Chemosphere 301 (2022) 134783

Contents lists available at ScienceDirect

Chemosphere

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

Impact of microplastics and ocean acidification on critical stages of sea urchin (*Paracentrotus lividus*) early development

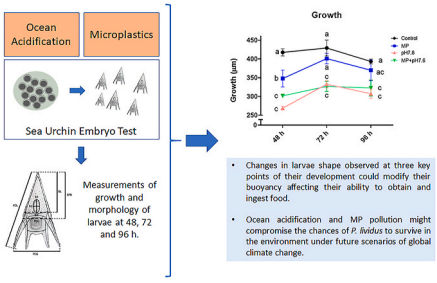
J.I. Bertucci *, A. Juez, J. Bellas

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

HIGHLIGHTS

- Ocean acidification and microplastics altered the morphology of *P. lividus* larvae.
- Ocean acidification and microplastics reduce growth of *P. lividus* larvae.
- Alterations occurred before and after larvae start to feed exogenously.
- The combined effect of both stressors on *P. lividus* morphology is non additive.
- SET is an ideal method to study the impact of ocean acidification at a lab scale.

GRAPHICAL ABSTRACT



ARTICLE INFO

Handling Editor: Willie Peijnenburg

Keywords:
Global climate change
Microplastics
Ocean acidification
Paracentrotus lividus
Sea urchin embryo test
Sea urchin

ABSTRACT

One of the major consequences of increasing atmospheric CO_2 is a phenomenon known as ocean acidification. This alteration of water chemistry can modulate the impact on marine organisms of other stressors also present in the environment, such as microplastics (MP). The objective of this work was to determine the combined impact of microplastic pollution and ocean acidification on the early development of *Paracentrotus lividus*. To study these multi-stressor impacts on development *P. lividus* the sea urchin embryo test (SET) was used. Newly fertilised embryos of *P. lividus* were exposed to a control treatment (filtered natural seawater), MP (3000 particles/mL), acidified sea water (pH = 7.6), and a combination of MP and acidification (3000 particles/mL + pH = 7.6). After 48, 72, and 96 h measurements of growth and morphometric parameters were taken. Results showed that ocean acidification and MP cause alterations in growth and larval morphology both before and after the larvae start to feed exogenously. The exposure to MP under conditions of ocean acidification did not produce any additional effect on growth, but differences were observed at the morphological level related to a decrease in the width of larvae at 48 h. Overall, changes in larvae shape observed at three key points of their development could modify their buoyancy affecting their ability to obtain and ingest food. Therefore, ocean acidification and MP pollution might compromise the chances of *P. lividus* to survive in the environment under future scenarios of global climate change.

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

https://doi.org/10.1016/j.chemosphere.2022.134783
Received 1 March 2022; Received in revised form 23 April 2022; Accepted 27 April 2022
Available online 30 April 2022

1. Introduction

Multiple anthropogenic activities are currently affecting the planet generating a global climate change. One of the consequences of global climate change is the decrease in seawater pH, known as ocean acidification. This phenomenon is generated by the increase in atmospheric CO_2 which leads to an increase in dissolved CO_2 in the ocean, decreasing seawater pH and perturbating the carbonate system (Gao et al., 2020). According to the prediction of the Food and Agriculture Organization of the United Nations (FAO), pH will decrease between 0.2 and 0.4 units by the end of this century (FAO, 2019) and this could have a great impact on marine life (Hall-Spencer et al., 2015). In this regard, it has been demonstrated that ocean acidification has the potential to reduce the calcification rate of marine calcifiers, such as corals, coccolithophores, or shellfish, and to exert a negative influence in a wide range of other processes such as growth and reproduction (Poloczanska et al., 2016). This reduction in seawater pH has been related to poor calcification in diatoms, generating physiological changes which diminish their population affecting also zooplankton communities (Gao et al., 2020). These alterations in phytoplankton and zooplankton have the potential to affect the entire ecosystem (Borics et al., 2021; Hébert et al., 2017; Vallina et al., 2017).

Another anthropogenic activity that is severely affecting the oceans is the improper management and disposal of plastic with the consequent plastic pollution (Plastics Europe, 2020). In particular, the amount of microplastics (MP; plastic particles under 5 mm) present in the aquatic environment is of increasing concern (Cole et al., 2011; Andrady, 2011). The generation and release of MP has been so pronounced in the last decade that it was predicted that by 2050 there will be more MP in the oceans than fish (Forum, 2016), representing a high risk to the marine environment (Khalid et al., 2021). Also, the ingestion and assimilation of MP at environmentally relevant concentrations has been shown to cause several adverse effects in aquatic life, such as stress, false feeling of satiation, reproductive complications, and reduced growth rate in several aquatic organisms (Andrady, 2017; Ma et al., 2020).

Therefore, the study of the impact of human activities on marine ecosystems has attracted considerable attention by the scientific community, aiming to determine the negative effects of the variety of stressors directly or indirectly released into the environment. This raises the necessity of experimental methods that allow analysing several stressors acting together in model organisms. Also, this implies the emulation of realistic scenarios aiming to truly determine the pressures organisms are exposed to in their environment (Tlili and Mouneyrac, 2021). These scenarios are often difficult to reproduce due to the high number of variables to adjust which, among other problems, requires experiments with a high number of treatments and replicates, considerably increasing their size and the space needed to carry them out (Boyd et al., 2018). The information obtained with model organisms allows a better understanding of the process under study but also might bring relevant information applicable to other organisms and, ultimately, to the ecosystem (Gao et al., 2020; Pérez-Ruzafa et al., 2018). Therefore, one of the current challenges in the field of ecotoxicology is to address the problem of determining the effects of a multiple-stressor environment on ecosystems through a suitable method to be carried out in the laboratory.

The sea urchin embryo test (SET) has been widely employed as a reference method to determine the effect of potential toxic agents in samples of seawater and sediments (Bellas and Beiras, 2005; Pereira et al., 2020; Rengifo et al., 2019; Sheppard Brennand et al., 2010; Trifuoggi et al., 2019). The SET is an excellent method presenting several advantages such as: high reproducibility, not using vertebrate animals, cost-effectiveness, low utilization of adult individuals, easiness to carry out in the laboratory or low requirements in terms of equipment, among others (Pagano et al., 2017). Sensitivity is also a key aspect when choosing a method for a toxicological assessment. In this regard, the SET has shown a high sensitivity to a wide range of physical and chemical pollutants (Beiras et al., 2001, 2012). Another approach commonly used in ecotoxicology is the sea urchin fertilization assay, but evidence suggests that the SET is a more suitable method for studying global climate change phenomena (Caetano et al., 2021; Pereira et al., 2020). The major drawback in the utilization of SET is the availability of gametes out of the spawning season, but this can be sorted by keeping adult sea urchins under culture conditions and inducing the gamete production with KCl injections (Luis et al., 2005). In addition, as sea urchins are key components of the ecosystem and second or third level predators, they are good indicators of the status of the ecosystem (Lawrence, 2013; Parra-Luna et al., 2020).

In this context, in a previous work we employed the SET to study the effect of MP and pH reduction under ocean warming conditions (Bertucci and Bellas, 2021). In that work, we firstly determined the best conditions of pH, temperature, and MP concentration to perform the bioassays. The water acidification method employed in Bertucci and Bellas (2021) does not take into consideration other changes in water chemistry, such as the decrease in alkalinity generated by the addition of a strong acid (Ulf et al., 2011). However, it allowed us to determine the effect of a higher concentration of protons in the media, which is one negative consequence of ocean acidification. Secondly, we observed that MP could aggravate the effect of pH reduction and that an increase in water temperature generated additional stress on *P. lividus* larvae after 48 h. It is our intention to initiate an organised study of this problem in the laboratory, developing all the necessary tools to analyse the combined effect of global climate change factors with that of other anthropogenic stressors. Therefore, in the present study, we aim to determine the effect of ocean acidification and MP on the growth and development of sea urchins, at critical stages of early development. To do this, we employ an easy method to acidify seawater recreating the ocean acidification conditions. Moreover, two informatic apps, developed by our group, that allow users to quickly process data on the growth and morphology of *P. lividus* larvae, are presented.

