Titanium oxide nanoparticles in the freshwater ecosystem: *Atya lanipes* **shrimp as a nanotoxicological model**

by

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Abstract

This dissertation specializes in studying and determining the environmental concentrations of an emerging pollutant of which we do not fully know, titanium oxide nanoparticles (TiO_2 NPs). Although we are aware of the extensive use and commercial production of this nanoparticle in the US and that there are various sources of contamination and their entry into rivers, we are almost completely unaware of the environmental concentrations that exist in Puerto Rico. There is little knowledge of the toxicological effects that could be associated with ecologically susceptible species. To date, there are no federal or state regulations for acceptable environmental concentrations in our rivers. This study aims to determine the actual status of urban and rural rivers in Puerto Rico related to the presence/absence of TiO_2 NPs and the specific fate and behavior in a laboratory-controlled environment. Also, we want to understand and measure how the presence of these nanoparticles in the freshwater ecosystem results in lethal and sublethal effects due to the toxicity of the TiO_2 NPs, on the life cycle of an endemic shrimp in Puerto Rico, *Atya lanipes*. The objectives of this dissertation are: 1) Determine the environmental concentrations of titanium oxide nanoparticles and the fate and behavior laboratory assessment, 2) Describe the early larval development description of the freshwater shrimp *Atya lanipes* holthuis, 1963 (Decapoda: Caridea: Atyidae), 3) Establish titanium oxide nanoparticles as emerging aquatic pollutants evaluating the nanotoxicity in the freshwater shrimp larvae *Atya lanipes,* and 4) Determine the neurotoxicity and oxidative stress development in adult *Atya lanipes* shrimp exposed to titanium oxide nanoparticles. Through field and laboratory studies, we obtained for the first time the concentrations of titanium oxide nanoparticles in sediment and dissolved titanium in water from the Rio Piedras and Sabana rivers. Also, we were

able to elucidate the first nine (9) larval stages of the *Atya lanipes* shrimp. Then, after acute and chronic exposures, we evaluated lethal and sublethal toxic effects in both larval and adult stages of the *Atya lanipes* shrimp. This study is an innovative one that contributes significantly to our scientific knowledge of the magnitude of the concentration of TiO_2 NP that we are facing. It provides a starting point to begin public policy and better management in the use and discharge of this type of nanomaterial.

Keywords: *Atyidae, emerging pollutants, nanoecotoxicology, Puerto Rico, Rio Piedras river, Sabana river, shrimp, streams, water quality, water pollution*

DEDICATION

This dissertation is dedicated to my immediate family, my husband Gabriel Vicente Vicente and my children Maia Vicente Cruz and Ares Vicente Cruz. Thank you all for being present at every moment of this long road. Also, to my Chair Advisor, Dr. Omar Pérez-Reyes for the guidance in my doctorate path and for believing in me all the time.

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CHAPTER I:

INTRODUCTION

The environment which is vital for human subsistence, faces new anthropogenic challenges every day. One of the most relevant environmental problems is aquatic pollution. Mainly, those aquatic ecosystems are defined as freshwater. This 0.4% of water availability represents our drinking water source; every day, chemicals and pollutants enter these ecosystems (Mishra & Dubey, 2023). Those emerging pollutants, of which there is no knowledge about the impacts they can cause on biodiversity and the functioning of the entire ecosystem, concern and alarm us the most as a scientific community.

The high population density, technological development, and the need to produce more and better products and services arecausing us to face environmental problems that we are not aware of, much less we know how to combat them. We live in a technological age. The human being has developed new knowledge and scientific developments, and this has allowed us to manipulate and produce more efficient substances or materials.

Nanotechnology, emerging since 1969, is a very recognized technological field with much commercial development around the world. It is defined as producing and manipulating matter at length scales of 1 to 100 nm in at least one dimension. In these tiny sizes, it is proved that matter can exert different physicochemical properties making them more reliable than in the natural environment of matter (Turan et al., 2019). Thanks to this, nanotechnology has enhanced products related to the food industry, medicine, cosmetics, electronics, water treatment and many

others (Khan & Khan, 2019). Its impact on society is on a large scale and may compromise the future development of our society.

Of the many types of nanomaterials, we can now define engineered nanomaterials that humans produce and their origins are not natural. For example, titanium oxide nanoparticles (TiO_2 NPs) are one of the most commercialized and produced in the world (Dedman et al., 2021). It is used in sunscreens, water treatment, cosmetics, food and packing, medicine, and others (Berardinelly & Parisi, 2021; Musial et al., 2020). The United States of America is the country with the most commercial production of TiO_2 nanoparticles. The average commercial production and uses of TiO_2 nanoparticles inthe United States . ranges from 7,800 to 38,000 tons/year. Europe consumption of the same nanomaterial is around 55 to 3,000 tons/year. Worldwide, the production and consumption are 550 to 5,550 tons/year (Nam et al., 2014). Considering this information, the excessive use of TiO_2 nanoparticles in the United States can predispose to several environmental impacts.

Recent studies are documenting the release of engineered nanoparticles into the aquatic environment via several routes, such assanitary discharges, domestics discharges, industrial discharges, andsunscreens use, (Tian et al., 2010; Mueller & Nowack, 2008; Bhatt & Tripathi, 2011). However, although we know this, we do not have robust knowledge about the possible environmental concentrations that we could face in Puerto Rico's rivers. In fact, many studies also recognize that there is no complete knowledge about the behaviors that these nanomaterials can present in the aquatic ecosystem (Bathi et al., 2021). We know that the water's physicochemical parameters can produce some nanoparticles' compositions and that these are destined to settle (cita). Not knowing the behavior of these contaminants in river water, it is important to fill the gap between the environmental concentrations in

river water in Puerto Rico, and the possible biological effects that it could cause in the biodiversity of these ecosystems. Therefore, it is necessary to study the ecologically susceptible populations. Many toxicity studies are carried out in organisms that are not environmentally susceptible to the presence of these nanomaterials or in some cases they are studied in organisms that inhabit other aquatic ecosystems that are not necessarily rivers (cita).

Freshwater ecosystems, specifically in the Caribbean and Puerto Rican rivers, differ from temperate rivers in that shrimp, not fish dominate the food web (cita).. The shrimp community have a leading ecological role in rivers. with ecological niches and complementary forms of feeding that enrich the ecosystem and keep it functional. In the case of the shrimp *Atya lanipes* Holthuis, 1963, a role of biofiltration of particles is attributed to it; this is considered a key role in the ecosystem. Water quality is improved when the species is present (cita). Like the other shrimp species in Puerto Rico, this shrimp has an amphidromous life cycle. This type of life cycle is characterized by the two different aquatic ecosystems to complete its life cycle. The adult shrimps release the larvae downstream normally in times of heavy rain. These larvae remain lecithotrophic (do not feed) for approximately 5 days until they reach the estuary. In the estuary, they develop as larvae until they metamorphose into a juvenile shrimp. The metamorphization time and the number of larval stages vary between species. Although the larval stages of other members of the family Atyidae have been described (*Atya innocuous and Atya scabra*) (Hunte, 1979; Vergara & Jiménez, 2008), to date only the larval stage I of the *Atya lanipes* shrimp is known in detail (Hunte, 1975).

Goals and objectives

Pointing out what was previously described about the aquatic contamination of freshwater bodies such as rivers, the need for conservation of the endemic shrimp species in Puerto Rico *Atya lanipes* and the gap that exists on their morphological development and possible interaction with pollutants both in the river as in the study in different stages of life leads us to formulate the following general aim: To determine the actual status of urban and rural rivers in Puerto Rico related to the presence/absence of TiO_2 NPs and the specific fate and behavior in laboratory-controlled environment. Also, we want to understand and measure how the presence of these nanoparticles in the freshwater ecosystem results in lethal and sublethal effects due to toxicity of the TiO_2 NPs, on the life cycle of an *Atya lanipes*. The specific objectives are: 1) Determine environmental concentrations of titanium oxide nanoparticles and dissolved titanium in sediment and water 2) Describe early larval development of the freshwater shrimp *Atya lanipes* Holthuis, 1963 (Decapoda: Caridea: Atyidae), 3) Determine titanium oxide nanoparticles toxicity freshwater shrimp larvae *Atya lanipes,* and 4) Determine the neurotoxicity and oxidative stress development in adult *Atya lanipes* shrimp exposed to titanium oxide nanoparticles.

To achieve this, we have proposed the following research questions with the following research tracks: 1) Environmental concentrations of TiO_2 nanoparticles in urban and non-urban rivers and understand the specific behavior of the nanomaterial in both water chemistry, a) What are the environmental concentrations of titanium oxide nanoparticles in the Piedras River (urban) and the Sabana River (non-urban)? 2) Fully knowing our study organism, a) What are the favorable environmental conditions to grow *Atya lanipes* larvae in the laboratory? b) How many stages can the development of the freshwater shrimp *Atya lanipes* be described?, 3) Early larval stage exposure to TiO_2 NPs, a) Does the exposure of TiO_2 in the early larval stage

exposure leads to lethal effects? b) Are there any sublethal effects observed as morphological changes of the larvae that affect their pigmentation, body structures or their size? c) Could an alteration in the normal movement of the larvae be observed? and 4) Related to the adult stage an exposure to TiO_2 NPs, a) Can exposure to titanium oxide nanoparticles in the adult stage of *Atya lanipes* trigger changes in movement? b) Due to acute to chronic exposure to titanium oxide nanoparticles, could adult *Atya lanipes* shrimp experience the development of oxidative stress? c) What is titanium oxide nanoparticles' effective concentration (EC_{50}) in adult *Atya lanipes* shrimp?

The proposed hypotheses are: 1) The presence of TiO_2 nanoparticles will be in higher concentration in the Rio Piedras River than in the Sabana river due to the greater anthropogenic impact and contamination, 2) The larval development of the freshwater shrimp *Atya lanipes* will be completed in estuarine conditions due to its amphidromy and will be completed between one to three months. Therefore, its development characteristics will be very similar to the shrimp of the Atyidae family previously described, such as *Atya innocuous*, 3) The exposure of TiO_2 NPs among *Atya lanipes* larvae would develop a toxic effect showing lethal and sublethal effects due to the photocatalytic activity and the production of oxidizing agents that damage neurons and other tissues, and 4) The acute exposure to titanium oxide nanoparticles will develop a toxic effect on the nervous system of the *Atya lanipes* shrimp that will produce hypoactivity behavior as has been determined in the larval stage of this species and in consequence we will find observed effects at very low TiO_2 NPs concentration due to the capacity of producing oxidative stress by Anatase form of this nanoparticle. Also, the development of oxidative stress will be evident

and will remain constant from acute to chronic exposure reflected in an increase in catalase enzyme activity.

The justification of this study is that it would bring innovation to the scientific field associated with the study of emerging pollutants such as TiO_2 NPs in ecosystems as valuable as freshwater. First, it will provide new knowledge about the effect of these nanoparticles (positive or negative) on an endemic shrimp, thus helping in the biodiversity conservation effort in Puerto Rico and the ecosystem management and maintenance. Also, this study may contribute to the need in the scientific literature to use the correct organisms in these nanotoxicological studies. Most nanotoxicological studies are performed on organisms dwelling in the water column. However, the benthic organisms play a key role in the functioning of the ecosystem and are the most vulnerable to being exposed to high concentrations of nanoparticles in the sediment (Garric & Thybaud, 2011). In this way, *Atya lanipes* is a shrimp that may be susceptible to the presence of the engineered nanoparticles in two different aquatic ecosystems: the estuary and the river. In addition, the way in which this shrimp feeds can help us to understand more assertively the biological effect on an organism that can be seen to be significantly affected by the presence of these nanoparticles not only in the water column but also in the sediment. Finally, there is a great need to do toxicological studies on nanoparticles since they are being used excessively, and the possible biological impacts are largely unknown. TiO_2 NPs are used in a wide variety of products and materials, and there are proposals to use them directly in freshwater ecosystems for "restoration" since they can degrade (catalyze with reactive oxygen species), pesticides, organic chemicals, among others. Therefore, understanding toxicological severity is a matter of great relevance today and for the future of our planet.

Summary of Chapters Content

Chapter two presents data on the environmental concentrations of titanium oxide nanoparticles in sediment for 2022 and the concentrations of dissolved titanium in water between 2021, 2022 and 2023 in the Rio Piedras and Sabana rivers. Also, the fate and behavior of the titanium oxide nanoparticles in Sabana and Rio Piedras rivers in laboratory-controlled environments are presented.

Chapter three presents the early larval development of the freshwater shrimp *Atya lanipes*. The nine described larval stages and the laboratory environmental conditions in which this development could be obtained are shown.

Chapter four describes the toxic effects of acute exposure (24, 48 and 78 h) to titanium oxide nanoparticles during the first and second life stages of the *Atya lanipes* shrimp.. Again, lethal and sublethal effects are observed.

Chapter five presents the toxic effects and the development of oxidative stress after chronic water exposure in adult *Atya lanipes.*. Neurotoxic effects and oxidative stress are observed mainly in gastrointestinal and nervous tissues.

Finally, in chapter six, the conclusions, limitations, and lines of future research are presented.

Literature Cited

Bathi, J. R., Moazeni, F., Upadhyayula, V. K., Chowdhury, I., Palchoudhury, S., Potts, G. E., & Gadhamshetty, V. (2021). Behavior of engineered nanoparticles in aquatic environmental samples: Current status and challenges. Science of the Total Environment, 793, 148560.

Berardinelli, A.; Parisi, F. TiO_2 in the food industry and cosmetics. In Titanium Dioxide (TiO_2) and Its Applications; Elsevier: Amsterdam, The Netherlands, 2021; pp 353–371.

Bhatt, I., & Tripathi, B. N. (2011). Interaction of engineered nanoparticles with various components of the environment and possible strategies for their risk assessment. Chemosphere, 82(3), 308-317.

Dedman, C.J.; King, A.M.; Christie-Oleza, J.A.; Davies, G.L. Environmentally relevant concentrations of titanium dioxide nanoparticles pose negligible risk to marine microbes. Environ. Sci. Nano 2021, 8, 1236–1255.

Hernández-Vergara, M. P., & Jiménez–Rojo, S. (2008). Desarrollo larval y supervivencia de *Atya scabra* (Crustacea: Decapoda: Atyidae), a diferentes salinidades de cultivo. Ciencia Pesquera, 16, 17-24.

Hunte, W. (1975). *Atya Lanipes* Holthuis, 1963, in Jamaica, Including Taxonomic Notes and Description of the First Larval Stage (Decapoda, Atyidae). Crustaceana, 28(1), 66-72.

Hunte, W. (1979). The complete larval development of the freshwater shrimp *Atya innocous* (Herbst) reared in the laboratory (Decapoda, Atyidae). Crustaceana. Supplement, 231-242.

Khan, I.; Saeed, K.; Khan, I. Nanoparticles: Properties, applications and toxicities. Arab. J. Chem. 2019, 12, 908–931.

Mishra, R. K., & Dubey, S. C. (2023). fresh water availability and it ‘s global challenge. Journal of Marine Science and Research, 2(1).

Mueller, N. C., & Nowack, B. (2008). Exposure modeling of engineered nanoparticles in the environment. Environmental science & technology, 42(12), 4447-4453.

Musial, J.; Krakowiak, R.; Mlynarczyk, D.T.; Goslinski, T.; Stanisz, B.J. Titanium dioxide nanoparticles in food and personal care products—What do we know about their safety? Nanomaterials 2020, 10, 1110. [Google Scholar] [CrossRef]

Nam, D. H., Lee, B. C., Eom, I. C., Kim, P., & Yeo, M. K. (2014). Uptake and bioaccumulation of titanium-and silver-nanoparticles in aquatic ecosystems. Molecular & Cellular Toxicology, 10, 9-17.

Tian, Y., Gao, B., Silvera-Batista, C., & Ziegler, K. J. (2010). Transport of engineered nanoparticles in saturated porous media. Journal of Nanoparticle Research, 12, 2371-2380.

Turan, N.B.; Erkan, H.S.; Engin, G.O.; Bilgili, M.S. Nanoparticles in the aquatic environment: Usage, properties, transformation and toxicity—A review. Process Saf. Environ. Prot. 2019, 130, 238–249. [Google Scholar] [CrossRef]

CHAPTER 2: ENVIRONMENTAL CONCENTRATIONS OF TITANIUM OXIDE NANOPARTICLES IN RIO PIEDRAS AND SABANA RIVERS

Abstract

The River Continuum Concept (Vannote et al., 1980), is sometimes applied to freshwater rivers in the tropics. However, tropical island rivers, such as those in Puerto Rico, are generally smaller that differ sometimes from the temperate zones and other areas. In fact, we often refer to our rivers as "streams" because of their small sizes. Also, the concept of the urban river syndrome is one that arises as a product of the direct and indirect impacts that urbanization has had on freshwater rivers. One of these impacts is the aquatic pollution. Titanium oxide is an emerging pollutant in aquatic environments and the scientific community is attentive to the ecological and environmental impacts. This study aims to determine the concentrations of TiO_2 NPs present in both water and sediments in two rivers in Puerto Rico that represent urban and non-urban areas. The methodology used was to transport water and sediment samples of the Rio Piedras River (urban) and Sabana rivers (non-urban) to the laboratory and use ICP-OES analytical technology to determine the environmental concentrations of TiO_2 NPs and dissolved titanium. The results showed the presence of the nanoparticles in sediment in both rivers. Rio Piedras showed an average of 47.34 mg/kg $\pm$ 7.30 and Sabana 48.30 mg/kg $\pm$ 13.1. In water, dissolved concentrations were 0.010 mg/L $\pm$ 0.01 for Rio Piedras and 0.005 mg/L $\pm$ 0.006. for Sabana river. One way ANOVA revealed that these differences were not significant between river sediment and water concentrations. The data obtained in this study are novel in our knowledge about environmental concentrations of TiO_2 NPs in rivers in Puerto Rico.

Keywords: *freshwater, nanomaterials, sediment pollution, water pollution*

INTRODUCTION

River ecosystem structure and function

Temperate freshwater ecosystems are described by the River Continuum Concept (Vannote et al., 1980). This concept has been very well studied in this part of the planet. It describes the hydrological part of the hydrographic basin based on the principle of dynamic balance between the hydrological, physical and biological parts. In this way, the river is defined as a fluvial ecosystem that can flow for hundreds or thousands of kilometers from its source at the head of the hydrographic basin until it empties into the sea. While the river flows, it manifests changes in water currents and their chemistry, causing different organisms to be distributed in microhabitats within the river ecosystem. The observable change from small streams to large rivers, characteristic of these ecosystems, allows the distribution of organisms according to local conditions (such as temperature, width of the stream, slope, availability of light in the bottom, current, and the amount of litter ingress), and to changes in the nature of the materials transported by the upstream (the downstream increase in fine particles and the decrease in coarse residues). This is why there is a relationship between the type of metabolism of organisms and the very properties of the ecosystem of natural rivers and streams (photosynthesis and respiration). Consequently, upstream animals are commonly (but not exclusively) concentrated in high areas of the watershed where the input of allochthonous material is continuous (greater presence of grinders and collectors). However, going downstream the riparian vegetation begins to change from large canopies to lower plants that allow more sunlight to enter and therefore increasing photosynthesis. Many organisms that prefer particulate fine organic matter have it available due to

the biological activity of the organisms that crushed the organic material in the upper parts of the basin. Lastly, in the lower parts of the hydrographic basin, we find a greater distribution of photosynthetic organisms and other organisms that feed on very fine particulate organic matter that arrives from the upper parts of the basin (allochthonous material). In general, the concept of the continuous river aims to present the ecosystems of natural rivers and streams that maintain notable and continuous changes in the downstream direction (Meza, 2017).

The River Continuum Concept (Vannote et al., 1980) has been applied to freshwater rivers in the tropics. However, the rivers in this area, such as Puerto Rico, are very small rivers (of low order) that differ greatly from the temperate zones and other areas. In fact, we often refer to our rivers as "streams" because of their small sizes. Also, in the rivers of Puerto Rico shrimp species dominate the food web, while in other insects dominate mainly (Ramirez, et al. 2009). On the other hand, the tropical zone differs from the temperate zone in temperature since the tropics maintain a relatively constant temperature and higher average air temperature throughout the year, while the temperate zone varies in seasons and temperatures. Also, the input of organic matter differs since the tropics are characterized by receiving the input of organic matter throughout the year (increasing during climatic events such as heavy rains and/or storms), while temperate rivers receive the input of organic matter mainly in autumn.

When it comes to freshwater ecosystems in the tropics, they can be described as fluvial ecosystems that, as they descend the slope, also undergo changes in currents and water chemistry, in addition to changes in riparian vegetation and in the deposition of materials through the river. In this way, it is that in a similar way, in tropical rivers, the distribution of various organisms in microhabitats throughout the

entire freshwater ecosystem can be observed. Mainly, we find that in the headwaters of the rivers shrimp dominates whilefish dominate further down the slope. However, shrimp generally dominate freshwater ecosystems in Puerto Rico, unlike other areas where it is mainly insects (Ramirez, et al. 2009).

Urban River Syndrome

The concept of the urban river syndrome arises as a product of the direct and indirect impacts that urbanization has had on freshwater rivers. After many studies conducted in various rivers around the world, mainly in temperate places, the patterns of impacts that each of these urbanized temperate rivers presented were established and described as symptoms. Identified symptoms include changes in hydrography, high concentrations of nutrients and contaminants, altered morphological channels, and reduced biodiversity (Walsh, et al. 2005; Kominkova, et al. 2007). In general, around the world, there is a lot of consistency in the symptoms shown in urban rivers, but the degree or intensity to which they appear can vary depending on local conditions (Kominkova, 2012). However, in general, symptoms affect hydrology, water and sediment chemistry, channel morphology, impacts on fish, invertebrate, and algae communities, and ecosystem processes (production, nutrient retention, metabolism net of the ecosystem, etc.) (Kominkova, 2012).

The concept of the urban river syndrome, established mainly for temperate rivers, also establishes symptoms or characteristics applicable to tropical rivers. The current information on the concept of the urban river syndrome in tropical rivers is very limited, and in early development. However, Ramírez et al. 2009, carried out a pertinent project to explain how the urban river syndrome occurs in Puerto Rico as an example for other tropical rivers due to the high presence of various ecosystems

on the island. In this way, our tropical island has representative ecosystems of other islands or continents in the tropical area (Ramírez, et al. 2009).

The case of tropical rivers is a very interesting one to analyze in this context because currently in Latin America, we have a greater number of people living in urban areas than rural ones. This urban increase has been shown to have great impacts on ecosystems. But many of these impacts are not being properly documented or managed effectively. This is why much of the information used as a guide is from non-tropical regions. (Dudgeon, 2000; Ramirez, et al. 2009).

Based on the work of Ramírez and collaborators in 2009, urban rivers in Puerto Rico show environmental problems observed in other regions of the planet, including the high concentrations of pollutants in these rivers. On the other hand, high levels of solutes in the water are consistent as urbanization increases in the hydrographic basin. In terms of nutrients and conductivity, they also increase with the increase in urban areas.

In urban rivers in Puerto Rico, a negative response of many taxa of aquatic invertebrate organisms is observed as urbanization increases (Ramirez, et al. 2009). Generally, in these urban rivers, the riparian vegetation is altered, affecting many insect species, making many of these species absent in some of these urban areas. However, some decapod shrimp species (another important group), adapts to living in urban environments, but there is still much more to learn about it.

Regarding the morphology of the channels of urban rivers in Puerto Rico, it is observable that these have highly altered channels in their morphology. This is due to excess channeling with concrete and other structures, straightening and filling of the channels urban developments for flood control. For its part, channel incisions are also observed in very urban areas. These canals handle large amounts of water and

are easily filled due to atmospheric events characteristic of the tropics, such as heavy rains and storms. Additionally, the coastal areas that were initially used for agriculture have been filled with accumulated sediment, making the channels in these areas much smaller, narrower, and shallower in the downstream direction and not larger, wider, and deeper as expected. This makes these canals unable to support or handle large water volumes and runoff causing major flooding in the city.

In tropical urban rivers, like in temperate rivers, changes in ecosystem processes are expected. The alteration of these processes can bring about a cascade of effects on the biodiversity. An example is the increase in the average water temperature of urban rivers of 25 ºC around the year. The impact of these high-water temperatures has been associated with microbial activity (Ramirez, et al. 2009).These microbes can carry out respiration up to approximately 28 ºC, after which their activity is reduced. However, these temperatures have been reached in the Río Piedras basin. In this way, it is anticipated that these temperatures may further affect many ecosystem processes.

TiO_2 NPs in aquatic ecosystems

Nanotechnology development refers to a special and purpose technology that is implemented at the nanoscale (1-100 micrometers in at least one dimension). It is well known that many elements at these nano sizes present better and applicable chemical properties than in their normal sizes. This characteristic is the reason that to date there are many applications of these nanomaterials in different sectors including technology, medicine, food industry, pharmaceutical industry, cosmetics, and others. In the United States of America (federal R&D), there is an increased tendency to invest about $20 billion in nanotechnology (2015). This tendency continues to grow (Buschan, 2017).

Currently, we are facing new aquatic contaminants that threaten not only the ecosystem's health, but also the communities that compose them are in danger due to possible adverse effects (cita). Scientific investigations are continually being conducted to assess and determine the presence of emerging aquatic contaminants. Recent studies documented the presence of nanomaterials into aquatic environments (Selck, et al., 2016). Especially for those nanomaterials that are very used and common in many industrial products, cosmetics, food, technology, and others (Mueller & Nowack, 2008; Wigginton et al., 2007).

However, although it is known that there are various routes of exposure of these nanoparticles in rivers and bodies of freshwater, no real estimate is known of how the concentrations of this nanomaterial are found in bodies of freshwater in Puerto Rico. This is because there is currently a limitation in studies that have determined the behavior and fate of nanoparticles in water-sediment systems and their environmental concentrations worldwide (Luo et al., 2017). Although this information is determined, there is a lack of standardization of analytic methods to determine the environmental concentrations of nanomaterials (they are expensive, inaccessible, or destructive for the desired samples) and the data available is not consistent.

This study aims to determine the concentrations of TiO_2 NPs present in both surface water and sediments in two rivers in Puerto Rico that represent an urban area and a non-urban area. We hypothesize that the environmental concentrations of TiO_2 NPs will be higher in an urban river such as Rio Piedras and lower in a non-urban river such as Sabana. Also, the water and sediment concentrations will be higher in the samples near the mouth. Finally, the dry months will present higher concentrations.

METHODS

Site selection

This study used two watersheds, the Sabana and the Rio Piedras rivers, to compare and analyze water and sediment samples in natural and laboratory settings. The Sabana River was used as a representation of a low urban impact basin (51% of secondary and primary forest; 6.2% of urban development) (Perez, et al. 2015), while the Rio Piedras River represented a river greatly impacted by urbanization (18.5% of secondary forest; 60% of urban development) (Perez, et al. 2015) (Figure 2.1).

The watershed of Rio Piedras, located in the northeastern area, represents the highest level of urbanization and anthropogenic impact than any other watershed in Puerto Rico Island (Figure 2.1 A). It has a population of 2 to 2.5 millions, being a highly populated area (second-largest city in the Caribbean) (Lugo et al., 2011; Ramírez et al., 2014). The watershed drains almost all the island's capital, San Juan (metropolitan area) (De Jesús-Crespo & Ramírez, 2011; Ramírez et al., 2009). The physical conditions of the daily average temperature of Rio Piedras watershed range from 24-30 °C (NCDC, 2009). It is mostly flat with a highest elevation of 200 m asl (De Jesús-Crespo & Ramírez, 2011). This represents a metropolitan area experiencing frequent runoff and floods yearly (Osterkamp 2001; De Jesús-Crespo & Ramírez, 2011). Although there is not enough scientific data, it is known that this basin is susceptible to illegal discharges and consequently to aquatic pollution. The specific land-cover of this urbanized watershed comprises 26.4% forest, 15.3% pasture, and 55.4% urban areas (Ramos, 2014). The riparian vegetation is characterized by grass and non-native species. The water and sediment samples were collected in the lower part of the watershed (18.416173, -66.078322).

Sabana watershed is located in the northeastern part of Puerto Rico Island (Figure 2.1 B) U.S. Forest Service manages the upper headwater of the watershed, and the most human-impacted zones are in the middle and lower parts of the watershed (Heartsill-Scalley & López-Marrero, 2014). Sabana watershed maintains a specific land-cover comprising 71.4% forest, 15.8% pasture, and 8.8% urban areas [Torres-Pérez & Pérez-Reyes, 2023; Ramos, 2014]. There are native riparian species in forested areas, while some grasses and fruit trees of non-native species are introduced in the watershed's high human densities density zones. The sampling was conducted in the lower part of the watershed (18.377329, -65.726071).

Natural water and sediment samples collection

Sampling points near the mouths were chosen to obtain the water and sediment samples from both rivers. Amber 1 L bottles were to collected the water by completely submerging the bottle (approximately 12 inches deep) and closing it underwater. . The samplings were performed on the same day for both rivers. We established two different localities/sampled sites in each river: High urban (HU) and low urban (LU) zones. The HU were the downstream zones, and the LU were the upstream zone of each rivers. The water samples were collected for the years 2021, 2022, and 2023; June 2021, January 2022, February 2022, April 2022, and February 2023). For the sediment samples, 0.5 L amber bottles were used. The sediment was collected, and the bottles were closed at the same depth at which the samples were obtained. The sediment samples were collected for January 2022, April 2022, and February 2023. Subsequently, all samples, water, and sediments, were stored in a refrigerator at 27°C until chemical analysis.

