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OCCURRENCE OF TETRODOTOXIN PRODUCING BACTERIA ON MARINE GASTROPODS OF THE NORTHERN COAST OF PORTUGAL

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List of Abbreviations

TTX: Tetrodotoxin

NaHO: Sodium hydroxide

rRNA: Ribosomal ribonucleic acid

DNA: Deoxyribonucleic acid

EDTA: Ethylenediaminetetraacetic acid

PCR: Polymerase chain reaction

Gib: *Gibbula umbilicalis*

Mono: *Monodonta turbinata*

S.W.: Surrounding waters

B.F.: Bio film

N.I.: Non interpretable

V.N.G.: Vila Nova de Gaia

Abstract

Tetrodotoxin is a low molecular weight neurotoxin present in a diverse range of taxa ranging from unicellular bacteria to evolved vertebrates (marine and terrestrial) that is responsible for a significative number of deaths annually, especially in the Indo-Pacific area. It has so far, no known antidote and is lethal to humans in very small dosages. Although this toxin's clinical effects are well known, its biological and synthesis pathways are still a highly controversial issue. Even though the majority of cases of intoxication happens in the oriental part of the globe we are witnessing a gradual appearance of the toxin's vectors in Mediterranean waters due partially to human intervention (the opening of the Suez Canal that allows the Lessepsian migration of invasive species from the Red Sea into the Mediterranean), and to the phenomenon of global warming that gradually raises the water temperature of non-tropical seas to levels inviting to foreign tropical species. The appearance of a case of poisoning by TTX in Spain in 2007 due to ingestion of a trumpet shell captured in Portuguese shores has raised the alarm to this possible public health issue until now unknown.

The work performed had as primary objective the detection of bacteria with the potential to produce TTX in the digestive tract of two marine gastropods recently shown to possess TTX that are common to the northern shore of Portugal. The laboratorial work was performed in LEGE (Ecotoxicology, Genomics and Evolution Laboratory) of CIIMAR (Interdisciplinary Center for Marine and Environmental Research). The bacterial samples were isolated and identified trough 16S rRNA gene amplification, which has shown that the intestinal microflora of the gastropods *Monodonta turbinata* and *Gibbula umbilicalis* is largely composed of bacterial groups known to produce TTX such as bacteria from the *Vibrio*, *Pseudoalteromonas*, *Shewanella*, *Photobacterium* and *Bacillus* genera. This study has further supported that there is a serious potential for the increase of TTX appearance in Portuguese coastal waters and as such has further increased the necessity to engage in more intensive work on this matter.

This work was partially financed by the ATLANTOX project and by the University of Porto IJUP project.

Resumo

A Tetrodotoxina é uma neurotoxina de baixo peso molecular presente num grande número de taxa distintos, desde bactérias unicelulares a vertebrados evoluídos (tanto marinhos como terrestres) que é responsavel anualmente por um número significativo de mortes, em especial em águas da zona Índico-Pacífica. Até à data não há qualquer antídoto conhecido e a sua letalidade para o homem verifica-se mesmo a muito baixas doses. Apesar dos seus sintomas clínicos serem bem conhecidos, o mecanismo biológico de síntese e funcionamento continua um tema de debate altamente controverso. Apesar de o maior número de casos de envenenamento por esta toxina acontecer na zona mais oriental do globo tem-se assistido a um progressivo aparecimento dos seus vectores mais comuns em águas Mediterrânicas devido em parte à intervenção humana (a abertura do Canal de Suez que permitiu a migração Lessepsiana de espécies invasoras vindas do Mar Vermelho para águas do Mar Mediterrâneo) mas também ao gradual aumento de temperatura das águas dos mares não tropicais devido ao fenómeno do aquecimento global, tornando-as convidativas a especies invasoras tropicais. O aparecimento de um caso de envenenamento por TTX em 2007 em territorio espanhol devido à ingestão de um búzio capturado em águas Portuguesas fez soar o alarme para este possivel problema de saúde pública, até agora ignorado.

O trabalho realizado teve como principal objetivo a deteção de bactérias com a conhecida capacidade de produzir TTX no trato digestivo de duas espécies de gastrópodes, recentemente reportados como possuíndo a toxina, que são comuns na costa Norte de Portugal. Todo o trabalho laboratorial foi feito no LEGE (Laboratório de Ecotoxicologia Genómica e Evolução) do CIIMAR (Centro Interdisciplinar de Investigação Marinha e Ambiental). As amostras bacterianas foram isoladas e depois identificadas recorrendo a amplificação do gene 16S rRNA, o que mostrou que a flora intestinal dos gastrópodes *Monodonta turbinata* e *Gibbula umbilicalis* é maioritariamente composta por bactérias com conhecida capacidade para produzir TTX, nomeadamente bactérias pertencentes aos generos *Vibrio*, *Pseudoalteromonas*, *Shewanella*, *Photobacterium* e *Bacillus*. Este estudo reforçou ainda mais a ideia que existe um sério risco de aumento de eventos de TTX na costa Portuguesa contribuindo para a necessidade de serem desenvolvidos novos e mais intensivos estudos sobre esta temática.

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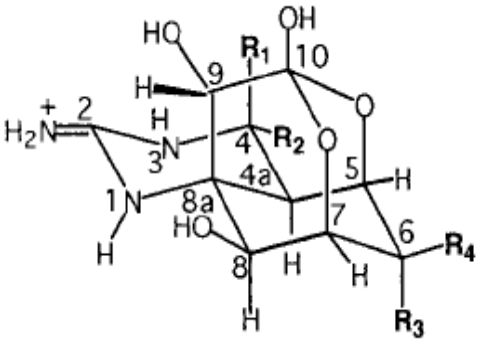
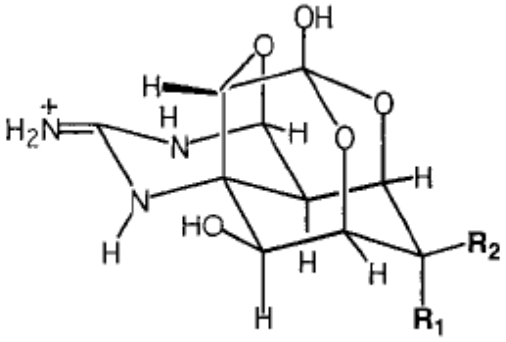
1.Introduction

1.1 *What is Tetrodotoxin?*

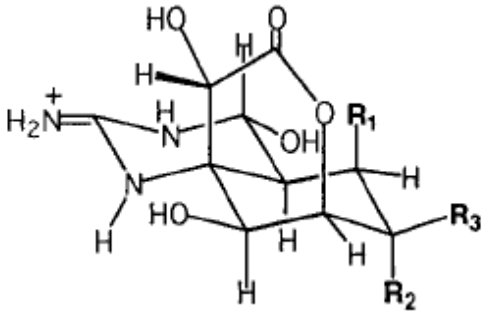
Tetrodotoxin (TTX) is one of the most potent neurotoxins known to man being more than 250 times more toxic than cyanide. The knowledge of its existence and negative effects date as far back as the expeditions of Cpt. Cook in 1774 (Beaglehole 1961), being TTX also one of the most powerful emetic agents in existence causing severe gastro-intestinal complications even at the lowest concentrations. Despite the already existing knowledge of the human physiological effects of TTX this toxin was only discovered as such in 1909 by the Japanese scientist Yashizumi Tahara (Tahara and Hirata 1909; Clark, Williams et al. 1999) associated with its most known vector the puffer fishes from family *Tetraontidae* (from where it also derives its designation) where it appears in conjunction with its analogues (Figure 1) (4-*epi* TTX; 4, 9-anhydro TTX; Tetrodonic acid; 6-*epi* TTX; 11 deoxyTTX/5-deoxyTTX; 11 norTTX-6(S)-ol/11-norTTX-6(R)-ol) (Jang and Yotsu-Yamashita 2006).



