrane) and the egg density. The fertilization success was always higher than 98% and the egg density was 7000 ± 200 eggs/mL. Within 30 min after fertilization, eggs were delivered into 50 mL vials containing the experimental treatments at a density of 30 embryos per mL. After 48 h of incubation at 20 °C in darkness, with no aeration, vials were fixed with 10 drops of 40% buffered formalin, and directly observed under an inverted microscope (Axiovert 40

CFL, Zeiss, Spain). Size and morphology measurements were determined in pictures taken with a DFK 42BUC03 camera system (Imagingsource®, Bremen, Germany).

To determine the optimum pH range for sea urchin embryos and selecting a pH condition to be used in the following experiments, seven pH conditions (6.5, 7.0, 7.2, 7.4, 7.6, 7.8, 8.0) were tested. The pH for each treatment was adjusted in FSW stock solutions (0.5 L) that were distributed in 50 mL vials (4 replicates per treatment). The bioassays were performed as described above. This trial was run in triplicate. To study the combined effect of MP and pH on *P. lividus* larvae, two concentrations of MP (1000 and 3000 particles/mL), two pH (8.1 and 7.6), and their combinations were tested. MP concentrations were chosen on the basis of previous work of the research group (unpublished results). Among the MP concentrations known to provoke an effect on larval growth those within the same magnitude of the levels measured in highly polluted areas were selected (e.g., Gorokhova, 2015; Kang et al., 2015; Zhao et al., 2015). The pH was adjusted in the FSW and MP stock solutions prior to the beginning of the experiment. Solutions were distributed in 50 mL vials (4 replicates per treatment) and incubated during 48 h as described above. The combined effect of temperature, pH, and MP (1000 and 3000 particles/mL) on *P. lividus* larval growth and development was also studied. A temperature of 24 °C was chosen to represent temperature stress according to FAO (FAO, 2019) and IPCC (Krinner et al., 2013) predictions for the global warming scenario at the end of the 21st century, and a control temperature of 20 °C was used.

2.4. Measurement of growth and morphometric parameters

Growth measurements were taken as described in Beiras et al. (2012) and morphometric parameters were adapted from Dorey et al. (2013). A scheme of how measurements were taken is shown in Fig. 1 **A.** The longest postoral arm (POL) was used to determine growth in 35 larvae per replicate. To determine alterations in the body shape, measurements of body width (BW), total body length (BL; measured from the top of the larvae to the end of the stomach), body width in the zone of the posterodorsal arms (PDG) and the postoral arms's gap (POG), were measured in 10 larvae per replicate. The stomach volume was calculated in 10 larvae per replicate as $SV = 4/3\pi \times [(S1 + S2) / 4]^3$, in which S1 and S2 represent the vertical and horizontal diameter of the stomach (Fig. 1 **A**). Growth was calculated using the web app "Growth Calculator", developed by our group and freely available at https://jignacio-ieo2020.shinyapps.io/Growth-Calculator-1/, and following the considerations explained in Saco-Álvarez et al. (2010).

The coefficient of variation (CV) which represents the data dispersion was calculated for each replicate in a treatment as: SD (R) / M (R) × 100. In which SD is the standard deviation, M is the mean and R is the data on growth of each replicate.

2.5. Degree-days

As the biological responses in sea urchin larvae depend on temperature, the larval growth must be expressed considering thermal differences among treatments (20 °C and 24 °C). To take into account the positive effect on growth provoked by the temperature increase, in the second experiment we calculated the larval growth per degree-day (DD) according to Young and Young (1998): $DD = ([T_{max} + T_{min}] / 2) - T_0$. Where DD are the degree-days accumulated in 24 h, T_{max} and T_{min} are the daily maximum and minimum temperatures (in this study, $T_{max} = T_{min}$), and T_0 is the temperature corresponding to 0 growth rate for this species (6.8 °C according to Beiras et al., 2001).

2.6. Statistical analysis

Growth and morphometric data were analyzed for normality and homogeneity of variances. Data were then analyzed by one-way ANOVA or two-way ANOVA, followed by the Tukey post-hoc test at a significance level of $p < 0.05$. All tests were performed using the Growth Calculator and Infostat software (Version 2008; Di Rienzo et al., 2013) for the two-way ANOVA analysis.

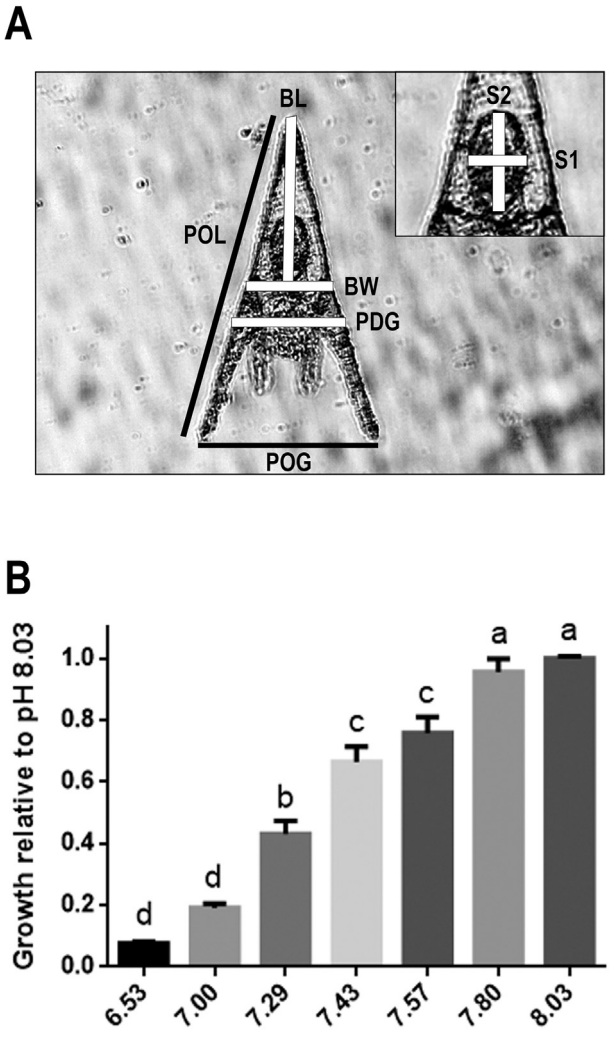


Fig. 1. Representation of how measurements were taken in 48 h *P. lividus* larvae (A), and length of *P. lividus* larvae 48 h after hatching at different water pH compared to control pH = 8.03 (B). Different letters denote statistical differences between groups assessed by one-way ANOVA follow by Tukey post hoc analysis test ($p < 0.05$). POL: longest postoral arm. BW: body width. BL: total body length. PDG: body width in the zone of the posterodorsal arms. POG: postoral arms's gap. S1 and S2 represent the vertical and horizontal diameter of the stomach.

