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Sudden peak in tetrodotoxin in French oysters during the summer of 2021: Source investigation using microscopy, metabarcoding and droplet digital PCR

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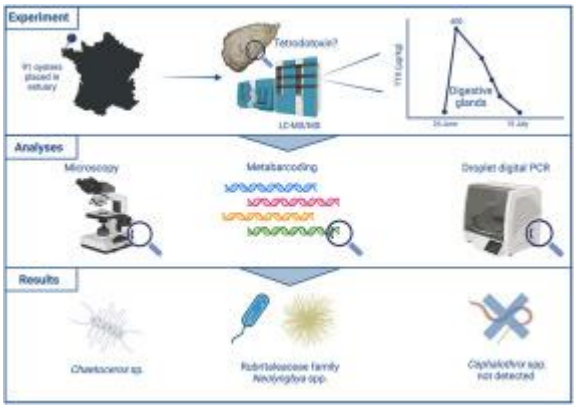
Abstract :

Tetrodotoxin (TTX) is a potent neurotoxin causing human intoxications from contaminated seafood worldwide and is of emerging concern in Europe. Shellfish have been shown to contain varying TTX concentrations globally, with concentrations typically higher in Pacific oysters Crassostrea gigas in Europe. Despite many decades of research, the source of TTX remains unknown, with bacterial or algal origins having been suggested. The aim of this study was to identify potential source organisms causing TTX contamination in Pacific oysters in French coastal waters, using three different techniques. Oysters were deployed in cages from April to September 2021 in an estuary where TTX was previously detected. Microscopic analyses of water samples were used to investigate potential microalgal blooms present prior or during the peak in TTX. Differences in the bacterial communities from oyster digestive glands (DG) and remaining flesh were explored using metabarcoding, and lastly, droplet digital PCR assays were developed to investigate the presence of Cephalothrix sp., one European TTX-bearing species in the DG of toxic C. gigas. Oysters analysed by liquid chromatography-tandem mass spectrometry contained quantifiable levels of TTX over a three-week period (24 June–15 July 2021), with concentrations decreasing in the DG from 424 µg/kg for the first detection to 101 µg/kg (equivalent to 74 to 17 µg/kg of total flesh), and trace levels being detected until August 13, 2021. These concentrations are the first report of the European TTX guidance levels being exceeded in French shellfish. Microscopy revealed that some microalgae bloomed during the TTX peak, (e.g., Chaetoceros spp., reaching 40,000 cells/L). Prokaryotic metabarcoding showed increases in abundance of Rubritaleaceae (genus Persicirhabdus) and Neolyngbya, before and during the TTX peak. Both phyla have previously been described as possible TTX-producers and should be investigated further. Droplet digital PCR analyses were negative for the targeted TTX-bearing genus Cephalothrix.

Graphical abstract



Highlights

► Tetrodotoxin exceeded European guidance value in French oysters (74 µg/kg whole flesh). ► Microscopic analyses suggested co-occurrence of TTX with *Chaetoceros* spp. ► Metabarcoding detected the bacterial family Rubritaleaceae and cyanobacteria *Neolyngbya* spp. before and during the TTX peak.

Keywords : Biotoxin, Bivalves, Cyanobacteria, ddPCR, Metabarcoding, Microalgae, Microbiome

1. Introduction

Tetrodotoxin (TTX) is one of the most potent naturally occurring marine toxins and ingestion of TTX-contaminated seafood results in numerous, sometimes fatal, human intoxications every year (Bagnis et al., 1970; Hwang and Noguchi, 2007; Katikou et al., 2022). TTX has a surprisingly broad distribution across highly diverged marine, freshwater and terrestrial taxonomic groups, including 14 classes of eukaryotes and five bacteria (Bane et al., 2014; Biessy et al., 2019; Chau et al., 2013). Because of its widespread occurrence in unrelated taxa, there is considerable debate about its ecological role and potential source organisms (Williams, 2010). TTX has been reported in at least 21 species of edible bivalves and gastropods from ten countries since the 1980's (Biessy et al., 2019). No regulatory limit for TTX in bivalves exists despite a scientific opinion published in 2017 by the European Food Safety Authority (EFSA), recommending a concentration below 44 µg TTX equivalent per kg of shellfish meat, based on a 400 g portion size (Knutsen et al., 2017). In the last decade, many European countries have reported the occurrence of TTXs in bivalve mollusks, with most confirmations from the Netherlands, Spain, Italy, England, Greece and France (Blanco et al., 2019; Bordin et al., 2021; Dell'Aversano et al., 2019; Gerssen et al., 2018; Hort et al., 2020; Leão et al., 2018; Réveillon et al., 2021; Turner et al., 2015; Turner et al., 2017; Vlamis et al., 2015). In France, TTX has been found in mussels (*Mytilus edulis* and *M. galloprovincialis*) and common whelk (*Buccinum undatum)* from the Mediterranean and Atlantic coasts (Hort et al., 2020). Oysters (*Crassostrea gigas*) and cockles (*Ruditapes philippinarum*) from four sites across the Normandy and Brittany regions also tested positive for TTX with concentrations reaching up to 32 µg/kg and events lasting a maximum of two weeks (Réveillon et al., 2021). Even though temperature is suggested to play a role in TTX occurrence in bivalves across the United Kingdom (Dhanji-Rapkova et al., 2023), as would be expected for a bacterial origin, this trend has not been confirmed at the European scale, with lower or similar TTX concentrations in shellfish reported from warmer waters (e.g., Greece and Italy; (Dell'Aversano et al., 2019; Vlamis et al., 2015).

To manage the risk associated with TTX in seafood, it is important to understand its origin, how it accumulates and is transported between organisms. The two most common hypotheses regarding the origin of TTX are that 1) it is produced by symbiotic bacteria (endogenous) or that 2) it is exogenously accumulated through the diet (e.g., bacteria, microalgae) (Chau et al., 2011). Exogenous or symbiotic bacteria are commonly suggested as the most likely TTX-producers, with a wide diversity of species and strains implicated. There are reports in the literature of at

least 150 TTX-producing bacterial strains (Katikou, 2019; Magarlamov et al., 2017). *Vibrio*, *Bacillus*, *Pseudomonas*, *Actinomyces* and *Micrococcus* are the most commonly reported bacterial genera associated with TTX production, however, only one study has claimed to successfully maintain TTX-producing bacteria in culture (Melnikova et al., 2019). When bacterial cultures reported to produce TTX were analysed using modern highly-specific analytical methods (e.g., liquid-chromatography with tandem mass spectrometry), no evidence of TTX production was found and potentially indicated contamination from the starting material (Biessy et al., 2019; Katikou et al., 2022). There has been evidence of production of TTX-like substance by microalgae in one study suggesting that the dinoflagellate *Alexandrium tamarense* produced TTX (Kodama et al., 1996) and more recently, the presence of high concentrations of the dinoflagellate *Prorocentrum minimum* (now *P. cordatum)* was potentially linked to TTX accumulation in Greek shellfish (Vlamis et al., 2015). These links between microalgae and TTX production have not been confirmed to date and despite sparse evidence on microalgal origin, there is a rationale behind the role of microplankton in TTX accumulation in bivalves, in particular with the preferential accumulation of TTX in the digestive systems of marine organisms (Dhanji-Rapkova et al., 2021). This alone forms a basis for studying microalgal and microbial communities in water and the digestive glands of shellfish before, during and after periods of TTX accumulation.