Water and sediment samples analyses

The chemical analytic methods used in this study was ICP-EOS. This technique only detects titanium. However, if we detect titanium in the digested samples, it is accurate to state that the samples do contain Ti-NPs. Whatever is detected on the water only, it can be correlated to soluble titanium.

ICP-OES for detecting titanium (Ti) in water and sediment samples

Titanium standard solutions preparations

Solutions of 15 mL of Ti 10 ppm and 50 mL of Ti 100 ppb (~500 uL of Ti 10 ppm + 49 500 uL of Nitric Acid 2%) solutions were prepared. Using standard solution in laboratory, the following solutions (15 mL of Ti) were made: 50 ppb (7.5 mL of 100 ppb and 7.5mL of HNO_3 2%), 25 ppb (3.75 mL of 100 ppb and 11.25mL of HNO_3 2%), 20 ppb (3 mL of 100 ppb and 12 mL of HNO_3 2%), 15 ppb (2.25 mL of 100 ppb and 12.75 mL of HNO_3 2%), 10 ppb (1.5 mL of 100 ppb and 13.5 mL of HNO_3 2%), 5 ppb (750 uL of 100 ppm and 250 uL of HNO_3 2%), and 2.5 ppb (375uL of 100 ppb and 14 625 uL of HNO_3 2%). After the standard solutions more nitric acid 2% (3 L to 4 L) (Info: 57.17 mL in 2 L or 28.6 mL in 1 L) was added.

Sediment protocol for ICP-OES analysis

The sediment samples were weighted (1.000 g) in an analytical balance. The digestion process was conducted using the EPA procedures (5 min to 95 °C, 20 minutes at this temperature and then freezing) in a digestion microwave. In each sediment sample, 9 ml of HNO_3 and 2 ml of HCl concentrated were added previously. After digestion, the samples were filtered, and deionized water was added to a final volume of 50 mL. (Roughly 12.6% HNO_3 and 1.4% HCl in the

background; this translates also to 20,000 ppm of sediment concentration). The final digested samples were stored at the refrigerator at 27 °C until chemical analysis.

Before the chemical analysis, samples were diluted to ~133 ppm. Centrifugation of the samples (already in 50 mL Falcon tubes) and collect 10 uL of the supernatant solution. Then, the 10 uL samples were transferred in a 1.5mL Eppendorf tube and added 1490uL of HNO_3 2%. A rest for ~2 hours. Samples were diluted to ~100 vppb. Using a 15mL Falcon Tube, we added 14 989 uL of Nitric acid and centrifuged once more the samples (in 1.5mL Eppendorf tubes) and transferred 11 uL of the supernatant solution to a Falcon tube with nitric acid. The samples rested for 24 hours.

Water protocol for ICP-OES analysis

For the water samples preparation adjusted from EPA method 200.7, pp 31-32, for untreated water was used. For 25 mL of water, we added 500 uL nitric acid concentrated and 250uL of HCl concentrated.Then, we refluxed and evaporated the water sample (avoiding vigorous boiling) until it was roughly 2 mL of sample. The samples were cooled and transferred to a 50mL flask, filled with nitric acid 2% and let settle overnight). Next, the samples were centrifuged ensuring there were no solids formed A sample transferring to a 15 mL Falcon tube was conducted.

Titanium method preparation in ICP-OES

Validation of the samples is properly written, and extra nitric acid 2% is needed for the instrument wash cycles. The full method was conducted with the autosampler. Samples were entered manually to the autosampler.

Statistical Analyses

Descriptive statistics were used to compare the titanium oxide and dissolved titanium concentrations in water and sediment samples. A full-nested ANOVA was performed with Tukey test to compare each river, months of sampling and locality with titanium oxide and dissolved titanium concentrations in water and sediment. Finally, a one-way ANOVA was performed to compare the concentrations of TiO_2 NPs and each variable of month, locality, and river. Analyses were performed in MINITAB.

RESULTS

Natural Sediment

Environmental concentrations of TiO_2 NPs were present and documented in the high urban impact and in the low urban impact rivers in Puerto Rico (Figure 2.2). For both rivers in Puerto Rico, we found a mean concentration of TiO_2 NPs of about 47.81 mg/kg $\pm$ 7.15. The minimum concentration detected was 9.80 mg/kg and the maximum was 100.24 mg/kg. In terms of the sampling months from January 2022, February 2022 and April 2022, the average concentrations of TiO_2 NPs in the sediment of both rivers were 70.9 mg/kg $\pm$ 11.0, 30.17 + 6.87 and 42.4 mg/kg $\pm$ 10.1 respectively. The same sampling months showed concentrations ranging from 53.3 - 100.2 mg/kg, 9.80 - 38.55 and 23.8 - 64.3 mg/kg (Figure 2.3).

In the Rio Piedras River, an average of 47.34 mg/kg $\pm$ 7.30 was observed. The nanoparticles concentrations range from 23.75 mg/kg to 75.42 mg/kg. The High Urban (HU) and Low Urban (LU) were the Rio Piedras River localities. In the LU locality, we obtained a TiO_2 NPs average concentration of 56.3 mg/kg $\pm$ 10.7, with concentrations ranging from 38.5 to 75.4 mg/kg. For the Rio Piedras River HU, the average concentration was 38.41 mg/kg $\pm$ 8.52, with a concentration range from

23.75 to 53.26 mg/kg. Sabana River showed an average of 48.30 mg/kg ± 13.1 of TiO_2 NPs. The concentrations range from 9.8 mg/kg to 100.2 mg/kg. In the LU locality, an average of 51.01 mg/kg ± 8.90 and in the HU was 45.6 mg/kg ± 27.8. For the same localities, the TiO_2 NPs concentrations ranged from 34.12 mg/kg to 64.34 mg/kg (LU) and from 9.80 mg/kg to 100.2 mg/kg (HU). For both rivers the largest concentration of TiO_2 NPs was found in January.

In general, the higher concentrations of TiO_2 NPs were in January 2022 for both rivers. In Sabana river for this month we observed a higher concentration in the HU sample site than in the HU of Rio Piedras. Also, this value was the highest concentration found in both rivers. For February 2022 and April 2022, in Sabana river concentrations were higher in the LU than in the HU. Rio Piedras rivers showed higher concentrations of TiO_2 NPs in the HU than in the LU as was expected (Figure 2.4).

The one-way ANOVA for the comparison of month and TiO_2 NPs concentration was significant (ANOVA, $F_{(2,11)}= 4.82$; $p < 0.05$). For the River and TiO_2 NPs concentration, the ANOVA showed a $p > 0.05$. Finally, for the locality and TiO_2 NPs concentration the ANOVA showed a $p > 0.05$.

Natural waters

The concentrations observed in the natural waters of Rio Piedras and Sabana rivers were smaller than those observed in the sediment for the same rivers. In both rivers, the average concentration of dissolved titanium was 0.00757 mg/L ± 0.003 with a minimum concentration of 0.000 mg/L to 0.03 mg/L. For March and June 2021, we observed mean concentrations of 0.020 mg/L ± 0.009 and 0.013 mg/L ± 0.002. In the same months of 2021, the concentration varies from 0.011 mg/L to 0.030 mg/L and from 0.011 mg/L to 0.015 mg/L. In the year 2022, we documented

concentrations in January and April 2022. The average concentration in January 2022 was 0.01 mg/L ± 0.004 with a minimum concentration of 0.007 mg/L to 0.015 mg/L. Otherwise, in April 2022 the average concentration was 0.001 mg/L ± 0.001 with values concentration ranging from 0.000 mg/L to 0.003 mg/L. Finally, for February 2023, we do not document concentrations of dissolved titanium in natural waters (Figure 2.5).

In Rio Piedras River specifically, the mean concentration of dissolved titanium was 0.010 mg/L ± 0.01. This river's concentration varies from 0.00 mg/L to 0.03 mg/L. The titanium concentrations for this river were found in March and June 2021 and in January 2022. The concentrations of February 2022, April 2022, and February 2023 were not found. In March 2021 the mean concentration was 0.029 mg/L ± 0.000. In June 2021, titanium concentration average was 0.015 mg/L ± 0.00, and for January 2022, 0.015 mg/L ± 0.00.

Sabana River showed concentrations of dissolved titanium on an average of 0.005 mg/L + 0.006. These concentrations vary from 0.000 mg/L to 0.011 mg/L. Interestingly, we found concentrations of titanium in water in this river for the months of March 2021, June 2021, January 2022, and April 2022. For March 2021, the mean concentration was 0.011 mg/L + 0.000. June 2021 presented an average concentration of 0.011 mg/L + 0.000. Also, January 2022 was documented as 0.007 mg/L of dissolved titanium. Finally, April 2022 showed an average concentration of 0.003 mg/L.

The mean higher concentration was found in March 2021 for both rivers. Following June 2021 and January 2022. The concentration was not perceptible or significant for the rest of the months of February 2022, April 2022, and February 2023

The nested ANOVA for the dissolved titanium concentration and the rivers and months comparison showed a $p > 0.05$.

DISCUSSION

This study determined the presence and the environmental concentrations of presumably titanium oxide nanoparticles and dissolved titanium in sediments and river waters in Puerto Rico. The presence of this type of nanomaterial is relevant for the scientific concern about the possible ecological and environmental impacts that they may have on the freshwater ecosystem. This is because this nanoparticle causes cytotoxicity and genotoxicity and can generate reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2), hydroxyl radicals (OH-), or singlet oxygen (O_2) (Fenoglio et al., 2013; Mu et al., 2014).

It is common for the highest concentration of pollutants to be found in urbanized and highly populated rivers. However, this study found that both urban and non-urban rivers present concentrations of titanium oxide nanoparticles and titanium dissolved in water. This demonstrates the potential impact of these emerging contaminants, such as nanoparticles, which may differ from those previously described for other aquatic contaminants. This environmental problem is exacerbated when we know there are many gaps regarding nanomaterials and their action in aquatic systems that are poorly understood (Peijnenburg et al., 2015).

Titanium is the ninth most abundant element in the earth's crust. It is a natural mineral element of igneous and sedimentary rocks (Gázquez et al., 2014). It is normally found in the oxide form as rutile. However, concentrations due to this weathering have been determined to be only 1-3 ug/L (Correa, 2010). To date, titanium is a type of nanomaterial that is widely used in the commercial area due to

its unique physical and chemical properties in nano sizes as the titanium oxide nanoparticle. This chemical interaction is because of the great affinity that titanium has for oxygen, several oxidation states of titanium can be produced including TiO_2. Due to its extensive use, titanium oxide nanoparticles are known to reach rivers and other bodies of water. The direct and indirect ways in which these nanoparticles can reach rivers are industrial products, application through the wastewater stream, sunscreen uses, sewage sludge, bathing, engineering applications and others (Gao, J., et al., 2009; Ternes, T.A., et al., 2004; Oberdorster, E. 2004; Wang, J.X., et al., 2011). Also, U.S. is the country that has the highest commercial production and uses of TiO_2 nanoparticles 7800-38000 tons/year. While Europe consumption is around 55-3,000 tons/year and worldwide the number is 550-5500 tons/year (Nam et al., 2014).

The literature has determined the main sources of TiO_2 NPs that most frequently enter the freshwater ecosystem. It is precisely sunscreens (59% of the uses given to this nanoparticle is for these purposes) associated with nanoparticle entry into rivers. The other major source is paints and coatings (13% of nanoparticle uses) (Helgeist 2021). With the increase in the marketing and medical recommendations for the use of sunscreen with TiO_2 NPs and the common use of swimming pools, rivers, and beaches in Puerto Rico, it has been seen that the entry of the nanoparticle has continued to increase. Therefore, if the trend continues, the concentrations that could be found in the river sediment and water will increase.

Studies have determined environmental concentrations of TiO_2 NPs in surface waters of 0.002 ug/L in U.S. and 0.015 ug/L in Europe. In sediment, the environmental concentrations of TiO_2 NPs in U.S. of 53 µg/kg and in Europe of 358 µg/kg (Nam et al., 2014). However, in this study of Rio Piedras and Sabana rivers,

the concentrations in natural water and sediment were largely higher in mg/L and mg/kg units. Also, we documented very similar concentrations for both rivers in sediment and water samples. This data differs from those documented by Neal et al., 2011, in which they established higher concentrations in urban or industrial waters than in rural upstream areas. This difference could be since Sabana River, despite having an environmentally protected area, has become a highly populated and highly urbanized area when compared to other areas of the river. An example is the area of the Urbanization of Alamar where it is known that the river was channeled in the 1980's to build residences (Correa, 2010). For its part, the river has begun to return to its place of origin, and it is known that at least six houses are falling into the river, which is why large banks with erosion are observed. In the same way, the proximity of this urbanization can be an area of point-source contamination since the river is a recreation area and domestic discharges could be expected. Additionally, there are also local industries and businesses close to the Alamar urbanization. For its part, the LU area of the Sabana river also has residences close to some areas of the river. Also, because is a forested area, many people dump garbage, unusable equipment, and other materials into the river. All the above presents possibilities of contamination in this non-urban river with a high concentration of TiO_2 NPs in the sediment and the similarity of the concentrations of dissolved titanium in water with a totally urbanized river such as the Rio Piedras River.

The data obtained in this study are novel in our knowledge about environmental concentrations of TiO_2 NPs in rivers in Puerto Rico. These data can be the beginning of a study with a longer analysis time and a river with intermediate urban development could be added. In addition, there is a need to study our freshwater bodies by evaluating the presence of emerging pollutants such as

nanoparticles and thus be able to develop public policy to mitigate the possible environmental and ecological impacts they may cause.

This study has evidenced the relevance of the study of nanomaterials in rivers in Puerto Rico and provides a starting point for the development of new ecotoxicological studies with environmentally relevant concentrations. Also, we have shown that both in an urban river and in a non-urban river in Puerto Rico, we can find different environmental concentrations. Eliminating the concept of more urbanism, more pollution for nanomaterials as emerging aquatic pollutants. The case of nanomaterials must be treated in a particular way and with special attention. It has been shown that, even though we only know the amounts of production/use of this nanoparticle in the US, we present higher concentrations of TiO_2 NPs in sediment and water in Puerto Rico. Therefore, investigation and mitigation are essential as soon as possible to avoid future impacts on the ecosystem that may be irreversible. Finally, according to the scientific literature and our results, the general behavior of TiO_2 NPs in freshwater bodies is evidenced. Most of this nanoparticle settles, so it is essential to carry out toxicological studies in ecologically susceptible species to measure the possible impacts we will be facing.

REFERENCES CITED

Meza Rodríguez, D., Martínez Rivera, L. M., Mercado Silva, N., García de Jalón Lastra, D., González del Tánago del Río, M., Marchamalo Sacristán, M., & De la Mora Orozco, C. (2017). Natural flow regime of the Ayuquila-Armería River in western Mexico. Terra Latinoamericana, 35(3), 203-217.

Bhushan, B. (2017). Introduction to nanotechnology. Springer handbook of nanotechnology, 1-19.

De Jesús-Crespo, R.; Ramírez, A. Effects of Urbanization on Stream Physicochemistry and Macroinvertebrate Assemblages in a Tropical Urban Watershed in Puerto Rico. J. North Am. Benthol. Soc. 2011, 30, 739–750.

Dudgeon, D. (2000). Large-scale hydrological changes in tropical Asia: prospects for riverine biodiversity: the construction of large dams will have an impact on the biodiversity of tropical Asian rivers and their associated wetlands. BioScience, 50(9), 793-806.

Esteban Correa, L. M. (2010). Río Sabana Streambank Erosion Control and Restoration at Urb. Alamar, Luquillo, Puerto Rico. Civil Engineering;.

Fenoglio, I., Ponti, J., Alloa, E., Ghiazza, M., Corazzari, I., Capomaccio, R., Rembges, D., Simonetta, OB., & Rossi, F. (2013). Singlet oxygen plays a key role in the toxicity and DNA damage caused by nanometric TiO_2 in human keratinocytes. Nanoscale, 5(14), 6567-6576.

Gao, J., Youn, S., Hovsepyan, A., Laneza, V.L., Wang, Y., Bitton, G., Bonzongo, J.J. (2009). Dispersion and toxicity of selected manu-factured nanomaterials in natural river water samples: effects of water chemical composition. Environ. Sci. Technol., 43(1), 3322– 3328.

Gázquez, M. J., Bolívar, J. P., García-Tenorio García-Balmaseda, R., & Vaca, F. (2014). A review of the production cycle of titanium dioxide pigment.

Greathouse, E. A., & Pringle, C. M. (2006). Does the river continuum concept apply on a tropical island? Longitudinal variation in a Puerto Rican stream. Canadian Journal of Fisheries and Aquatic Sciences, 63(1), 134-152.

Heartsill-Scalley, T.; López-Marrero, T. Land-cover composition, water resources and land management in the watersheds of the Luquillo Mountains, northeastern Puerto Rico. Caribb. Geogr. 2014, 19, 43–68.

Heilgeist, S., Sekine, R., Sahin, O., & Stewart, R. A. (2021). Finding nano: Challenges involved in monitoring the presence and fate of engineered titanium dioxide nanoparticles in aquatic environments. Water, 13(5), 734.

Komínková, D. (2012). The urban stream syndrome–a mini-Review. The Open Environmental & Biological Monitoring Journal, 5(1).

Kominkova, D., Handova, Z., Nabelkova, J., & Caletkova, J. (2007). Urban Stream Syndrome and New Approach to Urban Stream Rehabilitation. Vodni Hospodarstvi, 57(2), 39.

Lugo, A.E.; Ramos, O.M.; Rodríguez, C.R. The Río Piedras Watershed and Its Surrounding Environment; International Institute of Tropical Forestry: San Juan, PR, USA, 2011.

Luo, M., Qi, X., Ren, T., Huang, Y., Keller, A. A., Wang, H., Wu, B., Jin, H., & Li, F. (2017). Heteroaggregation of CeO_2 and TiO_2 engineered nanoparticles in the aqueous phase: Application of turbiscan stability index and fluorescence excitation-emission matrix (EEM) spectra. Colloids and Surfaces A: Physicochemical and Engineering Aspects, 533, 9-19.

Minitab, LLC. (2021). Minitab. Retrieved from https://www.minitab.com

Mu, Q., Jiang, G., Chen, L., Zhou, H., Fourches, D., Tropsha, A., & Yan, B. (2014). Chemical basis of interactions between engineered nanoparticles and biological systems. Chemical reviews, 114(15), 7740-7781.

Mueller, N., Nowack, B., 2008. Exposure modeling of engineered nanoparticles in the environment, Environmental Science & Technology, DOI: 10.1021/ es7029637.

Nam, D. H., Lee, B. C., Eom, I. C., Kim, P., & Yeo, M. K. (2014). Uptake and bioaccumulation of titanium-and silver-nanoparticles in aquatic ecosystems. Molecular & Cellular Toxicology, 10, 9-17.

NCDC (National Climatic Data Center). 2009. Normal daily mean temperatures. National Oceanographic and Atmospheric Administration, Washington, DC. (Available at http://lwf.ncdc.noaa.gov/oa/climate/online/ccd/meantemp.html)

Neal, C., Jarvie, H., Rowland, P., Lawler, A., Sleep, D., & Scholefield, P. (2011). Titanium in UK rural, agricultural and urban/industrial rivers: Geogenic and anthropogenic colloidal/sub-colloidal sources and the significance of within-river retention. Science of the Total Environment, 409(10), 1843-1853

Oberdörster, E. (2004). Manufactured nanomaterials (fullerenes, C60) induce oxidative stress in the brain of juvenile largemouth bass. Environmental health perspectives, 112(10), 1058-1062.

Osterkamp, W. R. 2001. Earth surface processes, materials use, and urban development: A case study of the San Juan metropolitan area, northeastern Puerto Rico. Water Resources Division, US Geological Survey, Tucson, Arizona.

Peijnenburg, W. J., Baalousha, M., Chen, J., Chaudry, Q., Von der kammer, F., Kuhlbusch, T. A., Lead, J., Nickel, C., Quik, T.K., Renker, M., Wang, Z., & Koelmans, A. A. (2015). A review of the properties and processes determining the fate of

engineered nanomaterials in the aquatic environment. Critical Reviews in Environmental Science and Technology, 45(19), 2084-2134.

Pérez-Reyes, O., Crowl, T. A., & Covich, A. P. (2015). Effects of food supplies and water temperature on growth rates of two species of freshwater tropical shrimps. Freshwater Biology, 60(8), 1514-1524.

Ramírez, A., De Jesús-Crespo, R., Martinó-Cardona, D. M., Martínez-Rivera, N., & Burgos-Caraballo, S. (2009). Urban streams in Puerto Rico: what can we learn from the tropics?. Journal of the North American Benthological Society, 28(4), 1070-1079.

Ramírez, A.; Rosas, K.G.; Lugo, A.E.; Ramos-González, O.M. Spatio-temporal variation in stream water chemistry in a tropicalurban watershed. Ecol. Soc. 2014,19, 45.

Ramos-González, O.M. The green areas of San Juan, Puerto Rico. Ecol. Soc. 2014, 19, 21.

Selck, H., Handy, R. D., Fernandes, T. F., Klaine, S. J., & Petersen, E. J. (2016). Nanomaterials in the aquatic environment: A European Union–United States perspective on the status of ecotoxicity testing, research priorities, and challenges ahead. Environmental Toxicology and Chemistry, 35(5), 1055-1067.

Ternes, T. A., Joss, A., & Siegrist, H. (2004). Peer reviewed: scrutinizing pharmaceuticals and personal care products in wastewater treatment. Environmental science & technology, 38(20), 392A-399A.

Torres-Perez, W. X., & Perez-Reyes, O. (2023). Population Status of the Tropical Freshwater Shrimp *Xiphocaris elongata* in Urban and Forest Streams in Puerto Rico. Hydrobiology, 2(1), 277-288.

Walsh, C. J., Roy, A. H., Feminella, J. W., Cottingham, P. D., Groffman, P. M., & Morgan, R. P. (2005). The urban stream syndrome: current knowledge and the search for a cure. Journal of the North American Benthological Society, 24(3), 706-723.

Wang, J., Zhu, X., Zhang, X., et., al. (2011). Disruption of zebrafish (Danio rerio) reproduction upon chronic exposure to TiO_2 nanoparticles. Chemosphere, 83(4), 461-467.

Wigginton, N.S., Haus, K.L., Hochella, M.F., 2007. Aquatic environmental nanoparticles. Journal of Environmental Monitoring 9, 1306–1316.

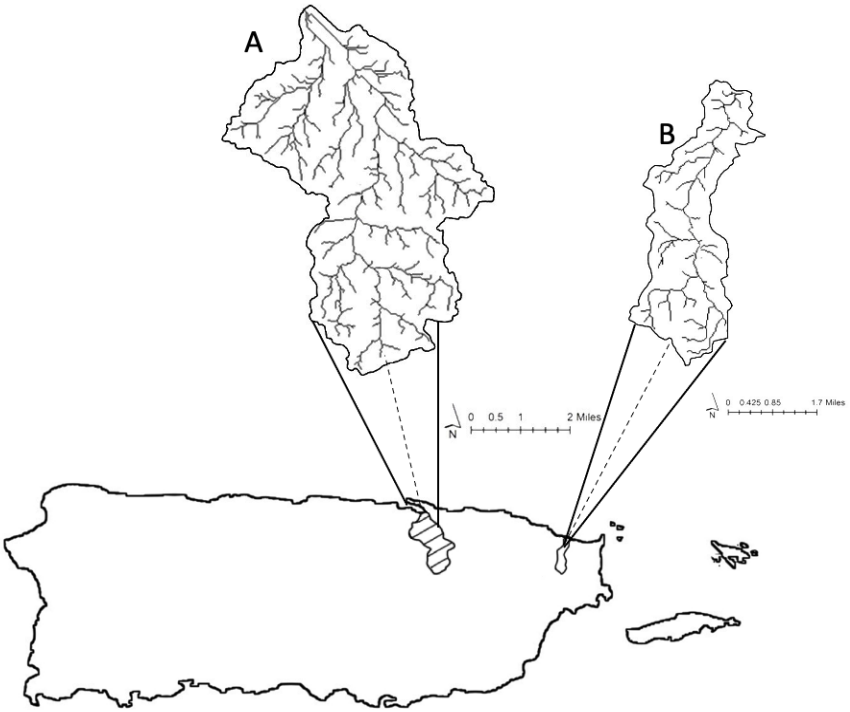


Figure 2.1 Location of the watersheds of the studied streams in the north coast of Puerto Rico. A) Río Piedras watershed (high urban impact basin) and B) Río Sabana watershed (low urban impact basin)

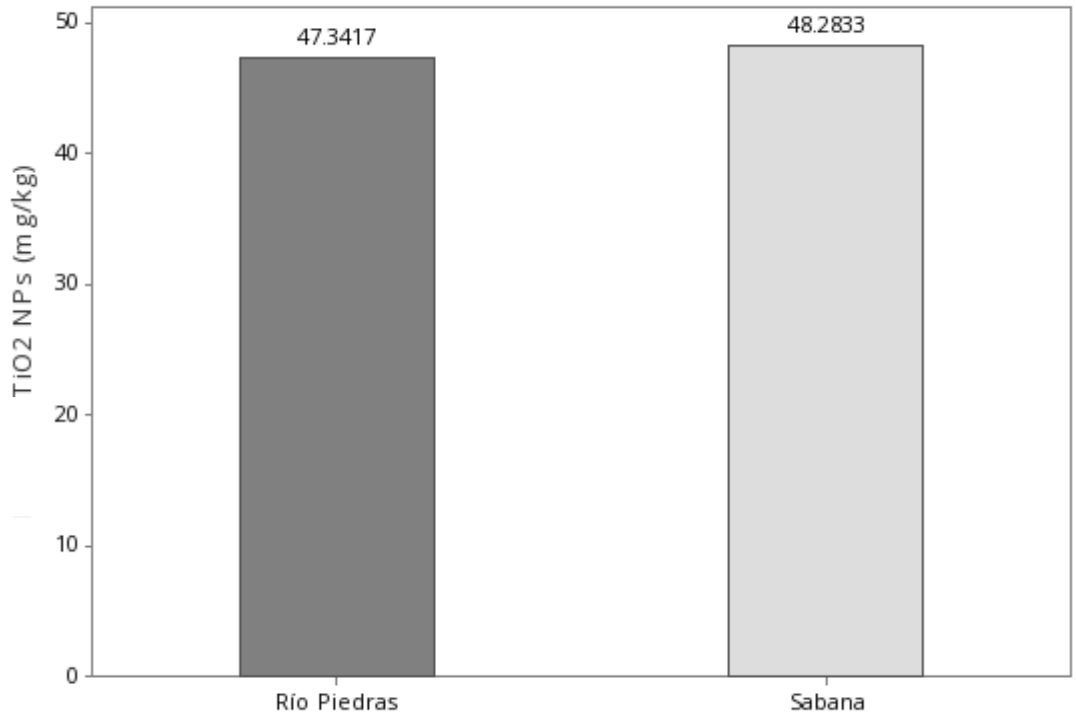


Figure 2.2 TiO_2 NPs concentration (mg/kg), in Rio Piedras and Sabana rivers pooled for time and localities. The black color represents Rio Piedras River and the gray Sabana River.

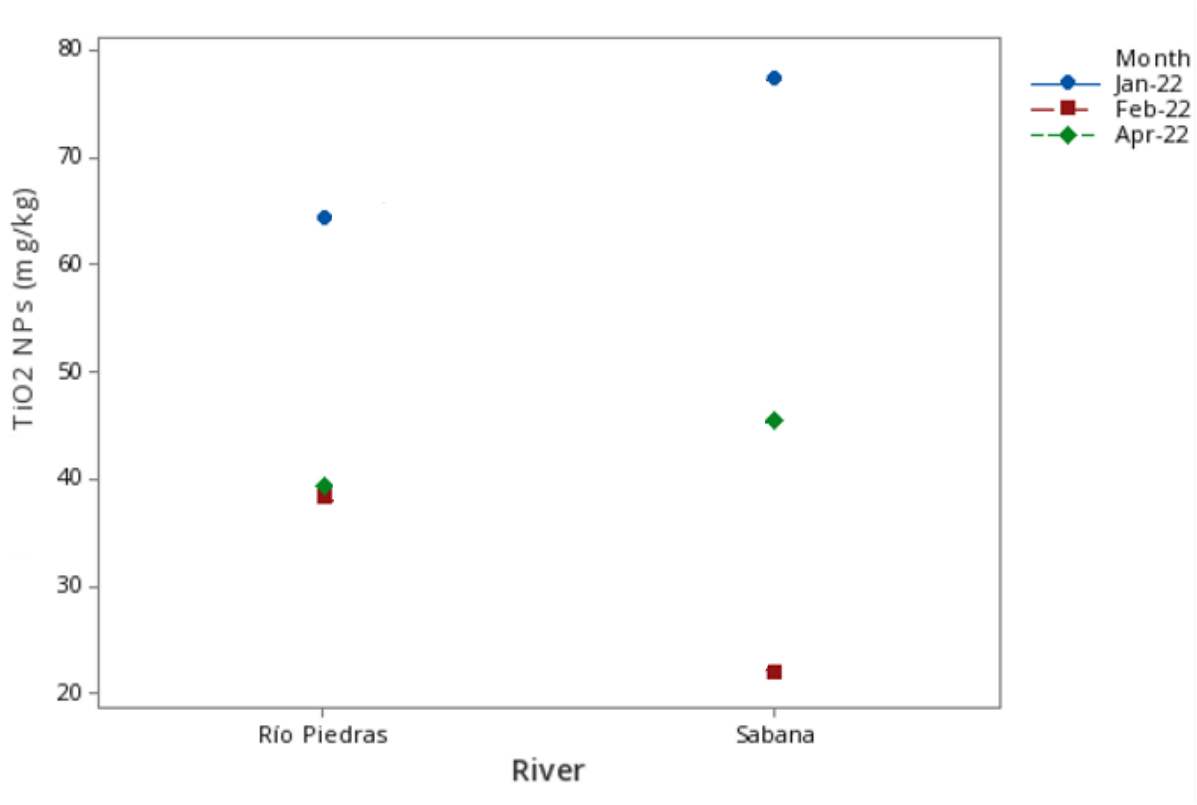


Figure 2.3 TiO_2 NPs concentration (mg/kg), in Sabana and Rio Piedras rivers by each month of sampled. The blue, green and red lines represent the average concentration of TiO_2 NPs in January 2022, February 2022 and April 2022.