3. Results

The optimal pH range for *P. lividus* embryos was determined (Fig. 1 **B**). Larvae resulting from embryos reared at pH below 7.6 showed a significantly delayed growth ($p < 0.05$ for pH 7.57–7.29 and $p < 0.01$ for pH 7.0 and 6.53), and at pH = 7.3 larvae showed a 50% decrease in length in comparison to the control (filtered seawater, pH = 8.03) treatment (Fig. 1 **B**). The inhibition of growth in embryos reared at pH = 7.6, was around 75% compared to the control, while non-significant effects were observed at pH values above 7.8 (Fig. 1 **B**). Based on these results, and in agreement with current FAO and IPCC predictions (FAO, 2019; Krinner et al., 2013), a threshold pH of 7.6 was chosen to study the combined effects of pH and MPs. In addition to the observed decrease in growth, exposure to a pH of 7.6 caused

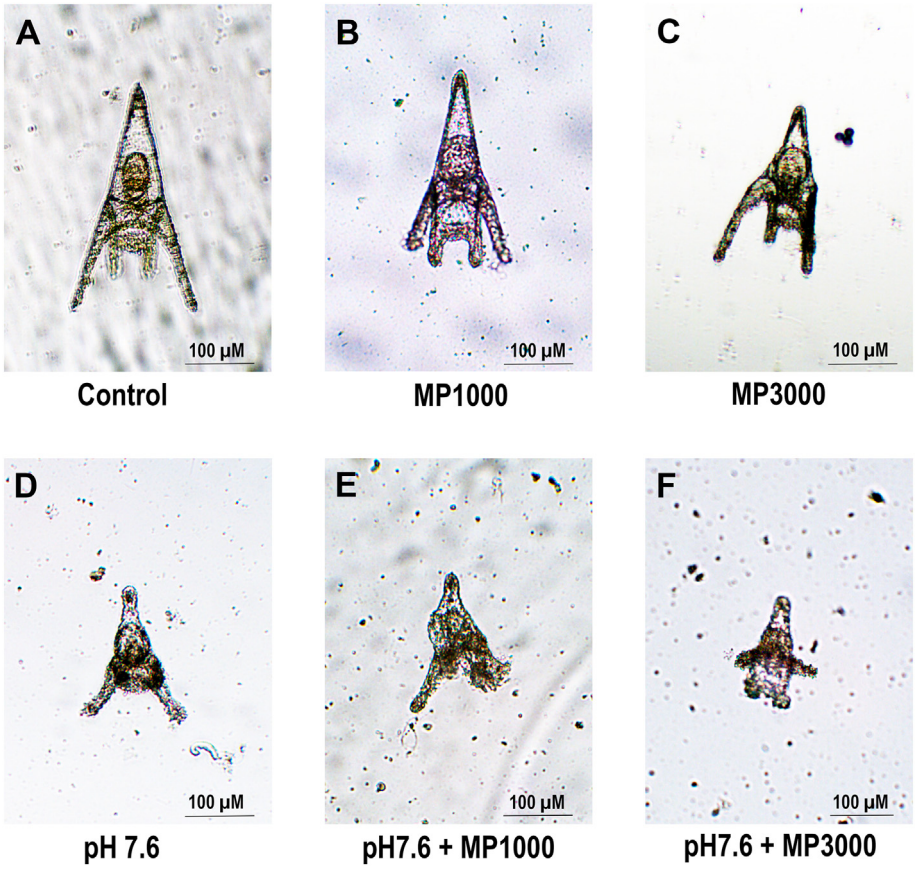


Fig. 2. Images of *P. lividus* larvae representing the most frequent deformities found in each treatment. (A) Control treatment (pH = 8.03 and no MPs). (B) Incubated with 1000 Particles/mL of MPs. (C) Incubated with 3000 Particles/mL of MPs. (D) Incubated at pH = 7.6. (E) Incubated at pH = 7.6 + 1000 Particles/mL of MPs. (F) Incubated at pH = 7.6 + 3000 Particles/mL of MPs.

relevant developmental effects, since 48-h pluteus larvae only develop two arms instead of the four arms developed by control 48-h pluteus larvae (Fig. 2A, D).

Regarding MP alone, exposure to 1000 particles /mL did not affect larval growth, but larvae resulting from embryos incubated at 3000 particles/mL showed a ca. 20% decrease in growth in comparison to controls ($p < 0.05$; Fig. 3 **A**). The exposure to MP also induced some developmental alterations such as the increase in the postoral arm separation or rounded vertices (Fig. 2 **B and C**).

The combined exposure of embryos to a pH of 7.6 and MP (1000 or 3000 particles/mL) during 48 h caused a significant decrease ($p < 0.05$) on larval growth compared to control (50% decrease) and pH = 7.6 (10% decrease) treatments (Fig. 3A). Severe morphological alterations were observed in those treatments, including the development of only two arms and several arm malformations (Fig. 2 E, F). The CV of pH + MP treatments increased around 50% ($p < 0.05$) in comparison to control and around 35% ($p < 0.05$) compared to pH and MP treatments alone (Table 1).

Morphometric parameters used to describe body shape were standardized to the BL (Fig. 3 **B to F**). Non-significant differences were observed in the relative stomach volume, except for the MP3000 treatment that increased the stomach volume compared to all the other treatments ($p < 0.05$; Fig. 3 **B**). The combined exposure of embryos to low pH and MP (1000 and 3000 particles/mL) caused a significant increase of 15% and 20% in the ratios BW/BL and PDG/BL ($p < 0.05$). The ratio POL/BL was slightly higher in larvae resulting from embryos exposed to MPs alone at both concentrations (1000 and 3000 particles/mL; $p < 0.05$; Fig. 3 **E**), whilst the ratio POG/BL showed a statistically significant increase in larvae resulting from embryos exposed to the combination of low pH and MP (1000 and 3000 particles/mL), in comparison to controls (15% and 20% of increase, respectively; $p < 0.05$). Non-significant effects were observed in ratios BW/BL, PDG/BL and POG/BL for pH and MP single exposures (Fig. 3 **C, D and F**).

