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Assessing the presence of marine toxins in bivalve molluscs from southwest India

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Abstract

The south west coast of India has been showing a steady increase in shellfish cultivation both for local consumption and fishery export, over recent years. Perna viridis and Crassostrea madrasensis are two species of bivalve molluscs which grow in some selected regions of southern Karnataka, close to the city of Mangalore. In the early 1980s, shellfish consumers in the region were affected by intoxication from Paralytic Shellfish Poison present in local bivalves (clams and oysters) resulting in hospitalisation of many, including one fatality. Since then, there have been no further reports of serious shellfish intoxication and there is little awareness of the risks from natural toxins and no routine monitoring programme in place to protect shellfish consumers. This study presents the findings from the first ever systematic assessment of the presence of marine toxins in mussels and oysters grown in four different shellfish harvesting areas in the region. Shellfish were collected and subjected to analysis for ASP, PSP and lipophilic toxins, as well as a suite of non-EU regulated toxins such as tetrodotoxin and selected cyclic imines. Results revealed the presence of low levels of PSP toxins in oysters throughout the study period. Overall, total toxicities reached a maximum of 10% of the EU regulatory limit of 800 µg STX eq/kg. Toxin profiles were similar to those reported from the 1980 outbreak. No evidence was found for significant levels of ASP and lipophilic toxins, although some cyclic imines were detected, including gymnodimine. The results indicated that the risk to shellfish consumers during this specific study period would have been low. However, with historical evidence for extremely high levels of PSP toxins in molluscs, there is a strong need for routine surveillance of shellfish production areas for marine toxins, in order to mitigate against human health impacts resulting from unexpected harmful algal blooms, with potentially devastating socio-economic consequences.

Keywords

Shellfish, India, Lipophilic toxins, Paralytic Shellfish Poisoning, Amnesic Shellfish Poisoning, LC-FLD, LC-UV, LC-MS/MS
**1. Introduction**

Marine biotoxins comprise various groups of naturally-occurring compounds present in Harmful Algal Blooms (HAB), a natural phenomenon caused by the overgrowth of marine phytoplankton (Visciano et al., 2016). Through filter feeding behaviour, bivalve molluscs can accumulate toxins from harmful algae (Landsberg, 2002; Hallegraeef, 2003; Llewellyn et al., 2006; Deeds et al., 2008). Some groups of toxins are known to cause human sickness after being consumed (Mead et al., 1999; Erdner et al., 2008). ASP is caused by domoic acid (DA), a cyclic tricarboxylic amino acid, and potentially other toxic DA isomers. Following human consumption of DA-contaminated shellfish, symptoms can be gastrointestinal and/or neurological, leading potentially to fatalities (Jeffrey et al., 2004). In comparison, Paralytic Shellfish Toxins (PST) comprise a family of more than 50, mostly hydrophilic, structural analogues of the tetrahydropurine saxitoxin (Wiese et al., 2010). Following ingestion by humans, these highly potent neurotoxins can induce symptoms such as nausea, numbness, breathing difficulties, paralysis, and at high enough concentrations, death (EFSA, 2009a).

Tetrodotoxin (TTX) produces a near-identical toxic response in mammals as saxitoxin and its presence has recently been proven in Asian (Kodama et al., 1993; McNabb et al., 2014) and European bivalve molluscs (Turner et al., 2015a, Vlamis et al., 2015). Lipophilic toxins (LTs) include compounds such as the DSP toxins: okadaic acid (OA), dinophysis toxin-1 and -2 (DTX1 and DTX2), including their ester derivatives (often termed DTX3), the azaspiracids (AZAs), yessotoxins (YTXs), pectenotoxins (PTXs) and a number of cyclic imines including the spirolides (SPXs) and gymnodimine (GYM) (McNabb et al., 2005). The acute effects of DSP and AZP are less severe than the effects from PSP and ASP, with no known fatalities resulting from intoxication following ingestion of any of the regulated lipophilic toxins (Blanco et al., 2005). A range of toxicological effects have however been reported, including tumour promotion and carcinogenicity, so serious long-term health effects cannot be discounted following exposure to DSP toxins (Valdiglesias et al., 2013). Cyclic imines are known to be fast acting toxins following direct injection into mice, but there is no evidence for acute oral toxicity to date in humans (EFSA, 2010; Hess et al., 2013).

