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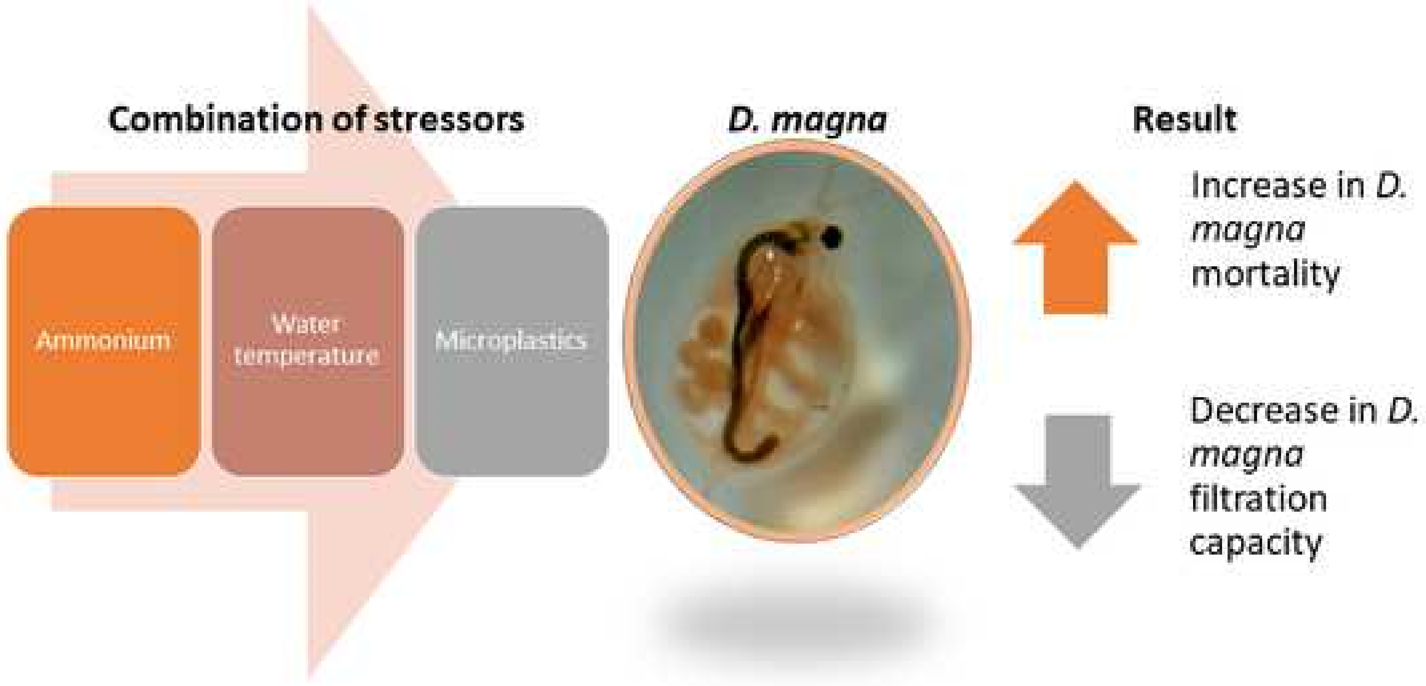
Combination of stressors
Ammonium
Water temperature
Microplastics
D. magna
Result
Increase in *D. magna* mortality
Decrease in *D. magna* filtration capacity

Synergistic effects of water temperature, microplastics and ammonium as second and third order stressors on *Daphnia magna*

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Abstract

Daphnids, including the water flea *Daphnia magna,* can be exploited for wastewater treatment purposes, given that they are filter feeder organisms that are able to remove suspended particles from water. The presence of pollutants, such as microplastics and chemicals, might be considered stressors and modify the behaviour and survival of *D. magna* individuals. The impact of the cumulative pollutants that regulate the fate of living organisms has yet to be fully determined. Here we present the effect of double and triple combinations of stressors on the behaviour of *D. magna*. The impact of water temperature, ammonium and polystyrene microplastics on the filtration capacity and survival of *D. magna* is studied. Water temperatures of 15 °C, 20 °C and 25 °C, microplastic-to-food ratios of 25% and 75%, and ammonium concentrations of 10 and 30 mg N-NH_4^+ L^{-1} are tested after making dual and triple combinations of the parameters. A synergistic effect between water temperature and ammonium is normally observed but not in the case of the lower values of ammonium concentration and temperature. The combination of three stressors (water temperature, microplastics and ammonium) is also found to be synergistic, producing the greatest impact on *D. magna* filtration capacity and reducing their survival. In comparison with the effect of the two stressor conditions, the combination of the three stressors caused a reduction of between 13.1% and 91.7% in the $t_{50\%}$ time (the time required for a 50% reduction in the *D. magna* filtration capacity) and a reduction of between 4.8% and 54.5% in TD50 (the time for 50% mortality).

Keywords: *Daphnia magna*, microplastics, *Daphnia* filtration, survival, wastewater tertiary treatment.

Main findings of the manuscript:

The coupling of three stressors (ammonium, microplastics and water temperature) resulted in a decrease in the *D. magna* filtration capacity and an increase in the *D. magna* mortality. The combination of stressors was found to act synergistically in all the cases studied, except those for the double combination of low ammonium concentration with low water temperature.

1. Introduction

Daphnids are planktonic crustaceans belonging to the Cladocera order that feed on small suspended particles with diameters below 30 μm (Burns, 1969; Pau et al., 2013), which make them suitable for the removal of suspended particles in wastewater as an alternative to conventional clarifiers. In addition, it has also been reported that daphnids act as disinfectants by reducing bacterial loads (Pous et al., 2020; Serra et al., 2014; Serra and Colomer, 2016). Such a low-cost, nature-based and sustainable tertiary treatment could contribute to promoting water reuse in zones where both water and economic resources are scarce. Although small suspended particles, bacteria and nutrients are removed from secondary effluents in a *Daphnia*-based reactor, the vulnerability of *D. magna* to different stressors, including water temperature and nutrients, might compromise the use of *D. magna* for wastewater tertiary treatments. Whilst our interest in evaluating the effect of these stressors on *Daphnia* is for their application in the treatment of wastewater, the findings will also be of ecotoxicological interest.