	R1	R2	R3	R4
TTX	H	H	OH	CH_2OH
4-*epi*TTX	OH	H	OH	CH_2OH
6-*epi*TTX	H	OH	CH_2OH	OH
11-deoxyTTX	H	OH	OH	CH_3
11-norTTX-6(S)-ol	H	OH	OH	H
11-norTTX-6(R)-ol	H	OH	H	OH
11-oxoTTX	H	OH	OH	CHO



	R1	R2
4,9-anhydroTTX	OH	CH_2OH
6-epi-4,9-anhydroTTX	CH_2OH	OH



	R1	R2	R3
5-deoxyTTX	H	OH	C_2OH
5,6,11-trideoxyTTX	H	H	CH_2

Figure 1: Chemical structures of TTX and its analogues (Shoji, Yotsu-Yamashita et al. 2001)

TTX is a heterocyclic molecule, which despite already being successfully synthetized in laboratory still has a mysterious biosynthetic pathway in the organism, (Yotsu-Yamashita and Mebs 2001; Kono, Matsui et al. 2008) with six hydroxyl groups, a guanidinium group and a pyridine ring with additional attached ring systems, at physiological pH it presents a positive charge (Soong and Venkatesh 2006). It presents also some distinctive physical characteristics; it is presented (when pure) as a white odorless powder with no taste, resistant to heat up to temperatures around 220ºC. It is degraded in solutions with extreme levels of pH being soluble in aqueous solutions of weak organic acids (Kao 1986).

The modus operandi of TTX was firstly described in 1964 Narahashi and collaborators (Narahashi, Moore et al. 1964) that discovered using lobster nervous cells and utilizing the voltage clamp technique that the toxin acted selectively on the extracellular parts of the axonal cells of the nervous system, contrarily to common anesthetics that affect the intracellular part of the cells. The toxin exerts its effect by binding to the receptor-site 1 of voltage-gated sodium channels blocking the outer pore, interrupting the electric emission potential in the muscular cell causing the principal symptom of TTX poisoning, paralysis leading ultimately to death by respiratory arrest (Cestele and Catterall 2000; Choudhary, Yotsu-Yamashita et al. 2003; Scheib, McLay et al. 2006). Although extremely toxic and potentially lethal TTX has also been studied for its therapeutic possibilities namely in terminal patients were its paralyzing activity can be used as a pain mitigator (Kao 1986; Marcil, Walczak et al. 2006; Hagen, Fisher et al. 2007).

1.2 *TTX Occurrence in animal vectors*

When it was first discovered, TTX was thought to be an endogenous defense mechanism of the animal family in which it was discovered, the family Tetraontidae that includes among other species, the puffer fish (*Fugu*) where the toxin acts as both a defense mechanism and a pheromone being released onto the water during female ovulation in order to attract males of the species (Matsumura 1995) and is encountered mostly on the skin liver and ovaries. This exclusivity theory was eventually put to the test when Mosher and collaborators in 1964 (Mosher, Fischer et al. 1964) discovered that TTX was also present in the eggs of the newt *Taricha torosa*. At this point, a long list of other marine and terrestrial organisms from greatly distinct taxa also containing TTX was discovered, from the marine gastropods *Charonia sauliae (Narita, Noguchi et al. 1981)*, *Tutufa lissostoma (Noguchi, Maruyama et al. 1984)*, *Babylonia japonica (Noguchi, Maruyama et al. 1981), Rapasa rapiformis*, *Rapana venosa venosa (Hwang, Lu et al. 1991)*, *Natica lineata*, *Natica Vitellus*, *Polinices didyma (Hwang, Chueh et al. 1990)*, *Niotha Clathrata*, *Zeuxis scalaris*, *Zeuxis siquijorencis (Narita, Noguchi et al. 1984; Hwang, Lu et al. 1991)*, *Oliva mineacea*, *Oliva mustelina*, *Oliva nirasei (Hwang, Tsai et al. 2003),* to the crustacean *Atergatis floridus* (Noguchi, Jeon et al. 1986), the echinoderm *Astropecten polyacanthus* (Narita, Matsubara et al. 1987) to the cephalopod *Octopus maculosus* (Hwang, Arakawa et al. 1989).

The discovery of the neurotoxin in such taxonomically diverse species opened the debate as to what is in fact the production point of TTX. This theme has three major theories. The bioaccumulation theory proposed that TTX was acquired through the trophic chain in the diet of predators like the starfish *Astropecten scoparius* (Lin and Hwang 2001; Kono, Matsui et al. 2008) (Figure 2). The Endogenous approach stated that the production of TTX was inherent to *Fugu* puffer fishes and was supported by the tests perform by Matsumura who artificially cultivated ovulated oocytes resulting in the increase of TTX in the embryos (Lehman, Brodie et al. 2004). The symbiont theory on the other hand supported that TTX production was in fact not produced by the animals themselves but by bacteria living in a symbiosis relation with them (Noguchi, Hwang et al. 1987; Yotsu, Yamazaki et al. 1987).

The bacterial origin of TTX theory is today widely supported due to the growing number of bacterial species reported to have the capability to produce the toxin. Since the first reported isolation of bacteria capable of producing TTX and anhydroTTX, *Vibrio* sp. extracted from the digestive tract of the *Atergatis Floridus* crab (Noguchi, Jeon et al. 1986), bacteria of the species *Vibrio alginolyticus* (Narita, Matsubara et al. 1987), *Pseudomonas* sp. (Yasumoto, Yasumura et al. 1986; Noguchi, Hwang et al. 1987; Yotsu,

Yamazaki et al. 1987), *Alteromonas* sp., *Bacillus* sp. (Hwang, Arakawa et al. 1989), *Aeromonas* sp., *Flavobacterium* sp. (Hwang, Cheng et al. 1994), *Microbacterium Arabinogalactanolyticum*, *Serratia marcescens* (Yu, Yu et al. 2004), *V. parahaemolyticus* and *Plesiomonas* sp. (Cheng, Hwang et al. 1995) were also reported to have the same capacity. The appearance of TTX production capable bacteria is not however restricted to animal symbionts as there are reports of their occurrence in either salt or fresh water sediments as well (Kogure, Do et al. 1988; Do, Kogure et al. 1990; Do, Kogure et al. 1991).

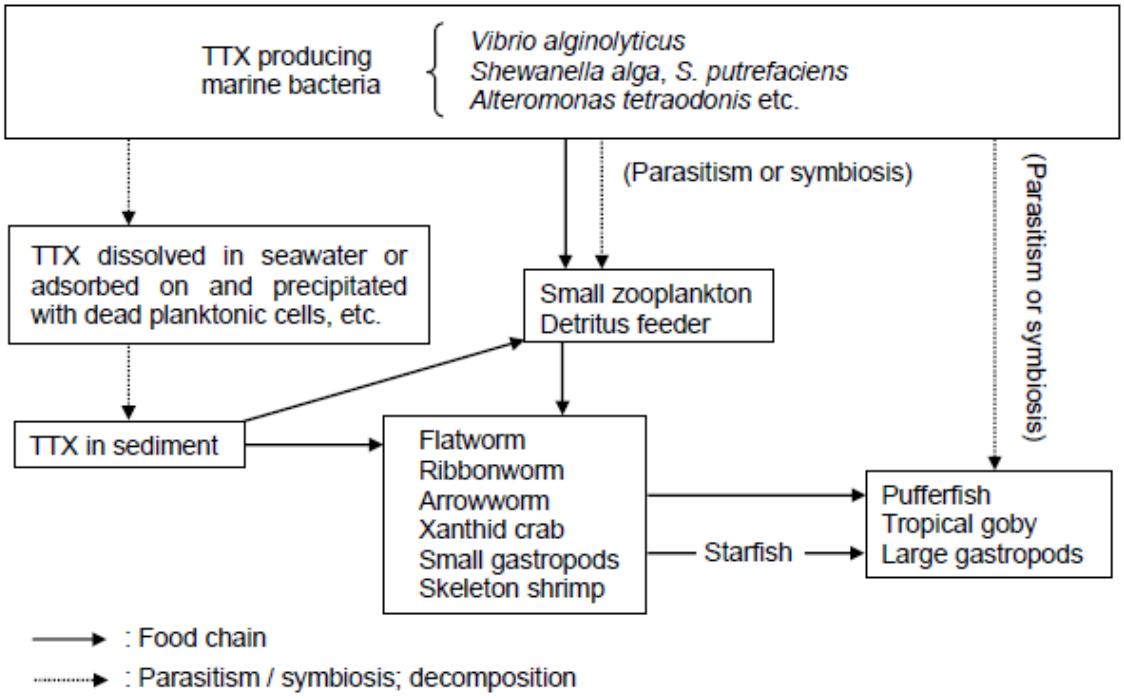


Figure 2: Mechanism of TTX accumulation in marine animals (adapted from (Noguchi and Arakawa 2008))

1.3 *Influence of climate changes on the distribution of TTX.*

The close relation between TTX and the tropical environment where most of the species capable of its production inhabit, has led to that the large majority of the reported cases of poisoning from this toxin are confined to the southwest Asian area, especially in Japan were the regular consumption of *Fugu* related cuisine leads to most of the registered events. Despite this initial geographical limitation a visible increase of TTX intoxication cases in Mediterranean waters were such cases should be unlikely is occurring.