2. Materials and methods

2.1. Biological material

P. lividus were collected from a natural population inhabiting the outer part of the Ría of Vigo (Galicia, NW Iberian Peninsula) in the late spring (May to June). Around 20 males and 20 females were maintained in 100 L tanks, with a natural photoperiod and natural seawater in flow-through conditions (temperature 14 ± 2 °C, pH = 8.1 ± 0.1) until its utilization. Individuals were fed daily with green and brown algae (*Ulva lactuca*, *Laminaria* sp.).

2.2. Microplastic reagent preparation

Solutions were prepared according to Bellas and Gil (2020) and Bertucci and Bellas (2021), with slight modifications. Data about MP and MP solution preparation is reported following the criteria of de Ruijter et al. (2020). The MP employed were high density 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. MP stock was purchased from Cospheric (California, USA). Polystyrene was selected as is one of the MP most frequently found in oceans (Andrady, 2017). The particle size used is in accordance with the average size found in the ocean (Andrady, 2011). MP suspensions were prepared by addition of 1 mg of powder in 1 L of filtered seawater (1 μm), sterilised 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 Partikel mess und Analyse systeme GmbH - S4031GO, Rutesheim, Germany) and constituted the stock suspension of MP used to prepare the experimental treatments. The concentration of MP used in the experiments (3000 particles/mL) is 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) and was selected based on a previous work from our group (Bertucci and Bellas, 2021).

2.3. Water acidification protocol

Experimental treatments were acidified following the recommendations of Ulf et al. (2011). According to this guide and to the prediction of the FAO, by the end of this century the dissolved inorganic carbon (DIC) will increase in 126 $\times$ 10^{-6} mol per Kg of sea water (SW). Considering the SW density is close to 1 g/mL, this increase on DIC was achieved adding 126 $\times$ 10^{-6} mol of $NaHCO_3$ (Merck KGaA, Darmstadt, Germany) to 1 L of FSW (stock solution). This addition will also increase the Total Alkalinity (TA) of the stock solution in 126 $\times$ 10^{-6} equivalents. Therefore, 126 $\times$ 10^{-6} equivalents from a strong acid (HCl, PanReac, Barcelona, Spain) were added to the stock solution to compensate the TA increase. The pH was checked during 20 min after stabilization and the TA was measured (HI755, Hanna instruments, Madrid, Spain) 10 min before and after the acid addition to confirm that no changes were provoked by the treatment. TA, pH, O_2, and temperature were measured in water samples from each treatment at 0, 48, 72 and 96 h. Parameters related to carbonate chemistry such as concentration of dissolved CO_2, concentration of dissolved inorganic carbon (DIC), partial pressure of CO_2 (pCO_2), concentration of HCO_3^-, concentration of $CO_3^=$, aragonite saturation state (Ω Aragonite), and calcite saturation state (Ω Calcite) were calculated using the seacarb function in r (Gattuso et al., 2021).

2.4. Experimental procedures for the SET

The SET was adapted from the methods described by Saco-Álvarez et al. (2010). Gametes were collected from a single pair of mature *P. lividus* individuals to avoid genetic variation. Approximately 1 mL of oocytes were placed in a measuring cylinder containing 40 mL of FSW and fertilised with 10 μL of undiluted sperm. Five minutes later, three aliquots of 25 μL were observed at the microscope to determine fertilization success (indicated by the presence of a fertilization membrane) and the egg quantity. Fertilization success was 100% and egg density was 4000 ± 200 eggs/mL. Within 30 min after fertilization, eggs were delivered into 250 mL bottles containing the experimental treatments at a density of 30 embryos per mL. Bottles were tightly caped to avoid gas exchange. Each treatment was run in 4 replicates and consists of a control treatment with FSW ("Control"), MP at a density of 1000 particles/mL ("MP"), acidified FSW ("pH7.6"), and a combination of both MP 1000 particles/mL and acidified FSW ("MP + pH7.6"). Conditions of pH and Temperature (T) were the same in all treatments (pH = 8.1, T = 20 °C) except for treatment "pH7.6" in which pH was decreased (Table 1). Experimental solutions were renewed after 48 h. At 48 and 72 h larvae from all treatments were fed with 3750 cells/mL of *Isochrysis galbana* and 3750 cells/mL of *Tetraselmis chuii* (PAMAS Partikel mess und Analyse systeme GmbH - S4031GO, Rutesheim, Germany). After 48, 72, and 96 h of incubation at 20 °C, with no aeration, aliquots of 10 mL were transferred to 35 mL vials and fixed with 10 drops of 40% buffered formalin. Samples were kept in darkness until their analysis under an inverted microscope (Axiovert 40 CFL, Zeiss, Spain).

Growth measurements were taken as described in Beiras et al. (2012) and morphometric parameters were recorded as in Bertucci and Bellas (2021). Morphological measurements are shown in Fig. 1 A. Growth of larvae was calculated as the total length of larvae minus the diameter of eggs, in 35 larvae per replicate. To determine alterations in the body shape, measurements of body width (BW), longest postoral arm (POL), supra body length (SPR), 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 = 4/3π × [(S1 + S2)/4]3, in which S1 and S2 represent the vertical and horizontal diameter of the stomach (Fig. 1 A).

Table 1
Water physicochemical parameters at 0, 48, and 96 h of incubation. Oxygen, pH, temperature and alkalinity were measured, the concentrations of total phosphate and silicate were set to 0. All the other parameters were calculated using the seacarb package v.2.1.5 in r.