In 2009, *Paphies australis*, an endemic and abundant clam regularly harvested for food in New Zealand, was reported to accumulate up to 800 µg TTX/kg (McNabb et al., 2014). Recent research investigated the source of TTX in *P. australis* (Biessy, 2021) and metabarcoding was used to explore the microbiome of *P. australis* temporally and spatially. The results highlighted a new lead in the search for TTX producers: marine cyanobacteria. Cyanobacteria, especially picocyanobacteria from the *Cyanobium*, *Synechococcus*, *Pleurocapsa*, and *Prochlorococcus* genera were present in the digestive glands and siphons of *P. australis* containing TTX but were absent from the organs of non-toxic clams (Biessy et al., 2020). Multiple researchers have explored the source of TTX at higher trophic levels over the last decades (Noguchi et al., 2006) and it has been hypothesized that different organisms accumulate TTX through the food web (Matsumura, 1998). Some examples include the trumpet shell *Charonia sauliae* that accumulated TTX by ingesting toxic starfish (Noguchi et al., 1982), or the sea slug *Pleurobranchaea maculata* that likely accumulated TTX from a TTX-containing marine flatworm *Stylochoplana* sp. found in its stomach (Salvitti et al., 2015), or the non-toxic pufferfish *Takifugu obscurus* that accumulated TTX in their liver and skin feeding on wild TTX-containing gastropods (Zhang et al., 2020). The accumulation process can be fast, as for the sea slug *P. maculata* maintained in

aquaria that accumulated TTX within one hour of being fed a prepared TTX-containing food source (Khor et al., 2013). Interestingly, Okabe et al. (2019) found DNA from the toxic flatworm *Planocera multitentaculata* in the intestine of the pufferfish *T. niphobles*. Further investigation showed that the pufferfish were accumulating TTX from consuming the flatworms' highly toxic eggs. Vlasenko and Magarlamov (2020) recently found high concentrations of TTX in the intestine of the marine nemertean *Cephalothrix* cf. *simula* and Malykin et al. (2023) demonstrated that during the spawning season of *Cephalothrix* species, their eggs can be source of TTX for filter-feeding shellfish. Lastly, high TTX concentrations were detected in the midgut gland of the Japanese scallop *Chlamys farreri* subsp. *akazara* and the source was likely the larvae of the flatworm, *P. multitentaculata* (Yasukawa et al., 2023). This is of importance as it highlights that TTX-bearing eggs from TTX-bearing organisms can be filtered and be the source of TTX accumulation in shellfish. In Europe, one TTX-bearing invasive species that has been detected in the last decade is the ribbon worm *Cephalothrix simula* (Turner et al., 2018). This species is a motile and commonly reported aquatic TTX-bearing species in Europe. Therefore, a detection assay was developed to assess if potentially toxic eggs of *C. simula* could accumulate in filter-feeding shellfish, resulting in the accumulation of TTX in the French oysters.

To date, TTX concentrations in French shellfish were found to peak for a few weeks in early summer (Réveillon et al., 2021) but data remain sparse for determining environmental dynamics due to low frequency of the national surveillance of emerging toxins (Amzil et al., 2023; Hort et al., 2020). However, the reasons for TTX variability over time and its source organisms in shellfish remain unknown. The overarching goal of the present study was to investigate the source of TTX in a French shellfish production site. Non-toxic adult Pacific oysters, *Crassostrea gigas*, purchased from a farm, were placed in cages in the Loperhet estuary where a previous peak of TTX had been observed. The approach of the study was to link TTX-concentrations observed in the oysters by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) to potential source organisms by: 1) characterising the eukaryotic microalgae in the surrounding waters and the bacterial communities in different organs of *C. gigas,* sourced weekly or fortnightly from the targeted estuary, using microalgal cell counts and metabarcoding techniques respectively; and to 2) develop a targeted assay to detect potential sources of TTX in *C. gigas*. In this case, a droplet digital PCR assay (Hindson et al., 2011) was developed to detect genetic material from *Cephalothrix* sp.

2. Material and Methods

2.1. Field experimental set-up and sampling

Triploid Pacific oysters *Crassostrea gigas* (n = 91) grown from spat and of similar size were purchased from an oyster farm (Vrennig, Aber Wrach, France), and were placed in oyster bags at Loperhet, France (48.34388889, -4.300555556; Figure 1) on April 1, 2021. Oysters were harvested 13 times, fortnightly from 11 May 2021, weekly in June and July 2021 (except on the 18th of June when the tides did not allow for sampling) and fortnightly in August and September 2021. At each sampling point, *C. gigas* (n = 6) were harvested for metabarcoding analysis (n = 3) and LC-MS/MS analysis of TTX analogues (3 individuals were pooled to have n = 1 LC-MS/MS analysis per sample). Immediately upon arrival in the laboratory, three individuals were aseptically dissected for genetic and toxin analysis into digestive glands (DG, including the intestine) and remaining flesh (RF), which were placed into individual five mL sterile screw cap tubes that were filled with RNA*later*™ (ThermoFisher, MA, USA) and kept cold (4 °C) until further analysis.

Salinity and water temperature were recorded as part of the REPHY programme (2022) at the "Pointe du Château" site (Figure 1).

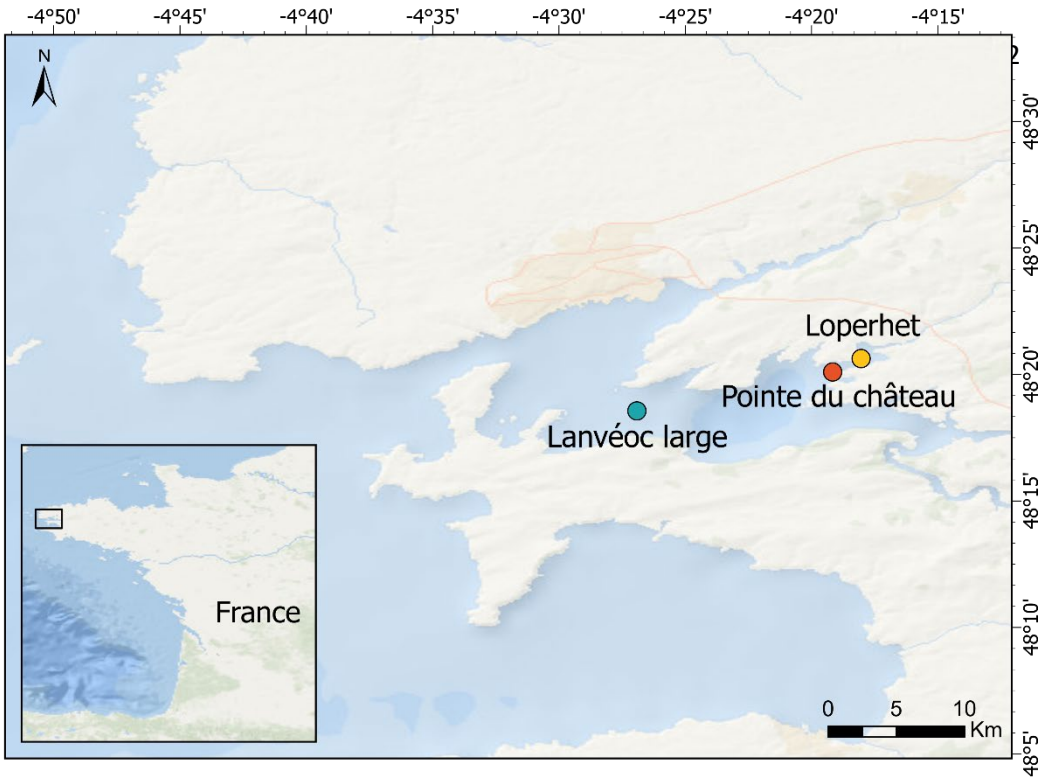


Figure 1 | Map of sampling sites. Loperhet = estuary where oysters were placed for the study; Pointe du Château = site where water temperature and salinity were recorded; Lanvéoc large = site where water samples were collected for microscopy analysis.

2.2. Toxin extractions and LC-MS/MS analysis

Extraction of TTXs from shellfish was performed following the method described in Réveillon et al. (2021). For each oyster sample (n = 3), DG and RF were extracted separately. Briefly, acetic acid (500 µL of 1% Hipersolv CHROMANORM for LC-MS, Aventor™, UK) was added to *C. gigas* (200 mg) before grinding with glass beads (Ø 150–250 µm, VWR) in a mixer mill (10 min, 30 Hz; Retsch MM400 mixer Mill, ThermoFisher). After centrifugation (15,000 × g, 10 min), 250 µL of the supernatant was subsequently collected and filtered with a 3 kDa cut-off (Pall Corporation, NY, USA) before analysis.

