Marine Biology (2019) 166:82
https://doi.org/10.1007/s00227-019-3529-x

ORIGINAL PAPER

The invasive sea slug *Pleurobranchaea maculata* is a vector of two potent neurotoxins in coasts of Argentina

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Received: 30 August 2018 / Accepted: 14 May 2019

Abstract

Toxic exotic organisms can have profound effect as new vectors of keystone compounds in non-native areas. In recent years the invasive sea slug *Pleurobranchaea maculata* has been reported as thriving along the oriental coasts of South America. The same species had been previously found to contain high levels of tetrodotoxins (TTXs) in its native range. With the aim of determining toxin contents for the introduced individuals we performed mouse bioassays (MBA) and liquid chromatography tandem–mass spectrometry analyses (LC–MS/MS) in three distant populations (−38°02′11″, −57°31′28″; −40°29′59″, −60°14′09″; −42°44′15″, −65°01′40″) and followed the temporal variation in toxin contents in one of them, from June 2014 to January 2015. Relative low levels of TTXs were detected jointly with high levels of PSTs. This is the first identification of TTXs in the temperate coasts of the southwestern Atlantic and the first detection of PSTs in a pleurobranch, in both adults and eggs. Concentrations of PSTs and TTXs varied widely among individuals and populations, and through time. Our results provide new hints on the origin and acquisition mechanisms of these toxins in *P. maculata* and highlight the risk posed by the introduction of this new vector of potent neurotoxins for seafood safety and marine communities in the invaded area.

Introduction

Toxins are traditionally seen as chemical defenses only, although also have role as signals, antioxidants and osmoregulatory molecules mediating a variety of interactions between organisms (Zimmer et al. 2006; Zimmer and Ferrer 2007; Derby and Aggio 2011). A few distinct toxins that are relatively rare in the environment yet perform multiple functions with impact in the community stronger than expected from its relative abundance. Such compounds have been termed 'keystone molecules' in analogy to the ecologic concept of keystone species (Zimmer and Ferrer 2007; Derby and Aggio 2011). Two guanidine alkaloids, saxitoxin (STX) and tetrodotoxin (TTX) are notable examples that occur naturally in a wide variety of taxa (Cusick and Sayler 2013; Ferrer and Zimmer 2013; Bane et al. 2014). These two compounds have different chemical structures but share activity as high-affinity neurotoxins that selectively block the voltage-gated sodium channels, inhibiting propagation of action potentials in muscle and nerve cells (Soong and Venkatesh 2006), with often fatal effect to humans at even extremely low doses and no antidote so far (Noguchi and Arakawa 2008).

Responsible Editor: E. Briski.

Reviewed by Undisclosed experts.

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s00227-019-3529-x) contains supplementary material, which is available to authorized users.

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Published online: 27 May 2019

Saxitoxin is actually the parent molecule in a class of compounds collectively referred as paralytic shellfish toxins (PSTs), due to its relation to the paralytic shellfish poisoning. The group is formed by dozens of structurally related neurotoxins, including saxitoxin itself (STX), neosaxitoxin (NEO), gonyautoxins (GTXs) and decarbamoylsaxitoxin (dcSTX) among others (Wiese et al. 2010). PSTs are mainly produced by dinoflagellates and then bio-accumulated throughout the food chain (Hall et al. 1990; Llewellyn et al. 2006; Wiese et al. 2010). Hence, local blooms of PSTs-producing dinoflagellates pose a risk for human health and seafood safety with large economic impact at a global scale. Thousands of cases of paralytic shellfish poisonings are registered per year globally, which around 15% are fatal (Cusick and Sayler 2013).

Tetrodotoxin is also the parent molecule of a group of closely related compounds grouped under the name tetrodotoxins (TTXs). These toxins are notorious for being the causative of the puffer fish poisoning, where it was first detected (Alcaraz et al. 1999; Noguchi et al. 2011). TTXs are known to also occur in a wide variety of phylogenetically distant marine invertebrates (Llewellyn and Endean 1989; Freitas et al. 1996; Ito et al. 2003; Lopes et al. 2013; Asakawa et al. 2013; Turner et al. 2015a), fish other than puffer fish (Noguchi et al. 1986; Yasumoto et al. 1986; Katikou et al. 2009), newts and frogs (Noguchi and Arakawa 2008). TTXs is produced by diverse bacteria (Noguchi et al. 1986; Yasumoto et al. 1986; Wu et al. 2005) and passed through metazoans by feeding, although the existence of alternate pathways and mechanisms through which TTXs are incorporated in the food web remains controversial (Magarlamov et al. 2017). Episodes of TTX poisoning were historically concentrated in warm water regions in the Pacific and Indian oceans, but in the last decade spread to a variety of organisms from more temperate waters such that it is currently considered an 'emerging marine toxin' (Bane et al. 2014).

In 2009 we detected the introduction of the sea slug *Pleurobranchaea maculata* in Argentina, which is currently distributed along more than 2000 km shoreline (Farias et al. 2015, 2016; Battini et al. 2019). The species is native to New Zealand (NZ) and southeastern Australia, but was also mentioned in China, Sri Lanka and Japan (Willan 1983); although Asian records require further taxonomic confirmation. A recent genetic study pointed to NZ populations as the likely origin of the invasion based on mtDNA data (Yıldırım et al. 2018). In its native area *P. maculata* is found from 0 to 300 m depth (Willan 1983), often at high densities and associated to bivalve beds (Taylor et al. 2011, 2015). Although described primarily as generalist consumers and scavengers (Khor et al. 2014; Taylor et al. 2015; Salvitti et al. 2016; Bökenhans et al. 2018), these slugs are also voracious selective predators that can significantly alter the local abundance of their prey (Ottaway 1977).