	Time (h)	Control	MP	pH7.6	MP + pH7.6
O_2 Mol × kg^{-1}	0	0.00023 ± 0.00001	0.00023 ± 0.00001	0.00022 ± 0.00001	0.00022 ± 0.00001
	48	0.00021 ± 0.00001	0.00021 ± 0.00001	0.00021 ± 0.00001	0.00019 ± 0.00001
	96	0.00022 ± 0.00001	0.00021 ± 0.00001	0.00022 ± 0.00001	0.00021 ± 0.00001
pH	0	8.16 ± 0.004 (a)	8.16 ± 0.004 (a)	7.73 ± 0.004 (b)	7.7 ± 0.01 (b)
	48	8.160 ± 0.0005 (a)	8.05 ± 0.008 (a)	7.66 ± 0.008 (b)	7.7 ± 0.01 (b)
	96	8.07 ± 0.006 (a)	8.02 ± 0.002 (a)	7.7 ± 0.02 (b)	7.69 ± 0.008 (b)
TA Mol × kg^{-1}	0	0.00252 ± 0.00001	0.00252 ± 0.00001	0.00246 ± 0.00001	0.00246 ± 0.00001
	48	0.00250 ± 0.00001	0.00250 ± 0.00001	0.00250 ± 0.00001	0.00250 ± 0.00001
	96	0.00251 ± 0.00001	0.002510 ± 0.000005	0.00251 ± 0.00001	0.00251 ± 0.00005
CO_2 Mol × kg^{-1}	0	1.06E-05 ± 0.0000001 (a)	1.06381E-05 ± 1E-07 (a)	3.2E-05 ± 3E-07 (b)	3.3E-05 ± 8E-07 (b)
	48	1.0257E-05 ± 0.0000001 (a)	1.4738E-05 ± 3E-07 (a)	3.9E-05 ± 8E-07 (b)	4.2E-05 ± 1E-06 (b)
	96	1.3281E-05 ± 0.0000001 (a)	1.53653E-05 ± 2E-07 (a)	3.2E-05 ± 2E-06 (b)	3.6E-05 ± 7E-07 (b)
DIC Mol × kg^{-1}	0	0.002121 ± 0.000002	0.0021919 ± 2E-06	0.0023482 ± 1E-06	0.0023501 ± 3E-06
	48	0.002167 ± 0.000003	0.0022959 ± 4E-06	0.0024121 ± 3E-06	0.0025716 ± 4E-06
	96	0.002230 ± 0.000005	0.002280 ± 3E-05	0.002389 ± 2E-05	0.0023909 ± 6E-06
pCO_2 μatm	0	326 ± 1 (a)	326 ± 1 (a)	1000 ± 10 (b)	1014 ± 25 (b)
	48	314 ± 5 (a)	447 ± 10 (a)	1189 ± 25 (b)	1292 ± 40 (b)
	96	414 ± 5 (a)	480 ± 10 (a)	1003 ± 60 (b)	1137 ± 25 (b)
HCO_3^- Mol × kg^{-1}	0	0.001940 ± 0.000005	0.001940 ± 5E-06	0.0022136 ± 1E-06	0.0022161 ± 5E-06
	48	0.001914 ± 0.000005	0.002082 ± 5E-06	0.0022829 ± 5E-06	0.0024347 ± 5E-06
	96	0.002007 ± 0.000005	0.002073 ± 1E-05	0.002248 ± 1E-05	0.0022584 ± 5E-06
$CO_3^=$ Mol × kg^{-1}	0	0.000240 ± 0.000001 (a)	0.0002405 ± 1E-06 (a)	0.0001020 ± 1E-06 (b)	0.0001009 ± 1E-06 (b)
	48	0.000242 ± 0.000001 (a)	0.0001989 ± 1E-06 (a)	0.000090 ± 1E-06 (b)	0.000094 ± 1E-06 (b)
	96	0.000208 ± 0.000001 (a)	0.0001921 ± 1E-06 (a)	0.0001090 ± 5E-06 (b)	0.0000960 ± 1E-06 (b)
Ω Aragonite	0	3.78 ± 0.01 (a)	3.78 ± 0.01 (a)	1.60 ± 0.01 (b)	1.59 ± 0.05 (b)
	48	3.81 ± 0.05 (a)	3.12 ± 0.05 (a)	1.41 ± 0.01 (b)	1.48 ± 0.05 (b)
	96	3.28 ± 0.05 (a)	3.03 ± 0.05 (a)	1.72 ± 0.05 (b)	1.51 ± 0.01 (b)
Ω Calcite	0	5.84 ± 0.05 (a)	5.84 ± 0.05 (a)	2.47 ± 0.01 (b)	2.45 ± 0.05 (b)
	48	5.90 ± 0.05 (a)	4.82 ± 0.05 (a)	2.18 ± 0.05 (b)	2.29 ± 0.05 (b)
	96	5.06 ± 0.05 (a)	4.67 ± 0.05 (a)	2.6 ± 0.1 (b)	2.33 ± 0.05 (b)

Data represents the mean value and standard error from the 4 replicates. (TA: Total Alkalinity. DIC: Dissolved Inorganic Carbon. Different letters denote statistical differences between treatments.

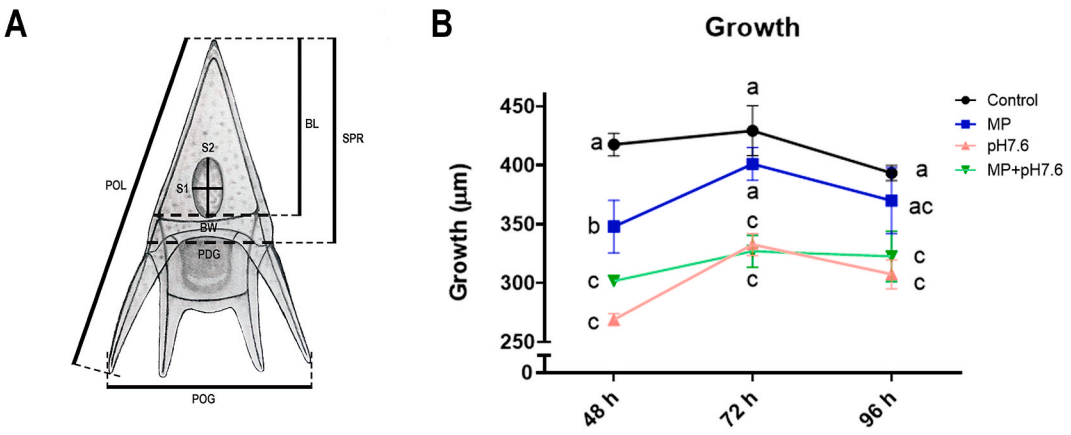


Fig. 1. (A) Morphological measurements of *P. lividus* larvae. POL: longest postoral arm. BL: total body length. SPR: supra body length. BW: body width. PDG: body width in the zone of the posterodorsal arms. POG: postoral arms' gap. S1 and S2 represent the vertical and horizontal diameter of the stomach. (B) Growth of *P. lividus* larvae under different microplastic and ocean acidification treatments at 48 h, 72 h, and 96 h. Growth expressed in µm relative to the mean size of embryos. Different letters denote statistical differences between groups assessed by one-way ANOVA follow by Tukey post hoc analysis test ($p < 0.05$).

2.5. Statistical analysis

Growth data were analysed 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). Larval length data collected with the imageJ software (.txt files), were uploaded and visualized for checking and resume statistics. Once data was uploaded and names were assigned to each treatment, the program generated bar graphs on growth, growth relative to the control treatment, and results from Normality (Shapiro-Wilk) and Homogeneity tests (Levene). The tab "Statistics" allows to perform an ANOVA and a Tukey test (if data is normally distributed). Alternatively, if the data did not follow a normal distribution, they were normalised and the ANOVA and Tukey tests were run again. This app, also contains an extra tab called "IC50" to do the IC50 (half-maximal inhibitory concentration) and EC50 (median effective concentration) calculations in concentration-response experiments. Morphological measures were expressed as a ratio between each measurement and "BL" using the Morpheus app, designed by our group and freely available at https://jignacio-ieo2020.shinyapps.io/Morpheus/. In this app, the morphological parameters of the larvae obtained in each treatment were uploaded in a.txt or.csv file and easily visualized together in the tab called "Data". Then, after names were assigned to each treatment, uploaded data was used to calculate the ratio between each parameter and "BL" and bar graphs were generated. Overall, the apps help to analyse large amounts of data and contribute to facilitate the use of the SET for toxicological determinations.

PCA-analysis and PCA-graphs were conducted and made in R (ggbiplot package, R Core Team, 2018). In this analysis, the 'variables' were the values of growth and morphometric ratios and the 'individuals' were the treatments (Control, MP, pH7.6 and MP + pH7.6) at 48, 72 and 96 h.

3. Results

3.1. Water physicochemical parameters

Water physicochemical parameters for each treatment, at different incubation times, are listed in Table 1. Control and MP treatments have a pH value around 8.1 while pH7.6 and MP + pH7.6 have pH values between 7.6 and 7.7. The DIC values in control and MP treatments were in the range of 0.0022–0.0023 mol × kg^{-1} and in pH7.6 and MP + pH7.6 treatments that value increased to 0.0024–0.0026 mol × kg^{-1}. The TA remains constant for all treatments at a value of 0.0025 mol × kg^{-1}. Dissolved oxygen does not change between treatments nor among incubation times. The omega (Ω) aragonite and calcite, which indicates the aragonite and calcite saturation state, are lower in pH7.6 and MP + pH7.6 treatments.

3.2. Growth and morphometric parameters

Growth of larvae reared under different treatments at 48, 72 and 96 h are represented in Fig. 1 B. At 48 h, larvae treated with MP showed a reduction in growth compared to the control group. Larvae from treatments pH7.6 and pH7.6 + MP showed a reduced growth compared to control group and compared to MP group. After 72 h and 96 h, larvae treated with pH7.6 and pH7.6 + MP showed a reduction in growth compared to control and to MP treatments.

After 48 h of incubation, BW/BL, PDG/BL, and POG/BL ratios were higher for larvae reared at pH 7.6 than for larvae from controls or any of the other treatments (Fig. 2 B, C, E). The BW/BL ratio in larvae from the pH7.6 + MP treatment was higher than in the control and MP treatments. No differences between treatments were observed in SPR/BL, POL/BL and SV/BL ratios (Fig. 2 A, D and F).