*Pseudo-nitzschia* spp. are the causative organisms for production of DA leading potentially to ASP (Bates et al., 1989; Lundholm et al., 1994). Paralytic shellfish toxins (PST) are produced by several species of phytoplankton including *Alexandrium* spp., *Gymnodinium catenatum* and *Pyrodinium bahamense* (van Dolah, 2000). Phytoplankton responsible for DSP include *Prorocentrum lima*, and a range of *Dinophysis* species (Yasumoto et al., 1980; Morton et al., 2009; Reguera et al., 2014). Yessotoxins are known to be produced by *Protoceratium reticulatum* and *Lingulodinium polyedrum* (Visciano et al., 2016). Azaspiracids, the most recently discovered of the regulated marine toxin classes, are now known to be produced by the dinoflagellate *Azadinium spinosum* (Krock et al., 2009a; Tillmann et al., 2009) together with a number of other species of *Azadinium* (Tillmann et al., 2010, 2011). Algal imines such as gymnodimine, pinnatoxins and spirolides have been isolated from dinoflagellates *Gymnodinium* sp., *Vulcanodinium rugosum* and A. *ostenfeldi/peruvianum* respectively (Hu et al., 2001, Moestrup et al., 2009; Seki et al., 1995).

As opposed to all the dinoflagellate sources for these toxins, TTX and a number of related analogues (TTXs) are shown to be produced by a range of marine bacterial species. Genera proposed include *Vibrio, Bacillus, Aeromonas, Alteromonas, and Pseudomonas* (Yasumoto et al., 1988; Wu et al., 2005; Noguchi et al., 2006, 2008; Wang et al., 2008; Chau et al., 2011, Turner et al., 2015a), although links to occurrence of *Prorocentrum cordatum/minimum* have been recently hypothesised (Vlamis et al., 2015).

Along the coast of India there have been reports of the occurrence of several phytoplankton species. These include PSP toxin producing species such as *Alexandrium* spp.,
including *A. tamarense*, *A. minutum* and *A. catenella*, and *Gymnodinum catenatum*. Among identified DSP toxin producers were *Dinophysis* species, such as *D. caudata*, *D. acuta* and *D. acuminata*. DA producers were represented here by *Pseudonitzchia* spp. A PSP outbreak has been reported previously from the Mangalore region of SW India, which resulted in human intoxication including one fatality (Karunasagar et al., 1984; Karunasagar et al., 1990; Segar et al., 1989). Two other PSP outbreaks have also been reported following consumption of toxic bivalves, with one in 1981 from Kalpakham, near Chennai, on the east Indian coast involving a low number of people (unpublished data) and a second in September 1998 from Vizhijam, near Trivandrum, when over 500 people were hospitalised and at least five deaths were reported (Karunasagar et al., 1998). To date there have been no reported occurrences of ASP or DSP intoxication in humans anywhere in India. With an absence of any routine regulatory monitoring programme for shellfish toxins in India, there is a scarcity of data describing the prevalence of marine toxin occurrence in shellfish.

The objectives of this study were therefore to assess the presence of domoic acid, paralytic shellfish toxins, tetrodotoxin and lipophilic toxins in mussels and oysters harvested in the marine waters of Mangalore, SW India. The assessment included the analysis of shellfish species harvested over a period of 13 months from four different shellfish harvesting beds in the Mangalore region. The detection of any hydrophilic or lipophilic biotoxins would provide links to toxic phytoplankton previously reported in Indian waters together with evidence for the potential risk to shellfish consumers from a wide range of natural shellfish toxins.

2. Materials and methods

2.1 Samples

The southern Karnataka coastline consists of long stretches of wide sandy beaches with a few rocky outcrops bisected by several major rivers originating from the western Ghats. Where these discharge into the Arabian Sea they form a network of estuaries, wetlands, mudflats and mangroves, often sheltered from the ocean itself behind sandspits (Sowmya and Jayappa, 2016). At several places along the coast, rich natural beds of *P. viridis* occur in the intertidal and subtidal rocky areas (Sasikumar and Krishnamoorthy, 2010; Sasikumar and Krishnakumar, 2011; Sasikumar et al., 2011). Oysters are less abundant, being present in only some of the major estuarine areas (Rao and Rao, 1985). 110 samples of shellfish tissue were analysed during this study, consisting of both green mussels (*Perna viridis*) and Indian backwater oysters (*Crassostrea madrasensis*). The four marine monitoring sites incorporated in the study were Gangoli, Mulki, Sasthana and Someshwar (Figure 1). At Gangoli, mussels were collected from the Panchagangavali estuary and at Someshwar from the open coast. Oysters were collected from the Padukere (Sasthana) and Nandini (Mulki) estuarine areas. Shellfish were collected using the same methods twice a month over the 13-month study period (Table S1). Typically, 25-50 individuals were collected for each sample. The samples were transported to the laboratory of the Department of Fishery Microbiology, College of Fisheries, Mangalore and were frozen, until required for sample processing.

