MULTIGENERATION IMPACTS ON *DAPHNIA MAGNA* OF CARBON NANOMATERIALS WITH DIFFERING CORE STRUCTURES AND FUNCTIONALIZATIONS

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(Submitted 24 June 2013; Returned for Revision 15 August 2013; Accepted 23 October 2013)

Abstract: Several classes of contaminants have been shown to have multigenerational impacts once a parental generation has been exposed. Acute and chronic toxicity are described for several types of nanomaterials in the literature; however, no information is available on the impact of nanomaterials on future generations of organisms after the exposure is removed. In the present study, the authors examined the impacts of carbon nanomaterials (CNMs), including fullerenes (C₆₀), single-walled carbon nanotubes (SWCNTs), and multiwalled carbon nanotubes (MWCNTs) with neutral, positive, and negative functional groups to F₁ and F₂ generation daphnids after an F₀ exposure. Data from the present study indicate that multigenerational toxicity is present with certain nanomaterial exposures and is highly dependent on the surface chemistry of the nanomaterial. Many CNMs that showed toxicity to exposed F₀ daphnids in previous experiments did not induce multigenerational toxicity. Certain nanomaterials, however, such as C₆₀-malonate, SWCNTs, SWCNT-CONH₂, and MWCNTs, caused a significant decrease in either survival or reproduction in F₁ daphnids; and SWCNT-CONH₂ decreased reproduction out to the F₂ generation. Impacts of nanomaterials on F₁ and F₂ size were small and lacked clear patterns, indicating that CNMs have minimal multigenerational impacts on size. Industries should take into account how surface chemistry influences nanomaterial toxicity to future generations of organisms to create sustainable nanomaterials that do not harm freshwater ecosystems.

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Keywords: Aquatic toxicology, Nanocotoxicology, Nanomaterial, Reproductive toxicology, Multigenerational toxicology

Epigenetics

INTRODUCTION

Engineered nanomaterials are emerging contaminants that have novel physical and chemical properties. They have already been widely commercialized in today’s marketplace despite the uncertainties regarding how they will interact with biological systems [1]. Most notably, carbon nanomaterials (CNMs) have been synthesized with a particularly wide array of shapes and functionalities for applications in medicine, clothing, cosmetics, electronics, and polymer composites. As the production and application of CNMs increase, the likelihood that CNMs will end up in the environment and in aquatic systems also increases [2].

Multigenerational impacts of parental exposure to chemicals have been demonstrated for several other classes of compounds, including endocrine-disrupting chemicals, perfluoro-octane sulfonic acids, perfluoro-octanoic acids, and heavy metals [3–5]. These impacts include elevated mortality and decreased size and reproduction in second- and third-generation offspring of various organisms, including rats, *Daphnia magna*, and Japanese medaka. Continuous exposure of multiple generations of organisms to a chemical may cause physiological changes to support adaptation or acclimatization [6–8]. In addition, a single exposure of parent organisms to a chemical can result in exposure of offspring to the chemical during sensitive prenatal stages of development, which can lead to significant adverse outcomes later in life; and these effects can show up a generation or more after the exposure is removed [9].

There are growing data for acute and chronic toxicity for a variety of nanomaterials [10]; however, no data are available on the multigenerational impacts of nanomaterials to whole organisms after the exposure has been removed. In vitro assays have demonstrated that nanomaterials can induce changes to the epigenome (DNA methylation, histone modifications, and gene silencing by noncoding RNAs) [11,12], suggesting the possibility of impacts to future generations. If the effects of nanomaterial exposure are transferred to future generations of organisms, there could be long-term ecological consequences. Therefore, an understanding of how nanomaterial exposure will affect populations of organisms across generations, even after the nanomaterial exposure has been removed is essential to increase our knowledge about the long-term ecological impacts of nanomaterials, and it is relevant for any scenarios where remediation is necessary [13].

The current study investigated the multigenerational response of future generations of *D. magna* from parental (F₀) exposure to multiple types of CNMs that differ in core structure and surface functionalization. *Daphnia* are a model aquatic invertebrate for toxicity assays because of their Holarctic distribution in freshwater systems, parthenogenetic reproductive strategy, and the quantity of data regarding their life history and responses to environmental stressors. They are ideal for multigenerational studies because they are genetic clones, allowing the potential for epigenomic impacts to be measured. In our previous study, we demonstrated that nanoparticle structure and functionalization influence particle toxicity to exposed *Daphnia* (F₀) [14]. In the present study, the consequences of F₀...
exposures to future generations of daphnids (F₁ and F₂) are evaluated, using impacts on survival, reproduction, and adult size as adverse outcome end points.