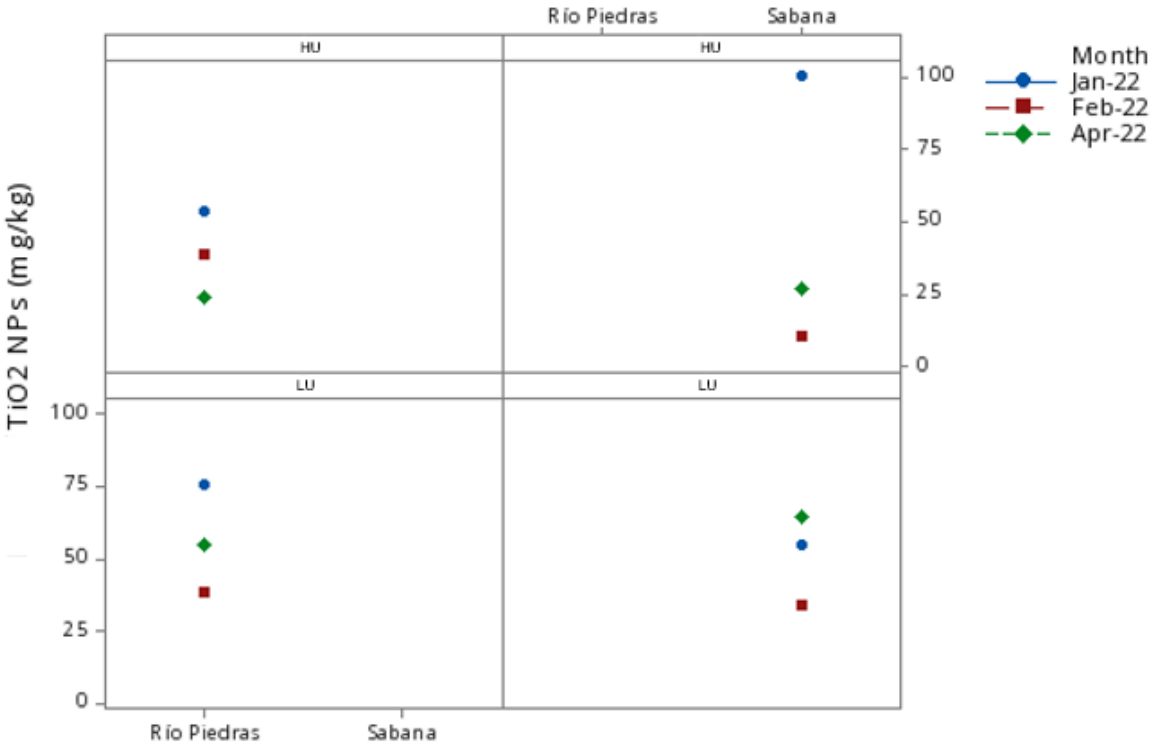


Figure 2.4 TiO_2 NPs (mg/kg) for Sabana and Rio Piedras rivers by month and the sampled localities, HU and LU. The blue, red and green dots represent the average concentration of TiO_2 NPs in each month by the HU and LU localities for Rio Piedras and Sabana rivers.

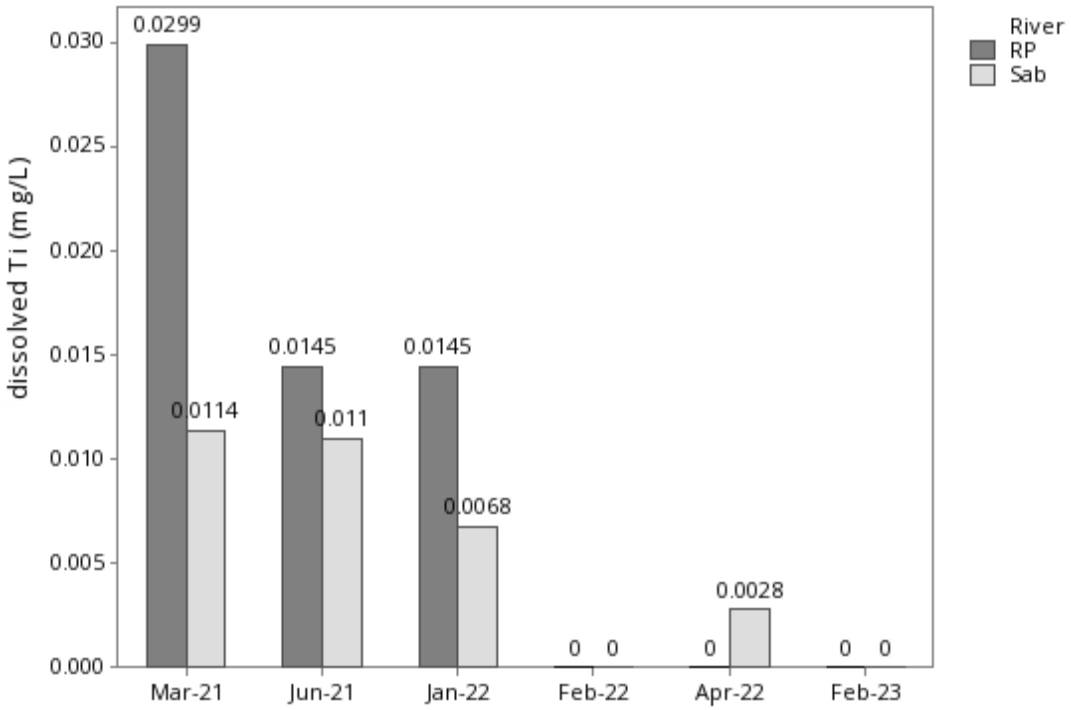


Figure 2.5 Dissolved titanium concentrations in Rio Piedras and Sabana rivers in each sampled month. The black dots represent Rio Piedras River and the grays Sabana River.

CHAPTER 3: EARLY LARVAL DEVELOPMENT DESCRIPTION OF THE FRESHWATER SHRIMP *Atya lanipes* Holthuis, 1963 (DECAPODA: CARIDEA: ATYIDAE)

Abstract

The family Atyidae is composed of species whose existence has been known since the seventeenth century. Within the family Atyidae, in Puerto Rico we find shrimp such as *Atya scabra*, *Atya innocous* Herbst 1792 and *Atya lanipes* Holthuis 1963. Of these three species, *Atya lanipes* is the most primitive and prefers microhabitats that possess fast but not very turbulent currents. *Atya lanipes*, is a freshwater shrimp widespread in the Caribbean zone, (Puerto Rico, Jamaica, St Thomas, Cuba, and Hispaniola). *Atya lanipes* is a scraper/filter feeder shrimp with an amphidromous complex life cycle. Hunte 1975 described the first zoea larval stage for *Atya lanipes*. However, to date, no scientific literature describes this species' early larval development after the first stage. This study aims to document the early larval development of *Atya lanipes* under laboratory conditions and to compare its larval development with other previously described species of the Atyidae family. Our study determined that the best environmental condition for *Atya lanipes* (although with very high mortality) was 30 ppm, constant gentle aeration and 27 °C. Larval development description was obtained using a Stereo Microscope, taking daily photos and videos of larvae development. Also, we preserved in ethanol 3-5 larvae per day for further morphological analysis. Finally, the larval development sketches were made using photos of each larval development. We identified nine stages to describe the early larval development of *Atya lanipes* shrimp. The early larval stages of *Atya lanipes* differ primarily in interstage larval size, appearance, and development of the telson,

appendage appearance, growth of antennae and antennules, and pigmentation. This study represents the first study that describes the larval development of the native shrimp *Atya lanipes*.

Keywords: *early shrimp development, freshwater shrimp, larval description, shrimp metamorphosis*

INTRODUCTION

Atyidae species biodiversity and life-cycle

As far as the family of Atyidae is concerned, these shrimps show characteristic adaptations that give them a greater degree of success in their survival in the microhabitats in which they are found. The family Atyidae is composed of species whose existence has been known since the seventeenth century. According to Hobbs, 1982, the first species Atyidae was described in 1648 by Marcgrave whom he called "Guaricuru", in Brazil. Subsequently, the description of different species of this family such as *Atya scabra* Leach 1816, was continued. Within the family Atyidae, in Puerto Rico we find shrimp such as *Atya scabra*, *Atya innocous* Herbst 1792 and *Atya lanipes* Holthuis 1963. Of these three species, *Atya lanipes* is the most primitive and is found in microhabitats that possess fast but not very turbulent currents (Chace and Hobbs 1969; Hobbs 1982). For its part, *Atya scabra* is the most complex species of all and is found in distinctive habitats such as waterfalls and strong water currents. The Atyidae are very similar to each other in terms of their taxonomic characteristics and the differences are minimal (Hobbs, 1982). Today, this family of Atyidae is known to be the dominant organisms in tropical rivers where the abundance of fish and other predators is limited. Finally, *Atya innocous* is the intermediate species in complexity between the three species and one that is quite open to microhabitats of strong currents but as *Atya scabra*, lives in habitat that are deeper and with slower current. It is common to find *Atya innocous* it is common coexisting in microhabitats with *Atya lanipes* and *Atya scabra*.

The morphological differences between these three species of Atyidae reside mainly (but not only) in the shape and size of the pereiopods. Pereiopods are limbs that shrimp use to "walk" and are different from pleopods that have the function of

swimming and in the case of females, keeping their eggs. In the case of *Atya lanipes*, its pereiopods are fine, *Atya innocous* has thicker pereiopods, but *Atya scabra* has much thicker ones than *Atya lanipes* and *Atya innocous*. However, between *Atya scabra* and *Atya innocous* other morphological characteristics differentiate them such as the antennules' peduncle that in the case of *Atya innocous* is more prominent than in *Atya scabra*. Also, the rostrum is used to differentiate the three species of atyids mentioned (Perez-Reyes et al. 2013; Chace and Hobbs 1969).

On the other hand, a functional morphology study conducted by Fryer (1977) in Dominica, clearly described the characteristics of the chelas of these organisms that present a modified form. The modification presented by these chelas (second pair of pereiopods), is that the terminal cells of the chelipeds are composed distally with tiny denticles. However, Atyidae species such as *Atya lanipes* and *Atya innocuous* also have a series of fan-like structures in the denticles that allow them to scrape to feed. Also, *Atya lanipes* present distal mushrooms that are known as brushes or fans (Hobbs, 1982).

All these modifications have been important to colonize, survive and be a successful species in the freshwater ecosystems in Puerto Rico. For example, these species live mainly in microhabitats of fast and/or turbulent currents. Therefore, they need to feed, and keep their eggs in the pleopods, among other activities of their life cycle, coping with the physical action of currents and avoiding the effects of gravity on the slope. In this way, these chelae provide them with an evolutionary advantage to filter the water and scrape the rocks to feed successfully. In the island of Puerto Rico, there are about nine (9) Atyidae species in the rivers (Perez-Reyes et al.

2013). *Atya lanipes* and *Atya innocuous* are shrimp that feed by filtration or by scraping the surfaces of rocks. However, *Atya scabra* is mainly fed by filtration. In this way, the modified chelae give them the ability to feed efficiently on microscopic algae and fine organic material. Also, these species are very important in sediment recovery, having an important role in preventing eutrophication (accumulation of nutrients) (Crowl, et al. 2001).

On the other hand, the life cycle of the Atyidae is an amphidromous one. This amphidromous life cycle in freshwater shrimp represents a very important relationship between river headwaters and estuarine/marine environments (Bauer 2013; 2011; Benstead, et al. 2000). This type of life cycle occurs when gravid females release larvae in the upper reaches of the river (headwaters); larvae in the early stage of life (i.e., newborn larvae) passively move to coastal/estuarine environments where they develop and metamorphose into post larvae that subsequently migrate upstream as juveniles to complete their adult life cycle (Benstead, et. al. 2000). The larval stages of many of these atyids species have been described by different scientists under laboratory conditions (Table 3.1). The number of larval stages can vary between species of Atyidae but the number tends to be between at least seven stages to twelve approximately. An example is *Atya innocuous*, where Wayne Hunte in 1979, managed to describe twelve larval stages (total development time 76-119 days). On the other hand, once these species overcome the larval stages, they become juveniles (post-larva or megalopa), who undertake one of the most important migrations of their life cycle: to return to the headwaters of the rivers to complete their adulthood and reproduce. This juvenile stage, in turn, can also present several juvenile stages that vary from species to

species. Subsequently, the adult Atyidae are in the upper parts of the river where they remain until the end of their life cycle (Bauer 2013, Bauer 2011).

Atya lanipes Holthuis 1963

Atya lanipes (Figure 3.1) is a freshwater shrimp widespread in the Caribbean zone, (Puerto Rico, Jamaica, St Thomas, Cuba, and Hispaniola) (Hobbs & Hart, 1982). *Atya lanipes* is a scraper/filter feeder shrimp with an amphidromous complex life cycle (Benstead, et. al., 2000). In this life cycle, migrations are ecologically important because they are temporarily variable components of different ecosystems (Bauer, 2013), which affects the habitat, productivity, and trophic relationships at different times of the year (Bauer, 2013). Crowl, among others (2001), demonstrate that *Atya lanipes* affect detrital processing and illustrate its potential importance in diversity and nutrient availability in the food web. Likewise, *Atya lanipes* is very important in the removal of sediments which have an important role in the eutrophication (nutrients accumulation) in this aquatic environment.

Hunte 1975 described the first zoea first larval stage for *Atya lanipes* shrimp. However, to date, no scientific literature describes this species' early larval development after the first stage. The larvae morphological development description of *Atya lanipes* species is beneficial to compare with other Atyidae species such as *Atya innocuous and A. scabra,* which had previous description of its larval stages (Table 3.1). Also, describing *Atya lanipes* early larval development could be useful to understand the evolutionary process of Atyidae species in the larval morphological development. This study aims to describe the early stages of *Atya lanipes* larvae rearing them in a laboratory environment.

This study aims to document the early larval development of the *Atya lanipes* shrimp species under laboratory conditions and to compare its larval development

with other previously described species of the Atyidae family. We hypothesize that the larval development of *Atya lanipes* will be similar (9-12 stages) to that of *Atya innocuous* and *Atya scabra* (family Atyidae) and will be achieved under estuarine environmental conditions.

MATERIALS AND METHODS

The ovigerous specimens of *Atya lanipes* shrimp were collected using baited Minnow traps at Buruquena stream (18.321207, -65.819389) at El Verde Field Station, Río Grande Puerto Rico. Collected shrimp were transported with constant aeration to the University of Puerto Rico at Río Piedras. Gravid females were placed individually in an aquarium with one Liter of dechlorinated water with heavy aeration until the eggs hatched.

The egg hatching usually occurred at night, and the free-swimming zoea larvae were collected early in the morning (8:00 AM). The larvae were reared in an aquarium with constant aeration and a photoperiod with 14 hours of light and 10 hours of darkness. The temperature of the water bath of the aquariums in which the larvae were placed, was around 27 ºC, and the water salinity was 30 parts per thousand (seawater diluted with dechlorinated water). We placed 100 larvae per one Liter of water.

Larval development was obtained using a Stereo Microscope (3.5X-90X LED Trinocular Zoom + 14MP USB 3.0 Digital Camera), taking daily photos and videos of larvae development. Also, we preserved in ethanol 3-5 larvae per day for further morphological analysis. Finally, the larval development sketches were made using photos of each larval development. Also, we used video recordings of details of

appendages and other parts of the larva that were not clearly appreciated in images of live and moving larvae.

RESULTS

Laboratory rearing conditions

To achieve larval development of *Atya lanipes*, multiple environmental conditions were attempted. These conditions varied in salinity, temperature, and aeration of the aquariums where the larvae grew. The environmental conditions that were characterized by having 0.0 ppm salinity and temperatures below 23 °C, in the presence or absence of aeration, no larval development was observed. The larvae lived for 7-12 days without going to the second larval stage. Alternatively, variable salinity concentrations (from 5-30 ppm) in the absence or presence of aeration and with a temperature below 23 °C, showed very little larval development. It was only observed that the larvae managed to pass to the second larval stage, but they all died in 7-12 days. However, those environmental conditions that included high salinity (25, 30, 32 ppm), in the presence or absence of aeration, and temperatures between 24-27 °C showed rapid larval development. However, our study determined that the best environmental condition for *Atya lanipes* (although with very high mortality) was 30ppm, constant gentle aeration and 27 °C. For the latter, obtaining the most extensive development possible in our experiments was possible. The light/night conditions were the same in all the cases studied (14 hours of light/10 hours of darkness)

Larval development

The larvae morphological development was assessed until the last mortality recorded at 32 days after the eclosion. The larvae mortality was high especially after

the first seven days, twelve days and post twenty-five days. Only one larva survived until day 32. In this way, it was impossible to acclimate to freshwater to complete the metamorphosis into a juvenile shrimp.

We identified nine stages to describe the early larvae development of *Atya lanipes* shrimp. The early larval stages of *Atya lanipes* differ primarily in interstage larval size, appearance, and development of the telson, appendage appearance, growth of antennae and antennules, and pigmentation.

First larval stage (1 to 3 days)

The first stage of *Atya lanipes* is a free-swimming zoea, characterized by a transparent coloration in the larvae. They present some red chromatophores distributed in their telson, abdominal area (segment 5), and antennular peduncle on the carapace posterior to each eye. Chromatophores usually present numerous dendrites. The larva also presents its lipids content near the head, varying in green, yellow, and brown colors. The gastrointestinal tract is observed with a clear appearance. They have a bent body in segment 3. First larval stage presented large and sessile eyes. Antennular peduncle with a flagellum that also has three plumose setae. Antennular structures with twelve plumose setae. Antennas with a pair of twelve plumose setae. Three pairs of pleopods with four plumose setae were seen. The telson is triangular, with the posterior margin broad and strongly notched in the middle. The posterior margins with six pairs of plumose setae and one pair of spines. The innermost pair touched at the tips. Regarding behavior, the first larval stage presented planktonic movement (in the water column). They are phototactic. During this stage, the larvae are lecithotrophic (Figure 3.2).

Second larval stage (3 to 8 days)

This second stage is characterized or clearly defined by presenting large, stalked eyes. They are still immobile. Also, greater pigmentation is observed in segment 5, as well as in the telson, carapace, and ventral and posterior parts of the eyes. Segments 1 to 4 remain transparent. The antennae are like stage I, and the antennule are elongated with 2 segments and a flagellum that presents 3 plumose setae. Telson presented 8 pairs of plumose setae maintaining the triangular shape. The larvae continue to be lecithotrophic with planktonic behavior and phototaxis (Figure 3.3).

Third larval stage (9 to 10 days)

All characteristics observed in stage two remain. However, greater pigmentation is observed throughout the larva. Significantly, the fifth abdominal segment becomes redder colored with shades endings. In this stage, abdominal segments 1 to 4 that previously remained transparent present red lines demonstrating the initial pigmentation of the area. Telson is elongated with the emergence of an exopod that begins to be observed. Two distal red chromatophores are seen on both sides of the telson with shade endings. The third pereiopod presents two plumose setae. Antennule with 3 segments and with a pair of chromatophores in the second segment. During this stage, the larvae presented nutritive activity since the presence of food was observed in their gastrointestinal tract. This is a critical stage because many larvae die from day seven to the day eight (Figure 3.4).

Fourth larval stage (10 to 12 days)

Larvae pigmentation remains like the third stage. Nevertheless, new chromatophores are seen: one in the initial ventral part of the telson, two bigger in the distal part of the carapace, and one in the ventral part of the carapace. Likewise,

the pair of distal chromatophores in the telson is now more red-pigmented, with a perfect circular form without shades. The lines between the third and fourth abdominal segments present another chromatophore. Antennules remain with three segments but now with a pair of chromatophores in each line between segments. Two telson exopods came to be present. A spine in each pleopod is seen (Figure 3.5).

Fifth larval stage (11 to 14 days)

During this stage, the chromatophores pigmentation increases in the previously mentioned regions. The most important changes are precisely the telson development. The exopod of the telson came separated from the tip. These exopods also have six plumose setae on each side. The tip of the telson remains has a pair of eight plumose setae. Another impressive development regards the pleopods; there are four on each side of the larva. Finally, the antennules peduncles are long and narrow and maintain the three segments that characterized the previous stages but although we know that the presence of an outer flagellum broad and short and a short plumose seta at its apex were present in previous stages, is in this stage that we observed them clearly in the pictures we took (Figure 3.6).

Sixth larval stage (14 to 18 days)

Mobile eyes. Larvae present benthic behavior with significant time in the corners of the aquariums. Rostrum is more prolonged and narrower than previous stages. Pleopods with another spine. The exopods of the telson are longer than the previous stage. Carapace is elongated (Figure 3.7).

Seventh larval stage (18 to 21 days)

Abdominal segment number two is shorter, while the third segment is larger. The larva presents a notable curve, as is seen at the juvenile shrimp stage. Carapace is longer. The eyes are more separated and mobile. The tip of the telson presents a shorter plumose seta. Telson endopods are present with a pair of four plumose setae (Figure 3.8).

Eight larval stage (21 to 25 days)

Abdominal segments are shorter except for the third segment. Telson exopods and endopods are larger. The telson tip is long and narrow with a rectangular form (with a slightly triangular form) and the plumose setae is shorter (Figure 3.9).

Ninth larval stage (25 to 32 days)

Larvae are larger than in previous stages. Telson exopods and endopods with similar sizes. The telson tip is completely rectangular with very short plumose setae (Figure 3.10).

DISCUSSION AND CONCLUSION

Atya lanipes larvae hatched as free swimming zoeae in 0 ppm salinity water. This is like other atyids like *Atya innocous* (Hunte, 1979a; Hunte, 1979b). Laboratory culture of the *Atya lanipes* larvae demonstrates that two parameters are important: salinity and temperature. According to our results, a salinity of 30 ppm and a temperature of 27 °C showed the highest larval development, although with a high mortality rate. Only five larvae completed stage seven, and only one developed to stage 9. These environmental variables are like those suggested by Cruz & Altson, 1992 for aquaculture of *Atya lanipes* and *Atya scabra*. Due to the last larva's death, we could not start the acclimatization process to freshwater. For this reason, we do

not complete the metamorphosis. This study confirmed the lecithotrophy of this species, larvae evidence of starting to eat at the third stage (8-11 days after hatching).

The morphological development of *Atya lanipes* was characterized by a progressive increase in the pigmentation of the larvae as the molts occurred. The telson had significant changes an increase in plumose setae, the development of exopods and endopods with their respective plumose setae that were maintained with the same number of these from the emergence of the structures until the nine stages. Likewise, a triangular-shaped telson was observed during the first larval stages that continued to decrease this shape until presenting a rectangular shape that coincided with a reduction in the sizes of the plumose setae.

The antennular peduncles showed an increase in segmentation from the second larval stage that was maintained until stage nine. However, they were elongating at each stage. The antennae remained with the same structure and only became elongated and narrow.

Another significant morphological development was the abdominal segments. In the early stages, these were kept to a similar size and shape. Already in more progressive stages, a change was found in segment three, which was larger than the others. This coincided with the change in characteristic morphology of juvenile and adult shrimp and with the beginning of benthic behavior.

In terms of pleopods, we observed three of them at the first stages on each larval side. At the fifth stage, we observed four pleopods. Additionally, during the 1-3 stages, the pleopods showed only four plumose setae. After this, the pleopods showed one spine in each one. During the sixth stage onwards, they present two spines.

This study demonstrated that the early larval development of *Atya lanipes* is like that previously described for other atyid species, such as *Atya innocuous*. The differences are in the days required for each molt and the time of the observed structures. Also, as inn *Atya Innocuous*, the ninth larval stage was accessed after 43-62 days, but *Atya lanipes* was accessed at 27-32 days. This data suggests that *Atya lanipes* presents a faster larval development.

This study represents the first study that describes the larval development of the native shrimp *Atya lanipes*. We will continue the process until we describe the total development of the species. It is suggested as a continuation of this study to use the environmental variables reported in this study to take measurements of the total length of the larvae at each stage and with more sophisticated microscopy equipment to integrate descriptions of the mandibles and maxillipeds.

Literature cited:

Bauer, R. T. (2011). Amphidromy and migrations of freshwater shrimps. I. Costs, benefits, evolutionary origins, and an unusual case of amphidromy. In New frontiers in crustacean biology (pp. 145-156).

Bauer, R. T. (2013). Amphidromy in shrimps: a life cycle between rivers and the sea. Latin American Journal of Aquatic Research, 41(4), 633-650.

Benstead, J. P., March, J. G., & Pringle, C. M. (2000). Estuarine larval development and upstream post-larval migration of freshwater shrimps in two tropical rivers of Puerto Rico. Biotropica, 545-548.

Chace Jr, F. A., & Hobbs Jr, H. H. (1969). Bredin-Archbold-Smithsonian biological survey of Dominica: the freshwater and terrestrial decapod crustaceans of the West Indies with special reference to Dominica. Bulletin of the United States National Museum.

Crowl, T. A., McDowell, W. H., Covich, A. P., & Johnson, S. L. (2001). Freshwater shrimp effects on detrital processing and nutrients in a tropical headwater stream. Ecology, 82(3), 775-783.

Cruz-Soltero, S., & Alston, D. E. (1992). Status report on research with *Atya lanipes* and *A. scabra* in Puerto Rico.

Fryer, G. (1977). Studies on the functional morphology and ecology of the atyid prawns of Dominica. Philosophical Transactions of the Royal Society of London. B, Biological Sciences, 277(952), 57-129.

Hobbs, H.H. & C.W. Hart. 1982. The shrimp genus *Atya* (Decapoda: Atyidae). Smit. Contri. Zool. 364: 1-143. https://www.fs.usda.gov/Internet/FSE_DOCUMENTS/stelprdb5338955.pdf

Hunte, W. (1975). *Atya Lanipes* Holthuis, 1963, in Jamaica, Including Taxonomic Notes and Description of the First Larval Stage (Decapoda, Atyidae). Crustaceana, 28(1), 66-72.

Hunte, W. (1979). The complete larval development of the freshwater shrimp Atya innocous (Herbst) reared in the laboratory (Decapoda, Atyidae). Crustaceana. Supplement, 231-242.

Perez-Reyes, O., Crowl, T. A., Hernandez-Garcia, P. J., Ledesma-Fuste, R., Villar-Fornes, F. A., & Covich, A. P. (2013). Freshwater decapods of Puerto Rico: a checklist and reports of new localities. Zootaxa, 3717(3), 329-344.

Walsh, C. J. (1993). Larval development of Paratya australiensis Kemp, 1917 (Decapoda: Caridea: Atyidae), reared in the laboratory, with comparisons of fecundity and egg and larval size between estuarine and riverine environments. Journal of Crustacean Biology, 13(3), 456-480.

Table 3.1 Summary of the larval atyids species stages that have been described by different scientists under laboratory conditions.

Locatity	*Species*	*Reference*
Jamaica	*Atya innocous*	Hunte 1975, 1977
Puerto Rico	*Atya scabra*	Soltero 1991
México	*Atya scabra*	Hernandez-Vergara & Jimenez-Rojo 2008
Brazil	*Atya scabra*	Herrera-Correa et al., 2013
Brazil	*Atya scabra*	Galvao & Bueno 2000

Figure 3.1 The Freshwater shrimp *Atya lanipes* Holthuis 1963.

