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Nanozinc as an emerging nanopollutant: sublethal effects on the invasive freshwater bivalve *Limnoperna fortunei* (Dunker, 1857)

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ABSTRACT. Zinc oxide nanoparticles (ZnONP) are among the most widely produced nanomaterials worldwide given their unique properties, despite their nanotoxicity has been poorly addressed. In parallel, environmental concern has risen because of increasing amounts of them reaching the environmental matrices, whereas the aquatic ones are one of the main final sinks. As key non-target species, we aimed to expose the invasive freshwater bivalve *Limnoperna fortunei* to sublethal concentrations of ZnONP (0, 0.025, 0.25, and 2.5 mg/L) to evaluate tissue damage and oxidative stress-related markers in the soft tissue. After a 96 h-exposure, the alkaline phosphatase enzyme activity increased after 0.025 mg/L, and the alanine aminotransferase activity decreased at 2.5 mg/L. Aspartate aminotransferase enzyme activity also decreased at 0.25 and 2.5 mg/L. In terms of oxidative stress, only superoxide dismutase activity increased after exposure to the lowest nanozinc concentration. We concluded that nanozinc may pose a threat to the aquatic biota in a context that lacks proper regulation and control for nanopollutants, and that the toxicity mechanisms in this species are mainly related to tissue damage, even at environmentally relevant concentrations, as the lowest one tested.

Keywords: biomarkers, oxidative stress, tissue damage, zinc oxide nanoparticles

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1. Introduction

Nanotechnology is a field of study under expansion that involves the synthesis and manipulation of materials (nanomaterials, NM) at a scale of 1 to 100 nm. Among the wide variety of NM, metallic-based ones have been of particular interest due to their unique properties in terms of catalysis, optics, and antimicrobial action (Pal and Pareek, 2025). Zinc oxide nanoparticles (ZnONP) are one of the most applied NM worldwide, after silica- and titanium-based NM. For example, they are extensively used in solar cells, optoelectronic devices, biomedicine, antibacterial materials, and personal care products (Wu et al., 2025). Their global annual production reaches up to 36,000 tons, and the predicted environmental concentrations are in the low range of 0.17 μg/L (Ale et al., 2024). However, through their multiple applications, the NM are released into the environment in increasing amounts. In this sense, it was proven that NM are released during all their life cycle; therefore, rising concern about their environmental fate, and interaction with non-target biota is in need to be addressed (Santos-Rasera et al., 2022). Particularly, when reaching the aquatic environments, ZnONP suffer from multiple transformations, which will depend on other factors (e.g., temperature, UV irradiation, or presence of organic matter), such as dissolution (and Zn ion release), agglomeration, and further sedimentation. The latter may enhance the bioavailability for benthic organisms; however, only a few studies were conducted on this animal group (Yung et al., 2014).

There is some robust evidence that ZnONP exert deleterious effects on aquatic biota (Abdel-Halim et al., 2020; Marisa et al., 2016a; Saidani et al., 2019; Zhao et al., 2016); however, these particles were studied to a lesser extent in terms of ecotoxicology (in comparison with, for example, Ag- or Ti-based NM) (Gutierrez et al., 2021). Mollusks were considered particularly sensitive to nanopollutants given both their filter-feeding and sessile habits (Skawina et al., 2024). Furthermore,

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according to the available bibliography, freshwater mollusks were even less studied in comparison with the marine ones, altough the toxicity mechanisms of any NM were proved to differ according to the ionic strength of the media. In other words, among other environmental factors, the ultimate nanotoxicity of freshwater environments differs extensively from the marine ones (Petosa et al., 2010; Yung et al., 2014). Therefore, there is currently a need for further research on the ecotoxicology of nanozinc, with emphasis on freshwater biota.

