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Emergent marine toxins risk assessment using molecular and chemical approaches

Y García-Cazorla and V Vasconcelos

Interdisciplinary Centre of Marine and Environmental Research (CIIMAR), Portugal

Abstract

Cyanobacteria harmful blooms represent a deviation to the normal equilibrium in planktonic communities involving a rapid and uncontrolled growth. Owing to the capacity to produce toxins as secondary metabolites, cyanobacteria may cause huge economic losses in the fishing and aquaculture industries and poisoning incidents to humans due to their accumulation in the food chain. The conditions which promote toxic blooms have not yet been fully understood, but climate change and anthropogenic intervention are pointed as significant factors. For the detection of toxins in edible marine organisms, the establishment of international regulations and compulsory surveillance has been probed as exceptionally effective. However, not regulation nor monitoring have been settled concerning emergent marine toxins. In the light of this scenario, it becomes essential to apply fast and reliable surveillance methodologies for the early detection of cyanobacterial blooms as well as the occurrence of emergent marine toxins. Shotgun metagenomic sequencing has potential to become a powerful diagnostic tool in the fields of food safety and One Health surveillance. This culture-independent approach overcomes limitations of traditional microbiological techniques; it allows a quick and accurate assessment of a complex microbial community, including quantitative identification and functional characterisation, in a single experiment. In the framework of the EU-FORA fellowship, with the final goal of evaluate metagenomics as a promising risk assessment tool, the fellow worked on the development of an innovative workflow through state-of-the-art molecular and chemical analytical procedures. This work programme aims to evaluate the occurrence of emergent marine toxins and the producing organisms in Cabo Verde coastal cyanobacteria blooms. Our results show the outstanding potential of a holistic metagenomic approach for the risk assessment of emergent marine toxins and the producing organisms. Additionally, we have also highlighted its value for the identification and evaluation of secondary metabolites as natural bioactive compounds with biotechnological and industrial interest.

Keywords: metagenomics, shotgun sequencing, bioinformatics, emergent marine toxins, cyanobacteria, harmful algal blooms, surveillance and risk assessment

Correspondence: eu-fora@efsa.europa.eu

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1. Introduction

Cyanobacteria, also known as blue-green algae, are a diverse group of Gram-negative photosynthetic prokaryotes with a great ecological importance. They colonise a wide heterogenous range of ecosystems and are a major component of the phytoplankton (Moreira et al., 2013). Their ability to produce toxins as secondary metabolites may lead to the development of harmful blooms causing huge economic losses in the fishing and aquaculture industries and poisoning incidents to humans due to their accumulation in the food-chain (Anderson et al., 2002). Nevertheless, cyanobacteria have also attracted an increasing interest owing to the production of a large number of natural bioactive compounds (e.g. alkaloids, non-ribosomal peptides, polyketides) with promising biotechnological and biomedical applications (Leão et al., 2012).

Harmful algal blooms (HABs) are unpredictable due to their erratic nature. Episodes of human poisoning caused by HABs have been commonly recorded in the last century mostly because of the lack of regulated monitoring programs. The establishment of international regulations and compulsory surveillance led to the detection of these toxins in edible marine organisms and today the cases of human poisonings in Europe are sporadic, usually because of violated health authorities' regulations imposing the closure of harvesting areas and seafood commercialisation (Regulation (EC) No 853/ 2004; 854/2004; 15/2011, 786/2013).

However, the situation is different when we refer to emergent marine toxins (e.g. ciguatera fish poisoning, cyclic imines, pufferfish poisoning, neurotoxic sellfish poisoning), which are not yet regulated nor monitored regularly in Europe. Climate change combined with human intervention in the ecosystem aid the migration and establishment of new toxic species typical from warmer waters into more temperate areas such as the European Union (EU), where they have been already reported (Otero et al., 2010; García-Altares et al., 2014). This fact highlights the essential need of a European surveillance plan to monitor and track these emergent toxins and toxin-producing organisms in marine ecosystems. A systematic compilation of data will provide the input needed to perform knowledge-based risk assessment which allow the regulatory authorities stablish measures to protect the consumers and advise the fish production sector.

At this regard, the present work programme aims to develop an innovative holistic approach to sample and analyse emergent marine toxins and their producing organisms both on marine ecosystems and food matrixes. With its power to comprehensively detect and analyse entire microbial communities in a single experiment, metagenomics-based methods stand out in addressing this need (Josić et al., 2017; Campos et al., 2020). This culture-independent methodology has the potential to overcomes limitations of the classical microbiology techniques which are time-consuming and target only specific subsets of microbes. Therefore, shotgun metagenomic sequencing and the subsequent computational analysis of the sequences represent a powerful tool capable of provide an exhaustive quantitative picture of the genetic and metabolic diversity encoded in these complex samples (Sharpton, 2014). Additionally, it has the potential to open access to untapped genetic resources for the screening and identification of genes encoding new toxins and bioactive compounds (Quince et al., 2017).