After 72 h, larvae from the pH7.6 treatment presented a lower POL/BL ratio, compared with control and MP (Fig. 3 D). The POG/BL ratio was lower in larvae from the MP treatment compared with controls and all the other treatments (Fig. 3 E). No differences were found in SPR/BL, BW/BL, PDG/BL, and SV/BL ratios after 72 h of incubation, although an increasing trend was observed for SV/BL in MP, pH7.6 and MP + pH7.6 treatments (Fig. 3A and B, C, F).

After 96 h, no significant differences between treatments were observed in the SPR/BL ratio (Fig. 4 A). However, MP, pH7.6 and MP + pH7.6 treatments provoked a decrease in BW/BL, PDG/BL, POG/BL and SV/BL ratios compared to controls after 96 h of incubation (Fig. 4B and C, E, F). The POL/BL ratio was lower in larvae treated with MP compared to controls, but higher than larvae from pH7.6 and MP + pH7.6 treatments (Fig. 4 D).

The PCA analysis shows that component 1 (PC1, horizontal axis) and component 2 (PC2, vertical axis) explain 45% and 21.8% of the variance of the data, respectively (Fig. 5). The first component is defined by high loadings of the variables POG, PDG, and BW (Fig. 5 A, Sup. Table 1), indicating that this component corresponds mainly to the width of the larvae (Fig. 1 A). The second component is defined by high loadings oft Growth and POL (Fig. 5 A, Sup. Table 1), indicating that this component corresponds mainly to the growth and body length of the larvae (Fig. 1 A). The variables POG, PDG and BW are positively correlated as well as Growth and POL (Fig. 5 A). Factors (cases) were clustered by experimental treatment (Fig. 5 B) and by exposure time (Fig. 5 C) in ellipses (95% confidence). The cluster formed by control is plotted parallel to

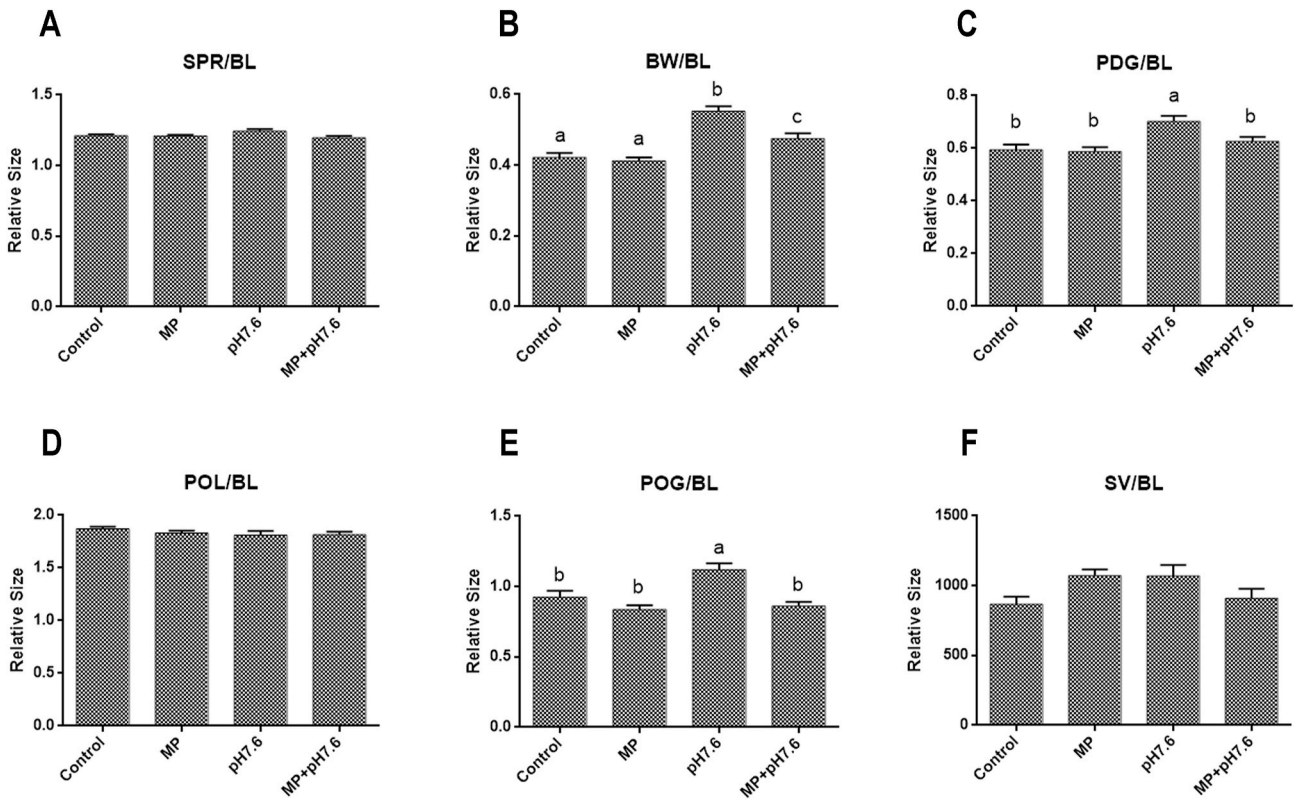


Fig. 2. Ratios between morphological parameters and the body length of *P. lividus* larvae after 48 h of incubation with different treatments. Supra body length (SPR), body width (BW), longest postoral arm (POL), total body length (BL), body width in the zone of the posterodorsal arms (PDG) and the postoral arms's gap (POG). (A) Supra body length relative to BL. (B) Body width relative to BL. (C) Width in the zone of posterodorsal arms relative to BL. (D) Length of the longest postoral arm relative to BL. (E) Width of postoral arms's gap relative to BL. (F) Stomach Volume relative to BL. MP: 3000 particles/mL. Different letters denote statistical differences between groups assessed by one-way ANOVA follow by Tukey post hoc analysis test ($p < 0.05$).

PC1 and separated from the other clusters, with cases mostly distributed between negative and positive values of this axis and in the positive side of PC2. The cluster MP is separated from pH7.6 and control clusters but overlaps with pH7.6 + MP. Cluster formed by MP is located parallel to PC2 with the majority of cases in the positive side of this axis and in the negative side of PC1. Clusters of pH7.6 and pH7.6 + MP are overlapped and located on the negative side of PC2 and distributed among the negative and positive side of PC1 (Fig. 5 B). When cases were clustered by time of exposure, groups formed by 48 h and 96 h were overlapped and mainly located in the negative side of PC2. The cluster that corresponds to 72 h of exposure was located in the positive side of PC1 and of PC2 and completely separated from the other clusters (Fig. 5 B).

4. Discussion

4.1. Acidification method for SET

The method for water acidification was adapted from Ulf et al. (2011) which recommended an increase in DIC using HCO_3^-, followed by the addition of a strong acid (HCl) which blocks the increase in TA. This method is recommended for closed systems to avoid the gas exchange that can modify the carbon chemistry. The overall idea is to emulate the ocean acidification conditions predicted by FAO (FAO, 2019) in terms of DIC-increase and pH-reduction, but without altering the TA. According to the results presented here, this method allows to easily recreate these conditions with minimum requirements in the laboratory. Thus, unlike other approaches which require more sophisticated equipment (e.g., CO_2 bubble system coupled with a pH-meter and pCO_2-meter; Foo et al., 2020; Johnson et al., 2020; Rodríguez et al., 2017; Sheppard Brennand et al., 2010; Stumpp et al., 2012), this is an ideal method to study the impact of ocean acidification using a simple procedure such as the SET. The addition of live food, such as phytoplankton, to the media, can increase the dissolved organic compounds derived from photosynthesis that contribute to the TA, especially when high biomass is added (Ulf et al., 2011). But no variations were found in the water parameters neither at 72 nor 96 h. According to our results, the acidification method proposed by Ulf et al. (2011) combined with the SET has the potential to be used in the study of ocean acidification at laboratory scale during 48 h (the standard SET incubation time, Beiras et al., 2012; Pagano et al., 2017; Saco-Álvarez et al., 2010), but also for longer times.