Oxidative stress is the most reported toxicity mechanism from nanozinc (and most NM) exposure. For example, the freshwater mussel *Elliptio complanata* showed altered antioxidant enzyme activities and lipid peroxidation in the soft tissue (apart from weight loss) after exposure to ZnONP (Gagné et al., 2013). Similarly, the freshwater snail *Lymnaea luteola* showed altered antioxidant enzyme activities and DNA damage in the digestive gland (Ali et al., 2012). Finaly, DNA damage, altered antioxidant enzyme activities, oxidative damage in lipids and proteins, and bioaccumulation were evidenced in the *Limnoperna fortunei* bivalve exposed to high concentrations of ZnONP (Girardello et al., 2021). Other less-studied biomarkers in the nanotoxicology field are tissue damage-related enzymes. For example, serum from fish exposed to ZnONP had low transaminase activity (Rasheed et al., 2023), but the tissue of the freshwater snail *Biomphalaria alexandrina* showed increased activity (Fahmy et al., 2014).

The golden mussel *Limnoperna fortunei* (Dunker, 1857) is native to Southeast Asia, being highly recognized for invading the aquatic ecosystems worldwide (Barbosa, 2014). Their wide availability, easy adaptation to laboratory conditions, and sensitivity have made them promising sentinel species for assessing early warning responses (Vereycken and Aldridge, 2023). This work includes an independent preliminary study that was conducted prior to another recently published work that has also employed *L. fortunei* as test species and nanozinc exposures (Ale et al., 2025). In order to deepen the understanding of this emerging nanopollutant, the present study aims to evaluate the sublethal effects of ZnONP in terms of oxidative stress and tissue damage in this freshwater bivalve species.

2. Materials and methods

2.1. Zinc oxide nanoparticles (ZnONP)

The particles were purchased from Sigma-Aldrich® (Product number 721077), guaranteeing their purity and stability. According to the Certificate of Analysis, the particles resulted correctly dispersed in the media (H_2O), pH 8.9, and their size was reported as 40 nm. The stock concentration was 19% wt.

The complete particle characterization has been reported in Ale et al. (2025). The transmission electron microscopy (TEM) analysis revealed that the ZnONP had an average primary diameter of approximately 27 nm with some degree of agglomeration. On the other hand, analysis by dynamic light scattering (DLS) confirmed the ZnONP aggregation in aqueous suspension by showing large agglomerates (630 ± 207 nm). Finally, the zeta potential was reported as -26.09 ± 0.79 mV, suggesting moderate colloidal stability.

2.2. Bivalves and exposure conditions

In March 2024 (summer season), the bivalves (adults, $n = 25$) were manually collected from a dock on the shore of Río Santa Fe (a secondary channel of the Middle Paraná River, 31°38'34.90" S; 60°41'6.22" W). This bioassay was conducted independently and in parallel to the study reported in Ale et al. (2025), using organisms collected at different time points. All experimental procedures, from bivalve collection to biomarker determination, were performed separately. The acclimatation procedure was described by Cazenave et al. (2025). After the bivalves were transferred to the laboratory (in plastic containers with natural river water), they were carefully separated and brushed to remove the biofilm. For the first 24 h, they were kept in a medium composed of half natural river water and the other half with dechlorinated tap water, always with oxygenation, at 27 ± 1ºC by employing incubators. The temperature was based on the mean temperature in their habitat in summer (Iriondo and Paira, 2007). Then, the bivalves were kept in dechlorinated tap water for 48 h, with a 16/8 h photoperiod (light/darkness) and algae-based food (*Tetradesmus obliquus* algae culture) *ad libitum* until 24 h before starting the final experiments. When an individual was attached and showed signs of valve activity in response to physical stimuli, it was considered healthy. At the beginning of the experiment, the organisms' mean length and weight were 29.33 ± 2.31 mm and 1.83 ± 0.42 g, respectively (and they were also weighed at the end of it).

2.3. Experimental design

The lowest concentration of ZnONP tested was chosen based on the previously reported Zn environmental concentration for urban surface and municipal effluents (0.010-0.050 mg/L) (Clara et al., 2012; Gagnon et al., 2014). For the higher concentrations, it was contemplated that non-lethal nanozinc concentrations for the test species (*L. fortunei*) were in the range of 1-50 mg/L (Girardello et al., 2021).