MATERIALS AND METHODS

Nanomaterial preparation and characterization

Six CNMs were synthesized by J. Chen at the University of Wisconsin-Milwaukee. These particles include C₆₀-βCD (derivative 1), C₆₀- amino (derivative 2), C₆₀-amino-γCD (derivative 3), C₆₀-malonic acid (derivative 4), C₆₀-malonate (derivative 5), and C₆₀-malonate-γCD (derivative 6) (Figure 1). Beta- and gamma-cyclodextrins (βCD and γCD, respectively) were ground with fullerenes in an agate mortar to yield derivatives 1, 3, and 6. Particles were suspended in deionized water by 1-h bath sonication in the absence of solvents and surfactants because this has been shown to change how the particles interact with organisms [15]. The smallest average diameter was observed with C₆₀-malonate-γCD particles (105 nm), followed by C₆₀-βCD (107 nm), C₆₀-amino (142 nm), C₆₀-amino-γCD (152 nm), and C₆₀-amino-γCD (175 nm). The most stable of these particle types were C₆₀-malonate and C₆₀-malonate-γCD, with more negative zeta (ζ) potential values (ζ potentials of −63.8 mV, −58.07 mV, and −52.04 mV), followed by fullerenes (C₆₀-OH and C₆₀ ζ potentials of −54.02 mV and −39.6 mV) and unfunctionalized nanotubes (SWCNT and MWCNT ζ potentials of 23.07 mV and 22.98 mV). Analysis by inductively coupled plasma mass spectroscopy indicated the presence of 9.49 ppb and 34.6 ppb iron in C₆₀ and C₆₀-OH suspensions, 0.1 ppb strontium in C₆₀ suspensions, and 6.88 ppb copper in C₆₀-OH suspensions (Supplemental Data, Table S1). Nickel was present in all carbon nanotube suspensions. The highest nickel concentration was found in SWCNT (368 ppb), followed by SWCNT-COOH (212 ppb), MWCNT (151 ppb), SWCNT-CONH₂ (60 ppb), and SWCNT-PEG (60 ppb) (Supplemental Data, Table S2). A sample of the catalyst that was used to synthesize the carbon nanotubes was obtained directly from the manufacturer (Carbon Solutions), and acute and chronic toxicity experiments with this catalyst indicated that it does not influence daphnid mortality, reproduction, and adult size at the concentrations in treatments.

Daphnia cultures

Daphnia magna were obtained from cultures in the R.D. Klaper laboratory at the University of Wisconsin-Milwaukee School of Freshwater Sciences and maintained in a 16:8-h light: dark cycle at a temperature of 20 °C in moderately hard reconstituted water [16]. Cultures were fed a combination of freshwater algae (Selenastrum capricornutum) and alfalfa glycol (PEG) functionalized SWCNTs (Carbon Solutions), and multiwalled carbon nanotubes (MWCNTs; NanoAmor). The average diameters for C₆₀ and C₆₀-OH were 141 nm and 144 nm, respectively. Average diameters for nanotubes ranged from 800 nm to more than 2 microns; however, because of the high aspect ratio of the nanotubes, the size of the aggregates is not uniform and some aggregate sizes are smaller and larger than these reported average diameters. Functionalized nanotubes (SWCNT-COOH, SWCNT-PEG, and SWCNT-CONH₂) were the most stable in suspension with Milli-Q water (ζ potential of −61 mV, −58.07 mV, and −52.04 mV), followed by fullerenes (C₆₀-OH and C₆₀ ζ potentials of −54.02 mV and −39.6 mV) and unfunctionalized nanotubes (SWCNT and MWCNT ζ potentials of 23.07 mV and 22.98 mV). Analysis by inductively coupled plasma mass spectroscopy indicated the presence of 9.49 ppb and 34.6 ppb iron in C₆₀ and C₆₀-OH suspensions, 0.1 ppb strontium in C₆₀ suspensions, and 6.88 ppb copper in C₆₀-OH suspensions (Supplemental Data, Table S1). Nickel was present in all carbon nanotube suspensions. The highest nickel concentration was found in SWCNT (368 ppb), followed by SWCNT-COOH (212 ppb), MWCNT (151 ppb), SWCNT-CONH₂ (60 ppb), and SWCNT-PEG (60 ppb) (Supplemental Data, Table S2). A sample of the catalyst that was used to synthesize the carbon nanotubes was obtained directly from the manufacturer (Carbon Solutions), and acute and chronic toxicity experiments with this catalyst indicated that it does not influence daphnid mortality, reproduction, and adult size at the concentrations in treatments.