2.2 Reagents and chemicals

Certified reference toxins for PST, DA and LTs were obtained from the Institute of Biotoxin Metrology at the National Research Council of Canada (NRCC, Halifax, Nova Scotia, Canada). TTX CRM was obtained from Cifga (Lugo, Spain). Microcystins and nodularin were obtained from Enzo Life Sciences, Exeter, UK. All reagents for preparation of LC-MS/MS mobile phases were LC-MS grade, and those used for LC-UV were HPLC grade or better. Trifluoroacetic acid (≥99% purity), glacial acetic acid (≥99% purity), formic acid...
(≥99% purity) and 25% ammonia (NH₄) were all LC-MS grade and purchased from Sigma-Aldrich (Poole, Dorset, UK).

2.3 Shellfish extraction

For each sample, a suitable number of individuals were shucked to generate a minimum of 100 g shellfish tissue. Shellfish meat was homogenized and sub-samples taken for each of the extraction methods. For each batch of samples extracted, a procedural blank consisting of deionised water was prepared. Extracts were stored (-20 °C) until shipped in one batch to the Cefas laboratory for toxin analysis. Extracts were received after three days of transportation in good condition with temperatures maintained < 0°C.

PSP and TTX extraction was conducted using the method of Turner et al. (2015c). 5±0.01g of each sample was extracted in 5 mL of 1% acetic acid in polypropylene centrifuge tubes. The tissues and solvents were vortexed for 90 s before adding capped tubes to a boiling water bath for 5 mins ± 10 s. Samples were subsequently cooled by placing in cold running water for a minimum of 5 mins. After cooling, tubes were vortexed (90 s) and centrifuged for 10 minutes at 4500 rpm, prior to decanting the supernatant into a 15 mL tube.

LT extraction was conducted using a scaled-down version of EURL (2015). 1±0.01g of each homogenised shellfish tissue sample was added to a 15 mL centrifuge tube. 4.5 mL of 100% methanol was transferred to the homogenate and the tubes capped before vortex mixing for 3 min. Extracts were centrifuged at 4500 rpm for 8 min at 20°C. The supernatant was decanted into a new 15 mL tube for each sample extract and PB, before adding a second 4.5 mL aliquot of 100% methanol to the tube containing the pellet. The shellfish solvent mix was again vortex-mixed, centrifuged and the supernatants from both extraction steps combined before diluting to a total volume of 10 mL.

ASP extraction was conducted using a method based on that of Quilliam et al., 1995. 2±0.01 g of each homogenised shellfish tissue sample was weighed into a 15 mL polypropylene centrifuge tube. 4 mL of 50/50 (v/v) methanol/water was pipetted into sample tubes and vortexed for 2 min. Extracts were then centrifuged (3500 rpm) for 20 min at 20°C. The supernatant for each shellfish sample and PB was transferred into separate 15 mL polypropylene tubes. A further 4 mL aliquot of 50/50 (v/v) methanol/water was added to the shellfish pellet tube, vortexed and centrifuged, before decanting into the tube containing the first supernatant. The supernatant was diluted to a total volume of 10 mL with 50/50 (v/v) methanol/water and gently shaken until thoroughly mixed.

