Ecotoxicity of Nanoparticles in Aquatic Environments: A Review Based on Multivariate Statistics of Meta-Data

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Abstract
Evaluation of environmental effects due to exposure to nanoparticles is a still partially unexplored frontier of research, although increasing use of these substances leads to the presumption of a notable increase in their emission in the near future. Current knowledge of possible impacts of nanoparticle emission on aquatic ecosystems (e.g. lagoons, estuaries, marine coasts) is not yet exhaustive in terms of the responses of aquatic species from different trophic levels to exposure to various nanoparticles types (different substances and particle sizes). This paper aims to collect and discuss recent data on ecotoxicological effects observed in aquatic species and to analyse, on a multivariate statistical basis, meta-data collected to evaluate relationships between nanoparticle size and ecotoxicological responses observed in several aquatic species with regard to the most commonly used substances (TiO₂, ZnO).

Keywords: Nanoparticles; Meta-data analysis; Water pollution; Ecotoxicological responses; Aquatic species

Introduction
Industrial interest in nanoparticles is rapidly growing due to the wide variety of technological applications of nanomaterials in numerous sectors representing a sort of new industrial revolution [1,2]. Pharmaceutical and personal care products (i.e. cosmetics and sunscreens), water disinfection, IT and electronics, plastics, ceramics, glass, cement, rubber, lubricants, paints, pigment and foods contain nanoparticles [3]. An inventory of nanotechnology-based consumer products introduced to the market was carried out by the US-based Woodrow Wilson Center [4] to gauge the exponential growth of nanomaterials in commerce, estimated at 2000 tons in 2004 and expected to grow to 58,000 tons in the period 2011-2020 [5]. Intentional release of nanoparticles is also increasing. Treatment of SiO₂ nanoparticles with cationic surfactants (e.g., cetlyl-pyridinium chloride) has proven potential to make nano-oxides into superior sorbents with a partition mechanism for the sorptive removal of organic contaminants from wastewater [6]. These and similar results have led to increased intentional release of nanoparticles for in situ remediation purposes, as recently documented by experiments performed for recuperation of contaminated soil [7] and groundwater [8]. Scientific interest in risks associated with nanoparticle exposure has increased exponentially from 1999 to today, as well documented by the increased number of articles published per year on this theme within the period 1999-2012 [9]. The term "nanoparticle" refers to a chemically heterogeneous group of pollutants characterized by dimensions falling within the 1-100 nm range [10]. Nanoparticles are thus not a discrete class of substances; rather, 'nanomaterial' is an umbrella term for a range of substances that derive unusual functionality from their small size [11]. In spite of their heterogeneity, nanoparticles have similar physical properties such as high surface/volume ratio and aggregation in water [12], and increased uptake and interaction with biota [13].

Nanoparticles can originate from natural (biogenic, geogenic, atmospheric, pyrogenic) and anthropogenic (by-products, manufactured, engineered) sources. As detailed in the literature [14], naturally produced nanoparticles may include humic and fulvic acids, fullerenes, organic acids, carbon nanotubes, nanospheres and metals (Ag, Au, Fe-oxides) while manmade nanoparticles would include Carbon Black, fullerenes, functionalized fullerenes, polyethyleneglycol, Platinum, TiO₂, SiO₂, metal phosphates, zeolites, and ceramics. Manufactured nanoparticles could be harmful for exposed species [12]. Ultrafine nanoparticles (particle size < 100 nm) could originate from urban motor traffic, and exposure to them may severely impact human health. This is a significant risk in Asian cities, where the average level of outdoor exposure is about four times greater than in European ones [15]. Furthermore, recent research evidenced that for certain materials, the reduction in particle size from bulk to nanoparticle induced toxicity, with attention focused on risks associated with their dispersion [16].

Nanoparticles could be carried from emission sources towards aquatic environments as via various routes, including atmospheric outfalls, solid surface leaching, hot-spot industrial or urban emissions from municipal wastewater treatment plants or electro thermal plants [17], although intentional release for water purification purposes is on the increase [6].

The best available technologies do not yet allow us to correctly quantify environmental levels of nanoparticles in environmental matrices [18], but the presence of nanoparticles in the environment is expected, and although there are still major knowledge gaps (e.g. nanomaterial production, application and release) that affect estimations performed by means of modeling, the order of magnitude of environmental concentrations can be modeled [19]. In 2009, Gottschalk et al. [20] modeled for the Europe expected levels of nanoparticles in air lower than 0.0005 mg/m³. On the contrary, authors modeled higher values in surface water (0.010 mg/L) and notable accumulation in sediments (2.90 μg/kg) and sludge from municipal wastewater treatment plants (17.1 μg/kg).

