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Relationship between imposex and levels of organotin substances in the sediment

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Sammanfattning

Marine Monitoring AB har på uppdrag av Havs och vattenmyndigheten sammanställt kompletterande information till indikator *8.2A Effekter av organiska tennföreningar på snäckor (imposex)* gällande koncentrationer av tennorganiska föreningar i sedimenten vid samtliga stationer som övervakas med avseende på imposex. Därefter har sambandet mellan koncentrationer av tributyltenn (TBT) samt summan av TBT (ΣBT) och dess nedbrytningsprodukter (DBT, MBT) i sedimentet och responsen i form av imposex (VDSI; IMPF%) hos stor tusensnäcka (*Peringia ulvae*) samt stor nätsnäcka (*Tritia nitida*) analyserats. För stor tusensnäcka undersöktes även effekten av sedimenttyp och typ av station (punktkälla, naturhamn och referensstation). Imposex är känt för att vara mycket starkt kopplat till exponering för organiska tennföreningar och främst TBT. Resultatet av analysen har därför även kopplats till vilken skyddsnivå som fås hos snäckorna av det nuvarande gränsvärdet (EQS) för TBT i sediment (1,6 µg/kg ts uttryckt som TOC 5%). Men även föreslaget tröskelvärde om VDSI 0,1 för att god status skall uppnås hos stor tusensnäcka har diskuterats utifrån dessa resultat.

Signifikanta korrelationer har hittats mellan VDSI-, IMPF%- och TBT-koncentrationer i sedimentet hos båda arterna, såväl som mellan VDSI, IMPF% och koncentrationer i vävnad hos nätsnäckor. Det finns också signifikanta positiva samband mellan TBT-koncentrationer i sediment och vävnad för nätsnäcka. Flera av korrelationerna är dock svaga, särskilt för stor tusensnäcka. Den svaga korrelationen för båda arterna kan vara en effekt av få provtagningstillfällen när det gäller sediment och det finns en möjlighet att korrelationerna ökar över tiden med mer sedimentprovtagning. Ett starkt samband ses dock mellan andelen drabbade honor (IMPF%) och typ av station för stor tusensnäcka, där honorna vid punktkällor är mer drabbade än vid naturliga hamnar och referensstationer. Liknande korrelationer ses för VDSI, dock inte lika starka.

Avsikten med EQS_{TBT} är att skydda arter som lever i sedimentet från att påverkas av TBT-exponering. Koncentrationen av TBT i sedimentet i denna studie är över EQS_{TBT} uttryckt som 5 % TOC vid de flesta stationerna. Resultat från stationerna, speciellt referenserna, visar bättre anpassning till data om 1,6 µg/kg ts används utan normalisering. Detta beror troligen på att de flesta av referensstationerna är belägna i områden med gyttjefria sediment (< 2 % TOC), och flera av dem hade halter av TBT under kvantifieringsgränsen. Normalisering till 5 % TOC för dessa stationer kommer utan tvekan att leda till en överskattning av TBT-koncentrationen. För att undvika detta problem föreslås det att inte normalisera för gyttjefria sediment (< 2 % TOC). För det motsatta problemet, dvs

underskattning av EQS_{TBT} vid normalisering till 5 % TOC, av ett sediment med hög halt av organiskt kol, rekommenderas det att bortse från normaliserade värden under EQS_{TBT} om den icke-normaliserade koncentrationen av TBT i sedimentet är över 1,6 µg /kg ts och att betrakta halten som över EQS_{TBT}.

Avseende föreslaget tröskelvärde för stor tusensnäcka om 0,1 indikerar resultatet att det är satt till en korrekt nivå men att använda IMPF% som stödparameter kan vara ett sätt att förbättra bedömningen av imposex hos stor tusensnäcka och därmed öka tillförlitligheten i helhetsbedömningen.

Slutligen föreslås det fortsatt analys av TBT och dess nedbrytningsprodukter DBT och MBT i sedimentet tillsammans med innehållet av TOC vid stationer med stor tusensnäcka, för att undersöka om en större datamängd kan ge större precision och öka förklaringskraften hos förhållandet mellan VDSI/IMPF% och TBT i sedimentet.

Summary

Marine Monitoring AB has at the request of the Swedish Agency for Marine and Water Management (Havs- och Vattenmyndigheten) compiled supplementary information with respect to *indicator 8.2A Effects of organotin compounds on snails (imposex)* regarding concentrations of organotin compounds in the sediments at all stations where imposex in snails is monitored. The relationships between concentrations of tributyltin (TBT) and the sum of TBT (ΣBT) and its derivatives (DBT, MBT) in sediment, and the imposex response (VDSI, IMPF %) in the larver spire shell (*Peringia ulvae*) and the netted dog whelk (*Tritia nitida*), were subsequently analyzed. In the larver spire shell, the effects of sediment class and station type (source, natural harbour and reference site) were also examined. The link between imposex and exposure to organotin compound, in particular TBT, is well established. The results of the analyses have therefore also been considered with respect to the protection afforded the snails by the current threshold (EQS) for concentrations of TBT in sediment (1.6 μg/kg dw, expressed as 5 % TOC). The proposed threshold of VDSI 0.1 to achieve good status in the larver spire shell is also discussed in the context of the results.

In both species, significant correlations were found between VDSI, IMPF %, and concentrations of TBT in sediment, as well as between VDSI, IMPF % and concentrations of TBT in tissue in the netted dog whelk. Moreover, there was a significant and positive relationship between the concentrations of TBT in sediment and in tissue of the netted dog whelk. However, modelled relationships are often weak, especially with respect to the larver spire shell. The weak relationships may be a consequence of few sediment sampling occasions, and it is possible that a larger sample size, obtained by continued sampling, will produce stronger relationships. In the larver spire shell, there was an effect of station type on the percentage of affected females (IMPF %), with females in source stations being more affected than females in either natural harbours or reference sites. Similar but somewhat weaker patterns were seen for VDSI.

The purpose of EQS_{TBT} is to protect species inhabiting the sediment from exposure to TBT. In this study, concentrations of TBT in sediment exceed EQS_{TBT}, expressed as 5 % TOC, at most stations. However, scrutinizing the results with respect to VDSI and IMPF % indicates that many stations, especially the reference sites, show a better fit to imposex data if 1.6 μg/kg dw is used without TOC normalization. Many reference sites are located in areas with non-muddy sediments (< 2 % TOC), and in several sites the concentrations of TBT were below the detection limit. Under these conditions, normalizing concentrations to 5 % TOC will undoubtedly

overestimate the TBT concentration. To avoid this problem, it is suggested that normalization is not performed for non-muddy sediments (< 2 % TOC). For the opposite problem, i.e. underestimating EQS_{TBT} when normalizing to 5 % TOC because the sediment has a high fraction of organic carbon, it is recommended that normalized values below EQS_{TBT} are disregarded if the non-normalized concentration of TBT in the sediment exceeds 1.6 μg/kg dw, and to consider the concentration in sediment as being above EQS_{TBT}.

The results of the analyses indicate that the proposed threshold of VDSI 0.1 for the larver spire shell is correctly set. The results also indicate that IMPF% may be used as a complementary parameter in order to improve the assessment of imposex in the larver spire shell and thus improve the reliability of the overall assessment.

Finally, it is suggested that analyses of TBT and its derivatives DBT and MBT in sediment continues, in combination with the TOC content at stations where the larver spire shell is monitored, to establish whether a larger sample size will allow for greater precision and explanatory power in the analyses of the relationship between VDSI/IMPF % and concentrations of TBT in sediment.

1 Introduction

Tributyltin (TBT) and other organotin compounds have long been used as additives in antifouling paints. However, TBT is toxic already at very low concentrations and can cause serious damage to marine life. It interferes with the endocrine system, altering and disrupting the production of hormones responsible for development, growth and reproduction in animals. TBT is also bioaccumulated, and levels can be high in top predators (Strand *et al*, 2005; Law *et al*, 2012). Among other things, it can block the enzyme that converts the male sex hormone testosterone to the female sex hormone estrogen, leading to very high levels of stored testosterone. In many gastropod species this causes the growth of a pseudo penis and/or a vas deferens in females, an effect called imposex.

Imposex has proven to be a sensitive biomarker for the determination of the degree of environmental organotin and especially TBT pollution in coastal waters. Several surveys, including national monitoring activities, have also shown the usefulness of imposex as an indicator for environmental assessment of TBT pollution in the Baltic Sea, Kattegat and Skagerrak (Schulte-Oehlmann et al. 1997; Bauer et al., 1997; Strand & Jacobsen 2002; Strand et al. 2006; Gercken & Sordyl 2007; Magnusson 2011; 2011; 2012 and 2015; Hansen 2012).

As a result of the serious effects on marine life, many countries have since the mid-80s introduced restrictions on the use of TBT-based paints on boats smaller than 25 meters, and from 2008 there is also a global ban for use on boats bigger than 25 meters. However, this does not mean that the contaminants will disappear from the marine environment within the foreseeable future. Organotin binds strongly to particles in the sediment and the water column. It takes days to months for TBT in the water column to degrade, and in anaerobic sediments the half-time can be several years.

Despite the global ban on the use of TBT-based paints, research has also revealed that paint can still be found on the hull of leisure boats and larger ships (Ytreberg et al 2017). Furthermore, Uc-Peraz et al (2021) revealed that TBT-based products are still being manufactured in the United States and offered for sale. Results from the Swedish environmental monitoring programme of offshore sediments also indicates that TBT levels in sediment frequently exceed the Swedish national threshold value for sediment (1.6 µg/kg dry weight; HVMFS 2019: 25) (Josefsson & Apler, 2019). TBT is also one of the substances that contributes most to the failure to achieve *good* chemical status in water bodies in Sweden.

1.1 Assignment

Marine Monitoring AB has been commissioned by the Swedish Agency Marine and Water Management (SwAM) to compile supplementary information to the indicator *8.2A Effects of organotin compounds (imposex).* This indicator is used to evaluate criterion D8C2 *Effects of hazard substances* under the descriptor *8 Contaminants - Concentrations of contaminants are at levels not giving rise to pollution effects,* in the national implementation of the *EU Marine Strategy Framework Directive (MSFD)*. TBT and imposex is also used in the regional status assessments of the North East Atlantic (OSPAR) and the Baltic Sea (HELCOM).

The purpose of the assignment has been to compile measured concentrations of organotin compounds in the sediments at Swedish sampling stations for monitoring imposex, and then investigate the relationship between concentrations in the sediments and the occurrence of imposex in gastropod snails. Comparisons have also been made regarding the *Environmental Quality Standard* for TBT in sediment (EQS_{TBT}) and the threshold value for Imposex.

Based on the results, the following themes are discussed:

1. The relationship between the levels of organic tin compounds in the sediments and the division of the sampling areas for imposex into reference, natural harbour, and point source.
2. The relationship between concentrations and response in the snails (VDSI, and / or percentage of population, IMPF%).
3. Protection level obtained from the current EQS_{TBT} value of 1.6 μg/kg *dw* normalized to 5 % TOC regarding the presence of imposex in the snails.
4. Possible recommendations for adjustments to indicator 8.2A with respect to, for example, the threshold value and assessment method.
5. Other conclusions from the analyses linked to the monitoring and assessment of the indicator.

The assignment is financed by SwAM through grant *1:11 Actions for the marine and aquatic environment*. This grant is an important tool for achieving Agenda 2030 and the decided environmental objective. It supports the implementation of several directives regarding *water management*, *marine environment*, *area protection, species protection* and *marine planning*. It also supports HELCOM's Baltic Sea Action Plan and is significant for the fisheries policy.