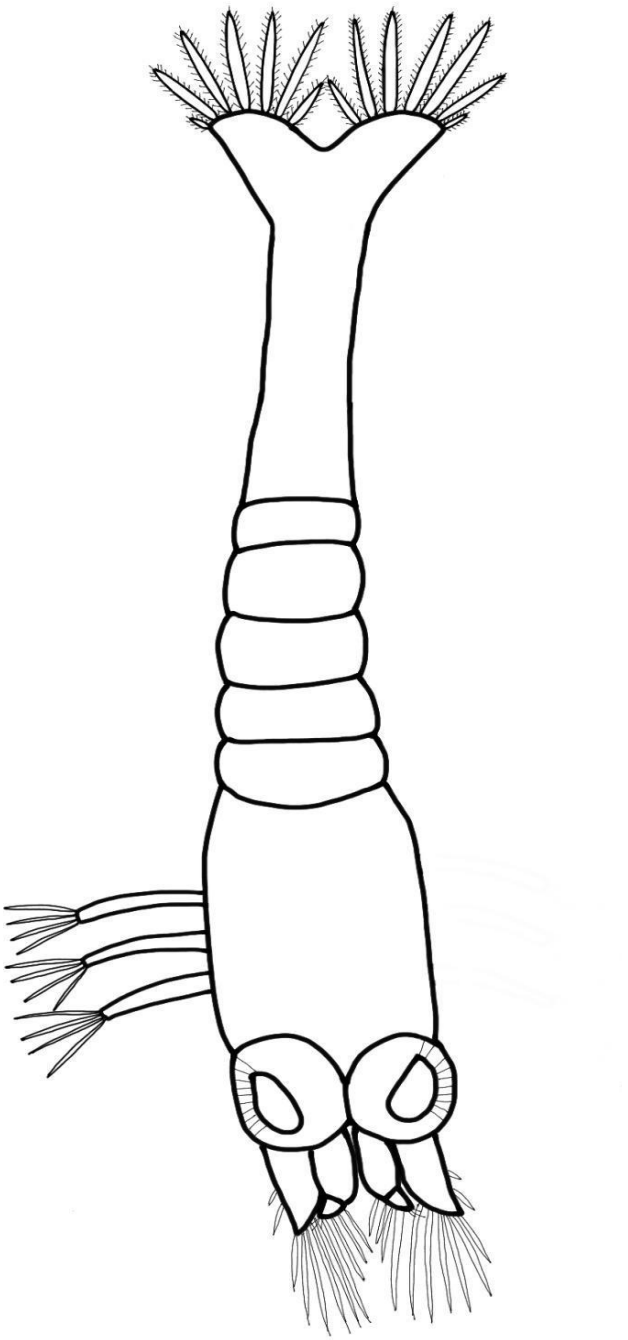


Figure 3.2 Dorsal view of the first larval stage development of *Atya lanipes*

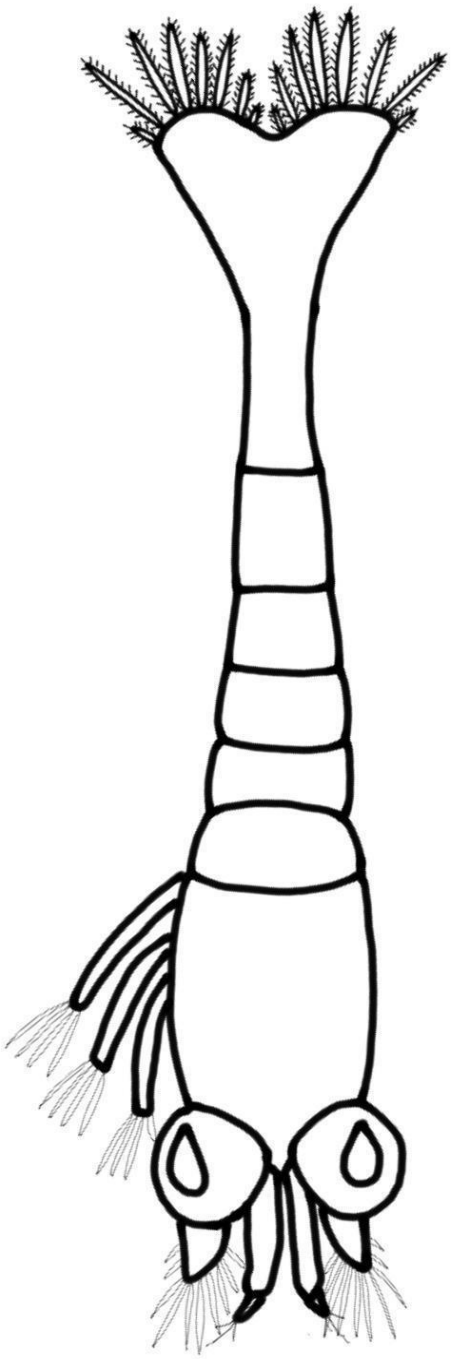


Figure 3.3 Dorsal view of the second larval stage development of *Atya lanipes*

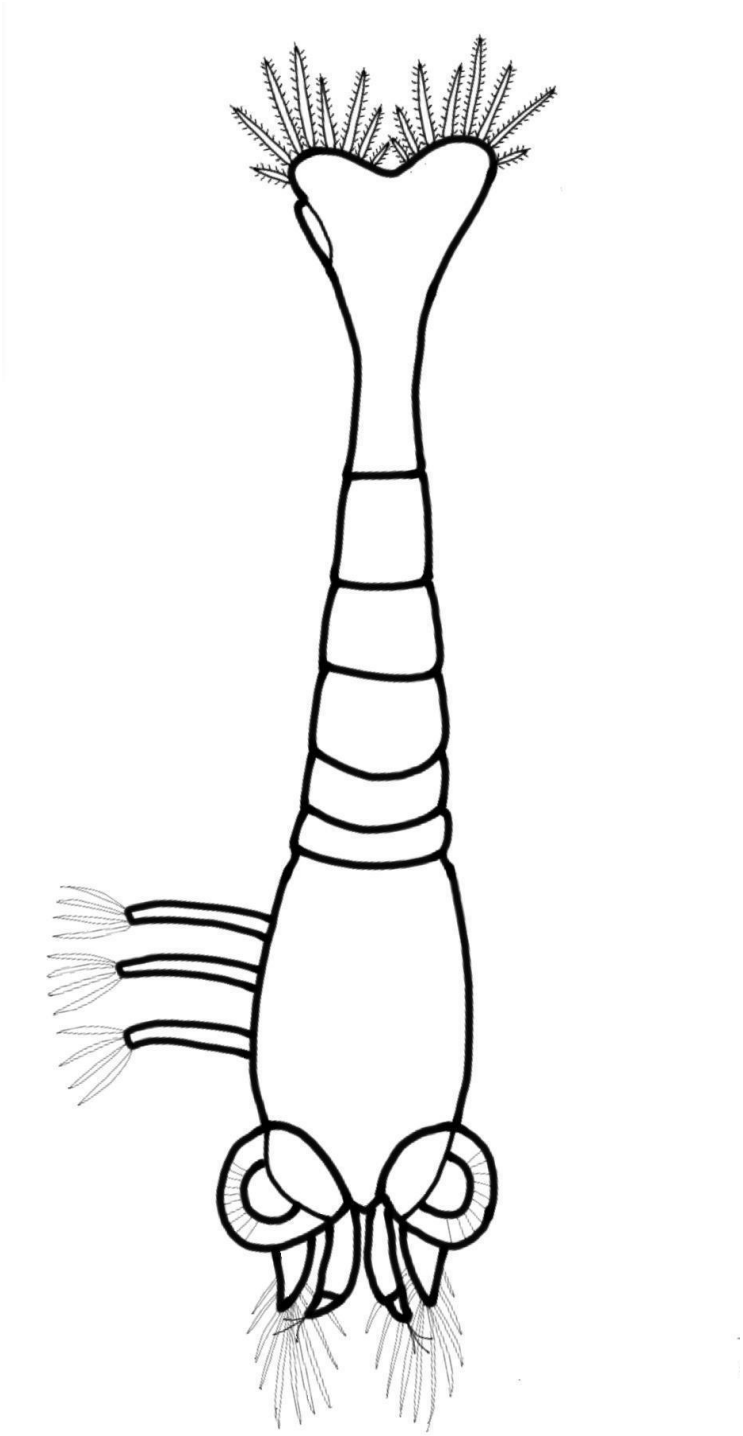


Figure 3.4 Dorsal view of the third larval stage development of *Atya lanipes*

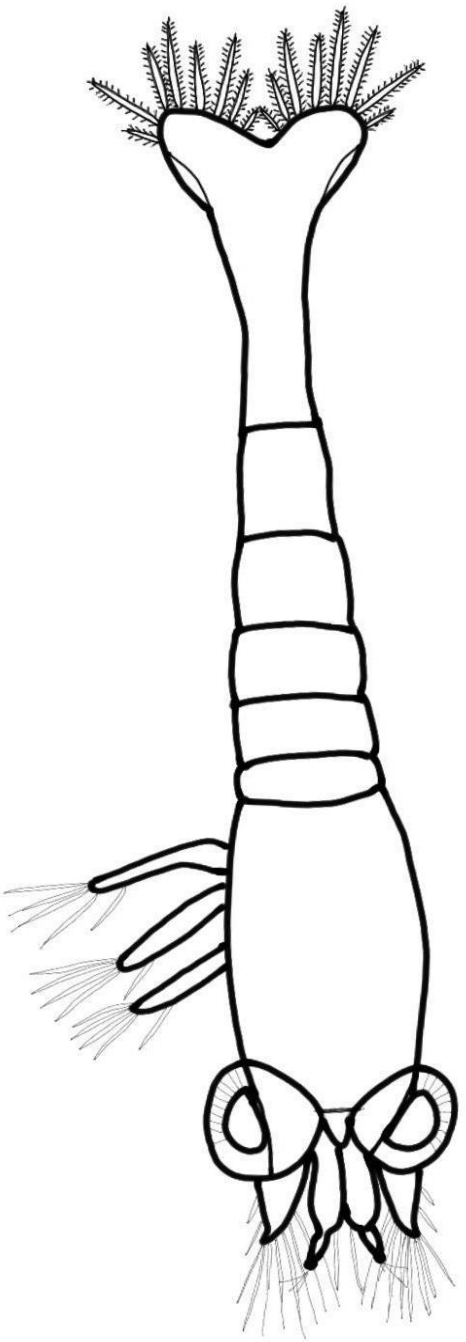


Figure 3.5 Dorsal view of the fourth larval stage development of *Atya lanipes*

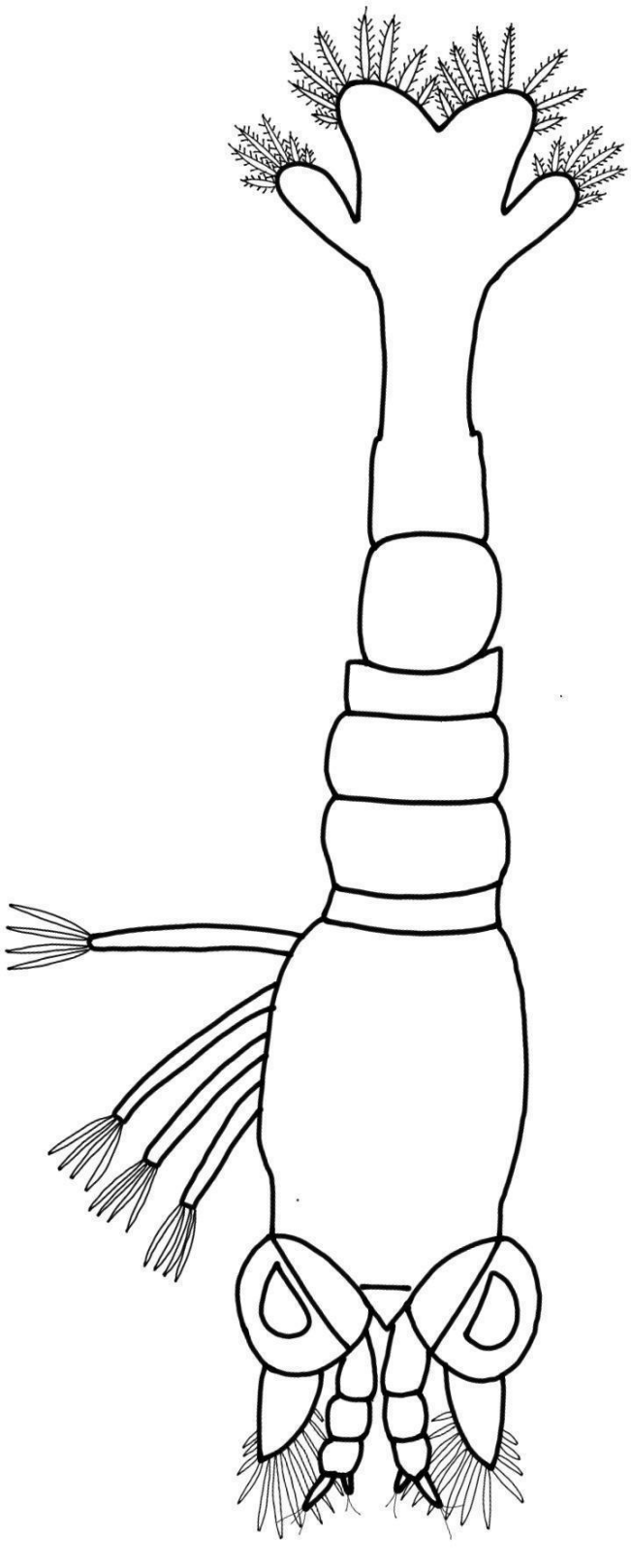


Figure 3.6 Dorsal view of the fifth larval stage development of *Atya lanipes*

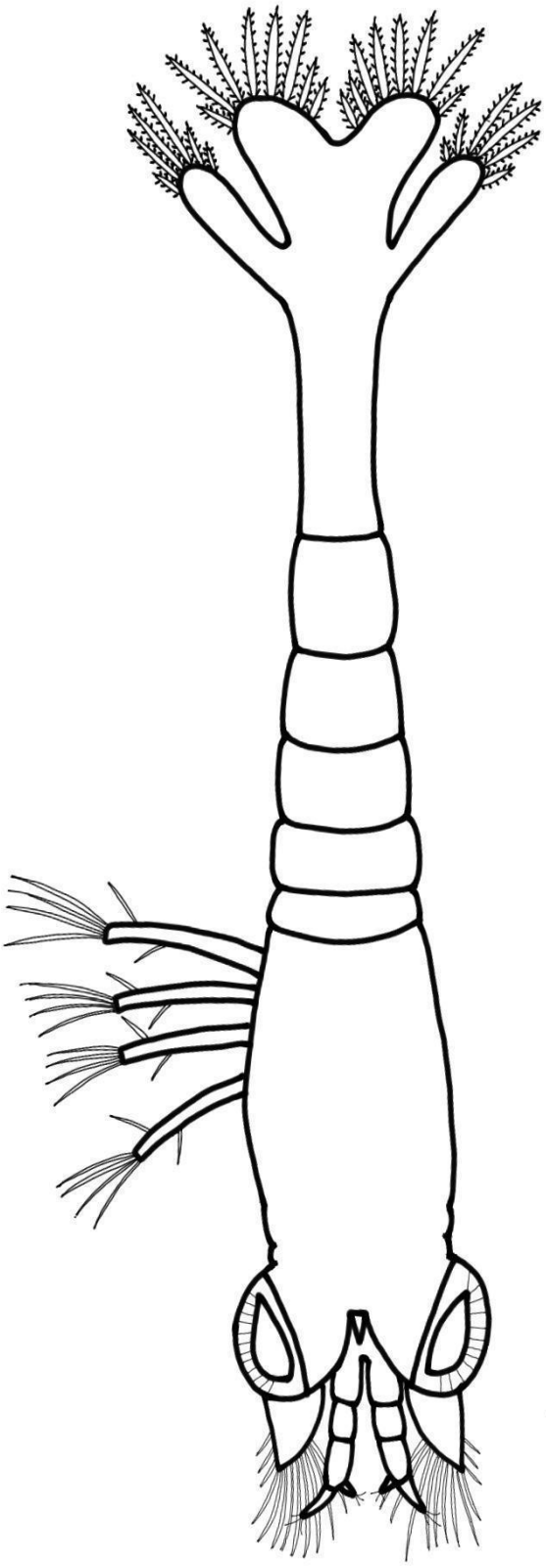


Figure 3.7 Dorsal view of the sixth larval stage development of *Atya lanipes*

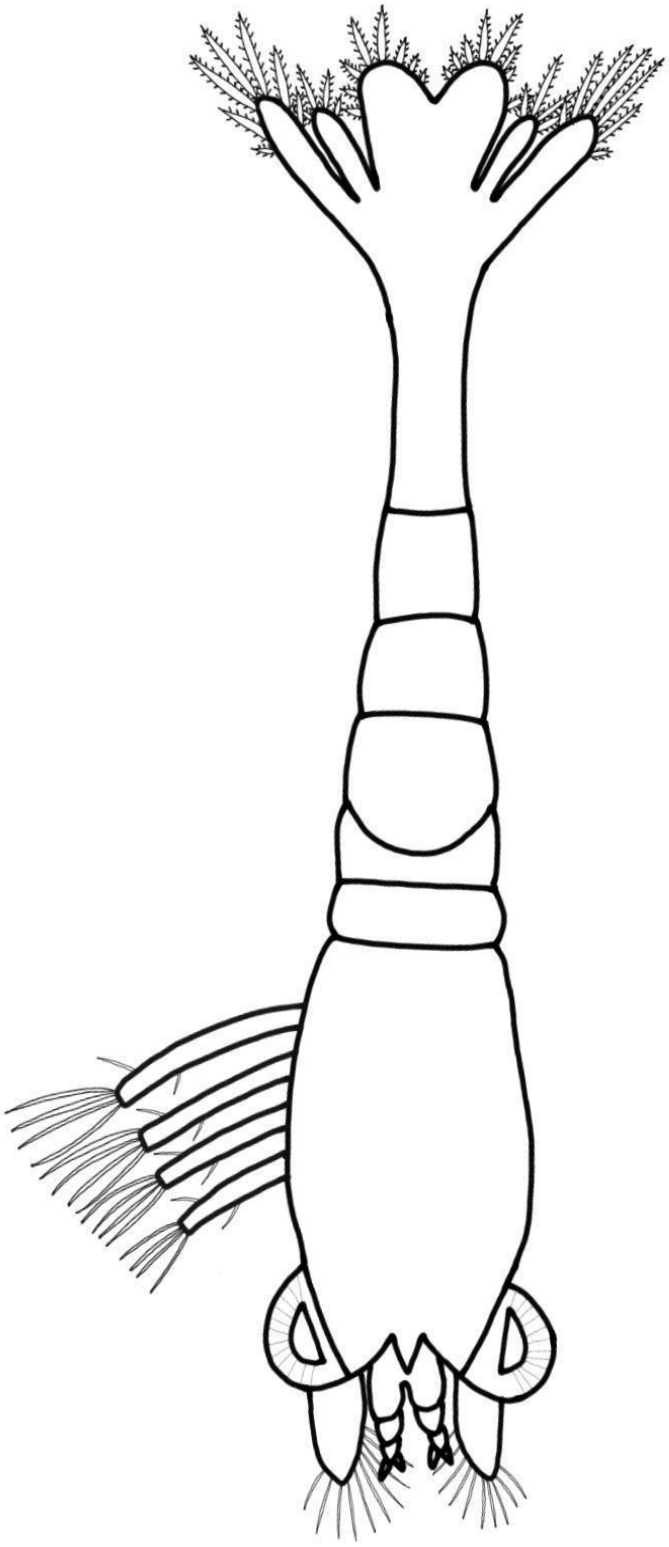


Figure 3.8 Dorsal view of the seventh larval stage development of *Atya lanipes*

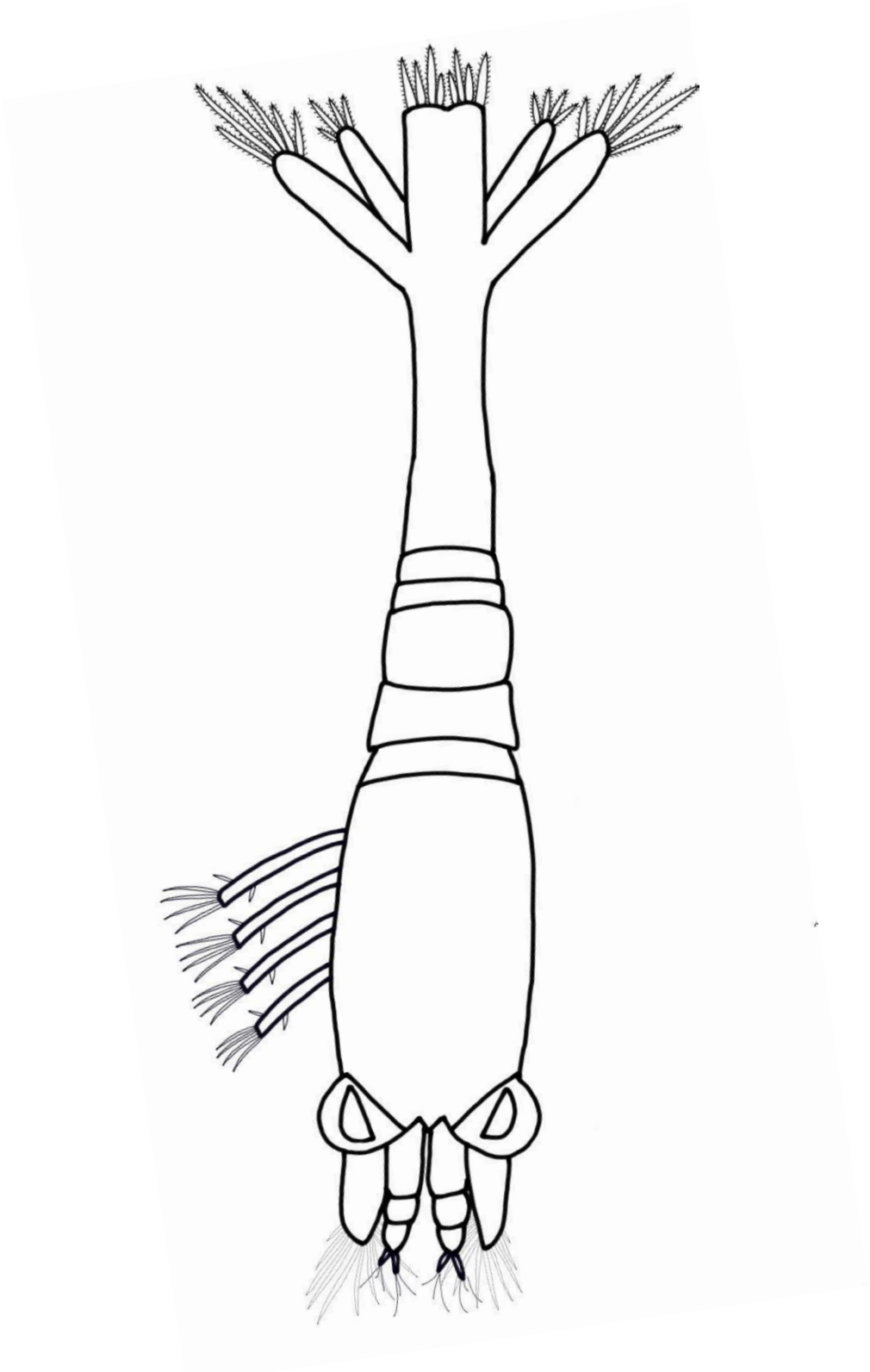


Figure 3.9 Dorsal view of the eight larval stage development of *Atya lanipes*

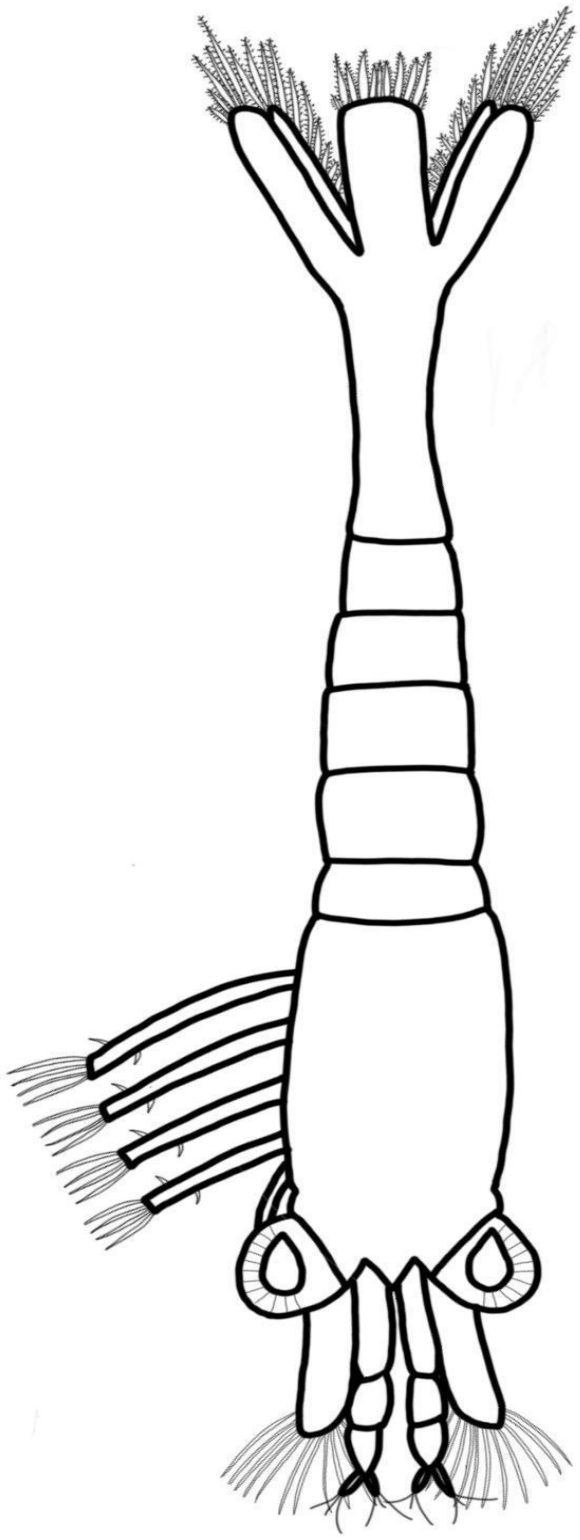


Figure 3.10 Dorsal view of the ninth larval stage development of *Atya lanipes*

CHAPTER 4: TITANIUM OXIDE NANOPARTICLES AS EMERGING AQUATIC POLLUTANTS: AN EVALUATION OF THE NANOTOXICITY IN THE FRESHWATER SHRIMP LARVAE *Atya lanipes*

Abstract

Nanoparticles are man-made materials defined as materials smaller than 100 nm in at least one dimension. Titanium oxide nanoparticles are of great interest because of their extensive use in self-care products. There is a lack of nanotoxicological studies of TiO_2 NPs in benthic organisms to have evidence about the effects of these pollutants in freshwater ecosystems. *Atya lanipes* is a scraper/filter that can provide a good nano-toxicological model. This study aims to determine how the TiO_2 NPs can develop a toxic effect in the larvae of the *Atya lanipes* shrimp and to document lethal and sublethal effects after acute exposures to TiO_2 NPs suspensions of: 0.0, 1.0, 10.0, 50.0, 100.0, and 150.0 mg/L. The results show that early exposure to TiO_2 NPs in *Atya lanipes* creates an increase in mortality at 48 and 72 h exposures, hypoactivity in movements, and morphological changes, such as less pigmentation and the presence of edema in exposed larvae. In conclusion, TiO_2 NPs are toxic contaminants in the larval stage of the *Atya lanipes*. It is necessary to regulate these nanoparticles for purposes of the conservation of aquatic biodiversity, especially for freshwater shrimp larvae and likely many other larvae of filter-feeding species.

Keywords: *aquatic toxicity; Atyidae; bioassays; Caribbean streams; ecotoxicology; macroinvertebrate; nanoparticles; nanotoxicology; tropics*

1. Introduction

With the rapid development of the nanotechnology industry, the world community is increasingly aware of the environmental impacts of manufactured nanoparticles (NPs) in biological systems [1,2]. NPs are man-made materials smaller than 100 nm in at least one dimension [3]. These particles have a greater surface/volume ratio and unique physicochemical properties compared to their normal forms and sizes [4,5].

Titanium oxide is one type of nanoparticle of great interest because it is used in paints and coatings as a self-cleaning, antimicrobial, antifouling agent, and in cosmetics as a UV absorber [6]. In addition, these NPs are energy semiconductors that exhibit photocatalytic activities [7]. From 2006 to 2010, the commercial production of titanium oxide nanoparticles (TiO2 NPs) was 5000 metric tons per year, increasing to more than 10,000 metric tons from 2011 to 2014, with an estimated 2.5 million metric tons by 2025 [8]

Nevertheless, there is a lack of standardized quantification of TiO_2 NPs in aquatic ecosystems and of the environmental concentration in many aquatic ecosystems [9]. This information gap includes the freshwater ecosystems in Puerto Rico and in the wider Caribbean region. Data from European studies have determined concentrations in freshwater ranging from 0.015 24.5 micrograms/L [10,11]. In contrast, concentrations in soils that exceed 100 micrograms/L have also been reported [12]. However, concentrations of titanium (including TiO_2 NPs) were found in the United States and Canada in a study involving 15 rivers. Concentrations of titanium in natural river waters range between 0.5 and 15 µg/L, and in soils and sediments, they range between 10 and 100 g/kg with an average of less than 5 g/kg [13]. Consequently, there is an urgent challenge to evaluate the toxicological effects

of TiO_2 NPs in the laboratory using environmental concentrations of this nanomaterial.

Some studies have determined the neurotoxic effects of TiO_2 NPs in different organisms. These neurotoxic effects are caused by the production of radical oxygen species in TiO_2 NPs. This type of NP causes oxidative stress in different tissues, including the brain/nervous system, causing neural damage. Moreover, TiO_2 NPs can be translocated and transported to other body organs [14–16]. Nevertheless, the toxicity effects in morphological development and mortality are not often clearly reported.

In this study, we used the larvae of the freshwater shrimp *Atya lanipes* Holthuis, 1963 (*A. lanipes*) to test the effect of TiO_2 NPs in their development, behavior, and survival. The *A. lanipes* is a good model to study the toxicity of the nanoparticles present in aquatic environments due to it being a scraper/filter feeder shrimp that lives part of its life as a planktonic organism until it reaches the post-larval stage, where it lives as a benthonic organism. This complex life cycle is called the amphidromous life cycle and is similar to all the shrimp families (Atyidae, Palaemonidae, and Xiphocarididae) that inhabit Puerto Rico and many of the Caribbean streams. This amphidromous life cycle represents an important connection between the headwaters and estuaries [17,18]. When females release the larvae (zoea larvae) in the upper reaches of a river, the first stage (i.e., newly hatched larvae) drift passively to coastal environments where they develop and metamorphose into post-larvae that subsequently migrate back upstream to the headwaters where they mature and reproduce as adult shrimp [19]. If the TiO_2 NPs are present in the estuarine/marine environment as the result of anthropogenic pollution, *A. lanipes* larvae could be affected by the possible toxic effects. In this part

of the life cycle, the interactions between the larvae and the nanoparticles will be in the water column due to the planktonic larval behavior. This early life cycle exposure to TiO_2 NPs in *A. lanipes* larvae can affect upstream migrations, population dynamics, and the species' survival.