Due to the potential risks and the presence of considerable gaps in our knowledge, the evaluation of effects induced on biota by the emission of nanoparticles in the environment represents a new challenge for current ecotoxicological research. In aquatic environments, the problem is much more complex because nanoparticles can significantly change their structure, shape, and size as a result of aggregation, solubilisation or adsorption phenomena [21]. However, data on both levels and effects of nanoparticles in aquatic environments are...
still lacking, and risk evaluation for environmental conservation and human health purposes is difficult to perform.

This paper collects and discusses recent advances in research on the ecotoxicity of nanoparticles in aquatic environments. It also proposes results obtained by a preliminary multivariate statistical analysis on meta-data reported by recent ecotoxicological studies on dose-dependent responses of aquatic species from different trophic levels exposed to different classes of nanoparticle substances of commercial interest (TiO$_2$, ZnO). The principal aim is to highlight relationships among ecotoxicological responses and i) trophic level of the species tested; ii) type of nanoparticle; iii) nanoparticle size. Ecotoxicological responses to exposure in freshwater and marine species for the same substances are also compared.

Materials and Methods

Selection of data

Meta-data were collected following extensive analysis of recent literature on nanoparticles published on international platforms and databases. In this paper, only studies performed on aquatic species that provide detailed information on factors considered of specific interest in this study are included. Meta-data were considered suitable for the statistical analysis if the following criteria were met: i) acute toxicity responses were reported; ii) tests were performed on aquatic species; iii) experimental details were described (e.g. maximum exposure dose tested, pre-treatments of nanoparticles, etc.); iv) EC$_{50}$ value was calculated for each considered endpoint. Meta-data failing to meet these criteria were excluded from the statistical analysis and treated separately as descriptive (but not statistical) observations. Authors are conscious that meta-data analysis assumes higher relevance when a large number of data are available, in a large range of experimental condition. The relative little number of published papers on this specific focus reduces the powerfullness of statistics but allow some preliminary results that permit highlighting the importance of considered factors routing future researches to improve knowledge with the aim to fill specific gaps highlighted by this study. At all results from 27 researches resulted useful for the purposes of this paper.

Considered factors & statistical analyses

Statistical analysis (n=27) was performed testing different factors as possible responsible of the observed variability reported by the literature collected in this paper.

A recent paper reports that high acute toxicity in various species is expected at low mg/L levels of considered nanoparticles, although responses are highly dependent on certain factors such as: species exposed, physical-chemical properties of materials (including nanoparticle size) and sample treatments [2]. In spite of that, no studies test the significance of these factors on nanoparticle toxicity. Among possible factors of interests, we tested four factors for which statistical analysis if the following criteria were met: i) acute toxicity responses were reported; ii) tests were performed on aquatic species; iii) experimental details were described (e.g. maximum exposure dose tested, pre-treatments of nanoparticles, etc.; iv) EC$_{50}$ value was calculated for each considered endpoint. Meta-data failing to meet these criteria were excluded from the statistical analysis and treated separately as descriptive (but not statistical) observations. Authors are conscious that meta-data analysis assumes higher relevance when a large number of data are available, in a large range of experimental condition. The relative little number of published papers on this specific focus reduces the powerfullness of statistics but allow some preliminary results that permit highlighting the importance of considered factors routing future researches to improve knowledge with the aim to fill specific gaps highlighted by this study. At all results from 27 researches resulted useful for the purposes of this paper.

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Samples treatments (seven levels, fixed). “Treatment” factor include different preliminary treatment of nanoparticles before and/or during ecotoxicological tests. These data are often lacking in considered literature; iv) “Species” (seven levels, fixed). Considered species are both freshwater (D. magna, D. rerio, T. platyurus, C. carpio) and marine (V. fischeri, P. tricornutum, B. plicatilis) species and owns to different trophic levels (bacteria, algae, crustacean and fish). Tests on “Species” factor are performed as single species and grouped according to the trophic level and the ecosystem (freshwater vs marine).