LC-MS/MS analyses were performed using an Ultra-Fast Liquid Chromatography system (Nexera, Shimadzu, Kyoto, Japan) coupled to a triple-quadrupole mass spectrometer (5500 QTRAP, Sciex, MA, USA). The parameters of both LC and MS systems were the same as described in Réveillon et al. (2021), as were the limits of detection (LOD) and quantification (LOQ) (5 and 12.5 µg/kg of total flesh). Matrix effect and extraction recovery were defined to estimate the total yield (matrix effect x extraction recovery) that was used to correct TTX concentration in DG (yield 74%, corrective factor of 1.35) and RF (yield 129%, corrective factor 0.775).

Concentrations of TTX obtained in DG and RF were converted into µg/kg of total flesh equivalent by using the percentage of DG and RF mass over the total flesh in addition to the concentration of TTX obtained in each tissue (for example: DG of the oyster sample of June 24 represented 14.2% of total mass and had a concentration of 424 µg/kg while RF represented 85.8% and had a concentration of 16 µg/kg, corresponding finally to (424.3 x 0.142) + (16 x (0.858) = 60 + 14 = 74 µg/kg of total flesh equivalent).

2.3. Microscopy analysis of microalgae in plankton

Water samples were collected at the site 'Lanvéoc large' (48.309405, -4.44852, Figure 1) using a 5-L Niskin bottle at the subsurface (0–1 m) for light microscopy counts of microalgae. Samples were fixed with acidic Lugol's Iodine solution (0.1% final concentration) (Andersen and Throndsen, 2003) and stored at 4 °C until examination. Total assemblages were counted using an inverted microscope (Zeiss, Axio Observer) following REPHY guidelines (REPHY, 2022). Samples were gently homogenized before settling in 10 mL sub-sample for > 12 h in counting

chambers (Utermohl, 1958) (Hydro-Bios, Alenholz, Germany). The lower limit of quantification was 100 cells/L. Data is publicly available (REPHY, 2022).

2.4. Molecular analysis

2.4.1. DNA extractions, PCR and metabarcoding

Dissected organs were removed from RNA*later*™ and were individually placed in the tube containing bashing beads of the DNA extraction kit (DNeasy Powersoil Pro kit, Qiagen, MD, USA), using sterile technique. The DNA was then extracted following the manufacturer's instructions using an automated homogenizer (1600 MiniG Automated Tissue Homogenizer and Cell Lyser, SPEX SamplePrep, NJ, USA) and a robotic workstation for DNA extraction (QIAcube, Qiagen). Negative extraction controls were performed every 23 samples. PCR amplification was undertaken targeting the V3 and V4 regions of the 16S ribosomal RNA (rRNA) gene using the primer sets as described in Klindworth et al. (2013): Bact341F- 5'- CCT ACG GGN GGC WGC AG-3' and Bact785R- 5'-GAC TAC HVG GGT ATC TAA TCC-3'. Primer sets were modified to include Illumina™ overhang adaptors following the dual-indexing method from Kozich et al. (2013).

PCR reactions were undertaken in triplicate with 450 nM of each primer, 25 µL of 2X MyFi™ Mix (Bioline, UK), ca. 5 ng of DNA, and sterile water for a total reaction volume of 50 µL. The cycling conditions were: 95 °C for 5 min, followed by 30 cycles of 94 °C for 30 s, 54 °C for 30 s, 72 °C for 45 s, and a final extension of 72 °C for 7 min. Triplicates of PCR products were pooled and visualized on 1.5% agarose gel with Red Safe™ DNA Loading Dye (Herogen Biotech, USA) and UV illumination. PCR negatives (RNA/DNA-free water Life Technologies) were run to assess for contamination during the PCR steps. The PCR products were purified, cleaned of primer dimers and normalized using SequalPrep Normalisation plate (ThermoFisher, MA, USA), and submitted to Auckland Genomics (University of Auckland, New Zealand) for library preparation. Sequencing adapters and sample-specific indices were added to each amplicon via a second round of PCR using the Nextera™ Index kit (Illumina Inc., USA). Amplicons were pooled into a single library and paired-end sequences (2 × 250 bp) generated on a MiSeq® instrument. The sequencing libraries were prepared following the Illumina 16S Metagenomics Library Prep manual with the exception that after the indexing PCR, five µL of each sample (including three water samples acting as sequencing blank) were pooled and a single clean-up was undertaken on the pool instead of samples being individually cleaned. Quality

control was undertaken using a bioanalyzer before the library was diluted to 4 nM and denatured. A 15% PhiX spike was used, and the final loading concentration was 7 ρM. Sequence data were automatically demultiplexed using MiSeq® Reporter (version 2, Illumina Inc.), and forward and reverse reads assigned to samples. Raw sequence reads were deposited in the National Centre for Biotechnology Information (NCBI) short read archive under the accession number PRJNA1029659 (reviewer link: https://dataview.ncbi.nlm.nih.gov/object/PRJNA1029659?reviewer=eb944as8eah290qvflpdcijcfn).

2.4.2. Bioinformatics

Raw reads were processed, subsequent to primers being removed with *cutadapt* (Martin, 2011), using the *DADA2* package (Callahan et al., 2016) within R. Reads were truncated to 228 and 230 bp and filtered with a maxEE (maximum number of "expected errors") of 2 and 4 for forward and reverse reads respectively (reads not reaching this threshold were discarded). *DADA2* constructs a parametric error matrix (based on the first 10^8 bps in the dataset), the samples are dereplicated and sequence variants for the forward and reverse reads are inferred based on the derived error profiles from the samples. Singletons observed in the inference step were discarded. Subsequently, paired-end reads were merged with a maximum mismatch of 1 bp and a required minimum overlap of 10 bp. Forward and reverse reads, which did not merge were not included in further analysis. Chimeras were removed using the function removeBimeraDenovo. The resulting chimera-checked, merged Amplicon Sequence Variants (ASV) were classified taxonomically using the rdp classifier (Wang, Garrity, Tiedje, & Cole, 2007) with a bootstrap of 70 against the Cyanoseq (Lefler et al., 2023) version of the SILVA v138 reference database (Pruesse et al., 2007). This database updated the taxonomic classification of the cyanobacteria, compared with the default SILVA database. The results were parsed into a table using the *phyloseq* package (McMurdie and Holmes, 2013), and reads assigned as eukaryotes, chloroplasts and mitochondria were removed. Negative controls were assessed, and the maximum read number of each contaminating ASVs in a control sample was subtracted from the samples. To facilitate comparison, samples were subsampled to an even depth of 8214 sequences.

Multivariate patterns were assessed with PERMANOVA undertaken using adonis2 in vegan (Oksanen et al., 2019) with one factor and three levels (Pre-TTX, Peak TTX and post-TTX) using Bray-Curtis distance matrices. Composition plots were plotted using ggplot and ggnested

(Wickham, 2016) at the genus level showing only those taxa that were present at a relative abundance of 2% (bacteria) or 0.5% (cyanobacteria) in at least one sample. ASVs that were present in higher abundance in the TTX peak period were identified by subtracting the maximum number of reads for the respective ASVs present in samples outside the TTX period away from those samples during the peak TTX period. ASVs with read counts greater than 0 were determined to be more abundant in the TTX period and plotted in a similar fashion as the previous composition plots.

2.4.3. Droplet digital PCR assay development

Droplet digital PCR was conducted using an automated droplet generator (QX200 Droplet Digital PCR System, Bio-Rad) to determine the presence or absence of potential TTX sources, *Cephalothrix* sp., in the digestive glands of *C. gigas* samples. DNA extracts were the same as used for metabarcoding analyses. Primers and gBlocks to detect *Cephalothrix* sp. from the digestive glands of *C. gigas* were designed for this study (Table 1). Each ddPCR reaction included 1 µL of 450 nM of each primer, 10 µL Bio-Rad ddPCR Evagreen supermix, 2 µL DNA, and sterile water for a total reaction volume of 22 µL. The Bio-Rad QX200 droplet generator partitioned each reaction mixture into nanodroplets by combining 20 µL of the reaction mixture with 20 µL of Bio-Rad droplet oil. After processing, this resulted in a total nanodroplet volume of 40 µL, which was transferred to a PCR plate for amplification using the following cycling protocol: hold at 95 °C for 5 min, 45 cycles of 95 °C for 30 s, 60 °C for 60 s, one final cycle of signal stabilisation of 4 °C for 5 min and 90 °C for 5 min for the *Cephalothrix* sp. assay. The plate was then analysed on the QX200 instrument. For each ddPCR plate run, at least one negative control (RNA/DNA-free water Life Technologies), and one positive control (using the gBlocks designed for the assay, Table 1) were included.