1.2 Species of intrest

More than 120 species of gastropod snails around the world have been shown to be sensitive to TBT and exhibit different stages of imposex. The severity of the impact differs between species and is also dose related. For example, severe imposex in the dog whelk Nucella lapillus can lead to sterility in females with reproductive difficulties as a result on both individuals and population as a result. Nucella lapillus has, due to this sensitivity, been used in several monitoring programs (Norway, Iceland, Denmark etc.). However, because it is not very common in Swedish waters, the netted dog whelk (*Tritia nitida*) and the laver spire shell (*Peringia ulvae*) are instead used for monitoring purposes (Figure 1).

1.2.1 Laver spire shell (*Peringia ulvae*[1])

Laver spire shell, or mud snail, is a very small (3-5mm) snail that belongs to the family Hydrobiidae. It is a marine species but in contrast to the netted dog whelk, it has a high tolerance for brackish water and can therefore be found in the Baltic proper. It is not as sensitive to exposure of TBT as the netted dog whelk but is nonetheless the only species in the Baltic proper that shows stages of imposex. It has been used in Sweden since 2008 for monitoring purposes. Four different stages of imposex with two types of development can be seen, though the higher stages of imposex are rare. Sex change does not seem to occur, but the snails do exhibit a stage of sterility that may be related to exposure to TBT. Due to their small size, tissue analysis to verify if the exposure is on-going is not possible. Their life span is approximately two years (Fish and Fish 1974; Lassen and Clark 1979), which ensures that the biological data gathered represent the current situation.

Laver spire shell can be found at most locations. They feed on detritus and are also believed to consume either the macroalgae directly or the microflora covering its surface. Juveniles are highly mobile, while adult snails are more or less stationary. They produce planktotrophic larvae (veliger) when spawning, which settle after up to four weeks. This allows them to spread to new habitats and the mixing of geographically separate populations. They are also believed to be an important food source for seabirds and fish (Clay 1960).

[1] Also known as the mud snail. Many synonyms have been used in the past but *Hydrobia ulvae* is the only one used recently.

1.2.2 Netted dog whelk (*Tritia nitida*[2])

Netted dog whelk has been used for monitoring on the Swedish west coast since 2003. It is sensitive to exposure to TBT, even though neither TBT-induced sterility nor sex change seems to occur. They have a free-swimming larval stage, which enables recruitment from other populations. Due to this, populations of netted dog whelks can survive even in heavily polluted areas, for example near ports and shipyards. They are scavengers and can easily be caught in large numbers using baited traps.

Four stages of imposex can be distinguished, with two different types in stages 1, 3 and 4. Since imposex is an irreversible effect, the analysis of imposex on netted dog whelk is always followed by a tissue analysis of TBT to verify that they are subject to an on-going exposure.

Netted dog whelk can withstand salinities as low as 16 ppt and are commonly found on soft bottoms down to a depth of 15 meters, but they can also occur on sandy and rocky shores with patches of soft materials into which they can burrow. In Sweden they can be found from areas north of Bohuslän to the northern parts of Skåne.

Figure 1. Netted dog whelk to the left and laver spire shell to the right. Photo: Marina Magnusson©

[2] Several synonyms; *Nassarius nitida, Nassarius reticulata, Hinia nitida, Hinia reticulata, Tritia reticulata*

1.3 Description of the station network.

Environmental monitoring of the effects of OTC in netted dog whelk began on the Swedish west coast in 2003. Since then, stations within two impact gradients have been sampled on the west coast; Brofjorden and Gothenburg, each with four stations, together with the reference stations Kalvhagefjorden and Burholmarna, respectively. The reference stations for the gradients were sites chosen to represent lightly exploited spots on the west coast. The impact station at Glommen and the reference station at Gåsenabbe were added in 2009 (Figure 2).

There is also a corresponding monitoring program for the Baltic Proper that began in 2008, where the laver spire shell is used as an indicator species (Figure 2). Stations in the Baltic proper are divided into point sources (usually larger ports or marinas), reference stations and natural harbours. Natural harbours are generally naturally protected bays that often is used temporarily by leisure boats and usually lacks most facilities. At most stations in the Baltic Proper, monitoring has been continuous since 2008. Data from the monitoring programme is reported to the Geological Survey of Sweden (SGU), the Swedish national data host for environmental toxins in sediment and biota.

While gastropod snails are collected every year, sediment at each station was sampled in 2010 and 2019. The sediment characteristics differ between stations and the substrate ranges from sand to gyttja. The sediment has for this assignment been classified according to the organic carbon content (TOC) at each station in accordance with Table 1. TOC has been analysed in sediment samples from 2019. Grain size analysis has not been performed, but a visual inspection of the sediment when sampling supports the classification according to organic carbon content. Sampling depth, TOC-concentration, and a short description of the sediment substrate for each station is given in Table 2 and Table 3.

Table 1. Sediment classified according to the organic carbon content.

Organic carbon content (TOC %)	Nomenclature	Example
<2	Non-muddy sediment	Sand, gravel
2-6	Muddy sediment	Gyttja clay, gyttja silty clay, gyttja silt
6-20	Muddy sediment (e.g. clay-gyttja)	Clay-gyttja, Silty-clayey gyttja
>20	Gyttja	Coarse detritus gyttja, fine detritus gyttja

Figure 2. Map over sampling areas (1-7) and stations. Laver spire shell are collected from a point source, a natural harbor and a reference station in areas 1-4 and from a point source and a reference station in areas 5-7. Netted dog whelks are sampled at all the other stations. Glommen is a point source and Burholmen, Kalvhagefjord and Gåsenabbe serve as reference stations. Stations from Brofjorden and Göteborg are sampled along a gradient from a point source.

Table 2. Sampling depth, TOC-concentration and a short description of the sediment at each station where laver spire shell was collected. The area code refers to the area in Figure 2 and station ID is in accordance with the Swedish national station register.

Area	Station	Station ID	Type of station	Depth	TOC	Substrate
1	**Bullandö marina**	220322	Source	2-3 m	1.58	Non muddy
	Lökaö	226017	Natural harbour	1-2 m	<0.10	Non muddy
	Stora Bäckskär	220320	Reference	4-6 m	0.64	Non muddy
2	**Oxelösund**	225491	Source	2-3 m	5.10	Muddy
	Ringsöfladen	225492	Natural harbour	1-2 m	8.81	Clay-gyttja
	Kittelön	225451	Reference	1.5-2 m	8.55	Clay-gyttja
3	**Blankaholm marina**	213973	Source	3-3.5 m	24.00	Gyttja
	Vippholmen	210548	Natural harbour	2-3 m	12.80	Clay-gyttja
	Öre	213953	Reference	2-3 m	12.80	Clay-gyttja
4	**Hälleviks kaj**	204408	Source	1-2 m	4.88	Muddy
	Tjärö	204753	Natural harbour	1-2 m	13.30	Clay-gyttja
	Toseboviken	205167	Reference	0.5 m	0.26	Non muddy
5	**Simrishamn småbåtshamn**[1]	197768	Source	3-4 m	2.30	Muddy
	Simrishamn ref. [1]	197767	Reference	6-7 m	0.12	Non muddy
6	**Trelleborg hamn** [2]	245310	Source	2-3 m	-	Non muddy
	Trelleborg ref [2]	245310	Reference	2-3 m	-	Non muddy
7	**Råå hamn**	205544	Source	3-4 m	4.62	Muddy
	Råå ref.	206454	Reference	0.2 m	0.19	Non muddy

[1] Sediment sampled only in 2019

[2] Sediment sampled only in 2010

Table 3. Sampling depth, TOC-concentration and a short description of the sediment, at each station where netted dog whelk was collected. Station ID is in accordance with the Swedish national station register.

Station	Station ID	Type of station	Depth	TOC	Substrate
Göteborg 4	245408	Gradient	0.5-1.0 m	0.16	Non muddy
Göteborg 5	245407	Gradient	2-3 m	4.02	Muddy
Göteborg 6	245401	Gradient	2-3 m	3.42	Muddy
Göteborg 7	208441	Gradient	10-11 m	2.15	Muddy
Brofjorden 1	245446	Gradient	1.0-1.5 m	0.27	Non muddy
Brofjorden 2	224786	Gradient	3-5 m	0.45	Non muddy
Brofjorden 3	245450	Gradient	5-7 m	4.45	Muddy
Brofjorden 4	245448	Gradient	5-6 m	5.61	Muddy
Kalvhagefjorden 2	224785	Reference	2-3 m	1.75	Non muddy
Burholmen 5	245482	Reference	2-3 m	1.11	Non muddy
Glommen	210819	Source	1-1.5 m	3.02	Muddy
Gåsanabbe	211558	Reference	6-7 m	0.20	Non muddy

2 Method

Existing data produced within the national monitoring program *Biological effect monitoring of organic tin compounds* financed by the Swedish Environmental Protection Agency (EPA) has been used to complete this assignment. The surveys aim to annually document levels of organotin compounds and effects on the reproductive organs of snails along the Swedish west and east coasts with the intention of following up the ban on TBT-based antifouling paints and demonstrating long-term changes in the marine environment. Methods for the different parts of the survey are described briefly below.

2.1 Sampling of gastropod snails

Collection of laver spire shell is performed in the beginning of June by dragging a hand net with a fine mesh along the bottom. The sample is then sorted by hand in the field. To simplify the sorting, this is performed on a white surface (Figure 3). Snails are picked out with tweezers. If species determination is not possible in the field, all snails are kept in order to be determined under a dissecting microscope.

Traps baited with fish are used for sampling netted dog whelk, with the time of trap deployment ranging from 1-2 hours. Most stations with netted dog whelk are sampled during autumn, except for Glommen and Gåsenabbe which are sampled during summer. Dog whelks are sufficiently large to be easily identified and do not require additional sorting in the field.

A selection of 60-70 snails of uniform size from each station is brought back to the lab, alive and under cool conditions.

Figure 3. From left to right: collection of netted dog whelk with trap, trap with bait and netted dog whelks, dragging a hand net over the bottom, sorting mud snails. *Photo: Marina Magnusson©*.

2.2 Analysis of imposex

Monitoring of effects of OTC is performed according to the standard methodology described in *Biological effect monitoring of organotin compounds* (Naturvårdsverket, 2015), OSPAR (2008, 2013) and in a number of scientific publications (Stroben *et al*, 1992; Schulte-Oehlmann *et al*, 1997). Before the live gastropods are examined, they are anesthetized in a 7% $MgCl_2$-solution in order to obtain maximum muscle relaxation (Figure 4). From each station, 50 individuals are analysed with respect to shell height, VDS stage (VDS = vas deferens sequence) in females, and penis length in both females and males. In the netted dog whelk, the tissue in females is also analysed with respect to the content of ten organotin compounds. These variables are then used to calculate comparative measures for each station such as the VDSI and the percentage of affected females (IMPF%). Specimens parasitized by trematodes or other endoparasites are excluded from analysis.