4.2. Growth and morphology of larvae exposed to microplastics under an ocean acidification scenario

In a previous work (Bertucci and Bellas, 2021) we demonstrated the stunning effect of low pH on *P. lividus* larvae, but the acidification method employed did not consider the increase in TA nor in DIC. In the present work we emulate ocean acidification conditions similar to those predicted by FAO (FAO, 2019) for future global climate change scenarios. Our results show that ocean acidification could severely affect the growth of *P. lividus* larvae. Rather than low pH or increased DIC, the saturation state of $CaCO_3$ has been shown to drive the negative impacts of ocean acidification on marine calcifying organisms (Foo et al., 2020; Johnson et al., 2020; Rengifo et al., 2019; Rodríguez et al., 2017; Zhao et al., 2018). Under-saturated seawater ($\Omega < 1$, $CaCO_3$) might be corrosive to calcifying organisms (Scott et al., 2010); however, any reduction in Ω increases the metabolic costs of creating skeletal structures out of $CaCO_3$ (Branch et al., 2013). The observed reduction in Ω-calcite and Ω-aragonite in the pH7.6 treatment might explain the observed reduction in larval growth. A reduction of water pH *per-se* has been related to

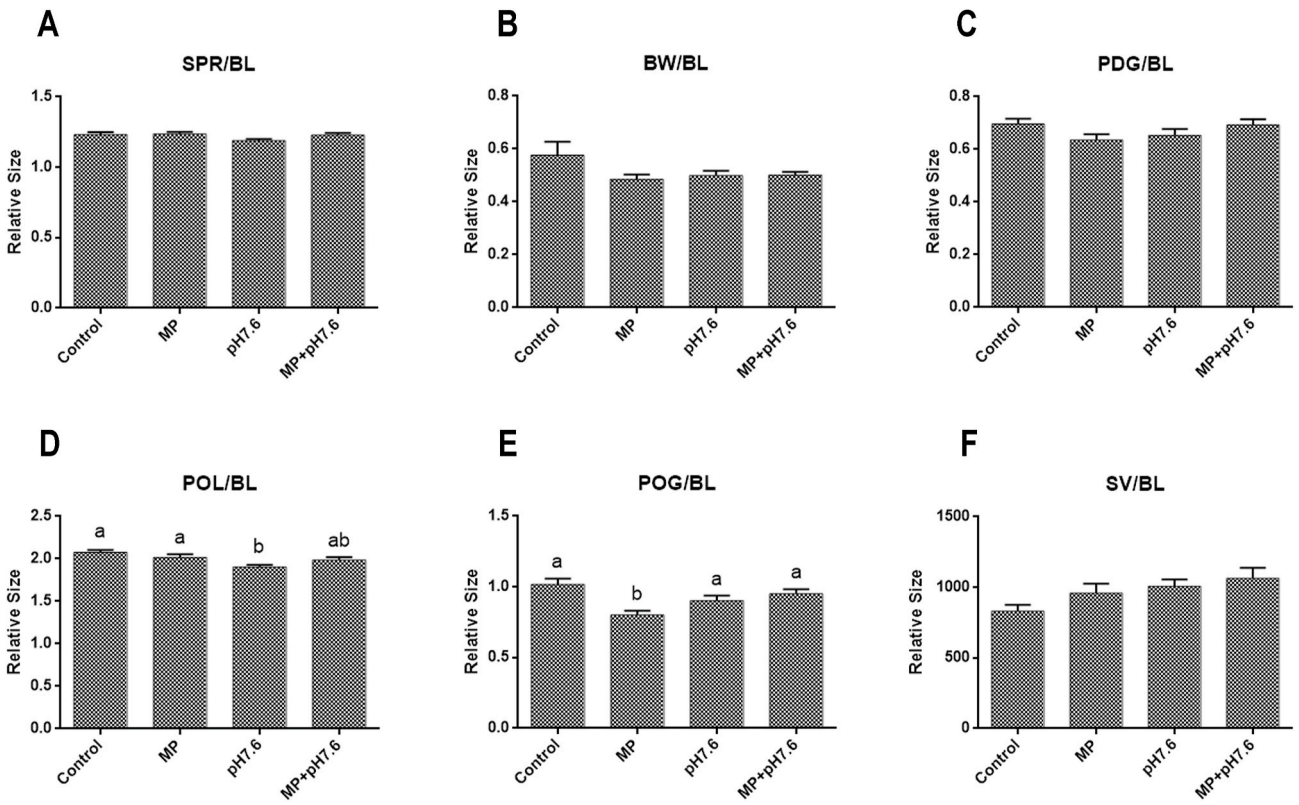


Fig. 3. Ratios between morphological parameters and the body length of *P. lividus* larvae after 72 h of incubation with different treatments. Supra body length (SPR), body width (BW), longest postoral arm (POL), total body length (BL), body width in the zone of the posterodorsal arms (PDG) and the postoral arms's gap (POG). (A) Supra body length relative to BL. (B) Body width relative to BL. (C) Width in the zone of posterodorsal arms relative to BL. (D) Length of the longest postoral arm relative to BL. (E) Width of postoral arms's gap relative to BL. (F) Stomach Volume relative to BL. MP: 3000 particles/mL. Different letters denote statistical differences between groups assessed by one-way ANOVA follow by Tukey post hoc analysis test ($p < 0.05$).

increased respiration rate, abnormal gene expression and oxidative response in sea urchin (Pagano et al., 2017). It was also found to generate morphological alterations and growth retardment in *P. lividus* larvae (Bertucci and Bellas, 2021). Sea urchin larvae are capable (within certain limits) of maintaining their inner pH despite external changes, but this consumes energy that is not available to anabolic processes. Consequently, the cost of maintenance of the inner conditions of the organism affects growth and increases mortality (Stumpp et al., 2012). Therefore, ocean acidification is a multifactorial problem (not only associated with the saturation state of $CaCO_3$) affecting several aspects of the organisms' biology.

The MP treatment generated a slight reduction in growth as it had been previously reported (Bertucci and Bellas, 2021) but, in this work we found that this reduction depends on the time of exposure. Supporting this result, the percentage of normal development of *Lytechinus variegatus* larvae was reduced in presence of virgin MP after 24 h of exposure (Nobre et al., 2015). Larvae from *Sphaerechinus granularis* did not show differences on growth compared to controls when incubated for 72 h with concentrations of MP of 0.1 and 1 mg/L (Trifuoggi et al., 2019). Although in Nobre et al. (2015) the time of exposure is lower compared with the present work, it seems that MP causes a higher impact at early stages of development in sea urchin. It has been demonstrated that *P. lividus* larvae are able to ingest MP after 48 h of exposure (Martínez-Gómez et al., 2017). Larvae from *Pseudechinus huttoni* exposed to MP (1000 particles/mL), exhibited a greater degree of oxidative damage compared to controls (Richardson et al., 2021). Authors concluded that oxidative damage was mostly related to MP ingestion, although the presence of MP in the media also exerts some level of damage. The mechanisms to avoid oxidative damage cost energy which is not available to growth and might explain the effect observed in our work in the first 48 h of exposure. Approximately 48 h post-hatching the energy reserves of the egg start to decrease, the digestive system of *P. lividus* larvae is fully developed and larvae start the digestion of phytoplankton (Gosselin and Jangoux, 1998). Additional energy uptake through feeding might temporarily counter the cost of growing in an adverse media. This is an interesting topic to investigate in the future and might help to elucidate the mechanisms of MP toxicity.

The combination of MP exposure and ocean acidification conditions did not produce any additional effect on growth than that caused by ocean acidification alone. This may be due to the type of interaction between both stressors. Antagonistic interactions have previously been reported in other species exposed to chemical and physical stressors (Dunne, 2010; Holmstrup et al., 2010). This kind of interaction is usually present when one of the stressors has a greater impact than the other (Darling et al., 2010). Concerning ocean acidification, Dong et al. (2020) found an enhanced tolerance to Cd driven by CO_2 acidification in *Phaeodactylum tricornutum*. Authors exposed the possibility that OA could change the biochemical mechanism that the diatom use to detoxify Cd. In a previous work (Bertucci and Bellas, 2021), we found a slight reduction in growth when larvae were treated with the combination of MP and low pH, compared with low pH alone. This might point out differences between the water acidification method employed in both experiments.