The experiment involved 250 mL aquaria with 0 (control), 0.025, 0.25, or 2.5 mg/L of ZnONP, and the exposure period was 96 hours. The conditions remained similar to the acclimatation procedure (dechlorinated tap water with constant aeration, 27 ± 1ºC, and a 16/8 h light/darkness photoperiod). The experimental unit was defined as an aquarium with five organisms, which was replicated three times per treatment. The particle administration was performed directly into the aquarium, before the corresponding dissolution with ultrapure water. Treatments were renewed every 24 hours, so ZnONP dissolutions were prepared daily from the stock to avoid particle transformation and/or agglomeration. The bivalves were not fed during the bioassay. The water conditions were always monitored, which remained similar before and after the renewal

procedure: pH = 7.66 ± 0.15 and conductivity = 165 ± 10 µS/cm. No mortality was recorded during the trial, except for the highest ZnONP concentration (2.5 mg/L), which was < 10%. At the end, the soft tissue of each organism was extracted and stored at -80ºC until the biomarker determinations.

2.4. Sublethal biomarker assessment

The soft tissue of each bivalve was homogenized to determine enzyme tissue damage according to Bacchetta et al. (2011) and oxidative stress markers following the technique proposed by Reglero et al. (2009) (n = 5 per biomarker type). Enzyme activities of alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (ALP) were measured through the methodology proposed by Reitman and Frankel (1957) with commercial kits (Wiener Lab®).

Oxidative stress was determined by measuring the antioxidant activities of superoxide dismutase (SOD) by the technique proposed by Misra and Fridovich (1972); catalase (CAT)–by following the procedure according to Beutler (1982), and glutathione S-transferase–by employing the technique suggested by Habig et al. (1974). Finally, lipid peroxidation levels (LPO) were calculated by the thiobarbituric acid reactive substances (TBARS) assay according to Yagi (1976). Each sample was measured in triplicate, and all the results were expressed in terms of protein content (Bradford, 1976).

2.5. Statistics

Data are reported as mean ± standard error. The Shapiro-Wilks and Levene's tests were applied to evaluate normality and homogeneity of variance, respectively. Variables without a normal distribution were transformed using log10 and tested again before parametric analysis. For statistical data comparisons among the treatments, 1-way ANOVA followed by Tukey post-test was used for normally distributed data, and the Kruskal-Wallis test–for non-normally distributed data. All statistical analysis was performed using the InfoStat software (Universidad Nacional de Córdoba, Argentina).

3. Results and discussion

At the end of the experiment (after 96 h), the bivalves were weighed again, and at the highest ZnONP concentration (2.5 mg/L), a significant increase in the weight was evidenced in comparison with the remaining treatments ($p = 0.0388$) (Table 1). Furthermore, at dissection time (when the mollusk valves were removed), the soft tissue of them was found to be light white compared to the other treatments (observational feature).

Additionally, we observed a white mucus secretion in the case of the highest exposure, which started after 48 h of exposure and became more obvious after 72 h (observational feature) (Fig. 1).

Interestingly, similar visualizations were made in this species but with exposure to titanium dioxide nanoparticles (TiO_2NP) (Manske Nunes et al., 2018). After microscopy analysis, the authors corroborated the presence of Ti in the mucus and explained that its production is a defense mechanism against the toxic effects caused by the nanopollutant. The white mucus secretion observed and the augmented weight of the exposed bivalves at 2.5 mg/L could confirm that the particles were actually captured by the organisms and potentially accumulated in the tissue. In this regard, Rojas Molina et al. (2010) explained that *L. fortunei* is capable of selectively ingest and retain particles spanning a broad size range (approximately 1-1000 µm), while smaller particles (≤ 1 µm) are typically aggregated and expelled as pseudofeces (Frau et al., 2016). However, when the particles lose stability and suffer from physical and chemical transformations such as ion dissolution, the released Zn ions may play a crucial role in metal bioaccumulation, as there is robust evidence that nanozinc can bioaccumulate in the tissue of freshwater and marine bivalves. For example, high levels of total Zn were found in *Dreissena bugensis* exposed to 50 µg ZnONP/L for 96 h (Auclair et al., 2020), and similar results were obtained for the *Unio tumidus*, *Xenostrobus secures*, and *Mytilus galloprovincialis* marine mussels exposed to ZnONP (Falfushynska et al., 2015; Hanna et al., 2013; Lai et al., 2023).