Ecosystems are being contaminated by a wide range of different pollutants, including ammonium and other nitrogen species from the agro-production sector (Green et al., 2004; Kremser and Schnug, 2002). It is worth noting that the effect of chemicals on biological organisms not only depends on their toxicity and concentrations, but also on their interaction with others factors such as water temperature and the presence of microplastics (Goussen et al., 2016). There are different theoretical approaches that attempt to explain the effect of the combination of different stressors, ranging from the view that the combined effect is equal to the sum of the individual effects (additive) to others that argue that either a combination may result in a reduced final effect (antagonistic) or, on the other hand, an enhanced final effect (synergistic) (Crain et al., 2008). Interactions between stressors that depart from the additive model are particularly important for ecological risk assessments (ERA) given that they deviate from predictions (Kimberly and Salice, 2014) and might have an impact on the efficiency of the wastewater treatment. Therefore, in order to obtain a realistic appreciation of the impact of stressors it is necessary to study them both as individual elements and as combinations (Lange and Marshall, 2017). Building on our previous work into the response of Daphnids to individual stressors in tertiary sewage treatment the present study investigates the effects of combinations of stressors. Single stressor effects on *D.magna* has been investigated in previous studies of our research group. In concrete, the effect of the increase in water temperature on the development of *D. magna* populations has been studied in the 11 °C to 29 °C range (Müller et al., 2018), with it being found that water temperatures above 29 °C are lethal for *Daphnia* after 5 d of exposure, with an optimum filtration capacity at 20 °C. Moreover, the use of fertilizers is especially harmful to *D. magna* individuals (Constable et al., 2003; Erisman et al., 2008). Sustained levels of ammonium above 30 mg $N\text{-}NH_4^+$ L^{-1} or nitrite concentrations above 6 mg $N\text{-}NO_2^-$ L^{-1} at optimal water temperatures of 20 °C have been found to be lethal for *D. magna* (Serra et al., 2019c) while other nutrients such as organic matter also are lethal at high concentrations (>250 mg COD L^{-1}) (Pous et al., 2020). However, the adverse effect of nutrients such as nitrogen or organic matter on *D. magna* might take place at lower concentrations when they are coupled with other stressors. For example, Maceda-Veiga et al. (2015) found that levels of nitrate above 250 mg NO_3^- L^{-1} (56.5 mg $N\text{-}NO_3^-$ L^{-1}) together with water temperatures of 26 °C reduced the filtration capacity, body size and fecundity in comparison with the experiments carried out with single stressors. Moreover, microplastics and other emerging contaminants from new consumer products (Browne et al., 2007; Thompson et al., 2010) and rising water temperature (global ~~warning~~warming) as a result of burning fossil fuels (O'Beirne et al., 2017) also affect ecosystems. Primary wastewater treatment has been found to reduce microplastic content by 78.3% (Hale et al., 2020). The resulting residues are normally sent to landfill sites where they are transferred to soils or aquatic ecosystems through run off (Jan Kole et al., 2017). The survival of *D.magna* under the presence of microplastics was found to sharply decrease as water temperature increased (Jaikumar et al., 2018), indicating that these stressors acted synergistically when they were combined. Therefore, the combination of multiple stressors on *D. magna* might determine their fate and the efficiency of the water treatment. Besides nutrient loads and water temperature, other possible stressors, whether identified or not, may play a role. It has recently been reported that microplastics in freshwater bodies have an adverse impact on living organisms, especially those that feed on particles (Andrady, 2011; Bosker et al., 2019; SAPEA, 2019). The effect of microplastics on *D. magna* differs considerably between studies (Bosker et al., 2019; Imhof et al., 2017; Martins and Guilhermino, 2018; Ogonowski et al., 2016). The increase in the ratio of microplastics to food (phytoplankton) has been found to reduce the *D. magna* filtration capacity and even cause their death after long exposure (above 7 d) (Colomer et al., 2019). The toxic effects of Cadmium on *D. magna* have been found to be variable depending on the food levels and the temperature of the water (Heugens et al., 2006) as have the effects of carbamate insecticide on *D. magna* (Cambronero et al., 2018). The influence of single, double and triple combinations of predation thread, parasitism and pesticide exposure on the behaviour of *D. magna* has also been studied and different antagonistic, synergistic and additive effects have been found (Coors and De Meester, 2008).

In this study, the impact of the combination of three stressors – microplastic particles (MP), ammonium and water temperature – on *D. magna* filtration capacity and survival is evaluated. The two-coupled and three-coupled stressor effect, with water temperature considered as a third stressor, will be compared in order to determine the precise manner in which the combination of stressors act and the quantification of these effects on *D. magna* population. .

2. Methods

2.1. *Daphnia magna.*

D. magna were obtained from laboratory cultures maintained for more than three years in three 40 L tanks of mineral water rich in calcium (35.7 mg L^{-1}) (Riessen et al., 2012). The water temperature in the tanks was maintained at 20.0 ± 0.5 °C and natural daylight photoperiods of 8 h light and 16 h darkness with a continuous air supply to avoid anoxia. *Daphnia* were fed three times a week with a mixture of dry spirulina powder (100% *Spirulina platensis*, KeyPharm, Belgium) and bakers' yeast (*Saccharomyces cerevisiae*, Mondeléz International, Spain) with a total particle concentration for each tank of 4.2 mg L^{-1}. One third of the water from each aquarium was renewed once every fifteen days (Müller et al., 2018; Serra et al., 2018; Serra et al., 2019a) with the same calcium-rich mineral water .