Table 1 | Sequences of primers and gBlock designed and used in this study.

Target	Sequence	NCBI accession number	Targeted gene region
***Cephalothrix* sp,**			
Fw_Cephalothrix	GTAGTTGACCTCCTTCAGGTATTG		
R_Cephalothrix	TCCTCCTAAACGATCTCCTTC		
gblock_C_simula	CCCTAGGATGGAGTTAGGTTGTAGTTGAC CTCCTTCAGGTATTGAGCCTTTAAATCCTT TTTCGGTACCTTTATTGAATACTGCTGTTC TTTTGGCTTCTGGAATTACTGTAACCTGAT CTCATCATTCTATAATAGAAGGAGATCGT TTAGGAGGAATTCAA GGTTTAATTTTAAC	NC_01282 1.1	Mitochondrial cytochrome c oxidase subunit III (Cox3)

3. Results

3.1. Tetrodotoxin analysis and environmental parameters

Tetrodotoxin was detected in *C. gigas* samples for seven weeks (three weeks above the LOQ) over the sampling period (24 June to 13 August 2021), with a rapid accumulation between 11 (< limit of detection) and 24th June (peak at 424 and 16 µg/kg in DG and RF, respectively) which then decreased for the following six weeks (Figure 2A). On June 24, 81% of the total TTX budget was in the digestive gland. The TTX in the total flesh equivalent was 74 µg/kg on June 24. TTX was the only congener in all positive samples. Temperature and salinity were relatively stable over the TTX peak (< 1 °C and 2 salinity units for six weeks prior and three weeks after TTX peak, while the temperature increased by ~2 °C after July 15, Figure 2B). A Distance-based ReDundancy Analysis (dbRDA) showed no significant relationship between TTX concentrations and environmental parameters (data not shown).

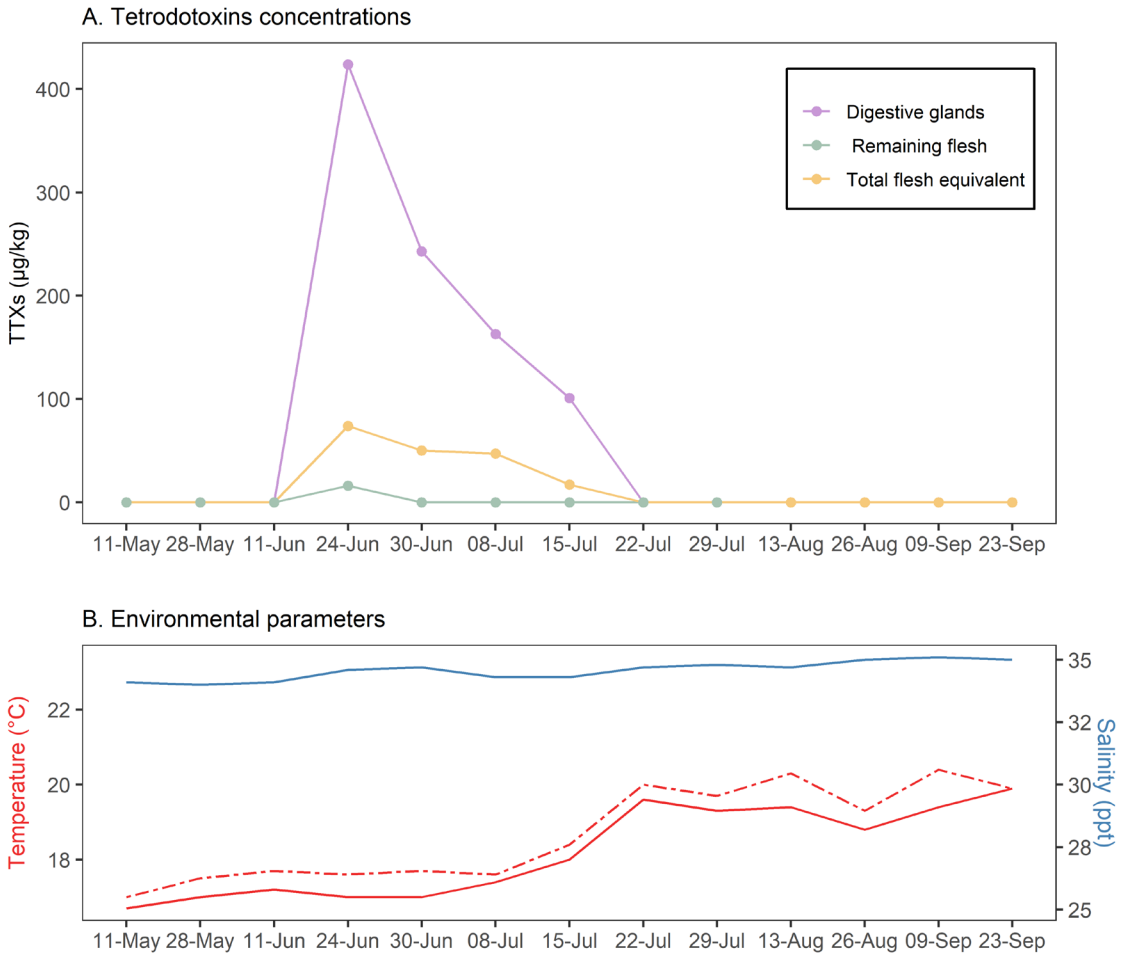


Figure 2 | A) Tetrodotoxin concentrations in the oysters *Crassostrea gigas* collected from the Loperhet site over a five-month period, determined using liquid chromatography-mass spectrometry (LC-MS/MS; n = 3 individuals blended together for n = 1 LC-MS/MS analysis). **B**). Temperatures (solid line = average daily temperatures, dotted line = maximum daily temperatures) and salinity at the Pointe du Château site (data retrieved from REPHY, (2022)).

3.2. Microscopy analysis

The phytoplankton counts from the Lanvéoc station (17 km from the cages deployed, Figure 1) showed that dinoflagellates and diatoms were abundant during the study period (Figure 3). Abundance of some species increased (>1,000 cells per litre threshold was used for this study) just prior to the peak in TTX concentration (displayed in grey, Figure 2). This includes the dinoflagellate genera *Diplopsalis*, *Gyrodinium*, *Katodinium*, *Protoperidinium* and *Tripos*, and the order of Peridianiales, and diatoms of the genera *Chaetoceros* and *Pseudo-nitzschia*. Interestingly, diatoms from the genus *Chaetoceros* bloomed (reaching 40,000 cells per litre) the same week as *C. gigas* tested positive for TTX for the first time.