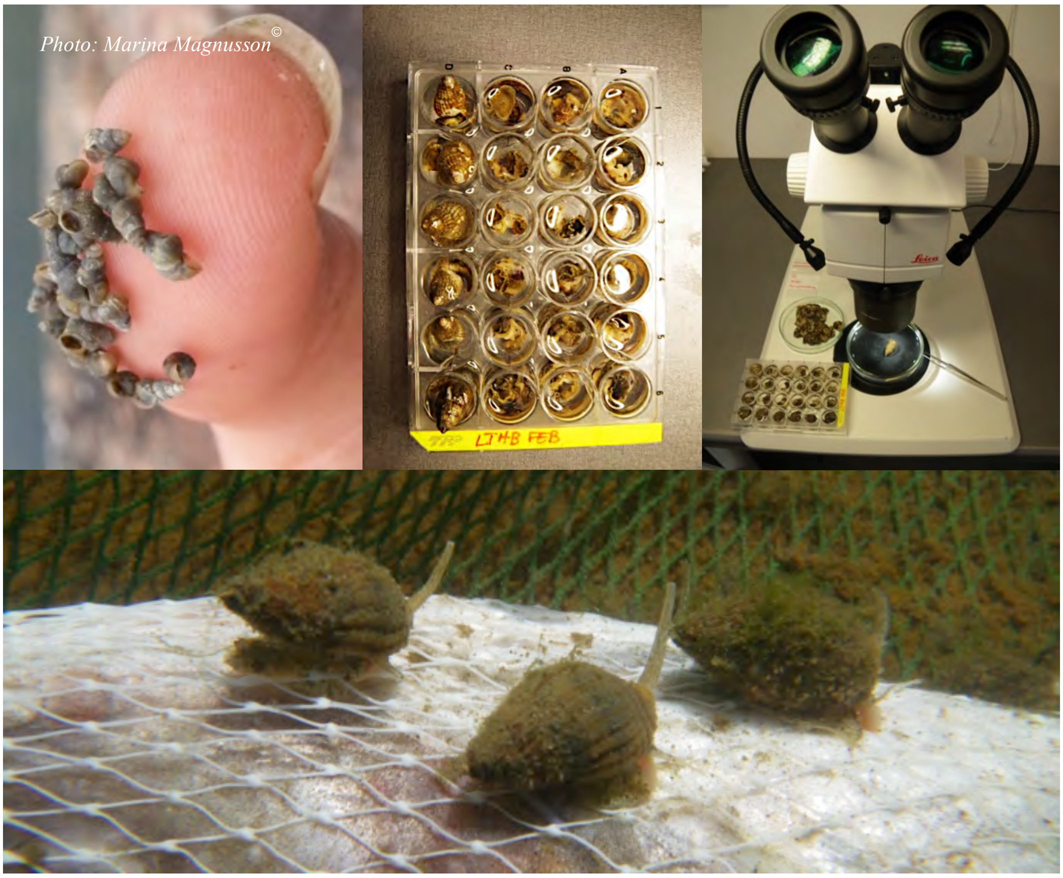

Figure 4. Laver spire shell to the left, netted dog whelk prepared for analysis of imposex in the middle and to the right and up close in the baited trap in the picture below. *Photo: Marina Magnusson*©.

2.3 Sediment sampling

Sediment sampling took place in 2010 and 2019 at all stations that were part of the national monitoring program regarding imposex. Methodology for sediment sampling was performed largely according to Undersökningstyp: Sediment - basundersökning (SwAM, 2016). However, it should be noted that this sediment sampling method recommends accumulation conditions, such that sediment is not resuspended and rearranged. Since the purpose of this assignment was to sample the sediment at the same places that the netted dog whelks and laver spire shells were collected, this necessarily meant that sediment samples varied between accumulation and transport conditions.

A grab sampler with a lid at the top that made it possible to collect a sediment sample with an undisturbed surface (0-2 cm) was used. For each station, five samples were pooled and sent to ALS Scandinavia AB for chemical analysis. The pooled sediment samples were packed in separate glass jars (Figure 5) and stored frozen, pending transport to the laboratory.

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Figure 5. Sediment samples from the stations Vippholmen, Blankaholm and Öre. *Photo: Marina Magnusson©*.

2.4 Chemical analysis

After analysis of imposex, a joint sample of all the analysed females from each station was prepared. Besides the biological variables, ten different organotin compounds were measured in the tissue as well as in the sediment from each station (Table 4). Please note that only the result for TBT and its degradation products (DBT, MBT) are used in this assignment. The method used for these analyses is described briefly below. Data from the chemical analysis is reported to the SGU.

Table 4. Analysed organotin compounds in sediment and pooled samples of netted dog whelk.

Monobutyltin (MBT)	Monophenyltin (MPhT)	Monooktyltin (MOT)	Tetrabutyltin (TTBT)
Dibutyltin (DBT)	Diphenyltin (DPhT)	Dioctyltin (DOT)	Tricyclohexyltin (TCHT)
Tributyltin (TBT)	Triphenyltin (TPhT)		

2.4.1 Tissue analysis of netted dog whelks

Tissue samples from netted dog whelks collected in 2010 and 2019 were analysed by GBA Group in Germany, a subcontractor to ALS Scandinavia AB. Determination of organotin compounds were carried out in accordance with §64 LFGB L 10.00-9 and measurement was performed with GC-FPD. Determination of dry weight (*dw*) was done according to a modified version of method §64 LFGB L 06.00-3.

2.4.2 Sediment analysis

Sediment samples collected in 2010 were analysed by GBA Group, a subcontractor to ALS Scandinavia AB. The samples were first homogenized, shaken with MeOH/hexane and then subjected to purification and derivatization. GC-FPD was used for determination of the concentrations of organotin compounds.

Sediment samples collected in 2019 were analysed by ALS Scandinavia AB in Luleå. Determination of organotin compounds were done according to method ISO 23161: 2011 with acid extraction and the analysis was performed with GC-ICP-MS. In addition, TOC was analysed according to SS-EN 15934.

2.5 Statistical analysis

The statistical analysis investigated the relationship between imposex (VDSI; IMPF%) in the gastropods laver spire shell and netted dog whelk and concentrations of the contaminant tributyltin (TBT) and the sum (ΣBT) of TBT and its degradation products (DBT, MBT), measured as μg/kg dry weight. For the *Peringia* samples, analyses also examined the effect of sediment type and type of station (point source, natural harbour, and reference station).

2.5.1 Type of sediment and station

To examine the effect of sediment, the substrate in all sampling stations were classified according to their total organic carbon content (TOC as % dry weight) (Leonardsson, 2005), in which TOC < 2 is classified as non-muddy, 2 < TOC < 6 as muddy, 6 < TOC < 20 as clay-gyttja, and TOC > 20 as gyttja (Table 1).

All four substrate types were found in the laver spire shell sampling sites, while non-muddy and muddy sediments were found in the netted dog whelk sampling sites. Note that it was assumed in the analyses that the substrate remained the same throughout the years at each sampling station, despite TOC only being measured in one year.

Sampling sites in the laver spire shell programme were classified as *point source*, *natural harbour* and *reference* station. In the netted dog whelk programme the sampling sites along gradients from the source were designated as *gradients*, while the additional reference sites were designated as such.

2.5.2 Available data

Data on VDSI and IMPF% are available from 2003-2020. For each year and station, VDSI was calculated as the average VDSI based on the number of females found in a sample of 50 individuals. This means that there is a variation in number of females for each station and over time. Tissue concentrations of TBT and ΣBT are also available from 2003-2020 but only for netted dog whelk. Concentrations of TBT and ΣBT in tissue were not normally distributed and were therefore $\log_{10}$-transformed prior to analyses.

Data on sediment concentrations of TBT and ΣBT were only available from the years 2010 and 2019. Concentrations of TBT and ΣBT were normalized to 5% total organic carbon content (TOC). As TOC only was available for 2019, these values were also used to normalize the 2010 data at the same stations. When values were indicated as below the quantification limit of TBT (<0.1) a value corresponding to 50% of the limit was used in the analysis (i.e. 0.05). All sediment analyses were performed on both non-normalized and normalized sediment concentrations.

Concentrations of TBT and ΣBT in sediment were not normally distributed and were therefore $\log_{10}$-transformed prior to analyses.

2.5.3 Regression analyses

The relationships between VDSI and IMPF% and concentrations of TBT and ΣBT in sediment from the years 2010 and 2019, and the relationships between sediment and tissue concentrations of TBT and ΣBT of netted dog whelk from years 2010 and 2019, were investigated by linear regression analyses. Each relationship was modelled with and without substrate as a factor, without interaction. The effect of substrate was tested by performing a likelihood ratio test between the regression models with and without sediment. The likelihood ratio test is significant (i.e. $p<0.05$) if the regression that includes substrate yields a significantly better fit to the data. For laver spire shell 31 measurements were used, and for netted dog whelk 24 measurements were used. Owing to the small sample size, the effect of station was not accounted for.

The relationships between VDSI and IMPF% with concentrations of TBT and ΣBT in tissue of netted dog whelk were investigated for all available years (2003-2020) by linear regression analyses with sampling station as random effect. Each relationship was modelled with and without substrate as a factor, with interaction. A total of 185 measurements were used in these regressions. The relationships between type of station (point source, natural harbour, and reference stations) and VDSI and IMPF% in laver spire shell were investigated in 1-way Anovas with sampling station as a random effect. A Tukey post-hoc test was performed to identify significant differences between station types. A total of 202 measurements were used in these regressions.

For laver spire shell, the regressions of the relationships between VDSI and sediment concentrations of TBT, and IMPF% and sediment concentrations of TBT were bootstrapped (n=10 0000) in an effort to obtain greater estimate precision. However, the confidence intervals of the bootstrapped estimates were only slightly narrower and did not affect significance. These results will therefore not be further discussed.

All analyses were done in R v. 4.1.2 (R Core Team 2021). For mixed effects models, p-values were calculated using Satterthwaite's approximation (package lmerTest, Kuznetsova et al 2017) and R^2-values as Nakagawa's conditional and marginal R^2 (conditional R^2 accounts for the variance of both fixed and random effects while marginal R^2 only considers variance of the fixed effects; package performance, Lüdecke et al 2021).

3 Results

The relationship between imposex in the gastropod laver spire shell and netted dog whelk and concentrations of contaminants TBT and Σ BT in both non-normalized and normalized sediment and tissue measured as μg/kg *dw* were investigated. The effect of different sediment substrate (Sub) and type of station (point source, natural harbour, and reference stations) were also examined. The result from the analysis for each species is presented below and original data can be obtained from the data host Geological Survey of Sweden (SGU).

3.1 Laver spire shell

All analyses displayed significant positive relationships between VDSI, IMPF%, and concentrations in the sediment with exception of the regression for VDSI-ΣBTsed with substrate (Sub) as a variable (Table 5). However, the slope and the R^2-value indicate a substantial amount of variation in imposex parameters not explained by sediment concentrations. Using non-normalized contra normalized sediment concentrations generally yielded regression models with slightly better fit (higher R^2) and lower p-values. There were significantly higher levels of both VDSI and IMPF% in point source stations compared to both natural harbours and reference stations, but the difference between natural harbours and reference stations was not significant.

Table 5. Summary of regression analyses for laverspire shell. Analyses were performed on both normalized and non-normalized concentrations of TBT and ΣBT. Abbreviations sed and tis refer to sediment and tissue, respectively. pLogLik refers to the p-value of the log-likelihood ratio test between model with and without substrate (Sub). Significant p-values are marked with green text.

Regression analyses		Non-normalized				Normalized			
Relationship	Variables	Slope	P	R^2	pLogLik	Slope	P	R^2	pLogLik
VDSI-TBTsed	TBTsed, Sub	0.073	0.03	0.33	0.365	0.067	0.039	0.31	0.113
VDSI-TBTsed	TBTsed	0.13	0.0046	0.25		0.11	0.029	0.15	
VDSI-ΣBTsed	ΣBTsed, Sub	0.041	0.053	0.29	0.175	0.0097	0.061	0.28	0.0395
VDSI-ΣBTsed	ΣBTsed	0.14	0.027	0.16		0.079	0.21	0.055	
IMPF-TBTsed	TBTsed, Sub	3.1	0.0019	0.47	0.00953	2.6	0.0023	0.46	0.0101
IMPF-TBTsed	TBTsed	7.5	0.0055	0.24		7.8	0.0071	0.22	
IMPF-ΣBTsed	ΣBTsed, Sub	3.4	0.0021	0.46	0.0058	1.9	0.0028	0.45	0.00303
IMPF-ΣBTsed	ΣBTsed	8.9	0.011	0.21		7.8	0.027	0.16	
Mixed effect models									
Relationship	Variables	Slope	P	R^2	TukeyHSD				
VDSI-Locality	Locality		0.014	0.43,0.31	S-N, S-R sig, N-R ns				
IMPF-Locality	Locality		0.014	0.92,0.39	S-N, S-R sig, N-R ns				

For the mixed effects models (VDSI-locality and IMPF-locality), p-values are based on the Satterthwaite's method and R^2-values are the conditional and marginal R^2-values (Nakagawa et al 2017), S=source; N=natural harbour; R=reference).