Pleurobranchaea maculata remained little known until being implicated in a spate of dog poisonings caused by the ingestion of TTX-bearing slugs washed onto beaches of Auckland, NZ (McNabb et al. 2010). In the light of this, we performed preliminary mouse bioassays (MBA) with a few individuals from Argentina, and detected neurotoxic activity similar to that found in NZ (Farias et al. 2015). Marine neurotoxins are well known in the temperate waters of the southwestern Atlantic due to the human syndrome named paralytic shellfish poisoning and its association with the consumption of shellfish exposed to harmful algal blooms. However, the possible introduction and spread of a new potent neurotoxin as resulting from the invasion of *P. maculata* raise serious concern for the potential impact on the local environment and human health.

Here we identified the neurotoxins present in the non-native populations of *P. maculata*, and studied their spatial and temporal variations in contrast to described for populations in the native area. The significance of our findings is discussed in the context of what is known about the origin, accumulation mechanisms and possible consequences of the introduction of this toxin bearing slugs for the local ecosystems and human health.

Materials and methods

Sampling

Individuals of *P. maculata* (Fig. 1a) were collected from three locations separated more than 300 km apart in Argentina (Fig. 1b): (1) Mar del Plata (38°02′11.1″S 57°31′27.9″W, 2–7 m depth, SCUBA diving), (2) off Buenos Aires (40°29′59″S 60°14′09″W, 60–75 m depth, bottom trawling onboard the R/V Puerto Deseado- CONICET); and (3) Puerto Madryn (42°44′15.22″S 65°01′40.27″W, 3–5 m depth, SCUBA diving). Samplings consisted in the collection of 10–40 individuals and were performed in May and December 2014 in Mar del Plata, in August 2014 off Buenos Aires, and on a monthly basis from June 2014 to January 2015 in Puerto Madryn. During sampling in Mar del Plata in December 2014, one individual was found laying a coiled mucilaginous strand containing eggs (Fig. 1c) which was included in further toxin analyses.

Detection of toxicity by mouse bioassays (MBAs)

Preliminary assessments for neurotoxins in our samples were performed by mouse bioassays (MBAs) to select samples to be sent out for more detailed analysis. To this, specimens were frozen and transported to the Marine Biotoxins Laboratory of the National Service of Agri-Food Health and Quality (SENASA) based in Mar del Plata, Argentina. Following

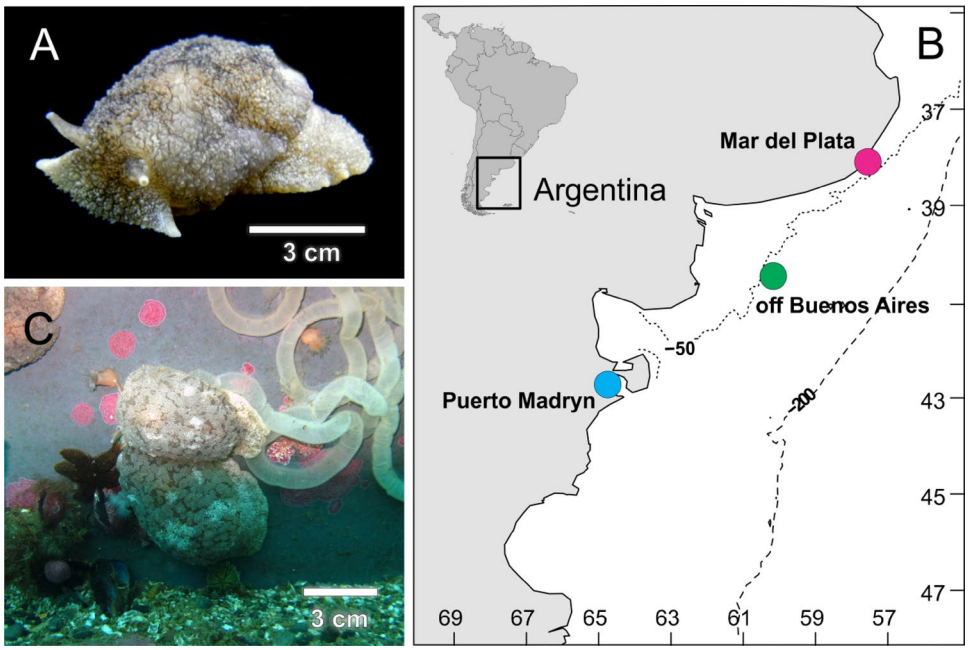

Fig. 1 **a** Habitus of a mature *Pleurobranchaea maculata*. **b** Sampling sites along the invaded area in coasts of the Argentine Sea, SW Atlantic Ocean. **c** Two individuals of *Pleurobranchaea maculata* laying eggs (white string adhered to the rock)

the AOAC 959.08 protocol for PSP analysis (Anon 2005), a minimum of 10 slugs from each sampling were separated, defrosted and homogenized, and 100 g of homogenate mixed with 100 mL 0.1 M hydrochloric acid using an Ultra-Turrax disperser. The pH was adjusted to 3.0–3.5. The extract was heated and subsequently boiled gently for 5 min, cooled in cold water to room temperature, and the pH readjusted to 3.0–4.0 if required. Acidic extracts were centrifuged and the supernatants were used for the bioassays. MBAs were performed using triplicate albino mice CF1 strain (body weight ranging 18–22 g). Sample toxicities were calculated from the median death times of the mice and expressed in terms of micrograms STX di-HCl equivalents per kilogram of tissue. The conversion factor (CF) value applied was 0.19. The limit of detection (LOD) of the bioassay was found to range from 300 to 350 µg STX di-HCl eq kg^{-1}. The remaining unprocessed slugs and homogenates were stored frozen (− 20 °C).