Univariate statistical analyses were performed using the GraphPad Prism (GraphPad Software, San Diego California USA, www.graphpad.com) package, while multivariate analyses were performed using Primer v6.0 software (Primer-E Ltd., Plymouth Marine Laboratory, UK) following the approach reported by Clarke and Warwick. Collected acute toxicity data were pre-treated by square root and successive log(x+1) transformation and normalization procedure. One-way ANOSIM (ANalysis Of SIMilarities) R statistic was run to test hypotheses of differences between groups of samples according to the previously detailed factors defined a priori, using permutation/randomization methods on the Euclidean resemblance matrix and performing 9,999 runs. ANOSIM test was applied to evaluate the effect on acute toxicity responses due to each considered factor. Further methodological criteria on multivariate approaches are widely reported in the literature (Benedetti-Cecchi, 2004; Renzi et al., 2013).

Results

Qualitative analysis

Recently (2008-2013) measured ecotoxicological responses to exposure to ZnO and TiO$_2$ on aquatic species considered are reported in Table 1. Based on a qualitative comparison of meta-data, a difference of toxicological responses is difficult to be observed due to the great heterogeneity of tested conditions. In spite of that, from a direct comparison performed for the same species (i.e. D. magna) exposed at comparable particle size and sample treatments, EC$_{50}$ seems to be higher for ZnO. A specie-specificity of response is also evidenced.

In Figure 1, relationships between “Size” factor and EC$_{50}$ are represented for ZnO. In this case, a clear species-specificity (effect due to the “Species” factor) in ecotoxicological responses is observed (e.g. a single given “Size” is able to produce significantly different ecotoxicological responses in terms of acute toxicity in fishes D. rerio and c. carpio). A relationship between toxicity and the “Size” factor is observed, with the exclusion of D. magna.

In Figure 2, relationships between “Size” factor and EC$_{50}$ are represented for TiO$_2$.

In this case as well, a clear effect due to the “Size” factor is observed, but there is also a specie-specificity of responses due to the exposure to the same “Size”, as evidenced by P. tricornutum (algae) and B. plicatilis (rotifer): the former seems to be much more sensitive to exposure than the latter. Furthermore, concerning D. magna, a clear effect due to the “Treatment” factor is also observed.

Multivariate statistics

Overall, multivariate statistical analysis performed on the meta-database shows: i) a clear effect due to the “Type” factor, based on the variance of ecotoxicological responses observed (ANOSIM Test performed imposing 9,999 permutations, Global R of 0.297, P: 0.02%, number of permuted statistics greater than or equal to Global R: 1); ii) a low-significant effect due to the “Treatment” factor (ANOSIM Test performed imposing 9,999 permutations, Global R of 0.140, P: 16.4%,
number of permuted statistics greater than or equal to Global R: 1637); iii) not significant differences due to the “Species” factor (ANOSIM Test performed imposing 9,999 permutations, Global R of -0.034, P: 54.1%, number of permuted statistics greater than or equal to Global R: 5404).

Absence of significance is also recorded grouping species according to ecosystem (freshwater vs marine) or trophic level; iv) a significant effect due to the “Size” factor is reported considered the whole size range only within the same “Species”, the same “Type” and the same “Treatment” (ANOSIM Test performed imposing 9,999 permutations, Global R of 0.979, P: 0.01%, number of permuted statistics greater than or equal to Global R: 0). In spite of that, low-significant effect due to the “Size” factor is recorded considering the minimum size of nanoparticle (ANOSIM Test performed imposing 9,999 permutations, Global R of 0.182, P: 10.2%, number of permuted statistics greater than or equal to Global R: 1022)

### Discussion

#### Straightness and laciness of the proposed approach

The principal straight of this approach is represented to the chance to put in a single space a multivariate reality and to test significance due to factors a priori defined by the operator. This variability is otherwise difficult to be taken into account without preconditioning by the human mind. To give a practical example of that, if we try to analyse by a multivariate statistic, on the contrary, is able to consider contextually the multidimensional variability that human mind is not able to taken into account. In spite of that the straightness of this approach is also its laciness. In fact, any statistic approximates the reality that increases its correspondence to the reality with the number of data considered. A statistical approach needs to allow to large quantities of data to increase powerfulness of the test but unluckily data on ecotoxicological effects in aquatic species are few and a sensible reduction of the available data is performed in this paper (n=27) due to standardization needing of the large variety of ecotoxicological tests. In fact, ecotoxicological results are highly experimentally conditioned because numerous variables are able to significantly affect obtained data. For this reason, considered literature support observations and measurements with a large number of experimental details such as the exposure time, sample pretreatments, light exposure, toxicant exposure route, etc. Concerning nanoparticles, additive internal variability of obtained results is possible due to difficulties occurring during manipulations and exposure tests. Furthermore, obtained results can be significantly different due to the considered endpoint and to the EC calculation method. In this study only EC_{50} results are considered. Furthermore, concerning the factor “Species”, some species are more represented than others (i.e. *D. magna, D. rerio*) in our dataset and this can significantly affect statistics by overweight reasons.