The combined effects of temperature with pH and MP are shown in Fig. 4. Since the pH was the variable that caused the most significant effects on growth, the effect of increasing temperature was also studied in combination with low pH alone. Increasing the temperature from 20 °C to 24 °C resulted in an increase of 15% in growth ($p < 0.05$), that can be observed also in the pH 7.6 (8% of increase; $p < 0.05$) and pH 7.6 + MP3000 (15% of increase; $p < 0.05$) treatments but not in the pH 7.6 + MP1000 treatment (Fig. 4 A). The relative stomach volume decreased around 15% with the increase of temperature ($p < 0.05$). This decrease was also observed in the pH 7.6, pH 7.6 + MP1000 and pH 7.6 + MP3000 treatments ($p < 0.05$), although the decrease observed at the pH 7.6 treatment was not statistically significant (Fig. 4 B). The ratio BW/BL was not affected by the increase in temperature (Fig. 4 C). The PDG/BL ratio only showed a slight (8%) but significant increase ($p < 0.05$) with temperature at the pH 7.6 + MP1000 treatment (Fig. 4 D). A slightly increased POL/BL ratio was observed in response to the elevated water temperature in all organisms exposed to a decrease of pH or to MPs ($p < 0.05$; Fig. 4 **E**). Such increase was around 5% in larvae exposed to pH 7.6 which is higher than in controls, and the highest effect of temperature on the POL/BL ratio was observed in those larvae resulting from embryos exposed to pH 7.6 + MPs, in which the increase was around 10% ($p < 0.05$; Fig. 4 E). The increase in water temperature caused a decrease in the POG/BL ratio but no effects were observed in the pH 7.6 treatment. On the other hand, the increase in temperature led to an increase ($p < 0.05$) in the POG/BL ratio for pH 7.6 + MP but decreased for controls (Fig. 4 **F**).

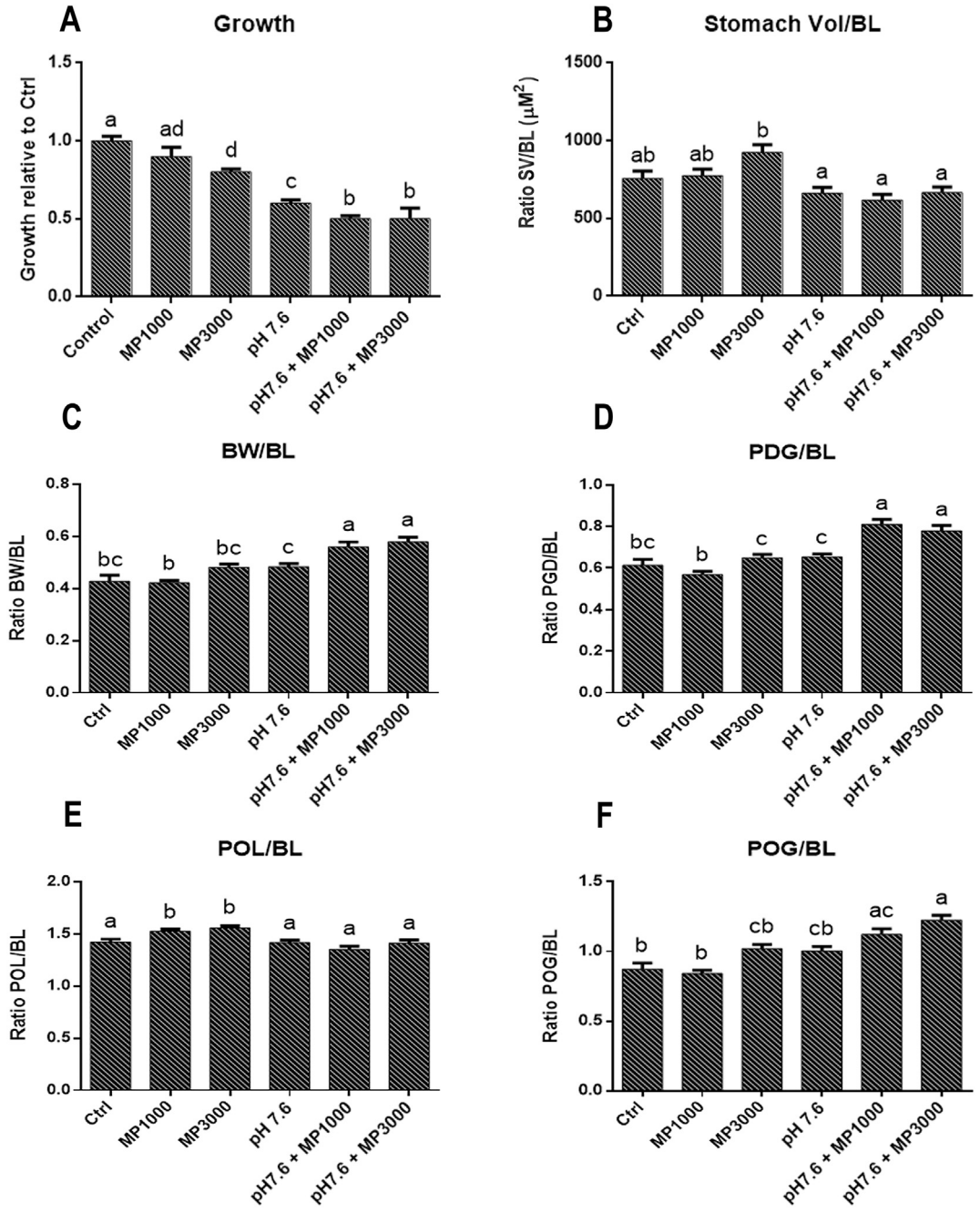


Fig. 3. Growth and ratios between body parameters and the body length of *P. lividus* larvae after 48 h of incubation with different treatments. Body width (BW), body length (POL), total body length (BL), body width in the zone of the posterodorsal arms (PDG) and the postoral arms's gap (POG). (A) Growth of larvae relative to control group. (B) Stomach Volume relative to BL. (C) Body width relative to BL. (D) Width of posterodorsal arms relative to BL. (E) Body length relative to BL. (F) Width of postoral arms's gap relative to BL. MP1000: 1000 particles/mL. MP3000: 3000 particles/mL. Different letters denote statistical differences between groups assessed by one-way ANOVA follow by Tukey post hoc analysis test ($p < 0.05$).

Fig. 5 shows the larval growth expressed per DD. Those larvae reared at 24 °C presented a lower growth per DD than those reared at 20 °C in all the treatments ($p < 0.05$). Ratios from morphometric parameters expressed per DD are represented in the supplementary material. Overall, the increase in temperature from 20 °C to 24 °C generated lower morphometric parameters in all treatments (Fig. S1).