Figure 1. Fullerene structures synthesized at the University of Wisconsin-Milwaukee: derivative 1: C₆₀-β-cyclodextrin (βCD); derivative 2: C₆₀-amino; derivative 3: C₆₀-amino-γ-cyclodextrin (γCD); derivative 4: C₆₀-malonic acid (which was a precursor used to synthesize derivatives 5 and 6); derivative 5: C₆₀-malonate; derivative 6: C₆₀-malonate-γCD. Derivative 4 was not used for toxicity investigations.
Multigenerational impacts of nanomaterials on *Daphnia* (Medicago sativa). Adult females were chosen from stock cultures for breeding purposes and maintained in 500-mL beakers at a constant population of 1 *Daphnia*/80 mL moderately hard reconstituted water.

**Multigeneration assays**

The F₀-generation daphnids were exposed to 0 ppm, 10 ppm, and 50 ppm concentrations of CNMs obtained from commercial sources and 0 ppm, 1 ppm, and 5 ppm for those nanomaterials that were synthesized by J. Chen because of limitations in quantity as well as higher toxicity found with a few of these nanomaterials [14]. The maximum concentrations chosen reflected exposure levels that were determined to be sublethal, based on a series of median lethal concentration values calculated from acute exposures of *Daphnia* to nanomaterials in previous work in our laboratory [14,17]. Additional controls with βCD and γCD were conducted to evaluate the potential toxicity of these surface attachments. Five F₁-generation female daphnids were chosen from second or third broods of F₀ daphnids. The F₁ daphnids were born in the exposure medium but placed in control moderately hard reconstituted water within 24 h. Five F₂-generation female daphnids were then chosen from second or third broods of F₁ daphnids. The F₁ and F₂ generations of daphnids were raised in control medium (moderately hard reconstituted water only) for 21 d with static renewal where medium was replenished 3 times per week.

Mortality and reproductive output were measured during medium changes. Daphnid size was measured as the length of the daphnid from the top of the head to the base of the apical spine at day 21. Experiments met the mortality and reproduction requirements of controls outlined by the Organisation for Economic Co-operation and Development Guidelines for the Testing of Chemicals [18]. Changes in population density and food availability were eliminated with removal of proportionate volumes of medium and food from the exposures as mortality occurred. Daphnids were kept at a concentration of 1 daphnid/20 mL medium with a food concentration of 400,000 algal cells/mL medium. Total reproductive output was calculated for the number of surviving individuals at the time of measurement and then reported as the average number of neonates produced per surviving individual.

**Statistical analysis**

Effects of nanomaterials on daphnid mortality, reproduction, and adult size were compared with those of controls by *t*-test or by nonparametric Mann-Whitney *U*-test. The effects of nanomaterials on daphnid mortality, reproduction, and size were compared across treatments within each generation. Values were determined to be significant at *p* < 0.05.

**RESULTS**

**Multigenerational impact of CNMs on mortality**

Multigenerational effects on survival were observed for some CNM treatments in F₁ daphnid generations (Figure 2). Of the unfunctionalized nanoparticle types, MWCNTs decreased the survival rate of F₁ daphnids, compared with controls (77.2% survival, *U* = 27, *p* < 0.05) (Figure 2A). Although unfunctionalized C₆₀ did not significantly impact survival, some types of surface chemistries were found to increase the toxicity of C₆₀ to *Daphnia*. This includes 10 ppm C₆₀-βCD (84% survival, *U* = 18, *p* < 0.05) and 5 ppm C₆₀-malonate (64% survival, *U* = 18, *p* < 0.05) (Figure 2B). None of these treatments decreased survival of F₀ daphnids in our previous experiment; however, MWCNTs at the higher 50 ppm concentration were found to decrease F₀ survival [14].