Toxin identification and quantification by liquid chromatography tandem–mass spectrometry (LC–MS/MS)

With the aim of identifying and quantifying hydrophilic toxins present in slugs' tissues, liquid chromatography tandem-mass spectrometry (LC–MS/MS) analyses were performed on individual specimens, and homogenates of pooled whole animals remaining from the previous MBAs. To that, samples were defrosted and then the individuals homogenized using an UltraTurrax® blender. All homogenized tissues were subjected to a single dispersive extraction using 1% acetic acid, as detailed by Boundy et al. (2015) and validated by Turner et al. (2015b). Then 5.0 ± 0.1 g of homogenized tissues were weighed into a centrifuge tube followed by the addition of 5.0 mL of 1% acetic acid. The mixture was vortex-mixed for 90 s at 2500 rpm, before being placed into a boiling water bath for 5 min. Samples were subsequently cooled for 5 min in cold running water, before further vortex-mixing for 90 s (2500 rpm). Samples were then centrifuged at 4500 rpm for 10 min, before pipetting a 1.0 mL aliquot into a 5 mL polypropylene tube and adding 5 µL 25% ammonia before clean-up. A Gilson Aspec XL-4 solid-phase extraction (SPE) liquid handler was used for automated SPE de-salting clean-up of both acetic acid extracts prepared at CEFAS, and HCl extracts prepared previously for the MBAs. SPE was performed using Supelclean ENVI-Carb 250 mg/3 mL SPE cartridges. Cartridges were conditioned and 400 µL of acidic extract was loaded onto the cartridge, followed by a 700 µL of deionised water wash (Boundy et al. 2015). Sample extracts were eluted and collected following the addition of 2 mL 20% MeCN + 0.25% acetic acid. SPE eluants were vortex-mixed prior to dilution of 100 µL aliquots in 700 µL polypropylene autosampler vials with 300 µL MeCN.

Hydrophilic interaction chromatography tandem-mass spectrometry analysis (HILIC-MS/MS) was conducted using a Waters (Manchester, UK) Xevo TQ-S tandem quadrupole mass spectrometer (MS/MS) coupled to a Waters Acquity UHPLC. Chromatography was conducted using a 1.7 µm, 2.1 × 150 mm Waters Acquity BEH Amide UPLC column in conjunction with a Waters VanGuard BEH Amide guard cartridge. The columns were held at + 60 °C, with samples held in the autosampler at + 10 °C. The sample injection volume was 2 µL. Mobile phases, mass spectrometer conditions and MRM transitions were those described by Turner et al. (2015b). The

HILIC-MS PST method involves the direct quantitation of fourteen saxitoxin analogues against toxin standards available as certified reference standards. Five additional analogues (C3, C4, dcGTX1, dcGTX4 and GTX6) were incorporated into the method, with quantitation performed using the calibrations generated from their nearest structural analogue, using experimentally determined relative response factors (Boundy et al. 2015). External standards were prepared in cleaned-up and diluted blank acetic acid extracts of shellfish. Toxicity equivalence factors (TEFs) for PST were those described by Turner et al. (2015b). Individual toxin concentrations were calculated and total toxicity summed from the individual concentration contributions from all quantified toxins and quoted in terms of μg STX di-HCl eq kg^{-1}.

Analysis of TTX and associated TTX analogues gg y was conducted on the same cleaned-up and diluted acidic extracts on the same instrumentation and mobile phases using the method of Turner et al. (2017). Additional analogues (4-epi-TTX, 5,6, 11-trideoxy TTX; 11-nor TTX-6-ol; 4,9-anhydro TTX; 5-deoxy TTX/11-deoxy TTX) were incorporated into the method, with semi-quantitation performed using the TTX calibration. Confirmation of the detection of TTX analogues was performed using the comparison of primary and secondary MRM peaks against those present in the analytical standard and the quality control samples. The ratio between the two MRMs was also used for confirmation purposes. No toxicity equivalence factors (TEFs) were applied to calculations of TTX analogues, given the current absence of any formal published recommendations. Hence, concentrations were expressed as μg TTX kg^{-1}. For the quantitation of TTXs in samples, toxin concentrations were not adjusted for recovery (Turner et al. 2017).

Statistical tests

Correlations were assessed using Pearson's product-moment correlation and the alternative hypothesis of true correlation not equal to 0 tested under a two-sided *t* test. Assumptions of homogeneity of variances and normality were tested using Levenes's and Shapiro–Wilk's tests, respectively. Differences in toxin concentration among sampling dates were assessed using a one-way ANOVA with Welch's correction for unbalanced samples. Due to unequal sample sizes, post hoc pairwise comparisons between successive months were carried out using the Games-Howell test (Games and Howell 1976). Statistical analyses and graphs were performed using the R program (R Core Team 2016).