3.1.1 VDSI and concentration of TBT in sediment

There is a significant positive relationship between VDSI in laver spire shell, non-normalized concentration of TBT_{sed} and the substrate (p=0.03). The effect of the substrate is not significant (pLogLik=0.365), though it slightly improves fit of the model (R^2 = 0.33 vs R^2 = 0.25) (Table 5, Figure 6). Similar results can be seen for the relationship between VDSI and non-normalized concentration of ΣBT_{sed}, where the relationship is significant (p=0.027) when substrate is excluded. Inspection of the slopes and the R^2-values indicates that the TBT concentration in the non-normalized sediment better explains the level of VDSI than ΣBT_{sed}, as the variation in the concentration of non-normalized TBT_{sed} explains 25% of the variation in VDSI, compared to 16% explained by the non-normalized concentration of ΣBT_{sed} (Figure 6). Normalizing the concentration of TBT_{sed} and ΣBT_{sed} to 5% TOC did not improve any of the relationships found with non-normalized concentrations (Figure 7).

Very few stations are below the suggested VDSI threshold value in laver spire shell, however, several stations are close to it. The EQS_{TBT} (1.6 μg/kg *dw*) refers to concentrations of sediment normalized against 5% TOC, and only one observation from 2010 and two from 2019 fall below this limit. All of these are reference stations (Figure 7). There are, for both years, stations where the concentration of TBT in the sediment has been below the reporting limit. Half of the reported value has then been used, to avoid that the TBT content is underestimated, which in connection with the normalization of the sediment to 5% TOC may have led to the content being overestimated instead. If the same limit for TBT is used for non-normalized sediments, then most of the reference stations from both 2010 and 2019 falls below the limit (Figure 6).

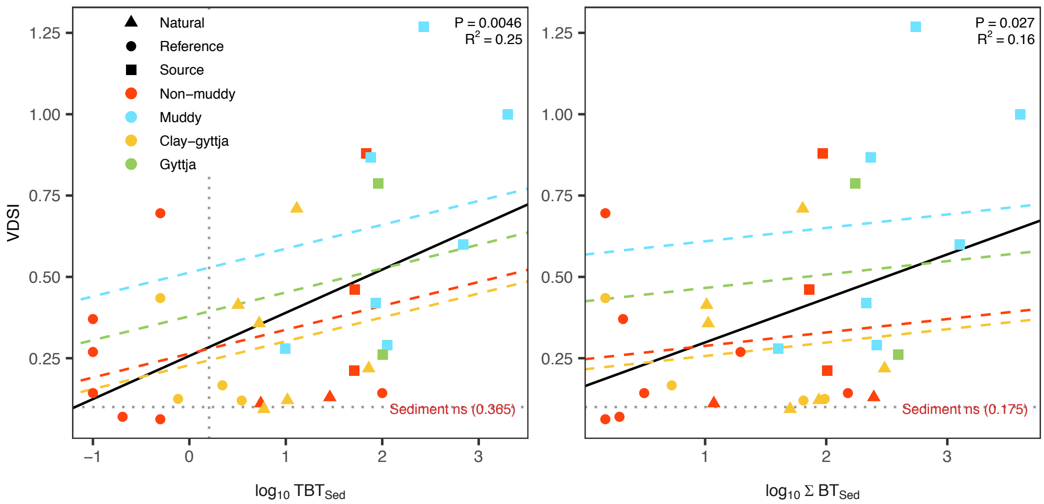

Figure 6. Relationship between non-normalized concentrations of $TBT_{sediment}$ and VDSI in laver spire shell to the left and between $\Sigma BT_{sediment}$ and VDSI to the right. Solid black line shows the relationship modelled without substrate effect and dashed coloured lines show the relationship modelled with substrate effects. Likelihood ratio test of the nested models show that the effect of substrate is not significant. Dashed grey vertical line corresponds to the threshold value of TBT-concentration of 1.6 μg/kg *dw.* and dashed grey horizontal line corresponds to the threshold value for VDSI t.

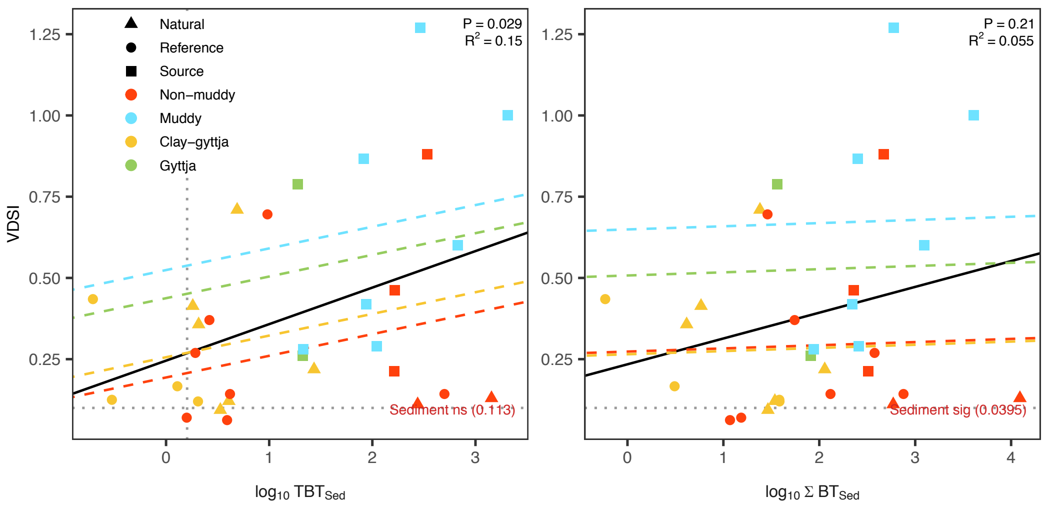

Figure 7. Relationship between normalized (5 % TOC) concentration of $TBT_{sediment}$ and VDSI in laver spire shell to the left and between $\Sigma BT_{sediment}$ and VDSI to the right. Solid black line shows the relationship modelled without substrate effect and dashed coloured lines show the relationship modelled with substrate effects. Likelihood ratio test of the nested models show that the effect of substrate is not significant. Dashed grey vertical line corresponds to EQS_{TBT} of 1.6 μg/kg *dw.* and dashed grey horizontal line corresponds to VDSI limit for good status.

3.1.2 IMPF (%) and concentration of TBT in sediment

There is a significant positive relationship between IMPF%, concentration of non-normalized TBT_{sed} and the substrate (p=0.0019). Substrate type has a significant effect (pLogLik = 0.00953) and improves fit of the model considerably (R^2 = 0.47 vs R^2 = 0.24) (Table 5, Figure 8). Similar results can be seen for the relationship between IMPF% and concentration of ΣBT_{sed}, which is significant (p=0.0021) and where the effect of substrate type is significant (pLogLik = 0.0058) and improves the fit of the model (R^2 = 0.21 vs R^2 = 0.46). Inspection of the slopes and the R^2-values indicates that the TBT concentration in the sediment and the sum of TBT and its degradation products DBT and MBT (ΣBT_{sed}) explain the variation in IMPF% equally well (R^2 = 0.47 and R^2 = 0.46, respectively, when accounting for substrate type, and R^2 = 0.24 and R^2 = 0.21 when not) (Figure 8).

Normalizing the concentration of TBT_{sed} and ΣBT_{sed} to 5% TOC did not improve any of the relationships found when using non-normalized concentrations (Figure 9).

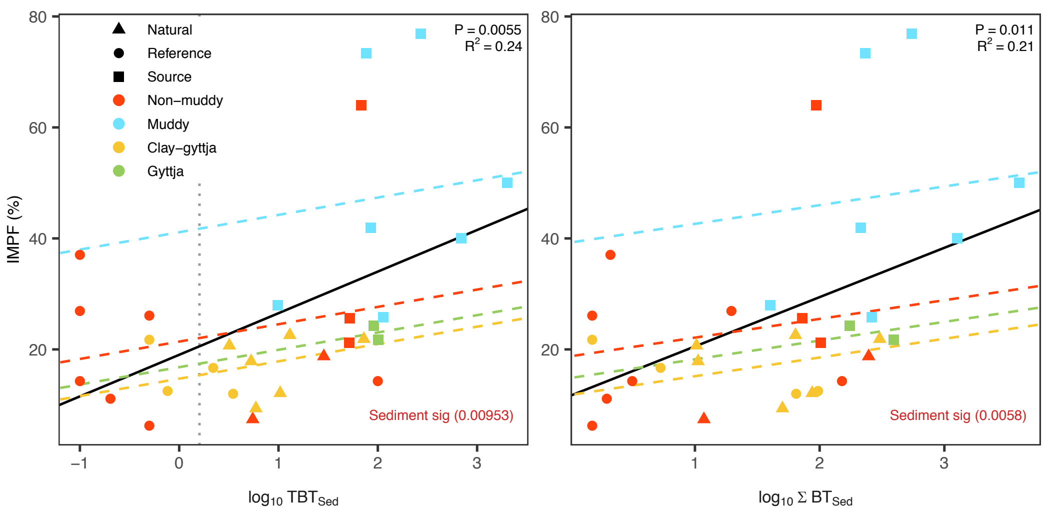

Figure 8. Relationship between non-normalized concentrations of $TBT_{sediment}$ and IMPF% in laver spire shell to the left and between $\Sigma BT_{sediment}$ and IMPF% to the right. Solid black line shows the relationship modelled without substrate effect and dashed coloured lines show the relationship modelled with substrate effects. Likelihood ratio test of the nested models show that the effect of substrate is not significant. Dashed grey vertical line corresponds to a TBT-concentration of 1.6 μg/kg *dw*.

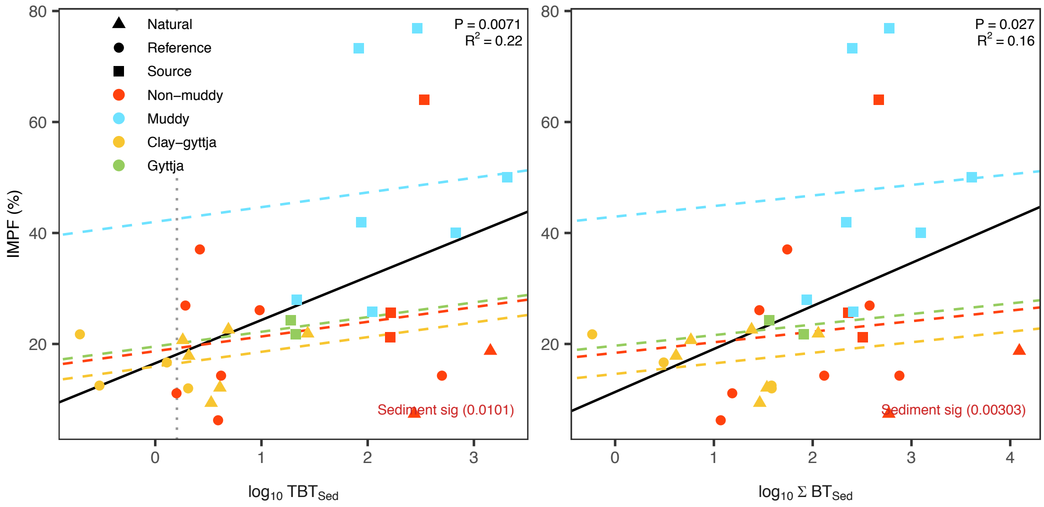

Figure 9. Relationship between normalized (5 % TOC) concentration of $TBT_{sediment}$ and IMPF% in laver spire shell to the left and between $\Sigma BT_{sediment}$ and VDSI to the right. Solid black line shows the relationship modelled without substrate effect and dashed coloured lines show the relationship modelled with substrate effects. Likelihood ratio test of the nested models show that the effect of substrate is not significant. Dashed grey vertical line corresponds to EQS_{TBT} of 1.6 μg/kg *dw*.

