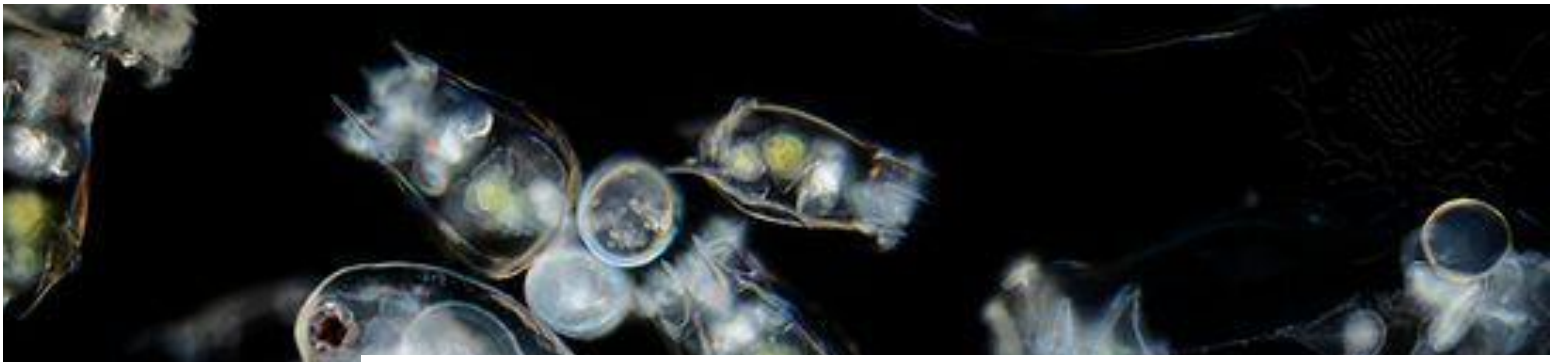

Initial evaluation of the combined effects of nanoplastics and 17α-ethinylestradiol on Daphnia magna

TOBIAS HENRIKSSON 2017

MVEC02 EXAMENSARBETE FÖR KANDIDATEXAMEN 15 HP

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Tobias Henriksson

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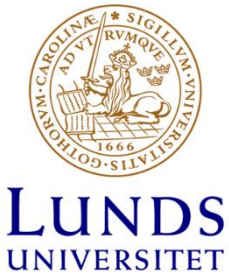

Tillåten uppsatslängd, antal ord för olika uppsatstyper

Nivå	Hp	Högsta antal ord (exklusive referenser)
Kandidat	15	8000
Magister	15	8000
Master	15	8000
Master	30 - 45	16 000

Maxlängden för antal ord får inte överskridas!

Tobias Henriksson

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Lunds universitet
Lund 2017

Abstract

Exposure of combined pollutants is as of today not a very well-researched field of environmental science due to the complex interactions that may occur. Nanoplastic particles (NP) in particular are lacking studies due to the relatively new interest in the subject. In this study, a small experiment in a lab environment was performed that observed the combined effects of polysterene NP and 17α-ethinylestradiol (EE2), a common steroid found in many contraceptive products, on the commonly used zooplankton *Daphnia magna*. Observations were made on mortality, reproduction, and neonate growth. When exposed to NP alone, a trend of increased reproduction by the initial set of adults, and a subsequent reduction of growth of the following neonates, to the point of these not being able to produce eggs of their own, could be observed. These effects could not be observed among the specimens treated only with EE2 or the combination of both EE2 and NP, implying an antagonistic interaction between the two pollutants. Lastly, this study implores further research on combined effects of pollutants, and the effects of NP, to improve future environmental risk assessments.