4. Discussion

In this work we studied the effect of pH, temperature, and MP on *P. lividus* larvae aiming to estimate potential combined effects of these stressors in a further scenario of climate change. The results presented here demonstrate that a pH level within the range of the lower limit of the FAO's and IPCC predictions (FAO, 2019; Krinner et al., 2013) can cause alterations in the growth of *P. lividus* 4-arm pluteus larvae. In a scenario of climate change, such alterations are presumably related to variations in the capacity of individuals to calcify their structures in response to a lower pH and increased pCO_2 generated by the increase in the atmospheric CO_2 (Foo et al., 2020; Johnson et al., 2020; Kurihara and Shirayama, 2004; Rengifo et al., 2019; Rodríguez et al., 2017; Sheppard Brennand et al., 2010; Zhan et al., 2016). In this work the water pH was decreased without a direct increase of the pCO2, so we conclude that lower pH provokes alterations in growth and development that might not be totally related to disruptions in the calcification

Table 1
Coefficient of variation (CV) of the growth of larvae resulting from embryos exposed to different pH and microplastic treatments.

Treatment	CV ± SE
Ctrl	8 ± 2
MP1000	10,2 ± 0,9
MP3000	12 ± 2
pH 7.6	13 ± 2
pH 7.6 + MP1000	17 ± 2 *
pH 7.6 + MP3000	18 ± 1 *

MP1000: 1000 particles/mL, MP3000: 3000 particles/mL, SE: Standard Error. (*) Represents significant differences compared to control treatment (One Way ANOVA, $p < 0.05$).

process. Negative effects on growth and morphology were also found in early stages of the sea urchin *Hemicentrotus pulcherrimus* exposed to filtered seawater acidified with CO_2 or HCl. The response of *H. pulcherrimus* in terms of developmental speed and pluteus larval morphology were similar for CO_2- and HCl- acidified seawater (Kurihara and Shirayama, 2004). According to Stumpp et al. (2012), sea urchin pluteus larvae are capable of maintaining their inner pH despite external changes, within certain limits, but this demands an energetic cost that could affect their growth and increase their mortality. Therefore, the 48 h-sea urchin larvae in the lecithotrophic phase (derive their nutrition from energy stored in the egg itself) employed in this work might be using part of the energy stored in the egg to maintain the pH homeostasis having less energy available to grow and develop. Lower pH was also related to increased respiration rate, abnormal gene expression and oxidative response (for a review see Pagano et al., 2017), which indicates that ocean acidification is a multifactorial problem affecting several aspects of the sea urchin biology.

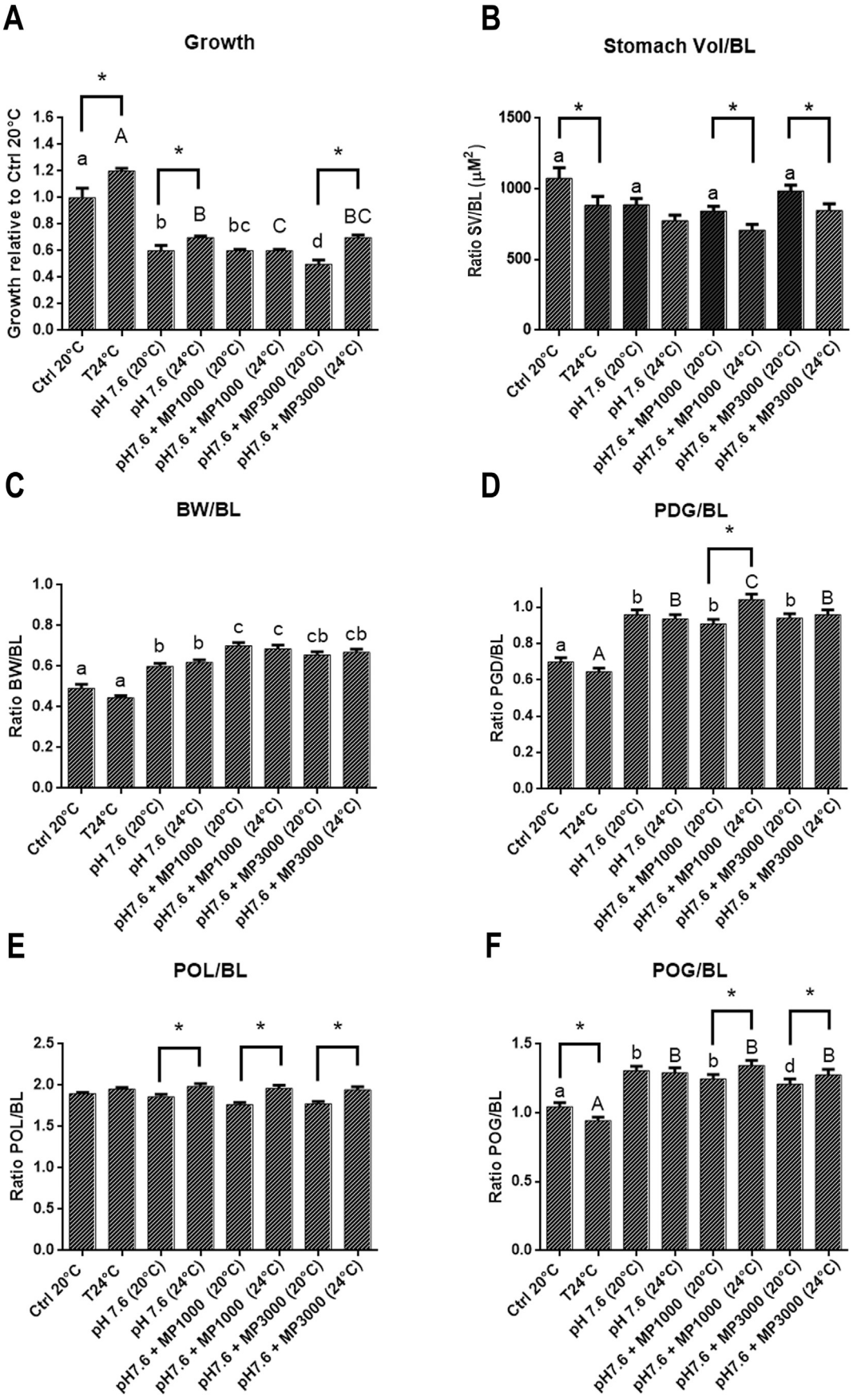
An increase in water temperature leads to a significant increase in larval growth, but when this parameter is expressed per DD the lower growth provoked by the thermal stress as is evidenced in all treatments. This means that larvae reared at 24 °C are not growing as much as they could. This delay in growth per DD indicates that although larval length increases, maybe due to an activation of metabolism (Ulbricht and Pritchard, 1972), temperature is causing a stress which might affect other aspects of growth. In that way, we found that this apparently beneficial effect of temperature is accompanied by several morphological alterations reflected in the abnormal morphometric ratios. The combined effect of pH and temperature was studied in other sea urchin species (for a review see Byrne and Hernández, 2020). For some species the pH has the stronger effect, while for others the temperature is the dominant factor. For example, in the sea urchin *Tripneustes gratilla* pH has the dominant effect and an increase in water temperature can increase growth partially counteracting the effect of acidification (Sheppard Brennand et al., 2010). On the counterpart, in *Heliocidaris erythrogramma* the decrease in pH does not produce major effects in terms of development, but temperature affects the normal embryo and larval development (Byrne et al., 2009). As a general rule, the combination of both parameters generates a stress that provokes a delay in growth and/or several complications on their development (Morley et al., 2016; Pereira et al., 2020; Wolfe et al., 2013). Taken together, our results suggest that growth and development of *P. lividus* larvae can be severely affected in further scenarios of ocean warming and acidification as those predicted by FAO and IPCC (FAO, 2019; Krinner et al., 2013).