This study aims to determine how the TiO_2 NPs can develop a toxic effect in the larvae of the *A. lanipes* shrimp that results in lethal effects, such as mortality, and sublethal effects, such as changes in behavior and morphological development. We hypothesized that the exposure of TiO_2 NPs among *A. lanipes* larvae would develop a toxic effect showing lethal and sublethal effects due to the photocatalytic activity and the production of oxidizing agents that damage neurons and other tissues.

2. Materials & Methods

2.1 Characterization of Titanium Oxide nanoparticles (TiO_2 NPs*)*

Titanium oxide nano powder (Sigma Aldrich Chemical Company St. Louis, MO, USA, titanium IV oxide, Anatase nanopowder) was used to conduct the experiments. The characterization method was an S4700 II Cold Field Emission Gun Scanning Electron Microscope cFEG SEM. Titanium oxide powder was spread on weighing paper and gently picked up by a sticky carbon surface on the top of aluminum stubs. An S4700 II cFEG SEM (Hitachi High Technologies-America) with a silicon drift EDX detector (Oxford Instruments, X-MaxN, UK) was used to measure the surface morphology, elemental composition, and distribution of elements. All the SEM data were obtained at an acceleration voltage of 10 kV, and the images were collected with a secondary-electron detector. The elemental mapping and energy spectra were acquired with Aztec tools (Oxford Instruments, UK). The elemental analysis through the energy dispersive spectrum indicates the presence of titanium,

oxygen, carbon, and sulfur elements (61, 36.1, 2.6, and 0.3 wt%, respectively). The observed size of the titanium oxide particle varied from 1 to 4 micron long

2.2. TiO_2 NPs Suspension Preparation and Physical Dispersion before the Exposure

Before the TiO_2 NPs suspensions were prepared, we dispersed the powder using a magnetic stirrer at a maximum speed for 30 min (the nanoparticles were not sonicated because the aim was to evaluate realistic environmental conditions). Then, the TiO_2 NPs suspensions were prepared one hour before the bioassay. We prepared suspension concentrations of 0.0, 1.0, 10.0, 50.0, 100.0, and 150.0 mg/L (mg of the weighted TiO_2 NPs in 1 L of water) in amber bottles covered with black tape to avoid nanoparticle–light interaction at 10 parts per million (ppm) of salinity. The seawater was pasteurized, filtered, and diluted with dechlorinated water to emulate natural estuary ecosystem water chemical conditions.

2.3 Atya lanipes ***gravid specimens collection***

The gravid females of *A.lanipes* were collected using baited minnow traps in the Buruquena Stream (18.321207, −65.819389) at the El Verde Field Station, Rio Grande, Puerto Rico (Figure 4.3). Baited traps (the bait was dry cat food) were set in different pools along the stream and removed 24 h later. The collected gravid shrimp were identified [20] and transported in a cooler under constant aeration to the laboratory. The gravid shrimp were transferred individually to glass tanks (15 cm × 15 cm × 15 cm) with 1 L of dechlorinated water and constant aeration. The shrimp were fed with commercial fish flakes (Tetra TetraMin Tropical Flakes) until the larval eclosion. After the larval eclosion, we separated the larvae from the adult shrimp, and the bioassay started. The duration was 8:00–10:00 AM.

2.4 Atya lanipes ***larvae bioassay***

For the bioassays, we used the protocol of Solis et al. (1993) [21]. *A. lanipes* zoea larvae hatched under normal laboratory conditions and were exposed to various TiO_2 NPs concentrations. The suspension concentrations of 0.0, 1.0, 10.0, 50.0, 100.0, and 150.0 mg of TiO_2 NPs/L were tested (N = 25 for each concentration). For each treatment, we used individual culture plates that contained six cells. This was duplicated for each exposure time individually. Replicates were made for each treatment and exposure time. In this way, each exposure time for the six treatments had 18 culture plates. In the culture plates of each concentration, 10 larvae were transferred to each cell of the six-well culture plate with 10.0 mL of each suspension concentration. Then, the six-well culture plates were transferred into a water bath with two thermometers. A temperature of 26 ± 1 ◦C was maintained for the bioassay exposure. During the entire experiment, the larvae were kept with no food and aeration. The light/dark photoperiod was 14:10 h (day:night). The exposures were carried out independently at 24, 48, and 72 h for all the nanoparticle suspension concentrations and the control group. The bioassay was conducted with different *A. lanipes* larvae samples to have genetic variation.

2.5 Mortality Analysis

Mortality was recorded as the following: (1) cessation of swimming or any movement by the larvae; (2) no phototaxis behavior; (3) change in color from transparent to white/gray. For each concentration, we analyzed a 40-larvae sample size. A mortality assessment was conducted for 5 min. We counted the number of larvae classified as “dead” following the previous definition for 24, 48, and 72 h of exposure.

2.6 Movement analysis

The movement was assessed 24, 48, and 72 h after exposure to the different TiO_2 NPs suspension concentrations using video recordings of the individual larva under free swimming behavior to avoid group interactions. The larvae were placed in a single well of ten well plates (5.8 cm width × 12.6 cm length) [22]. This single-well plate was a circular plastic container 14 mm in diameter, and the depth was 1 mm (Falcon Plastics, California). The recordings were obtained using a stereo microscope with a digital camera (3.5 × -90 × LED Trinocular Zoom + 14 MP USB 3.0 Digital Camera). Each larva was acclimated for 90 s and their movement was recorded for 60 secs. We analyzed a sample size of larvae (N = 25) for each nanoparticle concentration and the control group. The room was kept dark and silent to prevent movement caused by phototaxis or sound. The only light available was the microscope light at a minimum level and covered with a red cellophane to prevent the movement of the larva because they do not detect red light. The recordings were analyzed using the Loligo® Systems- Lolitrack 5. The movement variables obtained from the software were average speed (mm/s) and average acceleration (mm/s^2).

2.7 Morphological Development analysis

To evaluate the morphological development of the larvae, we measured the total length of each larva in the control and exposure groups for all concentrations and exposure times. The total length was measured from the post-orbit margin to the end of the telson, excluding setae [23]. To measure the length of the larvae, we used a stereo microscope (3.5×-90× LED Trinocular Zoom + 14 MP USB 3.0 Digital

Camera) with measuring software installed on a computer. Edemas and changes in pigmentation were classified as presence or absence.

2.8 Statistical analysis

The statistical analysis and graphs were completed using PAST software (2001). Data are presented as the mean ± standard error of the mean, considering each six-well plate as an experimental unit. For biological responses such as behavior and total length, we performed a one-way ANOVA followed by the Tukey test to compare each exposure time separately. The comparisons of morphological development were performed using observed/not observed parameters (edema and/or pigmentation changes). For mortality, we calculated the mortality rate (%) (number of death larvae/unit sample size), for each concentration for the three periods of exposure.

3. Results

3.1. Mortality rate

The mortality rate for each TiO_2 NPs concentration at 24, 48, and 72 h exposure provided relevant information. At 24 h of exposure, no dead larvae were observed in any TiO_2 NPs concentration exposures or the control group. However, after 48 and 72 h, the mortality rate increased in the exposed group, specifically within 72 h of exposure (Figure 4.7). However, we did not see a consistent relationship between mortality and the concentrations of TiO_2 NPs tested. The aforementioned is confirmed in the concentration of 150 mg/L where a decrease in mortality occurred rather than an increase for 48 and 72 h of exposure.

3.2. Movement Assessment

The analysis of movement after 24, 48, and 72 h of exposure to TiO_2 NPs showed significant changes leading to hypoactivity behavior for both variables in the exposed

groups. During the 24 h exposure, we observed a reduction in the average speed and average acceleration of the larvae in the exposed groups compared with the control group showing hypoactivity movement (Figure 4.8).

The average speed (with a Box–Cox transformation; N = 25) observed by the larvae in the control group was 0.32 mm/s ± 0.03 with a minimum of 0.00009 mm/s and a maximum of 0.46 mm/s during the 24 h of exposure. For the different concentrations of TiO_2 NPs in the exposed group, we observed average speeds of 0.22 ± 0.02, 0.14 ± 0.02, 0.16 ± 0.03, 0.13 ± 0.02, and 0.13 ± 0.03 mm/s for the concentrations of 1.0, 10.0, 50.0, 100.0, and 150.0 mg/L, respectively. Moreover, for the exposed groups, the speed of the larvae ranged between 0 mm/s and 0.34 mm/s. The one-way ANOVA for the comparison of the average speed variable for the 24 h of exposure of the larvae in the control group and the exposed larvae showed a significant difference ($F_{(5,144)} = 9.08$; $p < 0.01$) among groups. Consequently, the Tukey test showed a $p < 0.01$ among the exposed groups from 10 to 150 mg/L of TiO_2 NPs.

The average acceleration of the larvae (with a Box–Cox transformation; N = 25) was 0.38 ± 0.24 mm/s^2 with a minimum of −2.35 mm/s^2 and a maximum of 1.95 mm/s^2 for the control group during the 24 h of exposure. The average acceleration in the exposure of the concentrations of TiO_2 NPs were −0.27 ± 0.19, −0.82 ± 0.20, −0.65 ± 0.22, −0.89 ± 0.19, and −0.93 ± 0.23 mm/s2 for the concentrations of 1.0, 10.0, 50.0, 100.0, and 150.0 mg/L, respectively. Moreover, for the exposed groups, we observed a minimum average acceleration of −2.97 mm/s^2 and a maximum of 1.50 mm/s^2. The one-way ANOVA for the comparison of the average acceleration variable for the 24 h of exposure of the larvae in the control group and the exposed larvae showed a significant difference ($F_{(5,144)} = 5.61$; $p < 0.01$) between groups.

Consequently, Tukey test analysis showed values of $p < 0.01$ among exposed groups between 10 and 150 mg/L of TiO_2 NPs, indicating similarities between exposed groups except for the control group. In the exposure of larvae for 48 h to TiO_2 NPs, we observed significant results for both locomotion variables. The one-way ANOVA to compare the effects of TiO_2 NPs on the speed ($F_{(5,144)} = 7.7$; $p < 0.01$) and acceleration ($F_{(5,144)} = 7.88$; $p < 0.01$) of the larvae were highly significant. To analyze this exposure time, we used the Tukey test for significant differences among the concentrations of TiO_2 NPs of 10.0, 50.0, 100.0, and 150.0 mg/L. No significant differences were found in comparison with the control group. Consequently, at 72 h of exposure, we observed no significant differences between the exposed groups and the control (speed: $F_{(5,144)} = 1.13$; $p = 0.35$) (acceleration: $F_{(5,144)} = 1.37$; $p = 0.24$).

3.3. Morphological development

The morphological assessment consisted of total length measures, changes in the presence/absence of pigmentation, and edema in larvae at 24, 48, and 72 h of exposure to TiO_2 NPs. For the larval pigmentation, we observed less pigmentation in the abdominal area in some exposed groups and with some exposure times (Figure 4.9)

In contrast, we found an edema in the abdominal area of the larvae during the 72 h exposure to the TiO_2 NPs concentration of 150.0 mg/L (Figure 4.10). We did not find another case of edema in another period of exposure or concentration of TiO_2 NPs.

No significant differences in total length among the control and exposed groups were observed (Figure 4.11). Nevertheless, we found that, at 72 h of exposure, two concentrations (50.0 and 100.0 mg/L) of TiO_2 NPs showed statistical

significance compared to the control group means. The one-way ANOVA analysis showed total length: $F_{(5,144)} = 3.907$; $p < 0.01$. Consequently, the Tukey test showed similarities between the 50.0 and 100.0 mg/L of TiO_2 NPs ($p < 0.05$).

Discussion

Nanomaterials are an important emerging aquatic contaminant [24,25,26,27] that create potentially wide-ranging ecological impacts [28]. Studies with TiO_2 NPs have found that these nanoparticles interact with other metals and are stored in sediments, acting as an adsorptive agent. Furthermore, it has been shown that TiO_2 NPs can exacerbate the bioavailability and toxicity of organic pollutants and pesticides, serving as an adsorptive agent [29]. To date, several studies at a global scale are focused on using the photocatalysis (formation of oxidative agents) of TiO_2 NPs for the remediation and treatment of wastewater. Many of these studies do not consider the possible toxicity to aquatic organisms [30,31]. Another action of TiO_2 NPs in freshwater ecosystems is in the trophic transfer process. A recent study demonstrates that dietary TiO_2 NPs exposure in some species may constitute a significant route for higher trophic level bioaccumulation [32]. It is essential to understand the biological impacts that these emerging pollutants can cause in benthic organisms because they interact mainly with the sediment and have complex life cycles (diadromy/amphidromy).

The study of the development of the *A. lanipes* larvae is not yet completely understood, but, as in other well-studied species, the early developmental stages are most often sensitive to environmental contaminants. For example, in the developing nervous system, the toxicant stress can have a detrimental impact in the early stage with persistent effects into adulthood [33]. Our study demonstrates that the TiO_2 NPs

can harm the nervous system in the larval stages of *A. lanipes.* During the first 24 h of exposure, we observed a reduction in the locomotion of the larvae evidencing hypoactivity movement similar to previous studies with zebrafish larvae and neotropical tadpoles where the individuals reduced their velocity and mobility behaviors after exposures to TiO_2 NPs [33,34]. After 48 and 72 h of exposure, we observed variability in the hypoactive response of the shrimp larvae in the movement analyses. This result leads us to propose two possible explanations. The first is related to a possible change in the behavior of the TiO_2 NPs. Nanomaterials change their physicochemical properties depending on the environment in which they are found (salinity, temperature, light, biological interaction, etc.). Therefore, they can also create environmental changes over time, resulting in a variable toxic effect for the zoea larvae of *A. lanipes*. Another possible response to this variability is that *A. lanipes* larvae can develop from one larval stage to another in a few days (in 2–3 days they can go from zoea I to zoea II stage). In a period of 48 and 72 h of exposure, we observed larvae in the zoea I stage and others in the zoea II stage, which was more common at 72 h of exposure. Thus, the larvae of *A. lanipes* could have differences in susceptibility to the toxicity of TiO_2 NPs in different larval stages. In general, these changes in *A. lanipes* zoea larvae may represent a possible long-term impact on the development of adult shrimp and present undesirable ecological consequences. From upstream migrations after metamorphosis in the estuarine environment to adult activities, such as reproduction, food acquisition, and evasion of predation, can be affected by TiO_2 NPs.

A limitation in the movement analysis was that the larvae were recorded in a different container than the cells in which they were exposed. Moreover, having recorded the larvae in a round container, the total distance variable could not be

added because, in the data analysis, the program did not differentiate the distance traveled by the larvae when they were in the corner of the container due to the reflection of the water. Consequently, only the variables of velocity and acceleration were considered. The sample used for the movement analysis was only 25 larvae because they were recorded individually in a single microscope. Finally, the TiO_2 NPs content on the cells in the culture dishes at the exposure time of each bioassay was not analyzed to determine the movement or final locations of the concentrations of the TiO_2 NPs suspensions (agglomeration, dissolution, flocculation, etc.).

The results of this study demonstrated that an acute exposure to TiO_2 NPs in the freshwater shrimp *A. lanipes* larvae represents a lethal effect. Mortality was evident after 48 and 72 h of exposure. The data showed different susceptibility to nanoparticles in different larval stages because, in *A. lanipes* larvae, their development is rapid. This may explain the variability in mortality between 48 and 72 h of exposure, especially when we observed a decrease in mortality in both exposure times in the 150 mg/L suspension concentration.

Regarding the sublethal effects of TiO_2 NPs, we observed the loss of pigmentation in exposed larvae. Like other atyids shrimp, the pigment that develops in the larvae of *A. lanipes* is known as chromatophores [35]. These pigments are important for the development of the characteristic dark color of adult atyids that begins from the pre-larval stages [36]. These cells provide many important functions in atyids, such as controlling dissolved oxygen during periods of oxygen supersaturation, and they protect the larva and the adult shrimp from the harmful effects of low oxygen concentrations [36]. However, when there are changes in the chromatophores, this is due to indicators of many factors, among which are homeostatic instability in response to low oxygen concentrations, pathological

manifestations, and expressions that can indicate degrees of stress at a biological level [36]. For the pigment to disperse and express itself, the hormonal action of the red pigment-concentrating hormone (RPCH) is required, which induces pigment aggregation in shrimp chromatophores due to an internal or external increase in Ca2+ at the cellular level [36]. In oxidative stress, there is substantive evidence that the calcium homeostasis is altered, and some cells die (apoptosis) [37]. This explains why we found that larvae exposed to TiO_2 NPs (reactive oxygen species generators) showed a change in pigmentation. There is a possibility of oxidative stress inducing changes in cellular Ca^{2+} flux homeostasis and an impact on pigment dispersion/aggregation.

The presence of edemas in the abdominal region in exposed larvae is common in studies related to the toxicity of TiO_2 NPs, as is the case with zebrafish larvae [38,39]. However, in our study, for the three exposure periods 24, 48, and 72 h, we only observed one larva with an edema in the abdominal area close to the beginning of the telson. These data suggest that, for *A. lanipes*, unlike zebrafish, the development of edemas is not as relevant. We do not rule out the need for more repetition and validation of this analysis.

The data presented in this study are novel in helping to understand the toxicity levels of TiO_2 NPs in an ecologically susceptible species that play key roles in the biofiltration of natural organic particles [40,41,42,43]. The most obvious sublethal effect was in the locomotion analysis. It was shown that acute exposure to TiO_2 NPs can alter the normal locomotion of the larva, indicating neurobehavioral effects that could persist in other life stages of the shrimp. We demonstrated that this emerging contaminant could cause a significant biological impact by reducing locomotion, producing morphological changes, and, later, the death of the organism. A reduction

in the number of larvae that metamorphose into juveniles and adults could lead to a reduction in the number of individuals in the population and to changes in their ecological role in the ecosystem.

This study provides new knowledge about the effect of TiO_2 NPs in the larvae of a Caribbean freshwater shrimp that helps to keep flowing waters clear of suspended particles. Sustaining biodiversity and ecosystem management is critical for maintaining ecological integrity. Moreover, our results contribute to the need in the scientific literature to use the most effective organisms that are ecologically susceptible to the presence of TiO_2 NPs in aquatic environments. These nano ecotoxicological studies need to include native benthic as well as pelagic organisms [44]. The *A. lanipes* is a shrimp that can be susceptible to the presence of engineered nanoparticles in the estuary during its larval stages.

Literature cited:

1. Vance, M.E.; Kuiken, T.; Vejerano, E.P.; McGinnis, S.P.; Hochella, M.F., Jr.; Rejeski, D.; Hull, M.S. Nanotechnology in the real world: Redeveloping the nanomaterial consumer products inventory. *Beilstein J. Nanotechnol.* 2015, *6*, 1769–1780.
2. Colvin, V.L. The potential environmental impact of engineered nanomaterials. *Nat. Biotechnol.* 2003, *21*, 1166–1170.
3. Turan, N.B.; Erkan, H.S.; Engin, G.O.; Bilgili, M.S. Nanoparticles in the aquatic environment: Usage, properties, transformation and toxicity—A review. *Process Saf. Environ. Prot.* 2019, *130*, 238–249.
4. Maurer-Jones, M.A.; Gunsolus, I.L.; Murphy, C.J.; Haynes, C.L. Toxicity of engineered nanoparticles in the environment. *Anal. Chem.* 2013, *85*, 3036–3049.
5. Khan, I.; Saeed, K.; Khan, I. Nanoparticles: Properties, applications and toxicities. *Arab. J. Chem.* 2019, *12*, 908–931.
6. Berardinelli, A.; Parisi, F. TiO2 in the food industry and cosmetics. In *Titanium Dioxide (TiO_2) and Its Applications*; Elsevier: Amsterdam, The Netherlands, 2021; pp. 353–371.
7. Verma, S.K. *Engineered Nanomaterials and Phytonanotechnology: Challenges for Plant Sustainability*; Elsevier: Amsterdam, The Netherlands, 2019; Volume 87.
8. Dedman, C.J.; King, A.M.; Christie-Oleza, J.A.; Davies, G.L. Environmentally relevant concentrations of titanium dioxide nanoparticles pose negligible risk to marine microbes. *Environ. Sci. Nano* 2021, *8*, 1236–1255.

9. Musial, J.; Krakowiak, R.; Mlynarczyk, D.T.; Goslinski, T.; Stanisz, B.J. Titanium dioxide nanoparticles in food and personal care products—What do we know about their safety? *Nanomaterials* 2020, *10*, 1110.
10. Gottschalk, F.; Sonderer, T.; Scholz, R.W.; Nowack, B. Possibilities and limitations of modeling environmental exposure to engineered nanomaterials by probabilistic material flow analysis. *Environ. Toxicol. Chem.* 2010, *29*, 1036–1048.
11. Batley, G.; McLaughlin, M.J. *Fate of Manufactured Nanomaterials in the Australian Environment*; CSIRO Land and Water: Clayton, Australia, 2010.
12. Gottschalk, F.; Sun, T.; Nowack, B. Environmental concentrations of engineered nanomaterials: Review of modeling and analytical studies. *Environ. Pollut.* 2013, *181*, 287–300.
13. Titanium-Environmental Health Criteria 24. Available online: https://apps.who.int/iris/bitstream/handle/10665/37269/9241540842-eng.pdf (accessed on 13 August 2022).
14. Song, B.; Liu, X.; Wei, L.; Shao, L. A review on potential neurotoxicity of titanium dioxide nanoparticles. *Nanoscale Res. Lett.* 2015, *10*, 342.
15. Lin, D.; Ji, J.; Long, Z.; Yang, K.; Wu, F. The influence of dissolved and surface-bound humic acid on the toxicity of TiO2 nanoparticles to *Chlorella* sp. *Water Res.* 2012, *46*, 4477–4487.
16. Chen, T.H.; Lin, C.Y.; Tseng, M.C. Behavioral effects of titanium dioxide nanoparticles on larval zebrafish (*Danio rerio*). *Mar. Pollut.* 2011, *63*, 303–308.

17. Pérez-Reyes, O.; Crowl, T.A.; Covich, A.P. Comparison of decapod communities across an urban-forest land use gradient in Puerto Rican streams. *Urban Ecosyst.* 2016, *19*, 181–203.

18. Benstead, J.P.; March, J.G.; Pringle, C.M. Estuarine larval development and upstream post-larval migration of freshwater shrimps in two tropical rivers of Puerto Rico. *Biotropica* 2000, *32*, 545–548.

19. Bauer, R.T. Amphidromy in shrimps: A life cycle between rivers and the sea. *Lat. Am. J. Aquat. Res.* 2013, *41*, 633–650.

20. Perez-Reyes, O.; Crowl, T.A.; Hernandez-Garcia, P.J.; Ledesma-Fuste, R.; Villar-Fornes, F.A.; Covich, A.P. Freshwater decapods of Puerto Rico: A checklist and reports of new localities. *Zootaxa* 2013, *3717*, 329–344.

21. Solis, P.N.; Wright, C.W.; Anderson, M.M.; Gupta, M.P.; Phillipson, J.D. A microwell cytotoxicity assay using *Artemia salina* (brine shrimp). *Planta Med.* 1993, *59*, 250–252.

22. Ingebretson, J.J.; Masino, M.A. Quantification of locomotor activity in larval zebrafish: Considerations for the design of high-throughput behavioral studies. *Front. Neural Circuits* 2013, *7*, 109.

23. Anastasiadou, C.; Ntakis, A.; Leonardos, I.D. Larval development of the freshwater shrimp *Atyaephyra desmarestii* (Millet, 1831) sensu lato (Decapoda: Caridea: Atyidae) and morphological maturation from juveniles to adults. *Zootaxa* 2011, *2877*, 41–54.

24. Farre, M.; Gajda-Schrantz, K.; Kantiani, L.; Barcelo, D. Ecotoxicity and analysis of nanomaterials in the aquatic environment. *Anal. Bioanal. Chem.* 2009, *393*, 81–95.

25. Klaine, S.; Alvarez, P.; Batley, G.; Fernandes, T.; Handy, R.; Lyon, D.; Mahendra, S.; McLaughlin, M.J.; Lead, J.R. Nanomaterials in the environment: Behavior, fate, bioavailability, and effects. *Environ. Toxicol. Chem. Int. J.* 2008, *27*, 1825–1851.

26. Navarro, E.; Baun, A.; Behra, R.; Hartmann, N.B.; Filser, J.; Miao, A.J.; Quigg, A.; Santschi, P.H.; Sigg, L. Environmental behavior and ecotoxicity of engineered nanoparticles to algae, plants, and fungi. *Ecotoxicology* 2008, *17*, 372–386.

27. Nel, A.; Xia, T.; Madler, L.; Li, N. Toxic potential of materials at the nanolevel. *Science* 2006, *311*, 622–627.

28. Miller, R.J.; Bennett, S.; Keller, A.A.; Pease, S.; Lenihan, H.S. TiO2 Nano-particles Are Phototoxic to Marine Phytoplankton. *PLoS ONE* 2012, *7*, e30321.

29. Fang, Q.; Shi, X.; Zhang, L.; Wang, Q.; Wang, X.; Guo, Y.; Zhou, B. Effect of titanium dioxide nanoparticles on the bioavailability, metabolism, and toxicity of pentachlorophenol in zebrafish larvae. *J. Hazard. Mater.* 2015, *283*, 897–904.

30. Lazar, M.A.; Varghese, S.; Nair, S.S. Photocatalytic water treatment by titanium dioxide: Recent updates. *Catalysts* 2012, *2*, 572–601.

31. Li, Z.; Deng, S.; Yu, G.; Huang, J.; Lim, V.C. As (V) and as (III) removal from water by a Ce–Ti oxide adsorbent: Behavior and mechanism. *Chem. Eng.* 2010, *161*, 106–113.

32. Zhu, X.; Wang, J.; Zhang, X.; Chang, Y.; Chen, Y. Trophic transfer of TiO2 nano-particles from *Daphnia* to zebrafish in a simplified freshwater. *Chemosphere* 2010, *79*, 928–933.

33. Fan, C.Y.; Cowden, J.; Simmons, S.O.; Padilla, S.; Ramabhadran, R. Gene expression changes in developing zebrafish as potential markers for rapid developmental neurotoxicity screening. *Neurotoxicol. Teratol.* 2010, *32*, 91–98.

34. do Amaral, D.F.; Guerra, V.; Almeida, K.L.; Signorelli, L.; Rocha, T.L.; de Melo e Silva, D. Titanium dioxide nanoparticles as a risk factor for the health of Neotropical tadpoles: A case study of Dendropsophus minutus (Anura: Hylidae). *Environ. Sci. Pollut. Res.* 2022, *29*, 50515–50529.

35. Hunte, W. The Complete Larval Development of the Freshwater Shrimp *Atya innocous* (Herbst) Reared in the Laboratory (Decapoda, Atyidae). Crustaceana, Supplement, no 5, 1979, 231–242. Available online: http://www.jstor.org/stable/25027505 (accessed on 13 August 2022).

36. Ribeiro, M.; Campbell, J. Calcium movements during pigment aggregation in freshwater shrimp chromatophores. *Pigment. Cell Res.* 2007, *20*, 70–77.

37. Mattson, M. Oxidative stress, perturbed calcium homeostasis, and immune dysfunction in Alzheimer's disease. *J. Neurovirol.* 2002, *8*, 539–550.

38. Ma, H.; Diamond, S. Phototoxicity of TiO2 nanoparticles to zebrafish (*Danio rerio*) is dependent on life stage. *Toxicol. Chem.* 2013, *32*, 2139–2143.

39. Liu, J.; Fan, D.; Wang, L.; Shi, L.I.L.I.; Ding, J.; Chen, Y.; Shen, S. Effects of ZnO, CuO, Au, and TiO2 nanoparticles on *Daphnia magna* and early life stages of zebrafish *Danio rerio*. *Environ. Prot. Eng.* 2014, *40*, 139–149.

40. Covich, A.P. Atyid shrimp in the headwaters of the Luquillo Mountains, Puerto Rico: Filter feeding in natural and artificial streams. *Verh. Int. Ver. Für Angew. Limnol.* 1988, *23*, 2108–2113.

41. Covich, A.P.; Palmer, M.A.; Crowl, T.A. The role of benthic invertebrate species in freshwater ecosystems—Zoobenthic species influence energy flows and nutrient cycling. *BioScience* 1999, *49*, 119–127.

42. Covich, A.P.; Ewel, K.C.; Hall, R.O.; Giller, P.E.; Goedkoop, W.; Merritt, D.M. Ecosystem services provided by freshwater benthos. In *Sustaining Biodiversity and Ecosystem Services in Soils and Sediments*; Chapter 3; Wall, D.H., Ed.; Island Press: Washington, DC, USA, 2004; pp. 45–72.

43. Crowl, T.A.; McDowell, W.H.; Covich, A.P.; Johnson, S. Species-specific responses in leaf litter processing in a tropical headwater stream (Puerto Rico). *Ecology* 2001, *82*, 775–783.

44. Garric, J.; Thybaud, E. Toxicological Models Part B: Environmental Models. In *Nanoethics and Nanotoxicology*; Springer: Berlin/Heidelberg, Germany, 2011; pp. 379–396.