This new phenomenon of displaced TTX detection cases and the global increase of water temperatures can be linked to both the increase of the presence of TTX vectors in Mediterranean waters, and the increase of TTX contents in said vector. The appearance of TTX vectors on previously unrelated areas can be traced back as far as 1902 with the first reports of Lessepian migrations where animals supposedly confined to the Red Sea were appearing in the Mediterranean Sea with the construction of the Suez Canal. These migrations are a growing phenomenon accompanying closely the increase in global temperature (Lasram and Mouillot 2009). Among the most significant migrations is that of fishes from the *Lagocephalus* family*,* known vectors for TTX, such as *Lagocephalus sceleratus* (Akyol, Unal et al. 2005), *L. suezensis* (Mouneimne 1977) and *L. lagocephalus* (Saoudi, Abdelmouleh et al. 2008). Concerning the case of *L. sceleratus,* one of the most recent Lessepian species discovered, it has been related to cases of intoxication (Bentur, Ashkar et al. 2008) along the Mediterranean shore and its presence in the artisanal fishing captures is an increasing observation.

Published works are still inconsistent when relating an increase in water temperature with the increase of TTX content in its vectors although some studies exist that do so as is the case with the work of Matsumoto in 2007 (Matsumoto, Nagashima et al. 2007) that clearly relates an increased intake rate of TTX into the liver tissue of *Takifugu rubripes* with the increase in environmental temperature, however this relation between temperature variation and toxicity potential of TTX remains an obscure them as well as the mechanisms involving excretion and accumulation of the toxin on puffer fish. The seasonal rise in environmental temperatures is thought to be a determining factor in the variation of toxicity of the TTX vectors, as such several studies have been performed in order to establish a valid correlation between the two, tests performed in specimens of Taiwanese gobies were performed and showed that a small percentage of the animals had a measurable TTX content between August 1996 to July 1998 being observable a both regional and seasonal variation of the toxin with higher values from March to November (Lin, Hwang et al. 2000). These test results differ with those from another study

performed during an annual period (August 2000 to August 2001) in Indonesia on the puffer fish *Lagocephalus lunaris*, this study showed that the animals remained toxic through 9 months (March to November) with the toxicity peak being observed in August with 100% of the test animals possessing the toxin (Brillantes, Samosorn et al. 2003). Still there is an obvious lack of these systematic studies and more research is required in order to establish any kind of definitive hypothesis on the seasonal variance of TTX vector toxicity.

Although TTX is a well studied toxin from an epidemiologic perspective, it's molecular and cellular mechanics are still very much a mystery. Bacteria of the microflora of several animals are thought to be responsible for the production of TTX; as such studies on their kinetics are needed to understand their possible variation in today's changing weather patterns. One of the scarce studies to relate changes in water temperature with changes in the bacterial content of TTX vectors (in this case the puffer fish *Fugu niphobles*) demonstrated that the bacterial content of the skin, gills and intestines of the fish was in fact affected by thermic variations. Specifically the identification of bacteria from the genus *Vibrio*, known producers of TTX, was positive in temperatures of 20º and 29ºC but negative at 10ºC, these results were further confirmed when the same bacteria in laboratorial conditions were cultivated, yet again all of the strains were able to grow at 20º and 29ºC but very few were able to do so at 10ºC suggesting their preference for higher water temperatures (Sugita, Iwata et al. 1989).

1.4 *Occurrence of TTX poisoning cases*

Even though TTX poisoning events are being now reported in previously uncontaminated water the majority of the intoxication events are still largely exclusively to the Southeast Asia where the consumption of Fugu (the major TTX vector) is considered a national delicacy.

The minimal lethal dose of TTX for an average human is of 2g of toxin the severity of the symptoms presented varying with the individual health condition and amount of toxin ingested (Wang, Yu et al. 2008). On 1941 the symptoms of TTX poisoning were described by Fukuda and Tani as being groupable in four phases (Table 1) of symptom severity (Fukuda and Tani 1941). Prevention is the best "cure" against TTX poisoning as there is yet no antidote for this potent neurotoxin, although some possible mitigation chemicals have been suggested in order to treat TTX poisoning most of them are largely ineffective. The only course of treatment available today involves keeping the affected patient alive through the first 24 hours after intoxication with ventilation, hemodynamic support and

cardiac monitorization being required the patient's hospital internment (Hommel, Hulin et al. 1992; Ravaonindrina, Andriamaso et al. 2001).

Table 1: Symptomatic phases of TTX poisoning (adapted from Fukuda 1941)

First phase	Oral paraesthesia, occasional gastrointestinal symptoms.
Second phase	Advanced paraesthesia, paralysis of extremities with reflexes still intact.
Third phase	Muscular incoordination, dysphagia, respiratory distress, precordial pain, cyanosis, drop of blood pressure, victim still conscient.
Fourth phase	Mental faculties impaired, total respiratory paralysis, extreme drop in blood pressure, death.

1.4.1 *Eastern Asia*

The eastern zone of the Asian continent is especially relevant in cases of TTX poisoning as it is the zone where the largest number of events is reported due mostly to the consumption of, sometimes poorly prepared, puffer fish based food. In Japan the increasing number of TTX poisoning cases (Noguchi and Ebesu 2001) as made the Japanese government to publish a public guideline for edible pufferfish in 1983, updated in 1993 and 1995, and to prohibit the sale and consumption of pufferfish liver in restaurants and markets, even so the mortality rate due to TTX poisoning is still high (Table 2) due mostly to the consumption of homemade "kimo" (*Fugu* liver) dishes.

Table 2: Cases of pufferfish poisoning in Japan from 2003 to 2007

Year	Number of cases	Number of patients	Deaths	Mortality (%)
2003	28	35	3	8.6
2004	43	58	2	3.4
2005	40	49	2	4.1
2006	25	32	1	3.1
2007	24	38	2	5.3

In Taiwan and China waters TTX poisoning cases tend to be less frequent as the ingestion of *Fugu* related products is less frequent, even so such cases exist. In April 2001 a group of middle aged Chinese fishermen was admitted in to hospital care with symptoms of pufferfish poisoning after eating a pufferfish later identified as *Lagocephalus lunaris*. After as little as 3 hours of ingestion symptoms were first observed, within a day one of the men had died, three days past the remaining group was discharged without further complications (How, Chern et al. 2003).

In Taiwan, Chunghua province, a case of TTX poisoning was reported following the ingestion of a fish by a group of 5 persons (4 men 58 to 64 years old and a 46 year old woman) in January 2000. Symptoms included coma, paralysis ataxia and asphyxia. After a week of internment all patients were released uneventfully although two of the men needed mechanical respirators and intravenous fluids during commitment due to the severity of their symptoms. The animal responsible for this event was then identified molecularly and morphologically as being a specimen of the puffer *Takifugu niphobles (*Hwang and Noguchi 2007*)*.

1.4.2 *Europe and Mediterranean Sea*

Initially a phenomenon thought to be contained to the eastern Asian region of the globe, the appearance of TTX bearing organisms and consequent cases of intoxication in humans in these more western waters are gaining increased significance (Bentur, Ashkar et al. 2008; Fernandez-Ortega, Morales-de los Santos et al. 2010). Cases of terrestrial animals (newts belonging to the genus *Triturus*) as well as gastropods such as the sea shell *Caronia lampas lampas* (a carnivorous species confined to Mediterranean coasts) caught off the coast of Algarve or fish such as the specimens of Lagocephalus sceleratus (an invasive Lessepsian species migrated from the Red Sea through the Suez channel) caught off the coasts of Egypt, Greece and Israel (Yotsu-Yamashita, Mebs et al. 2007; Bentur, Ashkar et al. 2008; Fernandez-Ortega, Morales-de los Santos et al. 2010).