Table 1. Final weight of *L. fortunei* (whole organism) exposed to 0, 0.025, 0.25, and 2.5 mg ZnONP/L. The values are expressed as means ± SE. Means not sharing the same superscript (A or B) are significantly different at $p < 0.05$.

Treatment (mg ZnONP/L)	Final weight (t = 96 h) (g)
Control	1.48 ± 0.09 ^{A}
0.025	1.87 ± 0.13 ^{AB}
0.25	1.82 ± 0.20 ^{AB}
2.5	2.15 ± 0.16 ^{B}

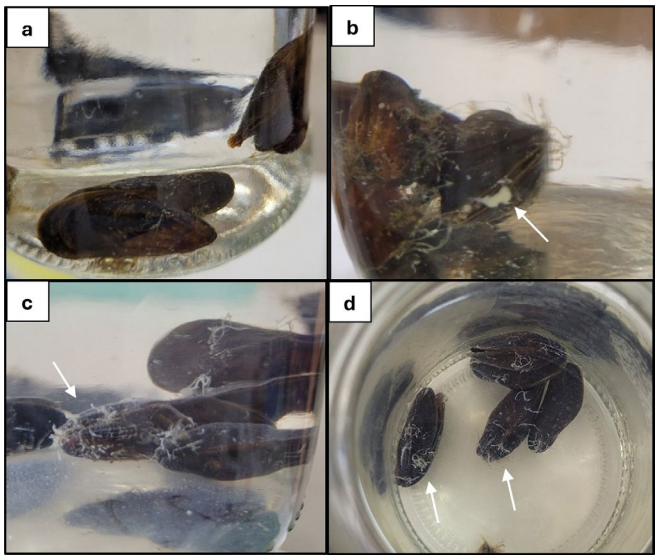

Fig.1. Individuals of *L. fortunei* belonging to (a) the control group (after 72 h) and exposed to 2.5 mg ZnONP/L for (b) 48 h and (c, d) 72 h. White arrows show the light white mucus secretion of the bivalves (the remaining treatments are not shown, as they visually resembled the control group).

Fig. 2 shows the tissue-damage-related enzyme activities in the soft tissue of *L. fortunei* exposed to ZnONP. Both ALT and AST enzyme activities decreased after the highest ZnONP exposure (2.5 mg/L) ($p = 0.0409$ and $p = 0.0295$, respectively), meanwhile ALP increased after the lowest concentration tested (0.025 mg/L) ($p = 0.0331$).

Enzyme tissue damage-related biomarkers showed to be particularly sensitive to nanozinc exposure. However, it is challenging to discuss our findings, as, to the best of our knowledge, ZnONP-related ecotoxicity has not been addressed in the soft tissue of bivalves. Our recent study (Ale et al., 2025) reported comparative nanozinc toxicity in terms of different temperatures, and for the one similar to the employed in this study (27 ºC), similar tendencies for AST decreased enzyme activity were also evidenced at all the concentrations tested. Conversely, discrepant results were found with ALT activity; while no effects were observed in the other study, which employed a lower ZnONP concentration. Here, we evidence an inhibition of the enzyme activity in soft tissue of bivalves exposed to the highest concentration (2.5 mg ZnONP/L). In this regard, the augmented particle (and metal) concentration could have exceeded the enzyme capacity to avoid tissue damage, or even the active site of the enzyme could also have been affected, causing its activity inhibition. For ALP, here we evidence an increased enzyme activity at the lowest concentration tested (0.025 mg/L); however, in Ale et al. (2025) its activity has been found to be decreased, suggesting that ecotoxicity of ZnONP is clearly affected by the biological responses of the test organisms. Although all organisms were collected from the same sampling site (but during different collection events, specifically in different months during the summer of 2024), complex variations in their habitat could be attributed to their discrepant sensitivity, as these organisms are considered tolerant to changes in river flow- and climate-related variables. In this regard, further studies of nanopollutant exposures to freshwater bivalves are highly necessary.