A recent research evidences that natural light exposure should take into account at least to some degree during ecotoxicological tests. In fact, the photocatalytic properties of some nanoparticles “Types”, as well as TiO_{2}, can increase toxicity to aquatic biota [22]. In spite of that, the lacking of specific data does not allow us to consider this factor. In

<table>
<thead>
<tr>
<th>Type</th>
<th>Species</th>
<th>Min Size (nm)</th>
<th>Treatment</th>
<th>Principal endpoint measured</th>
<th>EC_{50} (mg/L)</th>
<th>Notes</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>TiO_{2}</td>
<td><em>D. magna</em></td>
<td>30</td>
<td>Solvent (THF)</td>
<td>Behavioral and physiological changes</td>
<td>NC</td>
<td></td>
<td>[57]</td>
</tr>
<tr>
<td>TiO_{2}</td>
<td><em>D. magna</em></td>
<td>10</td>
<td>THF preparation</td>
<td>Acute toxicity</td>
<td>5.5</td>
<td>LC_{50} = 10 mg/L</td>
<td>[58]</td>
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<td><em>D. magna</em></td>
<td>10</td>
<td>ultrasonic dispersion</td>
<td>Acute toxicity</td>
<td>NC</td>
<td></td>
<td>[58]</td>
</tr>
<tr>
<td>TiO_{2}</td>
<td><em>D. magna</em></td>
<td>25</td>
<td>Unknown</td>
<td>Acute toxicity, accumulation</td>
<td>NC</td>
<td>Molting frequency increased</td>
<td>[59]</td>
</tr>
<tr>
<td>TiO_{2}</td>
<td><em>D. magna</em></td>
<td>66</td>
<td>vigourous shaking</td>
<td>Acute toxicity</td>
<td>&gt;20</td>
<td>EC_{50} = 20 mg/L</td>
<td>[43]</td>
</tr>
<tr>
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<td><em>D. magna</em></td>
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<td>Unknown</td>
<td>Acute toxicity</td>
<td>&gt;100</td>
<td></td>
<td>[60]</td>
</tr>
<tr>
<td>TiO_{2}</td>
<td><em>V. fischeri</em></td>
<td>&lt;100</td>
<td>Unknown</td>
<td>Bioluminescence reduction (Microtox®)</td>
<td>100</td>
<td>EC_{50} (15 min)</td>
<td>[54]</td>
</tr>
<tr>
<td>TiO_{2}</td>
<td><em>P. tricornutum</em></td>
<td>15</td>
<td>Unknown</td>
<td>Growth inhibition</td>
<td>10.91</td>
<td>EC_{50} (72h)</td>
<td>[55]</td>
</tr>
<tr>
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<td><em>P. tricornutum</em></td>
<td>25</td>
<td>Unknown</td>
<td>Growth inhibition</td>
<td>11.30</td>
<td>EC_{50} (72h)</td>
<td>[55]</td>
</tr>
<tr>
<td>TiO_{2}</td>
<td><em>P. tricornutum</em></td>
<td>32</td>
<td>Unknown</td>
<td>Growth inhibition</td>
<td>14.30</td>
<td>EC_{50} (72h)</td>
<td>[55]</td>
</tr>
<tr>
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<td><em>B. plicatilis</em></td>
<td>15</td>
<td>Unknown</td>
<td>Lethality</td>
<td>5.37</td>
<td>EC_{50} (72h)</td>
<td>[55]</td>
</tr>
<tr>
<td>TiO_{2}</td>
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<td>25</td>
<td>Unknown</td>
<td>Lethality</td>
<td>10.43</td>
<td>EC_{50} (72h)</td>
<td>[55]</td>
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<tr>
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<td><em>B. plicatilis</em></td>
<td>32</td>
<td>Unknown</td>
<td>Lethality</td>
<td>267.3</td>
<td>EC_{50} (72h)</td>
<td>[55]</td>
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<tr>
<td>ZnO</td>
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<td>67</td>
<td>vigourous shaking</td>
<td>Acute toxicity</td>
<td>&lt;0.5</td>
<td>EC_{50} = 0.5 mg/L</td>
<td>[43]</td>
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<td><em>D. magna</em></td>
<td>70</td>
<td>artificial freshwater</td>
<td>Acute toxicity</td>
<td>2.6</td>
<td></td>
<td>[27]</td>
</tr>
<tr>
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<td><em>D. magna</em></td>
<td>70</td>
<td>natural river water-min</td>
<td>Acute toxicity</td>
<td>1.7</td>
<td></td>
<td>[27]</td>
</tr>
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<td><em>D. magna</em></td>
<td>70</td>
<td>natural river water-max</td>
<td>Acute toxicity</td>
<td>9.0</td>
<td></td>
<td>[27]</td>
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<td><em>D. magna</em></td>
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<td>Unknown</td>
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<td>3.2</td>
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<td>[2]</td>
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<td><em>D. magna</em></td>
<td>&lt;200</td>
<td>Unknown</td>
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<td>7.5</td>
<td></td>
<td>[2]</td>
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<tr>
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<td><em>D. magna</em></td>
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<td>Unknown</td>
<td>Acute toxicity</td>
<td>1.51</td>
<td></td>
<td>[2]</td>
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<tr>
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<td><em>D. magna</em></td>
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<td>Unknown</td>
<td>Acute toxicity</td>
<td>2.6</td>
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<td>[2]</td>
</tr>
<tr>
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<td>Chronic effect</td>
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<td>[2]</td>
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<td>Unknown</td>
<td>Chronic effect</td>
<td>1.0</td>
<td></td>
<td>[62]</td>
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<td><em>D. magna</em></td>
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<td>Chronic effect</td>
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<td></td>
<td>[62]</td>
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<td>-</td>
<td>Unknown</td>
<td>Lethality</td>
<td>4.9</td>
<td>EC_{50} (96h)</td>
<td>[61]</td>
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<td><em>T. platyurus</em></td>
<td>50</td>
<td>Unknown</td>
<td>Lethality</td>
<td>0.14</td>
<td>LC_{50} (48h)</td>
<td>[27]</td>
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<tr>
<td>ZnO</td>
<td><em>C. carpio</em></td>
<td>-</td>
<td>Unknown</td>
<td>Lethality and oxidative stress induction</td>
<td>&gt;50</td>
<td>50 mg/L induction of oxidative stress</td>
<td>[56]</td>
</tr>
</tbody>
</table>