Since the filtration of *D. magna* individuals correlates with their body length (Serra et al., 2019b) and with the knowledge that their maximum body length is achieved in the first two weeks of life (Wickramarathna et al., 2014), all laboratory tests were carried out with 10 day old *D. magna* individuals. In order to control the age and length of the *D.magna* individuals in the tests, ephippia eggs from the laboratory tanks were hatched and left in darkness for nine days and then continuously exposed to light. The new-born *D. magna* were fed daily with spirulina until day 7 of the experiment. The mean *D. magna* body length, measured from the base of the spine to their head, was of 1.6 ± 0.1 mm. Their length was analysed at the beginning of each experiment and was monitored daily by the analysis of images from a video recording of 10 *D. magna* with ImageJ software (Serra et al., 2019a). Experiments were conducted at a photoperiod of 12 h of light and 12 h of darkness. The spirulina suspension was prepared by mixing 1 g of dry spirulina powder (with a density of 1.153 $g{\cdot}mL^{-1}$) with 1 L of mineral water for 1 min. This mixture was left unstirred for 60 minutes to allow the larger particles to settle. The supernatant volume was then used as the spirulina suspension to feed *D. magna*. All laboratory experiments, protocols and analyses with *D. magna* were carried out in accordance with the OCDE Test

Guidelines for Chemicals (OECD/OCDE, 2011) and the Spanish government's Sampling and laboratory protocol of benthonic invertebrates in lagoons (code ML-L-I-2013, 2013, Ministry of Agriculture, Food and Environment, Spain).

2.2. *Experimental set up*

For the experiments performed at water temperatures of 15 °C and 25 °C, *D. magna* were taken from the laboratory at 20 °C and acclimated for two days at the required testing water temperatures (15 °C and 25 °C). 1 L plexiglas containers placed in isolated chambers were externally thermostated to maintain the water temperature. Three replicates were performed for each experiment in containers with initial *D. magna* concentrations of 50 ind L^{-1}. Dead individuals were replaced by~~-~~ individuals that were pre-acclimated to the different conditions being tested. In each replicate, 50 mL of food (spirulina suspension) was added to 950 mL of mineral water with the following chemical composition: total dissolved solids = 206 mg L^{-1}, bicarbonate (HCO_3^-) = 165 mg L^{-1}, sulphates (SO_4^{2-})= 3.7 mg L^{-1}, chloride (Cl^-) = 18.8 mg L^{-1}, calcium (Ca^{2+}) = 78 mg L^{-1}, magnesium (Mg^{2+}) = 16.5 mg L^{-1}, sodium (Na^+) = 8.3 mg $L^{-1}$and silica (SiO_2) = 27.1 mg L^{-1}), resulting in a particle concentration of 8.4 ± 0.5 mg L^{-1} (8.0 ± 0.5 µL L^{-1}).

Polystyrene spherical microparticles with a density of 1.05 g mL^{-1} (SonTek/Xylem Inc., San Diego, USA) were used in the experiments with microplastics and their concentrations were in line with the range of microplastics concentrations found in urban wastewater treatment plants (Sun et al., 2019). Hence, two initial concentrations of microplastics with volume concentrations of 2.0 ± 0.1 µL L^{-1} (10^4 particles of MP L^{-1}) and 6.0 ± 0.5 µL L^{-1} (3×10^4 particles of MP L^{-1}) were prepared from a stock suspension. The particle volume concentration was measured with the laser particle size analyser Lisst-100x (Sequoia Inc.) that operates with particle diameters between 2.5 and 500 µm (Serra et al., 2001). In both experiments, *Daphnia* were fed with spirulina that was added to the microplastic suspension until reaching an initial volume concentration of 8 µL L^{-1} of particles (microplastics + Spirulina) in each of the two experiments, corresponding to a 25 % microplastics:75 % food ratio and to a 75% microplastics:25 % food ratio. In the experiment with 75% of food, the concentration of spirulina was ~~2.1~~6.1± 0.2 µL L^{-1} ~~mg L^{-1}~~ and for the experiment with 25% of food it was 1.9± 0.3 µL L^{-1}~~6.3 mg L^{-1}~~. The concentration of food used was of the same order as that used in the laboratory tanks. The media for all the experiments was renewed daily with the corresponding food to microplastics ratio.

The results of the particle volume distributions obtained with the laser particle analyser show that both spirulina and microplastics in the initial volume concentration tested had particles within the ingestible range of *D. magna* (<30 μm), presenting similar distributions in the 5-100 μm range (Figure 1). However, MP had a median diameter of 21.6 μm whereas spirulina particles had a median of 30.5 μm. Spirulina also had particles with diameters >100 μm and diameters <5 μm were found for MP. The volume concentration was used as the key parameter to characterize the concentration of food and MP as we have used in previous studies (Colomer et al., 2019; Müller et al., 2018; Serra et al., 2019b, 2018).

Since *D. magna* primarily feed on particles with diameters <30 μm, they are expected to feed on both MP and spirulina since they do not distinguish between different qualities of food (Arruda et al., 1983; Gliwicz, 1990). The volume concentration of particles within the range of 2.5 to 30 μm was calculated as the sum of all the particle concentrations measured in this range and used as a proxy to evaluate particle removal by *D. magna* (Burns, 1969; Pau et al., 2013).

In the experiments dealing with ammonium as a stressor to *D. magna*, nitrogen dosages in the form of ammonium (N-NH_4^+) with concentrations of 10 and 30 mg N-NH_4^+ L^{-1} were obtained from NH_4Cl by adding the amount required to reach the desired concentration into a beaker containing mineral water. The range of ammonium concentrations tested corresponded to the range of concentrations found in wastewater (Metcalf et al., 2002).

In the case of experiments with a combination of two stressors where one of these was water temperature (i.e. for temperatures of 15 °C and 25 °C), *D. magna* individuals were previously acclimated to the required temperature over a two-day period. The second stressor, N-NH_4^+ or MP, was introduced the next day following the previously described procedure. For experiments with MP and NH_4^+ at 20 °C, the desired NH_4^+ concentration was obtained by adding solid NH_4Cl to 950 mL of mineral water followed by 50 mL of the corresponding amounts of MP and spirulina in order to achieve the desired MP:food in the final solution. Experiments with three stressors were all carried out at a water temperature of 25 °C. *Daphnia* were first acclimated for two days to this temperature and on the following day the two other stressors were introduced together following the procedure explained above.