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Introduction

The synthetic estrogen 17α-ethinylestradiol (EE2) used in many contraceptive products (National Center for Biotechnology Information), and plastic particles in the size range of up to a few hundred nanometers, also known as nanoplastic particles or nanoplastics (NP), might not seem like they have many things in common. However, they both share the attribute of being difficult to treat efficiently in traditional wastewater treatment plants (Besseling et al. 2017, Luna et al. 2015). This means that high amounts of both pollutants originating from personal care products are released into the recipients of the wastewater treatment plants where they can then cause effects on the local biota. NP in this sense is especially difficult to stop, as it also originates from larger plastic objects that degrade after being released into the environment, causing small diffuse sources of NP wherever plastics may be found (Besseling et al. 2014). In 2013 it was estimated that 12% by length of Europe's rivers would reach concentrations greater than the proposed 0.035 ng/L environmental quality standard for EE2 (Johnson et al. 2013). By itself, EE2 has shown to decrease the amounts of neonates produced per female *Daphnia magna* at environmentally relevant concentrations (Luna et al. 2015, Goto and Hiromi 2003). Additionally, a study on other cyclopoids and calanoids have shown that EE2 exert distinctive effects on detoxifying and apoptotic systems which will affect physiological mechanisms, in turn reducing fitness (Souza et al. 2013). Meanwhile, according to a study by Besseling et al. (2014) *D. magna* exposed to polystyrene NP (~70 nm) at concentrations between 0.22-103 mg/L showed a decrease in number and body size of neonates, as well as an increase in malformation among exposed neonates. However, the authors do note that these concentrations are higher than what would be expected in the environment.

No study, to our knowledge, has been performed directly testing the combined effects of NP and EE2 on living organisms, but there are some predictions that can be made based on previous research. A study by Park et al. (2010) demonstrated that the presence nC60 (a nano-sized aggregate of fullerene) decreased the bioavailability of EE2 via adsorption, meaning that the EE2 adhered to the surface of nC60, causing the uptake by zebrafish to decrease. Additionally, a study by Farkas et al. (2016) showed that the interactive effects of nanoparticles of silver and EE2 had limited effects on juvenile turbot (*Scophthalmus maximus*), with only high concentrations having a synergistic effect, although silver nanoparticles may differ drastically compared to plastic nanoparticles as they are of different bulk

materials. In lack of further information about nanoplastics, we turn to microplastics instead as it would not be unreasonable to expect similar properties between the two. Oliveira et al. (2013) conducted a study that evaluated the single and combined effects of microplastic polyethylene particles (1-5 µm) and the organic compound pyrene on common goby (*Pomatoschistus microps*). Their results showed that microplastics delayed pyrene-induced fish mortality, and microplastics also increased the concentration of the pyrene metabolites in the fish bile. The authors concluded that this suggests that there are toxicologically relevant interactions between the two compounds, however, they could not determine any of the mechanisms behind these interactions based on their methods. If these mechanisms are due to adsorption of pyrene onto the microplastics then similar results could be expected to be seen with EE2 as they share similar calculated solubility (XLogP3 for EE2: 3.7, and for pyrene: 4.9 (National Center for Biotechnology Information)).

As there is a distinct lack of knowledge in this area of research, this study aims to evaluate the combined effects of EE2 and NP on the zooplankton *D. magna* on population-level endpoints to provide a basis for further studies. The questions posed are firstly "Are there any observable combined effects between EE2 and NP on *D. magna*?"; and secondly "What kind of combined effects does EE2 and NP pose on *D. magna* (additive, synergistic, antagonistic)?".

Method

Organism: The freshwater zooplankton Daphnia magna, also known as the common water flea, was chosen for the experiment due to its common use in toxicity studies, as well as its availability. The specimens used were taken from a population originating from Lake Bysjön that have been kept under controlled laboratory conditions for over 100 generations at Lund University.

Compounds: For every test, the same concentrations of the two compounds were used. EE2 was diluted from a stock solution of 0.2 g/L, dissolved in dimethyl sulfoxide (a common organosulfur solvent) to a new stock with a concentration of 0.01 mg/L. This stock was then used to create the further diluted concentrations in the replicates, at 0.1 µg/L, which has shown to have a significant effect on population growth rate in a previous study (Luna et al. 2015). The experiment uses polysterene NP, taken from a stock solution of 50 000 mg/L, designed to be 80 nm and negatively charged at a concentration of 5 mg/L in all tests, which has also shown to have an observable effect on population growth rate (Besseling et al. 2014). The NP were dialyzed against ddH20 during 2 days, with the water being changed twice. The experiment used copper-free tap water taken from the Ecology building, Lund University. While using freshwater prepared according to ISO standards was considered, it was decided against using this due to the known risk of NP interacting with the dissolved salt ions that freshwater would contain.