Results

Toxin analyses

A total of 13 pooled samples, 59 individual specimens and 1 egg strand were analyzed by LC–MS/MS. A breakdown of all the samples can be found in Table S1 of the Supplementary Material. TTXs were present in 34 of the 71 cases analyzed whereas PSTs were detected in all individual and pooled samples. Both toxin groups were present in the same specimen in 44% of the cases, with no correlation between total TTXs and PSTs at the individual level (Pearson's coefficient = − 0.094; Two sided *t* test: $t = -0.82$, $df = 76$, $p = 0.41$) (Fig. 2).

Spatial and individual variability in neurotoxin concentrations in *Pleurobranchaea maculata*

The levels of TTXs and PSTs showed high geographical variation in terms of both the total amount of toxins and the relative proportion of their different analogs (Fig. 3). The PSTs concentrations in individuals collected off Buenos Aires in August 2014 were up to three orders of magnitude higher than those taken in Puerto Madryn during the same period; whilst the individuals collected in Mar del Plata during December 2012 have relatively smaller amounts of PSTs, but much higher levels of TTXs (up to two orders of magnitude) compared with those collected in Puerto Madryn at the same month. In Mar del Plata both toxin groups were detected jointly in all samples and at the individual level the concentrations of TTXs ($n = 8$, mean = 183.8 μg kg^{-1}, min = 3.2 μg kg^{-1}, max = 626 μg kg^{-1}) exceeded those of PSTs ($n = 8$, mean = 59.6 μg STX eq kg^{-1}, min = 11.5 μg STX eq kg^{-1}, max = 205 μg STX eq kg^{-1}) in 63% of cases, including an egg strand containing 13 μg STX eq. kg^{-1} of PSTs and 81.3 μg kg^{-1} of TTXs. Conversely, in Puerto Madryn while PSTs were also present in all samples, TTXs was not detected in 53.6% of cases and among those individuals containing both toxin groups, 95% had higher concentrations of PSTs ($n = 56$, mean = 177.4, min = 6.3, max = 869.6) than TTXs ($n = 56$, mean = 5.7, min = 0, max = 145). Lastly, slugs sampled off Buenos Aires all had high levels of PSTs ($n = 12$, mean = 3934.6 μg eq STX kg^{-1}, min = 431.2, max = 8873), but TTXs was absent or below the detection limits in all cases.

In terms of toxin profiles, the PST analogue saxitoxin was largely the principal component in the three populations analyzed, followed by dcSTX and minor proportions in Puerto Madryn of doSTX and GTX1-5, which varied with time and among individuals (Figs. 3,

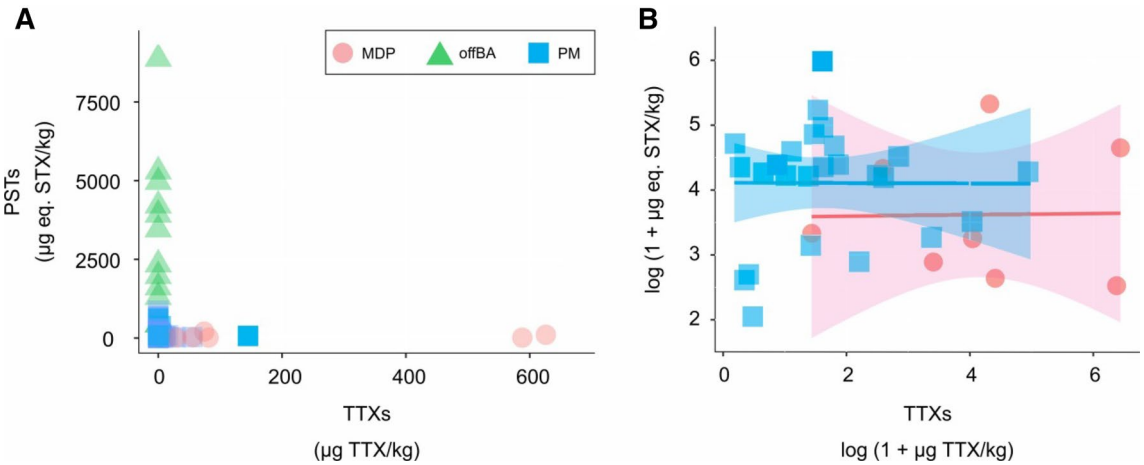

Fig. 2 Relationship between the contents of PSTs and TTXs at individual level as determined by LC–MS/MS analysis. **a** Untransformed data of all individuals analyzed from the three sites sampled. Although PSTs were detected in all samples, only 34 individuals had detectable levels of TTXs. **b** Log transformed data. The samples from off Buenos Aires are not included as none of them contained TTXs. Correlation lines were calculated for each site and appear in the same color, with the shading areas representing one standard error. *MDP* Mar del Plata, *offBA* off Buenos Aires, *PM* Puerto Madryn