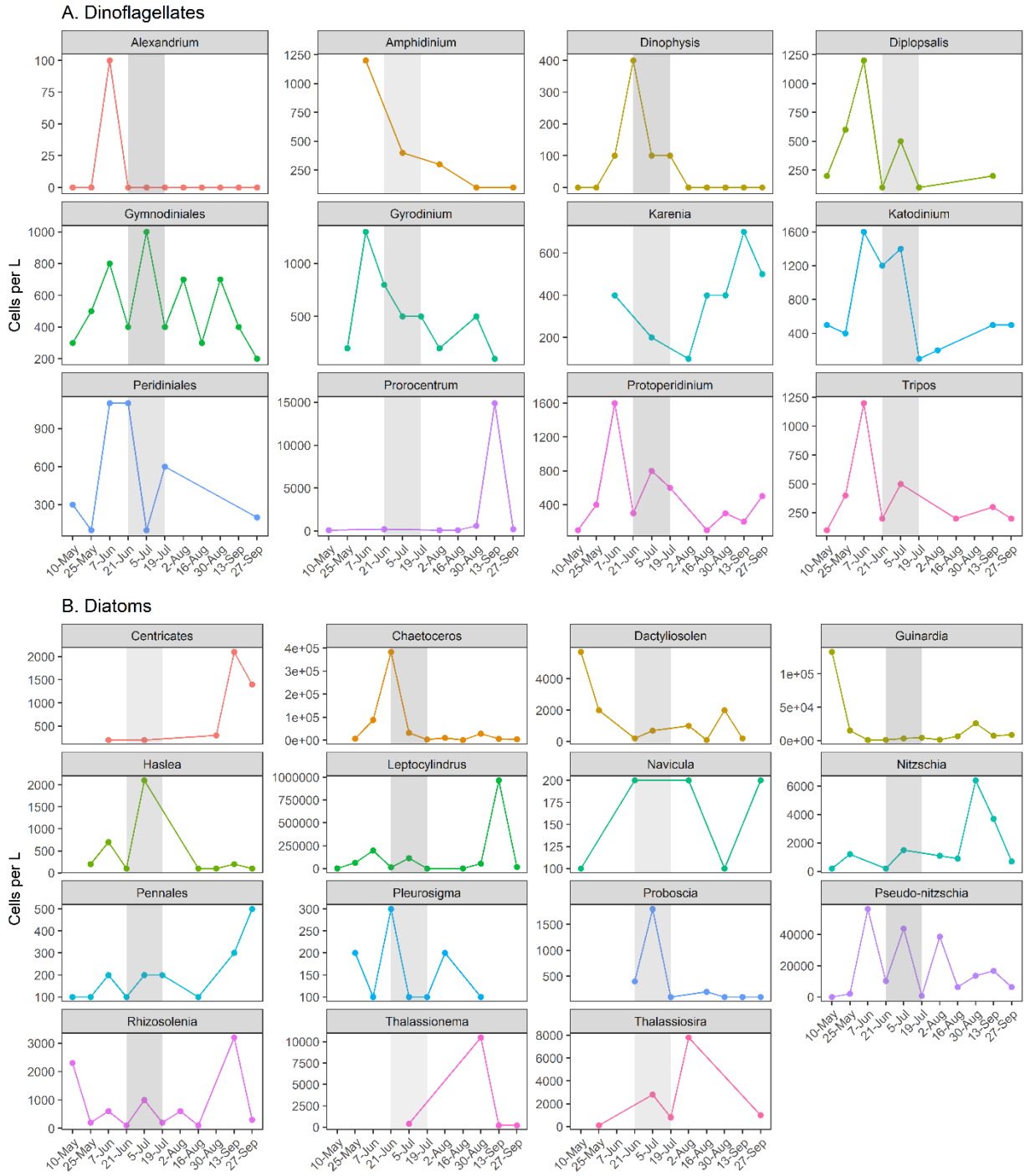


Figure 3 | Microscopic observations and counts (cells per litre) of **A**) dinoflagellates and **B**) diatoms species in the Lanvéoc large station near the study site. The grey area represents the period of quantifiable TTX concentrations. Data retrieved from REPHY (2022).

3.3. Metabarcoding results

3.3.1. Bacterial analysis

A total of 2,165 ASVs were identified in the 16S rRNA gene sequencing with samples rarefied to an even depth of 8,214 reads. Multivariate analysis (PERMANOVA) showed a non-significant difference in community composition amongst time periods (pre-TTX peak, during TTX peak and post TTX-peak) for bacteria in both the digestive gland (Bray–Curtis: P = 0.088, F = 1.508) and the remaining flesh (Bray–Curtis: P = 0.275, F = 1.182; Figure 4).

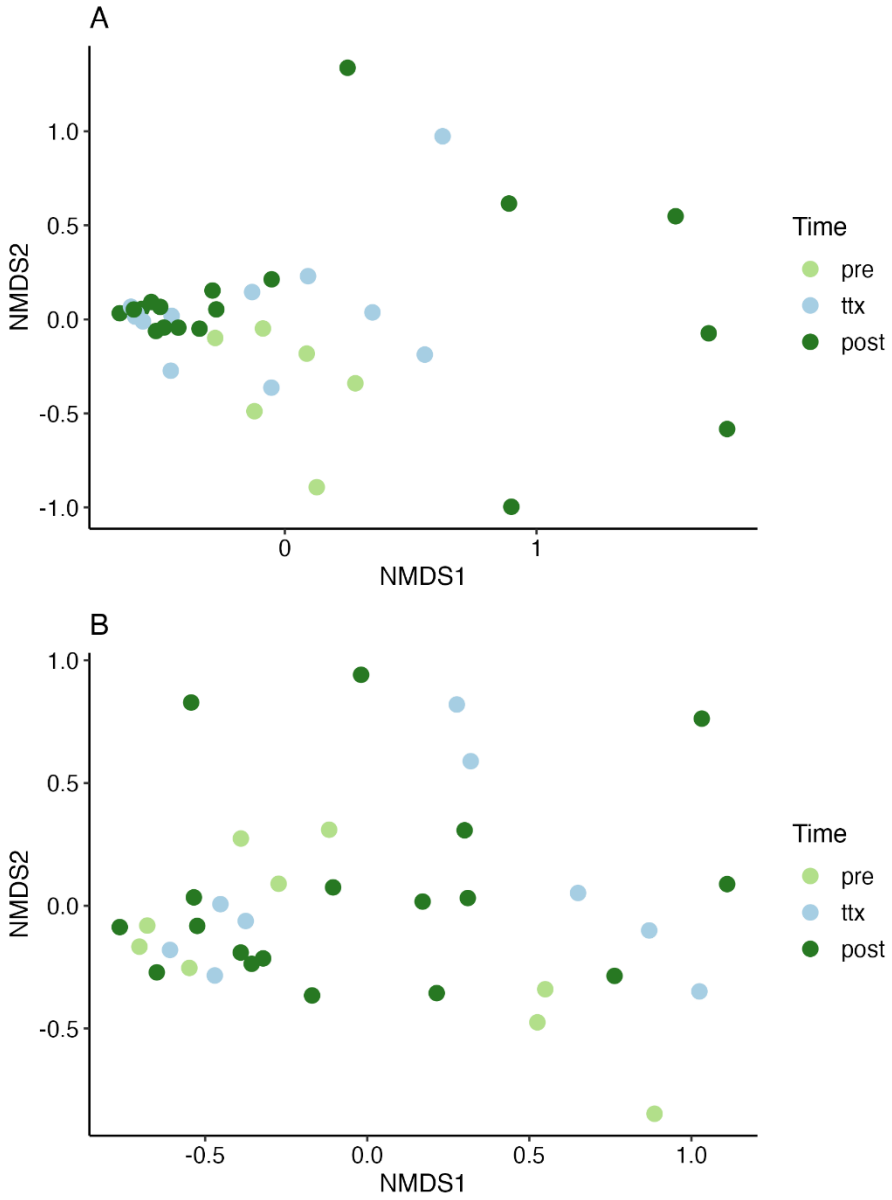


Figure 4: Multivariate analysis (NMDS) showing the distribution of samples across the time period for A) the digestive gland and B) the remaining flesh.

The bacterial composition showed that the genus *Mycoplasma* (family: Mycoplasmataceae) was the dominant component in the microbiomes of *C. gigas* over the whole period, with the exception of 24 June 2021, ranging from 17% to 93% in the digestive gland and between 25% and 91% in the rest. The family Vibrionaceae was also present in the microbiome throughout the experiment in both the digestive gland and the rest. Changes were noted in the genera of Vibrionaceae with *Aliivibrio* being less prevalent after the TTX peak (grey area, Figure 5) and with *Vibrio* having a higher relative abundance during the peak (especially in the rest) and afterwards. There was a large increase in the genus *Persicirhabdus* (family: Rubritaleaceae) at the start of the peak in TTX concentrations in the digestive gland (grey area, Figure 5A) reaching a relative abundance of 54% on June 24, and with a peak in relative abundance (41%) in the rest prior to the TTX peak (Figure 5B). Other genera that increased with the TTX peak included *Sulfuvorum* at the start of the peak and the genera *Geminobacterium*, *Haloferul*a, *Myxosarcina*, *Neolyngbya*, *Pseudahrensia, Roseibacilus and Pseudoaltermonas* mid-TTX peak in the digestive glands (30 June 2021, Figure 5A).