The concentrations of MP used in this study are higher than average concentrations found in the marine environment (1.2–2.5 MP/m^3 for the Northeast Atlantic, (Frias et al., 2014; Kanhai et al., 2017; Lusher et al., 2015). But they are closer to maximum concentrations measured in polluted estuaries and coastal environments (100–15,600 MP/m^3; Gorokhova, 2015; Kang et al., 2015; Zhao et al., 2015). It must also be considered that MP concentrations in the marine environment are probably being underestimated (Lindeque et al., 2020). Although the effects of single exposure to MP were not as pronounced as those of pH, we found that MP alone can cause a reduction in larval growth and slight alterations in their morphology. Since embryos and larvae used in this study are not capable to intake food, MP ingestion was discarded as a factor that could contribute to the observed reduction of growth. However, other studies showed that the exposure of embryos of the sea urchin *Sphaerechinus granularis* to polystyrene and polymethyl methacrylate microparticles, provoked developmental, cytogenetic and genotoxic effects (Trifuoggi et al., 2019). Moreover, 0.1 μM polystyrene microbeads caused alterations in the swimming activity in *P. lividus* 24-h larvae (Gambardella et al., 2018), which could partly explain the observed growth reduction. Also, in agreement with the results presented here, a study carried out with *P. lividus* pluteus larvae (Piccardo et al., 2020) showed that polyethylene MP of the same particle-size range as those used in this work can cause a reduction in growth of around 20%. The exposure to MP together with a reduction in water pH provoked a significant decrease in growth compared to the exposure to low pH alone. According to the CV, which is a statistical measure of the dispersion of data around the mean, the combination of low pH and MP increases the differences between the larval growth inside each treatment. These results might indicate that MP are adding an extra stress to embryos exposed to low pH affecting their normal development. This interaction between pH and MP in detriment of sea urchin embryo and larval growth was also described by (Piccardo et al., 2020), which found that a reduction in water pH aggravates the effect of MPs.

The impact of low pH and MP on the relative stomach volume seems to be dependent of temperature since a reduction on this ratio was observed at 24 °C compared to control temperature. This result is confirmed when data is expressed per DD (Fig. S1 **B**) evidencing that temperature is the main factor driving this reduction. A decrease in stomach -length and -width in response to high temperatures was previously reported in *Strongylocentrotus intermedius* (Zhao et al., 2018). This might generate complications for larvae to feed and process food properly in further stages of their development, although more experiments should be carried out to corroborate it. All the ratios studied in this work were altered by pH and temperature which is in concordance with other multi-stressor studies that emulate scenarios of climate change (Byrne et al., 2009; Byrne and Hernández, 2020; Delorme and Sewell, 2014; Zhao et al., 2018).

Overall, in this work we demonstrated that MP could aggravate the effect of a decreased pH and increased temperature in *P. lividus* larvae, causing a lower growth and an altered development, including alterations in the morphometric parameters. These effects are non-additive, probably because a decreased pH alone cause a high impact on growth, being the dominant stressor, and that negative effect could not be much aggravated by MP. This type of interaction between stressors was described by Darling et al. (2010) as a non-additive antagonism, present when one of the stressors has a dominant or greater impact than the others. These effects occur in the first 48 h after hatching, the beginning of the so-called planktonic stage in which the larvae start to feed independently (Gosselin and Jangoux, 1998). Although after 30 days the larvae structures are reabsorbed leading to the adult form of the sea urchin, during the planktonic stage the size and morphology of *P. lividus* larvae are crucial to ensure their buoyancy and therefore their survival. For instance, swimming is essential to move through

Fig. 4. Ratios between body parameters and the body length of *P. lividus* larvae after 48 h of incubation with different treatments at two temperatures (20 °C and 24 °C). (A) Growth of larvae relative to control group. (B) Stomach Volume relative to BL. (C) Body width relative to BL. (D) Width of posterodorsal arms relative to BL. (E) Body length relative to BL. (F) Width of postoral arms's gap relative to BL. MP1000: 1000 particles/mL. MP3000: 3000 particles/mL. Different letters denote statistical differences between groups within a temperature (20 °C or 24 °C) and asterisks denotes differences between temperatures within the same treatment assessed by Two-way ANOVA test ($p < 0.05$).


A
Growth
Growth relative to Ctrl 20°C
B
Stomach Vol/BL
Ratio SV/BL (μM²)
C
BW/BL
Ratio BW/BL
D
PDG/BL
Ratio PGD/BL
E
POL/BL
Ratio POL/BL
F
POG/BL
Ratio POG/BL
Ctrl 20°C
T24°C
pH 7.6 (20°C)
pH 7.6 (24°C)
pH7.6 + MP1000 (20°C)
pH7.6 + MP1000 (24°C)
pH7.6 + MP3000 (20°C)
pH7.6 + MP3000 (24°C)