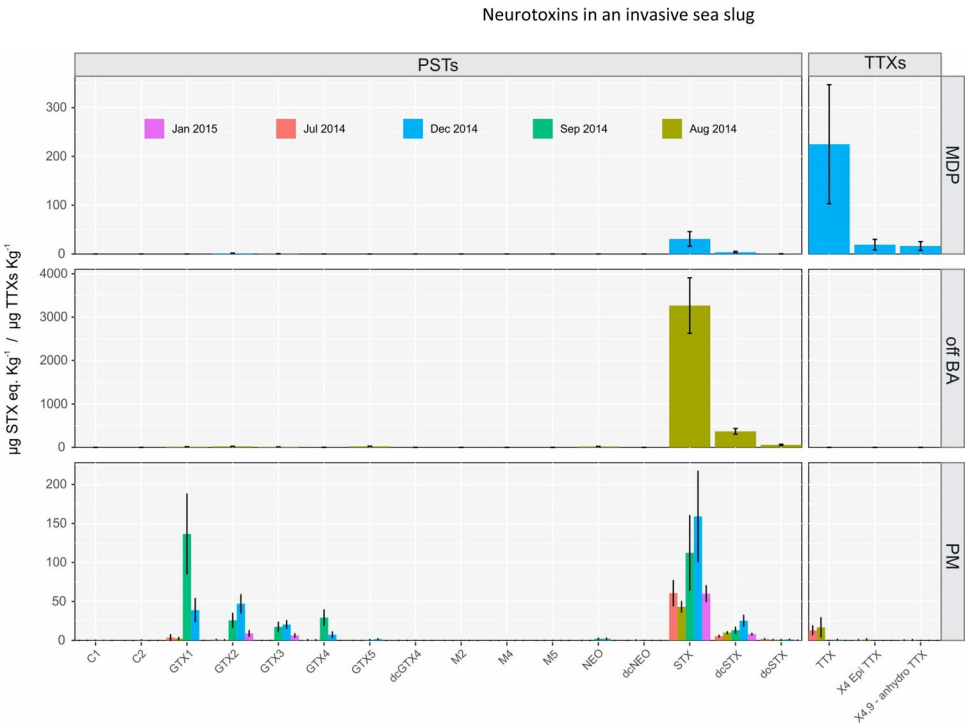

Fig. 3 Spatial variation in concentrations of the different analogs of PSTs and TTXs found in *Pleurobranchaea maculata* from Argentina, as determined by LC–MS/MS analysis. Bar heights are mean values and whiskers represent ± SE. For comparisons note that bar colors represent sampling dates and that the vertical axis scale differs among sites

4, respectively). With the exception of Mar del Plata, traces of other analogs were also quantified, mostly NEO and dcNEO, but also C1&2 and M2. When comparing among sites along the same months, the toxin profiles from Puerto Madryn were notably different to those from the other two areas, with higher contribution of GTXs and other analogs to the total PSTs (Fig. 4). Although the analogs GTX6, C3&4, and dcGTX1&4 were incorporated into the detection method they were not detected in any of the samples. Regarding TTXs analogs, they were almost exclusively represented by TTX itself, with traces of only two variants 4-epiTTX and 4,9-anyhdroTTX present in some samples (Figs. 3, 4).

