Synthesis and characterization of TiO_2 nanoparticle and study of its impact on aquatic organism

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Abstract

The rapid development of nanotechnology or nanotoxicology is stimulating research on the potential health hazards and environmental impacts of manufactured nanomaterials (MNMs) and nanoparticles (NPs). This paper focused on the identification of *Daphnia pulex* from the environment and TiO_2 nanoparticles synthesis, characterization and the 24 h acute toxicity of water suspensions of TiO_2 NPs to *Daphnia pulex*, using mortality as toxicological endpoints. The results show that the acute toxicities of 0, 5, 50, 100, 150, 200, 210, 220, 225 and 230 ppm are dose dependent or concentration level. The Lethal Concentration (LC50) values for mortality 24h is observed at 218.79ppm. *D. pulex* were found to ingest nanoparticles from the test solutions through feeding and their behavioral and morphological effects indicates that the potential ecotoxicological and environmental health of these NPs cannot be ignored.

Keywords: *Daphnia pulex*, Nanoparticles, TiO_2, Toxicity, LC50.

1. Introduction

Nanoparticles as particles with at least one dimension smaller than 1 micron, $1x10^{-9}$ m (1-100 nm). Nanotoxicology was proposed as a new branch of toxicology to address the adverse health effects caused by nanoparticles. The increased usage of nanoparticles in commercial products such as sunscreens and cosmetics, coating and paints may reach the environment intentionally or accidentally [1]. It is estimated that the worldwide production of TiO_2 nanoparticle will reach 2.5 million tons by 2025 [2]. In fact it has become a top anteriority in governments, the private sector and the public all over the world [3 - 5]. As well as most industrial products, NPs are expected come into the aquatic environment, and many of these particles are bioavailable and can demonstrate toxicity [6]. Most attention has thus far been committed to the toxicology and health implications of NP [7-10], while the behavior of NP in the environment [11-12] and their ecotoxicology [6, 13, 7]. Research on the potential environment and health impacts of NPs is essential to protect the environment and to assure a sustainable nanotechnology industry [13 – 15].

The freshwater planktonic microcrustacean *Daphnia* is a universal freshwater dweller in ponds and lakes [16]. *Daphnia* are keystone species in freshwater food chains and food webs, and an excellent bio indicator species for use in environmental monitoring of pollutants [17 – 18]. Accordingly, it has been routinely employed as a model organism for toxicology, ecology, ecotoxicology, and evolutionary biology [19 – 20]. Daphnids naturally ingest particulates from the water column or from the sediment [21] and have been shown to readily take up and accumulate NPs in the gut within 6–12 h after exposure [22 – 25].

This has been demonstrated in a number of studies with *Daphnia sp*. and different types of nanoparticle or agglomerates e.g. Lovern et al. [24], Baun et al. [25,26], Petersen et al. [27], Zhu et al. [28], Croteau et al. [29], Hartmann et al. [30] and Hu et al. [31]. As a part of the digestion process *Daphnia sp*. are known to take in water [32] thus small particles can directly be taken up from the water column [33].

A significant noesis gap still exists considering all expressions of environment toxicology related to NPs [34]. While more information about the ecotoxicity of NPs continues to become available, because studies have been conducted on a limited number of MNMs, and in a small number of aquatic species [1], compared to many metal oxide nanoparticles such as TiO_2, ZnO, SiO_2 [35]. Research on the toxic effects of these NPs on aquatic organisms reported in the literature has largely focused on metals and metal oxides such as titanium dioxide [36 – 38].

In recent years, titanium dioxide nanoparticles (TiO_2 NPs) have been widely used in industrial and consumer products due to their stronger catalytic activity. These nanomaterials made its path in the public domain through the waste of these product. Concerns have been raised that these same properties of TiO_2 NPs may present unique bioactivity and challenges to human health [39].

There are few published studies of the toxicity of TiO_2 using freshwater invertebrates as test organisms. While some studies reported acute effects of TiO_2 nanoparticles [40 – 43] on daphnids, whereas Griffitt et al. [44] found no measurable toxic effects on *Daphnia pulex*.

The present work aimed to investigate the toxic effects of TiO_2 NPs on the crustaceans *Daphnia pulex* collected from Guntur pond, Tiruchirappalli in order to assess the responses of NP exposure in vivo. We have compared our toxicity data on TiO_2 with the literature reports and discussed the suitability of aquatic crustaceans for hazard identification of engineered nanoparticles.