This sudden increase in the number of TTX containing species in European and Mediterranean waters has caused an increase in intoxication reports as well. In Egypt a family of seven individuals was admitted into the Suez Hospital in late 2004 with symptoms of TTX poisoning after consuming pufferfish bought at the ports of Attaka. All patients were closely monitored for the following six hours and left the hospital uneventfully on the 6th day after internment. Toxicological analysis of the fish's flesh and description given by the family confirmed the ingestion of poisonous fish containing TTX. Another case of TTX poisoning, the first encountered in Europe, was reported on October 2007 in southern Spain when a 49 year old male was admitted into a local hospital with

clear signs of TTX poisoning, namely nausea and vomiting, difficulty in speech articulation, breathing difficulties and in keeping his pupils open, all of these symptoms appeared 2 minutes after ingestion of a specimen of the seashell *Caronia lampas lampas* bought of a market in Malaga but caught off the Algarvian coast. The victim recovered fully 72 hours after intoxication. Toxicity tests performed on the tissue of the trumpet shell and on the blood and urine of the patient confirmed the presence of TTX and its analogs especially on the man's urine as it is known that the excretory system is the major route of TTX detoxification (Fernandez-Ortega, Morales-de los Santos et al. 2010).

The global increase of temperatures due to global warming and the crescent rate of invasion from species usually confined do the Red Sea into Mediterranean waters with the consequent increase in TTX poisoning cases brings to light the relevance of new, more profound studies on the kinetics and ecology of TTX and its vectors in order to comprehend and possibly avoid future fatalities due to poisoning with the toxin.

2. Objectives

The main objectives of this work were:

1. To isolate bacterial species part of the intestinal flora of marine gastropods of the Northern Portuguese coastal areas.

2. To identify bacterial species with TTX production potential utilizing molecular methods.

3. To study the toxicity effects in marine invertebrate's larvae exposed to the isolated bacteria.

3. Material & Methods

3.1 Sampling

For the present study, live gastropod specimens were collected from five beaches along the northwestern coast of Portugal: Aguda (41º 2' 58,35'' N, 8º 39' 19,22'' W) , Memória (41º 13’ 50.96'' N; 8º 43’ 18.09” W), Angeiras (41º 15’ 49.06” N; 8º 43’ 48.43” W), Valadares (41º 06’ 02.91” N; 8º 39’ 08.18” W) and São Bartolomeu do Mar (41º 34’ 26.90” N; 8º 47’ 53.54” W) (Fig.1). The analyzed beaches were selected due, not only to their relative accessibility, but also for their representativity of the zone and for their large extension of rocky intertidal substrate which provides the ideal collection conditions for the marine gastropods (Figure 3).

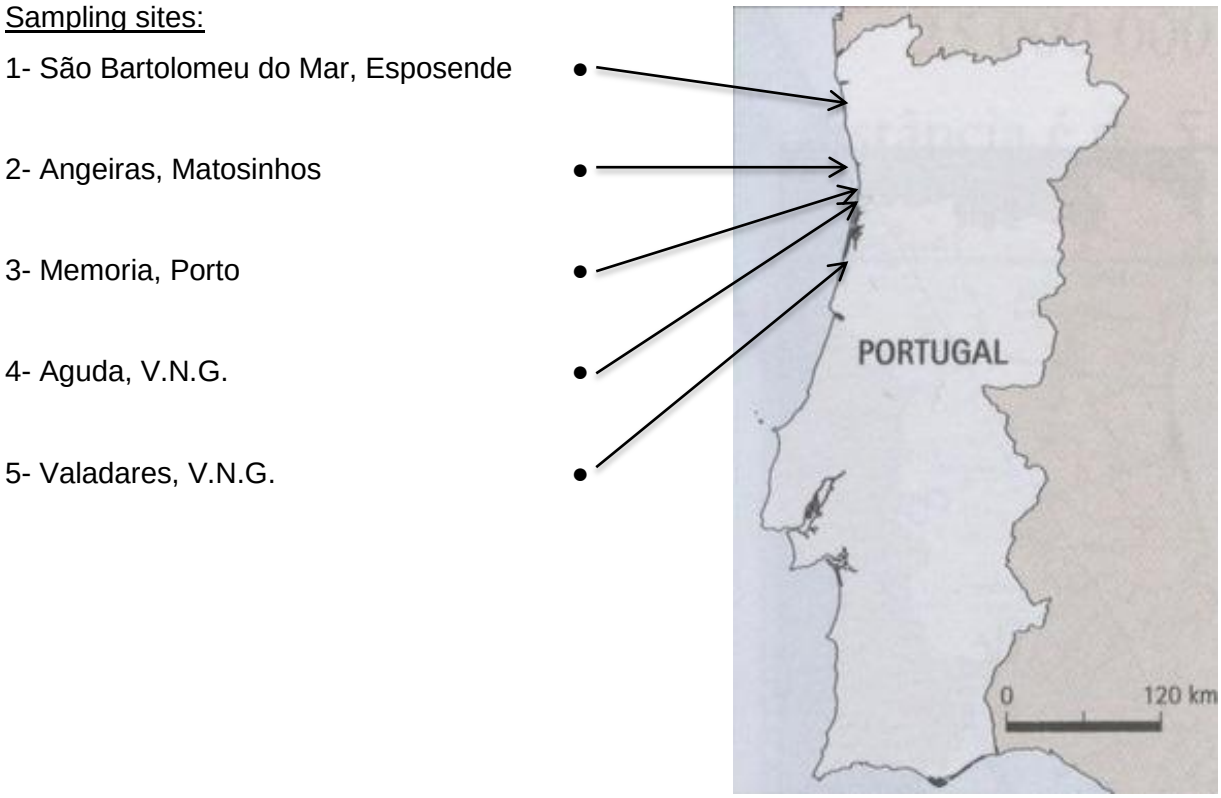


Figure 3: Sampling site locations.

Samplings were performed on monthly intervals at the referred beaches on their corresponding low tide hours (Table 3).

Specimens of both analyzed gastropod species, *Monodonta Turbinata* and *Gibbula Umbilicalis* (Figure 4) were collected from the intertidal rocky area of the beaches and transported securely in a cooler to the laboratory. Using plastic 100mL plastic cups the water surrounding the collected animals was also sampled along with biofilm samples obtained by scraping said film with a knife and collecting it in a sterile Falcon tube.

Figure 4: Examples of collected species *Monodonta Turbinata* (left) and *Gibbula umbilicalis* (right)

Figure 5: Valadares beach intertidal area

3.2 Sample treatment:

3.2.1 *Tissue excision and preparation*

At the laboratory the animals were subjected to triage being selected of each of the sampled species ten individuals of sufficient size to obtain a representative weight (Table 3) of viscera for analysis. The excision and preparation of the viscera was achieved following the protocol for bacterial extraction and growth proposed by Wu (2005), with some adaptations, through the use of a vice and other properly sterilized equipment (sterilized with ethanol 70%). From each animal the muscle tissue was discarded, the viscera collected and then homogenized in filtered, using 0,45µm filters (Schleicher & Schuell®), sterile marine water through maceration using a sterilized pestle. The obtained homogenate was then diluted in sterile marine water to a final concentration of 0.025g of tissue per milliliter of sea water (Wu, Yang et al. 2005).

3.2.2 *Bacterial isolation and growth*

To achieve desired bacterial growth 0.5ml of the homogenate obtained on the previous step was then inoculated in Petri dishes containing marine agar medium (Difco®), prepared accordingly to the manufacturer's instructions with pH value set to 7.2 using a pH measurer (Jenway®) and a NaHO solution (1M). The solution was then heat sterilized in autoclave (AJC® Uniclave 88) at 1atm, 120 ºC during 15 minutes. The inoculated bacteria were incubated for 5 to 6 days in an incubator (Binder®) at constant temperature of 22 ºC. Bacterial isolation was then achieved through macroscopical observation of differing colonial morphologic characteristics being each unique colony isolated to a fresh marine agar petri dish using streak techniques, this procedure was executed under aseptic conditions in a laminar flux chamber (Crumair® 9005-FL) previously sterilized with UV radiation and using discardable sterile loops to manipulate the bacteria.

The Petri dishes containing the isolated bacterial cultures were then also grown in an incubator at 22 ºC for 4 to 6 days in order to achieve optimal growth. Re-isolations were performed as necessary using the same method until full isolation.

In order to obtain a good amount of bacterial biomass to enable further testing the isolated bacterial colonies were inoculated in 300 ml of autoclaved liquid culture medium composed of 5 g Peptone (Sigma®) and 5 g Sodium Chloride per liter of filtered sterile water, the cultures were then grown on an incubator at 22 ºC for 6 to 7 days until optimal growth was observed. Biomass was collected through centrifugation of the cultures on a refrigerated centrifuge (Thermo™ Legend RT) at 4600 rpm and 4 ºC for 20 minutes. The

supernatant was then discarded and the pellets stored at -20 ºC in a cryoconservant solution (20% Glycerol) until further analysis (Wu, Yang et al. 2005).