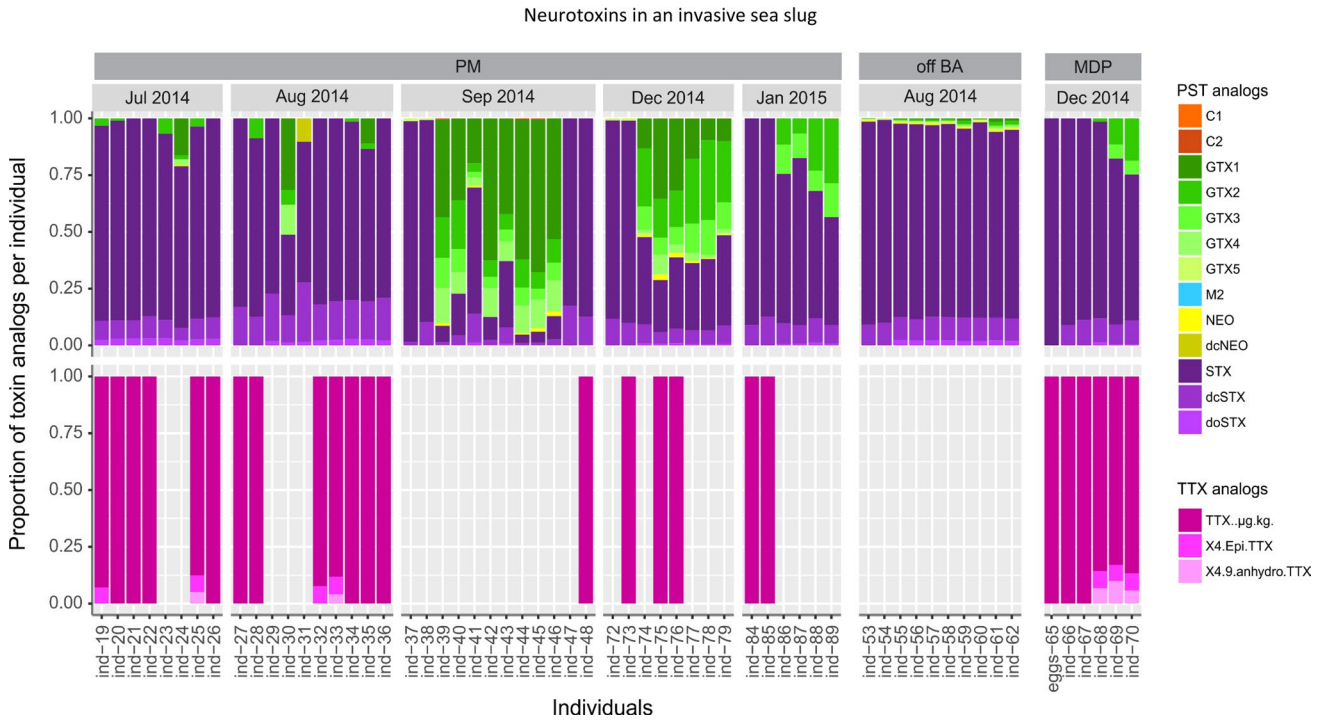

Fig. 4 Individual variation of the relative proportions of the analogs of PSTs and TTXs, grouped by sampled month and area. Each bar represents the relative content of toxin analogs in an individual sample as quantified by LC MS/MS analysis. All the analogs detectable in this study (i.e., those for which toxin standards were available) are included in the legend independently of having been found or not among the analyzed samples. *PM* Puerto Madryn, *off BA* off Buenos Aires, *MDP* Mar del Plata. Note that the sample numbered 65, collected in December 2014 in Mar del Plata, is an egg mass

Temporal Variation in toxin concentrations in *Pleurobranchaea maculata* from Puerto Madryn

A total of 134 individuals were collected in Puerto Madryn on a monthly basis, with the number of individuals sampled varying between months (Table S1). From this, 90 individuals were used for the MBAs and the remaining 44 analyzed individually by LC–MS/MS.

PSTs were present in all individuals throughout the entire sampling period. The higher averages and maxima of total PSTs occurred at the end of September ($n = 13$; mean = 368 µg STX eq kg^{-1}, ranging 33–870 µg STX eq kg^{-1}) and mid-December ($n = 9$; mean = 280 µg STX eq kg^{-1}, ranging 96–616 µg STX eq kg^{-1}) (Fig. 5). The period of increase in total PSTs coincides with a clear change in the toxin profiles in which the relative proportion of STX diminished reaching a minimum in September, when it was replaced mostly by GTX variants, before increasing again in December and January (Fig. 4 and Table S1). The relative proportions of other analogs such as doSTX, and to a lesser extent dcSTX, NEO and dcNEO, also varied throughout the sampling period although at very low relative concentrations.

In contrast, TTXs were detected in only half of the slugs tested, and at comparatively much lower levels. Games-Howell pairwise comparisons confirmed that the highest TTX levels occurred in mid-July ($n = 9$; mean = 12.6 m ranging 0–55 µg kg^{-1}) and the end of August ($n = 11$; mean = 17, ranging 0–145 µg kg^{-1}), being the mean levels not significantly different from 0 for the rest of the year (Fig. 5).

Discussion

Spatial and individual variability in neurotoxin concentrations in *Pleurobranchaea maculata*

There was a wide inter-population and inter-individual variation in the levels of TTXs and PSTs among the samples of the invasive *P. maculata*. Such variability is common among toxic species of disparate taxa (Miyazawa et al. 1985; Dao et al. 2009; Bane et al. 2014) and reflects differences in either the availability of external sources or the capability to produce or accumulate the toxins. Among opisthobranches de novo synthesis of toxins is rare (Wägele and Klussmann-Kolb 2005), and particularly in *P. maculata* most evidences point to trophic transference and bioaccumulation as the source for the large amounts of TTXs registered in some individuals (Wood et al. 2012b;

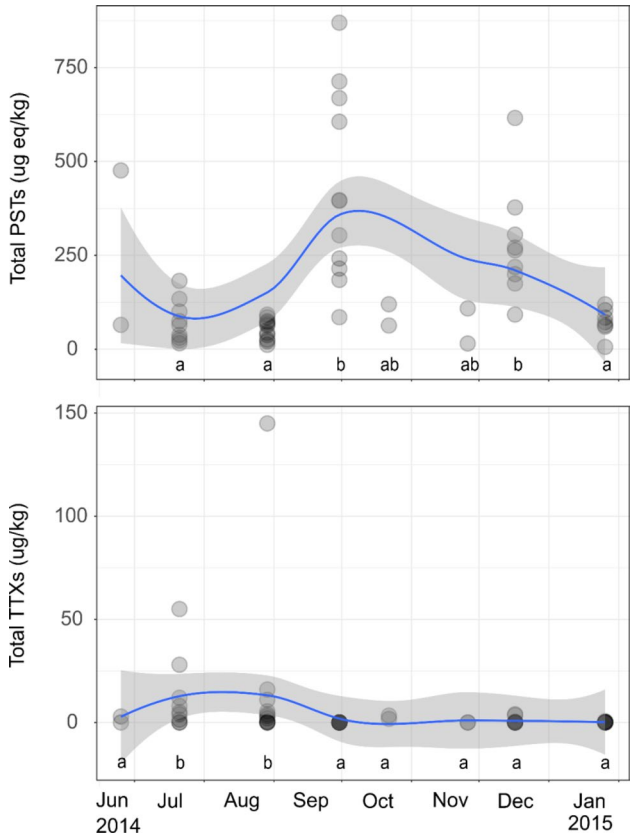

Fig. 5 Temporal variation in toxin concentrations in *Pleurobranchaea maculata* collected in Puerto Madryn (Chubut, Argentina) from June 2014 to January 2015. Each point is the per-individual total concentration of Tetrodotoxins (TTXs) and Paralytic Shellfish Toxins (PSTs) as quantified by LC–MS/MS analysis. Different letters mean statistically significant differences between samplings according to the Games-Howell pairwise comparisons test with alpha = 0.05. The line is a loess smoothed local polynomial regression and the grey shading represents one standard error