Cabo Verde is a diverse group of volcanic islands located in the Tropical Eastern Atlantic, in the Sahel region of Africa. The environmental characteristics of this archipelago provide niche conditions for a wide variety of cyanobacteria. Due to the scarce information existing at the date in what concerns cyanobacteria, their toxins and natural products, these underexplored habitats are an interesting and promising location for the development of this work programme (Semedo-Aguiar et al., 2018). Environmental samples have been collected and analysed following an integrated metagenomic approach which allows to assess the taxonomic biodiversity of the samples and the presence of genes of interest (including cyanotoxins and secondary metabolites with biotechnological potential). The final objective was obtaining results that will allow gathering new data on the distribution of emergent marine toxins in the region, detect new vectors, new toxin producing species and map the current state of this problem, essential steps all of them for a risk assessment analysis which study the potential food-chain implications of these cyanobacteria.

2. Description of work programme

The work programme proposed by CIIMAR to the EU-FORA fellow was framing in the context of the project EMERTOX, funded by EU Horizon 2020 Framework Research Programme. This project focus on the study of emergent marine toxins and the producing organisms in the North Atlantic and Mediterranean, with the aim of mapping the actual situation, developing new approaches to assess

their occurrence and predicting the possible future scenarios based on molecular data (routes of dispersion) and modelling in the framework of global environmental changes.

Regarding the inexistent legislation on emergent marine toxins and the producing organisms, the implementation of this project and development of a global surveillance and monitoring system, may provide insights into the mechanisms of emerging and spread of marine toxins and help identify hotspots and novel vectors. Thus, food risk assessment and knowledge-based interventions could be developed to reduce the spread and dispersal of marine toxins throughout the food chain.

With the objective of contributing to the data gathering and innovation of monitoring tools, the fellow worked on the development of an innovative workflow through state-of-the-art molecular and chemical analytical procedures.

2.1. Aims

The aims of the work programme were:

i) to broad the proficiency of the fellow with the food and feed safety risk assessment and surveillance methodologies, including hazard identification, hazard characterisation, exposure assessment and risk characterisation.
ii) to gain first-hand experience in the execution of qualitative and quantitative risk assessment and epidemiological surveillance based on molecular and chemical approaches in relation to emergent marine toxins and the producing organisms.
iii) to develop and validate a novel workflow (including collection, normalisation and analysis of data) for the identification and characterisation of emergent marine toxins and their producing organisms in environmental samples based on the potential of next-generation sequencing (NGS), shotgun metagenomic sequencing and computational analysis for One Health surveillance and food safety risk assessment.
iv) to contribute to the raise of awareness across Europe regarding emerging marine toxins and their producing organisms in the food chain.

2.2. Activities and methodology

The activities and methodology described below were in line with the aims of the work programme proposed by CIIMAR, rooted in the improvement of systematic collection and evaluation of data to facilitate the food risk assessment of emergent marine toxins and their producing organisms. As study model, the research project is dedicated to examine the biodiversity, toxicity and biotechnological potential of cyanobacteria from Cabo Verde islands following a molecular approximation relied on shotgun metagenomic sequencing and computational bioinformatic analysis.

2.2.1. Sampling, DNA extraction and shotgun metagenomic sequencing

Sampling points were stablished at specific locations in São Vicente and Santo Antão islands (Cabo Verde archipelago). Two millilitres of samples of cyanobacterial mat growing on sediment were collected in each sampling point using sterile plastic syringes. Care was taken to target only the mat, with sediment found embedded in the mat matrix. For preservation, cyanobacterial mat samples were snap-frozen in tubes containing an aqueous, nontoxic reagent that rapidly permeates tissue to stabilise and protect molecular integrity (RNA*later*, Thermo Fisher Scientific). Upon arrival to the laboratory, samples were stored at −80°C until processing.

A total of 10 metagenomic samples from Cabo Verde were studied during this work programme. After checking different approximations and optimisation of the DNA extraction procedure, a spin column silica-based methodology which requires no phenol or chloroform extraction was selected. DNA was extracted from 200 mg wet weight of each homogenised sample, using the NZY Plant/Fungi gDNA Isolation Kit (NZYtech) according to manufacturer's instructions, including a first step of cell lysis and RNA removal, followed by a clarification step of the crude lysate and the final genomic DNA (gDNA) purification.