Experimental design A: Experiment A was designed to observe the survivability of *D. magna* and how many neonates are produced per female. The design used 100 mL glass test tubes with one specimen each, at 4 different treatments, Control, EE2, NP and EE2+NP (C, EE, NP and X respectively); each with 6 replicates for a total of 24 test tubes (Fig. 1a). The experiment ran for 3 weeks, with feeding of 0.5 mL algal culture (dominated by the green algae, *Scenedesmus sp*) three times a week. At every time of feeding, data was collected on total number of the original adult *D. magna*, and total number of neonates.

Experimental design B: Experiment B was designed to observe how second and third generations are affected by the treatments. This design used 500 mL glass beakers with 5 different treatments, each with 3 replicates for a total of 15 beakers (Fig. 1b), each with 5 specimens. The treatments were the same as in experimental design A with an additional treatment, named time shift (TS), this one only containing EE2 for the first half of the experiment, with NP being added 11 days in. The experiment ran for approximately 4 weeks, with feeding of 2 mL algal

culture three times a week. Due to the natural degradation of EE2, the water was changed on the 11th day of the experiment, increasing the concentration of EE2 to be closer to the original exposure concentration in a pulse-like exposure pattern. Once again, at every time of feeding, data was collected on total number of the original adult *D. magna*, and total number of neonates.

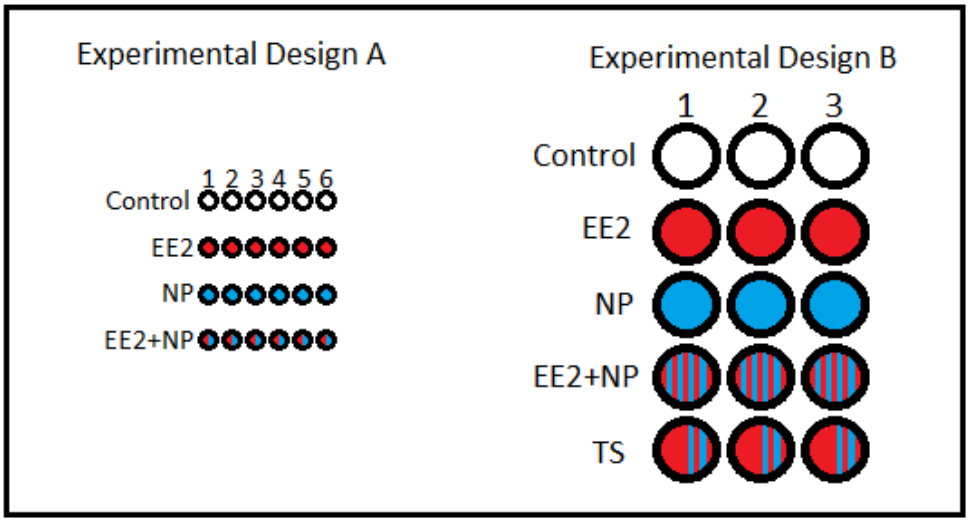

Figure 1

Visual representation of the two experimental designs A and B, with each circle representing an individual microcosm (test tube or beaker).

Data Analysis: The data collected on number of original adults and number of neonates was analyzed with a two-way repeated measures ANOVA using SPSS Statistics version 22. The within-subject variables were set as a factor of days, with one level for each day, and the between-subject factor set as the exposure treatment. A post-hoc Turkey test was subsequently performed in order to see if there were any difference between the treatment groups, significance level was set to $p \leq 0.05$.

Results

Experiment A: No significant difference between the treatments when testing for adults were found (two-way repeated measures ANOVA; df=3, p=0.739), nor was there a significant difference in the treatments when testing for neonates (df=3, p=0.223). In the subsequent post-hoc Turkey performed for both groups, among the adults, no significant differences could be observed ($p>0.742$ among all treatments). Likewise, no significant differences could be observed among the neonates, with the largest difference being between NP and C, and NP and X ($p=0.193$, and $p=394$ respectively, rest being $p>0.583$). When regarding only the mean values, a trend of NP being generally higher than the other treatments could be observed (Fig. 2 and 3).