2.4 Clean-up and analysis

SPE clean-up of acetic acid extracts prior to analysis for PST and TTX was performed following the method of Boundy et al., (2015). SPE eluents were vortex-mixed and diluted 3:1 with acetonitrile in polypropylene LCMS-grade autosampler vials, before placing into the autosampler (set at +10°C) for analysis using an Acquity I-Class UPLC system coupled to a Waters Xevo TQ-S tandem mass spectrometer (Waters, Manchester, UK). UPLC was conducted using a 1.7 µm, 2.1x150 mm Waters Acquity BEH Amide UPLC column in conjunction with a Waters VanGuard BEH Amide guard cartridge, held at +60°C. Chromatographic and MS/MS parameters used were exactly those detailed by the validated method of Turner et al., 2015c (Table 1). Samples were run together with six-point external calibration solutions prepared from CRM stocks. Toxicity equivalence factors (TEFs) and relative response factors (RRFs) for PST were those described by Turner et al., 2015c (Table 2). For TTX analysis the modified method of Turner et al., (2017a) was followed, with detection conducted using six-level calibration standards prepared from TTX stock solution. Method performance characteristics are those reported by Turner et al., 2015c and Turner et al., 2017a.
Methanolic extracts for each sample was thawed and filtered through a 0.2 µm nylon syringe filter and an aliquot taken for LC-MS/MS analysis of LT. A second 1.0 mL aliquot of the raw extract was transferred into a 2 mL screw capped vial for alkaline hydrolysis, by adding 125 µL of 2.5 M NaOH. After vortex mixing, the vial was heated to 76 ± 2 °C for 40 min, cooled to room temperature before the addition of 125 µL of 2.5 M HCl. The hydrolysed extract was then ready for LC-MS/MS analysis, using an Acquity Ultra Performance Liquid Chromatography (UPLC) system coupled to a Waters Xevo TQ tandem mass spectrometer. UPLC was performed using a Waters BEH C18 column (50 x 2.1 mm, 1.7 µm) with a VanGuard BEH C18 (5 x 2.1 mm, 1.7 µm) guard cartridge. The analytical method used was as described by Turner and Goya, 2015 (Table 1). Toxin concentrations were quantified against six-point external calibrations prepared from NRCC standards. Concentrations of free toxins were determined in non-hydrolysed extracts, with hydrolysed extracts used for assessment of total OA-group toxins (free plus esterified toxins). LTs were confirmed as being detected when both the quantitative and qualifier MRM transitions were present at the expected toxin retention time, with a concentration above the method limit of quantitation, taken in this study as 4 µg/kg per toxin.

The 50/50 (v/v) methanol/water extracts were filtered through 0.2 µm syringe nylon membrane filters into glass autosampler vials. Chromatographic separation for ASP analysis was conducted using a Phenomenex (Manchester, UK) Kinetex PFP 5.0 µm 4.6 x 150 mm HPLC column. LC-UV analysis was performed using Agilent 1100/1200 modules (Agilent, Manchester, UK): quaternary pump, vacuum degasser, autosampler, column over and UV-diode array detector (242 nm). Samples were run alongside external calibration standards for detection and quantitation purposes, with a method LOQ equivalent to 0.2 mg domoic acid per kg shellfish tissue.

3. Results

3.1 PSP and TTX toxins

3.1.1 Total PST and TTX

PST were detected in all four shellfish harvesting areas during the study, in both mussel and oyster samples. The highest concentrations were quantified in oysters from Mulki and Sasthana, with values reaching > 75 µg STX eq/kg in both sites, with a maximum concentration of 82 µg STX eq/kg in oysters from Sasthana, collected in December 2015. Significantly lower total PST concentrations were obtained in the mussels collected from both Gangoli and Someshwar, with the highest concentration ~ 8 µg STX eq/kg in the mussels collected from Gangoli during December 2014. Figure 2 illustrates the temporal variability in total PST quantified in both species across the four sites. At both oyster sites, very low (< 5µg STX eq/kg) levels of PST were presented between December 2014 and March 2015. Subsequently from the end of March 2015 onwards, at both sites, a sudden increase in PSP toxicity was found, with toxins remaining in the flesh consistently until the end of the study period in January 2016. Much lower levels were quantified in the mussels from the two other sites, with the highest concentrations determined in shellfish harvested during early 2015. No TTX was detected in any of the samples from any of the four shellfish harvesting areas.

3.1.2 PST profiles

Oyster samples from Mulki and Sasthana were found to contain a range of PST analogues, including C1&2, GTX2&3, GTX1&4, dcSTX, STX and GTX5. No C3&4, dcGTX2&3, dcGTX1&4, NEO, dcNEO or doSTX was detected in any of the shellfish samples. In terms of toxicity equivalents, the profiles were dominated by GTX1 (mean proportion ~60%), followed by GTX4, GTX2, GTX3 and dcSTX around the same proportion.
(mean ~ 10-15%). The N-sulfocarbamoyl analogues, C1&2 and GTX5 were present at lower relative levels, with mean proportions around 4-6%. Figure 3 illustrates the mean toxin profiles from November 2014 to January 2016 in oysters from each of the two harvesting areas. The results indicate the near identical profiles at both sites. Due to the overall low toxicity in the mussel samples, the toxin profiles proportions were not determined. However, toxins detected included dcSTX, STX, GTX2, C1 and C2. Notably GTX1, the dominant PST congener in the oyster samples, was not detected.

### 3.2 Lipophilic toxins

Analysis of methanolic extracts of mussels and oysters showed a near complete absence of regulated lipophilic marine toxins from the four study areas. No MRM peaks were identified for any of the OA-group toxins, AZAs and YTXs. Esterified OA-group toxins were absent in the hydrolysed extracts. The only LT identified was PTX2, present at very low concentrations (0.4 µg/kg) in one oyster sample from Mulki harvested in Jan 2015. No other shellfish samples from this study contained PTX2 or any other pectenotoxins.