2.3. *Filtration capacity and survival.*

The *D. magna* filtration capacity (F, in mL ind^{-1} L^{-1}) was determined from $F = \frac{k_{Dph}}{C_{Dph}}$ (Pau et al., 2013), where C_{Dph} is the *D. magna* concentration and k_{Dph} is the rate of particle removal by *D. magna* (Pau et al., 2013). To quantify the rate of particle removal from the suspensions with *D. magna* individuals, measurements of the particle concentrations at different time intervals were made with a laser particle analyser. The ingestion rate of *D. magna* for all experiments was determined after 4 h since filtration by the *D. magna* individuals caused an exponential decrease in suspended particle concentrations (Pau et al., 2013). In order to only consider the reduction in the particle concentrations due to the *D. magna* filtration activity , control experiments were carried out without *Daphnia* to evaluate the reduction due to the sedimentation (Pau et al. 2013).

For the case of two or three stressors, the theoretical filtration for an additive behaviour ($F^{add}_{x,y,z}$, where x, y, z are the stressors considered) was calculated as the sum of the *D. magna* filtration capacity in the presence of each of these single stressors ($F^{add}_{x,y,z} = F_x + F_y + F_z$) when acting alone. Therefore, the experimental value of the filtration of combined stressors ($F^{exp}_{x,y,z}$) was compared to the theoretical filtration (additive behaviour) and the percentage of difference (ΔF(%)) was calculated as:

$$\Delta F(\%) = \frac{\left(F^{add}_{x,y,z} - F^{exp}_{x,y,z}\right)}{F^{add}_{x,y,z}} 100 \quad (4)$$

Therefore, ΔF(%)>0 indicates that the theoretical filtration of either two or three stressors is greater than the experimental filtration measured in the presence of the combined stressors, indicating that in such cases they act synergistically. In contrast, ΔF(%)=0 indicates that $F^{add}_{x,y,z} = F^{exp}_{x,y,z}$, i.e. that the combination of stressors have an accumulative effect. Cases with ΔF(%)<0 indicate that the experimental filtration is greater than the accumulation and, hence, that the effect is antagonistic.

The time, $t_{50\%}$ in days, at which $F^{exp}_{x,y,z}(t_{50\%}) = (1/2)\, F^{exp}_{x,y,z}(0)$ was calculated by fitting the seven-day experimental results to a second order polynomial. Low values of $t_{50\%}$ correspond to synergistic stressors while high values of $t_{50\%}$ correspond to an accumulative behaviour of the combined stressors.

D. magna individuals were counted every day in each of the replicates of all experiments and the time required to reduce the number of individuals by 50% (TD50) was calculated from the temporal evolution of the number of *D. magna* per litre.

2.4. *Statistical analysis*

The Shapiro-Wilk test was conducted to check for data normality and it was transformed accordingly to fulfil the normal distribution criteria. The Levene test was conducted to test data homogeneity. After these tests, a three-way analysis of variance (ANOVA) was carried out to study the effects of single, double and triple stressors on both TD50 and $t_{50\%}$ (results presented in Table 2).

3. Results

3.1 Survival of D. magna when faced with different stressors

The half time of survival, TD50, varied between the combination of stressors, with significant differences for all the cases (Table 2). In general, the combination of three stressors significantly reduced the TD50 (Figure 2a and 2b) in comparison with the combination of two stressors. For example, for an ammonium concentration of 10 mgN-NH_4^+ L^{-1} and 75% microplastics at 25 ºC, the TD50 was lower than for the experiments carried out at the same water temperature and microplastic ratio without ammonium (Figure 2a). The same applied in the case of a high ammonium concentration of 30 mgN-NH_4^+ L^{-1}. However, TD50 for 30 mgN-NH_4^+ L^{-1} was lower than all the cases of 10 mgN-NH_4^+ L^{-1}. TD50 for experiments carried out at 25% MP was also greater than that for 75%MP (Figure 2b). For all the experiments, TD50 at 25 °C was always lower than that for 15 °C (Figures 2a and 2b).

3.2 Effect of the combination of stressors on the D. magna filtration capacity

The results obtained are discussed by comparing the differences between the theoretical filtration, calculated by assuming an accumulative effect of each stressor, and the experimental filtration capacity of Daphnids, as already explained in the methodology (section 2.3). The percentage difference ΔF between the experimental filtration and the theoretical one increased with time for all the cases except for the combination of the lower ammonium concentration (10 mgN-NH_4^+.L^{-1}) and the lower water temperature (15 °C), which remained nearly constant over 7 d (Figure 3a), indicating that no synergy between stressors was produced. However, ΔF increased with time for the experiments carried out with the higher ammonium concentration (30 mgN-NH_4^+ L^{-1}) and the two microplastic ratios of 25% and 75% at 15 °C (Figure 3a). The fastest increase in ΔF at this water temperature was obtained for experiments performed with 30 mgN-NH_4^+ L^{-1}, followed by the two experiments with microplastics. For the experiments carried out at 25 °C (Figure 3b), ΔF also increased with time following the same sequence as was found for 15 °C (Figure 3a). However, in the case of 10 mgN-NH_4^+ L^{-1} a slight increase over time was obtained at 25 °C that was not observed in the case of 15 °C (Figure 3a).

The combination of two stressors (N-NH_4^+ and MP) at different concentrations at the optimal water temperature of 20 °C was found to act synergistically (Figure 3c). When water temperature was warmer, 25 °C (Figure 3d), ΔF for the combination of three stressors (N-NH_4^+ and MP plus T) indicated a stronger synergistic effect on the *Daphnia* filtration capacity. For the two water temperatures of 20 °C and 25 °C, the combination of a high ammonium concentration of 30 mgN-NH_4^+ L^{-1} and a high microplastic ratio of 75% resulted in the greatest increase in ΔF over time, followed by the combinations of 30 mgN-NH_4^+ $L^{-1}$and 25% of MP, 10 mgN-NH_4^+ $L^{-1}$and 75% of MP, and 10 mgN-NH_4^+ L^{-1} and 25% of MP (Figures 3c and 3d). However, the ΔF obtained for the experiments with the water temperature of 25 °C (Figure 3d) produced the fastest change with ΔF >50% in less than 28h except for the case of 25% of MP and 10 mgN-NH_4^+ L^{-1} that reached 50% after 72h.