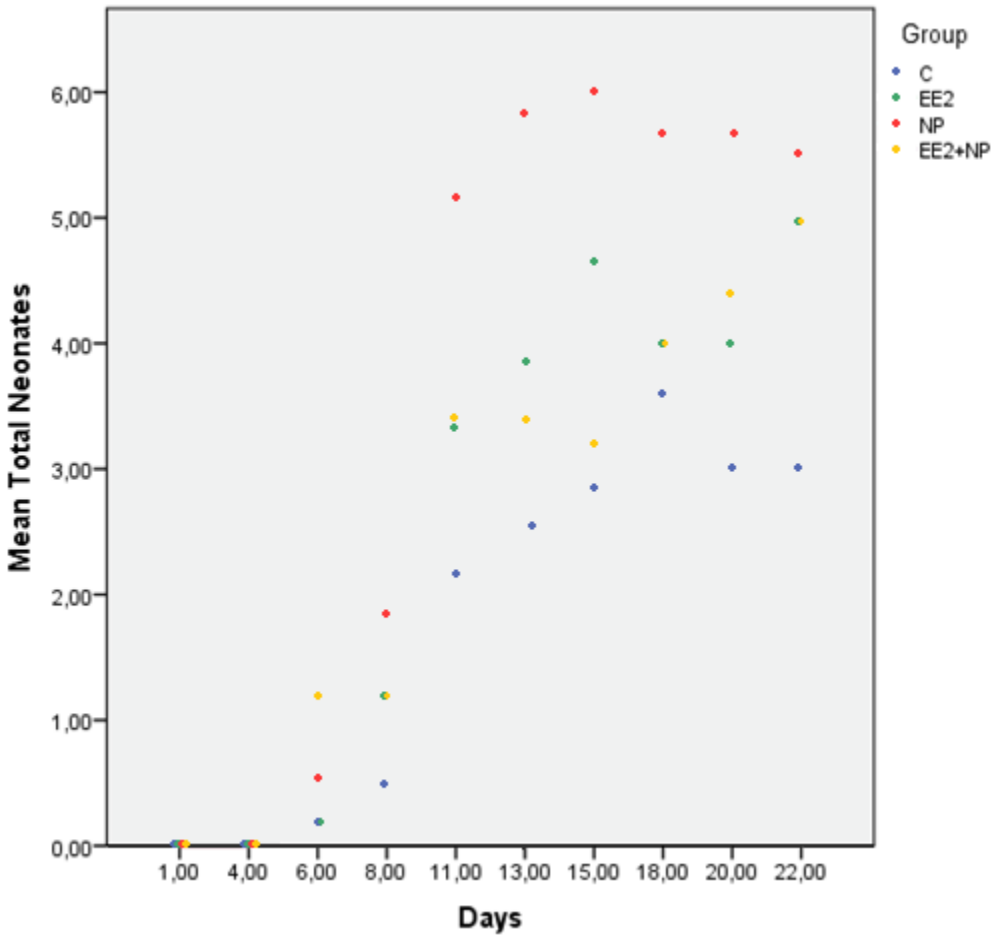

Figure 2

Mean total numbers of neonates recorded in Experiment A over the 22 days tested. Error bars were not included due to their size cluttering the figure and making it more difficult to read.