2. Materials and methods

2.1. Identification of *D. pulex*

Guntur pond (Fig.1) is a seasonal pond situated in the Tiruchirappalli - Pudukkottai high way, Tiruchirappalli, Tamilnadu, India. The area of the pond is about 1.3 hectares. The pond is fed by a canal branching out from new Kattalai high level channel of river Kaveri and cultured for over a year in the laboratory prior to the experiments. *D. pulex* filtered through 0.45µm membrane filter [45], identified using standard protocol [46].

2.2. Synthesis and Characterization of TiO_2 nanoparticles:

Amorphous titania was prepared by hydrolysis of titanium (IV) isopropoxide. In a typical preparation, a solution of 2-propanol was added dropwise to a solution of titanium (IV) isopropoxide (Aldrich, 97%) in 2-propanol under stirring at room temperature. The solution was stirred overnight (~12 h) and the white product so precipitated was recovered by centrifugation. The white product so obtained was calcined at 350°C and 500°C for 2 h to get TiO_2 nanoparticles. TiO_2 nanoparticles characterization through powder X-ray diffraction (XRD), and Micro-XRF were performed in the Analytical Chemistry Division, Mod-Labs, Bhabha Atomic Research Centre (Mumbai). Fourier Transformed Infrared Spectroscopy (FTIR) and Ultra Violet-visible Spectroscopy studies were also performed.

2.3 Culture media

Daphnids were acclimatized in the Environmental Research laboratory, Jamal Mohamed College, Tiruchirappalli and the water parameters were maintained [47]. *D. pulex* was cultured at 22±2^{0}C [48] in 2 litre plastic jar containing a feed of bakery yeast 0.2g/L. The light cycle was 12 h light: 12 h dark [49]. The *D. pulex* (filter feeders) are fed three times a week with a suspension of dry yeast 0.2g/L [50]. The water was changed in three times a week [51-52].

2.4 Acute toxicity studies

24h acute toxicity tests were performed in the *D. pulex* [53-54]. Test solutions were prepared immediately prior to use for diluting the stocks cited above with filtered water (The membrane filters (Somar) AXIVA white cellulose Nitrate 47 MM). In this procedure, the stock solution (30min) mixture was continuously Ultra sonication cleaner (50/60Hz) [42, 52] to maintain the suspension at as stable a concentration as possible. The present test engaged a completely random design consisting of 0, 5, 50, 100, 200, 220 and 230 ppm and a control group per test. Twelve arbitrarily (randomly) selected neonates (<24h old) [55] were placed in a 100mL glass exposure beaker containing 75 mL of test solution [54] modified test solution level. Three replicate were applied per treatment. Food was not provided during the exposure [56] and all tests were conducted inside lab at a constant pH (7-8) and temperature (22±2^{0}C) [48] with a natural light–dark cycle the observation after an every six hours, for 24h.The mortality of the individuals in each container were evaluated using a Leica microscope equipped with a digital camera (Leica, Germany). The uptake and absorption of nanoparticles in *D. pulex* were observed and authenticated using a microscope with digital camera.

2.5 Statistical analysis

The Lethal concentration- LC50 (24h) - were expressed by Probit analysis using the SPSS software package for windows (version 17.0) and sensible interpretations were made following.

3. Results

3.1 *D. pulex*

As an identification morphology and reproduction aspects (Fig.2). Cyclical parthenogenesis is the ancestral breeding system of the genus Daphnia as shown by its dominance as compared to other crustaceans. Under favorable conditions, cladocerans typically reproduce by parthenogenesis. Parthenogenetic females produce eggs that are carried in brood chamber, which is located between the body and carapace. The eggs mature in the brood pouch, where they are particularly nutrified by maternal secretions of nutritive fluid. The eggs develop into neonates and ultimately to new parthenogenetic females and so on. The different sized neonates can be used as live feed to the different economically important animals such as shrimps and fishes.

3.2. Nanoparticles characterization

3.2.1. XRD Studies

XRD analysis confirms the formation of TiO_2. Broadening of the peaks in XRD (Fig.3) suggests the formation of nanoparticles, however the size was calculated using Sherrer formula considering the FWHM of the most intense peak. The size calculated was 29 nm. This was verified by measuring size by zeta sizer nano ZS which gave a mean diameter of 28.2 nm.