3.1.3 Correlation between VDSI, IMPF (%) and type of station

Both the levels of VDSI and IMPF% at point source stations were significantly higher than both natural harbours (VDSI: P = 0.028, IMPF: P = 0.014) and reference stations (VDSI: P = 0.0051, IMPF: P = 0.009) , but there was no significant difference between natural harbours and reference stations (VDSI: P = 0.97, IMPF: 0.96) (Table 5, Figure 10). For IMPF%, the conditional R^2 = 0.92 (i.e. accounting for both random effect of sampling site and the fixed effect of station type) while the marginal R^2 = 0.39 (i.e. accounting only for the fixed effect of type station), indicating a strong effect of sampling station, in addition to the station type. By comparison, the corresponding R^2 values for VDSI were 0.33 and 0.27, i.e. a more reduced effect of sampling station.

Note that both natural harbours and reference stations are just above the VDSI threshold value and that there are very few stations below.

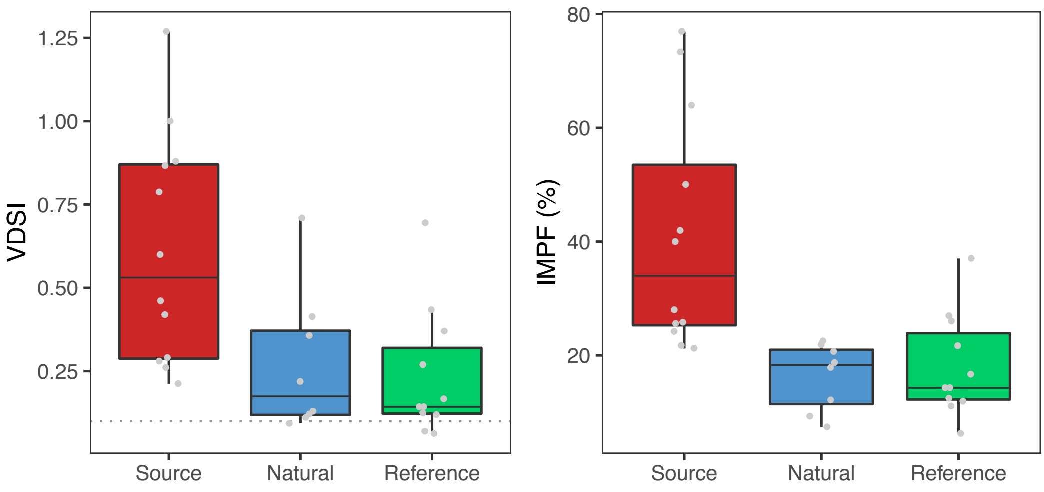

Figure 10. Relationship between locality (source, natural harbour, and reference stations), and VDSI in laver spire shell to the left and for IMPF to the right. Tukey HSD shows significant differences between point source and natural harbour, and point source and reference stations, but no difference between natural harbour and reference stations. Grey dots correspond to sampled stations and dashed grey horizontal line corresponds to VDSI limit for good status.

3.2 Netted dog whelk

All analyses revealed significant positive relationships between VDSI, IMPF%, and concentrations in the sediment, as well as between VDSI, IMPF% and concentrations in tissue of netted dog whelk (Table 6). There were also significant positive relationships between concentrations in the sediment and tissue for netted dog whelk. Comparisons between regressions based on non-normalized concentrations in sediment and regressions based on normalized concentrations indicate that substrate type more frequently has a significant effect in the latter regressions, and then improved the fit (R^2) of the regression model considerably.

For the mixed effects regression models (VDSI and IMPF% against tissue concentrations), the conditional R^2 (which accounts for the variance of both the fixed and the random effects, i.e. which includes the effect of sampling site) were as expected higher than the marginal R^2 (which only accounts for the variance in the fixed effects, i.e. TBT and ΣBT concentrations and substrate), but marginal R^2 was still around 50% in most analyses. Nevertheless, the effect of sampling site is not negligible.

Table 6. Summary of regression analyses for netted dog whelk. Analyses were performed on both normalized and non-normalized concentrations of TBT and ΣBT. Abbreviations sed and tis refer to sediment and tissue, respectively. pLogLik is the p-value of the log-likelihood ratio test between model with and without substrate (Sub). Significant p-values are marked with green text.

Regression analyses		**Non-normalized**				**Normalized**			
Relationship	Variables	Slope	P	R^2	pLogLik	Slope	P	R^2	pLogLik
VDSI-TBTsed	TBTsed,Sub	0.41	0.012	0.34	0.777	0.42	0.0051	0.4	0.0363
VDSI-TBTsed	TBTsed	0.46	0.0027	0.34		0.53	0.0094	0.27	
VDSI-ΣBTsed	ΣBTsed, Sub	0.26	0.033	0.28	0.31	0.32	0.016	0.32	0.0131
VDSI-ΣBTsed	ΣBTsed	0.43	0.014	0.24		0.38	0.087	0.13	
IMPF-TBTsed	TBTsed, Sub	14	0.0065	0.38	0.485	14	0.004	0.41	0.01
IMPF-TBTsed	TBTsed	19	0.0017	0.37		19	0.02	0.22	
IMPF-ΣBTsed	ΣBTsed, Sub	2.4	0.023	0.3	0.0604	5.6	0.018	0.32	0.00354
IMPF-ΣBTsed	ΣBTsed	15	0.036	0.18		8.5	0.34	0.041	
VDSI-TBTtis	TBTtis, Sub	0.91-1.6	0.0022	0.7,0.49	0.0135				
VDSI-TBTtis	TBTtis	1.2	<0.001	0.63,0.47					
VDSI-ΣBTtis	ΣBTtis, Sub	1.2-1.6	0.041	0.76,0.54	0.166				
VDSI-ΣBTtis	ΣBTtis	1.4	<0.001	0.73,0.55					
IMPF-TBTtis	TBTtis, Sub	26-49	<0.001	0.73,0.49	0.00289				
IMPF-TBTtis	TBTtis	36	<0.001	0.65,0.43					
IMPF-ΣBTtis	ΣBTtis, Sub	37-51	0.021	0.8,0.54	0.0732				
IMPF-ΣBTtis	ΣBTtis	44	<0.001	0.76,0.53					
TBTtis-TBTsed	TBTsed, Sub	0.3	<0.001	0.57	0.182	0.32	<0.001	0.63	0.000034
TBTtis-TBTsed	TBTsed	0.45	<0.001	0.54		0.44	0.0041	0.32	
ΣBTtis -ΣBTsed	ΣBTsed, Sub	0.28	<0.001	0.52	0.118	0.3	<0.001	0.57	0.000023
ΣBTtis -ΣBTsed	ΣBTsed	0.45	<0.001	0.46		0.36	0.024	0.21	

For the mixed effects models (VDSI-TBTtis, VDSI-ΣBTtis, IMPF-ΣBTtis, and IMPF-ΣBTtis), p-values are based on the Satterthwaite's method and R^2-values are the conditional and marginal R^2-values (Nakagawa et al 2017). Multiple values for slope refer to the range of estimated slopes (see respective figure).

3.2.1 VDSI and concentration of TBT in sediment

There is a significant positive relationship between VDSI in netted dog whelk, concentration of non-normalized TBT_{sed} (p=0.0027) (Table 6, Figure 11). The effect of the substrate is not significant (pLogLik=0.777) and does not improve the fit of the model (R^2 = 0.34). Similar results are seen for the relationship between VDSI and concentration of ΣBT_{sed}, which reveals a significant relationship (p=0.014), and again the effect of the sediment is not significant (pLogLik = 0.49) and only slightly improves the fit (R^2 = 0.28 vs R^2 = 0.24) (Figure 11). The slope and the R^2-value indicate that the TBT concentration in the sediment explains slightly more of the variation in VDSI than the sum of TBT and its degradation products DBT and MBT (ΣBT_{sed}), as the variation in the concentration of TBT_{sed} explains 34% of the variation VDSI, compared to 24% by the variation in the concentration of ΣBT_{sed}.

Normalizing the concentration of TBT_{sed} and ΣBT_{sed} to 5% TOC only displayed that substrate type had a significant effect and improved the fit (R^2) of the regression model considerably (Figure 12).

It is noticeable that all reference stations are found below the threshold value for VDSI. The EQS_{TBT} in the sediment (1.6 μg/kg dw) refers to concentrations of normalized sediment against 5% TOC, which most of the stations exceeds (Figure 12), despite showing low measured concentrations. If the same limit for TBT is used for non-normalized sediments, then all reference stations are below the limit (Figure 11).

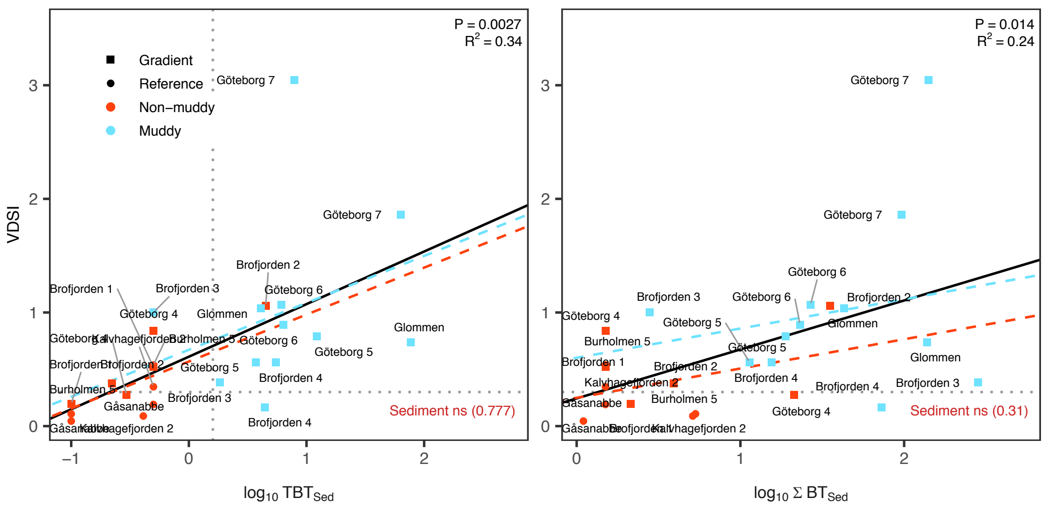

Figure 11. Relationship between non-normalized concentrations of $TBT_{sediment}$ and VDSI in netted dog whelk to the left and between VDSI and $\Sigma BT_{sediment}$ to the right. Solid black line shows the relationship modelled without substrate effect and dashed coloured lines show the relationship modelled with substrate effects. Likelihood ratio test of the nested models show that the effect of substrate is not significant. Dashed grey vertical line corresponds to a TBT-concentration of 1.6 μg/kg *dw* and dashed grey horizontal line corresponds to VDSI threshold value. Points are labelled with station name.