Notes: NC = not calculable.

**Table 1:** Ecotoxicological responses measured in aquatic species.
Daphnia magna, a sensitive crustacean, is significantly affected by salinity and ionic strength. The toxicity of nanoparticles in freshwater ecosystems is not easy to extrapolate to marine ones due to the fact that toxicity in bacteria is strongly affected by water chemistry. In fact, increasing pH, HPO$_4^{2-}$, and dissolved organic matter in water solutions reduces the concentration of free Zn$^{2+}$, lowering the toxicity of ZnO-nanoparticles. In addition, both Ca and Mg ions dramatically reduce the toxicity of Zn ions in bacteria, while no significant effects are observed for Na and K ions.

Results reported in this study are overrepresented by freshwater species and tests performed to evaluate ecotoxicological differences between freshwater and marine species are not significant. The absence of significant differences between freshwater and marine species allows us to perform analyses of a single database. In spite of that, the effect of considered factors could be different in aquatic environments due to their salinity and should be tested in the near future with the increasing of scientific knowledge.

### Tested factors

**Type:** Different toxicity responses observed depending on the “Type” factor is the most solid output of the statistical approach applied and this is related to their different mechanisms of action even if literature is not lacking. ZnO results much more toxic than TiO$_2$ for the aquatic species considered. Most of actual knowledge on ecotoxicological effects induced by exposure to ZnO nanoparticles is available on bacteria, and data on other biological taxa are improving even if literature is still lacking. ZnO results much more toxic than TiO$_2$ for the aquatic species considered.