The extracted gDNA quality was evaluated by agarose gel electrophoresis (1% agarose, 150V, 40min), NanoDrop Spectrophotometer and Qubit Fluorometer (Qubit dsDNA BR Assay Kit, Thermo Fisher Scientific) to determine DNA integrity, concentration and potential contaminations.

Shotgun sequencing was performed on a DNBseq™ sequencing platform by BGI (Beijing Genomics Institute). This system is powered by combinatorial Probe-Anchor Synthesis (cPAS), that combines linear isothermal Rolling-Circle Amplification (RCA) and DNA Nanoball (DNB) technology with stepwise

sequencing using DNA polymerase on patterned array flow cells, followed by fluorescent high-resolution digital imaging analysis (Goodwin et al., 2016; Natarajan et al., 2019). After gDNA fragmentation, library preparation and sequencing, short paired-end reads (150 bp) were generated with a deep of 5Gb (~ 40 million reads) per sample.

2.2.2. Computational data analysis: bioinformatics and statistics

Quality control

After sequencing, the raw reads were filtered to obtain clean reads. Data filtering includes removing adaptor sequences, contamination and low-quality reads as follow:

1) Filter adapter: delete the entire read if more than 25% match the adapter sequence.
2) Filter low-quality data: delete the entire read if there are more than 50% bases having a quality value lower than 20 and if the read is shorter than 150 bp.
3) Remove N: delete the entire read if there are more than 3% N in the read.
4) Filter out duplication.

Adapters were removed with the bioinformatic tool Cutadapt (version 3.5) (Martin, 2011). Reads were quality trimmed to a minimum quality score threshold of 20 and reads shorter than 150 bp were removed using Trimmomatic software (version 0.38) (Bolger et al., 2014). The final quality of the cleaned reads was assessed with the tool optimised for high throughput sequence data FastQC (version 0.11.9) (Andrews, n.d.).

Assembly-free taxonomic profiling

In order to determine the phylogenetic diversity of the microbial community present in each sample, the cleaned set of reads obtained after quality trimming were analysed under the pipeline MetaPhIAN (version 3.0.13) (Beghini et al., 2021). The minimum total nucleotide length for the markers in a clade for estimating the abundance without considering sub-clade abundance was set in 2000. The minimum mapping quality value (MAPQ) was set in 5 and the mapping was performed under the 'very sensitive' parameter with BowTie2, an ultrafast and memory-efficient tool for aligning sequencing reads to long reference sequences.

The taxonomically annotated reads allowed as to obtain an accurate estimation of organismal relative abundance (in terms of cells) at species-level resolution.

Assembly-free profiling of functional potential

With the aim of describing the metabolic potential of the microbial communities present in our samples, we utilised the tool HUMAnN2 (version 0.11.1) (Abubucker et al., 2012) with default parameters, a query coverage threshold for nucleotide alignments of 90 and using the UniRef90 cluster database. Paired-end reads were concatenated prior to their use in this pipeline because HUMAnN does not support paired end data. Gene families were clustered with the Kyoto Encyclopedia of Genes and Genomes (KEGG) Orthology database into functional orthologs.

We obtained the functional profiling and the abundance of genes and microbial pathways from our shotgun metagenomic sequencing data.

***De novo* assembly**

To improve the detection of toxins and secondary metabolites in our samples, the high-quality cleaned paired-end reads from each sample were undependably assembled *de novo* into contigs, following by scaffolding of these generated contigs, applying the pipeline metaSPAdes (version 3.15.3) (Nurk et al., 2017) with K-mer sizes of 33, 55, 77, 99 and 127. After assembly, only scaffolds exceeding 1 kb in length were retained for downstream analysis in order to avoid binning errors and ambiguous gene annotation from shorter contigs.

The assembled contiguous genome fragments were clustered into different linages (bins) using depth-of-coverage, nucleotide composition, and marker genes with the pipeline MaxBin2 (version 2.2.4) (Wu et al., 2016). The resulting draft Metagenome Assembled Genomes (MAGs) were quality-checked using QUAST (version 4.4) (Gurevich et al., 2013) to generate and verify assembly statistics, compare results from different workflows and preclude the inclusion of misassembles from our analysis.

MAGs taxonomic profiling and search for genes of interest

Taxonomic assignment to each draft MAG was done using GTDB-tk (version 1.7.0) (Chaumeil et al., 2020) and annotated using RASTtk (version 1.073) (Aziz et al., 2008; Overbeek et al., 2014; Brettin et al., 2015).

MAGs were additionally explored for the identification, annotation and analysis of secondary metabolite biosynthetic gene clusters (BGCs) using the antiSMASH web server pipeline (version 6.0.1) (Blin et al., 2021) under strict setting. To determine the genetic novelty of the BGCs identified, we performed homology searches against the NCBInt database using NCBI BLAST+.