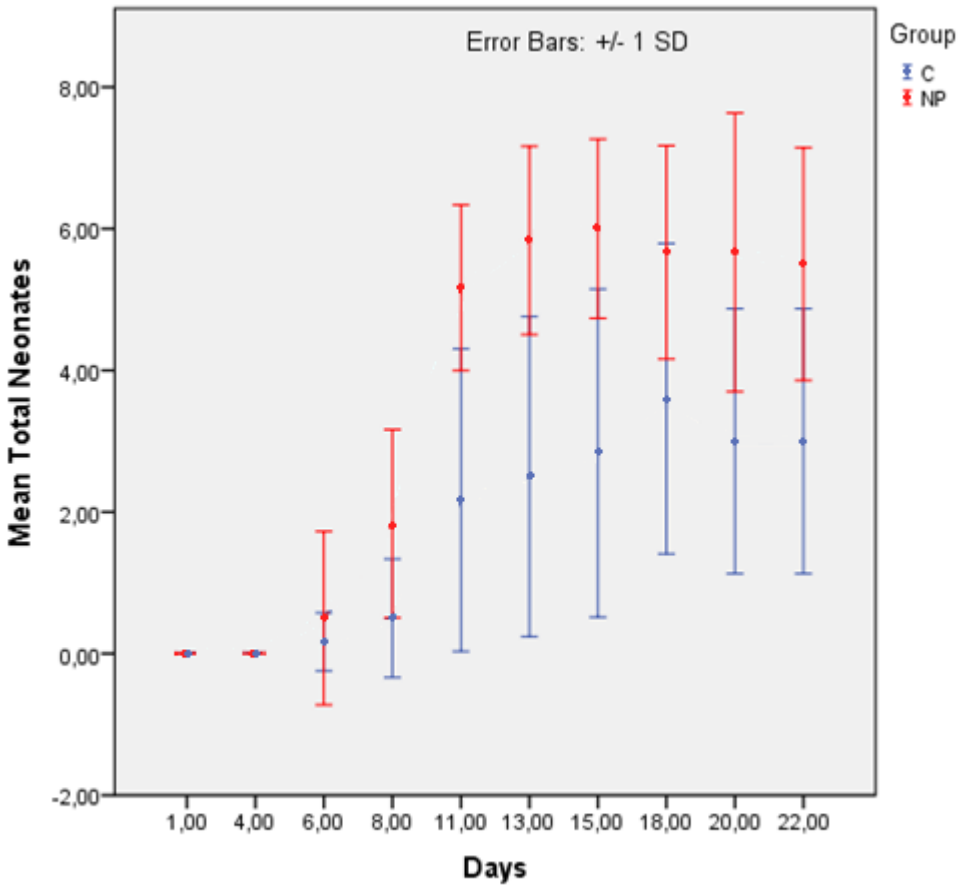

Figure 3

Mean total numbers of neonates in treatment NP and control recorded in Experiment A over the 22 days tested with error bars of 1 standard deviation. EE2 and EE2+NP not shown due to its consistent overlap with C.

Experiment B: A trend among the adult treatment groups could be observed (two-way repeated measure ANOVA; df=4, p=0.102), however, no significant differences were found among the neonates (df=4, p=0.514). In the subsequent post-hoc Turkey test, among the adults, no significant differences were observed among the groups, with NP and C, and NP and X showing the highest difference (p=0.120 and p=0.136 respectively, p>0.275 among the rest). No significant difference could be observed among the neonates either (p>0.515 among all).

The first generation of neonates in the NP exposure treatments remained small and never reached a stage of producing eggs of their own, despite maintaining a neonate population for up to 21 days. This is in contrast to the other groups where the first generation of neonates produced eggs after approximately 4-9 days after hatching (Tbl. 1).

Table 1

Number of days between the second generation hatching to when this generation began carrying visible eggs. Red cells instead display how long the neonates lived before the experiment ended, as these never produced eggs. Some treatment groups are missing as these were terminated early due to all specimen dying before producing any neonates.

Group	C 1	C 3	EE2 1	EE2 2	NP 1	NP 2	NP 3	EE2+NP 3	TS 1	TS 2	TS 3
Days	7	4	4	9	21	12	12	9	12	7	4

Discussion

In this study, the exposure of EE2, polysterene nanoparticles and the combination of EE2 and NP on *D. magna* was evaluated. Above all else, the implications of the effects NP have on the *D. magna* neonates, being an initial increase in reproduction, but the subsequent lack of neonate growth, stand out as the most important results of the study, together with the lack of similar effects being seen when NP is combined with EE2.

Based on the results, a significant effect on mortality rate could not be observed in any treatment. This was to be expected since the treatments used sub-lethal concentrations of EE2 and NP (Besseling et al. 2014, Luna et al. 2015). More surprising is that there seemed to be a universal positive effect on reproduction across all treatments. However, none of the treatments proved to have a statistically significant difference to the control ($p > 0.05$) with NP being the only treatment showing a trend of being different. This poses the question of why the treatments seemed to either have no, or have a positive effect on reproduction.