3.3 Genotypic characterization

The 16S rRNA gene (codifier for the ribosomal RNA) is a widely used bacterial identification tool due to its ubiquitous distribution through the various prokaryote groups (Vandamme, Pot et al. 1996; Coenye and Vandamme 2003), serving sometimes as an identifier event at gender or species level being only a small amount of genetic material required in order to perform a valid identification through PCR reactions. Possibly the most important characteristic of the 16S rRNA gene as a tool of genetic identification is its structural independence from culture or growth methods, this (in conjunction with the aforementioned advantages) makes this gene an invaluable tool in the identification of unknown bacteria.

3.3.1 *DNA extraction:*

Of the previously collected bacterial biomass small aliquots of 100µL were used to perform the extraction of bacterial DNA. This extraction was achieved utilizing an *Invitrogen*® genomic extraction kit using the Gram- procedure suggested by the manufacturer. To achieve DNA extraction an Eppendorf® 5415R centrifuge and an Eppendorf® termomixer compact were used. Small alterations were made in the proposed protocol in order to achieve optimal DNA yield, namely the 4 hours digestion period recommended was extended to a 24 hour period.

The success of the extraction procedure was verified by a preliminary run of the DNA product on a 1.5% agarose gel (Molecular Biology Agarose, Biorad) (Davis, Kuehl et al. 1994) running in Tris-Acetate EDTA solution (TAE 1%, BioRad – 40mM Tris, 20mM acetic acid, 1 mM EDTA, pH: 8.3) using a Sub-Cell GT (BioRad®) electrophoresis tray, each well was loaded with 2 µL DNA product and 1 µL 1x loading dye (Nucleic acid sample loading buffer 5x, Biorad – 50 mM Tris-HCl, pH: 8.0, 25% glycerol, 5 mM EDTA, 0.2% Bromophenol blue and 0.2% Xylene Cyanol FF). To perform the electrophoresis the voltage utilized was of 110V for 30 min.

The molecular ruler utilized was of 1Kb (Bioline®), DNA purity and concentration was then estimated through fluorescence of Ethidium Bromide (10mg/ml, Biorad) irradiated with UV light (Cleaver Scientific® UV transluminator). A successful extraction was denoted by the appearance of highly Fluorescent DNA products on the gel visualization.

Table 3: Date, locale, and fresh weight of viscera collected per sampling

Sampling site	Time of sampling	Fresh weight of viscera extracted (g)	
Aguda	November 2010	*Gibbula Umbilicalis*	1.0
		Monodonta Turbinata	2.7
Memória	January 2011	*Gibbula Umbilicalis*	0.8
		Monodonta Turbinata	3.0
Angeiras	February 2011	*Gibbula Umbilicalis*	0.8
		Monodonta Turbinata	2.6
Memória	March 2011	*Gibbula Umbilicalis*	0.9
		Monodonta Turbinata	2.0
Valadares	April 2011	*Gibbula Umbilicalis*	0.5
		Monodonta Turbinata	2.1
Memória	May 2011	*Gibbula Umbilicalis*	0.7
		Monodonta Turbinata	2.0
São Bartolomeu do Mar	June 2011	*Gibbula Umbilicalis*	0.6
		Monodonta Turbinata	1.6

3.3.2 *PCR amplification of the 16S rRNA gene*

Amplification of the 16S rRNA gene of the extracted bacteria, was achieved through 20µL reactions using the primers 8F (5'-AGAGTTT GATCMTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTTACGACTT-3') (Lane, Pace et al. 1985) which are generic bacterial primers used widely for sequencing of the 16S gene.

After the confirmation of successful gene amplification (through visualization in agarose gel) the PCR reaction was scaled up to 40 µL per reaction for sequencing.

Each of the initial 20 µL reaction contained, 2 µL 1X PCR buffer (Bioline, 10X NH_4 Reaction Buffer), 2 µL dNTP's (Bioline 2,5mM), 1 µL $MgCL_2$ (Bioline 50 mM), 1 µL of each primer (10 pM), 0.1 µL Taq DNA polymerase (Bioline), 11.9 µL of autoclaved ultrapure water and 1 µL of DNA sample.

In order to achieve amplification the following program, suggested by Wang (Wang, Yu et al. 2008) was utilized on the thermocycler (Biometra® Professional multigradient thermocycler), initial denaturation at 94 ºC for 10 min, followed by 25 cycles of 94 ºC for 1 min, 57 ºC for 1 min and 72 ºC for 2 min, and a final extension step at 72 ºC for 10 min. The PCR tubes were stored at 4 ºC until further use.

Verification of successful amplification of 16S rRNA gene was obtained through electrophoresis by running 2 µL of each PCR product loaded with 1 µL of loading dye on a 1.5% agarose gel then ran at 110V for 30 minutes using a 1Kb marker in order to measure the obtained band which, in order to confirm correct amplification, should be approximately 780bp, for positive control a strain of isolated *Microcystis aeruginosa* M6 was utilized.

Purification of PCR products for sequencing was then performed using an *Invitrogen*® DNA purification Kit (Purelink™ Quick gel extraction and PCR purification Combo kit) using the instructions supplied by the manufacturer but using 25 µL of purified water instead of the 50 µL proposed, again confirmation of purification was achieved by running 1 µL of each of the PCR products on an agarose gel. The purified product was sent, together with 5 µL of each 10 pM/µL primer to the sequencing company MACROGEN® to obtain the genetic code of each sample.

The obtained sequences were then analyzed utilizing the programs Sequence Scanner™ (Applied Biosystems®), FinchTV™ (Geospiza®) and Bioedit in order to ascertain the quality of the unknown sequences. In order to identify the isolated bacterial species the consensus sequence obtained by alignment of the forward and reverse sequences (Supplementary information) was inserted onto the BLASTn online tool (Basic Local Alignment and Search Tool for nucleotide) part of NCBI's (National Centre for Biotechnological Information) GenBank < http://www.ncbi.nlm.nih.gov/ > in order to find

the species or genera of the isolated bacteria. Only the maximum similarity percentage and maximum score results were considered as a valid result.

3.4 Artemia Bioassays

These tests were performed following the methods proposed by Metcalf et al. (2002) with some adjustments. The brine shrimp eggs (Ocean Nutrition™) were hatched in a solution of artificial salt water (30 g salt (Tropical Marine Center) in 1 L of ultrapure water (1 g eggs/L Salt Water)). The conditions of luminosity and airing and temperature were maintained constant through the hatching period. The incubation system used is represented below (Fig. 6). In order to perform the toxicity tests 10 nauplii (contained in 100 µl incubation solution) were transferred to 24-well cell culture plates (COSTAR®) each with 900 µl of different concentrations (0.9; 0.68; 0.45; 0.23; 0.09 mg/ml) of the test solution obtained from the bacterial biomass collected in the preceding steps. An extra test was also performed utilizing a duplicate of each concentration tested above but subjecting the cells to ultra sonication (70 Hz, 1 min) (Vibra Cell, Sonic & Materials, Reagente 5, Portugal) before plating. Each of the plates was then wrapped in tinfoil and incubated under the above mention conditions.

Death occurrence was registered every 3, 24, 72 hours for each plate, the animals were then sacrificed with 100 µl of 5% (v/v) Lugol solution followed by a final counting. Triplicates were made per concentration for all samples (Metcalf, Lindsay et al. 2002).

Figure 6: Incubation system.

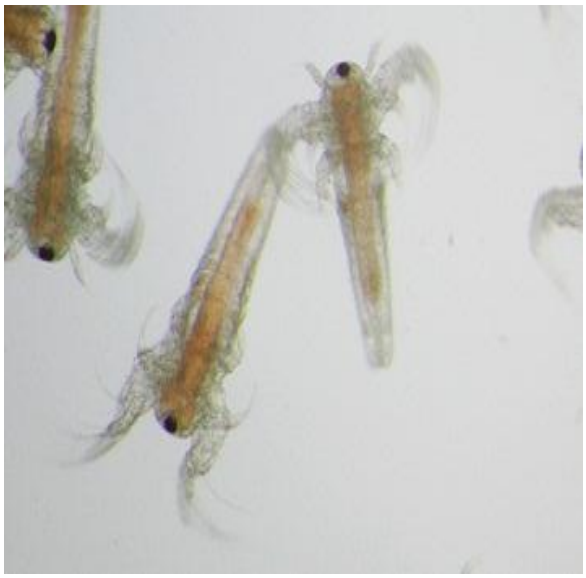


Figure 7: *Artemia Salina*

4. Results

4.1 *Sample collection and bacterial isolation*

From the five beaches sampled, a total of 51 bacterial colonies were isolated (Table 4), 9 colonies from Aguda beach (V.N.G.), 7 colonies from Memória beach (Porto), 7 colonies from Angeiras beach (Matosinhos), 4 colonies from a second sampling of Memória beach, 9 colonies from Valadares beach (V.N.G.) based on morphological parameters of size, colonial structure and visual characteristics.