The 3 cyclic imines (CIs) analysed in these samples were SPX1 (13-desMeC spirolide), SPXG (20-Me SPXG spirolide) and GYM (gymnodimine). Of these three, SPX1 and GYM were identified, with 42 samples (~38%) containing detectable levels of SPX1 and all 110 containing GYM. Concentrations of SPX1 were low ranging from 1.7-2.0 µg/kg. Figure 4 summarises the GYM concentrations in both shellfish species throughout the year-long study, with the higher levels found in oysters in comparison to mussels. Concentrations in oysters ranged between 9.0 and 40.2 µg/kg, with elevated values between Nov 2014 to Jan 2015 (mean 24.4 µg/kg). Mussels contained GYM at lower and more consistent concentrations throughout the study (4.7-9.5 µg/kg; mean = 6.8 µg/kg).

### 3.3 ASP

Out of the 100 bivalve mollusc samples analysed in this study, only two showed trace levels of DA. One mussel sample from Gangoli, collected in Nov 2014 showed DA at 0.16 mg/kg, and an oyster sample harvested from Mulki in Feb 2015 presented a similar level of 0.18 mg/kg. Both results were below method LOQ and close to the LOD (0.2 mg/kg). No other samples showed chromatographic peaks indicative of DA.

### 4. Discussion

In relation to the PST regulatory action limit of 800 µg STX eq/kg, the maximum concentrations of PST determined in this study were low. The highest concentrations of toxins quantified reach approximately 10% of action limit, thereby representing a low overall risk to shellfish consumers based on the data generated in this study. The recent work of Turner et al., (2016), showed evidence for low PST uptake (maximum 31 µg STX eq/kg) in mussels in mesocosms containing *Alexandrium minutum* at 100,000 cells/L held at conditions (temperature 28°C and 32°C; salinity 35 PSU and 31 PSU) similar to the environmental conditions recorded in Mangalore during this study (Table S1). The highest concentrations were determined in oysters from Mulki and Sasthana, in comparison to the mussels from Gangoli and Someshwar. Without any of the sites containing both shellfish species, however, it is not clear whether the significant differences recorded are due to the differences in toxin uptake rates between the species, or relate more to the conditions at individual sites. Previous reports of PSP in shellfish from this region showed PSP toxicity rising to 1200 µg STX eq/kg in oysters (*Crassostrea cucullata*) and 3400 µg STX eq/kg in clams (*Meretrix casta*) (Karunasagar et al., 1984). Several PSP intoxications in humans were reported including one fatality. Cooked clams obtained from the homes of affected people and clams collected from the natural bed were analysed by MBA and found to contain PSP at a level of 3370 µg STX
The results from this study show the almost uniform presence of PST in oysters between April and December 2015. Blooms of dinoflagellates along the west coast of India are thought by some authors to proliferate between September and October, although this may relate in part to the lower number of phytoplankton analyses conducted during monsoon season (D’Silva et al., 2012). Other authors, however, have evidenced a dominance of diatoms in the water column until December, with dinoflagellates increasing their overall contribution during February to March (Asplund et al., 2011). Mean toxin profiles in oysters from both shellfish harvesting areas were nearly identical, with a clear dominance of GTX1, together with the presence of other gonyautoxins (GTX2-5), dcSTX, STX and C1&2. Toxin profiles determined from the 1983 outbreak samples showed a similar dominance of gonyautoxins (GTX1-4) and C1&2, as well as lower concentrations of STX and dcSTX. In addition, the results showed the presence of NEO and dcGTX2&3, as well as C3&4, toxins not detected in this study (Karunasagar et al., 1990). These differences may relate to the higher overall toxicity levels found in the 1983 samples in comparison to those from the current study. In addition, the analysis of the outbreak samples was performed using a post-column oxidation LC-FLD method, so may have been subject to interferences for some of the toxins present at low concentrations. Finally, there may have been species-related differences in the toxin profiles as a consequence of bacterial or enzymatic toxin transformation within tissues (Bricelj and Shumway, 1998; Cembella et al., 1994; Jaime et al., 2007; Oshima, 1995; Sakamoto et al., 2000; Sato et al., 2000; Wiese et al., 2010; Turner et al., 2012).