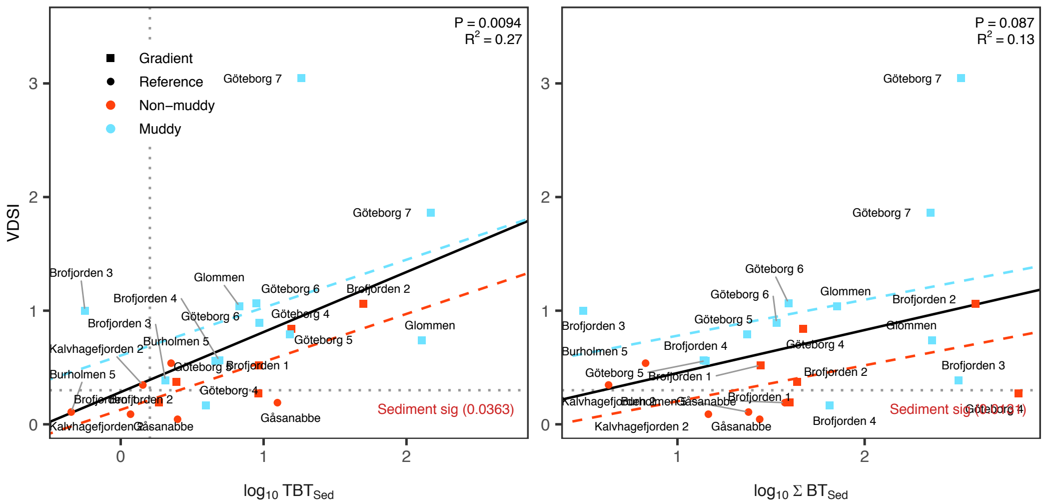

Figure 12. Relationship between normalized (5 % TOC) concentrations of $TBT_{sediment}$ and VDSI in netted dog whelk to the left and between VDSI and $\Sigma BT_{sediment}$ to the right. Solid black line shows the relationship modelled without substrate effect and dashed coloured lines show the relationship modelled with substrate effects. Likelihood ratio test of the nested models show that the effect of substrate is not significant. Dashed grey vertical line corresponds to EQS_{TBT} of 1.6 μg/kg *dw* and dashed grey horizontal line corresponds to VDSI threshold value. Points are labelled with station name.

3.2.2 IMPF (%) and concentration of TBT in sediment

There is a significant positive relationship between IMPF% in netted dog whelk, concentration of non-normalized TBT_{sed} and the substrate (p=0.0065) (Table 6, Figure 13). However, the effect of the substrate is not significant (pLogLik=0.485), and only marginally improves the fit of the model (R^2 = 0.38 vs R^2 = 0.37). Similar results are seen for the relationship between IMPF% and concentration of non-normalized ΣBT_{sed} (Figure 13), where the relationship is significant (p=0.023), and while the substrate is only borderline significant (pLogLik = 0.0604) it does improve the fit of the model (R^2 = 0.3 vs R^2 = 0.18). Inspection of the slope and the R^2-value indicates that the variation of TBT concentration in the sediment explains more of the variation in IMPF% (R^2 = 0.37) compared to that explained by the variation of TBT and its degradation products DBT and MBT (ΣBT_{sed}) (R^2 = 0.18).

Normalizing the concentration of TBT_{sed} and ΣBT_{sed} to 5% TOC displayed that the substrate type had a significant effect and improved the fit (R^2) of the regression model considerably (Figure 14, Table 6).

All reference stations are below the EQS_{TBT} when applied for non-normalized sediments (Figure 13). Several reference stations, nonetheless, exceeds this limit when used in normalized sediments (Figure 14).

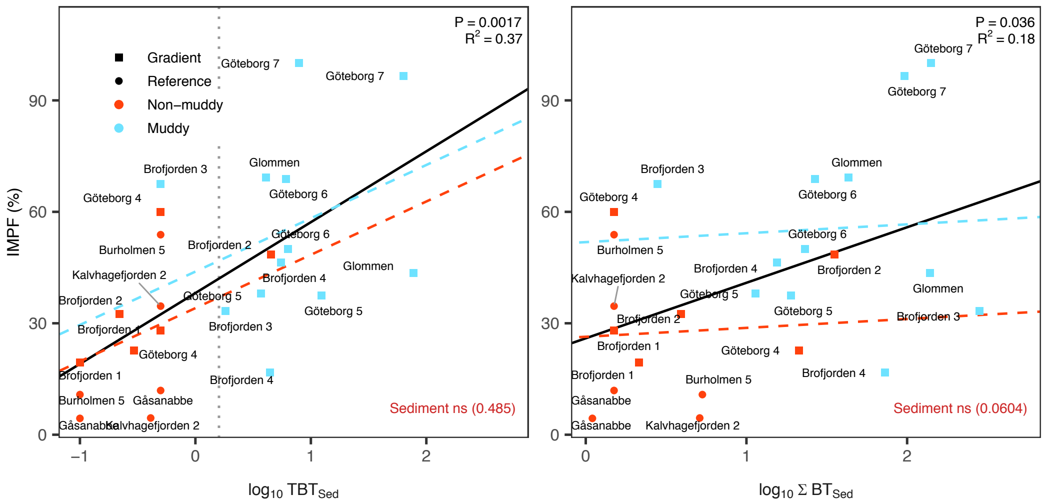

Figure 13. Relationship between non-normalized concentrations of $TBT_{sediment}$ and IMPF% in netted dog whelk to the left and between IMPF% and $\Sigma BT_{sediment}$ to the right. Solid black line shows the relationship modelled without substrate effect and dashed coloured lines show the relationship modelled with substrate effects. Likelihood ratio test of the nested models show that the effect of substrate is not significant. Dashed grey vertical line corresponds to TBT-concentration of 1.6 μg/kg *dw*. Points are labelled with station name.

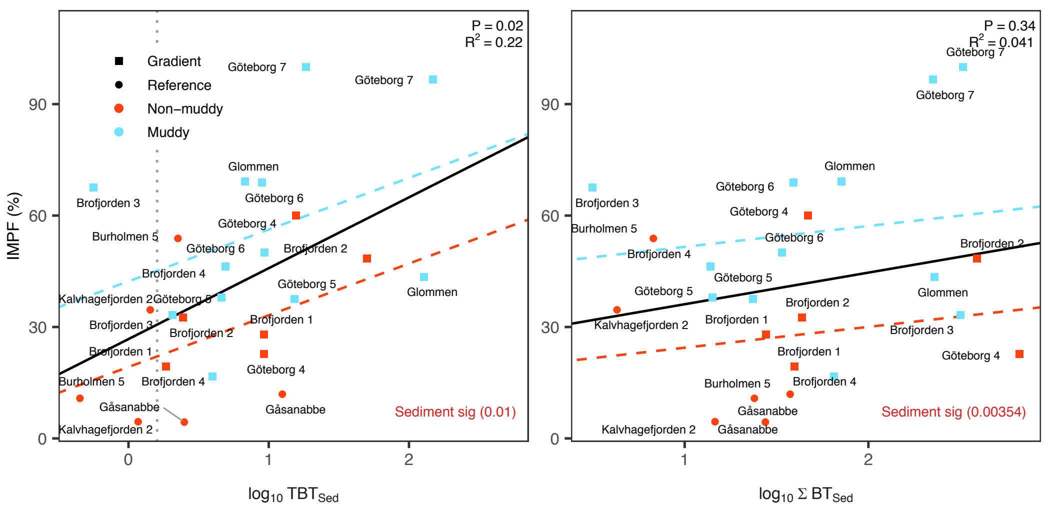

Figure 14. Relationship between normalized (5 % TOC) concentrations of $TBT_{sediment}$ and IMPF% in netted dog whelk to the left and between IMPF% and $\Sigma BT_{sediment}$ to the right. Solid black line shows the relationship modelled without substrate effect and dashed coloured lines show the relationship modelled with substrate effects. Likelihood ratio test of the nested models show that the effect of substrate is not significant. Dashed grey vertical line corresponds to EQS_{TBT} of 1.6 μg/kg. Points are labelled with station name.

3.2.3 VDSI and concentration of TBT in tissue

There is a significant positive relationship between VDSI in netted dog whelk, concentration of TBT_{tis} and the substrate (p=0.0022) (Table 6, Figure 15). The effect of the substrate is significant (pLogLik=0.0135), though only slightly improves the fit of the model (conditional R^2 = 0.70 and marginal R^2 = 0.49 vs conditional R^2 = 0.63 and marginal R^2 = 0.47). The relationship between VDSI and concentration of ΣBT_{tis} is also significant (P < 0.001) (Figure 15), though the substrate does not have a significant effect (pLogLik = 0.144) and has little impact on the fit of the model (conditional R^2 = 0.76 and marginal R^2 = 0.54 vs conditional R^2 = 0.73 and marginal R^2 = 0.55). Inspection of the slope and the R^2-value indicate that the variation in VDSI is slightly better explained by the variation in the sum of TBT and its degradation products DBT and MBT (ΣBT_{tis}) in the tissue (conditional R^2 = 0.73 and marginal R^2 = 0.55), and the variation in the concentration of TBT_{tis} (conditional R^2 = 0.63 and marginal R^2 = 0.47).

It is noticeable that most of the reference stations are found below or close to the threshold value for VDSI.

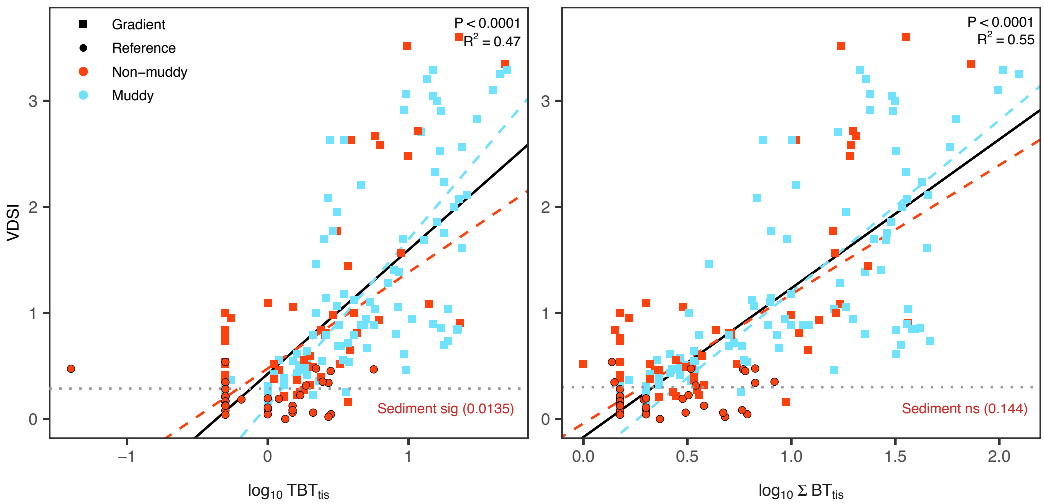

Figure 15. Relationship between concentrations of TBT_{tissue} and VDSI in netted dog whelk to the left and between VDSI and ΣBT_{tissue} to the right. Solid black line shows the relationship modelled without substrate effect and dashed coloured lines show the relationship modelled with substrate effects. Dashed grey horzintal line corresponds to VDSI limit for good status. Likelihood ratio test of the nested models show that the effect of substrate is significant for VDSI and TBT_{tissue} but not VDSI and ΣBT_{tissue}.