Salvitti et al. 2015a). However, and particularly, when the toxin concentration in slug's tissues is very low, secondary sources as the incidental intake of TTXs producer bacteria while feeding, or even the endogenous production via endosymbiosis could not be discarded (Wood et al. 2012a; Khor et al. 2014; Salvitti et al. 2015b, c, 2016).

In Argentina the levels of TTXs were low compared to that of toxic populations in NZ (Wood et al. 2012b). Despite this, in both native and invaded areas there was a common pattern of wide spatial variation in TTXs levels with toxic and non-toxic individuals co-occurring. Studies in NZ reported marked differences in toxicity between northern and southern populations (McNabb et al. 2010; Wood et al. 2012b; Salvitti et al. 2016). A recent study found no evidence of genetic differences that may explain the spatial correlation in toxicity. Instead, the regional differences in the prevalence of TTXs seem to be determined by the distinct bio-oceanographic features of each island and the consequent availabilities of TTXs sources.

Similarly to NZ, the three locations in Argentina differ markedly in oceanographic and ecological features that could explain the toxin profiles reported here. Individuals with the highest PSTs contents were collected off Buenos Aires on natural beds of the Patagonian scallop *Zygochlamys patagonica*, which are often exposed to harmful algal blooms of the dinoflagellate *Alexandrium* spp. known to produce PSTs (Carreto et al. 1996; Gayoso 2001; Montoya et al. 2018). In fact, large amounts of these toxins were detected in the viscera of *Z. patagonica* in natural beds off Buenos Aires (Ciocco et al. 2006; AG and AT, unpublished data) and a recent study confirmed that *P. maculata* readily consumes the exposed soft tissues from bivalves that are open after damaged or dead (Taylor et al. 2015). In contrast, the prevalence of TTXs over PSTs in Mar del Plata may result from the low energy conditions found within the confined port, that favor the accumulation of fine sediments and particulate organic matter where TTX-producing bacteria and spores can flourish (Pratheepa and Vasconcelos 2013 and references therein), while inhibits the incidence of local blooms of microalgae by restricting nutrient circulation and water exchange. Lastly, the specimens from Puerto Madryn come from an open port located inside the Nuevo gulf, a large body of water of about 70 km long and deeper than the adjacent continental shelf, which circulation is tide-dominated (Mazio et al. 2004). Within the gulf, blooms of the PSTs produced by *Alexandrium* occur sporadically with strong interannual variation (Gayoso 2001). Hence, the presence of both toxin groups at comparable levels could be explained by the lack of barriers restricting the exposure of the slugs and their filter feeding preys to harmful algal blooms, in combination with the relative low-energy conditions favoring the deposit and accumulation of organic matter and bacteria.

Regarding the inter-individual variation, there are at least four alternatives, non-exclusive hypotheses to explain it. A first explanation is related to differences in body mass, under the reasoning that if the toxins are synthesized endogenously then larger individuals would produce more toxins. Likewise but under the hypothesis of an exogenous source, larger individuals might accumulate more toxins by consuming more or larger toxin-bearing preys, including cannibalism over smaller toxic conspecifics, which is a common behavior of *P. maculata*. However, these scenarios would result into a positive correlation between toxin concentration and body mass that was not corroborated by previous studies on this species (Wood et al. 2012b). A third possibility is that the exogenous sources of toxins are randomly distributed within the environment or among individual preys, so that differences in toxin profiles at the individual level would result simply by chance. In fact, variable rates of toxin accumulation by primary consumers are well documented (Bricelj

et al. 2012). Lastly, differences in the ability to produce, sequester and accumulate toxins may result directly from inter-individual genetic variation (MacQuarrie and Bricelj 2008). Further experiments must be performed to test these hypotheses.

Temporal variation in toxin concentrations in *Pleurobranchaea maculata* from Puerto Madryn

In samples collected in Puerto Madryn the average level of TTXs increased slightly during the austral winter, from mid-July to the end of August, being virtually absent during the rest of the year. In turn, PSTs appear delayed with respect to TTXs, with the maximum at the beginning of the spring, then decreasing through the successive months. Accordingly, nutrient concentrations within the gulf peak in winter followed by a phase of depletion in late spring and summer, when dinoflagellates tend to be abundant (Gayoso 2001).