3.2.2. Micro-XRF Analysis (Soil Mode)

The spatial distribution and speciation of TiO_2 in the soil mode was studied using micro-XRF. Figure 4 show the soil mode with TiO_2 NPs and blue colour for K. Moreover, the Ti/TiO_2 NPs found their way soil mode. The tricolor micro-XRF map showed Ti at high concentration in the soil mode. Accumulation of toxic elements in soil mode has been reported for heavy metals such as Table. 1. Ti, Ba and Zr.

3.2.3. Fourier Transformed Infrared Spectroscopy studies

Fig.5. shows the FTIR spectra of titanium dioxide nanoparticles in which the peaks appear at 3403 and 2921 cm^{-1}, are due to stretching and bending vibration of the –OH group. The absorption band at 1630 $cm^{−1}$ was caused by bending vibration of coordinated H_2O as well as from the Ti–OH and were indicated to stretching of titanium carboxilate, which formed from TTIP and ethanol as precursors[58]. The bending vibrational mode of water may appear as shoulders on the spectrum such as 3403.23 $cm^{−1}$.The peaks at 497.42cm^{-1}, 688.32cm^{-1} to 775.84cm^{-1}shows bending and stretching mode of Ti-O vibrations [59].

3.2.4. Ultra Violet-visible Spectroscopy studies

Fig.6. shows the UV spectra of TiO_2 Nanoparticles. In this spectroscopy graph is plotted between Absorbance and wave number. A strong absorption peak in the UV region is observed at the wavelenght of 322 nm.

3.3. Acute toxicity of NPs to *D. pulex*

The results showed that the acute toxicities of TiO_2 NPs to D. pulex enhanced with increasing particle concentration demonstrating dose dependency. A concentration of 218.79 ppm caused 50% mortality. The nanoscale TiO_2 also exhibited a gradual increase in toxicity toward *D. pulex*, with 50% mortality occurring in the 218.79ppm exposure group (Fig.7) based on the probit analysis.

3.3. Uptake and assimilation of NPs by *D. pulex*:

Prominent amounts of dark material were found in the gut tract of *D. pulex* after NPs exposure and the control. All NPs tested in this study distinctly can be ingested in *D. pulex* a accumulating could occur in the gut (Fig.8a, b). This occurred within 24 h of the commencing of the exposure. In some events, the consumption and assimilation of NPs were significant plenty to prevent ambulation of the daphnids through the water coloumn. These results hint that aquatic exposures to such NPs by aquatic organisms could lay a risk of bioaccumulation, particularly for filter feeding copepods such as *D. pulex, D. magna* etc……,

4. Discussion

Identification of *D. pulex* is very difficult because more 200 species around the world have similar structure, behaviour, feeding habitat an ecosystem. Ecotoxicity studies with nTiO_2 on *D. magna* often focused on exposure concentration, the physicochemical properties of nanoparticles, and NPs pre-treatment. Hund-Rinke and Simon [41] reported that nTiO_2 (25 nm or 100 nm) concentrations of less than 3 mg L^{-1} exhibited little effect on the immobilization of daphnids, and the toxicity of nTiO_2 under preillumination by simulated sunlight seemed to be higher than that of non-illuminated $nTiO_2$.

Titanium dioxide NPs were toxic to *D. magna* having LC50 value of 0.016 mg/ml and nanoparticle size is 10nm [60] and Nanoparticle size is 21nm, LC 50 value-1.19mg/l [61]. Present study results as (24 h) LC50 of $nTiO_2$ were calculated to be as high as 218.79ppm result finding and nanoparticles size is 29nm.

Toxicity can be affected also by particle number, charge, size and size distribution, surface area, structure and shape [62, 63]. When the size of nanoparticles increases, the toxicity level decreases.