3.2.4 IMPF (%) and concentration of TBT in tissue

There is a significant positive relationship between IMPF% in netted dog whelk, concentration of TBT_{tis} and the substrate ($p < 0.001$) (Table 6, Figure 16). The effect of the substrate is significant (pLogLik=0.00289), though it only slightly improves the fit of the model (conditional $R^2 = 0.73$, marginal $R^2 = 0.49$ versus conditional $R^2 = 0.65$, marginal $R^2 = 0.43$). Similar results are seen for the relationship between IMPF% and concentration of ΣBT_{tis} (Figure 16), where the relationship is significant (p=0.021), and the effect of substrate is borderline significant (pLogLik = 0.0732). Inspection of the slope and the R^2-value indicates that the variation in the sum of TBT and its degradation products DBT and MBT (ΣBT_{tis}) in the tissue explains slightly more of the variation in IMPF%, compared to the variation in the concentration of TBT_{tis} (conditional $R^2 = 0.76$, marginal $R^2 = 0.53$ versus conditional $R^2 = 0.65$, marginal $R^2 = 0.43$).

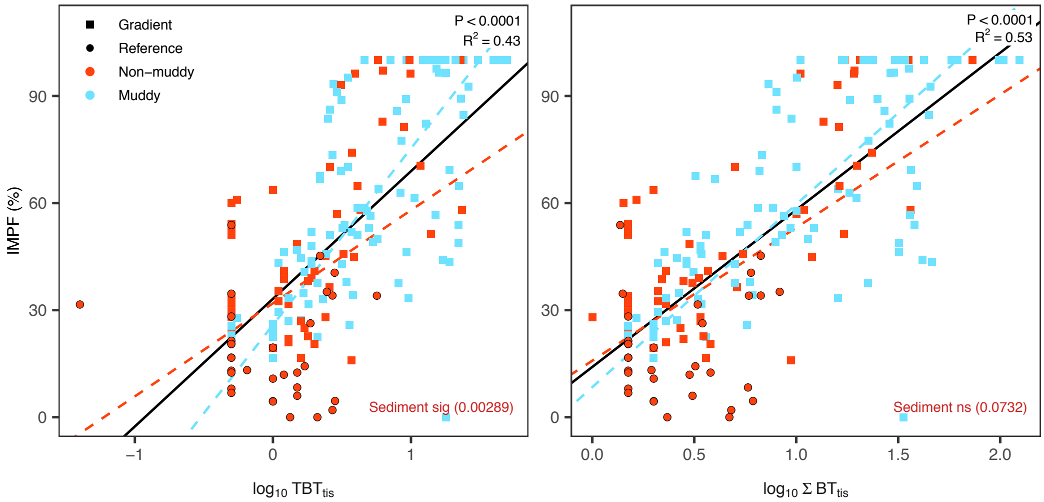

Figure 16. Relationship between concentrations of TBT_{tissue} and IMPF% in netted dog whelk to the left and between IMPF% and ΣBT_{tissue} to the right. Solid black line shows the relationship modelled without substrate effect and dashed coloured lines show the relationship modelled with substrate effects. Likelihood ratio test of the nested models show that the effect of substrate is significant for IMPF% and TBT_{tissue} but not for IMPF% and ΣBT_{tissue}.

3.2.5 Correlation between TBT in tissue and in sediment

The relationship between concentration of TBT_{tis} in netted dog whelk and the concentration of non-normalized TBT_{sed} and the substrate is significant ($p<0.001$), though the effect of substrate is not significant (pLogLik=0.182), and the effect of substrate on the fit of the regression model is slight ($R^2 = 0.57$ versus $R^2 = 0.54$) (Table 6, Figure 17). Similar results are seen for the relationship between ΣBT_{tis} and concentration of ΣBT_{sed}, where the relationship is significant ($p<0.001$) but the effect of substrate is not (pLogLik = 0.118) with very slightly improved fit ($R^2 = 0.52$ vs $R^2 = 0.46$).

Normalization of the concentration of TBT_{sed} and ΣBT_{sed} to 5% TOC gives a significant effect of the substrate which considerably improves the fit (R^2) of the regression model (Table 6, Figure 18).

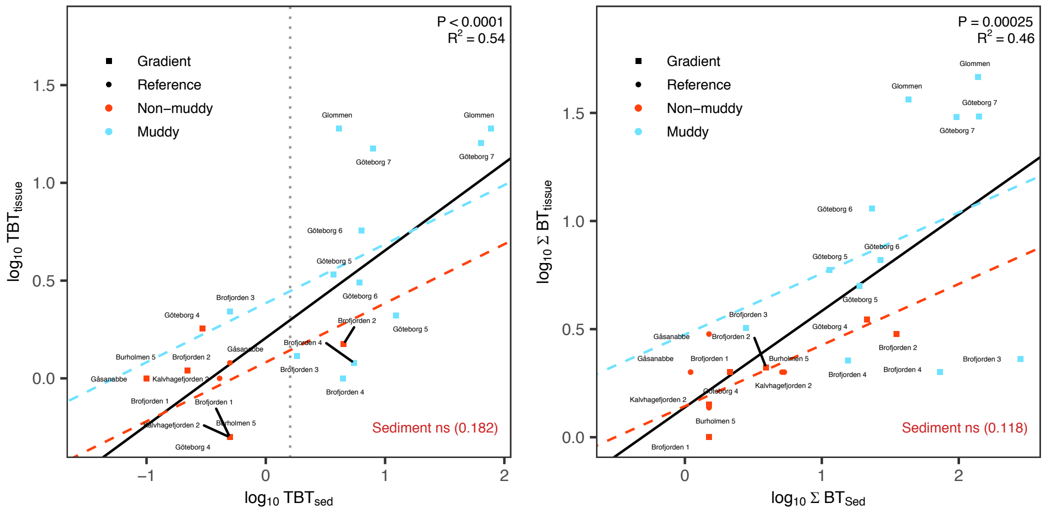

Figure 17. Relationship between non-normalized concentrations of $TBT_{sediment}$ and TBT_{tissue} in netted dog whelk to the left and between $\Sigma BT_{sediment}$ and ΣBT_{tissue} to the right. Solid black line shows the relationship modelled without substrate effect and dashed coloured lines show the relationship modelled with substrate effects. Likelihood ratio test of the nested models show that the effect of substrate is not significant. Dashed grey vertical line corresponds to EQS_{TBT} of 1.6 μg/kg. Points are labelled with station name.

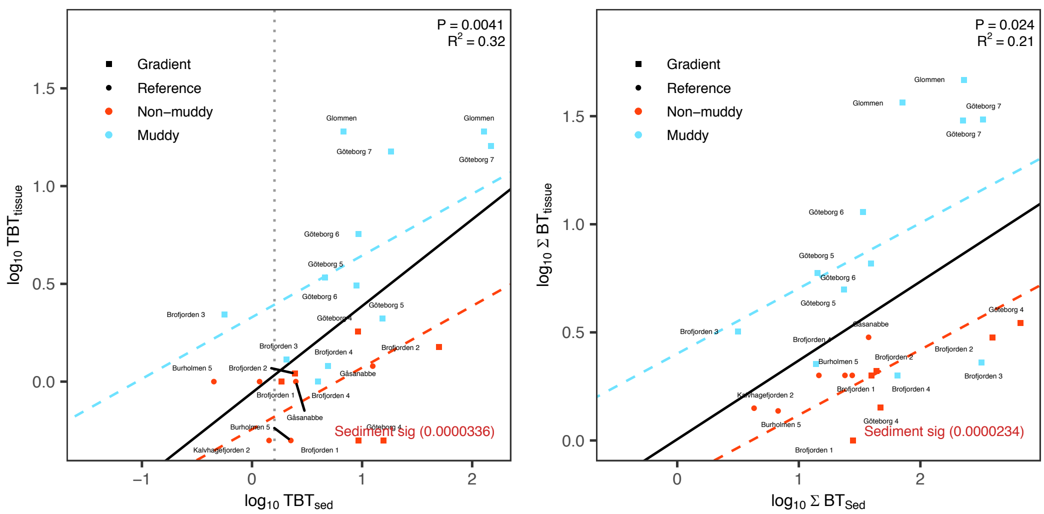

Figure 18. Relationship between normalized (5 % TOC) concentrations of $TBT_{sediment}$ and TBT_{tissue} in netted dog whelk to the left and between $\Sigma BT_{sediment}$ and ΣBT_{tissue} to the right. Solid black line shows the relationship modelled without substrate effect and dashed coloured lines show the relationship modelled with substrate effects. Likelihood ratio test of the nested models show that the effect of substrate is not significant. Dashed grey vertical line corresponds to EQSTBT of 1.6 μg/kg. Points are labelled with station name.

4 Discussion

Although TBT has been banned for many years in antifouling paint, high levels of the substance persist. The severe effects it causes are still a major problem in the marine environment and will remain so for a long time due to its ability to bind to sediment particles in combination with long degradation time. Today, the major source of TBT in the marine environment is believed to be leakage from contaminated sediments from shipping lanes, and from contaminated land and sediments in port areas and marinas. The latest status report from HELCOM (2018) reveals that the HELCOM core indicator *Tributyltin (TBT) and imposex* do not achieve *good* status in the HELCOM area. There are downward trends in VDSI which is supported by observations of TBT in tissue from blue mussels and from water measurements. This is to be expected as the use of TBT in antifouling paints has been banned for almost fifteen years. Nonetheless, threshold levels at most stations are still exceeded, and there is also concern about TBT in fish used for human consumption, since TBT can bioaccumulate in marine organisms. It is therefore important to maintain monitoring programmes, and also to establish how well the indicator *8.2A Effects of organotin compounds (imposex)* responds to concentrations of OTC, and especially TBT, in the sediment.

The purpose of the assignment was to compile measured concentrations of organotin compounds in the sediments at Swedish sampling stations for monitoring imposex and then investigate the relationship between concentrations in the sediments and the occurrence of imposex in gastropod snails. Based on the findings of this assignment, topics raised under chapter *1.1 Assignment* will be discussed further in the text below.

1. *The relationship between the levels of organic tin compounds in the sediments and the division of the sampling areas for imposex into reference, natural harbour, and point source.*

There is a strong correlation between IMPF% and type of station. Laver spire shell is significantly more affected by TBT at point sources than at natural harbours and reference stations. VDSI displayed a significant but not a strong relationship with type of station. An explanation as to why the relationship is weaker for VDSI than IMPF% may be that the laver spire shell rarely displays the higher stages of imposex even though the level of TBT in the sediment is high. However, sediment with high concentration of TBT affect more females, which results in a higher IMPF%.

The relationship between the levels of organic tin compounds in the sediments and the division of the sampling areas for imposex in *reference*, *natural harbour*, and *point source* has not been statistically investigated in this report. However, analyses of VDSI and IMPF% in relation to TBT in the sediment clearly show that lower concentrations of TBT are mostly found in reference stations and that the higher levels are mostly found at stations at point sources. Natural harbours are located approximately between point sources and reference stations. An explanation for this may be that reference stations have been placed in areas that that are far away from point sources and are difficult to access by boat, but also because the substrate in most of the reference stations is non-muddy. Non-muddy sediments such as sand are known to often have low concentrations of lipophilic hazardous substances. This is a function of the grain size and organic content; hazardous substances tend to bind to organic particles in the sediment, so more organic particles usually mean higher concentrations. A muddy substrate contains a great number of very fine particles and typically has a relatively high organic content, while non-muddy substrates such as sand have lower organic content and contain fewer and larger particles.

It is in this context important to remember that data used in the analysis of the relationship between VDSI and TBT in sediment is from 2010 and 2019. The global ban on TBT in antifouling paint came into effect in 2008, so it is likely that the impact of TBT was higher in 2010 than in 2019. This is also supported by the fact that most values above the regression lines are from 2010. Results from the national monitoring program also reveal significant improvement in VDSI for seven out of fourteen stations in the Baltic sea (Sveriges vattenmiljö). Some of these reference stations are also situated in very exposed areas, for example Råå ref, which is a sandy beach south of Barsebäck were TBT has not been detected in the sediment. This station is likely more affected by the shipping lanes through Öresund than TBT in the sediment. This also applies to Toseboviken in Blekinge county.