The above pattern in TTXs agree well with the observed in the only other population followed across time, in NZ, where there was maximum of TTXs levels during June – August, then falling to a minimum throughout the rest of the year (Wood et al. 2012b). In NZ, the period of maximum levels of TTX coincides to the laying season in austral winter, and considering that TTX was detected in the eggs strands, together with the fact that the highest TTX concentrations generally occur in the gonads (Wood et al. 2012a; Khor et al. 2014; Salvitti et al. 2015b) but diminish with successive spawns (McNabb et al. 2010), strongly suggests that TTX is involved in the defense of eggs and embryos (Wood et al. 2012a; Salvitti 2015 and references therein). In this regard, the variations of PSTs in the invaded population may also be related to the reproductive activity. We have confirmed here that at least the PST analog Saxitoxin can be also accumulated in the egg masses of *P. maculata*, likely transferred vertically from the adult to the eggs as reported for TTXs (Wood et al. 2012b). Different to what is known for NZ, in Puerto Madryn the egg masses are not present only in winter but also are frequently seen during spring and summer. Therefore, the increase in PSTs contents may be related to the supply of toxins to the eggs during the warmer seasons. Seasonal variations in the levels of toxins associated to the reproductive cycle, particularly on TTX, were already reported in puffer fishes (Sabrah et al. 2006; Ikeda et al. 2010), goby fishes (Tatsuno et al. 2013) and a flatworm (Yamada et al. 2017). As with *P. maculata*, in most of these organisms the toxin was subsequently found in the eggs and then assumed to play a defensive role for the progeny. However, with few exceptions (e.g., Itoi et al. 2014) the efficacy of TTX and PSTs as a predator deterrent still needs to be experimentally probed. At any case, these considerations must be taken carefully given the low number of samples analyzed in some months and the time coverage of our study, which did not span a whole year. In fact, the spring–summer peak in PSTs may be a by-product of the increased availability of exogenous sources of the toxins due to blooms of toxic dinoflagellates during early spring and summer (Esteves et al. 1992; Gayoso 2001).

Interestingly, the temporal variations in the overall toxin contents (Fig. 5) correlate with changes on the relative proportions of toxin analogs (Figs. 3, 4). The higher proportion of gonyautoxins GTX 1/4 and GTX 2/3 from September to January may reflect increased relative availability in the environmental sources, selective uptake/elimination rates of the different compounds or biotransformation within the tissues as a detoxification mechanism (Bricelj et al. 1990; Blanco et al. 2003; Kwong et al. 2006; Qiu et al. 2018) and/or the selective endowing of STX to the eggs during the reproductive period. Further attempts to conclude on this point should assess the posed correlation between the reproductive status of the individuals and their toxin profiles, in simultaneous with their availability in the environment, and the experimental testing of the putative defensive role of TTXs and PSTs for the eggs, larvae and adults.

Outlook

We found that *P. maculata* is able to jointly accumulate PSTs and TTXs in adults and transfer them to the eggs. The herein represents the first finding of PSTs in a pleurobranch; and to the best of our knowledge also in opisthobranches *s.l.* Although there are reports of PSTs in marine gastropods from Argentina (Turner et al. 2014), its detection in the invasive *P. maculata* contrast with the previous negative screenings in NZ populations (McNabb et al. 2010; Wood et al. 2012b; Khor et al. 2014) where PSTs are common (MacKenzie 2014). In addition, the identification of TTXs in this study has expanded the knowledge of its geographical distribution to the temperate waters of the southwestern Atlantic (Bane et al. 2014; Lago et al. 2015). Our results open three main questions: (1) What is the impact and risk for human health posed by the introduction of this new vector of potent neurotoxins?; (2) Is there a single source and a shared mechanism for the production/assimilation of both groups of toxins?; and (3) Was TTX introduced jointly with *P. maculata* and transmitted vertically (thus indicating endogenous production) or was the toxin already present in sediment and/or biota in the invaded area, but not detected until now?. Whatever the case, the presence of *P. maculata* is of concern beyond the traditionally expected negative effects of invasive species, since represents the introduction of a new vector for two keystone neurotoxins. To determine whether TTXs are established in the local food webs with potential compromise to seafood safety, urgent studies are needed. The next step should be gathering basic knowledge on the sources and fate of PSTs and TTXs in the invaded

area by a combination of an extensive environmental screening for these two toxin groups, and experimental and field studies to determine the trophic position of *P. maculata* in the novel food web.

Acknowledgements We want to acknowledge Carolina Kelly and Sonia Ortega from SENASA's Mar del Plata Laboratory, for their help to perform mouse bioassays. We would like to thank Dr. Alejandro Bortolus (GEAC-IPEEC-CONICET) for encouraging us to undertake this study and for his contribution to the first discussions. We also thank two anonymous reviewers whose comments and criticism contributed significantly to improve this manuscript.

Authors contribution NF has performed the field sampling, designed the study, analyzed the data and written a first draft. AG performed the mouse bioassays. ES provided the individuals from Puerto Madryn. MDR and AT performed the LC–MS/MS analyses. AG, ES, SO, MDR and AT contributed to the writing of the manuscript.

Funding Field work was supported by ANPCyT-PICT 2016 # 1083, PIP CONICET 508 granted to ES. Toxin identification and quantifications were funded with Internal Cefas Seedcorn Funding (contract code DP345).

Compliance with ethical standards

Conflict of interest All authors declare that they have no conflict of interest.

Ethical approval All bioassays in this study have been conducted following the regulation 617/2002 for biological tests and animal facilities of the National Service of Agri-Food Health and Quality of Argentina (SENASA), and in fulfillment of the ISO/IEC 17,025 norm, that conforms to the Directive 2010/63/EU of the European Parliament, the Council of 22 September 2010 on the protection of animals used for scientific purposes and the Code of Ethics of the World Medical Association (Declaration of Helsinki) for animal experiments.

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