**Multigenerational impacts of CNMs on reproduction**

Select CNMs did have an impact on reproduction up to 2 generations after parental exposure. Carbon nanomaterial core structure was an important parameter that influenced multigenerational reproduction in daphnids. Of the unfunctionalized CNMs, only 50 ppm SWCNTs significantly decreased F₁ daphnid reproduction compared with controls (decrease of 23%, *t* = 2.767, *p* < 0.05; Figure 3A); but this effect was not observed in the F₂ generation. At a concentration of 50 ppm, C₆₀ reduced reproduction by 17% in F₂ daphnids ( *t* = −4.137, *p* < 0.05) (Figure 3B). Finally, 10 ppm and 50 ppm MWCNTs reduced reproduction in the F₁ and F₂ generations, but this was only significant for 10 ppm exposures in F₂ (decrease of 18%, *t* = −2.192, *p* < 0.05; Figure 3A and B). The addition of COOH or PEG surface chemistry to SWCNTs did not change reproduction compared with controls in any generation; however, SWCNT-CONH₂ significantly reduced reproduction in both F₁ (decrease of 17%, *t* = −6.351, *p* < 0.05) and F₂ (decrease of 17%, *t* = −3.956, *p* < 0.05) daphnids (Figure 4A and B), indicating that this surface chemistry increases the toxicity of SWCNTs to future generations of daphnids.

Some functionalized fullerenes had significant impacts on F₁ and F₂ reproduction. Although 10 ppm C₆₀ had no significant impact on multigenerational reproduction, reproduction in F₁ was significantly decreased by 5 ppm C₆₀-malonate (decrease of 9%, *t* = −2.361, *p* < 0.05) and increased by 10 ppm C₆₀-βCD (increase of 22%, *t* = 4.863, *p* < 0.05; Figure 5A). However, these effects were not found in the F₂ generation (Figure 5B). In
addition, impacts of 10 ppm C60-βCD on increased reproduction were not significantly different from those of 10 ppm C60, indicating that the attachment of βCD to C60 does not change the impacts of unfunctionalized fullerenes on F1 reproduction. Finally, 50 ppm C60-OH decreased F1 reproduction by 12% (t = −3.608, p < 0.05) and increased F2 reproduction by 10% (t = 2.336, p < 0.05).

Multigenerational impact of CNMs on adult size

Carbon nanomaterials also had a marginal multigenerational impact on daphnid size, and this was dependent on core structure and functionalization. At 10 ppm and 50 ppm, C60 significantly decreased F1 adult size by 5.5% and 4% (t = −4.083, p < 0.05 and t = 3.351, p < 0.05). For functionalized fullerenes, significant decreases in F1 size were observed for 10 ppm C60-OH, 5 ppm C60-amino, and 5 ppm C60-malonate (decreases of 4%, 5.8%, and 7%; t = 3.036, p < 0.05; t = −4.863, p < 0.05; t = −6.687, p < 0.05, respectively; Figure 6A); however, none of these treatments were significantly different from controls in the F2 generation (Figure 6B). In addition, none of these results were significantly different from F1 daphnids from C60 exposures, indicating that functionalization with these surface chemistries does not change the toxicity of unfunctionalized fullerenes to F1 daphnid size. In addition, SWCNT-CONH2 significantly decreased F1 adult size at a concentration of 50 ppm compared with controls (decrease of 5%, t = −6.439, p < 0.05). Increases in F1 size were observed with 10 ppm MWCNTs (increase of 2.8%, t = 2.374, p < 0.05) and 10 ppm SWCNT-CONH2 (increase of 6%, U = 3, p < 0.05). In the F2 generation, a decrease in adult size was observed for 10 ppm SWCNT-COOH (decrease of 4.9%, t = −2.876, p < 0.05) and increases in size were observed for 50 ppm C60 (increase of 1.7%, t = 2.003, p < 0.05), 50 ppm MWCNT (increase of 5%, t = 3.711, p < 0.05), and 10 ppm SWCNT-PEG (increase of 6.5%, t = 4.401, p < 0.05).
Multigenerational impacts of nanomaterials on Daphnia

FIGURE 6. Size impacts of functionalized fullerenes to (A) F1 and (B) F2 daphnids. Error bars indicate standard error. Values determined to be significant by t test at p < 0.05. BCD = beta-cyclodextrin.