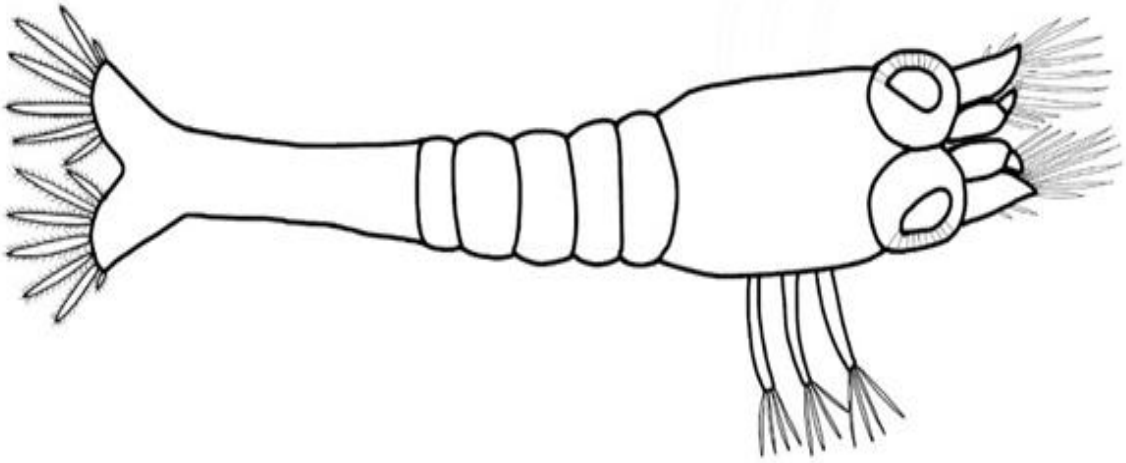


Figure 4.1 *Atya lanipes* zoea larvae stage; the organism used in the study.

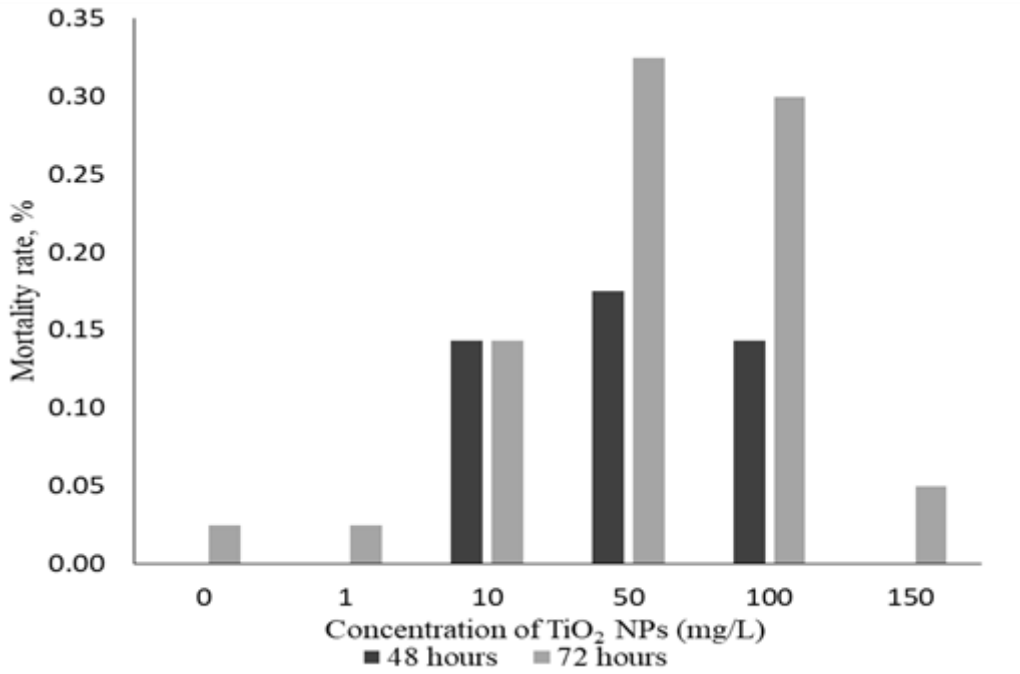
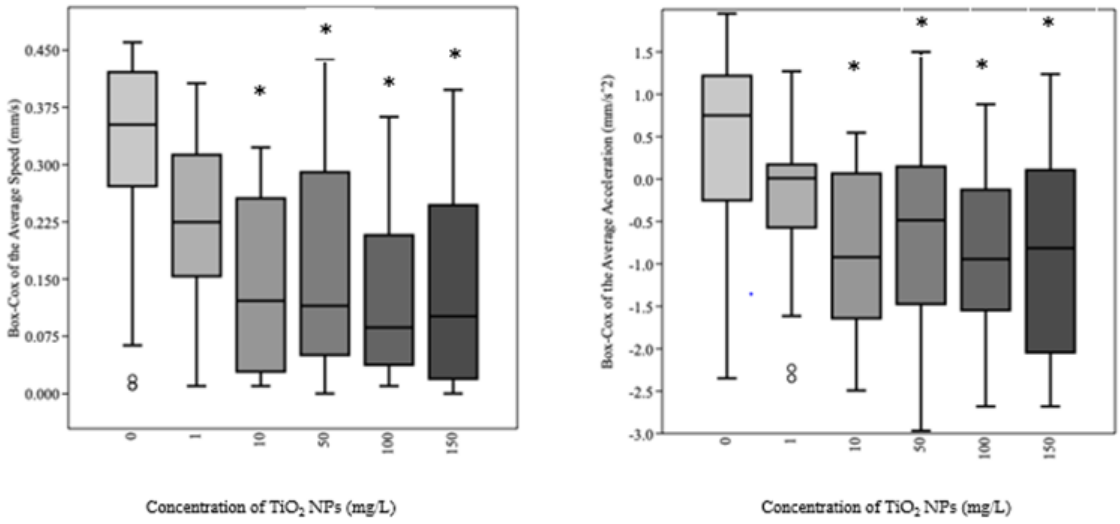


Figure 4.2 The Percentage of the mortality rate of *A. lanipes* larvae exposed to different concentrations of TiO_2 NPs after 24, 48, and 72 h of exposure (N=40 for each NPs concentrations). (Note: after 24 h of exposure, we observed no mortality in any exposed group and control).



(a) **(b)**

Figure 4.3. Movement larvae behavior assessment (N=25 for each concentration). a) Average speed (mm/s) and b) Average acceleration (mm/s2) of *A. lanipes* larvae exposed to different concentrations of TiO_2 NPs in an acute exposition of 24 h (* $p<0.01$).

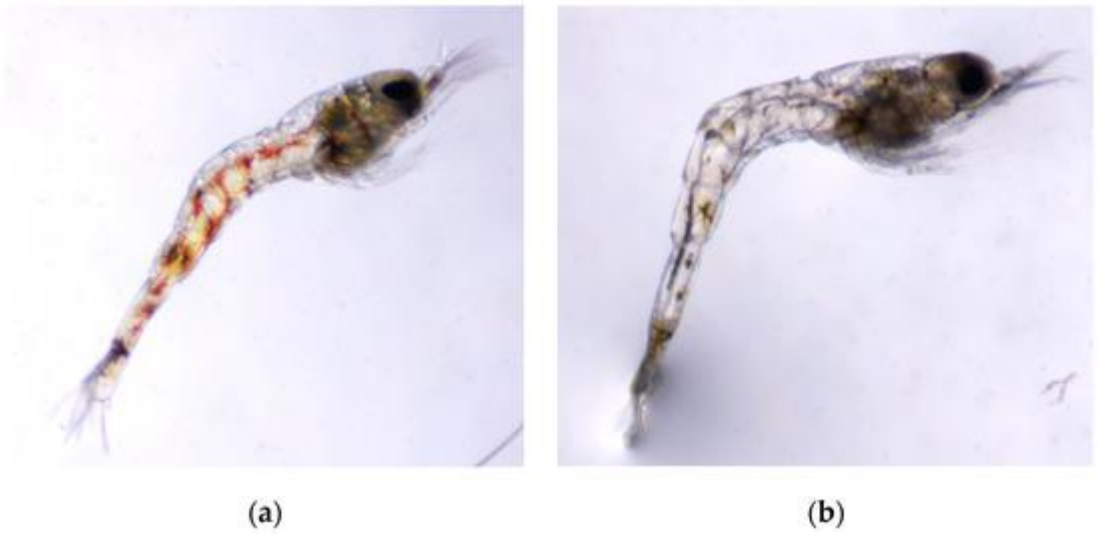


Figure 4.4 *A. lanipes* larva exposed to a suspension of TiO_2 NPs for 24 h showing changes in body pigmentation: (**a**) control *A. lanipes* larva, normal pigmentation along the abdomen; (**b**) *A. lanipes* larva exposed to 50 mg/L of TiO_2 NPs, loss of pigmentation because of exposure to TiO_2 NPs. The images were obtained using a stereo microscope (3.5 × -90 × LED Trinocular Zoom + 14 MP USB 3.0 Digital Camera). Both larvae were in the zoea I larval stage; the total length of the larvae is 1.3 mm.

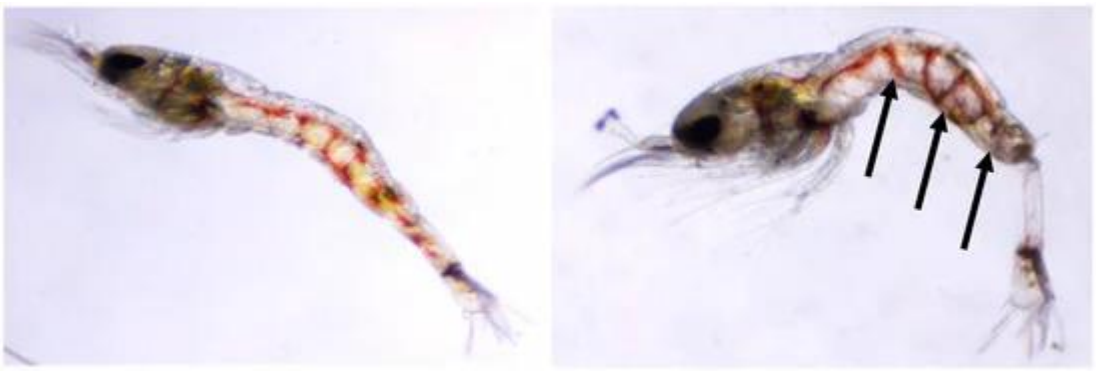


Figure 4.5 *A. lanipes* larvae exposed to TiO_2 NPs for 72 h showed edema development: (**a**) control *A. lanipes* larva; (**b**) *A. lanipes* larva exposed to 150 mg/L of TiO_2 NPs. The images were obtained using a stereo microscope (3.5×-90× LED Trinocular Zoom + 14 MP USB 3.0 Digital Camera). Both larvae were in the zoea I larval stage; the total length of the larvae is 1.3 mm.

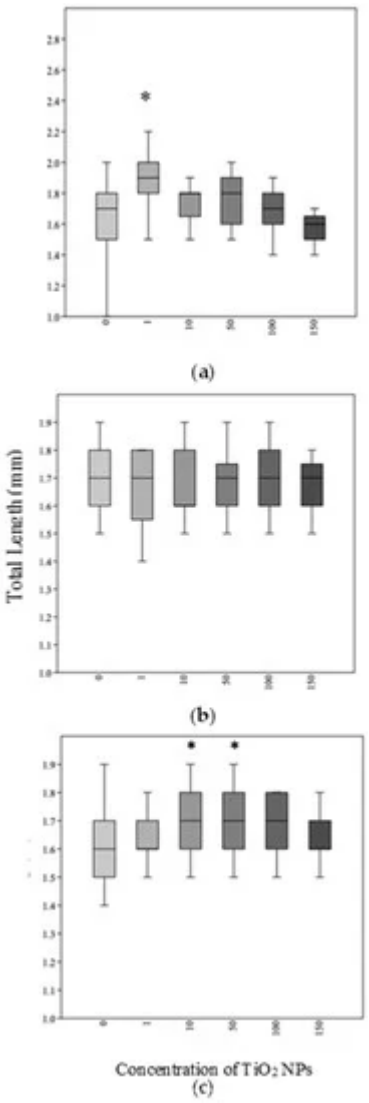


Figure 4.6. Total length (mm) of *A. lanipes* larvae exposed to different TiO_2 NPs concentrations for (a) 24 h (* $p < 0.01$), (b) 48 h (* $p < 0.01$), and (c) 72 h. (* $p < 0.05$).

CHAPTER 5: DETERMINE THE NEUROTOXICITY AND OXIDATIVE STRESS DEVELOPMENT IN ADULT *Atya lanipes* SHRIMP EXPOSED TO TITANIUM OXIDE NANOPARTICLES

Abstract

Titanium oxide is one type of nanoparticle composed of one titanium atom and two oxygen atoms that absorb One of its physical-chemical activities is photolysis, producing different reactive oxygen species (ROS). Due to their photocatalytic activities, titanium oxide nanoparticles (TiO_2 NPs) are of great interest because they are used in paints and coatings as self-cleaning, antimicrobial, and antifouling agents, food additive, and cosmetics as a UV-absorber. *Atya lanipes* affect detrital processing and illustrate the potential importance of diversity and nutrient availability to the rest of the food web. Also, *Atya lanipes* are essential in removing sediments from the streams, which have an important role in preventing eutrophication in this aquatic environment. For this reason, any contaminant that can both settle or accumulate/dissolve in the water could harm the species and alter their role in the ecosystem. This study aims to determine the toxic effect reflected in changes in behavior and development of oxidative stress that exposure to titanium oxide nanoparticles can develop in the adult life cycle of the *Atya lanipes* shrimp and to determine the dose-response index (EC_{50}), for behavioral variables after exposures to different TiO_2 NPs suspension concentrations. Adult shrimp were collected and acclimated for three days. The concentrations of TiO_2 NPs were: 0.0, 0.50, 1.0, 2.0 and 3.0 mg/L with two positive controls of 100 ug/L of titanium and 3 mg/L of TiO_2 NPs + 100 ug/L of titanium. After 24 h of exposure significant hypoactivity was documented. Also, for the EC_{50} a concentration of 0.146 mg/L was determined. Finally, after the chronic exposure to 10 mg/L of TiO_2 NPs and the dissection of the

gastrointestinal, gills and nervous tissues we found oxidative stress development in gastrointestinal and nervous tissues starting at 24 h of exposure. The toxic effects of this emerging aquatic pollutant from acute to chronic exposure are characterized by sublethal effects such as behavior changes and oxidative stress.

Keywords: *Acute exposure, catalase, chronic exposure, effective concentration, macroinvertebrate, nanotoxicity*

INTRODUCTION

Titanium oxide is one type of nanoparticle composed of one titanium atom and two oxygen atoms that absorb One of its physical-chemical activities is photolysis, producing different reactive oxygen species (ROS) (Wu et al., 2020; Tsang et al., 2019). In this way, it is a chemically inert and photocatalytic nanoparticle that reflects all the colors of the light spectrum (it reflects white light). Also, titanium dioxide is a white and fine powder (Skocaj et al., 2011); it can exist in different forms such: rutile, anatase, and brookite (Penn & Banfield, 1999; Zaban et al., 2000; Oskam et al., 2003). The anatase form is the most photocatalytic arrangement of this nanoparticle (Wang & Zhang, 2020; Kawahara et al., 2002).

Due to their photocatalytic activities, titanium oxide nanoparticles (TiO_2 NPs) are of great interest because they are used in paints and coatings as self-cleaning, antimicrobial, and antifouling agents, food additive, and cosmetics as a UV-absorber (NP's) (Berardinelli & Parisi, 2021; Verma, 2019). From 2006 to 2010, the commercial production of titanium oxide was 5000 metric tons per year; from 2011 to 2014, it was more than 10,000 metric tons, estimated to reach 2.5 million metric tons by 2025 (Dedman et al., 2021).

The rapid production and use of TiO_2 NPs result in a direct and indirect release into aquatic environments through bathing, industrial effluent, and engineering applications (Zahra et al., 2022; Kansara et al., 2022). In this way, one of the ecosystems affected by the presence of TiO_2 NPs is the freshwater ecosystem. The environmental concentrations of these NPs in freshwater ecosystems are variable in the scientific literature. However, the health of the river can be affected by TiO_2 NPs in ways that are not yet quantified. Nevertheless, some studies evidenced the presence of TiO_2 NPs in large concentrations in natural

surface waters and rivers (Gottschalk et al., 2013). However, most of the studies focus on determining TiO_2 NPs in surface waters, TiO_2 NPs in surface waters, and few studies have studied these in sediments. Despite the lack of standardized quantification of TiO_2 NPs in aquatic ecosystems, European studies have determined that the concentrations of TiO_2 NPs in the freshwater range from 0.015 - 24.5 micrograms/liter (Gottschalk et al., 2010; Batley & McLaughlin, 2010). On the other hand, studies have documented that soil concentrations may exceed 100 micrograms/liter (Gottschalk et al., 2013). However, concentrations of Titanium (including TiO_2 NPs), were found in the United States and Canada in a study involving 15 rivers. Concentrations of Titanium in water range from 0.5-15 micrograms/liter and, in some soils and sediments, about 10-100 g/kg with an average of less than 5 g/kg (World Health Organization, 1982).

In the Caribbean and Puerto Rico freshwater ecosystems, we have an important shrimp species named *Atya lanipes Holthuis 1963. Atya lanipes* is a scraper/filter feeder shrimp that live part of their life as a planktonic organism until they reach the youth stage. This amphidromous life cycle in the Caribbean freshwater shrimp represents an important relationship between the headwaters and estuaries (Benstead et al., 2000). Crowl, among others (2001), demonstrates that *Atya lanipes* affect detrital processing and illustrate the potential importance of diversity and nutrient availability to the rest of the food web. Also, *Atya lanipes* are essential in removing sediments from the streams, which have an important role in preventing eutrophication in this aquatic environment. For this reason, any contaminant that can both settle or accumulate/dissolve in the water could harm the species and alter their role in the ecosystem. Therefore, TiO_2 NPs, like other hydrophobic nanomaterials, tend to sedimentation as their fate in the aquatic

ecosystem. In this way, *Atya lanipes* will be susceptible to the presence of these engineered nanoparticles in two different aquatic ecosystems: the estuary and the river. This makes *Atya lanipes* an excellent nano-toxicological model.

Studies with TiO_2 NPs have found that these NPs interact with metals and are stored in sediments, acting as an adsorptive agent (Fang, 2018). Alternatively, it has been shown that TiO_2 NPs can exacerbate the bioavailability and neurotoxicity of organic pollutants and pesticides, serving as an adsorptive agent (Fang, 2018). There are several studies at a global level that are interested in using the photocatalysis (formation of oxidative agents) of TiO_2 NPs for remediation and treatment of wastewater, and many of these studies do not consider the possible toxicity to aquatic organisms (Li et al., 2010; Lazar et al., 2012).

Researchers have recognized the reaction between nanomaterials and biological systems in many ecotoxicology studies (Nel et al., 2006). However, most of these studies were carried out in bacteria, cell lines, and rodent animals (Zhu et al., 2011). Nevertheless, these studies reveal the development of oxidative stress as the principal biological effect. Oxidative stress is produced by free radicals, which contain one electoral unpaired and are highly reactive and capable of damaging molecules and transforming them into reactive molecules. This produces a redox reaction chain that damages cells and tissues in biological systems. Also, includes cell toxicity by oxidation of lipids, proteins, carbohydrates, and nucleotides (intracellular aggregates, mitochondrial dysfunction, excitotoxicity, and apoptosis). All biological systems have an antioxidant defense to avoid oxidative stress. An antioxidant is any substance present in low concentrations that delay or inhibit oxidation. The action of antioxidants is decreasing the concentration of oxidants, voiding the initiation of the chain reaction by “sweeping” (cover or stop a very high

chemical reactivity), the first free radicals to form, binding to metal ions to prevent the formation of reactive species; transforming peroxides into less reactive products; and topping the spread and increase of free radicals.

One example of a very important antioxidant enzyme is the catalase which acts as metabolizer, transforming the peroxides in H_2O and O_2. In this way, this enzyme minimizes the oxidative stress damage (Martínez & Arancibia, 2003).

Although the biological effect of oxidative stress by TiO_2 NPs is known as the dose-dependent toxicity index. Some studies focused on green algae (*Desmodesmus subspicatus*) have determined that EC_{50} = 44 mg/L (Hund-Rinke & Simon, 2006). Nothing is known from these dose-response toxicological analyses on the *Atya lanipes* shrimp for behavioral variables (another important sublethal effect). These indices are imperative to explore in this species due to the susceptibility of this species to contamination in bodies of water. Particularly for nanomaterials as we have previously discussed. It is necessary to determine toxicological indices for TiO_2 NPs of behavioral variables to analyze better environmental risk by this type of nanoparticles in freshwater ecosystems where this species inhabits (Zhu et al., 2011). Also, this shrimp needs a healthy nervous system to complete their complex life cycle (migrations), acquire food, avoid predation, and other ecological roles.

This study aims to determine the toxic effect reflected in changes in behavior and development of oxidative stress that exposure to titanium oxide nanoparticles can develop in the adult life cycle of the *Atya lanipes* shrimp and to determine the dose-response index (EC_{50}), for behavioral variables after exposures to different TiO_2 NPs suspension concentrations. We hypothesize that acute exposure to TiO_2 NPs will develop a toxic effect on the nervous system of the *Atya lanipes* shrimp that will produce hypoactivity behavior as has been determined in the larval stage of this

species, and in consequence, we will find observed effects at very low TiO_2 NPs concentration in mg/L due to the capacity of producing oxidative stress by Anatase form of this nanoparticle. A probit analysis for behavioral variables will show the toxicity of the TiO_2 NPs in very small concentrations. Also, the development of oxidative stress will be evident and remain constant from acute to chronic exposure, reflected in an increase in catalase enzyme activity.

MATERIALS AND METHODS

Characterization of Titanium Oxide nanoparticles suspensions (TiO_2 NPs)

Titanium Oxides nanopowder with a particle size of 25 nm (Sigma Aldrich Chemical Company St. Louis, MO, USA, Titanium IV Oxide, Anatase nanopowder) was used to conduct the experiments. The characterization method was S4700 II Cold Field Emission Gun Scanning Electron Microscope cFEG SEM). This way, the titanium oxide powder was spread on a weighing paper and gently picked up by the sticky carbon surface above the aluminum stubs. A S4700 II cFEG SEM (Hitachi High Technologies-America) with a silicon drift EDX detector (Oxford Instruments, X-Max^N, UK) was used to measure the surface morphology, elemental composition, and distribution of elements. All the SEM data reported were obtained at an acceleration voltage of 10kV, and the images were collected with a Secondary Electron detector. The elemental mapping and energy spectrums were acquired with Aztec tools (Oxford Instruments, UK). The elemental analysis through the energy dispersive spectrum indicates the presence of Ti,O, C, and S elements (61, 36.1, 2.6 & 0.3 wt% respectively) (Figure 4.1 & 4.2). The observed size of the Titanium oxide nanoparticle varies from 1 micron to 4 micron long.

Atya lanipes ***specimens collection***

Adults of *A. lanipes* specimens were collected and identified (Pérez et al., 2013), using baited minnow traps at Sonadora stream at El Verde Field Station, Rio Grande, Puerto Rico. Baited traps (bait was dry cat food) were set in different pools along the stream and removed 24 hours later. The collected shrimp were transported to the laboratory in a cooler under constant aeration. The shrimp were transferred individually to glass tanks (15 cm x 15 cm x 15cm) with 1 Liter of dechlorinated water and constant aeration. Animals were acclimated to 72-120 h (three to five days), in the laboratory environment prior to the bioassay.

The microcosm

Fifty microcosms (25-control and 25-experimental) were designed. They consisted in a square aquarium that included one L of dechlorinated water, 150 g of synthetic sediment previously sterilized with activated carbon and heated in an oven at a temperature of 50-60 °C, an air stone with and air pump, and LED lamps with a photoperiod of 10-hour light and 14-hours of darkness (controlled by an automatic timer). The water temperature was kept between 19-21 °C (Figure 5.1). These variables represented the environmental conditions in which *Atya lanipes* live in the wild.

Acute toxicity tests

Before the bioassay, we mixed the titanium oxide nano-powder with one L of dechlorinated and oxygenated (for 24-hr) water using a magnetic stirrer at maximum speed for 30 minutes. Subsequently, TiO_2 NPs were weighed to evaluate concentrations of 0.0, 0.50, 1.0, 2.0 and 3.0 mg/L. The nanoparticles were added into the fish tanks and let fall by gravity; the aquarium water was mixed with a glass stirrer to obtain homogeneity in the microcosm. For each treatment 25 replicates

were developed and 25 in the control. Positive controls for titanium (100 µg/ml) and the synergy between the TiO_2 NPs and titanium were prepared (3.0 mg/L; 100 µg/ml). The microcosms were set in an exposure bench covered with a piece of blue fabric to prevent the entry of external light and to control any possible contamination. Physicochemical parameters (temperature, salinity, dissolved oxygen, dissolved solids, and pH) of the water of each microcosm were taken before and after the exposure period.

After preparing the microcosms with their respective treatments, adults of *Atya lanipes* were randomly assigned to each treatment; previous measurements of the postorbital and cephalothorax of the shrimp were collected. Then, shrimp were individually introduced into the microcosms and left exposed to the treatments for an acute exposure of 24 hr.

Behavioral Analysis

After acute exposure to TiO_2 NPs for 24 h, a movement analysis was carried out. Each specimen of *Atya lanipes* in the experiment was removed from the tank and acclimatized for 5 minutes in a red plastic box with 2 liters of dechlorinated water previously oxygenated for 24 h (it was changed by new water for each shrimp analyzed to avoid contamination and minimize errors in the analysis). The shrimp were set in the red box with new water because the artificial sediment alters the video creating artifacts that result in errors in the video; in addition, the movement of the water TiO_2 NPs to the red box will result in the displacement of the nanoparticles that were set in the sediment. This acclimatization was done in the record room under total darkness and with no sound. Red lights were used to set up the tank and the camera; shrimp does not detect red light. After the acclimation period, the movement of the shrimp in the tank was recorded for 5 minutes using a camera (Go

Pro Hero 6®). The movement analysis was performed using the Loligo System (Figure 5.2).

Oxidative Stress Development Analysis

To analyze oxidative stress after chronic exposure (0-11 days) in *Atya lanipes*, we dissected five specimens previously exposed to each TiO_2 NPs concentration and the control. These shrimps were dissected every two days. The shrimp exposed to the nanoparticles were not fed during the 11 days. The *Atya lanipes* specimens were preserved by gradually putting the shrimp at a cold temperature to decrease their biological activity without causing strong stress that would alter our results. This was done in approximately five minutes; then we removed the gastrointestinal tract (gut), the ventral nervous system (nerve cord), and the gills (Figure 5.3).

We used the Catalase (CAT) Activity Assay kit (Catalog No: MBS2540413; Colorimetric method; sensitivity 0.27 U/ml), to determine the catalase enzymatic activity in each tissue for each analysis period. Then, we calculate the enzymatic activity in U/mgptot with the following formula: CAT activity = $\Delta A \times 32.5 / 1 \times V \times f/Cpr$ where: 32.5- reciprocal of slope, 1- Reaction time, ΔA- $OD_{Control}$ - OD_{Sample} , V- Volume of sample, mL, f- Dilution factor of sample before test, and C_{pr}-Concentration of protein in sample, gprot/L.

Statistical Analyses

Descriptive statistics were used to summarize the values of the physico-chemical parameters. To compare the physico-chemical parameter among treatments and control one-way ANOVA was done. The shrimp movement was compared among treatments by a one-way ANOVA and Tukey's tests were

performed. A Probit analysis was used to compare the toxicity levels in the movements. Lastly, for the analysis of the development of oxidative stress we used the formula suggested by the catalase kit. All the descriptive and statistical analyses were performed in Minitab 17 Statistical Software (Minitab 2021).

RESULTS

Specimens of Atya lanipes and physical-chemical parameters

The sample of 25 organisms for control and exposed groups was chosen to keep a similar shrimp size to standardize the bioassay and obtain reliable data. Cephalothorax length (CL), sizes between the control and TiO_2 NPs-exposed groups ranged from 14.6 to 16.9 mm on average, and the post-orbital length (POL), sizes were between 11.3 to 13.9 mm on average. Also, gravid shrimp were excluded from the bioassay. Shrimp were not fed during the bioassay.

To maintain the internal environmental conditions of the microcosms stable during the bioassay, we measured the physicochemical parameters of the water before and after the 24 h of exposure time to TiO_2 NPs. The temperature of the microcosm was the laboratory temperature which varied between 19-21°C for the entire exposure. The pH values of the pre-exposure time range from 8.0 to 8.6 in all tanks in the control group with an average of 8.2 $\pm$ 0.04. After the exposure, the control group presented pH values ranging from 7.5 to 8.4 with an average of 8.2 $\pm$ 0.04 for the pre and post-exposure time. The conductivity for the control group was on average 328.2 ms $\pm$ 19.0 with a range value of 99.0 to 446.0 ms in the pre-exposure time and an average of 341.5 ms $\pm$ 15.0 with a range value of 240.0 to 537.0 ms in the post-exposure time. The salinity remained relatively constant. In the control group, the average salinity was 0.2 ppm $\pm$ 0.01 with a minimum value of 0.1

to 0.2 ppm in the pre-exposure time. However, at the post-exposure, the average of salinity was 0.2 ppm ± 0.01 with a range of 0.1 to 0.2 ppm. For the dissolved oxygen, the control group presents an average of 8.7 mg/L ± 0.2 (with a range of 7.7-9.9 mg/L) in the pre-exposure and an average of 8.8 mg/L ± 0.1 (with a range of 8.1-10.4 mg/L) in the post-exposure time.