Memória beach was selected for triple sampling in order to detect possible variations of the bacterial content in the gut of the animals collected relating to changing seasonal conditions.

Table 4: Number of isolated bacterial colonies per sampling

Date of sampling	Site of Sampling	Collected Sample	Nº of species isolated
November 2010	Aguda	*Gibulla umbilicalis*	2
		Monodonta turbinata	2
		B.F.	2
		S.W.	3
January 2011	Memória	*Gibulla umbilicalis*	1
		Monodonta turbinata	2
		B.F.	2
		S.W.	2
February 2011	Angeiras	*Gibulla umbilicalis*	1
		Monodonta turbinata	2
		B.F.	2
		S.W.	2

Table 4: Number of isolated bacteria colonies per collection (continued)

Date of sampling	Site of Sampling	Collected Sample	Nº of species isolated
March 2011	Memória	*Gibbula umbilicalis*	1
		Monodonta turbinata	1
		B.F.	1
		S.W.	1
April 2011	Valadares	*Gibulla umbilicalis*	3
		Monodonta turbinata	2
		B.F.	2
		S.W.	2
May 2011	Memória	*Gibbula umbilicalis*	1
		Monodonta turbinata	3
		B.F.	1
		S.W.	1
June 2011	S. Bartolomeu do Mar	*Gibbula umbilicalis*	2
		Monodonta turbinata	2
		B.F.	1
		S.W.	2

4.2 *Genotypic analysis and identification*

The genomic DNA of all the 51 isolated was extracted initially. In order to achieve confirmation of DNA extraction a gel with the obtained products was run as shown in figure 8. The 16S rRNA content was then amplified utilizing the universal primer set 8F/1492R as shown in figure 9. A last run was executed to confirm successful purification the 16S rRNA amplified products as shown in figure 10.

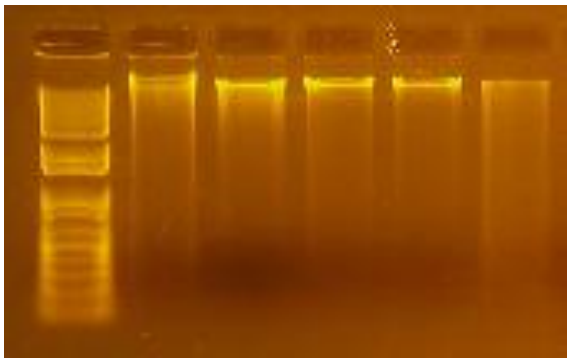


Figure 8: DNA extraction, Angeiras beach. Left to right; Mol. ruler, Mono I, Mono II, Gib I, BF I, BF II

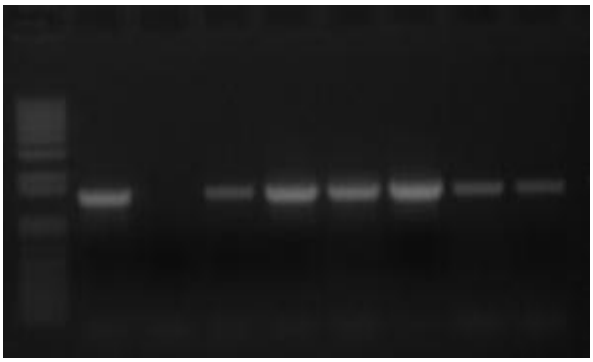


Figure 9: 16s RNA amplification product of Aguda beach. Left to right; Mol. ruler, PC, NC, Mono I, Mono II, Gib I, Gib II, BF I, BF II

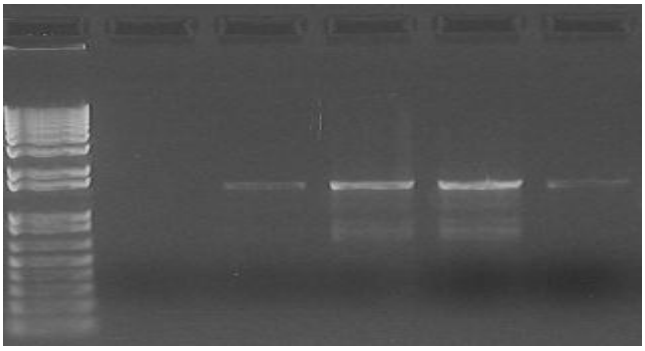


Figure 10: Confirmation of good rRNA purification from Angeiras beach. From left to right; Mol. ruler, NC, Mono I, Mono II, Gib I

The results obtained from the sequencing of the genome of each of the analyzed species were then inserted upon the BLAST database in order to obtain their identification (Table 5).

Table 5: Genotypic identification of isolated bacteria

Collection site	Collection code	Species	Accession number	Max. Identity value
	Gib 01	*Vibrio gigantis* strain S-15	JF412224	99%
	Gib 02	*Vibrio gigantis* strain S-34	JF412230	99%
	Mono 01	*Photobacterium sp.* Mj139	GQ454940	99%
	Mono 02	*Vibrio splendidus* isolate PB1-10rrnG	EU091331	99%
Aguda	B.F. 01	*Vibrio gallaecicus* strain CECT 7244	EU541605	99%
	B.F. 02	*Vibrio splendidus* isolate PB1-10rrnA	EU091325	99%
	S.W. 01	*Vibrio splendidus* isolate PB1-10rrnA	EU091325	99%
	S.W. 02	*Pseudoalteromonas* sp. RE2-11	AF539778	99%
	S.W. 03	*Vibrio splendidus* LGP32 chromosome 1	FM954972	99%

Table 5: Genotypic identification of isolated bacteria (continued)

Collection site	Collection code	Species	Accession number	Max. Identity value
	Gib 01	*Vibrio sp.* BSw21772	JF697302	100%
	Mono 01	*Vibrio sp.* V004	DQ146970	99%
	Mono 02	*Vibrio sp.* IRL552	GQ414576	99%
Memória (First Sampling)	B.F. 01	*Vibrio gigantis* strain S-7	JF412219	99%
	B.F. 02	*Pseudoalteromonas marina*	FR750954	100%
	S.W. 01	*Vibrio gigantis* strain S-34	JF412230	99%
	S.W. 02	*Vibrio sp.* BSi20140	DQ492722	99%

Table 5: Genotypic identification of isolated bacteria (continued)

Collection site	Collection code	Species	Accession number	Max. Identity value
Angeiras	Gib 01	*Vibrio sp.* B69	FN295820	99%
	Mono 01	*Vibrio sp.* B69	FN295820	99%
	Mono 02	*Vibrio sp.* BSw21405	FJ748512	98%
	B.F. 01	*Alteromonadaceae* bacterium P120	EU195925	97%
	B.F. 02	*Pseudoalteromonas sp.* BSw20104	EU365600	99%
	S.W. 01	*Vibrio tasmaniensis* strain Mj28	GQ455006	99%
	S.W. 02	*Vibrio splendidus* LGP32 chromosome 1	FM954972	98%
Memória (second sampling)	Gib 01	*Vibrio gigantis* strain S-15	JF412224	99%
	Mono 01	*Vibrio sp.* B69	FN295820	99%
	B.F. 01	*Pseudoalteromonas sp.* BR050	FJ889570	99%
	S.W. 01	*Pseudoalteromonas marina*	FR750954	99%

Table 5: Genotypic identification of isolated bacteria (continued)

Collection site	Collection code	Species	Accession number	Max. Identity value
	Gib 01	*Vibrio sp.* B69	FN295820	99%
	Gib 02	*Bacillus sp.* strain TZQ22	HQ143630	99%
	Gib 03	*Bacillus stratosphericus* strain GD65	HQ857755	99%
	Mono 01	*Vibrio tasmaniensis* strain Mj28	GQ455006	99%
Valadares	Mono 02	*Shewanella pacifica* strain UDC382	HM031976	98%
	B.F. 01	*Shewanella surugensis* strain c959	NR_040950	97%
	B.F. 02	*Bacillus sp.* JG-TB2	FR849914	99%
	S.W. 01	*Vibrio sp.* B69	FN295820	99%
	S.W. 02	*Alteromonadaceae* bacterium P120	EU195925	98%