DISCUSSION

Select CNMs exert a multigenerational effect on Daphnia survival, reproduction, and growth as exposure of the parent population of daphnids (F0) had a consequence for the F1 or F2 generations. The nature of the effect is dependent on the core nanomaterial structure and surface functionalization; however, the reason for the observed change in toxicity with specific surface chemistries is unclear. Others have proposed that surface charge plays a large role in toxicity [19,20], but the present data show toxicity associated with positive, negative, and neutral particle types. Similarly, the nanomaterials represented in the present study encompass a wide range of zeta potentials (ranging from $-60$ mV to $+23$ mV) and sizes (approximate diameters of 150 nm for fullerene particle types and diameters of several microns for carbon nanotubes) with no clear correlation between aggregation state and multigenerational impacts. It is possible that the interaction of the specific surface chemistry of the nanomaterials that reduced multigenerational reproduction in Daphnia led to specific interactions within the daphnid that need to be explored further, such as chemical-specific interactions with receptors in the organism [21], environmental or protein coronas that dictate interactions with the organism [22], or differences in genomic impacts of nanomaterials across generations. Results of the present study emphasize the importance of testing for multigenerational impacts of nanomaterials on sublethal endpoints because the results would not have been evident from single-generation assays.

Impacts of CNMs on F1 and F2 reproduction

Carbon nanomaterials impacted reproduction across generations, but this impact was specific to the type of nanomaterial to which the original organism was exposed. Reproduction of F1 was decreased by several treatments including 50 ppm SWCNT, SWCNT-CONH$_2$, C$_{60}$-OH, and 5 ppm C$_{60}$-malonate; and F2 reproduction was decreased by 50 ppm C$_{60}$ and SWCNT-CONH$_2$ treatments. The F1 daphnids were born in the nanomaterial medium and received a brief initial exposure to the nanomaterials as neonates; and Daphnia neonates are often pregnant when they were born, so it is possible that the F2 daphnids also received an initial exposure to nanomaterials during sensitive developmental stages.

Multigenerational nanomaterial toxicity could be mechanical in nature. The daphnid feeding current has been shown to be diverted to the brood chamber to oxygenate the neonates as they develop [23]; and if nanomaterials are present in this feeding current, they could disrupt the flow of oxygen and other nutrients to the embryos. The transfer of nanomaterials across the epithelial lining of the parent daphnid digestive tract to lipid storage compartments could also impact F1 daphnids. Lipid storage compartments are used for sustenance during periods of low food resources and for the synthesis of vitellogenin [24], which is an essential protein required for embryogenesis in Daphnia. Many nanomaterials have lipophilic properties, and it is possible for nanomaterials to accumulate in lipid storage compartments of Daphnia as they are ingested from feeding with subsequent impacts to vitellogenin synthesis and activity. Some CNMs have already been shown to inhibit protein activity [25] and disrupt membrane transport activities in the cell [26], and these actions could impede normal daphnid reproduction and growth.

Multigenerational reproductive toxicity could also result from changes in gene expression and genotoxicity [27]. Nanomaterials have been shown to generate oxidative stress in organisms [28,29], increase DNA damage [30,31], and induce immune system activity [32]. Molecular models also indicate the potential for CNMs to bind to DNA and alter DNA conformations [33]. Previous work in our laboratory showed differential expression of oxidative stress biomarkers glutathione-S-transferase and catalase in daphnids exposed to CNMs [17]. Other types of nanomaterials have also been shown to induce genetic changes in Daphnia as zinc oxide nanoparticles induced differential expression of multicystic, ferretin, and C1q genes [34]. Changes in gene expression have also been found in Caenorhabditis elegans after exposure to silver nanoparticles regarding expression of the SOD and Daf12 genes [35].