Titanium dioxides were within the same order of magnitude than those found and the previous study reported values of 50% mortality for 0.0055 mg/ml for filtered oxide titanium NPs. Differences in LC50 values between our and [42] study may be assigned to the different way of preparing NPs solutions. Lovern and Klaper [42] reported a very high acute (48 h) toxicity of filtered $nTiO_2$ (30 nm) to *D. magna* (LC50 = 5.5 mg L^{-1}), whereas $nTiO_2$ (100–500 nm) prepared by sonication at the highest concentration of 500 mg L^{-1} led to only 9% mortality and reported that a sonicated TiO_2 suspension at a concentration of 50-500mg L^{-1} did not show any toxicity to *D. magna* after 48h exposure, LC50 are more than 100 mg L^{-1}. Acute toxicity tests using *Daphnia* species have been performed so far under questionable conditions when the objective was to explore the photo activity of the TiO_2.

Titanium dioxide and hydrogenated fullerenes did not cause significant toxicity at levels lower than 500 ppm [64]. Nanoscale TiO_2 also exhibited a gradual increase in toxicity toward *D. magna*, with 100% immobilization and 90% mortality occurring in the 500mg/l exposure group [54]. TiO_2 nanoparticle prepared by vigorous shaking at the concentration of 20mg^{-1}(40% mortality) particle size was not found affect toxicity, TiO_2 particle size is (66nm,950nm,44nm) Adams et al .,2006.The $nTiO_2$ even at a low concentration of the 0.1 mg L^{-1} can cause reproduction inhibition on *D. magna*, and consequently, affect the population of *D. magna*. Moreover, growth inhibition and mortality of *D. magna* were also observed in low concentration ranges of 0.5–5 mg L^{-1}.

Similar results observed by Wiench et al. [38] i.e., enhanced toxicity by prolonged NP exposure, there are some discrepancies between our findings and theirs. The $nTiO_2$ may exert toxic effects directly on the organism since it has been reported recently that $nTiO_2$ can induce cytotoxicity and oxidative effects such as DNA damage in cells [65]. The Overview of *Daphnia* species studies on worldwide for acute toxicity and accumulation of engineered nanoparticles has been given in Table.2.

Daphnia feed by creating a water column with their appendages that funnels the water towards their mouth, as well as circulating oxygen-rich water into the carapace to facilitate respiration [69]. Due to the differences in product particle sizes and characteristics and pre-treatments of the test dispersions a direct comparison of the studies mentioned above with our test results is difficult and thus supports the controversies of Crane et al. [70] and Warheit [67], concerning the development of standard protocols and characterization for toxicological test using nano-scale. This lack of relationship observed between the acute toxicity of nanomaterials and their size is in agreement with a previous study in which all. The arises a urging need to revisit the prior eco-toxicity database for titanium oxide, and other metal oxide nanoparticles, where ionic toxicity has so far been neglected owing to their presumably "insoluble" nature.

Conclusion

In the present study Titanium dioxide (TiO_2) nanoparticles have been successfully synthesized using a wet chemical technique and characterized by Powder X-ray diffraction (XRD), Micro-XRF, Fourier Transformed Infrared Spectroscopy (FTIR) and Ultra Violet-visible Spectroscopy studies. The NPs used in this research have acute, dose- dependent ecotoxicological effects on freshwater zooplankton *D. pulex*. The results presented in this research suggest that the potential ecotoxicological and environmental health effects of NPs should not be neglected. This finding may have implications for long term studies of ENPs in *D. pulex*.