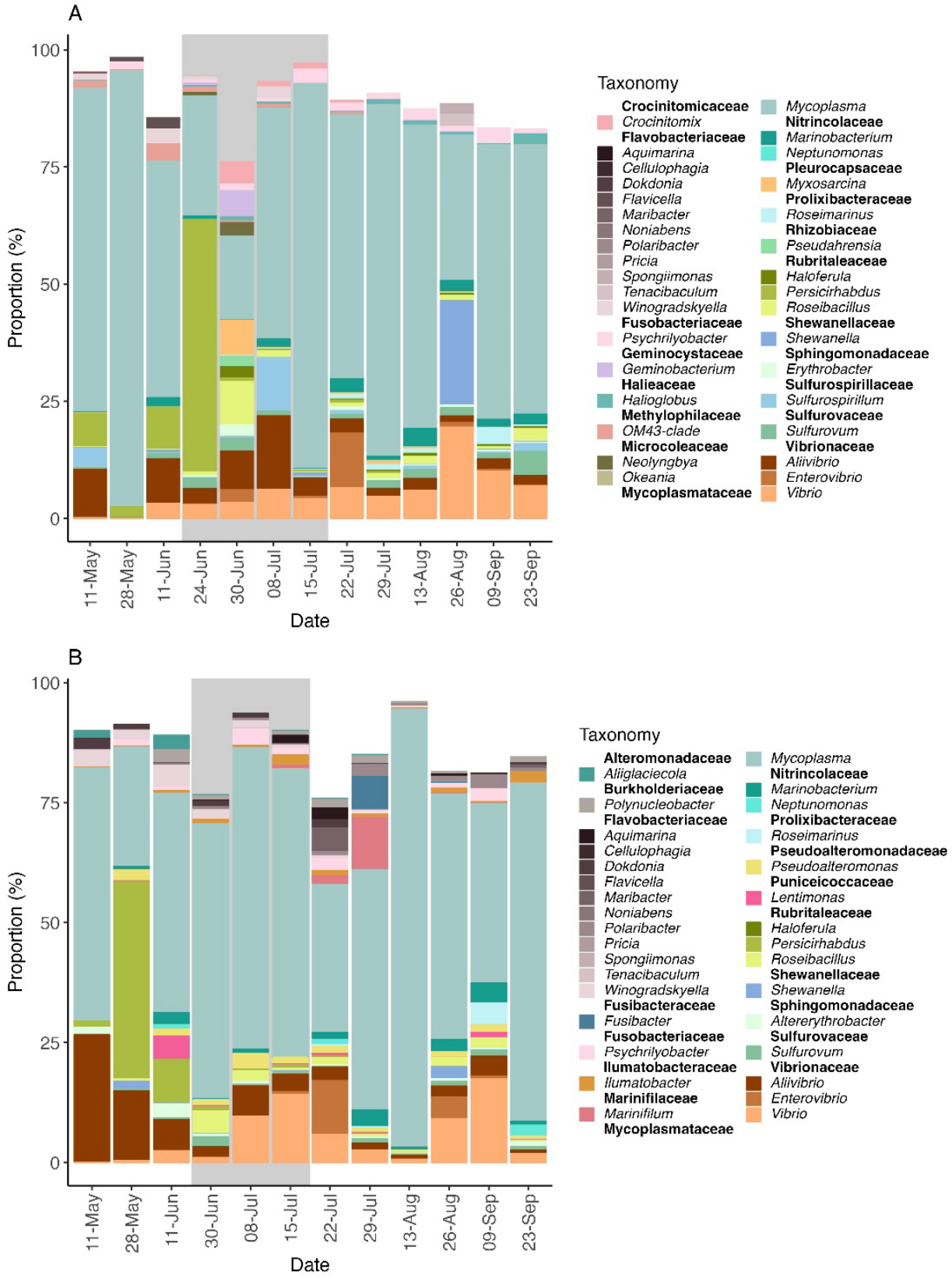


Figure 5 | Stacked barplots showing the relative abundance of bacterial 16S ribosomal RNA sequences in the digestive gland (**A**) and the rest (**B**), at the genus level (>2% proportion in at least one sample), that are present in *Crassostrea gigas* during the study period. The grey area corresponds to the period when *C. gigas* tested positive for TTX.

3.3.2. Cyanobacterial composition

The cyanobacterial community in *C. gigas* showed that in the digestive glands, *Parasynechococcus* was the dominant genus although it had a low relative abundance (2%) at the beginning of the TTX peak and reached a maximum relative abundance of 94% post TTX peak (Figure 6A). *Parasynechococcus* was not as abundant in the rest of *C. gigas,* with the exception of 9 September 2021, where it had a relative abundance of 96%. In the rest samples, *Zarconia* was observed to be the dominant cyanobacterial genus prior to the TTX peak (mean relative abundance: 76%; Figure 6B) although this genus was not prevalent in the digestive gland during the experiment. Species of the *Neolyngbya* genus appeared just before TTX increased, especially in the digestive gland.