At the time of the toxin profile identification in outbreak samples, authors used the qualitative toxin profile, in tandem with the findings of cysts morphologically similar to A. cohnorticula, to postulate that Alexandrium species was the probable causative organism for PSP occurrence (Karunasagar et al., 1990). Since then, the long-term monitoring of hydrographic parameters such as sea surface temperatures, rainfall, wind speed and water column mixing and phytoplankton occurrence. Nevertheless, whilst phytoplankton communities have been highly dynamic in the past decades, the presence of the potentially PSP-producing genera, Gymnodinium has been found on a regular basis (Godhe et al., 2015). G. catenatum itself was reported to occur both in planktonic and cyst forms in 1996 from waters in the Mangalore region (Godhe et al., 1996). A. minutum has also been found by microscopic and polymerase chain reaction (PCR) detection methods in field samples from Mangalore during 1999 (Godhe et al., 2001). Other toxin producing species identified along the west coast include A. minutum, A. tamarense and A. catenella (Shahi et al., 2015). Certainly the absence of PST analogues related to G. catenatum such as C3&4, GTX6 and dcGTX2&3 (Vale, 2010; Costa et al., 2015) in this study, indicates that the causative organisms in Mangalore during 2015 are possibly Alexandrium spp.

No evidence was found for the presence of TTX in any samples, even during December when V. parahaemolyticus abundance has been shown to be highest in this region (Rehnstam-Holm et al., 2014), although significant variability in V. parahaemolyticus abundance has been previously recorded even during times of stable water column temperature and salinity (Rehnstam-Holm and Godhe, 2012). It is noted however that oysters from this study were collected in the shallow sublittoral zone and mussels were collected by hand divers from deeper water sites. Consequently, none of the shellfish from this study were present in the intertidal zones, where exposure to the high temperatures during low tides may...
potentially result in the increase of bacterial levels, and therefore promote TTX production
(Turner et al., 2017b).

Domoic acid was detected at trace levels only (< 0.2 mg/mg), showing little evidence
for accumulation of toxins from DA-producing phytoplankton in this region. The presence of
organisms such as *Pseudonitzchia* sp. (Härnström et al., 2007; Shahi et al., 2015) and
*Nitzschia* sp. (Härnström et al., 2009; D’Silva et al., 2012; Shahi et al., 2015) has been
previously reported around the west coast of India during period of diatom dominance in the
water column, although the temporal variability in bloom occurrence has been highlighted
(Shahi et al., 2015) and the toxicity of such species from this region has never been tested. As
such the risk, until further toxicity assessment is conducted, should not be discounted.

The EU-regulated LTs were notable by their near-complete absence from both mussel
and oyster samples. This was surprising given the prevalence of at least six species of the
genus *Dinophysis* in ~40% of water samples around the coast over a long-term monitoring
period, between 1990 and 2010 (Godhe et al., 2015). The detection of trace amounts of the
pectenotoxin PTX2 in one sample indicates the presence of *D. acuminata* (Kamiyama and
Suzuki, 2009), but such a species is generally also associated with production of OA-group
toxins (Tango et al., 2004; Reguera et al., 2012, 2014). Species identified along the western
coast of India include *D. acuminata*, *D. caudata*, *D. miles*, *D. norvegica*, *D. tripos* and *D.
rotundata* (Shahi et al., 2015), with several of these associated with DSP toxin production.

Over a 21-year period of assessment, *Dinophysis* spp. were detected in 19 years (~90%), with
variable (moderate to high cell densities) between years. Moreover, cell counts were
positively correlated with sea surface temperatures (SST) during this period. The highest
presence of *Dinophysis* previously recorded was during 1996-1998, which coincided with the
strongest El Nino Southern Oscillation event of the 20th century (Godhe et al., 2015), during
which elevated SST resulted in a significant increase in net phytoplankton abundance. Mean
annual SST values were >30 °C during this period, before decreasing to ~29 °C around 2005
and then increasing to ~30 °C in 2010 (Godhe et al., 2015). During this study, SST ranged
from 26.0 °C to 29.5 °C, with a mean of 27.8 °C. Therefore, it is likely that lower cell densities
of *Dinophysis* spp. were present between 2014 and 2015, although it is noted that there is no
phytoplankton data available to our knowledge. *Dinophysis* species present in the marine
waters around Mangalore have not to date been cultured and tested for toxin production
capability. Until proven otherwise, it is to be inferred that the *Dinophysis* present around
Mangalore may potentially be non-toxic strains.