3. Conclusions

3.1. Conclusions of the research study

Shotgun metagenomic sequencing has been scrutinised as a promising technology for food risk assessment and One Health surveillance. This methodology allows to explore and link the phylogenetic and metabolic complexity encoded in uncultured microbial communities from environmental, agricultural and clinical settings. While there are still technical limitations and challenges that need to be solved, such as the detection of low abundance microorganisms, metagenomics has the potential to address with precision manifold issues concerning the global food supply chain, including pathogen detection, monitoring of toxins and antimicrobial resistance determinants, detection of fraudulent products and quality control. Considering the active development and evolution of this field, together with the rapid establishment of WGS in the food and feed safety risk assessment during the recent few years, we envisage a near future where omics-based tools will become an indispensable part of the food safety surveillance and risk assessment.

Applying this state-of-the-art sequencing technology and computational bioinformatic analysis, we successfully determined the taxonomic biodiversity in our samples, the presence of genes of interest and explore the production of toxins and secondary metabolites with biotechnological potential. Our samples from Cabo Verde Islands exhibited a broad compositional biodiversity and interesting production of novel BGCs. Altogether, this study evidences the potential for the discovery of novel bioactive compounds with forthcoming biotechnological and biomedical implementations, but it also highlights the plausible risks of the appearance of HABs and new emergent marine toxins in the Cabo Verde coastal marine areas. Taking into account the food chain implications of these HABs and the toxins produced, our results will grant the evaluation of occurrence, toxicity and exposure to HABs toxins in order to accomplish an evidence-based risk assessment covering the food and feed chains.

3.2. Conclusions of the EU-FORA Fellowship experience

The fellow was hosted by the Blue Biotechnology and Ecotoxicology team at CIIMAR. Her integration on such a multidisciplinary research group has provided her a unique opportunity to interact and create synergies with scientific experts in the fields of Global Changes and Ecosystems Services, Marine Biotechnology and Biology, Aquaculture and Seafood Quality.

The proposed work programme was an excellent fit with the experience and expectations of the fellow. It has been an opportunity to consolidate and broaden her knowledge on microbiological and chemical risk assessment from a One Health perspective, enabling her to gain valuable first-hand experience on shotgun metagenomics analysis, bioinformatic tools and *in silico* evaluation at all stages of the food safety assessment.

Throughout the year, the fellow had the opportunity to participate in diverse scientific discussions and seminars. Additionally, she attended several complementary trainings to acquire further skills needed for the accurate performance of the research project: *Foundations of One Health* (University of Calgary), *Introduction to Bioinformatics* (University of Calgary) and *Metagenomics applied to surveillance of pathogens and antimicrobial resistance* (Technical University of Denmark).

The preliminary results of this project have been presented with a format of oral communication during the 19th International Conference on Harmful Algae (ICHA) in Mexico. A full description of the tools, methodology and results will be reported in a manuscript currently under preparation.

The work programme together with these supplementary activities, and the high-level set of trainings provided by the EU-FORA programme (covering in detail all the different areas of food safety risk assessment, risk management, risk and crisis communication), have expanded the fellow expertise

selecting and applying risk assessment methodologies, collecting and analysing relevant data, using computer models in risk assessment, and providing effective risk and crisis communication.

In short, both the fellow and the supervisor value positively the capacity building opportunity bring by the fellowship programme. We agree that EU-FORA provides an exceptional framework for the building of scientific networks. It is a win-win scenario for the development of cooperation, exchange of high-level knowledge and professional experiences with the final goal of achieve a major harmonisation of risk assessment methodologies and practices in the farm-to-folk chain across Europe.

4. Disclaimer

The results obtained during the research study are intended to be published in scientific peer review journals. In order to avoid copyright claims, they have not been included in the present technical report.

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Abbreviations

BGC	Biosynthetic Genome Cluster
BLAST	Basic Local Alignment Search Tool
BR	broad range
CIIMAR	Interdisciplinary Centre of Marine and Environmental Research (Portugal)
cPAS	Combinational Probe-Anchor Synthesis
DNB	DNA Nanoball
dsDNA	double-stranded DNA
EU-FORA	European Food Risk Assessment Fellowship Programme
gDNA	genomic DNA
HABs	harmful algal blooms
ICHA	International Conference on Harmful Algae
KEGG	Kyoto Encyclopedia of Genes and Genomes
MAG	Metagenome-Assembled Genome
MAPQ	mapping quality
NGS	next generation sequencing
RCA	rolling-circle amplification