The NP treatment implied a trending difference to the control concerning the neonates, in both experimental set-ups. In the beakers, the neonates treated with NP grew at a very slow rate, without ever reaching a size large enough to reproduce again before they eventually began dying. This is in comparison to the control group, where the second generation of *D. magna* lived for approximately a week before they began carrying eggs. In short, NP seemed to increase reproduction in the already adult population, while decreasing the growth rate of the second generation, even to the point of not being able to reproduce again. A decreased growth rate was to be expected according to the results of Besseling et al. (2014), and a reduction in growth rate is a common phenomenon to observe among juvenile *D. magna*, often induced by different varying stress factors such as heavy metals, temperature change, oxidative stress, cyanobacteria and more (Bae et al. 2016, Heugens et al. 2006, pers. com. Alex Hegg 2017). However, no previous studies could be found that explained the increase in reproduction. A study by Pietrzak et al. (2010) stated that *D. magna* underwent a tradeoff of shortened lifespan for higher investments in early reproduction in environments of high food supply. It is possible that NP could cause a prolonged false feeling of satiation when ingested by the *D. magna*, which has been observed to be an effect of ingesting microplastic particles (Eerkes-Medrano et al. 2015), which in turn triggers the response of this tradeoff.

Interestingly, the NP showed a greater difference to the combined treatment of EE2+NP, than what the combined treatment did to the control. The combined treatment did not show the same increase in reproduction, nor did the neonates in the combined treatment seem to suffer from the same growth reduction as the ones exposed purely to NP. This would imply that whatever effect that NP has on *D. magna* is reduced or inhibited by EE2. This in turn would suggest an antagonistic reaction between the two compounds, as the addition of EE2 lowered the effects of NP, whereas an additive or synergistic effect would have shown the same, or higher effects between the NP and NP+EE2 treatments.

A parallel study by Emma Nilsson, investigating the chemical interaction of EE2 and NP, found there was indeed an interaction between NP and EE2. The interaction was greater in ISO-standard freshwater than tap water, and further still in water in which *D. magna* and algae was present. This could be a clue to where the biological effects observed in this study might come from, as an interaction between NP and EE2 might deactivate the NP, making it less harmful to the nearby organisms.

In the larger picture, these results stand to show the many unknowns that exist in environmental efforts. Our understanding of NP is still lacking, and much more needs to be done in this field of research. Both NP and EE2 provide their own share of issues to the environment, and in combination with other pollutants that are released directly or indirectly into the environment, the different kinds of potential ecological impacts are seemingly endless. There are methods of handling EE2 as well as other pharmaceuticals currently on the market that may be implemented in wastewater treatment plants, such as ozone treatment, or hydrogen peroxide activated through UV radiation (Martz 2012). However, the implementation of these are slow due to the cost of reworking already operational wastewater treatment plants. NP on the other hand comes in large part from diffuse sources as larger plastic articles degrade after being released into the environment (Besseling et al. 2014), rendering it impossible to treat on-site. Additionally, the methods of detecting and analyzing environmental concentrations of microplastics and subsequently NP are underdeveloped and unreliable (Naturvårdsverket, 2017), which in turn makes it difficult to conduct accurate environmental risk assessment that take into account NP.

Due to the lack of previous studies concerning the combined effects of NP with pharmaceutical compounds, further discussion into how these combined effects affect the natural environment is difficult to make. However, if continued studies into the subject show similar results, efforts should be made to raise awareness of the effects NP has on the environment, as well as the sources of where they come from. Seeing as a NP, as previously mentioned, comes in large part from diffuse sources of plastic litter degrading in the environment, it seems more likely that raised environmental awareness will prove to be more effective than regulatory intervention.

Conclusion

In conclusion, this study show that studies of long-term exposures of NP needs to be undertaken and that polysterene NP in combination with another pollutant can produce results wildly different from the original compounds by themselves. More studies need to be done on the impacts of combined pollutants in the environment to further improve environmental risk models, and research needs to be pushed to develop methods of cheaply and reliably perform environmental analyzes of NP concentrations.

Special Thanks

I would like to make a special thanks to Alexander Hegg for giving me a better understanding in what effects I should be looking at in the *D. magna*, to Per-Erik Isberg for saving me from tearing my hair out when working with SPSS statistics, to a special panda for her continued concern on the wellbeing of the zooplankton, and finally a huge thanks to my supervisor Lina Nikoleris for having the patience and time to put up with my endless stream of questions and requests.

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