When analysing the effect at the morphological level after 48 h of incubation, the combination of both stressors generated a decrease in the ratio BW/BL compared to the control, which means that larvae are wider than in controls. However, the most pronounced effect was caused by the water acidification (pH7.6 treatment). This indicates that the effect of both stressors on this parameter is again non additive, and maybe antagonistic. The acidification of water after 48 h generated an

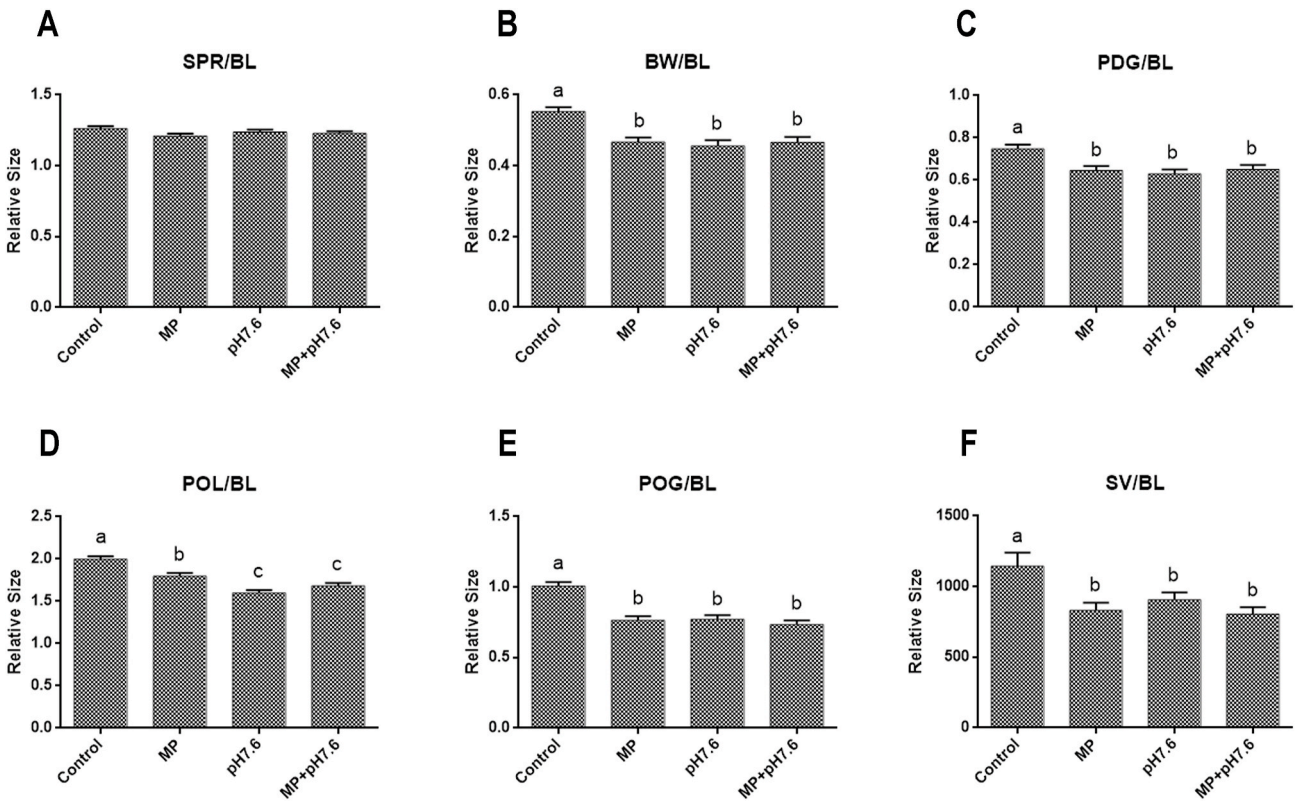


Fig. 4. Ratios between morphological parameters and the body length of *P. lividus* larvae after 96 h of incubation with different treatments. Supra body length (SPR), body width (BW), longest postoral arm (POL), total body length (BL), body width in the zone of the posterodorsal arms (PDG) and the postoral arms's gap (POG). (A) Supra body length relative to BL. (B) Body width relative to BL. (C) Width in the zone of posterodorsal arms relative to BL. (D) Length of the longest postoral arm relative to BL. (E) Width of postoral arms's gap relative to BL. (F) Stomach Volume relative to BL. MP: 3000 particles/mL. Different letters denote statistical differences between groups assessed by one-way ANOVA follow by Tukey post hoc analysis test ($p < 0.05$).

increase in the separation of larvae arms and therefore larvae from this treatment are wider than controls. After 72 h, the overall effect on larvae morphology was not as marked as at 48 h. The relative size of larvae arms seems to be shortened by acidification and MP increased the separation of the arms. Considering the acidification treatment, some morphological changes observed at 48 h seem to be mitigated after 72 h. As it was explained for growth, this would be attributed to the increase in the energy available to growth and develop caused by the ingestion of food together with the leftovers of the energy stores from the egg. But, larvae in the environment do not have the optimum amount of food as in the experimental conditions, so that this recovery effect might not be present in natural conditions. The only parameter altered by the pH7.6 treatment after 72 h was the POL/BL ratio, which means that larvae develop equally than the control, but the length of arms compared to the rest of body was altered. After 96 h, most of the treatments seem to alter the morphology of the larvae reducing the length, the size, and the separation of arms. Also, all the treatments reduced the stomach volume. Clearly, all of these perturbations start to have a marked effect on *P. lividus* larvae after 96 h and this might be related with feeding and development. As time post hatching increase, body structures are getting more complex and larger, which requires a higher amount of energy for maintenance (Marsh et al., 2013). A stressor present from the beginning of larval development may be a source of energy expenditure that is not available for growth and development, generating the adverse effects observed (Sokolova, 2013; Sokolova et al., 2012; Todgham and Hofmann, 2009). Overall, the change in the shape of *P. lividus* larvae can have a strong impact on their capacity for buoyancy and feeding and therefore, their chances to survive in their natural environment. Modifications in buoyancy could modify their position in the water column exposing larvae to suboptimal temperatures and light conditions (Hodin et al., 2016; Strathmann, 2006). Also, as larval food is usually available in a specific layer of the water column, changes on buoyancy can severely affect the ability of larvae to reach food (Strathmann, 2006). Moreover, the anatomical deformities observed after the time *P. lividus* larvae open their mouth could directly alter their ability to ingest food. This arises as an interesting topic to address in future studies.

Growth is one of the most important sublethal parameters to determine the effect of a stressor and therefore is one of the most employed end-point when performing the SET (Beiras et al., 2012; Qiao et al., 2003). A PCA analysis was performed to determine if some of the morphological parameters and growth at different times are correlated and if their alteration could have a predictive value (Jolliffe, 2005; Manton et al., 2005; Ringnér, 2008). This would allow using any of the parameters measured here at 48 h to predict the effect of a stressor at longer exposure times. The morphological parameter more related with growth is POL and therefore, it could be used to complement growth data. According with the PCA analysis, MP have a negative impact on parameters related to the width of larvae, while water acidification has a negative impact on growth and length of larvae. The presence of MP seems to attenuate the negative effect of water acidification on larval development. Effects provoked by all treatments at 48 h are related with those at 96 h. This might be due to the metabolic changes produced by the mouth opening around 72 h that was discussed previously. The impact of water acidification, MP, or their combination on growth and morphological parameters at 48 h seems to be a good indicator of what can be observed at 96 h.