The highest $t_{50\%}$ was at 10 mgN-NH_4^+ L^{-1}, followed by the experiments with 25% of MP, 75% of MP, and those carried out at the higher ammonium concentration of 30 mgN-NH_4^+ L^{-1}, respectively (Figure 4). The combination of three stressors gave lower $t_{50\%}$ than the combination of two stressors, indicating that the combination of three stressors was the most synergistic. Experiments carried out at water temperatures of 25 °C also had lower $t_{50\%}$ than experiments carried out at 15 °C. All the results obtained for $t_{50\%}$ presented significant differences (Table 2), indicating that single, double and triple stressors have significant effects on $t_{50\%}$.

3.3 Effect of the combination of stressors on D. magna growth rates

In the control experiments without the presence of either ammonium or MP and at 20 °C *D. magna* were observed to grow at a rate of 0.09 ± 0.02 mm d^{-1}. For experiments carried out at 25 °C they grew at a rate of 0.06 ± 0.02 mm d^{-1} and for experiments at 15 °C they grew at 0.07 ± 0.01 mm d^{-1} (Table 1). Despite these differences in growth rates were small, a one-way ANOVA analysis showed that they were significant ($p>0.05$). For the other experiments with double and triple combination of stressors, no *D. magna* growth rate was observed (Table 1).

4. Discussion

Cocktails of variables are likely to be found in the environment that might impact simultaneously on living organisms, which may operate synergistically, antagonistically or accumulatively (Crain et al., 2008; Cuevas et al., 2018). Although many studies are focused on the combination of two stressors, ~~the~~ just a few studies focus on the~~study of~~ the impact of three stressors ~~is unknown~~on *Daphnia magna* (Cambronero et al., 2018; Coors and De Meester, 2008; Heugens et al., 2006). The present study combines three different potential stressors and compares their combined effects with those of two stressors. The results show that the combination of microplastics and ammonium together with water temperature produced a synergistic effect on both *D. magna* filtration capacity and, at the same time, reduced the survival of individuals. The results also provide the thresholds for the combinations of these stressors that need to be considered when using *D. magna* as filtration organisms for wastewater treatment. The effect of the combination of ammonium, water temperature and microplastics was found to act synergistically in almost all cases. Although the tested concentrations for microplastics were higher than those found in the environment (Bayo et al., 2020; Mao et al., 2020; Rist et al., 2017), they are in the range of the concentrations found at the inlet of wastewater treatment plants ($1\text{-}10^4$ particles L^{-1}) (Sun et al., 2019). The concentrations of ammonium tested were also in the range of those found at the secondary effluent of sewage plants (Metcalf, 2002). Therefore, the results found here are representative of how *D. magna* behave in in tertiary wastewater treatment reactors.

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4.1 Combination of two stressors on *D. magna* filtering rate and survival

The increase in the percentage of suspended microplastics from 25% to 75% of MP resulted in a decrease in the filtration capacity and an increase in the mortality of *D. magna*. The blockage of the gut due to an accumulation of microplastics is the cause of the reduction in the food uptake, (Ogonoswki et al. 2016; Bosker et al. 2019).

The effect of the presence of microplastics coupled with ammonium produced a greater effect than the sum of each individual stressor, indicating that their combination resulted in a synergistic effect on the *D. magna* filtration. The greater reduction in the *Daphnia* filtration capacity for 75% of MP versus food compared with 25% of MP agrees with previous findings of reductions in filtration capacities, especially when the ratio MP:food was above 50% (Colomer et al. 2019). Previous studies have stated that zooplankton may show enhanced tolerance to some stressors depending on their diet (Gaudy et al., 2000; Maas et al., 2012; Seibel et al., 2012). Kim et al. (2017) studied the toxic effect of a mixture of nickel and microplastics on *D. magna*, finding that this combination always had a synergistic effect. The combination of the pesticide Carbaryl with a parasite also acted as synergistic stressors on *D. magna* survival (Coors and De Meester, 2008). Similar results have been obtained in the present study, where *D. magna* has been found to present synergistic responses in survival when different stressors act together.

Salinity acts as an environmental stressor for *D. magna* (Hall et al., 2013). However, when a parasite is introduced as a second stressor coupled with salinity, they act antagonistically on host survival and fecundity (Hall et al., 2013). In the present study, none of the combination of the stressors presented an antagonistic effect on *D. magna* filtration nor on *D. magna* survival. Other authors have speculated that high stressor levels might commonly produce synergistic effects whereas low stressor levels might produce antagonistic effects (Lange and Marshall, 2017). In the present study, microplastics and N-NH_4^+ still acted as synergistic stressors even at low concentrations (25 % MP and 10 mgN-NH_4^+ L^{-1}, respectively). Only in the case of low ammonium concentration (10 mgN-NH_4^+ L^{-1}) together with low temperature (15 °C) was the effect found to be accumulative.

These results of the *D. magna* growth rate were in agreement with the experiments carried out by Wickramarathna et al. (2014), where the mean *Daphnia* body length was found to grow at a rate of 0.08 mm d^{-1} at 20 °C. Therefore, during the seven days that the experiments lasted, we should have expected a growth of 0.56 mm in the *Daphnia* body length, which was not observed in any of the experiments conducted with the combination of two stressors, except for the double combination of 15 °C and 10 mgN-NH_4^+ L^{-1} (Table 1). The lack of growth of the *D. magna* was attributed to the impact of stressors on *D. magna* individuals, in line with findings when working with single stressors (Colomer et al., 2019; Serra et al., 2019c).