The positive control of titanium (100 micrograms/ml), we observed a pre-pH average of 8.2 ± 0.04 (range of 7.9-8.5) previous the exposure and an average pH of 8.1 ± 0.04 (range of 7.7-9) after the exposure. The TiO_2 NPs + titanium treatment showed a pH average of 8.2 ± 0.04 (8.0-8.5) before the exposure and an average of 8.1 + 0.04 (7.7-8.4) after exposure. Regarding the conductivity, the positive controls showed an average before and after the exposure of 332.4 ms + 15.8 and 367.2 ms + 21.0 with ranges of 203.0 to 447.0 ms and 231.0 to 560.0 ms. The titanium treatment showed an average of 340.4 ms ± 21.0 and 372.0 ms ± 22.4 with a range value of 213.0 ms to 448.0 ms and 233.0 ms to 520.0 ms, respectively. The average salinity, before exposure, was 0.2 ppm ± 0.01; 0.01 for both positive controls with values ranges of 0.1 to 0.2 ppm for titanium and the synergistic treatment. After the exposure time, the salinity was on average for titanium treatment and the synergistic treatment was the same as before the exposure (0.2 ppm ± 0.01). The dissolved oxygen in the positive controls was documented as a before-exposure average of 8.6 mg/L ± 0.1 (range of 7.6-9.7 mg/L) for titanium treatment and an average of 8.6 mg/L ± 0.1 (range of 7.6-9.5 mg/L). After the 24 h of exposure, the average oxygen content for the titanium treatment was 8.9 ± 0.1 mg/L (range of 8.3-9.6 mg/L) and an average of 8.9 ± 0.1 mg/L (range of 8.3-9.6 mg/L).

The different TiO_2 NPs exposures of 0.5, 1.0, 2.0 and 3.0 mg/L showed a pH average in the pre-exposure time of 7.8 ± 0.003, 8.3 ± 0.00, 7.8 ± 0.01, 8.2 ± 0.04

with ranges values of 7.7-7.7, 8.3-8.3, 7.8-7.9 and 7.9-8.5 respectively. Also, the pH averages of the same suspension concentrations for the post-exposure time were 7.7 ± 0.02, 7.7 ± 0.02, 7.7 ± 0.02, 8.1 ± 0.04 with ranges of 7.6-7.8, 7.7-7.9, 7.4-7.8 and 7.7-8.5 respectively. The average conductivity in the pre-exposure time for the TiO_2 NPs concentrations were 468.7 ± 1.0, 353.2 ± 1.0, 469.6 ± 3.6, 332.2 ± 17.3 ms (0.5, 1.0, 2.0 and 3.0 mg/L respectively). The conductivity ranges for the same concentrations were 460-470, 450-470, 450-490 ms and 350-674 ms. After the exposure, the averages of conductivity were 436.0 ± 9.1, 362.8 ± 2.5, 475.2 ± 4.2 and 337.4 ± 16.2 ms, with ranges of 370-470, 480-510, 450-500, 182-445 ms). The average salinity was 0.2 ± 0.001; 0.004; 0.0001; 0.002; 0.01 ppm, for all TiO_2 NPs concentrations in the pre-exposure time with ranges of 0.2-0.2, 0.2-0.2, 0.2-0.2 and 0.1-0.2 ppm (beginning with the smaller to the bigger concentration). The average salinity in the post-exposure time was 0.2 ± 0.004; 0.0001; 0.002; 0.01 ppm, remaining the same with ranges of 0.2-0.2, 0.2-0.3, 0.2-0.3 and 0.1-0.2 respectively. Finally, for the dissolve oxygen the averages in the pre-exposure were 9.2 ± 0.02, 9.3 ± 0.1, 9.5 ± 0.1 and 8.6 ± 0.1 mg/L with ranges of 9.1-9.3, 9.1-10.4, 9.1-9.9 and 7.7-9.6 mg/L for the concentration of 0.5, 1.0, 2.0 and 3.0 mg/L respectively. After the exposure the DO averages were 8.0 ± 0.1, 8.4 ± 0.1, 7.9 ± 0.1 and 8.9 ± 0.1 mg/L with ranges of 7.7-8.6, 8.2-8.9, 7.2-8.8 and 8.3-9.5 mg/L respectively.

The -way ANOVA for the physicochemical properties showed no significant differences between any variable pre and post-exposure time. There are no changes in pH, conductivity, and salinity measurements before and after exposure. However, dissolved oxygen measurements showed a decrease in groups exposed to TiO_2 NPs; this result was not observed in the controls. An increase in dissolved oxygen concentrations were observed after 24 h of exposure. This is considerably expected

due to the presence of the constant oxygen pump in each microcosm during the entire exposure. The temperature of the microcosm was the laboratory temperature which varies between 19-21°C (Table 5.1).

Movement assessment

The analysis of movement after the exposure of TiO_2 NPs for 24 h showed significant changes leading to hypoactivity behavior. The heat maps for the adult shrimp in the control group (0.0 mg/L of TiO_2 NPs), showed a preference for the corner of the box than any other location. The movement was limited to corners of the box. While the exposed group showed erratic preferences and less exploration movement, especially in the TiO_2 NPs exposure (Figure 5.4).

This hypoactivity characteristic in the movement assessment was statistically evaluated using the one-way ANOVA with a Tukey test. During the 24 h of exposure, total distance moved and active time (min) we observed significant differences between exposed and control group ($p < 0.05$) (Figure 5.5). The average of the total distance (N = 25) observed by the adult shrimp in the negative control group was 15372.6 mm +/- 2581.8 mm with a minimum of 592.1 mm and a maximum of 46759.8 mm during the 24 h of exposure. Also, for the positive controls of titanium, a total distance average of 21039.8 mm $\pm$ 10070.0 mm with a range of 403.7 mm to 254938.2 mm was observed. In the second positive control of the synergy (titanium + TiO_2 NPs), the average total distance was 16525.2 mm $\pm$ 4188.4 mm with a range of 272.5 mm to 95032.1 mm. For the different treatments of TiO_2 NPs, (0.5, 1.0, 2.0 & 3.0 mg/L), we observed a total distance average of 6072.7 $\pm$ 1150.8, 9644.3 $\pm$1585.3, 5429.7$\pm$626.0 and 6571.0 $\pm$ 1388.9 mm respectively. Moreover, the total distance moved for the exposed shrimp ranged from 7.1 mm to 35659.7 mm. The

one-way ANOVA for the comparison of the total distance moved variable for the 24 h of exposure of the adult shrimp *Atya lanipes* in the control group and the exposed group showed a significant difference ($F_{(6,167)} = 2.6$; $p < 0.05$) among groups. Tukey test did not show significant differences in this variable for any TiO_2 NPs suspension concentration and controls.

The active time (min) of the adult shrimp (N = 25) was 3.18 min $\pm$ 0.17 min with a minimum of 0.8 min and a maximum of 4.32 min for the negative control during the 24 h of exposure. Consequently, the active time for the positive controls was 2.74 min $\pm$ 0.22 min and 2.98 min $\pm$ 0.19 min with minimums of 0.6 and 1.51 and maximums of 4.77 and 4.84 min respectively. The exposed groups' average active time for the TiO_2 NPs suspension concentration of 0.5, 1.0, 2.0 and 3.0 mg/L were the following: 2.37 $\pm$ 0.18, 2.96 $\pm$ 0.18, 2.62 $\pm$ 0.17 and 2.54 $\pm$ 0.22 min. We observed minimums of 0.47, 0.85, 0.06 and 0.35 min with maximums of 3.90, 4.37, 4.02 and 4.26 min for the TiO_2 NPs suspension concentration of 0.5, 1.0, 2.0 and 3.0 mg/L respectively. The one-way ANOVA for the comparison of all TiO_2 NPs suspension concentrations and positive and negative controls for the active time variable after 24 h of exposure showed a significant difference ($F_{(6,167)} = 2.24$; $p < 0.05$) between groups. Tukey test analysis showed values of $p < 0.05$ among the treatment of 0.5 mg/L.

For the movement variable of active time a Probit Analysis was conducted. The suspension concentrations of TiO_2 NPs in adult *A. lanipes* were: 0.50, 1.0, 2.0 & 3.0 mg/L in an acute exposure of 24 h. The lognormal probability plot at 95% CI (-6.320 - 2 .424), showed a coefficient of 0.20 for the concentrations,

standard error = 0.19, Z= 1.03 and a *p* value = 0.30. Also, the regression data showed a chi-square = 0.025, Df = 2 and a pearson = 0.99. EC values for 50, 60, 70 and 90 % of events are presented in table 5.2. This data showed a median dose (EC_{50}), in the active time variable at a suspension concentration of 0.142 mg/L of TiO_2 NPs for *Atya lanipes* shrimp species in an exposure of 24 h (Figure 5.6). The EC_{60} represent a TiO_2 NPs concentration of 0.520 mg/L, EC_{70} represents 2.073 mg/L; TiO_2 NPs, EC_{80} presents 10.467 mg/L and the EC_{90} represents a concentration of TiO_2 NPs of 98.868 mg/L.

Oxidative stress assessment

Analysis of the development of oxidative stress defined as catalase enzyme activity in shrimp exposed to TiO_2NPs was compared against the unexposed shrimp. The dissections of the branchial, nervous, and gastrointestinal tissues were obtained from fresh tissues of 5 shrimp for each analysis time and for each NPs suspension concentration and control group, from an acute exposure (24, 72 and 120 h) to a chronic exposure (≥ 168 h). The average catalase activity for each tissue for the control group in each exposure analysis time to compare with the catalase activity of the exposed group during the exposure (from 24 h to 264 h). The average environmental conditions of the microcosms of the shrimp exposed for obtaining the fresh tissues are summarized in Table 2. During the exposure time of 0 - 264 h to TiO_2 NPs, the temperature varies between 18.5-19.9. The pH ranged from 5.6 to 7.7. A pH below 6.0 was seen in chronic exposures of 216 to 264 h of exposure. The conductivity varied from 0.3-0.5 µs. Dissolved oxygen in the exposure ranged from 7.8-9.2 mg/L and the salinity from 0.1-0.2 ppm.

The specimen sizes were determined by measuring their POS and CEF lengths (Table 5.4). The POS sizes of the samples ranged from 10-17 mm and the CEF from 11-22 mm. The averages of the *Atya lanipes* specimens for the POS size were 12.7 ± 1.3, 13.3 ± 1.3, 12.1 ± 1.0, 11.1 ± 0.2, 12.2 ±1.0, 12.0 ± 1.0 and 10.8 +/- 0.2 mm for the control group, 24 h, 72 h, 120 h, 168 h, 216 h and 264 h of exposure respectively. For the same exposure times and for the control group, the CEF averages were 14.3 ± 2.0, 16.6 ± 1.4, 14.6 ± 1.4, 13.6 ± 0.2, 14.2 ± 1.1, 14.9 ±1.2 and 14.0 ±0.4 mm respectively.

Catalase activity in gastrointestinal tissues between 24 to 264 h of exposure to 10 mg/L of TiO_2 NPs was accessed. The average catalytic activity of catalase in the control group was 10.97 +/- 0.2 U/mg prot. The catalase activity at each exposure time analysis (24, 72, 120, 168, 216 & 264 h) were: 0.79, 15.93, 27.94, 55.90, 19.5 & 17.77 U/mg prot respectively. After 24 h of exposure was approximately 0, which indicates an extreme shock and oxidative stress development in a short period of time. However, after 72 h of exposure to the 264 h of exposure catalase shows more enzymatic activity than the control group.

The analysis of catalase activity in the gills tissues shows that after 24 h of exposure, the enzyme activity was significantly higher than the control group (54.38 U/mgprot) for the exposed group and 18.09 +/- 0.2 U/mg prot for the control group). This result differed from the values we observed in the analysis of catalase activity in the gastrointestinal tissues. In this case, the enzymatic activity was higher after 24 h but after 72 h (22.48 U/mg prot), 120 h (28.87 U/mg prot), 216 h (22.60 U/mg prot) and 264 h (20.39 U/mg prot) the enzyme activity was like the control group. Except for the exposure time of 168 h when the enzyme activity was higher than in the 24 h of exposure (104.73 U/mg prot).

In the nervous tissues, the oxidative stress development was significant. In the control group the average enzyme activity was 258.04 +/- 0.3 U/mg prot. However, the catalase activity for each exposure time was the following: 69.44, 62.23, 31.70, 135.0, 24.01 & 147.02 U/mg prot for the exposures time of 24, 72, 120, 168, 216 and 264 h respectively (Figure 5.7).

DISCUSSION

This study assessed the neurotoxicity level of TiO_2 NPs in an acute exposure of 24 h in the species of *Atya lanipes* shrimp and documented the development of oxidative stress in acute to chronic exposure. To date, more studies must be carried out related to the biocompatibility and toxicity of engineering nanomaterials (Jafari et al., 2020; Demir, 2021). This is because the use continues to increase in many sectors globally including medicine, the food industry, and technology (Lehutso & Thwala, 2021). For its part, the biological and ecosystem interaction that nanomaterials can have has been important to define and prevent future impacts on ecosystems such as freshwater ones. Also, this data could help in the production, management, and future environmental regulation related to engineered nanoparticles. Considering its possible interaction with living organisms and its toxicity before its commercial use.

Species conservation continues to be a core issue in assessing nanomaterials in living organisms (Boraschi et al., 2020; Pandey & Jain, 2020). *Atya lanipes* is a very common macroinvertebrate found in the rivers of Puerto Rico and the Caribbean (Greathouse & Pringle, 2006). Therefore, conserving this species in this region is vital for the health of the ecosystem in general. Freshwater shrimp have important effects in controlling algal biomass and species composition, in the quality

and quantity of benthic organic matter, leaf decomposition rates, the quantity of epilithic fine sediments, and the abundance and biomass of benthic invertebrates (Crowl et al., 2001; March et al., 2002). In addition, the amphidromous life cycle of this species makes it a complex and a good study organism to understand the possible impacts that TiO_2 NPs can develop in these populations (Cruz & Pérez, 2023).

Most of the nanotoxicological studies have been carried out in organisms not environmentally exposed to nanomaterials in the aquatic environment where they live (Garric & Thybaud, 2011). However, in the communities and populations of freshwater shrimp, little has been done about this matter. Although it has been shown in studies related to other aquatic contaminants such as pesticides that these macroinvertebrates are a good toxicological model (Torres & Pérez, 2023). However, for the species of *Atya lanipes* and the toxicity of TiO_2 NPs, the effect on lethality and sublethal effects in larval stages (Cruz & Pérez, 2023), has been evaluated and documented. Regarding the movement of the larvae after acute exposure, a significant hypoactivity was observed in the exposed groups. In this study, in the adult stage of the *Atya lanipes* shrimp, we observed that after acute exposure of 24 h a hypoactive movement was obtained in the shrimp exposed to low concentrations of TiO_2 NPs. Especially, the heat maps that documented the “normal” movement of exploration of the *Atya lanipes* shrimp to be in the corners of the box rather than in the center. This behavior is expected in this species of shrimp since it is a normal mechanism to evade predation. Remaining in the center or still, can impact possible predation. Thus, not exposed to any treatment, healthy adult shrimp show constant movement in this direction for more than half of the recording time. However, shrimp exposed to titanium treatment or the synergy between titanium and titanium oxide

nanoparticles begin to show a decrease in this trajectory of exploration. Especially the shrimp exposed to 3 mg/L TiO_2 NPs that do not show movement within the recording box. Compared with other studies that evaluated animal behavior after exposure to this nanomaterial, neotropical tadpoles, and zebrafish also presented this characteristic hypoactivity (do Amaral et al., 2022; Chen et al., 2011).

Scientific investigations have shown the relevance of the sublethal effects that toxins can develop. In the past, we referred to toxicity primarily as the ability of contaminants to cause the death of the organisms under study. Today we know that sublethal effects can produce a great disparity in the organism's functioning that can be extrapolated to effects in the ecosystem where it develops. Therefore, it is necessary to know the probability that some percentage of the sample under study presents some characteristic or defined effect, in this case, a sublethal effect. When carrying out the probit analysis for the variable of active time of the shrimp, we obtained that the average index was of 0.142 mg/L. This innovative data demonstrates the susceptibility that this species can present before an acute exposure of only 24 h to TiO_2 NPs. The need to evaluate toxicological indices for sublethal effects for this species at different exposure times is evident.

In gene ral , acute toxicity studies (≤ 96h) in bacteria (*V. fischeri*), green algae (P*seudokirchneriella sub-capitata* and *Chydorus sphaericus*), some crustaceans (*D. magna* and *T. platyurus*) and fish embryos (*D. rerio*) have shown little or no toxicity when exposed to TiO_2 NPs (Heinlaan et al., 2008; Velzeboer et al., 2008; Griffittet al., 2008). Recent research shows that many emerging contaminants can present biochemical responses to exposure such as oxidative stress (as an example of sublethal effects), in acute exposures and perhaps we will not see a lethality for that same exposure time (Rodríguez-Ariza et al., 1993). However, one study evaluated

the nanotoxicity of TiO_2 in the freshwater shrimp of *Atya lanipes* in their zoea larval stages and the results showed that an exposure to these nanoparticles produce mortality after 48 h of exposure, edema and less pigmentation development and hypoactivity in the larvae movement (Cruz & Pérez, 2023).

The characteristic biological effect of TiO_2 NPs is the development of oxidative stress. Oxidative stress occurs when reactive oxygen species (ROS) are produced uncontrollably (Martínez & Arancibia, 2003). ROS are interrelated with many cellular mechanisms. Their properties of being highly reactive species allow them to react with many biomolecules, mislead and/or damage them including cellular apoptosis (Onose et al., 2022; Barnham et al., 2004). Fortunately, the cell has molecules and enzymes that have an antioxidant role. One of the most important is the enzyme catalase. The activity of this enzyme below or above the expected or normal values may indicate an increase in ROS and therefore the development of oxidative stress (Nandi et al., 2019). This excess of ROS in response to a xenobiotic contaminant can overwhelm the antioxidant mechanisms present in the organism (Shim et al., 2003). This can cause oxidative damage and loss of compensatory mechanisms and in this way, we can observe a suppression of antioxidant enzymatic activities (Zhang et al., 2004; Shim et al., 2003). In this study, a catalase enzymatic activity was observed in the groups exposed to TiO_2 NPs that was significantly different from the control group. It was shown that a 24 h acute exposure to TiO_2 NPs in the shrimp species *Atya lanipes* results in the development of oxidative stress in both gill, gastrointestinal, and nervous tissue.

This study has verified the neurotoxicity after an acute exposure of 24 h and the development of oxidative stress caused by TiO_2 NPs in the freshwater shrimp species *Atya lanipes*. The toxic effects of this emerging aquatic pollutant from acute

to chronic exposure are characterized by sublethal effects such as behavior changes and oxidative stress. Also, we suggest that freshwater shrimp are an excellent nanotoxicological model because of their life cycle and susceptibility to the presence of nanomaterials in the freshwater ecosystem and their ecological role in the biofiltration of natural organic particles in the freshwater ecosystem (Covich et al., 2004; Crowl et al., 2001; Covich et al., 1999; Covich, 1988). Future research should evaluate other sublethal effects such as bioaccumulation of the TiO_2 NPs in *Atya lanipes* shrimp to understand their biological routes.

Some limitations of this study are in the aspect of the assessment of the movement of the shrimp when exposed to TiO_2 NPs. It was not possible to record the shrimp in the same exposure tank due to the presence of artificial sediment that was part of the microcosm and because the system that was used to analyze the behavior of the shrimp did not recognize the sediment as different from the shrimp due to the dark color. from both. It was not possible to determine "locomotion", on the contrary, the "movement" of the shrimps was analyzed. Although it was recorded in a red box with red lights, which are particularly colors that shrimps do not detect and that promote their normal active behavior at night, the fact of removing them from exposure to fresh, clean, and oxygenated water limited us to determining locomotion. However, it was controlled that a short acclimatization period was allowed to avoid losing the effect of the TiO_2 NPs in the exposure medium. All other variables were successfully controlled to obtain a movement assessment after exposure to TiO_2 NPs.

This study contributes to the understanding of the nanotoxicity of TiO_2 NPs and provides a starting point to determine the importance of regulating this type of nanomaterial and controlling concentrations of this contaminant in the freshwater

ecosystem. These results are very relevant in the scientific community because they present data that has been little studied in the area of nano-ecotoxicology and promote research towards understanding the biocompatibility of these nanomaterials. At the same time, it shows us the need to prevent biological, ecological, and environmental impacts in the short, medium, and long term. With the purpose to conserve aquatic environments and biodiversity both in PR and in the world.

Literature cited:

Crowl, T.A., McDowell, W.H., Covich, A.P., and Johnson, S.L. 2001. Freshwater shrimp effects on detrital processing and nutrients in a tropical headwater stream. Ecology, 82: 775–783.

Hund-Rinke, K., Simon, M., 2006. Ecotoxic effect of photocatalytic active nanoparticles (TiO_2) on algae and daphnids. Environ. Sci. Pollut. Res. 13, 225–232.

Skocaj, M., Filipic, M., Petkovic, J., & Novak, S. (2011). Titanium dioxide in our everyday life; is it safe?. Radiology and oncology, 45(4), 227-247.

Barnham, K.J.; Masters, C.L.; Bush, A.I. Neurodegenerative diseases and oxidative stress. Nat. Rev. Drug Discov. 2004, 3, 205–214.

Batley, G., & McLaughlin, M. J. (2010). Fate of manufactured nanomaterials in the Australian environment. Clayton, Australia: CSIRO Land and Water.

Berardinelli, A.; Parisi, F. TiO_2 in the food industry and cosmetics. In Titanium Dioxide (TiO_2) and Its Applications; Elsevier: Amsterdam, The Netherlands, 2021; pp. 353–371.

Boraschi, D., Alijagic, A., Auguste, M., Barbero, F., Ferrari, E., Hernadi, S., ... & Pinsino, A. (2020). Addressing nanomaterial immunosafety by evaluating innate immunity across living species. Small, 16(21), 2000598.

Chen, T.H.; Lin, C.Y.; Tseng, M.C. Behavioral effects of titanium dioxide nanoparticles on larval zebrafish (*Danio rerio*). Mar. Pollut. 2011, 63, 303–308.

Covich, A.P. Atyid shrimp in the headwaters of the Luquillo Mountains, Puerto Rico: Filter feeding in natural and artificial streams. Verh. Int. Ver. Für Angew. Limnol. 1988, 23, 2108–2113.

Covich, A.P.; Ewel, K.C.; Hall, R.O.; Giller, P.E.; Goedkoop, W.; Merritt, D.M. Ecosystem services provided by freshwater benthos. In Sustaining Biodiversity and Ecosystem Services in Soils and Sediments; Chapter 3; Wall, D.H., Ed.; Island Press: Washington, DC, USA, 2004; pp. 45–72.

Covich, A.P.; Palmer, M.A.; Crowl, T.A. The role of benthic invertebrate species in freshwater ecosystems—Zoobenthic species influence energy flows and nutrient cycling. BioScience 1999, 49, 119–127.

Crowl, T.A.; McDowell, W.H.; Covich, A.P.; Johnson, S. Species-specific responses in leaf litter processing in a tropical headwater stream (Puerto Rico). Ecology 2001, 82, 775–783.

Cruz-Rosa, S., & Pérez-Reyes, O. (2023). Titanium Oxide Nanoparticles as Emerging Aquatic Pollutants: An Evaluation of the Nanotoxicity in the Freshwater Shrimp Larvae *Atya lanipes*. Ecologies, 4(1), 141-151. https://doi.org/10.3390/ecologies4010011

Dedman, C.J.; King, A.M.; Christie-Oleza, J.A.; Davies, G.L. Environmentally relevant concentrations of titanium dioxide nanoparticles pose negligible risk to marine microbes. Environ. Sci. Nano 2021, 8, 1236–1255.

Demir, E. (2021). A review on nanotoxicity and nanogenotoxicity of different shapes of nanomaterials. Journal of Applied Toxicology, 41(1), 118-147.

do Amaral, D.F.; Guerra, V.; Almeida, K.L.; Signorelli, L.; Rocha, T.L.; de Melo e Silva, D. Titanium dioxide nanoparticles as a risk factor for the health of Neotropical tadpoles: A case study of Dendropsophus minutus (Anura: *Hylidae*). Environ. Sci. Pollut. Res. 2022, 29, 50515–50529.

Fang, Q.; Shi, X.; Zhang, L.; Wang, Q.; Wang, X.; Guo, Y.; Zhou, B. Effect of titanium dioxide nanoparticles on the bioavailability, metabolism, and toxicity of pentachlorophenol in zebrafish larvae. J. Hazard. Mater. 2015, 283, 897–904.

Garric, J.; Thybaud, E. Toxicological Models Part B: Environmental Models. In Nanoethics and Nanotoxicology; Springer: Berlin/Heidelberg, Germany, 2011; pp. 379–396.

Gottschalk, F.; Sonderer, T.; Scholz, R.W.; Nowack, B. Possibilities and limitations of modeling environmental exposure to engineered nanomaterials by probabilistic material flow analysis. Environ. Toxicol. Chem. 2010, 29, 1036–1048

Gottschalk, F.; Sun, T.; Nowack, B. Environmental concentrations of engineered nanomaterials: Review of modeling and analytical studies. Environ. Pollut. 2013, 181, 287–300.

Greathouse, E. A., & Pringle, C. M. (2006). Does the river continuum concept apply on a tropical island? Longitudinal variation in a Puerto Rican stream. Canadian Journal of Fisheries and Aquatic Sciences, 63(1), 134-152.

Griffitt, R.J., Luo, J., Gao, J., Bonzongo, J.C., Barber, D.S., 2008. Effects of particle composition and species on toxicity of metallic nanomaterials in aquatic organisms. Environ. Toxicol. Chem. 27, 1972–1978.

Heinlaan, M., Ivask, A., Blinova, I., Dubourguier, H.-C., Kahru, A., 2008. Toxicity of nanosized and bulk ZnO, CuO and TiO_2 to bacteria *Vibrio fischeri* and crustaceans *Daphnia magna* and *Thamnocephalus platyurus*. Chemosphere 71, 1308–1316.

Jafari, S., Mahyad, B., Hashemzadeh, H., Janfaza, S., Gholikhani, T., & Tayebi, L. (2020). Biomedical applications of TiO_2 nanostructures: recent advances. International journal of nanomedicine, 3447-3470.

Kansara, K., Bolan, S., Radhakrishnan, D., Palanisami, T., Ala'a, H., Bolan, N., ... & Karakoti, A. (2022). A critical review on the role of abiotic factors on the transformation, environmental identity and toxicity of engineered nanomaterials in aquatic environment. Environmental Pollution, 296, 118726.

Kawahara, T., Konishi, Y., Tada, H., Tohge, N., Nishii, J., & Ito, S. (2002). A patterned TiO_2 (anatase)/TiO_2 (rutile) bilayer-type photocatalyst: effect of the anatase/rutile junction on the photocatalytic activity. Angewandte Chemie, 114(15), 2935-2937.

Lazar, M.A.; Varghese, S.; Nair, S.S. Photocatalytic water treatment by titanium dioxide: Recent updates. Catalysts 2012, 2, 572–601.

Lehutso, R. F., & Thwala, M. (2021). Assessment of nanopollution from commercial products in water environments. Nanomaterials, 11(10), 2537.

Li, Z.; Deng, S.; Yu, G.; Huang, J.; Lim, V.C. As (V) and as (III) removal from water by a Ce–Ti oxide adsorbent: Behavior and mechanism. Chem. Eng. 2010, 161, 106–113.

March, J.G., Pringle, C.M., Townsend, M.J., and Wilson, A.I. 2002. Effects of freshwater shrimp assemblages on benthic communities along an altitudinal gradient of a tropical island stream. Freshw. Biol. 47: 377–390.

Martínez, C. D., Vargas, C. R., & Arancibia, S. R. (2003). Estrés oxidativo y neurodegeneración. Rev Fac Med, 46(6), 229-235.

Nandi, A., Yan, L. J., Jana, C. K., & Das, N. (2019). Role of catalase in oxidative stress-and age-associated degenerative diseases. Oxidative medicine and cellular longevity, 2019.

Nel, A.; Xia, T.; Madler, L.; Li, N. Toxic potential of materials at the nanolevel. Science 2006, 311, 622–627.

Onose, G.; Anghelescu, A.; Blendea, D.; Ciobanu, V.; Daia, C.; Firan, F.C.; Oprea, M.; Spinu, A.; Popescu, C.; Ionescu, A.; et al. Cellular and Molecular Targets for Non-Invasive, Non-Pharmacological Therapeutic/Rehabilitative Interventions in Acute Ischemic Stroke. Int. J. Mol. Sci. 2022, 23, 907.

Oskam, G., Nellore, A., Penn, R. L., & Searson, P. C. (2003). The growth kinetics of TiO_2 nanoparticles from titanium (IV) alkoxide at high water/titanium ratio. The Journal of Physical Chemistry B, 107(8), 1734-1738.

Pandey, G., & Jain, P. (2020). Assessing the nanotechnology on the grounds of costs, benefits, and risks. Beni-Suef University Journal of Basic and Applied Sciences, 9, 1-10.

Penn, R. L., & Banfield, J. F. (1999). Morphology development and crystal growth in nanocrystalline aggregates under hydrothermal conditions: Insights from titania. Geochimica et cosmochimica acta, 63(10), 1549-1557.

Perez-Reyes, O.; Crowl, T.A.; Hernandez-Garcia, P.J.; Ledesma-Fuste, R.; Villar-Fornes, F.A.; Covich, A.P. Freshwater decapods of Puerto Rico: A checklist and reports of new localities. Zootaxa 2013, 3717, 329–344.

Rodriguez-Ariza, A., Peinado, J., Pueyo, C., Lopez-Barea, J., 1993. Biochemical indicators of oxidative stress in fish from polluted littoral areas. Can. J. Fish. Aquat. Sci. 50, 2568–2573.