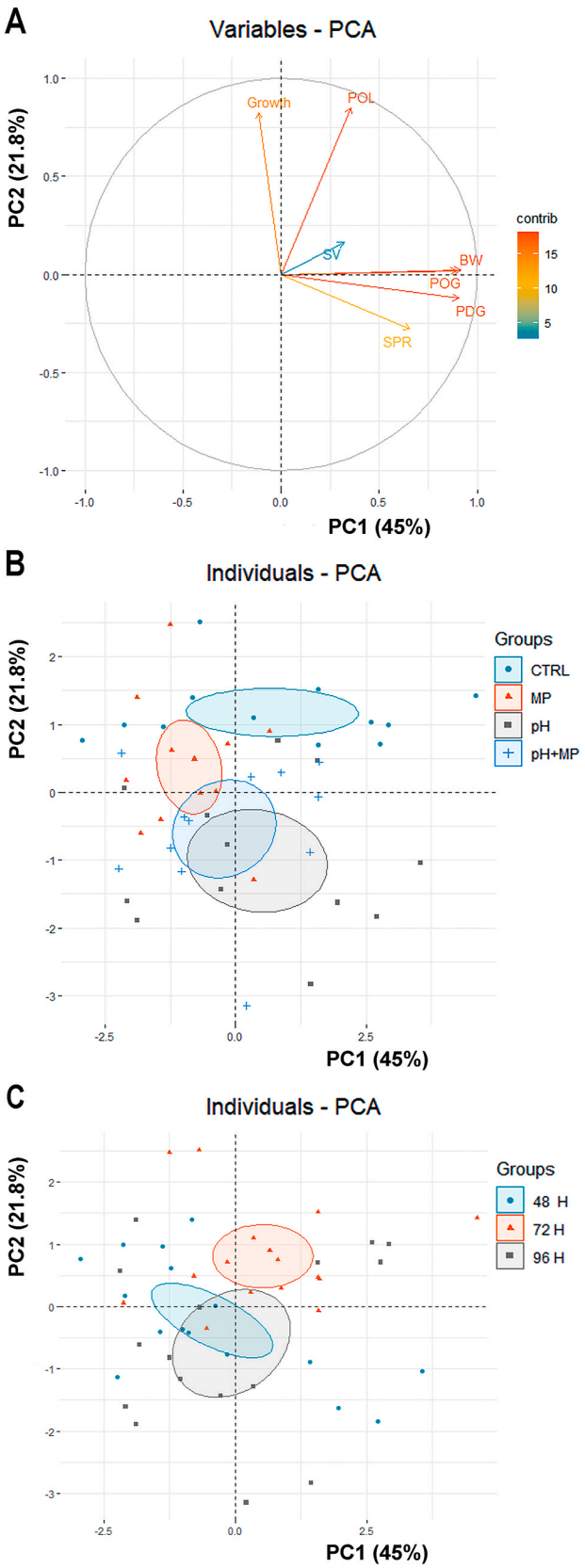


Fig. 5. (A) PCA plot of variables (morphologic parameters and growth). Colours in arrows indicate the contribution to each variable to both axes. (B) Cluster Plot of factors at 48, 72 and 96 h grouped by treatments. Colour ellipses indicate 95% of confidence. (C) Cluster Plot of factors at 48, 72 and 96 h grouped by time of exposure. Colour ellipses indicate 95% of confidence. PC1 and PC2 are represented by the horizontal and the vertical axis, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

5. Conclusion

This work is part of a larger framework that aims at assessing the use of the SET as a methodology for testing multi-stressor environments, particularly those including factors of global climate change. Through this approach, it has been demonstrated that ocean acidification and, to a lower extent, MP pollution, can cause alterations in the morphology and growth of *P. lividus* larvae. The combination of both MP and conditions of ocean acidification did not produce any additional effect on growth than that caused by ocean acidification alone. But some differences were observed at the morphological level. These negative effects are present at critical stages of their early development, including the moment in which larvae start to feed independently. Therefore, the chances of the sea urchin *P. lividus* to develop and survive in a predicted scenario of ocean acidification with an increasing concentration of MP are compromised, potentially threatening other components of the ecosystem.

Author contributions

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

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

Authors are grateful to Pedro Pousa and María Dolores Pampillón for his technical support in the experiments and to Pedro Pousa for drawing the larvae of *P. lividus* used in Fig. 1. This study was partially funded by the Spanish Government (Ministry of Science and Innovation) through the PLASMED Project (CTM2017-89701-C3-3-R). This project has received funding from the European Union's Horizon 2020 research and innovation programme under the Marie Skłodowska-Curie grant agreement No. 101027681.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2022.134783.

References

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

Andrady, A.L., 2017. The plastic in microplastics: a review. Mar. Pollut. Bull. 119, 12–22. https://doi.org/10.1016/j.marpolbul.2017.01.082.

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.

Bellas, J., Beiras, R., 2005. Embryotoxicity of the antifouling biocide zinc pyrithione to sea urchin (*Paracentrotus lividus*) and mussel (*Mytilus edulis*). Mar. Pollut. Bull. 50, 1382–1385. https://doi.org/10.1016/j.marpolbul.2005.06.010.

Bellas, J., Gil, I., 2020. Polyethylene microplastics increase the toxicity of chlorpyrifos to the marine copepod Acartia tonsa. Environ. Pollut. 260, 114059. https://doi.org/10.1016/j.envpol.2020.114059.

Bertucci, J.I., Bellas, J., 2021. Combined effect of microplastics and global warming factors on early growth and development of the sea urchin (Paracentrotus lividus). Sci. Total Environ. 782, 146888. https://doi.org/10.1016/j.scitotenv.2021.146888.

Borics, G., Abonyi, A., Salmaso, N., Ptacnik, R., 2021. Freshwater phytoplankton diversity: models, drivers and implications for ecosystem properties. Hydrobiologia 848, 53–75. https://doi.org/10.1007/s10750-020-04332-9.

Boyd, P.W., Collins, S., Dupont, S., Fabricius, K., Gattuso, J.P., Havenhand, J., Hutchins, D.A., Riebesell, U., Rintoul, M.S., Vichi, M., Biswas, H., Ciotti, A., Gao, K., Gehlen, M., Hurd, C.L., Kurihara, H., McGraw, C.M., Navarro, J.M., Nilsson, G.E., Passow, U., Pörtner, H.O., 2018. Experimental strategies to assess the biological ramifications of multiple drivers of global ocean change—a review. Global Change Biol. 24, 2239–2261. https://doi.org/10.1111/gcb.14102.

Branch, T.A., DeJoseph, B.M., Ray, L.J., Wagner, C.A., 2013. Impacts of ocean acidification on marine seafood. Trends Ecol. Evol. 28, 178–186. https://doi.org/10.1016/j.tree.2012.10.001.

Caetano, L.S., Pereira, T.M., Envangelista, J.D., et al., 2021. Impact on fertility rate and embryo-larval development due to the association acidification, ocean warming and lead contamination of a sea urchin *Echinometra lucunter* (echinodermata: echinoidea). Bull. Environ. Contam. Toxicol. 106, 923–928. https://doi.org/10.1007/s00128-021-03225-4.

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.

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 (2), 122–130. https://doi.org/10.1111/j.1755-263X.2009.00089.x.

de Ruijter, Vera N., Redondo-Hasselerharm, Paula E., Todd, Gouin, Koelmans, Albert A., 2020. Quality criteria for microplastic effect studies in the context of risk assessment: a critical review. Environ. Sci. Technol. 54 (19), 11692–11705. https://doi.org/10.1021/acs.est.0c03057.

Dong, F., Zhu, X., Qian, W., Wang, P., Wang, J., 2020. Combined effects of CO2-driven ocean acidification and Cd stress in the marine environment: enhanced tolerance of Phaeodactylum tricornutum to Cd exposure. Mar. Pollut. Bull. 150, 110594. https://doi.org/10.1016/j.marpolbul.2019.110594.

Dunne, R.P., 2010. Synergy or antagonism-interactions between stressors on coral reefs. Coral Reefs 29, 145–152. https://doi.org/10.1007/s00338-009-0569-6.

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.