Other studies of mollusks exposed to different stressors like metals and even nanoplastics identified alterations of these enzyme activities (Brandts et al., 2022; Kokhan et al., 2024; Li et al., 2012; Narvia and Rantamäki, 1997). In case of freshwater species, increased enzyme activities of transaminases (ALT and AST) and ALP were found in serum of *Cyprinus carpio* injected with ZnONP, and it was explained that the particles caused dysfunction in the kidney of fish (Rasheed et al., 2023). In terms of mollusks, an interesting study made on the freshwater snail *Biomphalaria alexandrina* exposed to 7.35 mg ZnONP/L evidenced transaminases and ALP increased both in hemolymph and soft tissue of the organisms. Such results were related to muscle damage, intestinal and hepatopancreatic injuries, and toxic hepatitis in the snail (Fahmy et al., 2014). This study clearly indicates damage in the soft tissue of *L. fortunei* after the highest nanozinc concentrations tested (0.25 and 2.5 mg/L).

Table 2 shows oxidative stress-related biomarkers. Only SOD activity was increased with exposure to the lowest ZnONP concentration (0.025 mg/L) ($p = 0.0394$), while no differences were found for the remaining treatments, neither for the other antioxidant enzymes nor peroxidation levels in comparison with the control group. In this context, we highlight the need for studies under realistic conditions, as the actual nanotoxicity could be underestimated.

Nanoparticles were closely associated with cytotoxicity by increasing intracellular reactive oxygen species (ROS) and the levels of the proinflammatory mediator; thus, the homeostatic redox state of the test organism becomes disrupted upon ROS induction by NP (Khanna et al., 2015). Their related biomarkers were widely reported throughout the available literature in terms of nanotoxicity in aquatic organisms (Ale et al., 2024; 2021; Cazenave et al., 2019; Gutierrez et al., 2021). In the case of ZnONP, it was explained that their metabolisms in cells generate ROS overproduction, leading to oxidative damage (Zhao et al., 2016). However, the LPO levels analyzed in soft tissue of *L. fortunei* showed no changes in comparison with the control group. Among the available literature, augmented LPO levels with ZnONP exposure were found at much higher concentrations than those tested in this study. For example, in a tissue of the freshwater snails *Lymnaea luteola* and *Biomphalaria alexandrina*, increased LPO levels (measured by malondialdehyde content, MDA) were evidenced at 7-32 mg ZnONP/L (Ali et al., 2012; Fahmy et al., 2014). Accordingly, Zhao et al. (2016) exposed the *Danio rerio* fish to increasing ZnONP concentration and found increased MDA levels only at higher concentrations (from 10 mg/L onwards,

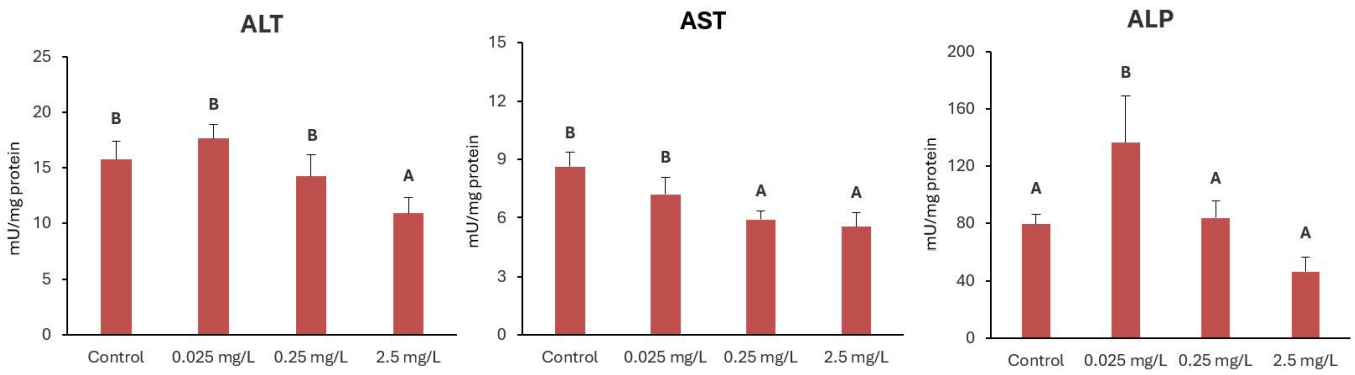

Fig.2. Tissue damage-related enzyme activities: alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (ALP) in soft tissue of *L. fortunei* exposed to 0, 0.025, 0.25, and 2.5 mg ZnONP/L for 96 h. The values are expressed as means ± SE. Means not sharing the same superscript (A or B) are significantly different at $p < 0.05$.

