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Imposex in *Nassarius nitidus* (Jeffreys, 1867) as a possible investigative tool to monitor butyltin contamination according to the Water Framework Directive: A case study in the Venice Lagoon (Italy)

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ABSTRACT

Butyltin (TBT, DBT, and MBT) effects on molluscs, especially endocrine disruption in bivalves and gastropods, have been widely investigated. Imposex, the superimposition of male characters onto female gonochoristic Caenogastropods, is the most studied biological effect of TBT. TBT compounds are among the priority hazardous substances within Directives 2000/60/EC (WFD) and 2008/105/EC. The Environmental Quality Standards (EQSs) set by the WFD for TBT are quite difficult to quantify by means of chemical analysis, without the use of expensive and high performance methods. Assuming that EQSs set for TBT were derived from evidence of imposex development at very low concentrations, this specific biomarker could be used as an indirect measure of assessing levels of bioavailable BTs. Therefore, this study aims to validate the use of imposex development as an investigative tool to monitor the bioavailable fraction of BTs within the WFD, by comparing imposex levels and BT concentrations in *Nassarius nitidus* from the Venice Lagoon.

BT concentrations and imposex levels in *N. nitidus*, collected in 2013, had decreased when compared to previous studies in the same area. Both VDSI and RPLI correlated positively with BT body burden in females, confirming that imposex is a valid tool to monitor bioavailable BTs. However, TBT is still a matter of concern in the Venice Lagoon, as TBT concentrations were still higher than its degradation products suggesting recent fresh TBT inputs in the studied area. To propose imposex levels as an indicator of the impact of BTs within the WFD, classification class boundaries and Ecological Quality Ratios were introduced.

As a preliminary attempt, imposex levels were also compared to the OSPAR Commission EcoQOs which linked imposex levels in *Nassarius reticulatus* with TBT concentrations in water. Based on this comparison the degree of imposex development in the Venice Lagoon suggested that TBT concentrations in water should be over the EQS-AA concentration and, at one site, also over the EQS-MAC.

From all the results obtained in this work, it appears that imposex evaluation in *N. reticulatus* could give information about the ecological status regarding BT compounds under the WFD and also be used to monitor their effects and support chemical analyses until more sensitive methods become available.

1. Introduction

Organotins, especially butyltins (BTs, that is mono-, di-, and tributyltin), have been used worldwide in industrial and agricultural applications giving rise to contamination of aquatic environments. More specifically tributyltin compounds (TBT) have been widely introduced into the marine and transitional environments through antifouling paints, as they were probably the most cost-effective biocide preventing biological fouling on boat hulls (Alzieu, 2000; Terlizzi et al., 2001). Once present in marine and transitional environments, TBT is persistent and extremely

Abbreviations: AA, Average Annual concentration; BT, butyltin; DBT, dibutyltin; DW, dry weight; EQR, Ecological Quality Ratios; EQS, Environmental Quality Standard; LOQ, limit of quantification; MAC, Maximum Allowable Concentration; MBT, monobutyltin; RPLI, Relative Penis Length Index; FPL, female penis length; TBT, tributyltin; VDS, Vas Deference Stage; VDSI, Vas Deference Sequence Index; WFD, European Water Framework Directive 2000/60/EC

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toxic to non-target organisms. In fact, TBT was defined as "probably the most toxic chemical compound ever deliberately introduced by societies into natural waters" (Goldberg, 1986).

Many industrialized countries started to restrict the use of TBT in the 1980s (Champ, 2000) and in the European Union the global ban was enacted by EC Regulation 782/2003 (EC, 2003), anticipating the introduction of the International Convention on the control of harmful antifouling systems on ships (AFS-Convention 2001) (IMO, 2001), i.e. the total interdiction on the application of organotin compounds on ships after 1st of July 2003 and the eradication of these compounds from ships from 1st January 2008. The AFS-Convention 2001 came into force in September 2008, banning the use of TBT-based antifouling systems almost all around the world, with nowadays 69 contracting States (IMO, 2015). Several studies concerning the temporal trends of BTs have been made in order to evaluate the effectiveness of the restrictions, showing that TBT and its metabolites (DBT and MBT) have been decreasing over recent years but they are still present in the marine environment (Sousa et al., 2007; Rato et al., 2009; Ruiz et al., 2008; Barroso et al., 2011; Cuevas et al., 2014). Nevertheless, even if antifouling paints are no longer the main source of BTs, considering the low rate of TBT degradation in sediments compared to water, sediments are an important reservoir and secondary source of these compounds: resuspension and desorptive processes could, indeed, remobilize them to the water column and make them easily accumulable by organisms (Santos et al., 2004; Sousa et al., 2007). Moreover TBT is still being manufactured and applied (Turner and Glegg, 2014).