The consistently low levels of the spirolide SPX-1 throughout the study samples is of
little if any consequence to human food safety, given the lack of evidence for oral toxicity
from cyclic imines (Richard et al., 2001; Davidson et al., 2015). Various *Alexandrium*
species have been identified as SPX producers, including *A. ostenfeldii* and more recently the
morphologically similar, but usually smaller, *A. peruvianum* (Cembella et al., 2000; Touzet et
al., 2008). *A. peruvianum* has been identified along the western coast of India (Shahi et al.,
2015) although the toxin concentrations determined in this study perhaps indicate that
phytoplankton producers are present at only very low densities, which in addition may not be
resolved from the presence of other *Alexandrium* species. Gymnodimine has been linked to
neurotoxicity in mice following i.p injection (Davidson et al., 2015) and has been isolated
from *Gymnodinium mikimotoi* (Seki et al., 1995), later renamed as *Karenia selliformis*
(Haywood et al., 2004). Production of GYM has also been demonstrated in European strains
of *A. ostenfeldii* (Salgado et al., 2015). To date GYM has been identified in shellfish from
Northern and Southern Africa, New Zealand (Krock et al., 2009; Davidson et al., 2015), and
more recently Mexico (Garcia-Mendoza et al., 2014). *Gymnodinium* spp. have previously
been reported as re-occurring in the water column of the study areas over the past few
decades (Godhe et al., 2015), particularly during the warmer months. As discussed in the
context of PST results, blooms of dinoflagellates in this region are generally at their
maximum density between September and October (D'Silva et al., 2012). GYM
concentrations in oysters, however, showed a maxima around December to January, 2-3
months after the expected peak of phytoplankton blooms. Moreover, the increase in GYM
was not observed during the end of 2015. The higher concentrations of GYM in oysters from
this study in comparison to mussels are interesting given the general consensus that many
marine toxins accumulate to significantly higher levels in mussels than many other species of
mollusc (e.g. Bricelj and Shumway, 1998). As with the PST results, the inter-species
differences for GYM may either relate to species-specific uptake effects or to differences in
the water column during shellfish feeding and toxin uptake.

Overall the results have indicated a relatively low level of risk from biotoxins for the
majority of the study period. With maximum total PST concentrations around 10% of the
current EU regulatory MPL of 800 µg STX eq/kg, no significant concentrations of regulated
lipophilic marine toxins and only trace levels of domoic acid detected, there is good evidence
that the shellfish grown and consumed during 2015 were relatively free from harmful toxins.
However, with past work showing significant inter-annual differences in toxin phytoplankton
production in Mangalore, more analysis on a larger number of samples would be required
over a longer time period to generate a better understanding of risk to shellfish consumers in
this region of India. Given the significant growth in the local shellfish industry including
international export, and the socio-economic impacts this brings to the region, it is critical
that routine monitoring of bivalve mollusc production areas is implemented, to help mitigate
against these potentially life-threatening natural toxins.

Acknowledgements
The authors are grateful for help with sample preparation provided by the technical staff at
the Department of Fishery Microbiology, College of Fisheries, Mangalore during this study.
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Table 1. MRM transitions used for LC-MS/MS detection and quantitation of PST, TTX and LT analogues, with primary (quantitative) transitions highlighted in bold