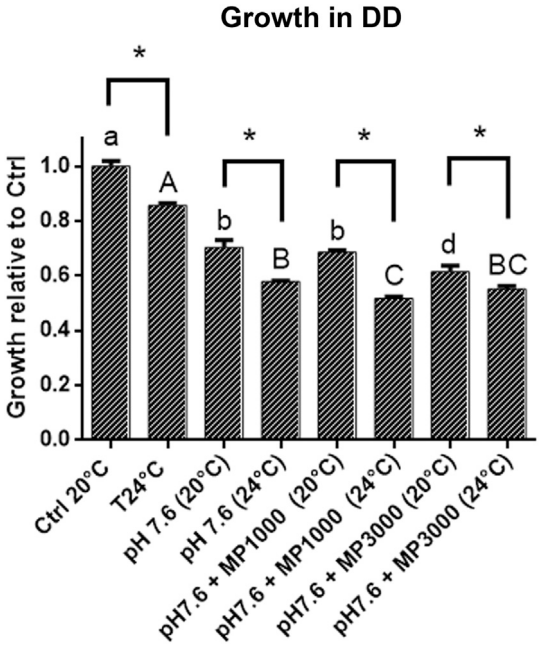


Fig. 5. Larvae growth expressed in a Degree Day basis (μm DD^{-1}) relative to control treatment. MP1000: 1000 particles/mL. MP3000: 3000 particles/mL. Different letters denote statistical differences between groups within a temperature (20 °C or 24 °C) and asterisks denotes differences between temperatures within the same treatment assessed by Two-way ANOVA test ($p < 0.05$).

the water column and to find new places rich in food (Strathmann, 2006). The evolutionary pressures shape the larvae morphology to fit within opposing functional constraints such as feeding ability and stability in the water column. Feeding structures generally require large surface area for particle capture, whereas stability relies upon minimal surface area (Hodin et al., 2016). Thus, deformities and altered growth can compromise both crucial activities and therefore the chance of survival of *P. lividus* larvae.

In conclusion, according to the results presented here, if actions are not urgently taken to modify the current tendency of ocean warming, ocean acidification, and microplastic pollution, the combined stress caused by them could threaten sea urchin populations leading to a potential impact on coastal ecosystems (Oliva et al., 2016).

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2021.146888.

CRediT authorship contribution statement

Juan Ignacio Bertucci: Conceptualization, Methodology, Investigation, Formal analysis, Visualization, Writing - Review & Editing. **Juan Bellas:** Supervision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

This study was partially funded by the Spanish Government (Ministry of Science and Innovation) through the PLASMED Project (CTM2017-89701-C3-3-R). Dr. Juan Ignacio Bertucci is a postdoctoral fellow from Juan de la Cierva Formación, call 2019 (Ministry of Science and Innovation, Spanish Government). Authors are grateful to Ainhoa Juez Zabala, María Dolores Pampillón Lorenzo, and Pedro Pousa Fernández for their helpful technical support.

References

Alava, J.J., Cheung, W.W.L., Ross, P.S., Sumaila, U.R., 2017. Climate change–contaminant interactions in marine food webs: toward a conceptual framework. Glob. Chang. Biol. 23, 3984–4001.

Andrady, A.L., 2011. Microplastics in the marine environment. Mar. Pollut. Bull. https://doi.org/10.1016/j.marpolbul.2011.05.030.

Beiras, R., Vázquez, E., Bellas, J., Lorenzo, J.I., Fernández, N., Macho, G., Mariño, J.C., Casas, L., 2001. Sea-urchin embryo bioassay for in situ evaluation of the biological quality of coastal seawater. Estuar. Coast. Shelf Sci. 52, 29–32. https://doi.org/10.1006/ecss.2000.0720.

Beiras, R., Durán, I., Bellas, J., 2012. Biological effects of contaminants : Paracentrotus lividus sea urchin embryo test with marine sediment elutriates. ICES Tech. Mar. Environ. Sci. 51 13pp.

Beiras, R., Bellas, J., Cachot, J., Cormier, B., Cousin, X., Engwall, M., Gambardella, C., Garaventa, F., Keiter, S., Le Bihanic, F., López-Ibáñez, S., Piazza, V., Rial, D., Tato, T., Vidal-Liñán, L., 2018. Ingestion and contact with polyethylene microplastics does not cause acute toxicity on marine zooplankton. J. Hazard. Mater. 360, 452–460. https://doi.org/10.1016/j.jhazmat.2018.07.101.

Bellas, J., Beiras, R., Vázquez, E., 2003. A standardisation of Ciona intestinalis (Chordata, Ascidiacea) embryo-larval bioassay for ecotoxicological studies. Water Res. 37, 4613–4622. https://doi.org/10.1016/S0043-1354(03)00396-8.

Byrne, M., Hernández, J.C., 2020. Sea urchins in a high CO2 world: impacts of climate warming and ocean acidification across life history stages. Developments in Aquaculture and Fisheries Science. Elsevier, pp. 281–297 https://doi.org/10.1016/B978-0-12-819570-3.00016-0.

Byrne, M., Ho, M., Selvakumaraswamy, P., Nguyen, H.D., Dworjanyn, S.A., Davis, A.R., 2009. Temperature, but not pH, compromises sea urchin fertilization and early development under near-future climate change scenarios. Proc. R. Soc. B Biol. Sci. 276, 1883–1888. https://doi.org/10.1098/rspb.2008.1935.

Castro, L.C., Cetina-Heredia, P., Roughan, M., Dworjanyn, S., Thibaut, L., Chamberlain, M.A., Feng, M., Vergés, A., 2020. Combined mechanistic modelling predicts changes in species distribution and increased co-occurrence of a tropical urchin herbivore and a habitat-forming temperate kelp. Divers. Distrib. 26, 1211–1226. https://doi.org/10.1111/ddi.13073.

Cole, M., Lindeque, P., Halsband, C., Galloway, T.S., 2011. Microplastics as contaminants in the marine environment: a review. Mar. Pollut. Bull. 62, 2588–2597. https://doi.org/10.1016/j.marpolbul.2011.09.025.

Cole, M., Lindeque, P., Fileman, E., Halsband, C., Galloway, T.S., 2015. The impact of polystyrene microplastics on feeding, function and fecundity in the marine copepod Calanus helgolandicus. Environ. Sci. Technol. 49, 1130–1137. https://doi.org/10.1021/es504525u.

Darling, E.S., McClanahan, T.R., Côté, I.M., 2010. Combined effects of two stressors on Kenyan coral reefs are additive or antagonistic, not synergistic. Conserv. Lett. 3, 122–130. https://doi.org/10.1111/j.1755-263X.2009.00089.x.

Delorme, N.J., Sewell, M.A., 2014. Temperature and salinity: two climate change stressors affecting early development of the New Zealand Sea urchin Evechinus chloroticus. Mar. Biol. 161, 1999–2009. https://doi.org/10.1007/s00227-014-2480-0.

Di Rienzo, J., Casanoves, F., Balzarini, M., Gonzalez, L., Tablada, M., Robledo, C., 2013. Infostat - Sofware estadístico. Universidad Nacional de Córdoba, Argentina [WWW Document]. Univ. Nac., Córdoba, Argentina http://www.infostat.com.ar/.