TBT effects on molluscs, especially endocrine disruption in bivalves and gastropods, have been widely investigated (see e.g. Salazar and Salazar (1996), Morcillo and Porte (1998) and Alzieu (2000)). Imposex, the superimposition of male characters onto female gonochoristic Caenogastropods (Smith, 1971), is one of the most studied TBT effects and it has been widely used as a specific biomarker to monitor TBT pollution, especially in species showing a good correlation between the degree of morphological alterations and the concentrations of TBT (Titley-O'Neal et al., 2011). Among these, *Nassarius nitidus* (Jeffreys, 1867), a common estuarine whelk, has been used as bioindicator of butyltin pollution (Pavoni et al., 2007; Berto et al., 2007; Rodríguez et al., 2009; Lahbib et al., 2011) and it is considered as good as the closely related species *Nassarius reticulatus* (Linnaeus, 1758) (Rodríguez et al., 2007; Cuevas et al., 2014).

Due to their persistence, toxicity and bioaccumulation properties, TBT compounds are among the priority hazardous substances according to the European Water Framework Directive 2000/60/EC (WFD) and its daughter Directive 2008/105/EC (EC, 2000, 2008). The WFD requires Member States to achieve good ecological and chemical status in water bodies by 2015 (EC, 2000). The chemical status is based on compliance with legally binding Environmental Quality Standards (EQSs) of priority substances laid down in the Directive 2008/105/EC (EC, 2008). The EQSs set for TBT compounds (expressed as cation) in marine and transitional waters are 0.2 ng/L and 1.5 ng/L, as Annual Average (AA) and Maximum Allowable Concentrations (MAC), respectively (EC, 2000, 2008). These values are quite difficult to detect and quantify by means of chemical analysis in accordance with the application of best available analytical techniques (see e.g. Italian Authority of the basin of Eastern Alps (2014)), unless the use of much more expensive and high performance methods are employed. As a consequence, the gap between analytical performance and EQSs set for TBT compounds could prevent the evaluation of water chemical status according to the WFD.

The WFD allows Member States to monitor parameters which are indicative of the status of each relevant quality element, both chemical and biological, in order to evaluate the ecological status of the water body. The WFD advocates the use of community structure traits specified in its Annex V as biological quality elements. Accordingly, biological monitoring must be presented as Ecological Quality Ratios (EQR). Moreover the WFD allows the use of any elements (for example more surveys on sediment or biota) and methods (e.g. biomarker or ecotoxicological tests) suitable for the problems under investigation. Assuming that EQSs set for TBT compounds were also derived from evidence of imposex development at very low concentrations (Axiak et al., 1995; Gibbs and Bryan, 1996; Alzieu, 2000 and authors therein), this specific biomarker could be proposed for assessing BT environmental contamination. Oehlmann (2002) proposed a baseline study on intersex (modification of the female pallial organs towards male morphological structures) in *Littorina littorea* with recommendations for biological TBT assessment criteria regarding the WFD; and also the WFD-United Kingdom Technical Advisory Group (WFD-UKTAG) suggested the use of indicator species under the general benthic invertebrate quality element, by using imposex in the common dog-whelk *Nucella lapillus* to assess the impact of TBT on the coastal waters of England, Northern Ireland, Scotland and Wales (WFD-UKTAG, 2014). Moreover, the European subgroup Chemical Monitoring and Emerging Pollutants (CMEP) under the WFD Common Implementation Strategy (WFD-CIS) has recently identified potential effect-based tools, such as biomarkers or bioassays, that could be used in the context of the different WFD monitoring programmes, linking chemical and ecological status assessment. Among biomarkers, imposex was proposed as a specific response to organic tin compounds (Wernersson et al., 2015). Finally, Laranjeiro et al. (2015) recently studied a multispecies approach for the assessment of the ecological status within the WFD in coastal and transitional waters of NW Portugal, and proposed new boundaries for the ecological status classes.

The Venice Lagoon is the largest wetland in Italy and one of the most important coastal ecosystems in the whole Mediterranean basin, with a total area of 550 km^2. Due to its importance and suitability, the Venice Lagoon was selected for Italy in forming the intercalibration network referred to in Directive 2000/60/EC (Commission Decision of 17 August 2005, 2005/646/EC). The Venice Lagoon has several sources of pollution: the industrial area of Porto Marghera, the dense urban sites of Venice and Chioggia, several watercourses that flow from the lagoon watershed and the intense maritime traffic (Maggi et al., 2012). Results of the WFD Operative Monitoring (2010–2012) of the Lagoon could not assess the chemical status for TBT due to the quantification limit (10 ngTBT/L) of the available analytical method which, in contrast to the regulations, is over the EQSs in water (Italian Authority of the basin of Eastern Alps, 2014).

In this context, the objective of this study was to evaluate the usefulness of imposex development as an investigative tool for assessing bioavailable BTs within the WFD, by comparing imposex intensity and BT concentrations in *N. nitidus* and by proposing new boundaries for the ecological status classes in transitional water regarding this specific biomarker. For this purpose, the Venice Lagoon was chosen as the study area.

2. Materials and methods

2.1. Samples collection and preparation

N. nitidus specimens were sampled from seven sites distributed throughout the Venice Lagoon (NE, Italy, Fig. 1) in June–July 2013.

The survey was designed to include areas exposed to a variety of pollutants from different anthropogenic sources, with particular reference to BTs. Indeed, sites were selected according to the availability of historical data regarding TBT contamination in

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