Interestingly, reproduction increased in F1 daphnids from 10 ppm C$_{60}$-BCD treatments. It is possible that cyclodextrins could be utilized for additional nutritional value with a consequence of increased reproduction in F1 daphnids as CNMs have been shown to utilize lipids that are non-covalently bound to SWCNTs for nutritional value in conditions of starvation [36]. However, reproduction in F1 Daphnia was not increased by BCD treatments alone. In addition, reproduction was not increased in 1 ppm C$_{60}$ treatments that were non-covalently bound to $\gamma$CD. Treatments with C$_{60}$-amino-$\gamma$CD and C$_{60}$-malonate-$\gamma$CD were too toxic for F0 daphnids to conduct multigenerational trials at concentrations higher than 1 ppm. The F1 and F2 daphnids from SWCNT-PEG treatments also exhibited trends for increased size and reproduction, and it is possible for daphnids to use PEG attachments for nutritional value in ways similar to the cyclodextrins discussed above.

Impacts of CNMs on F1 and F2 size

Some CNMs induced changes to the size of adult Daphnia. Although these changes were statistically significant, they were
relatively small in nature with potentially negligible biological implications to the overall fitness of the individual or population. This is in contrast to the 10% decrease in adult size of F₀ daphnids for many of these nanomaterial exposures in our previous study [14]. There was a slight decrease in size of F₁ daphnids from 10 ppm and 50 ppm CONH₂ exposures; however, F₂ daphnids exhibited sizes that were comparable to controls, indicating that daphnid populations can recover from this effect. Increased size was observed in F₁ and F₂ daphnids for some nanomaterial types in this experiment, and this could support the idea of a life-strategy shift to produce fewer neonates (reduced number of offspring) of higher quality (larger neonate size) in times of environmental stress [37]. Overall, no nanomaterial treatments impacted size by more than 6.5%, and these results suggest that CNMs do not have strong multigenerational impacts on daphnid size.

Potential for transgenerational toxicity

The multigenerational toxicity of CNMs investigated in the present study could also be explained by toxic impacts to the daphnid epigenome, suggesting the potential for transgenerational toxicity of nanomaterials. “Transgenerational toxicity” is defined as an exposure of a previous generation of organisms to a chemical that induces a change to the germ line that is propagated to future generations of organisms that never received a direct exposure to the chemical. Epigenetic impacts from DNA methylation and histone modifications have already been shown with other types of toxicants [38]. Changes in the epigenome are heritable and can appear in future generations of organisms even after the exposure is removed [39]. Many of the effects to Daphnia that were observed in the nanomaterial treatments across generations disappeared by the F₂ generation, indicating that most of the treatments do not likely have an epigenomic effect on daphnids. However, exposure of daphnids to 50 ppm SWCNT-CONH₂ resulted in changes to reproduction that were consistently decreased across F₀, F₁, and F₂ generations; and it is possible that this exposure could have transgenerational impacts on Daphnia. If exposure of Daphnia to SWCNT-CONH₂ resulted in epigenetic changes to germ-line cells, the effect would be observed in future generations, even after the exposure was removed. Future work will include an evaluation of genetic and epigenetic marks (DNA methylation) of F₂ and F₃ generations to observe whether any patterns arise regarding genetic or epigenetic expression and reduced reproduction for this particle type.

Impacts of CNMs on ecological viability of Daphnia

Daphnids play an essential role in aquatic food webs [40], and a sudden decrease in daphnid population viability over several generations could be detrimental to the balance of an aquatic ecosystem. The results seen in the present study describe survival and reproductive impacts of some nanomaterial types up to 20% that persisted past the initial F₀ exposure, and this could have important ecological consequences for population dynamics in natural environments. The present study calls for more detailed information on the types of surface chemistries that may be appropriate for creating nanomaterials that have lower toxicity across generations and are therefore more sustainable. Acquiring toxicity information about how a nanomaterial can influence sensitive early developmental stages of an organism and future generations of organisms is an essential component to understanding the potential ecological impacts of nanomaterials on ecosystems.

SUPPLEMENTAL DATA

Tables S1 and S2. (64 KB DOCX).

Acknowledgment—This research was supported by National Science Foundation funding (CBET 1134013) to R. Klaper. Special thanks go to individuals who helped with experiments: A. Nikolaus, J. Bozich, N. Neureuther, and B. Blalock. Special thanks also go to the committee members who offered expert advice on this research—R. Hutz, A. Udvardia, J. Kaster, and M. Carvan—and 2 anonymous reviewers.

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