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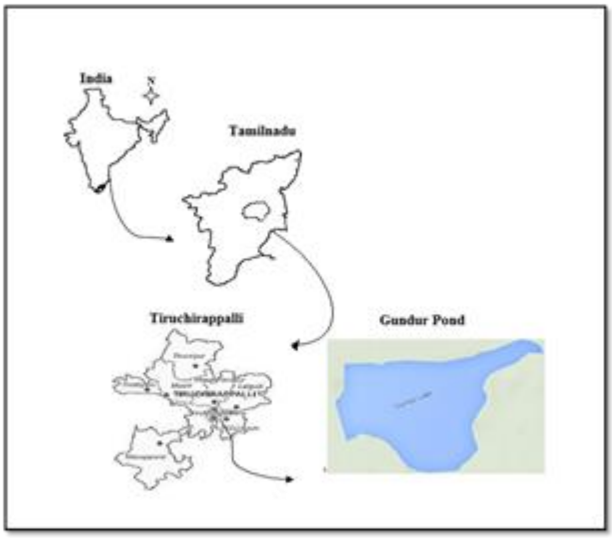
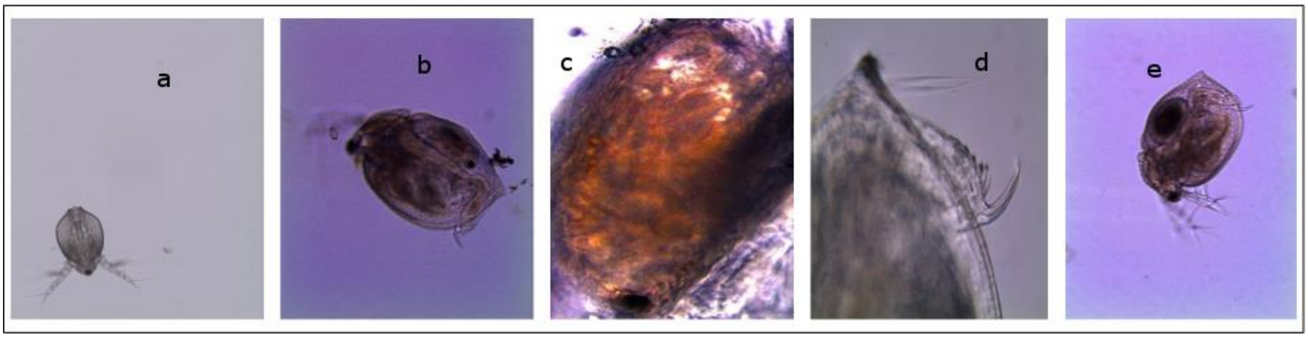


Fig.1. *Daphnia pulex* study area



(a) Neonate, (b) Parthenogenesis (new neonates formation), (c) Inside daphnia neonate, (d) Haploid egg formation, (e) Female *Daphnia pulex*

Fig.2. The figures sources observation in Leica microscope, Germany.

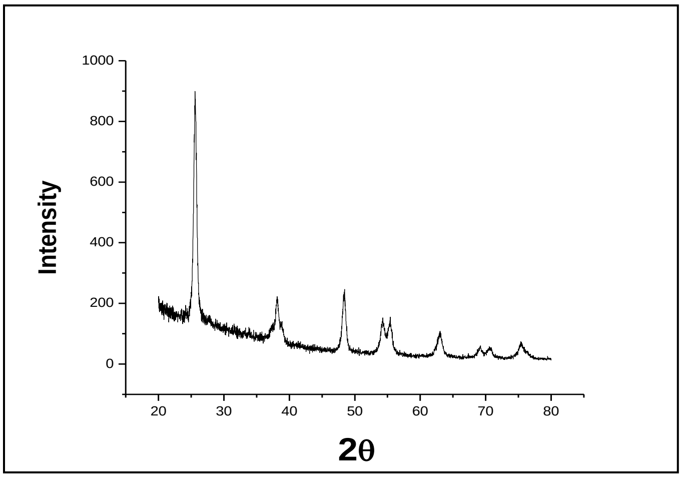


Fig.3. XRD of TiO2 NP

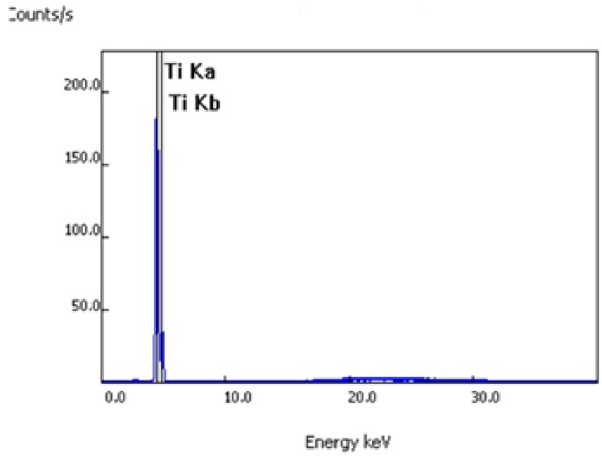


Fig.4. Micro-XRF Analysis TiO2 Nanoparticle (Soil Mode)