Table 5: Genotypic identification of isolated bacteria (continued)

Collection site	Collection code	Species	Accession number	Max. Identity value
Memoria (third sampling)	Gib 01	*Vibrio tapetis* strain CECT 4600	NR_026361	97%
	Mono 01	*Vibrio splendidus* isolate PB1-10rrnM	EU091337	98%
	Mono 02	*Shewanella surugensis* strain c959	NR_040950	98%
	Mono 03	*Vibrio sp.* P124	EU195936	99%
	B.F. 01	N.I.	N.I.	N.I.
	S.W. 01	*Vibrio gigantis* strain S-34	JF412230	99%
S. Bartolomeu do Mar	Gib 01	Mucus bacterium 59	AY654788	99%
	Gib 02	*Vibrio gigantis* strain S-34	JF412230	99%
	Mono 01	*Vibrio sp.* HNS024	JN128258	99%
	Mono 02	*Vibrio cyclitrophicus* strain LMG21359	DQ481610	100%
	BF 01	*Pseudoalteromonas sp.* RE1-12a	AF539781	99%
	SW 01	*Vibrio tasmaniensis* strain 04102	AM422801	99%
	SW 02	*Pseudoalteromonas sp.* RE1-12a	AF539781	99%

Of the 51 initially isolated species, 50 were successfully sequenced and identified. As indicated in table 5 the obtained maximum identity values varied in the range of 97 to 100 per cent which indicates good significance of the obtained alignments.

Due to extremely low quality of the obtained sequences, the sample corresponding to the biofilm collection of the third sampling of Memória beach was impossible to align properly and therefore identify.

The sequences utilized for identification purposes are presented in the supplementary information and are a result of manual manipulation of the original forward and reverse raw sequences to obtain the highest representativity possible and therefore more valid results. In table 5 a more brief representation of the identifications achieved is presented.

Table 6: Species identified

Sampling site	Sample	Species
Aguda	Gib 01	*Vibrio gigantis*
	Gib 02	*Vibrio gigantis*
	Mono 01	*Photobacterium sp.*
	Mono 02	*Vibrio splendidus*
	Bio 01	*Vibrio gallaecicus*
	Bio 02	*Vibrio splendidus*
	S.W. 01	*Vibrio splendidus*
	S.W. 02	*Pseudoalteromonas* sp.
	S.W. 03	*Vibrio splendidus*
Memória (1st Sampling)	Gib 01	*Vibrio sp.*
	Mono 01	*Vibrio sp.*
	Mono 02	*Vibrio sp.*
	B.F. 01	*Vibrio gigantis*
	B.F. 02	*Pseudoalteromonas marina*
	S.W. 01	*Vibrio gigantis*
	S.W. 02	*Vibrio sp.*
Angeiras	Gib 01	*Vibrio sp.*
	Mono 01	*Vibrio sp.*
	Mono 02	*Vibrio sp.*
	Bio 01	*Alteromonadaceae*
	Bio 02	*Pseudoalteromonas sp.*
	S.W. 01	*Vibrio tasmaniensis*
	S.W. 02	*Vibrio splendidus*

Table 6: Species identified (continued)

Sampling site	Sample	Species
Memória (2nd Sampling)	Gib 01	*Vibrio gigantis*
	Mono 01	*Vibrio sp.*
	B.F. 01	*Pseudoalteromonas sp.*
	S.W. 01	*Pseudoalteromonas marina*
Valadares	Gib 01	*Vibrio sp.*
	Gib 02	*Bacillus sp*
	Gib 03	*Bacillus stratosphericus*
	Mono 01	*Vibrio tasmaniensis*
	Mono 02	*Shewanella pacifica*
	B.F. 01	*Shewanella surugensis*
	B.F. 02	*Bacillus sp.*
	S.W. 01	*Vibrio sp.*
	S.W. 02	*Alteromonadaceae*
Memória (3rd Sampling)	Gib 01	*Vibrio tapetis*
	Mono 01	*Vibrio splendidus*
	Mono 02	*Shewanella surugensis*
	Mono 03	*Vibrio sp.*
	B.F. 01	N.I.
	S.W. 01	*Vibrio gigantis*
São Bartolomeu do Mar	Gib 01	Mucus bacterium
	Gib 02	*Vibrio gigantis*
	Mono 01	*Vibrio sp.*
	Mono 02	*Vibrio cyclitrophicus*
	BF 01	*Pseudoalteromonas sp.*
	SW 01	*Vibrio tasmaniensis*
	SW 02	*Pseudoalteromonas sp.*

Bacteria of the *Vibrio* genus were predominant with 30 positive identifications encountered; also found were bacteria contained in the *Pseudoalteromonas*, *Shewanella*, *Photobacterium*, *Alteromonadaceae* and *Bacillus* genera as well as a single identification of mucus bacteria.

4.3 *Artemia Bioassays*

Results of the toxicity test were negative as it was impossible to establish a valid concentration response relationship on any of the solutions tested.

The physical properties of the solutions used (turbidity and sedimentation rate mainly) made impossible the adjustment of the concentration parameters to higher values and as such the tests were not repeated.

5. Discussion

In regards to the sampling procedures of the analyzed species (Table 4), in all of the beaches visited for collection a large presence of both *Monodonta turbinata* and *Gibbula umbilicalis* were encountered regardless of the time of sampling (November 2010 to June 2011) however it was observed that *Monodonta turbinata* was the species with heaviest presence in all of the sampling events. The month of December 2010 was not subject to sampling due to time and weather restrictions.

Extraction and isolation of the bacteria obtained from the gut of the gastropods were achieved with relative ease through streaking with only a few cases of contamination promptly solved with further isolations. The bacterial colonies were isolated taking into consideration morphologic characteristics namely color, ranging from a milk like tone of white, to orange or bright yellow; colonial formation, round, radiating or amorphous mat like colonies and other punctual characteristics observed such as depth of penetration on the solid medium.

Biomass growth was found to reach optimal growth within 5 to 7 days of incubating at the mentioned parameters of temperature and luminosity. The volume of biomass collected from each of the samples through centrifugation (300 ml of peptone culture medium was used per sample) was within the 2 to 4 ml range, enough to enable genomic DNA extraction, toxicity testing and culture regrowth if further testing is to be performed, even so biomass waste upon collection of the centrifuged pellet was evident and must be considered for optimization.

The genotypic identification of the obtained bacteria was performed to specification and with good results the only exception being the colony extracted from the biofilm of the second sampling in Memória beach. This sample yielded a severely damaged reverse sequence which in turn made it impossible to perform the alignment required for proper identification.

The identification results were mostly successful as the large majority of the bacteria encountered are genera already found to be marine animal symbionts with the potential for TTX production as is the case of the *Vibrio (Noguchi, Jeon et al. 1986; Simidu, Noguchi et al. 1987)* and *Shewanella (Yasumoto, Yasumura et al. 1986; Matsui, Taketsugu et al. 1990)* groups of bacteria that have been shown to have the capability to synthetize TTX and anhydroTTX. Bacteria of the *Photobacterium* genera, namely *Photobacterium phosphoreum (Simidu, Noguchi et al. 1987)* have also been show to produce TTX although the identification attained relative to this bacteria in this study was not species specific being therefore inconclusive. *Pseudoalteromonas* is yet another

genus of bacterium known as a eukaryotic symbiont (Holmstrom and Kjelleberg 1999) and as a TTX producer (Simidu, Kita-Tsukamoto et al. 1990) that was encountered. The bacteria of the *Bacillus* genus that were found solely on Valadares beach are also reported TTX producers but are bacteria not typically found in marine environments (Do, Hamasaki et al. 1993) therefore raising questions as to their appearance in the biofilm and gut of the *Gibbula umbilicalis* specimens collected from that beach. Lastly, one of the bacterial samples analyzed was identified as a Mucus bacterium, a group of bacteria isolated form the mucus of the Mediterranean coral *Oculina patagonica*, not being a decisive identification per se this identification is nonetheless plausible within the aims of this study as a large majority of the bacteria contained in this group are part of the *Pseudomonas* and *Vibrio* genera.

Localization wise it bears to notice that even though bacteria of the *Vibrio* genus are widely distributed and can be found in all of the beaches sampled the remaining genera appear to be more localized with the appearance of *Photobacterium sp.* Restricted to Angeiras beach while the *Bacillus* and *Shewanella* bacterium were confined to Valadares beach.