Sani, M. A., Maleki, M., Eghbaljoo-Gharehgheshlaghi, H., Khezerlou, A., Mohammadian, E., Liu, Q., & Jafari, S. M. (2022). Titanium dioxide nanoparticles as multifunctional surface-active materials for smart/active nanocomposite packaging films. Advances in Colloid and Interface Science, 300, 102593.

Shim, I. S., Momose, Y., Yamamoto, A., Kim, D. W., & Usui, K. (2003). Inhibition of catalase activity by oxidative stress and its relationship to salicylic acid accumulation in plants. Plant Growth Regulation, 39, 285-292.

Titanium-Environmental Health Criteria 24. Available online: https://apps.who.int/iris/bitstream/handle/10665/37269/9241540842-eng.pdf

Torres-Perez, W. X., & Perez-Reyes, O. (2023). Population Status of the Tropical Freshwater Shrimp *Xiphocaris elongata* in Urban and Forest Streams in Puerto Rico. Hydrobiology, 2(1), 277-288.

Tsang, C. H. A., Li, K., Zeng, Y., Zhao, W., Zhang, T., Zhan, Y., Xie, R., Leung, D., & Huang, H. (2019). Titanium oxide based photocatalytic materials development and their role of in the air pollutants degradation: Overview and forecast. Environment International, 125, 200-228.

Velzeboer, I., Hendriks, A.J., Ragas, A.M., Van de Meent, D., 2008. Aquatic ecotoxicity tests of some nanomaterials. Environ. Toxicol. Chem. 27, 1942–1947.

Verma, S.K. Engineered Nanomaterials and Phytonanotechnology: Challenges for Plant Sustainability; Elsevier: Amsterdam, The Netherlands, 2019; Volume 87.

Wang, C., & Zhang, X. (2020). Anatase/Bronze TiO_2 heterojunction: enhanced photocatalysis and prospect in photothermal catalysis. Chemical Research in Chinese Universities, 36(6), 992-999.

Wu, X., Li, J., Xie, S., Duan, P., Zhang, H., Feng, J., ... & Wang, Y. (2020). Selectivity control in photocatalytic valorization of biomass-derived platform compounds by surface engineering of titanium oxide. Chem, 6(11), 3038-3053.

Zaban, A., Aruna, S. T., Tirosh, S., Gregg, B. A., & Mastai, Y. (2000). The effect of the preparation condition of TiO2 colloids on their surface structures. The Journal of Physical Chemistry B, 104(17), 4130-4133.

Zahra, Z., Habib, Z., Hyun, S., & Sajid, M. (2022). Nanowaste: another future waste, its sources, release mechanism, and removal strategies in the environment. Sustainability, 14(4), 2041.Zhang, J.F., Shen, H., Wang, X.R., Wu, J.C., Xue, Y.Q., 2004. Effects of chronic exposure of 2,4-dichlorophenol on the antioxidant system in liver of freshwater fish *Carassius auratus*. Chemosphere 55, 167–174.

Zhu, X., Zhou, J., & Cai, Z. (2011). The toxicity and oxidative stress of TiO_2 nanoparticles in marine abalone (*Haliotis diversicolor supertexta*). Marine pollution bulletin, 63(5-12), 334-338.

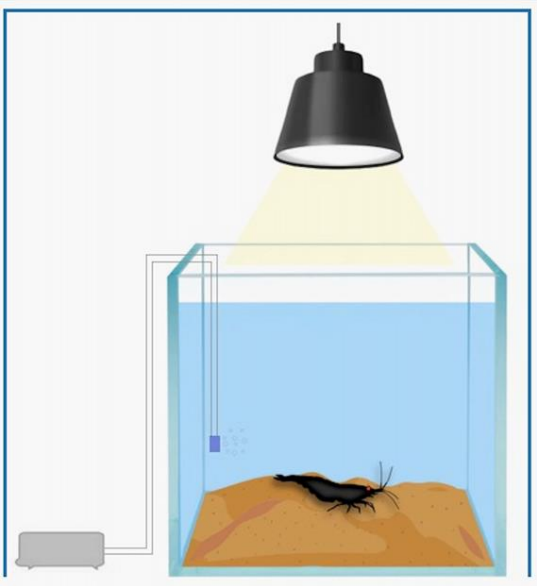
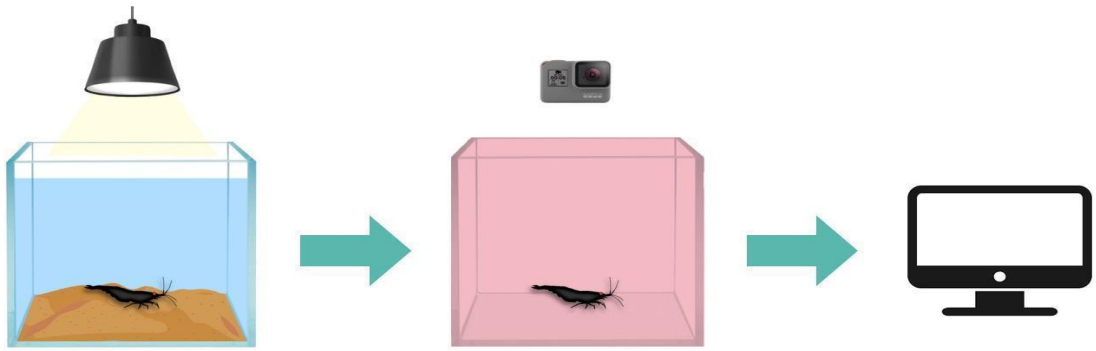


Figure 5.1 Microcosm: aquarium with 1 L of water, 150 g of artificial sediment, air stone and connected to the air pump, light, and one individual of *Atya lanipes* adult.



a) b) c)

Figure 5.2 Movement analysis performed on *Atya lanipes* exposed to different concentrations of TiO_2 NPs for 24 hours. a) the microcosm prepared for the bioassay with the *Atya lanipes* shrimp exposed to the nanoparticles, b) the acclimatization process of the shrimp in the recording area inside a red plastic box, c) the analysis of the movement collected in the videos in Loligo Systems®.

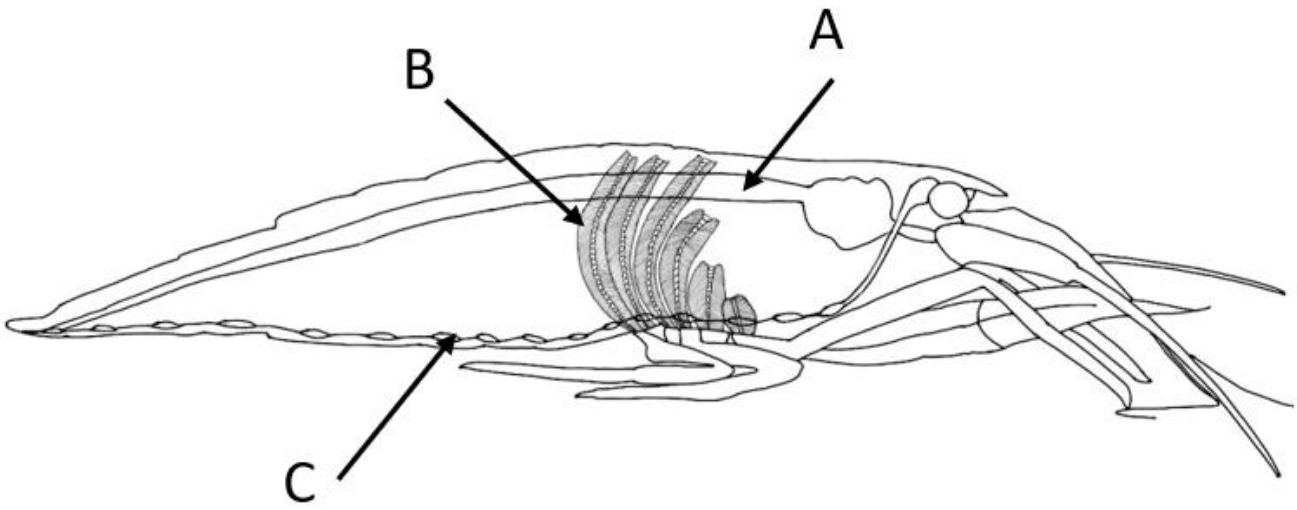


Figure 5.3 Diagram of *Atya lanipes* shrimp and the anatomy of the dissected tissues for the oxidative stress assessment. A) gut, B) gills and C) nerve cord.

Table 5.1 Average of the microcosm's chemical parameters during the assay (pre and post *Atya lanipes* exposures to TiO_2 NPs for 24 h), for each control and suspension concentrations of TiO_2 NPs.

Group	Time	pH	Conductivity (ms)	Salinity (ppm)	O_2, (mg/L)
0.0 mg/L; TiO_2 NPs	Pre	8.2 ± 0.04	328.2 ± 19.0	0.17 ± 0.01	8.7 ± 0.2
	Post	8.2 ± 0.04	341.5 ± 15.0	0.17 ± 0.01	8.8 ± 0.1
0.50 mg/L; TiO_2 NPs	Pre	7.8 ± 0.003	468.7 ± 1.0	0.23 ±0.001	9.2 ± 0.02
	Post	7.7 ± 0.02	436.0 ± 9.1	0.22 ± 0.004	8.0 ±0.1
1.0 mg/L; TiO_2 NPs	Pre	8.3 ± 0.00	353.2 ± 0.8	0.23 ±0.0001	9.3 ± 0.1
	Post	7.7 ± 0.02	362.8 ± 2.3	0.22 ±0.0001	8.4 ± 0.1
2.0 mg/L; TiO_2 NPs	Pre	7.8 ± 0.01	469.6 ± 3.6	0.23 ±0.002	9.5 ± 0.1

	Post	7.7 ± 0.02	475.2 ± 4.2	0.24 ±0.002	7.9 ± 0.1
3.0 mg/L; TiO_2 NPs	Pre	8.2 ± 0.04	332.2 ± 17.3	0.17 ± 0.01	8.6 ± 0.1
	Post	8.1 ± 0.04	337.4 ± 16.2	0.17 ± 0.01	8.9 ± 0.1
Titanium (100µg/ml)	Pre	8.2 ± 0.04	332.4 ± 16.0	0.17 ± 0.01	8.6 ± 0.1
	Post	8.1 ± 0.04	367.2 ± 21.0	0.18 ± 0.01	8.9 ± 0.1
TiO_2 NPs (3 mg/L) + Titanium (100µg/ml)	Pre	8.2 ± 0.04	340.4 ± 21.0	0.17 ± 0.01	8.6 ± 0.1
	Post	8.1 ± 0.04	371.8 ± 22.4	0.19 ± 0.01	8.9 ± 0.1

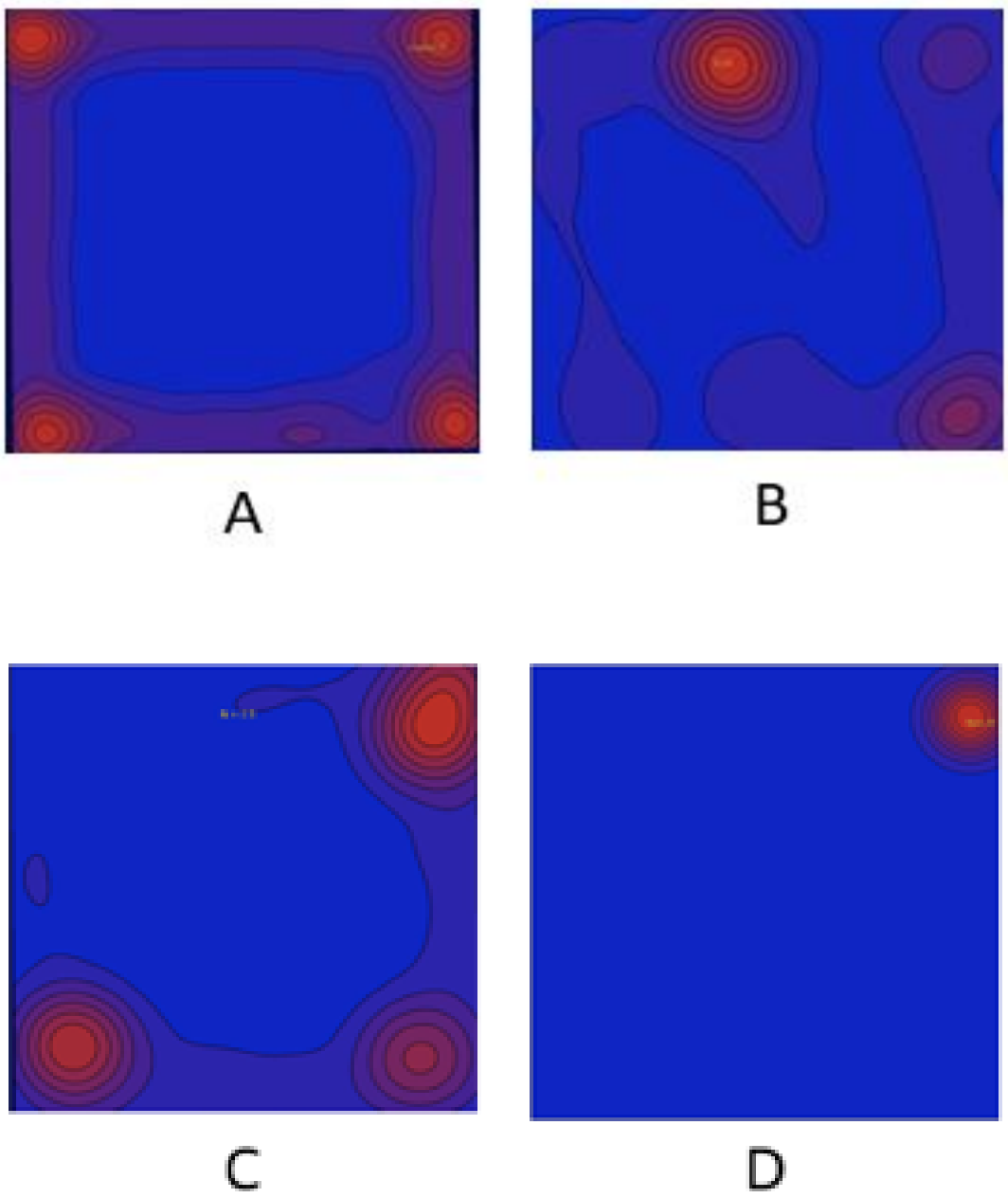


Figure 5.4 Heat maps illustrations for the adult shrimp *Atya lanipes* behavior in all treatments and control. Redness areas = more time in the place by the shrimp. A) Control, B) Titanium ions (100 micrograms/ml), C) Titanium (100 micrograms/ml) + TiO_2 NPs (3 mg/L), D) TiO_2 NPs (3 mg/L)

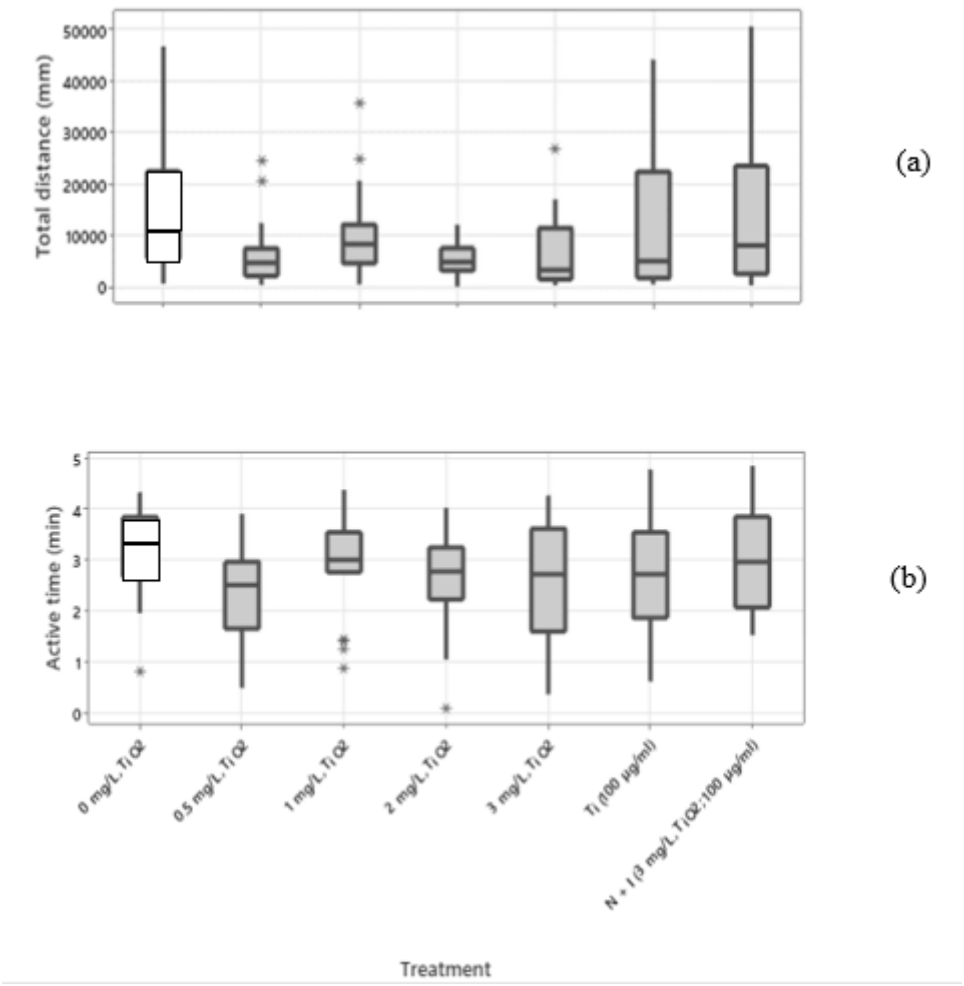


Figure 5.5 Movement adult shrimp *Atya lanipes* assessment (N=25 for each treatment). a) Total distance moved (mm) and b) Active time (min) of *A. lanipes* shrimp after acute exposure of 24 h of different TiO_2 NPs suspension concentrations, titanium exposure of 100µg/ml and the synergy of TiO_2 NPs and titanium (3 mg/L; 100µg/ml). * = outliers

Table 5.2. EC concentration of TiO_2 NPs (mg/L) for active time behavior variable of *Atya lanipes* shrimp exposed in an acute exposure of 24 h.

Point	***Concentration (mg/L)***
	24 h
EC 50	0.143
EC 60	0.520
EC 70	2.073
EC 80	10.467
EC 90	98.868

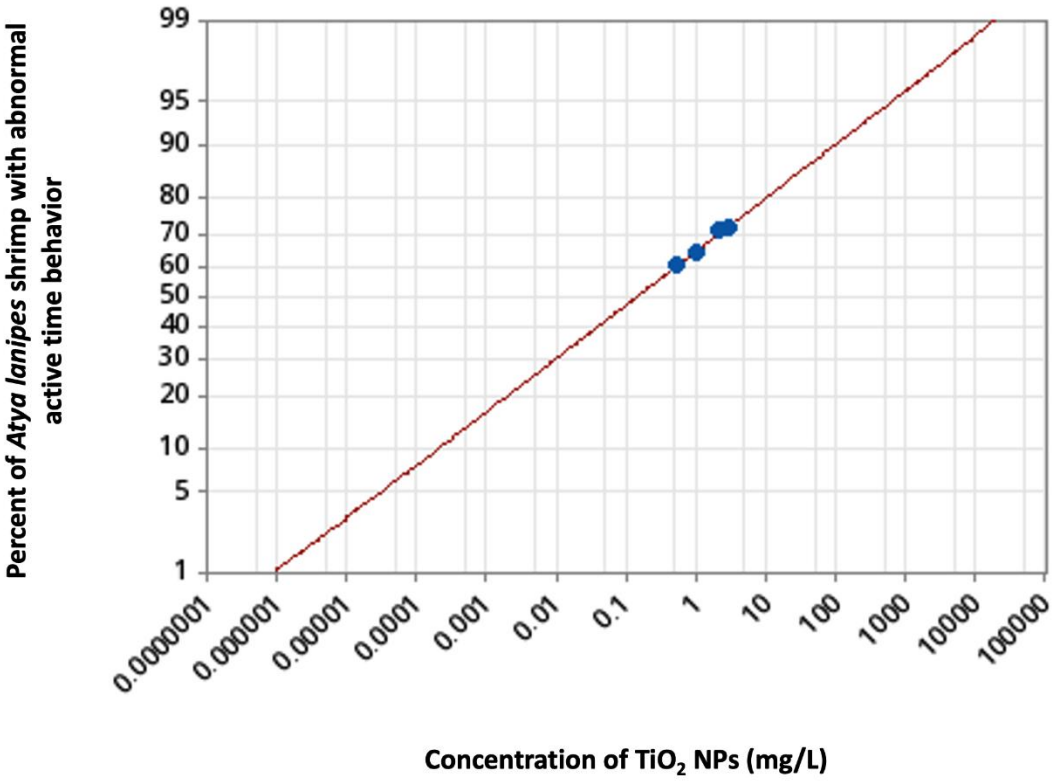


Figure 5.6 Lognormal probability plot at 95% CI of the active time variable for *Atya lanipes* species exposed to TiO_2 NPs in 24 h

Table 5.3. Average of the microcosm's chemical parameters during the bioassay for each exposure time and after exposure to 10 mg/L of TiO_2 NPs from 24 to 264 h.

Time (hours)	**Temperature (°C)**	**pH**	**Conductivity (µs)**	**DO (mg/L)**	**Salinity (ppm)**
Control group	19.4 ± 0.01	7.0 ± 0.1	0.4 ± 0.01	8.9 ± 0.04	0.2 ± 0.01
24	19.9 ± 0.02	7.1 ± 0.2	0.3 ± 0.004	8.3 ± 0.1	0.2 ± 0.01
72	18.5 ± 0.1	6.1 ± 0.1	0.4 ± 0.01	9.0 ± 0.1	0.2 ± 0.01
120	18.8 ± 0.02	6.8 ± 0.1	0.4 ± 0.0	8.7 ± 0.2	0.2 ± 0.001
168	18.6 ± 0.02	7.1 ± 0.1	0.4 ± 0.0	7.8 ± 0.01	0.2 ± 0.0
216	19.2 ± 0.1	5.8 ± 0.1	0.4 ± 0.01	8.4 ± 0.1	0.2 ± 0.004
264	18.7 ± 0.1	6.0 ± 0.04	0.4 ± 0.001	8.9 ± 0.04	0.2 ± 0.01

Table 5.4. Averages of the size of the dissected specimens in each exposure time were presented as postorbital and cephalothorax lengths.

Time of exposure (hours)	POS (mm)	CEF (mm)
Control	12.7 +/- 1.3	14.3 +/- 2.0
24	13.3 +/- 1.3	16.6 +/- 1.4
72	12.1 +/- 1.0	14.6 +/- 1.4
120	11.1 +/- 0.2	13.6 +/- 0.2
168	12.2 +/- 1.0	14.2 +/- 1.1
216	12.0 +/- 1.0	14.9 +/- 1.2
264	10.8 +/- 0.2	14.0 +/- 0.4

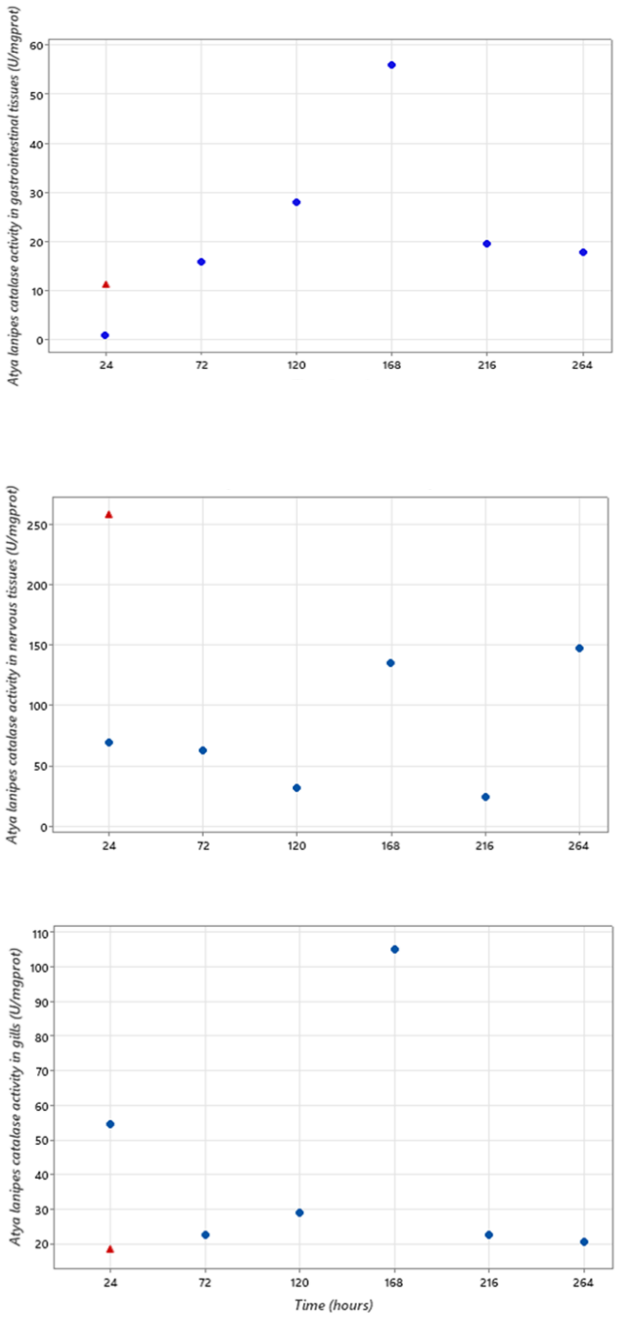


Figure 5.7 *Atya lanipes* Catalase activity in tissues between 24 to 264 hours of exposure to 10 mg/L of TiO_2 NPs. Red triangle is the average of catalase activity in the control group for the entire exposure. a) gastrointestinal tissues, b) nervous tissues, and c) gills tissues.

CHAPTER 6:

CONCLUSIONS

This doctoral dissertation research was focused on evaluating and understanding the freshwater aquatic emergent pollutants such as TiO_2 NPs and their possible ecological impacts on a very important species such as *Atya lanipes*. Our literature search for Puerto Rico found no scientific data that would have measured ambient concentrations of TiO_2 NPs in rivers on the island. In fact, globally, we know very little about TiO_2 NPs in rivers. These studies have been conducted in temperate zones, not the Caribbean. Without this information, we are left at a disadvantage, unrealistic toxicology investigations take place, and the gap we face widens.

This study provides specific information for Puerto Rico on TiO_2 NPs. To obtain more information and to be able to make statistical comparisons, we worked with sediment and water samples from two rivers urbanized to different degrees. These types of studies that evaluate concentrations in sediments as well as in water are few in the scientific literature. The results obtained in this objective provide us with a realistic panorama of the TiO_2 NPs concentrations in the rivers in Puerto Rico and document that there are no significant differences regarding the concentrations of this nanoparticle and the level of urbanism that a river may present in the region. island. Concentrations can be similar to higher in non-urban rivers in Puerto Rico. Having some knowledge of our river's water quality we can develop more realistic studies that evaluate the toxic effects of environmentally susceptible species. Additionally, this study provides a starting point for the beginning of mitigation and environmental management of concentrations in freshwater bodies such as rivers.

Regarding the toxicity assessment of the TiO_2 NPs in the two life stages of the shrimp, they elucidated knowledge that was not found in the scientific literature. Knowing the complex life cycle and being able to simulate the conditions of the estuarine and freshwater ecosystems under laboratory conditions and obtaining results on chronic water exposures in this shrimp, it was possible to document the relevance of sublethal and second fatal effects (larvae). For the larval stage, we now know that exposure to TiO_2 NPs under study conditions represents fatal effects after 48 and 72 h of exposure, morphological effects (edema and less pigmentation of the larvae), and effects on the nervous system statistically evidenced in hypoactivity. significant.

In the adult stage of the *Atya lanipes* shrimp and under simulated river environment conditions in microcosm, we were able to document sub-lethal effects in just a 24-h exposure. We document hypoactivity with an EC_{50} of 0.143 mg/L and the development of oxidative stress evident in overstimulation or repression of catalase activity in nervous, gastrointestinal, and gill tissues. The first two are the most pronounced. This development of oxidative stress was observed in the tissues from 24 h to 264 h (acute to chronic). *Atya lanipes* is a key species in the stream ecosystem and susceptible to the presence of TiO_2 NPs both in larval and adult stages; in the estuarine and freshwater ecosystem.

In general, this study is a pioneer in the documentation of the environmental concentrations of TiO_2 NPs in two rivers in Puerto Rico and in the biological effects on a species that is in contact with the sediment, the place where these types of nanoparticles are finally deposited. It is important to recognize that nanomaterials in aquatic ecosystems are a great threat to both water quality and species conservation. At a time when there are no environmental regulations for Puerto Rico

and EE.UU., it is imperative to continue scientific research in order not only to know the possible effects on aquatic suceptibles organisms, but also to carry out more exhaustive studies to know the environmental concentrations of titanium oxide nanoparticles in sediment and water in different rivers in Puerto Rico, using more efficient analytical methods. Also, geology studies are needed to help us understand the rates of titanium in the natural weathering of rocks in order to know the concentrations attributed to anthropogenic impacts. I suggest that federal and state agencies dedicated to water quality and the protection of native or endemic species should consider projecting research projects aimed at rivers nanoparticles pollution in order to develop good management and regulation of them and thus preserve the freshwater ecosystems on the Island.