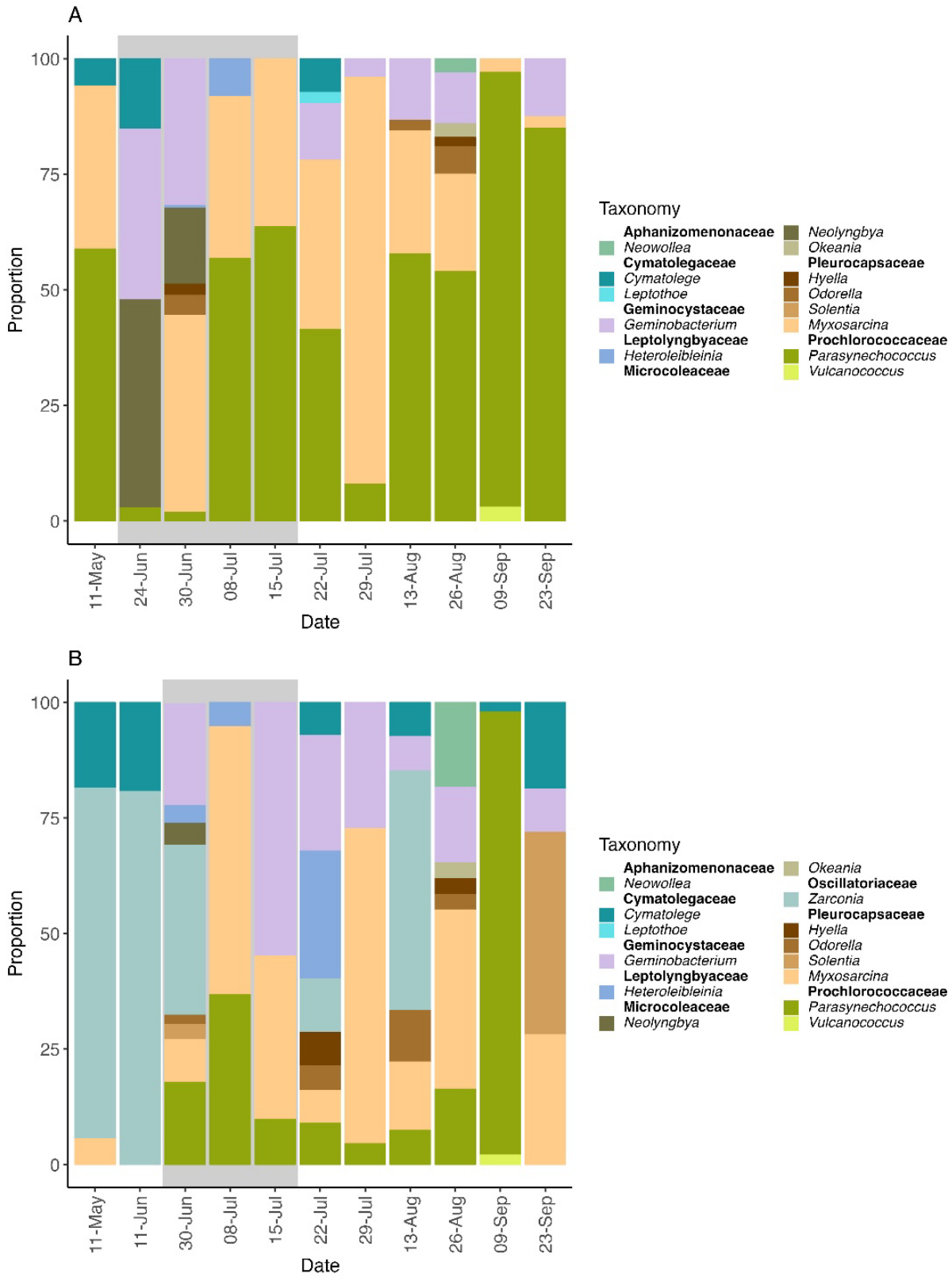


Figure 6 | Stacked barplots showing the relative abundance of cyanobacterial sequences in the digestive gland (**A**) and the rest (**B**), at the genus level (>2% proportion in at least one sample), that are present in *Crassostrea gigas* during the study period. The grey area corresponds to the period when *C. gigas* tested positive for TTX.

3.3.3. TTX-bearing bacterial composition

We investigated the ASVs that were more prevalent when *C. gigas* tested positive for TTX, by subtracting the core species present before and after the TTX peak period from those present during the peak of TTX (Figure 7). The most abundant bacterial genus in TTX-bearing *C. gigas*' digestive glands at the start of the peak was *Persicirhabdus* (4,294 reads) and disappeared completely after June 30. (Figure 7A). The other genera present were less abundant and varied between the sampling days although *Vibrio* increase during the TTX peak period, especially in the samples of remaining flesh.

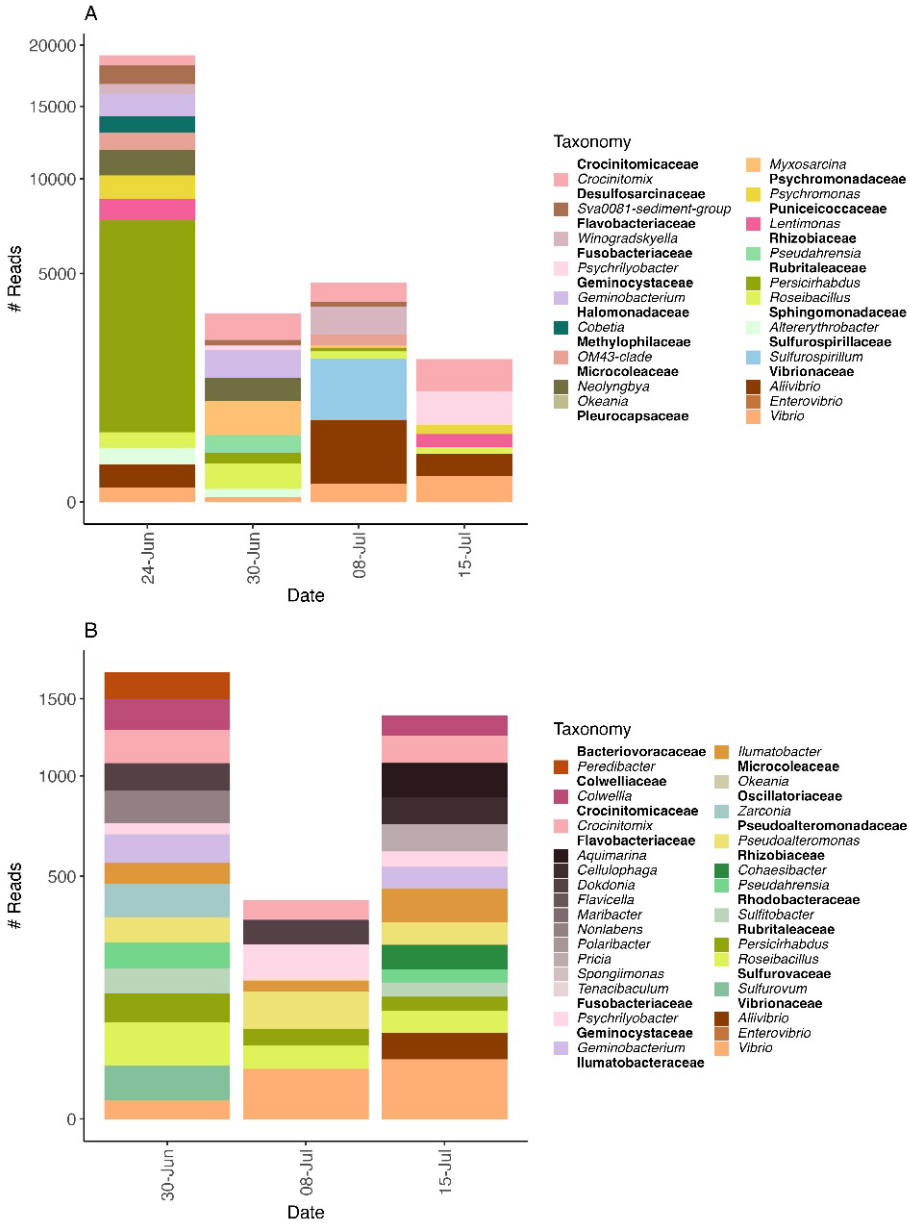


Figure 7 | Stacked barplots showing the relative abundance of bacterial 16S ribosomal RNA sequences for the digestive gland (**A**) and the rest (**B**), at the genus level (>1% of sample read number), that were only present in the tetrodotoxin-bearing *Crassostrea gigas* during the TTX peak.

3.4. Droplet digital PCR

The assay developed in this study targeting the genera of interest, *Cephalothrix,* was successful and amplified the designed gBlock. No *Cephalothrix* sp. DNA was detected in the digestive glands of *C. gigas*. Because of the lack of nemertean tissue samples acquired for this assay development, the specificity of the assay was not tested and should be considered as a limitation of this assay development.

4. Discussion

In this study, we aimed at identifying potential sources of TTX contamination in French Pacific oysters *C. gigas* that were placed in an estuary previously known for TTX presence over a short period of time in summer 2019 (Loperhet, Brittany, France, unpublished data). Seasonal differences in bacterial communities present in the digestive glands and other organs of *C. gigas* were explored, as well as phytoplankton differences in the surrounding waters, aimed at identifying any relationships with TTX-presence. To achieve this, the bacterial communities were characterized using metabarcoding in *C. gigas* placed in cages and harvested 13 times, weekly or fortnightly for five months, starting at the end of spring and finishing at the start of autumn. We also developed one detection assay for one of the TTX-bearing and invasive genera present in Europe, the ribbon worm *Cephalothrix* sp., to understand if it could be the source of TTX accumulation in European shellfish.

TTX was detected in *C. gigas* for a period of seven weeks (three weeks only > LOQ), with a rapid accumulation, from <5 µg/kg (i.e., LOD) on the 11th of June to 74 µg/kg on the 24th of June (424 µg/kg in the digestive gland and only 16 µg/kg in the remaining flesh) and a rapid depuration (50 µg/kg six days later and <5 µg/kg four weeks later in the digestive glands). These results classify *C. gigas* as a rapid detoxifier for TTX (Bricelj and Shumway, 1998; Guéguen et al., 2008; Lassus et al., 2000), and showed no migration between the organs, with the majority (i.e. >90% when considering LOD/LOQ) of TTX being in the digestive glands, similar to distribution between organs already reported for *C. gigas* (Dhanji-Rapkova et al., 2021). Oysters showed highest TTX levels in the digestive gland indicating that TTX in oysters likely comes from an exogenous source, feeding in particular, as has been previously established in other shellfish studies (Biessy, 2021; Biessy et al., 2021; Dhanji-Rapkova et al., 2023).