**Size:** A significant effect due to the “Size” factor is recorded in this study only by the exclusion of the other variability (imposing the test on same “Species”, “Type” and “Treatment” factors). This occurrence is probably due to the high internal variability of ecotoxicological responses associates to the “Size” factor. Nevertheless an important contribution to the absence of effects associated to “Size” factor in *D. magna* could represent a possible cause of observed results due to the over-weight of *D. magna* data on the whole database. On the contrary, the “Size” factor affects overall toxicity in algae species. *Dunaliella tertiolecta* shows EC$_{50}$ lower than 1.8 times when exposed to ZnO nanoparticles vs the bulk form (EC$_{50}$ = 1.94 mg/L, range 0.78–2.31 mg/L for nanoparticles compared; EC$_{50}$ = 3.57 mg/L, range 2.77–4.80 mg/L for bulk counterpart; EC$_{50}$ = 0.65 mg/L, range 0.36–0.70 mg/L for ZnCl$_2$) as reported by the literature [28]. Even in juvenile carp (*Cyprinus Carpio*), bioaccumulation and sub-chronical effects (oxidative stress and severe histopathological changes after 30 days of exposure) are significantly affected by the “Size” factor [29].

Concerning ZnO nanoparticles, the “Size” factor seems to significantly affect the release of Zn ions in water. Very high releases (four times higher) are observed for 4-7 nm particles diameter, while lower or similar releases were recorded for 15-130 nm [30]. The absence of significant differences between the embryological effects induced on zebrafish (*Danio rerio*) by ZnO nanoparticles and Zn ion exposure lead to the conclusion that the effects of nanoparticles are mainly related to the release of Zn ions [31].

### Treatment

From early approaches to physical-chemical characterization and ecotoxicological risks due to nanoparticles [32,33], some progress has been made concerning the assessment of...
environmental levels and biological effects of nanoparticles, although certain key aspects are still open to question and several knowledge gaps need to be filled to gain a thorough understanding of nanoparticle toxicity and carry out large-scale risk assessment and management strategies. One key aspect concerning ecotoxicological studies performed to evaluate environmental risks related to nanoparticles is the Treatment factor. Nanoparticles used in technical applications are functionalized; therefore studies performed on pristine nanoparticles are not representative of their environmental behaviour [14]. In a recent study, the effects of γ-alumina, α-alumina, modified TiO\(_2\) and commercial TiO\(_2\) nanoparticles on the survival, behavior, and early life stages of the freshwater snail Physa acuta (Draparnaud), an epic-benthic grazer on sediments, were evaluated. Although no mortalities were observed during the static 96 h test containing sediment spiked with 0.005–0.5 g/kg doses of both commercial and modified nanoparticles, a significant change in antioxidant levels which altered peroxidation was observed at 0.05–0.5 g/kg exposure to γ-alumina, α-alumina modified TiO\(_2\). Furthermore, the hatching percentage in test chambers at 0.5 g/kg is 50% less than that observed in controls [34]. The less significance reported for the “Treatment” factor is probably associated to the lacking of data even if the possible absence of significance could be associated to an absence or reduction of considered acute effects in some species.

**Species**: Differences due to the “Species” factor are not significant. Different ecotoxicological effects are reported in the literature in freshwater species owing to different trophic levels after TiO\(_2\) exposure. Some species evidence effective detoxification phenomena induced by exposure to lower doses that could reduce toxicological measured effects. For example, in freshwater algal species (Scenedesmus obliquus), although cytotoxicity is observed at higher doses, sub-lethal exposure (<1 mg/L) induced detoxification due to agglomeration–sedimentation processes exacerbated by algal interactions and by the exo-polymicro substances produced by the cells, which reduce nanoparticle reactivity, enhancing detoxification effects [35]. In spite of that, the absence of significant effect observed is due to the weight of D. magna responses on statistics. In fact, D. magna shows highly variable ecotoxicological responses ranging within 0.5<EC\(_{50}\) – EC\(_{20}\)>200 mg/L that are due to different factors that should be standardized during experiments.

As example, it has been demonstrated that in Daphnia sp., ingestion via the food chain is the principal route of nanoparticle toxicity. The presence of algae during exposure increased nanoparticle levels in the gut by a factor of 3. In the case of direct contact with the peritrophic membrane and the cuticle, depuration is not efficient to remove nanoparticles from the organisms. In this species, the shedding of the chitinous exoskeleton is the crucial mechanism governing the release of nanoparticles regardless of the feeding regime during exposure [36].