The sampling of Memória beach was made in three distinct periods in order to observe possible variations in the bacteria of the gastropods guts throughout the different seasons namely Winter (January), early Spring (March) and late Spring, early Summer (May). It was found that the content in *Vibrio* bacteria in the gut of the animals remained mostly unaltered throughout the sampling dates but there was a change in the content of Pseudoalteromonas bacteria found in the surrounding environment being present in January, peaking in March and disappearing in late May. Bacteria from the Shewanella genus were also found to be present during the sampling of May in the gut of the sampled *Monodonta turbinata* and absent in the months of January and March. The alteration in these two genera may be due to the changing in weather conditions especially temperature during the year.

No direct correlation was apparent between the bacterial content of the gut of the gastropods and that of the surrounding waters and biofilm analysis other than the fact that the microflora of the snail's intestines appears to be composed almost exclusively of bacteria of the Vibrio genus as on the other and a much greater variability was encountered on the surrounding environments, this may suggest that there is some sort of selectivity in the intake of bacteria from the exterior environment at a trophic level of the snail although further testing would be required in order to support this claim.

The toxicity tests with *Artemia salina* were negative as it was impossible to establish a congruent concentration-response relation amongst the tests performed. Tests with both intact and ruptured cells (using sonoporation) were performed in order to conclude whether the integrity of the cell itself affected its toxicity to the animals, again this testing was generally inconclusive although several samples shown a small increase in toxicity in the sonicated samples, the non-interpretable nature of these results made it impossible to definitively conclude as to this theme.

Even though the toxicity tests were concentration/time negative the mortality rates among the nauplii used in them are still relatively high in most samples, especially in the Surrounding water 02 sample from Aguda beach which showed approximately 80% mortality rates in high and medium concentrations as early as 24 hours after inoculation and 99% mortality rates at the 72 hour count, these mortality rates are then abruptly different from the ones reported in another work (Silva and Vasconcelos 2010) where the toxin was extracted with dichloromethane and then inoculated in wells with *Artemia* nauplii reporting totally negative results mortality wise. The high mortality rates observed in these tests may perhaps be due to the sedimentation of the bacterial cells which caused zones of great bacterial concentration on the wells causing visible locomotion difficulties on the animals and perhaps causing biological difficulties as well leading to their death.

6. Final Considerations and Future Perspectives

TTX is still a vastly disregarded toxin in Portuguese waters being the only species known to possess the toxin in national waters the sea snail *Charonia lampas lampas* and more recently the marine gastropods *Monodonta turbinata* and *Gibbula umbilicalis* and suspected the equinoderm *Paracentrotus lividus*.

The goals of this work were achieved:

- The culture and isolation of bacterial symbionts of marine gastropods of the Portuguese shore.
- The identification of the bacterial species most likely to cause the TTX positive results in the gastropods *Monodonta turbinata* and *Gibbula umbilicalis*.

Several bacteria reported in earlier studies to have the capability to produce TTX were isolated from two species of ubiquitous sea snails of the Portuguese shore, snails which have been confirmed to possess the potent neurotoxin. As such, and even though the presence of those bacteria does not directly implicate the presence of TTX it is a very significative evidence of such.

It was shown that the variations in seasonal temperature may in fact play a role in the bacterial symbionts present in an animal at a given time which in turn may lead to increased or decreased toxicity upon consumption by a predator.

This work has served to further illuminate the obscure theme of TTX presence in Portuguese waters hopefully being a stepping stone for future work, namely in more profound genetic studies to understand the biological pathways of the toxin and it's interaction with its vectors, or perhaps in order to further correlate bacterial content variations with toxicity changes in these animal.

It is a fact that TTX is present in National waters, it is now then more than ever, imperative do further our knowledge on this subject in order to prevent possible environmental hazards.

7. References

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8. Suplementary information

8.1 Bacterial 16S rRNA sequences

Aguda beach

Gib I

TATGCGGTACCAGCGGAACGACACTAACAATCCTTCGGGTGCGTTAATGGGCGTCGAGCGGCGGACGGGT
GAGTAATGCCTAGGAAATTGCCTTGATGTGGGGGATAACCATTGGAAACGATGGCTAATACCGCATAATG
CCTACGGGCCAAAGAGGGGGACCTTCGGGCCTCTCGCGTCAAGATATGCCTAGGTGGGATTAGCTAGTTG
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TACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTGGGCTGCAAAAGTAAGTGGGTAGTTT
AACCTATAGAGTATGACG

Gib II

GCAGTCGAGCGGAACGACACTAACAATCCTTCGGGTGCGTTAATGGGCGTCGAGCGGCGGACGGGTGAG
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CGGGCCAAAGAGGGGGACCTTCGGGCCTCTCGCGTCAAGATATGCCTAGGTGGGATTAGCTAGTTGGTGA
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Mono I

CATGCAGTCGAGCGGTAACAGGAATTAGCTTGCTAATTTGCTGACGAGCGGCGGACGGGTGAGTAATGCC
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Mono II

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B.F. I

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B.F. II

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S.W. I

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S.W. II

ATGGCAAGTCGAGCGGTAACATTTCTAGCTTGCTAGAAGATGACGAGCGGCGGACGGGTGAGTAATGCTT
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ACC

S.W. III

CAANCTACACATGCAGTCGAGCGGAACGACACTATATAGATATCTTCGGGTGCTGTTAATGGGCGTCGAG
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Memória beach (1st sampling)

Gib I

CTCGCGTCAAGATATGCCTAGGTGGGATTAGCTAGTTGGTGAGGTAATGGCTCACCAAGGCGACGATCCC
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Mono I

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Mono II

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B.F. I

TGCAGTCGAGCGGAGACGACACTAACAATCCTTCGGGTGCGTTAATGGGCGTCGAGCGGCGGACGGGTG
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B.F. II

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S.W. I

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S.W. II

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CGTTCCC

Angeiras beach

Gib I

TGCAGTCGAGCGGAACGACACTAACAGATATCTTCGGGTGAGTTAATGGGCGTCGAGCGGCGGACGGGT
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Mono I

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Mono II

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AGCCGCGGTAATACGGAGGGTGCGAGCGTTAATCGGAATTACTGGGCGTAAAGCGCATGCAGGTGGTTC
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B.F. I

GCCTACCATGCAGTTCGAGCGGTAACAGAAAGAAGCTTGCTTTCTTTGCTGACGAGCGGCGGAGGGGTGA
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B.F. II

ACACATGCAGTCGAGCGGTAACAGACAAGTAGCTTGCTACTTTGCTGACGAGCGGCGGACGGGTGAGTAA
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GTTA

S.W. I

CATGCAGTCGAGCGGAACGACACTATACAGATACCTTCGGGTGCGTTAATGGGCGTCGAGCGGCGGACG
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S.W. II

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GTGAATACGTTCCCGGGCCTTGTACAACACCGCCCGTCACACCATGGGGAGTGGGCTGCAAAAGGAAAGT
GGGTAAGTTTAACCCTTTCGGGGAGGACGCTCAC

Memória beach (2nd sampling)

Gib I

CATGCAGTCGAGCGGAACGACACTAACAATCCTTCGGGTGCGTTAATGGGCGTCGAGCGGCGGACGGGT
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Mono I

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CCATTCGGGGAG

B.F. I

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S.W. I

TGCAGTCGAGCGGTAACAGAAAGTAGCTTGCTACTTTGCTGACGAGCGGCGGACGGGTGAGTAATGCTTG
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Valadares beach

Gib 01

ACACATGCAGTCGAGCGGAACGACACTAACAATACCTTCGGGTGCGTTAATGGGCGTCGAGCGGCGGACG
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Gib 02

ATACATGCAGTCGAGCGGACAGAAGGGAGCTTGCTCCCGGATGTTAGCGGCGGACGGGTGAGTAACACG
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Gib 03

TGCAGTCGAGCGGACAGAAGGGAGCTTGCTCCCGGATGTTAGCGGCGGACGGGTGAGTAACACGTGGGT
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Mono 01

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Mono 02

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CTTCGGG

B.F. 01

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B.F. 02

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S.W. 01

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S.W. 02

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Memória beach (3rd sampling)

Gib 01

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Mono 01

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Mono 02

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GGAGGGCGTTA

Mono 03

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B.F. 01

N.I.

S.W. 01

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São Bartolomeu do Mar beach

Gib 01

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Gib 02

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Mono 01

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Mono 02

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B.F. 01

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S.W. 01

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S.W. 02

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