Forum, W.E., 2016. World Economic Forum Annual Meeting 2016: the Fourth Industrial Revolution, Davos-Klosters, Switzerland 20-23 January. World Econ. Forum 1–47.

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.

Gattuso, J.-P., Epitalon, J.-M., Lavigne, H., Orr, J., Gentili, B., Hagens, M., Hofmann, A., Rae, J., 2021. Seacarb: Seawater Carbonate Chemistry.

Gorokhova, E., 2015. Screening for microplastic particles in plankton samples: how to integrate marine litter assessment into existingmonitoring 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.

Hall-Spencer, J., Thorndyke, M., Dupont, S., 2015. Impact of ocean acidification on marine organisms—unifying principles and new paradigms. Water 7, 5592–5598. https://doi.org/10.3390/w7105592.

Hébert, M.P., Beisner, B.E., Maranger, R., 2017. Linking zooplankton communities to ecosystem functioning: toward an effect-Trait framework. J. Plankton Res. 39, 3–12. https://doi.org/10.1093/plankt/fbw068.

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.

Holmstrup, M., Bindesbøl, A.M., Oostingh, G.J., Duschl, A., Scheil, V., Köhler, H.R., Loureiro, S., Soares, A.M.V.M., Ferreira, A.L.G., Kienle, C., Gerhardt, A., Laskowski, R., Kramarz, P.E., Bayley, M., Svendsen, C., Spurgeon, D.J., 2010. Interactions between effects of environmental chemicals and natural stressors: a review. Sci. Total Environ. 408, 3746–3762. https://doi.org/10.1016/j.scitotenv.2009.10.067.

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. Biol. Ecol. 523, 151250. https://doi.org/10.1016/j.jembe.2019.151250.

Jolliffe, I., 2005. Principal component analysis. In: Encyclopedia of Statistics in Behavioral Science. John Wiley & Sons, Ltd, Chichester, UK, pp. 1215–1220. https://doi.org/10.1002/0470013192.bsa501.

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.

Khalid, N., Aqeel, M., Noman, A., Hashem, M., Mostafa, Y.S., Alhaithloul, H.A.S., Alghanem, S.M., 2021. Linking effects of microplastics to ecological impacts in marine environments. Chemosphere 264, 128541. https://doi.org/10.1016/J.CHEMOSPHERE.2020.128541.

Lawrence, J.M., 2013. Sea Urchins: Biology and Ecology. Academic Press.

Luis, O., Delgado, F., Gago, J., 2005. Year-round captive spawning performance of the sea urchin Paracentrotus lividus: relevance for the use of its larvae as live feed. Aquat. Living Resour. 18 (1), 45–54. https://doi.org/10.1051/alr:2005004.

Ma, H., Pu, S., Liu, S., Bai, Y., Mandal, S., Xing, B., 2020. Microplastics in aquatic environments: toxicity to trigger ecological consequences. Environ. Pollut. 261, 114089. https://doi.org/10.1016/j.envpol.2020.114089.

Manton, K.G., Lowrimore, G., Yashin, A., Kovtun, M., 2005. Cluster Analysis: overview. In: Encyclopedia of Statistics in Behavioral Science. John Wiley & Sons, Ltd, Chichester, UK, pp. 105–125. https://doi.org/10.1002/0470013192.bsa101.

Marsh, A.G., Powell, M.L., Watts, S.A., 2013. Biochemical and energy requirements of gonad development. Dev. Aquacult. Fish. Sci. 38, 45–57. https://doi.org/10.1016/B978-0-12-396491-5.00004-6.

Martínez-Gómez, C., León, V.M., Calles, S., Gomáriz-Olcina, M., Vethaak, A.D., 2017. The adverse effects of virgin microplastics on the fertilization and larval development of sea urchins. Mar. Environ. Res. 130, 69–76. https://doi.org/10.1016/j.marenvres.2017.06.016.

Nobre, C.R., Santana, M.F.M., Maluf, A., Cortez, F.S., Cesar, A., Pereira, C.D.S., Turra, A., 2015. Assessment of microplastic toxicity to embryonic development of the sea urchin Lytechinus variegatus (Echinodermata: echinoidea). Mar. Pollut. Bull. 92 (1–2), 99–104. https://doi.org/10.1016/j.marpolbul.2014.12.050.

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. Indicat. 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.

Pérez-Ruzafa, A., Pérez-Marcos, M., Marcos, C., 2018. From fish physiology to ecosystems management: keys for moving through biological levels of organization in detecting environmental changes and anticipate their consequences. Ecol. Indicat. 90, 334–345. https://doi.org/10.1016/j.ecolind.2018.03.019.

Plastics Europe, 2020. Plastics – the Facts 2020. An analysis of European plastics production, demand and waste data. PlasticEurope 16. https://plasticseurope.org/knowledge-hub/plastics-the-facts-2020/.

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.

Qiao, D., Nikitina, L.A., Buznikov, G.A., Lauder, J.M., Seidler, F.J., Slotkin, T.A., 2003. The sea urchin embryo as a model for mammalian developmental neurotoxicity: ontogenesis of the high-affinity choline transporter and its role in cholinergic trophic activity. Environ. Health Perspect. 111, 1730–1735. https://doi.org/10.1289/ehp.6429.

R Core Team, 2018. R: A Language and Environment for Statistical Computing.

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.

Richardson, C.R., Burritt, D.J., Allan, B.J.M., Lamare, M.D., 2021. Microplastic ingestion induces asymmetry and oxidative stress in larvae of the sea urchin Pseudechinus huttoni. Mar. Pollut. Bull. 168, 112369. https://doi.org/10.1016/j.marpolbul.2021.112369.

Ringnér, M., 2008. What is principal components analysis? Nat. Publ. Gr. 26, 303–304.

Rodríguez, A., Hernández, J.C., Brito, A., Clemente, S., 2017. Effects of ocean acidification on juveniles sea urchins: predator-prey interactions. J. Exp. Mar. Biol. Ecol. 493, 31–40. https://doi.org/10.1016/j.jembe.2017.04.005.

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.

Scott, K.A., Clarke, A., Falkner, K., Hamilton, J., Jones, E.P., Lee, C., Petrie, B., Prinsenberg, S., Starr, M., Yeats, P., Atlantic, N., Sound, S., Strait, B., Bay, B., Strait, D., 2010. Calcium carbonate saturation states in the waters of the Canadian arctic archipelago and the Labrador sea. J. Geophys. Res.: Ocean 115, 1–18. https://doi.org/10.1029/2009JC005917.

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.

Sokolova, I.M., 2013. Energy-limited tolerance to stress as a conceptual framework to integrate the effects of multiple stressors. Integr. Comp. Biol. 53, 597–608. https://doi.org/10.1093/icb/ict028.

Sokolova, I.M., Frederich, M., Bagwe, R., Lannig, G., Sukhotin, A.A., 2012. Energy homeostasis as an integrative tool for assessing limits of environmental stress tolerance in aquatic invertebrates. Mar. Environ. Res. 79, 1–15. https://doi.org/10.1016/j.marenvres.2012.04.003.

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.

Tlili, S., Mouneyrac, C., 2021. New challenges of marine ecotoxicology in a global change context. Mar. Pollut. Bull. 166 https://doi.org/10.1016/j.marpolbul.2021.112242.

Todgham, A.E., Hofmann, G.E., 2009. Transcriptomic response of sea urchin larvae Strongylocentrotus purpuratus to CO2-driven seawater acidification. J. Exp. Biol. 212, 2579–2594. https://doi.org/10.1242/jeb.032540.

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.

Ulf, R., Fabry, V.J., Lina, H., Gattuso, J.-P., 2011. Guide to Best Practices for Ocean Acidification Research and Data Reporting. https://doi.org/10.2777/66906.

Vallina, S.M., Cermeno, P., Dutkiewicz, S., Loreau, M., Montoya, J.M., 2017. Phytoplankton functional diversity increases ecosystem productivity and stability. Ecol. Model. 361, 184–196. https://doi.org/10.1016/j.ecolmodel.2017.06.020.

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.

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.