Results evidences that for ZnO nanoparticles, EC\(_{50}\) is inferior to 20 mg/L whatever the species (exception made for C. carpio), these data suggest the occurrence of some mechanism related to the body size of the tested species. In spite of that, the lacking of specific data does not allow us to explore on statistical basis this occurrence and our idea remains a hypothesis at the moment.

**Further factors of interest**

Even if the lacking of data does not allow including these results in our dataset, other possible factors of variability are reported by the literature.

In aquatic environments, nanoparticles undergo important structural transformations as well as changes in structure, shape, and size as a result of aggregation, solubilisation or adsorption phenomena [21]. These changes could affect both toxicity and behavior. A recent study [37] evidenced that even more than the “Size” factor, the surface charge of nanoparticles could significantly impact toxicological responses in aquatic species (Escherichia coli and Daphnia magna) and should be considered as important factor of variability. Furthermore, some recent research has shown that in terms of ecotoxicological and environmental issues, particle size is less key [38] than the specific surface area affecting toxicological responses [39].

Another important area in the field of nanoparticle research is the need to acquire a more complete knowledge of nanoparticles and complex matrix interactions.

A strong influence due to the presence of sulfur containing compounds, dissolved oxygen, pH, Cl\(_2\), organic compounds and lighting conditions has been reported [40]. Important effects on ZnO nanoparticles dissolution and toxicity in bacteria (E. coli) appear to be due to water chemistry [26]. In fact, humic and fulvic acids appear to have a variety of functional groups that allow them to form complexes with metal ions and interact with nanomaterial, changing their environmental behavior [41]. In natural environments, nanomaterials acquire a coating of humic/fulvic acids due to the pervasiveness of humic substances, and consequently the final toxicity of nanomaterials is significantly altered [42]. Furthermore, humic/fulvic acids, with some exceptions, affect aggregation properties, degradation processes and, consequently, toxicity [42,43]; in fact, hetero-aggregation processes modify sedimentation rates and dissolution of nanoparticles [44]. Combinatorial experiments on environmental conditions of water (i.e. organic acid type, organic acid concentration, pH, salt content, and electrolyte type) have evidenced important effects on the behaviour of nanoparticles [45].

Alginates - naturally occurring components of organic matter in natural soil - have been shown to significantly enhance nanoparticle toxicity on corn plants by increasing trace element accumulation in roots, reducing chlorophyll–a content and triggering overexpression of heat shock protein 70 [46]. These and similar [47] results highlight the importance to take into account factors related to physical-chemical characterization water in order to correctly evaluate the ecotoxicity of nanoparticles in aquatic environments.

Furthermore, nanoparticles have been found to act as a carrier of co-existing contaminants, and this interaction alters the toxicity of specific chemicals on D. magna [48].

Nanoparticles could affect the toxicity of chemical pollutants in a nanoparticle type-dependent way. For example, Cr(VI) toxicity on the freshwater algae Scenedesmus obliquus was notably reduced by the presence of 0.05 μg/mL of TiO\(_2\) nanoparticles, while the presence of Al\(_2\)O\(_3\) appeared to have no significant effect [49]. Other ecotoxicological interactions among water pollutants and nanoparticles are reported by recent in vitro studies. A study performed on cell cultures from fish species (Danio rerio) evidenced that fullerene C\(_{60}\) increases the intake of benzo-a-pyrene into hepatocyte cells, decreasing cell viability and impairing the detoxification response by phase II enzymes (i.e. GST) at the transcriptional level [50]. Exposure of the mussel species Mytilus galloprovincialis to both dioxins and nanoparticles demonstrates the occurrence of synergistic or antagonistic effects, depending on experimental condition, cell/tissue and type of measured response. In some cases, interactions may result from a significant increase in dioxin accumulation in whole mussel organisms in the presence of nanoparticles, indicating a Trojan horse effect [51].
In spite of these preliminary studies, synergic effects due to simultaneous exposure to nanoparticles and environmental pollutants are actually not yet well known, and further research should be carried out in the near future to fill this knowledge gap.