Dorey, N., Lançon, P., Thorndyke, M., Dupont, S., 2013. Assessing physiological tipping point of sea urchin larvae exposed to a broad range of pH. Glob. Chang. Biol. https://doi.org/10.1111/gcb.12276.

FAO, 2019. Deep-ocean climate change impacts on habitat, fish and fisheries. FAO Fisheries and Aquaculture Technical Paper No. 638.

Foo, S.A., Koweek, D.A., Munari, M., Gambi, M.C., Byrne, M., Caldeira, K., 2020. Responses of sea urchin larvae to field and laboratory acidification. Sci. Total Environ. 723, 138003. https://doi.org/10.1016/j.scitotenv.2020.138003.

Frias, J.P.G.L., Otero, V., Sobral, P., 2014. Evidence of microplastics in samples of zooplankton from Portuguese coastal waters. Mar. Environ. Res. 95, 89–95. https://doi.org/10.1016/j.marenvres.2014.01.001.

Gambardella, C., Morgana, S., Bramini, M., Rotini, A., Manfra, L., Migliore, L., Piazza, V., Garaventa, F., Faimali, M., 2018. Ecotoxicological effects of polystyrene microbeads in a battery of marine organisms belonging to different trophic levels. Mar. Environ. Res. 141, 313–321. https://doi.org/10.1016/j.marenvres.2018.09.023.

Gao, K., Gao, G., Wang, Y., Dupont, S., 2020. Impacts of ocean acidification under multiple stressors on typical organisms and ecological processes. Mar. Life Sci. Technol. 2, 279–291. https://doi.org/10.1007/s42995-020-00048-w.

Gorokhova, E., 2015. Screening for microplastic particles in plankton samples: how to integrate marine litter assessment into existing monitoring programs? Mar. Pollut. Bull. 99, 271–275. https://doi.org/10.1016/j.marpolbul.2015.07.056.

Gosselin, P., Jangoux, M., 1998. From competent larva to exotrophic juvenile: a morphofunctional study of the perimetamorphic period of Paracentrotus lividus (Echinodermata, Echinoida). Zoomorphology 118, 31–43. https://doi.org/10.1007/s004350050054.

Harding, S., 2016. Marine Debris: Understanding, Preventing and Mitigating the Significant Adverse Impacts on Marine and Coastal Biodiversity. CBD Technical Series.

Hodin, J., Lutek, K., Heyland, A., 2016. A newly identified left–right asymmetry in larval sea urchins. R. Soc. Open Sci. 3, 160139. https://doi.org/10.1098/rsos.160139.

Johnson, R., Harianto, J., Thomson, M., Byrne, M., 2020. The effects of long-term exposure to low pH on the skeletal microstructure of the sea urchin Heliocidaris erythrogramma. J. Exp. Mar. Bio. Ecol. 523, 151250. https://doi.org/10.1016/j.jembe.2019.151250.