4.2 Combination of the three stressors on *D. magna* filtration and survival

D. magna has been found to be ubiquitous at different latitudes and thrive in waters at a wide range of temperatures (Yampolsky et al., 2014), being able to filter particles at different light/darkness ratios (Serra et al., 2019a). However, in the present study, water temperature has been demonstrated to work as a potential third stressor when it is not at the optimal *D. magna* filtration temperature, which has been found to be 20 °C (Burns, 1969; Müller et al., 2018). For example, the combination of 10 mgN-NH_4^+ L^{-1} and 75 % MP at 25 °C resulted in a greater ΔF and lower TD50 than the same experiment carried out at 20 °C. These results indicate that *D. magna* become more vulnerable when they are subject to different stressors and out of their optimum temperature range (Engert et al., 2013). This result agrees with the results of the model presented by Goussen et al. (2016) for the combination of two stressors, where the presence of one chemical compound had a greater impact in the tropics than in a more temperate region, demonstrating the importance of temperature. In a study coupling the presence of humic substances with different temperatures on the behaviour of another Cladocera (*Moina macrocopa*), Engert et al. (2013) found that above optimum, temperatures can act antagonistically whereas temperatures below the optimum can have a synergistic role.

In the present study with *D. magna*, the two water temperatures studied of 25 °C and 15 °C had a synergistic effect on *Daphnia* filtration and survival. As Engert et al. (2013) also found (2013), it was important to determine in each individual case whether non-optimal water temperatures stress the organism and how this stress is manifested. As was found for the combination of two stressors, *D. magna* did not present body growth during the days that experiments were carried out when three stressors were combined (Table 1). The TD50 results for *D. magna* indicate that their survival was lower in warmer water temperatures (25 °C) but at the same concentration of MP or/and N-NH_4^+ as in cooler water temperatures (15 °C). This result is in agreement with the fact that, at warmer water temperatures, *Daphnia* have a higher food ingestion rate associated to a greater metabolic demand (Cambronero et al., 2018) and therefore, the presence of N-NH_4^+ or MP at these temperatures can be expected to enhance the ingestion of these contaminants, increasing the lethal effect on *D. magna*. However, at water temperatures of 25 °C, *Daphnia* have a lower filtration capacity than at 15 °C (Müller et al., 2018). Therefore, here the combination of warming and the presence of the water contaminant (MP and/or N-NH_4^+) had a synergistic effect.

Warmer water temperatures of 25 °C produce a greater presence of the non-ionized NH_3 form than a water temperature of 20 °C. For example, for the same pH=7.3, for the concentration of 30 mgN-NH_4^+ L^{-1} and 25 °C, the concentration of NH_3 was calculated and resulted 0.34 mgN-NH_3 L^{-1} while for a water temperature of 20 °C its concentration resulted of 0.23 mgN-NH_3 L^{-1}. These results are in line with Nørgaard et al. (2016), who found that a water temperature of 25 °C produced a feeding inhibition of 65% whereas at 20 °C it was 40%. Similarly, Chen et al. (2012)found that water temperatures above 25 °C and below 10 °C adversely affected *D. magna* populations, reducing their mobility. Further similarity with our results was found by Kimberly and Salice (2014) who studied the combination of temperature with cadmium on the survival of the snail *Physa pomilia*, finding that temperature increased the toxicant sensitivity of the snail against cadmium.

In the present study, the additional third stressor was found to produce an adverse effect on *Daphnia* filtration compared to the case of two stressors. For the three stressors experiments, the characteristic time $t_{50\%}$ was reduced in the range of 13.1% to 91.7% compared to the case of the combination of two stressors. The highest reduction corresponds to the experiments performed with the combination of 25 °C, 75 % MP and 30 mgN-NH_4^+ L^{-1}. In contrast, the lowest reduction corresponds to the combination 25 °C, 25 % MP and 10 mgN-NH_4^+ L^{-1}. An additional reduction in *D. magna* survival was also observed for the experiments with three stressors compared with the case of two stressors. In these cases, the characteristic TD50 time for the combination of three stressors was reduced in the range of 4.8% to 54.5% times that found for the combination of two stressors. Like it was found for $t_{50\%}$, the highest reduction in TD50 corresponds to the experiments performed with the combination of 25 °C, 75 % MP and 30 mgN-NH_4^+ L^{-1}. In contrast, the lowest reduction corresponds to the combination 25 °C, 25 % MP and 10 mgN-NH_4^+ L^{-1}. Therefore, non-optimal temperatures produce adverse effects, magnifying the effect of the combination of stressors on both *D. magna* survival and *D. magna* filtration. This is in accordance with previous results on the effect of temperature when working as a third stressor, increasing the toxic effects induced by variable food levels and cadmium (Heugens et al., 2006), or magnifying the adverse effects of carbamate insecticide and variable food levels (Cambronero et al., 2018).

5. Conclusion

In conclusion, the combination of ammonium, water temperature and microplastics resulted in a synergistic effect on both *D. magna* filtration capacity and survival. Although *D. magna* have been found to be resilient to the effect of a single stressor, they are unable to cope with the combination of two or three pollutants stressors. In addition, *D. magna* filtration capacity and survival responded to ammonium and microplastics differently depending on the water temperature, with higher temperatures increasing the vulnerability of the *Daphnia* population when faced with more than one stressor. From an environmental perspective, we can postulate that the increased nutrient loads, water temperatures and concentrations of microplastics forecasted for the coming years will have a highly significant impact on *D. magna* survival and activity in aquatic ecosystems. From a technological perspective, it is to be envisaged that the confluence of these different stressors will have an adverse effect on the efficiency of *Daphnia*-based tertiary wastewater treatment as they take on increasing importance in the environment.

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Authors' individual contributions: Teresa Serra: Conceptualization; Data curation; Formal analysis; Methodology; Writing – original draft; writing-review and editing; Visualization. Aina Barcelona: Methodology; Investigation. Narcís Pous: Writing – review & editing. Victòria Salvadó: Resources; Writing – review & editing. Jordi Colomer: Supervision; Validation; Writing – review & editing.

Figure Captions

Figure 1. Particle volume concentration of two initial suspensions with 100% of food (Spirulina) and 100% of MP. The green area represents the range of particles ingestible by *D. magna* with d<30 μm.