**Further endpoints**

Ecotoxicological data on aquatic species should be augmented to include further acute and chronic effects due to both long-term and low-dose exposure tests on a wider range of key species from different trophic levels. As evidenced by a recent study by Pettit and Lead [11], characterisation programmes of nanoparticles and their ecotoxicological effects must be considerably improved to meet the requirements of current and future regulatory frameworks for nanomaterials, with a specific focus on the new European Union REACH framework. At present, the use of biomarkers is still absent in most marine monitoring programs, and has not been included in the status assessment of the Water Framework Directive for monitoring in Europe. In particular, the details of the minimum physical-chemical characterization of nanomaterials necessary to fully interpret ecotoxicological data and characterization of test organisms pre- and post-exposure of the test organisms should be better explored and defined both in terms of methods and variables of concern. Furthermore, the determination of the presence of nanomaterials in the biological target of interest (gill, cell etc.) would provide additional information on uptake biokinetics. Significant methodological limitations must be overcome in order to measure nanoparticles in aquatic environments and quantify biological uptakes. In fact, even though a range of different methods are available (i.e. microscopy-based approaches, dynamic light scattering, and size separation approaches paired with detection methods such as inductively coupled plasma MS), significant disadvantages limit their resolution power, and some are unable to distinguish between nanoparticles and natural interferences; other techniques require sample preparation approaches that can introduce artifacts; and others are complex and time-consuming. For these reasons, natural levels are currently modeled on the basis of scientific knowledge and data on emission sources, and the development of powerful new techniques is still a future aim to better describe factors and processes affecting nanoparticle levels, distribution and fate in aquatic environments.

**Normative remarks**

Ecotoxicological tests and battery of species that have to be used for the evaluation of nanoparticles toxicity as well as samples pretreatments or treatments procedures are neighed neither sufficiently standardized nor sufficiently detailed to allow generalized considerations on statistical basis. These aspects as well as the endpoint recorded are not ruled but are actually an open field of scientific research.

A holistic approach is urgently needed to fill our knowledge gap regarding the safety of discharged nanoparticles [32]. Testing of nanomaterials in aquatic environments requires the development of improved OECD (Organisation of Economic Cooperation and Development). The majority of the OECD TG for chemicals is generally applicable for the testing of nanomaterials, with the exception of TG 105 (water solubility) and 106 (adsorption-desorption) [53]. New research must be carried out to develop and test new tools and emerging new endpoints - beyond traditional ones - that could begin to describe real nano-ecotoxicological effects on biota and trophic webs. The development of nano-relevant endpoints replacing $K_{oc}$, BioMagnification Factor or BioConcentration Factor and the identification of key parameters affecting the fate and behaviour of nanomaterials are key aims [53].

**Future developments**

Considered literature actually available on effects due to the exposure of aquatic environments to nanoparticles evidence some important gaps on scientific knowledge that should be joined in the near future. Some methodological aspects should be better detailed to allow the complete exploration of the effects due to the tested factors “Size” and “Treatments” and other factors of specific interest should be considered as source of variability of ecotoxicological responses in aquatic environments. Furthermore, more detailed researches are needed to better understand whether and how nanoparticle pollution could affect stochastic changes, successional changes and cyclical changes in aquatic ecosystems. A complete analysis of potential impacts of nanoparticle exposure on predator-prey interactions in aquatic environments and cascade effects induced by such exposure on the entire trophic web is completely lacking, with the exception of a single study performed on D. magna [37]. Relationships among sub-lethal nanoparticle levels and biodiversity of aquatic ecosystems have yet to be fully described. Better knowledge of exposure pathways (e.g. via sewage sludge) and long-term studies are important challenges for the near future.

Furthermore, some efforts should be made to develop tools and techniques to differentiate between different modes of action and to predict the relative importance of particle-induced toxicity, photo-induced toxicity, and dissolved associate ion effects. Augmenting our current knowledge of reactions among nanoparticles and natural matrices (water, sediments) could help us to select opportune variables to monitor, measure and standardize during in vitro exposure experiments (particle agglomeration, dissolution, precipitation, irradiation condition). Finally, but no less importantly, data on the effects induced on prey-predator interactions and on aquatic trophic webs should be explored.

**Conclusion**

This study represents a first and preliminary attempt to weight, on a multivariate statistical basis, different factors that literature suggest as able to affect TiO$_2$ and ZnO nanoparticles toxicity in aquatic environments. A severe lacking of useful data is recorded ($n=30$) and some results are probably affect by underweight, general knowledge on aquatic ecosystems is lacking, and efforts should be made in the near future to develop strategies, methods and approaches to fill this gap.

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**References**


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