Kang, J.H., Kwon, O.Y., Lee, K.W., Song, Y.K., Shim, W.J., 2015. Marine neustonic microplastics around the southeastern coast of Korea. Mar. Pollut. Bull. 96, 304–312. https://doi.org/10.1016/j.marpolbul.2015.04.054.
Kanhai, L.D.K., Officer, R., Lyashevska, O., Thompson, R.C., O'Connor, I., 2017. Microplastic abundance, distribution and composition along a latitudinal gradient in the Atlantic Ocean. Mar. Pollut. Bull. 115, 307–314. https://doi.org/10.1016/j.marpolbul.2016.12.025.
Krinner, G., Germany, F., Shongwe, M., Africa, S., France, S.B., Uk, B.B.B.B., Germany, V.B., Uk, O.B., France, C.B., Uk, R.C., Canada, M.E., Erich, M., Uk, R.W.L., Uk, S.L., Lucas, C., 2013. Long-term climate change: projections, commitments and irreversibility. Phys. Sci. Basis Work. Gr. I Contrib. to Fifth Assess. Rep. Intergov. Panel Clim. Chang., pp. 1029–1136 https://doi.org/10.1017/CBO9781107415324.024 9781107057
Kurihara, H., Shirayama, Y., 2004. Effects of increased atmospheric CO2 on sea urchin early development. Mar. Ecol. Prog. Ser. 274, 161–169. https://doi.org/10.3354/meps274161.
Lee, K.W., Shim, W.J., Kwon, O.Y., Kang, J.H., 2013. Size-dependent effects of micro polystyrene particles in the marine copepod tigriopus japonicus. Environ. Sci. Technol. 47, 11278–11283. https://doi.org/10.1021/es401932b.
Lindeque, P.K., Cole, M., Coppock, R.L., Lewis, C.N., Miller, R.Z., Watts, A.J.R., Wilson-McNeal, A., Wright, S.L., Galloway, T.S., 2020. Are we underestimating microplastic abundance in the marine environment? A comparison of microplastic capture with nets of different mesh-size. Environ. Pollut. 265, 114721. https://doi.org/10.1016/j.envpol.2020.114721.
Ling, S.D., Barrett, N.S., Edgar, G.J., 2018. Facilitation of Australia's southernmost reef-building coral by sea urchin herbivory. Coral Reefs 37, 1053–1073. https://doi.org/10.1007/s00338-018-1728-4.
Lusher, A.L., Tirelli, V., O'Connor, I., Officer, R., 2015. Microplastics in Arctic polar waters: the first reported values of particles in surface and sub-surface samples. Sci. Rep. 5, 14947.
Morley, S.A., Suckling, C.C., Clark, M.S., Cross, E.L., Peck, L.S., 2016. Long-term effects of altered pH and temperature on the feeding energetics of the Antarctic Sea urchin, Sterechinus neumayeri. Biodiversity 17, 34–45. https://doi.org/10.1080/14888386.2016.1174956.
Oliva, S., Farina, S., Pinna, S., Guala, I., Agnetta, D., Ariotti, P.A., Mura, F., Ceccherelli, G., 2016. Determinants of Paracentrotus lividus sea urchin recruitment under oligotrophic conditions: implications for conservation management. Mar. Environ. Res. 117, 13–20. https://doi.org/10.1016/j.marenvres.2016.02.013.
Pagano, G., Guida, M., Trifuoggi, M., 2017. Sea urchin bioassays in toxicity testing: I. Inorganics, organics, complex mixtures and natural products. Expert Opin. Environ. Biol. https://doi.org/10.4172/2325-9655.1000142.
Parra-Luna, M., Martín-Pozo, L., Hidalgo, F., Zafra-Gómez, A., 2020. Common sea urchin (Paracentrotus lividus) and sea cucumber of the genus Holothuria as bioindicators of pollution in the study of chemical contaminants in aquatic media. A revision. Ecol. Indic. 113, 106185. https://doi.org/10.1016/j.ecolind.2020.106185.
Pereira, T.M., Gnocchi, K.G., Merçon, J., Mendes, B., Lopes, B.M., Passos, L.S., Chippari Gomes, A.R., 2020. The success of the fertilization and early larval development of the tropical sea urchin Echinometra lucunter (Echinodermata: Echinoidea) is affected by the pH decrease and temperature increase. Mar. Environ. Res. 161, 105106. https://doi.org/10.1016/j.marenvres.2020.105106.
Perry, D., Staveley, T., Deyanova, D., Baden, S., Dupont, S., Hernroth, B., Wood, H., Björk, M., Gullström, M., 2019. Global environmental changes negatively impact temperate seagrass ecosystems. Ecosphere 10. https://doi.org/10.1002/ecs2.2986.
Piccardo, M., Provenza, F., Grazioli, E., Cavallo, A., Terlizzi, A., Renzi, M., 2020. PET microplastics toxicity on marine key species is influenced by pH, particle size and food variations. Sci. Total Environ. 715, 136947. https://doi.org/10.1016/j.scitotenv.2020.136947.
PlasticsEurope, 2015. Plastics - the facts 2015. An Analysis of European Plastics Production, Demand and Waste Data. Association of Plastics Manufacturers (PlasticsEurope).
Poloczanska, E.S., Burrows, M.T., Brown, C.J., García Molinos, J., Halpern, B.S., Hoegh-Guldberg, O., Kappel, C.V., Moore, P.J., Richardson, A.J., Schoeman, D.S., Sydeman, W.J., 2016. Responses of marine organisms to climate change across oceans. Front. Mar. Sci. 3, 1–21. https://doi.org/10.3389/fmars.2016.00062.
Rengifo, M.C., Agüera, A., Bouma, T., Zoudi, S.M., Flammang, P., Dubois, P., 2019. Ocean warming and acidification alter the behavioral response to flow of the sea urchin Paracentrotus lividus. Ecol. Evol. 9, 12128–12143. https://doi.org/10.1002/ece3.5678.
Rodríguez, A., Hernández, J.C., Brito, A., Clemente, S., 2017. Effects of ocean acidification on juveniles sea urchins: predator-prey interactions. J. Exp. Mar. Bio. Ecol. 493, 31–40. https://doi.org/10.1016/j.jembe.2017.04.005.
Ryan, P.G., Moore, C.J., Van Franeker, J.A., Moloney, C.L., 2009. Monitoring the abundance of plastic debris in the marine environment. Philos. Trans. R. Soc. B Biol. Sci. https://doi.org/10.1098/rstb.2008.0207.
Saco-Álvarez, L., Durán, I., Ignacio Lorenzo, J., Beiras, R., 2010. Methodological basis for the optimization of a marine sea-urchin embryo test (SET) for the ecological assessment of coastal water quality. Ecotoxicol. Environ. Saf. 73, 491–499. https://doi.org/10.1016/j.ecoenv.2010.01.018.
Sheppard Brennand, H., Soars, N., Dworjanyn, S.A., Davis, A.R., Byrne, M., 2010. Impact of ocean warming and ocean acidification on larval development and calcification in the sea urchin Tripneustes gratilla. PLoS One 5, e11372. https://doi.org/10.1371/journal.pone.0011372.
da Silva Souza, L., Pusceddu, F.H., Cortez, F.S., de Orte, M.R., Seabra, A.A., Cesar, A., Ribeiro, D.A., Del Valls Casillas, T.A., Pereira, C.D.S., 2019. Harmful effects of cocaine byproduct in the reproduction of sea urchin in different ocean acidification scenarios. Chemosphere 236, 124284. https://doi.org/10.1016/j.chemosphere.2019.07.015.
Strathmann, R.R., 2006. Good eaters, poor swimmers: compromises in larval form. Integr. Comp. Biol. 46, 312–322. https://doi.org/10.1093/icb/icj031.
Stumpp, M., Hu, M.Y., Melzner, F., Gutowska, M.A., Dorey, N., Himmerkus, N., Holtmann, W.C., Dupont, S.T., Thorndyke, M.C., Bleich, M., 2012. Acidified seawater impacts sea urchin larvae pH regulatory systems relevant for calcification. Proc. Natl. Acad. Sci. U. S. A. 109, 18192–18197. https://doi.org/10.1073/pnas.1209174109.
Trifuoggi, M., Pagano, G., Oral, R., Pavičić-Hamer, D., Burić, P., Kovačić, I., Siciliano, A., Toscanesi, M., Thomas, P.J., Paduano, L., Guida, M., Lyons, D.M., 2019. Microplastic-induced damage in early embryonal development of sea urchin Sphaerechinus granularis. Environ. Res. 179, 108815. https://doi.org/10.1016/j.envres.2019.108815.
Ulbricht, R.J., Pritchard, A.W., 1972. Effect of temperature on the metabolic rate of sea urchins. Biol. Bull. 142, 178–185.
Wolfe, K., Dworjanyn, S.A., Byrne, M., 2013. Effects of ocean warming and acidification on survival, growth and skeletal development in the early benthic juvenile sea urchin (Heliocidaris erythrogramma). Glob. Chang. Biol. 19, 2698–2707. https://doi.org/10.1111/gcb.12249.
Yamada, Y., Ikeda, T., 1999. Acute toxicity of lowered pH to some oceanic zooplankton. Plankt. Biol. Ecol. 46, 62–67.
Young, L.J., Young, J.H., 1998. Statistical Ecology. Springer, US, Boston, MA https://doi.org/10.1007/978-1-4757-2829-3.
Zhan, Y., Hu, W., Zhang, W., Liu, M., Duan, L., Huang, X., Chang, Y., Li, C., 2016. The impact of CO2 - driven ocean acidification on early development and calcification in the sea urchin Strongylocentrotus intermedius. Mar. Pollut. Bull. 112, 291–302. https://doi.org/10.1016/j.marpolbul.2016.08.003.
Zhao, S., Zhu, L., Li, D., 2015. Microplastic in three urban estuaries. China. Environ. Pollut. 206, 597–604. https://doi.org/10.1016/j.envpol.2015.08.027.
Zhao, C., Zhang, Lisheng, Shi, D., Ding, J., Yin, D., Sun, J., Zhang, B., Zhang, Lingling, Chang, Y., 2018. Transgenerational effects of ocean warming on the sea urchin Strongylocentrotus intermedius. Ecotoxicol. Environ. Saf. 151, 212–219. https://doi.org/10.1016/j.ecoenv.2018.01.014.