Analysis of the eukaryotic phytoplankton in the surrounding waters showed that the concentration of some microalgal species increased just prior to the peak in TTX concentrations. In particular, diatoms from the genus *Chaetoceros* bloomed the same week that TTX concentrations peaked, reaching 40,000 cells per litre. The diatom genus *Chaetoceros* is one of the most diverse and widespread groups among marine phytoplankton. Its species are distributed worldwide, and they often dominate in coastal ecosystems (Evensen, 1975; Guillard and Kilham, 1977). The genus includes over 200 different species, some of which are important bloom formers in both oceanic and coastal habitats (Assmy et al., 2008). Many *Chaetoceros* species are used as feed algae for seafood aquaculture (Gladue and Maxey, 1994) and there has not been any report of them producing harmful toxins to date. *Chaetoceros* species have only been reported to cause mechanical damage in fish due to the barbed setae becoming embedded in the lamellar epithelium of fish gills with resultant production of excessive amounts of mucus, resulting in asphyxia (Rolton et al., 2022). It is not likely that blooms of *Chaetoceros* spp. have ever been tested for TTX production, but this is worth investigating in the future, in this study's location and around the same time of year when peaks in TTX concentrations have been observed. Culturing and testing for TTX should also be extended to the dinoflagellate genera *Diplopsalis*, *Gyrodinium*, *Katodinium*, *Protoperidinium* and *Tripos*, and the diatoms from the genera *Pleurosigma* and *Pseudo-nitzschia* that were also present prior to the peak in TTX concentrations.

A parallel approach of this study was to investigate the bacterial and cyanobacterial microbiome of the oysters using metabarcoding. The bacterial communities of both DG and RF were dominated by species from the genus *Mycoplasma*, a genus that is also consistently associated with bivalves, often in high abundances (Aceves et al., 2018; Biessy et al., 2020; Lokmer and Wegner, 2015; Pierce and Ward, 2018). The family Vibrionaceae (Proteobacteria) was also abundant in all organs, which corroborates with previous studies of TTX-bearing bivalves (Biessy et al., 2020; Milan et al., 2018) and non-TTX-bearing bivalves (Aceves et al., 2018; Cho, 2019; Dubé et al., 2019). Vibrionaceae were present throughout the study and did not have a correlation with the peak in TTX concentration. Of note is the increase in the abundance of bacteria from the Rubritaleaceae family, in particular the genera *Persicirhabdus* before and during the TTX peak. Bacteria from the Rubritaleaceae are often associated with shellfish microbiomes (Biessy et al., 2020; Cho, 2019), showing different proportions likely due to differences in geographical origins (del Rio-Lavín et al., 2023). In a previous study, we found that Verrucomicrobia (four ASVs from the Rubritaleaceae family) were significantly and

positively correlated to TTX concentrations in TTX-bearing clams (Biessy et al., 2020). To the extent of our knowledge, species from the Rubritaleaceae family, in particular the genus *Persicirhabdus,* have not been tested for TTX production and are a great candidate for future work to identify the producer of TTX.

Previously, cyanobacteria have been hypothesized as potential TTX producers. Strains of the genera *Pleurocapsa*, *Cyanobium* and *Prochlorococcus* were detected in TTX-bearing clams in New Zealand, and the genus *Synechococcus* was found to be significantly correlated to higher TTX concentrations in these clams (Biessy et al., 2020). Another study showed that cyanobacteria from the Calotrichaceae lineage were found centred in the microbial co-occurrence network of toxic pufferfish along *Vibrio* species (Li et al., 2020). It was hypothesised that *Vibrio* and cyanobacteria may form a symbiotic relationship in the gut environment of toxic puffer fish (Li et al., 2020). In this study, species of *Neolyngbya* were detected in the oyster's digestive glands just before and at the start of the peak in TTX concentrations before completely disappearing when the TTX concentrations started to decrease. *Neolyngbya* spp. belong to the family Oscillatoriaceae and are described as marine filamentous cyanobacteria that are well known as producers of a rich diversity of secondary metabolites and toxins (Caires et al., 2018), and should be tested for TTX production in the future. Cyanobacterial ecotoxicology is particularly well documented in freshwater habitats and are known to produce a wide range of toxins (Jakubowska and Szeląg-Wasielewska, 2015; Rastogi et al., 2015), including paralytic shellfish toxins (PSTs). PSTs are potent neurotoxins produced by cyanobacteria and marine dinoflagellates and are known to exert the same toxic effect as TTX through an interaction with voltage gated sodium channels (Narahashi, 1988). Both toxins have been detected simultaneously in several aquatic species but toxicological studies on cyanobacteria have not been performed to the same extent in marine environments (Bane et al., 2014; Frazão et al., 2010). Marine cyanobacteria are thus an interesting lead for future studies aiming to identify TTX-producing bacterial species. It is worth noting that the eukaryotic communities (using metabarcoding targeting the V4 18S rRNA gene region) were also investigated in this study to identify potential eukaryotic TTX-producers. However, despite using *C. gigas* blocking primers previously developed by Clerissi et al. (2020), over 80% of sequence reads belonged to the class Bivalvia and thus the data was not robust enough to include in this study. It is also important to note that the metabarcoding analyses from this study was limited by the frequency of sampling. This study was designed to guarantee to capture the yearly peak of TTX but if this study was to be repeated, oysters should be harvested daily or at least weekly around the time of the TTX peak, and during

a shorter period (Mid-June until the end of July). Another limit to metabarcoding is the well-known challenge of taxonomic reference database and limited taxonomic resolution (Keck et al., 2023) and these were hopefully mitigated by the use of up-to-date databases such as *Cyanoseq* (Lefler et al., 2023), in particular for cyanobacteria taxonomy, and bioinformatic pipelines.

Lastly, we developed a droplet digital PCR assay aimed at investigating the presence or absence of *Cephalothrix,* a genus of interest. This genus is known to be TTX-bearing and has been detected in Europe in the last decades (Turner et al., 2018). It is possible that the eggs of this species, after spawning events, could accumulate in filter-feeding shellfish. This is particularly relevant because spawning of some organisms can be very regular (e.g., within a week for some fish (Cushing, 1969) and within a month for some marine worms (Bybee et al., 2007)), which is the case here with TTX concentrations in French shellfish found to peak for a few weeks in early summer (Réveillon et al., 2021). Due to the lack of positive tissue samples available for assay development, gBlocks were designed to use as positive controls. *Cephalothrix* sp. was not detected in the digestive glands of *C. gigas* and was thus not likely to have been the source of TTX contamination in the oysters. It is worth mentioning that due to the lack of positive and negative Nemertean tissues available, this assay is not fully developed (i.e., sensitivity, cross-reactivity with other species) and this result should be interpreted with care. This assay, once fully developed and validated, could be of broad interest globally to detect this invasive and toxic species.

5. Conclusion

The aim of this study was to investigate the potential source of TTX in French Pacific oysters *Crassostrea gigas* that were placed in an estuary previously reported to encounter TTX accumulation in shellfish. The microscopic observations of algae present during the TTX peak showed that a sudden bloom in *Chaetoceros* species co-occurred with the sudden appearance of TTX in oysters. Metabarcoding analysis suggested that prokaryotic microbiome components correlate with previous studies indicating that 1) bacteria from the Rubritaleaceae (in particular from the genus *Persicirhabdus*) were positively correlated to TTX concentrations in TTX-bearing shellfish and that 2) marine cyanobacteria, *Neolyngbya* in particular for this study, should be investigated as potential TTX producers. Culturing, isolating and testing these bacterial and cyanobacterial species and genera for toxin production would be a logical next step in the search to identify the source of TTX in marine organisms.

Data Availability Statement

The datasets presented in this study are available in online repositories. The names of the repository/repositories and accession number(s) can be found in the article.

Author Contributions

LB, KS, KNM, DR and PH conceived and designed the experiments. LB, LT, KNM, LL, AT and VS performed the experiments and TTX analysis. LB and JP undertook the metabarcoding analysis. All authors helped with the data interpretation and experiments, and the editing and writing of the manuscript.

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Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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