Figure 2. Acute TD50~~-~~ for *D. magna* under the different experimental conditions tested for ammonium combined with the other stressors in double or triple forms (a) and MP with the other stressors in double or triple forms (b). In each figure stressors have been ordered from triple combinations, to double at the optimum temperature of 20°C and then double with temperature as a stressor. Error bars represent the standard deviation obtained among the three replicas. All the results had *p*-values <0.01 except those corresponding to the triple combination of stressors that had a *p*-value of <0.05 (Table 2).

~~. Error bars represent the standard deviation of the mean of three replicates.~~

Figure 3. Percentage of difference (ΔF) between the filtration obtained experimentally and the filtration predicted by the additive model for the different experimental conditions tested for double stressors at different water temperatures of T=15 °C (a), T=25 °C (b), and at T=20 °C (c) and the combination of three stressors (d). The horizontal dashed line represents the percentage of 50% of difference in ΔF.

Figure 4. $t_{50\%}$ time for each of the experimental conditions tested. Stressors are ordered from triple combinations, to double at the optimum temperature of 20 °C and then double with temperature as a stressor. Error bars represent the standard of the three replicates. All the results had *p*-values <0.01 except those corresponding to the double combination of NH4+ and MP that had a *p*-value of <0.05 (Table 2).

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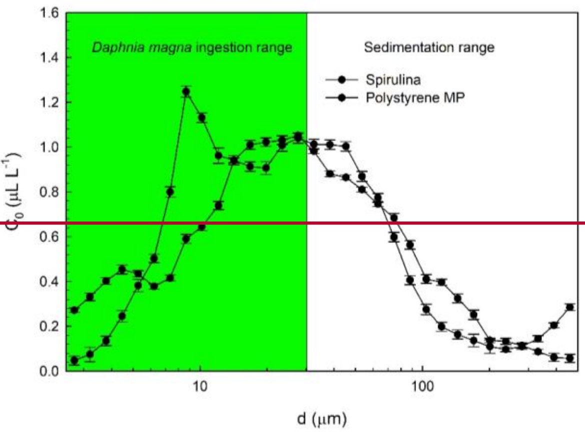

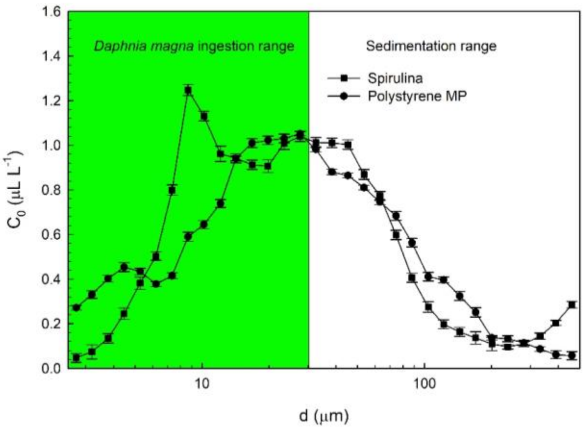

Figure 1. Particle volume concentration of two initial suspensions with 100% of food (Spirulina) and 100% of MP. The green area represents the range of particles ingestible by *D. magna* with d<30 μm.

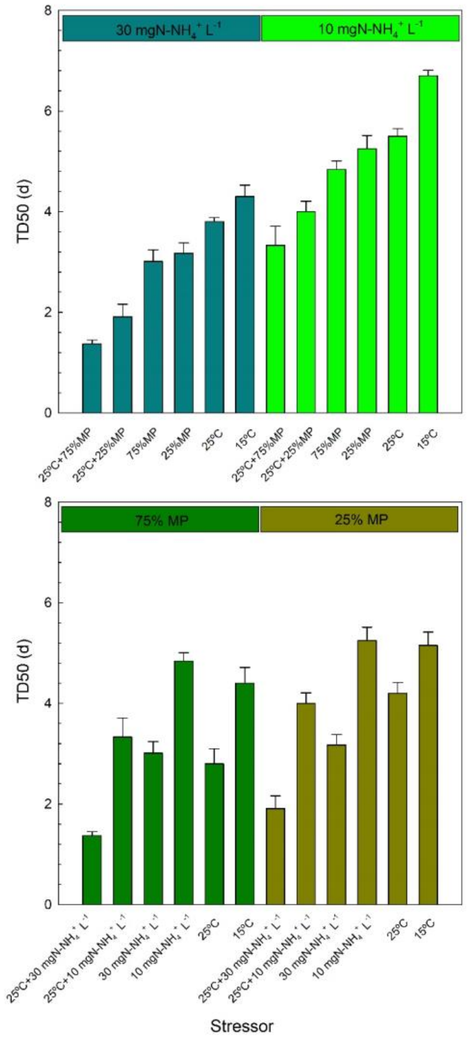

Figure 2. Acute TD50 for *D. magna* under the different experimental conditions tested of ammonium combined with the other stressors in double or triple forms (a) and MP with the other stressors in double or triple forms (b). In each figure stressors have been ordered from triple combinations, double at the optimum temperature of 20 °C and double with temperature being a stressor. Error bars represent the ~~mean~~ standard deviation obtained among the three replicas. All the results had *p*-values <0.01 except those corresponding to the triple combination of stressors that had a *p*-value of <0.05 (Table 2).