2. *The relationship between concentrations and response of the snails (VDSI, and / or percentage of population (IMPF %)).*

Imposex is known to be strongly linked to exposure to OTC, mainly TBT. Significant correlations have been found between VDSI, IMPF%, and concentrations in the sediment for both species, as well as between VDSI, IMPF% and concentrations in tissue from netted dog whelk. There are also significant positive relationships between concentrations in the sediment and tissue for netted dog whelk.

Correlations between the content of OTC in tissue and sediment were thus better explained by TBT than by ΣBT. Comparisons between regressions based on non-normalized concentrations in sediment and regressions based on normalized concentrations also revealed that non-normalized sediment concentrations often explained more of the variance (higher R^2). It also indicated that substrate type more frequently had a significant effect on regressions with normalized data which improved the fit (R^2) of the regression model. This is not surprising, since the substrate of the sediment was classified according to the organic carbon content (TOC).

However, the slope and the R^2-value in most regressions indicate TBT_{sed} only explain a small part of the variation in imposex. This may be an effect of the small number of data points, since TBT in the sediment has only been analysed for all stations on two occasions (2010 and 2019). The strongest relationships involving TBT_{sed} was seen for TBT_{tis} in the netted dog whelk, were 54% of the variation in the concentration of TBT in the tissue was explained by the variation in TBT concentration in the sediment. The concentration of TBT in the tissue was related to both VDSI and IMPF% and explained about 70 % of the variation of imposex in netted dog whelk. It should be noted, however, that the number of samples associated with each sediment type is low, and that station Göteborg 7, where sediment is classified as *muddy*, contributes some of the highest recorded values for both sediment concentrations, as well as VDSI and IMPF%. Moreover, no samples obtained from stations where substrate was classified as *non-muddy* showed high tissue concentrations. The effect of substrate must therefore be interpreted with caution.

The correlation between TBT_{sed} and VDSI and IMPF% for the laver spire shell were in general weak and the substrate only explained around 25% of the variation in imposex. Inclusion of substrate type, however, increased the explanatory power to 46 %. The strongest relationship for the laver spire shell was seen between IMPF% and station type, where 92 % of the variation of imposex in females was explained, when accounting for station. Notably, where sample size allowed station to be accounted for as a random factor (i.e. the analyses of the relationship between tissue concentrations and VDSI and IMPF%), sediment emerged as a significant factor.

To conclude, there is a relationship between concentrations of TBT in the sediment and VDSI and affected females in both species. The correlation is, however, weak especially for laver spire shell. The weak correlation for both species might be an effect of a limited number of sampling occasions, and there is a possibility that the correlations will strengthen over time as more sampling will expand the data set.

3. *Protection level obtained from the current EQS_{TBT} value of 1.6 µg/kg dw normalized to 5 % TOC regarding the presence of imposex in the snails.*

The current EQS_{TBT} in sediment (1.6 µg/kg *dw*), expressed as 5% organic carbon) was established by SwAM and included in the Swedish legislation in 2015. It is based on ecotoxicity studies on benthic organisms and is considered to be protective for both marine and freshwater species. It has also been adopted by Ospar as a sufficiently robust threshold value for assessing TBT in sediment for the next Quality Status Report (OSPAR, 2020).

Concentrations of TBT in the sediment in this study are above the EQS_{TBT} when expressed as 5% TOC at most of the stations. In 2019, only four stations out of 27 were below and all of them were reference stations. When comparing concentrations of TBT in non-normalized sediment to a TBT-concentration of 1.6 μg/kg *dw*, then the number of stations below the threshold rises to ten stations. Eight of these stations are from reference areas while the other two are from the outer locations in the two gradients on the west coast.

Most of the reference stations are located in areas with non-muddy sediments (< 2 % TOC) and several of them have concentrations of TBT that has been below the quantification limit (1,0 μg/kg TS). A value of half the limit has then been used for these stations, to avoid underestimating the TBT concentration, which in connection with the normalization of the sediment to 5% TOC have probably led to an overestimation of the true TBT content.

Normalizing the TBT concentration in the sediment to 5% TOC undoubtedly leads to overestimations of the concentration of TBT in sandy substrates with low TOC content. For example, Toseboviken, a reference station on a sandy beach in Blekinge county, where TOC in sediment corresponded to 0,26 %, results in a 20 times higher concentration of TBT when normalized against TOC. In sediments with levels of TOC >5%, concentrations are decreased after normalization, such as the point source Blankaholm where TOC in sediment is 24%, which means that the concentration of TBT in normalized sediments instead decreases by 79 %. Another example is Kittelön, where the non-normalized TBT concentration in the sediment was above the threshold value for TBT but below it when normalized to 5% TOC.

To avoid problems of overestimating the concentration of TBT in sediments, it is proposed to not normalize for non-muddy sediments (TOC <2 %; grain size >0.06 mm). Further, to avoid problems with underestimating during normalization to 5% TOC of a sediment with a high content of organic carbon, it is recommended to disregard normalized values below EQS_{TBT} if the TBT content of the non-normalized sediment is above 1.6 μg/kg *dw* and to consider the concentration to be above EQS_{TBT}.

Nonetheless, the purpose of EQS_{TBT} is to protect species living in the sediment from being affected by TBT exposure. It is therefore important to also analyse whether stations within the Swedish environmental monitoring on imposex achieve *good* status or not in accordance with MFSD. Most of the reference stations for netted dog whelk are close to or achieve the threshold value (VDSI

<0.3). When it comes to laver spire shell, there are a number of downward trends, but at present only a few stations achieve the threshold value (VDSI <0.1). If normalized TBT levels in the sediment are used, only three stations with laver spire shells achieve the EQS_{TBT}. However, none of these stations have a VDSI below 0.1. If non-normalized sediment values are used, most laver spire shell reference stations achieve the EQS_{TBT}, while two of them will also achieve the VDSI threshold value. The same applies to netted dog whelk, where out of the seven stations that achieve the threshold value for imposex, only two stations achieve the EQS_{TBT} in sediment if the concentrations are normalized. If the levels are not normalized, six out of seven stations achieve the EQS_{TBT}. To conclude, there is a better fit to the data if the EQS_{TBT} is used without normalization to 5% TOC.

4. Possible recommendations for adjustments to indicator 8.2A with respect to, for example, the threshold value and assessment method.

Assessment of the indicator *8.2A Effects of organotin compounds (imposex)* is performed per sampling station and measured VDSI representative of the assessment period is used for comparison with the threshold value. The threshold value is reached when the 95th percentile of measured VDSI is lower than the threshold value. The threshold value for netted dog whelk is VDSI 0.3, which is agreed within OSPAR (OSPAR, 2013) and used in OSPAR's assessment in 2017 of TBT and imposex (OSPAR, 2017). A VDSI for a station that is significantly below 0.3 is considered to indicate that the exposure to TBT is close to zero.

Imposex is known to be very strongly linked to exposure to OTC and mainly TBT. There are no studies that clearly show other causes, although the impact of parasites is often mentioned in this context. The fact that the threshold value for VDSI is not zero reflects an acceptance that other environmental factors can cause imposex (Strand, 2003). Thus, there is a built-in margin to the threshold value which reflects that 0-30% (VDSI 0.3) of the females in a population may display some form of imposex without being exposed to TBT.

The corresponding threshold value for laver spire shell is 0.1, which means that it is accepted that 0-10% of the females in a population displays imposex from natural causes. The threshold value for laver spire shell has been set lower than for netted dog whelk since the VDSI parameter in laver spire shell is not as sensitive to exposure to TBT as in other species. The more pronounced stages of imposex (VDS stage 3-6) are therefore rarely seen. However, the proportion of individuals (IMPF%) which displays some degree of imposex is still a sensitive endpoint. The threshold value is currently not agreed on within HELCOM but is included as a "test threshold value" in the assessment of TBT and imposex (HELCOM, 2018). Results from this assignment indicate that the suggested threshold for laver spire shell (0.1) is set at a correct level. Raising the threshold value to e.g. 0.3 could mean that stations such as point sources, despite a high percentage of imposex and sediment concentrations above EQS_{TBT}, achieve the threshold value. A further point to consider is using IMPF% as a supporting parameter for the assessment of imposex in laver spire shell to increase the reliability in the assessment.

5. Other conclusions from the analyses linked to the monitoring and assessment of the indicator.

Significant correlations have been observed, several of them with a relatively low degree of explanation (R^2), especially in connection with TBT concentration with sediment as a factor. The explanation for this may be that analyses are based on sediment data from only two sampling occasions. These occasions occurred nine years apart, while the TOC content was only analysed on the latest occasion. Furthermore, the results indicate that the TBT concentration in the sediments has decreased over time, and time has not been included as a factor in this investigation. There are therefore several sources of uncertainty and continuing the analysis of the concentration of TBT and its degradation products DBT and MBT in the sediment at stations with laver spire shell would likely be beneficial in order to obtain a larger data set and thus greater precision and explanatory power of the analyses.

5 Conclusions

- There is a strong correlation between percentage of affected females (IMPF%) and type of station for laver spire shell, and females are more affected at *point sources* than at *natural harbours* and *refere*nce stations. Similar correlations are seen for VDSI, although not as strong. An explanation for why the relationship is weaker for VDSI than IMPF% may be that the laver spire shell rarely displays the higher stages of imposex, even though the level of TBT in the sediment is high. However, a sediment with high concentrations of TBT affects more females, which results in a higher IMPF%.

- Imposex is known to be very strongly linked to exposure to OTC and mainly TBT. Significant correlations have been found between VDSI, IMPF%, and TBT concentrations in the sediment in both species, as well as between VDSI, IMPF% and concentrations in tissue from netted dog whelk. There are also significant positive relationships between TBT concentrations in the sediment and tissue for netted dog whelk. Several of the correlations are however weak, especially for laver spire shell. The weak correlation for both species might be an effect of few sampling occasions in regard to sediment and there is a possibility that correlations increase over time with more sediment sampling.

- Concentrations of TBT in the sediment in this study are above the EQS_{TBT} when expressed as 5% TOC in most of the stations. The meaning of EQS_{TBT} is to protect species living in the sediment from being affected by TBT exposure. Results from the stations, especially the references, reveal a better fit to the data if 1.6 μg/kg *dw* is used without normalization. This is probably linked the fact that most of the reference stations are located in areas with non-muddy sediments (< 2 % TOC), and several of them had concentrations of TBT below the quantification limit. Normalization to 5 % TOC for these stations will undoubtedly lead to an overestimation of the TBT- concentration. To avoid this problem, it is proposed not to normalize for *non-muddy* sediments (TOC <2%). For the opposite problem, i.e. underestimating EQS_{TBT} when normalization to 5% TOC, of a sediment with a high content of organic carbon, it is recommended to disregard normalized values below EQS_{TBT} if the TBT content of the non-normalized sediment is above 1.6 μg/kg *dw* and to consider the concentration to be above EQS_{TBT}.

- The threshold value for laver spire shell is 0.1. It is currently not agreed on within HELCOM but is included as a "test threshold value". The results in this assignment indicate that the suggested threshold is set to a correct level.. Using IMPF% as a supporting parameter may be a way to improve the assessment of imposex in laver spire shell and increase the reliability of the assessment.

- It is suggested to continue to analyse TBT and its degradation products DBT and MBT in the sediment together with the content of TOC at stations with laver spire shell, to investigate if a larger data set will produce greater precision and increase the explanatory power of the relationship between VDSI/IMPF% and TBT_{sed}.

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Marine Monitoring

Relationship between imposex and levels of organotin substances in the sediment

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