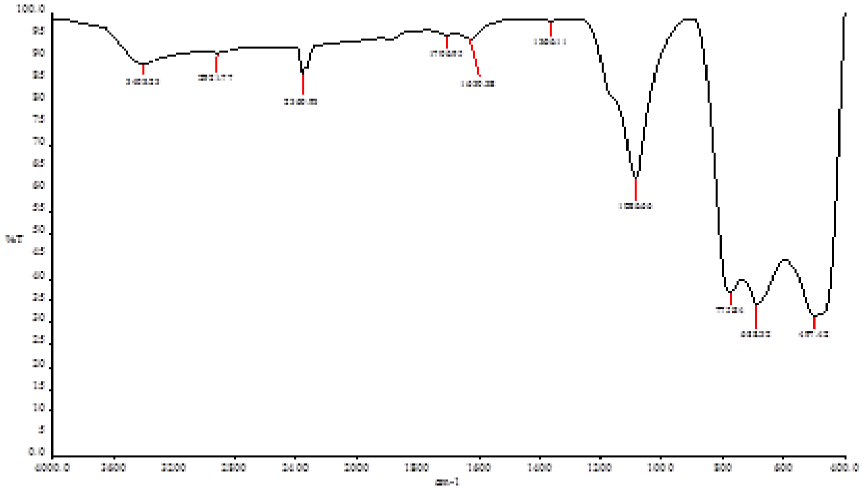


Fig.5. FTIR spectra o f Titanium dioxide Nanoparticles

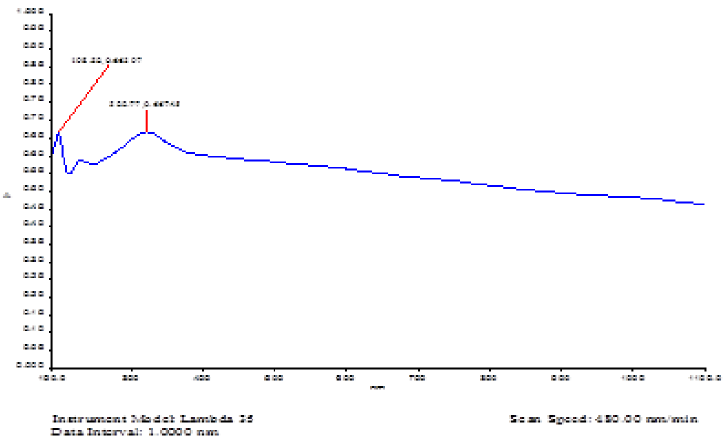


Fig.6. UV spectra of Titanium dioxide Nanoparticles

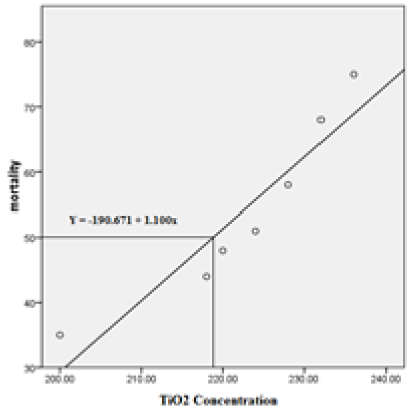
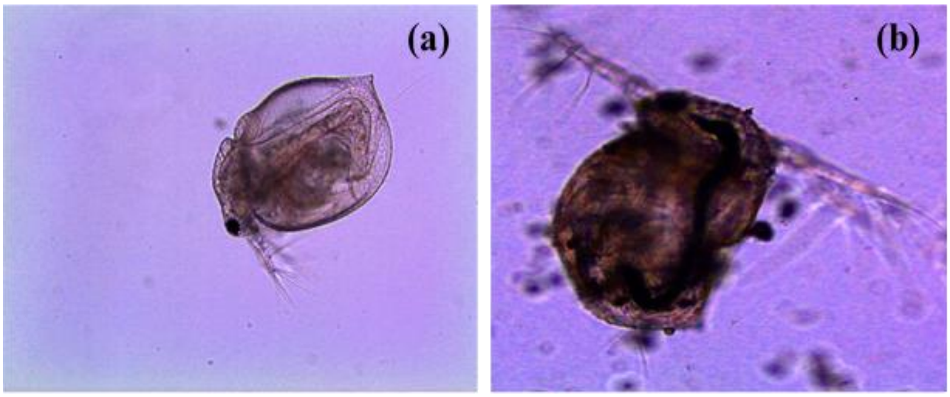