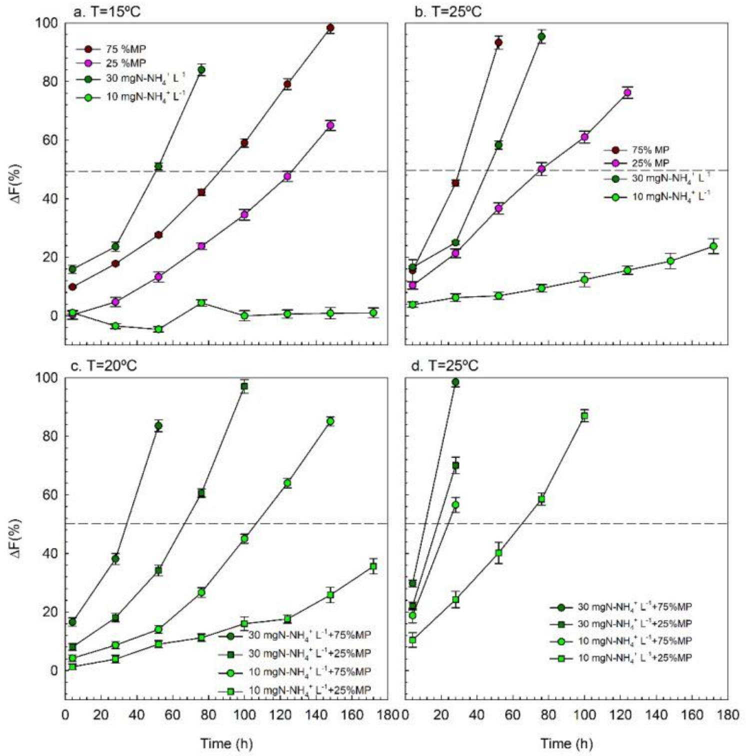

Figure 3. Percentage of difference (ΔF) between the filtration obtained experimentally and the filtration predicted by the additive model for the different experimental conditions tested of single stressors at different water temperatures of T=15 °C (a), T=25 °C (b), two stressors at T=20 °C (c) and the combination of three stressors (d). The horizontal dashed line represents the percentage of 50% of difference in ΔF.

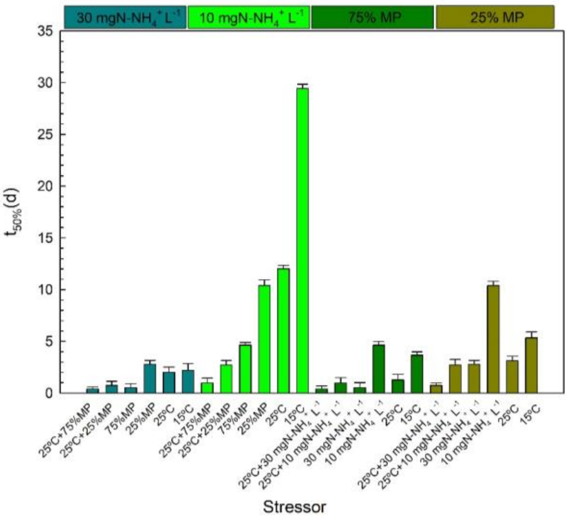

Figure 4. Time $t_{50\%}$ for each of the experimental conditions tested. In the figure, stressors have been ordered from triple combinations, double at the optimum temperature of 20 °C and double with temperature being a stressor. Error bars represent the standard deviation among the three replicas. All the results had *p*-values <0.01 except those corresponding to the double combination of NH4+ and MP that had a *p*-value of <0.05 (Table 2).

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Table 1. Summary of the experiments carried out with the different combination of the stressors considered and with the different dosages. The filtration of the first day of the experiment (F_{day1}, in ml ind^{-1} h^{-1}) and the *D. magna* growth rate (in mm d^{-1}).

Number of stressors	**Stressors tested**	**NH_4^+ content (mgN-NH_4^+ L^{-1})**	**MP(%)**	**T(°C)**	**F_{day1} (ml ind^{-1} h^{-1})**	**Growth rate (mm d^{-1})**
1	T	0	0	20	1.410 ± 0.002	0.09± 0.02
				25	0.888 ± 0.001	0.06± 0.02
				15	1.128 ± 0.003	0.07± 0.01
	MP	0	25	20	1.362 ± 0.001	0.00± 0.01
			75		0.940 ± 0.002	0.00± 0.01
	NH_4^+	10	0	20	1.388 ± 0.002	0.00± 0.01
		30			1.205 ± 0.004	0.00± 0.01
2	NH_4^+ + MP	10	25	20	0.816 ± 0.005	0.00± 0.01
		10	75		0.387 ± 0.004	0.01± 0.01
		30	25		0.674 ± 0.003	0.00± 0.01
		30	75		0.263 ± 0.001	0.00± 0.01
	NH_4^+ + T	10	0	15	1.070 ± 0.004	0.07± 0.01
		30		15	0.841 ± 0.003	0.00± 0.01
		10		25	0.839 ± 0.001	0.01± 0.01
		30		25	0.659 ± 0.005	0.00± 0.01
	MP + T	0	25	15	1.066 ± 0.004	0.00± 0.01
			75	15	0.586 ± 0.002	0.00± 0.01
			25	25	0.749 ± 0.003	0.01± 0.01
			75	25	0.321 ± 0.004	0.00± 0.01
3	NH_4^+ + MP + T	10	25	25	0.741 ± 0.005	0.01± 0.01

		10	75	25	0.332 ± 0.001	0.00± 0.01
		30	25	25	0.567 ± 0.002	0.00± 0.01
		30	75	25	0.223 ± 0.002	0.00± 0.01

Table 2. Degrees of freedom, F values and *p* values for the three-way ANOVA for the effects of the water temperature (T), the ammonium concentration (in mgN-NH_4^+ L^{-1}) and the percentage of microplastics (MP) and their double and triple interactions on the half time of survival (TD50) and on $t_{50\%}$ of *D. magna*.

	TD50			**$t_{50\%}$**		
	df	**F**	***p*-value**	**df**	**F**	**p-value**
NH_4^+	2	713.41	<0.01	2	388.87	<0.01
MP	2	787.24	<0.01	2	335.39	<0.01
T	2	355.11	<0.01	2	184.26	<0.01
NH_4^+×MP	4	53.54	<0.01	4	2.70	0.037
NH_4^+×T	4	77.21	<0.01	4	7.08	<0.01
T×MP	4	22.96	<0.01	4	14.10	<0.01
NH_4^+×MP×T	4	3.01	0.02	4	15.52	<0.01

Declaration of interests

☒ 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.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

The author statement has been also included in the manuscript file after the Acknowledgements section.

Authors' individual contributions: Teresa Serra: Conceptualization; Data curation; Formal analysis; Methodology; Writing – original draft; writing-review and editing; Visualization. Aina Barcelona: Methodology; Investigation. Narcís Pous: Writing – review & editing. Victòria Salvadó: Resources; Writing – review & editing. Jordi Colomer: Supervision; Validation; Writing – review & editing.