<table>
<thead>
<tr>
<th>Analogue</th>
<th>ESI+ Transition</th>
<th>ESI- Transition</th>
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<tbody>
<tr>
<td>STX</td>
<td>300.1&gt;204.1,138.0</td>
<td></td>
</tr>
<tr>
<td>NEO</td>
<td>316.1&gt;126.1,220.1</td>
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</tr>
<tr>
<td>dcSTX</td>
<td>257.1&gt;126.1,222.0</td>
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<tr>
<td>dcNEO</td>
<td>273.1&gt;126.1,225.1</td>
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<tr>
<td>doSTX</td>
<td>241.1&gt;60.0,206.1</td>
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<tr>
<td>TTX</td>
<td>320.1&gt;302.1,162.1</td>
<td>394.1&gt;351.1,333.1</td>
</tr>
<tr>
<td>GTX2</td>
<td>396.1&gt;298.1</td>
<td>394.1&gt;333.1</td>
</tr>
<tr>
<td>GTX3</td>
<td>396.1&gt;298.1</td>
<td>410.1&gt;367.1,349.1</td>
</tr>
<tr>
<td>GTX1</td>
<td>412.1&gt;314.1</td>
<td>410.1&gt;367.1</td>
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<tr>
<td>GTX4</td>
<td>380.1&gt;300.1</td>
<td>378.1&gt;122</td>
</tr>
<tr>
<td>GTX5</td>
<td>396.1&gt;316.1</td>
<td>394.1&gt;122</td>
</tr>
<tr>
<td>GTX6</td>
<td>353.1&gt;255.1</td>
<td>351.1&gt;164.0,333.1</td>
</tr>
<tr>
<td>dcGTX2</td>
<td>369.1&gt;271.1</td>
<td>367.1&gt;349.1</td>
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<tr>
<td>dcGTX3</td>
<td>396.1&gt;298.1</td>
<td>474.1&gt;122.0,351.1</td>
</tr>
<tr>
<td>dcGTX1</td>
<td>412.1&gt;332.1</td>
<td>490.1&gt;410.1</td>
</tr>
<tr>
<td>dcGTX4</td>
<td>412.1&gt;314.1</td>
<td>490.1&gt;392.1</td>
</tr>
<tr>
<td>C1</td>
<td>351.1&gt;164.0,333.1</td>
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</tr>
<tr>
<td>C2</td>
<td>396.1&gt;298.1</td>
<td>410.1&gt;367.1</td>
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<tr>
<td>C3</td>
<td>412.1&gt;332.1</td>
<td>490.1&gt;410.1</td>
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<td>C4</td>
<td>412.1&gt;314.1</td>
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<tr>
<td>OA, DTX2</td>
<td>803.5&gt;255.1,113</td>
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<td>YTX</td>
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<tr>
<td>45 OH YTX</td>
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<tr>
<td>45 OH homo YTX</td>
<td>585.5&gt;474.2,403.2</td>
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<tr>
<td>AZA1</td>
<td>842.5&gt;654.4,362.3</td>
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<td>AZA2</td>
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<td>828.5&gt;658.4,362.3</td>
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<td>PTX1, PTX11</td>
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<td>GYM</td>
<td>508.4&gt;136.1,162.1</td>
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<tr>
<td>20-Me SPX-G*</td>
<td>706.5&gt;164.2</td>
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*Only 1 MRM used for identification and quantitation
### Table 2. Toxicity equivalent factors (TEFs) used in study.

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</tr>
<tr>
<td>C3</td>
<td>0.02</td>
</tr>
<tr>
<td>C4</td>
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<tr>
<td>dcGTX2</td>
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</tr>
<tr>
<td>dcGTX3</td>
<td>0.4</td>
</tr>
<tr>
<td>dcGTX1</td>
<td>0.5⁺</td>
</tr>
<tr>
<td>dcGTX4</td>
<td>0.5⁺</td>
</tr>
<tr>
<td>GTX2</td>
<td>0.4</td>
</tr>
<tr>
<td>GTX3</td>
<td>0.6</td>
</tr>
<tr>
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</tr>
<tr>
<td>GTX4</td>
<td>0.7</td>
</tr>
<tr>
<td>GTX5</td>
<td>0.1</td>
</tr>
<tr>
<td>GTX6</td>
<td>0.1</td>
</tr>
<tr>
<td>doSTX</td>
<td>0.05²</td>
</tr>
<tr>
<td>dcSTX</td>
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</tr>
<tr>
<td>dcNEO</td>
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<tr>
<td>STX</td>
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<tr>
<td>NEO</td>
<td>1</td>
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<tr>
<td>OA</td>
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<tr>
<td>DTX1</td>
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<tr>
<td>DTX2</td>
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<td>PTX2</td>
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<td>AZA1</td>
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<td>AZA3</td>
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<td>45-OH homo YTX</td>
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</tbody>
</table>

1- dcGTX1 and dcGTX4 based on assumed toxicity equivalency factors (Sullivan, 1983)
2- doSTX toxicity equivalency factor (Turner et al., 2015b)
Figure 1. Map showing location of shellfish harvesting areas and photos of four marine monitoring points for bivalve molluscs sampled during this study a) Gangoli b) Mulki c) Someshwar d) Sasthana
Figure 2. Summary of total PST concentrations (µg STX eq/kg) quantified in mussels and oysters from four shellfish harvesting areas in Mangalore.
**Figure 3.** PST profiles in oysters from Mulki and Sasthana
Figure 4. Summary of GYM concentrations (µg/kg) quantified in mussels and oysters from four shellfish harvesting areas in Mangalore.
• First ever systematic study of Indian shellfish toxins
• Application of chemical detection monitoring
• Assessment of marine biotoxins
• PST temporal variability
• PST profile assessment
Ethical Statement

To whom it may concern,

All authors have agreed to this submission and the final manuscript has been seen by all authors. This paper has not been published and the authors will not permit its submission or publication elsewhere before it is accepted or declined by this journal.

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