Fig.7. Identification of lethal concentration of TiO2 NP on *D. pulex* by Probit analysis SPSS



Control TiO_2- 224ppm

Fig.8 a Control, b. 24h after exposure TiO2 Nanoparticle Death of daphnia pulex (ingestion on gut TiO2 nanoparticle level)

Detected	**Ppm**	+/-
Ti	>10%	3%
Ba	10208	1730
Zr	17	2

Table 1 Detected metals

S. No	Material	Particle size (Diameter)	Test species	Effect measured	Suspension preparation	Measurements	Reference
1	TiO_2 NPs	29nm	*D. pulex*	Acute toxicity	Sonication	LC50= 218.79ppm /224mg/L (50% mortality)	Present study
2	TiO_2 NPs	30nm	*D. magna*	Acute toxicity	Solvent (THF) and Sonication	EC50=not achieved, LC50 (5.5 mg/L)	Lovern et al. [43]
3	TiO_2 NPs	Degussa P25:25nm Hombikat UV 100: 100nm	*D. magna*	Acute toxicity	Sonication	Increase of immobilization of *D. magna* when stimulated with light	Hund-Rinke and Simon et al. [41]
4	TiO_2 NPs	66, 950,44 nm	*D. magna*	Acute toxicity	Vigorous shaking	TiO2 produced 40% mortality at 20 mg/L	Adams et al. [40]
5	TiO_2 NPs	21nm	*D. magna*	Toxicity and bioaccumulation of TiO_2 nanoparticle aggregates (Acute toxicity)	Sonication	LC50 Value: 2.02 mg L^{-1} and EC50 Value: 1.62 mg L^{-1}	Zhu et al. [54]
6	TiO_2 -coated NPs		*D. magna*	Acute toxicity		EC50>100mg/L	Baun et al. [26]
7	TiO_2 NPs		*D. pulex*	Chronic effects		40% mortality	Adams et al. [40]
8	TiO_2 NPs	21nm	*D. magna*	Acute and Chronic effects	Sonication	13% mortality (0.1 mg/L) and LC50 = 2.62 mg/L	Zhu et al. [28]
9	TiO_2 NPs	<100nm	*D. magna*	Acute and Chronic effects	Stirring	Acute toxicity = EC50 <100mg/L, 21 days (offspring) EC50 = 26.6 (n. d)[a] and mortality= 66.1	Wiench et al. [38]
10	TiO_2 NPs	10nm	*D. magna*	Acute toxicity		LC50= 0.016mg/ml	García et al.[60]
11	TiO_2 NPs	30nm	*D. magna*	Acute toxicity	Sonication	EC50=2mg/L	Hartmann et al.,[30]
12	Nano-TiO_2 and Graphene-TiO_2	21nm	*D. magna*	Toxicity	Sonication	LC50= Nano-TiO_2 (118mg/L^{-1})and Graphene TiO_2 (138mg/L^{-1})	Li et al. [56]
13	TiO_2 NPs	7 and 20nm	*D. magna and C. riparious*	Comet assay and Ecotoxicity assay	Sonication	DNA damage, Growth, Reproduction.	Lee et al., [66]
14	TiO_2 (Coated)	140nm in Water	*D. magna*	Acute toxicity	Not specified	EC50: >100mg/L^{-1}	Warheit [67]
15	TiO_2 NPs	10-20nm	*D. magna*	Acute toxicity	Solvent (THF) / Ultrasonic dispersion	EC50: 5.5mg/L^{-1}	Lovern and Klaper [42]
16	TiO_2 NPs	Less than 40nm	*D. magna*	Antioxidant enzyme assay and size fractionation	Sonication	LC50 Not calculated	Kim et al.2010
17	$nTiO_2$ and TiO_2/bulk	≤20 and 10,000nm	*D. magna*	Acute toxicities	Magnetic stirrer	LC50 Value:$nTiO_2$ (143.387mg/L), TiO_2/Bulk (>500mg/L	X. Zhu et al [54]

(*Nm=Nanometer, Different exposure time= 26, 48, 96 (hrs), LC50= Lethal Concentration (50% mortality), EC50= Median effective concentration.)

Table 2 Overview of *Daphnia* species studies on toxicity and accumulation of engineered nanoparticles