Table 2. Oxidative stress-related enzyme activities: superoxide dismutase (SOD), catalase (CAT), and glutathione S-transferase (GST), and lipid peroxidation levels (LPO) in soft tissue of *L. fortunei* exposed to 0, 0.025, 0.25, and 2.5 mg ZnONP/L for 96 h. The values are expressed as means ± SE. Means not sharing the same superscript (A or B) are significantly different at $p < 0.05$.

Treatment (mg ZnONP/L)	SOD (U/mg protein)	CAT (U/mg protein)	GST (mU/mg protein)	LPO (nmol TBARS/mg protein)
Control	348.3 ± 54.4 ^{A}	6.7 ± 0.7	72.0 ± 13.4	0.6 ± 0.2
0.025	608.3 ± 62.8 ^{B}	6.6 ± 0.9	98.3 ± 15.6	1.0 ± 0.1
0.25	446.1 ± 48.6 ^{AB}	6.6 ± 0.9	89.9 ± 8.9	0.9 ± 0.2
2.5	551.9 ± 32.7 ^{AB}	7.0 ± 0.7	90.3 ± 6.0	0.9 ± 0.1

up to 120 mg/L). Therefore, our tested concentrations may not generate enough oxidative damage to be evidenced after 96 h of exposure.

Concerning the measured antioxidant enzyme activities, only SOD was evidenced to differ from the control values (Table 2). Interestingly, the increase in its activity was only at the lowest ZnONP concentration assayed (0.025 mg/L), suggesting that the exerted nanotoxicity may not be concentration-dependent in this case. Therefore, studies that employ only high NP concentrations and do not consider environmentally relevant exposure conditions may underestimate the actual ecotoxicological scenario. In our previous work, we have also observed SOD activation with *L. fortunei* exposure to low ZnONP concentrations (Ale et al., 2025). One explanation for this result could be related to the lower dispersion of the NP at higher concentrations (and reduced bioavailability), as it was already proved for AgNP in the marine mussel *Saccostrea glomerata* (Carrazco-Quevedo et al., 2019). The importance of SOD in maintaining the homeostatic redox state lies in being the first one to handle oxyradicals, particularly, for catalyzing the dismutation of the superoxide radical O^{2-} to hydrogen peroxide (Zhao et al., 2016). Other studies of mussels also found increased SOD activities. In this regard, Falfushynska et al. (2015) exposed the freshwater mussel *Unio tumidus* to ZnONP for 14 days (concentration reported as 3.1 μM) and explained that SOD augmented activity was due to the ROS overproduction. Furthermore, under environmentally relevant nanozinc concentrations (1 and 10 μg/L), SOD activity increase was evidenced in gills and digestive gland of marine mussels *Ruditapes philippinarum* and *Xenostrobus secures* (Lai et al., 2023; Marisa et al., 2016b).

4. Conclusions

Nanotoxicity is induced in complex ways that are in urgent need of further investigation. The ultimate toxicology on test organisms will depend on the particle type, poorly assessed ZnONP (when compared to others like Ag- and Ti-based), the released media (freshwater or marine), intrinsic properties, and biology of the test organism, among others. The latter results are particularly important, as bivalves are not only filtering-feeding organisms but also vulnerable to further agglomeration and sedimentation of the NM due to their sessile habits. We conclude that *L. fortunei* was sensitive to the tested ZnONP, which could be considered low (in comparison to the available bibliography), even at the lowest concentration that is regarded as environmentally relevant. Finally, we highlight the urgent need for further studies assessing the toxicological implications associated with this nanopollutant, with emphasis on freshwater mollusks and realistic exposure conditions.

Conflict of interests

The authors declare no conflicts of interest.

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Author contributions

Conceptualization: A.A.; Methodology: all authors; Formal analysis: A.A.; Investigation: all authors; Writing - original draft preparation: A.A.; Writing - review and editing: all authors; Funding acquisition: A.A., F.R.M.; Resources: A.A., F.R.M., L.M.; Supervision: A.A., F.R.M.

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