Guidelines for monitoring of biological effect– imposex and intersex

1. Background

1.1 Introduction

Tributyltin (TBT) and other organotin compounds have long been used as additives in antifouling paints. The compounds are toxic even at very low concentrations (<1 ng/L), and can cause serious damage marine life. TBT interferes with the endocrine system, altering and disrupting the production of hormones responsible for development, growth and reproduction in animals. Among other things it blocks the enzyme that converts the male sex hormone testosterone to the female sex hormone estrogen, leading to very high levels of stored testosterone. The imposex (superimposition of penis and/or vas deferens on prosobranch females) and intersex (pathological alterations in the oviduct of littorinides and replacement of female by male organs) condition have been proved sensitive biomarkers 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 and intersex as indicators 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 2016; 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 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. The organotins bind strongly to particles in the sediment and the water column. Degradation of TBT in the water column takes days to months, in anaerobic sediments the half-time can be several years. Maintenance dredging in harbours and marinas constitutes a large problem due to the release of TBT from heavily contaminated sediments.

This monitoring guideline is modified after the JAMP guidelines (OSPAR 2008), the Danish national guidelines for monitoring (Strand 2013) and the Swedish guidelines for effect monitoring of organotin compounds (Magnusson 2015).

1.2 Purpose and aims

The aim of the monitoring programme is to perform spatial assessments and to detect long term changes in the marine environment as a result of the contamination with TBT and related organotin compounds.

2. Monitoring methods

2.1 Monitoring features

Imposex in dog whelk (*Nucella lapillus*) has been widely used as an indicator of TBT contamination and is an extremely sensitive indicator, sterilisation of females occur at concentrations of <1 ng/ L (Gibbs et al. 1988; Stroben et al. 1995). Due to the sensitivity for TBT and the resulting sterilisation, it has become threatened in many areas. Dog whelk is absent from the Baltic Sea, as well as the Kattegat and unusual in Skagerrak and southern parts of the North Sea, leading to the requirement of other effect monitoring species in these areas. Suitability as monitoring species depends on the distribution and the sensitivity for TBT. What kind of area (coastal or offshore) being studied is also of importance when choosing indicator species. Netted dog whelk (*Nassarius nitidus*) is common in Skagerrak, Kattegat, the Belt Sea and the Sound, on depths between 1 -15 m (Stroben et al 1992; Hansen 2012) and is recommended as indicator species for coastal areas and point sources. Common whelk (*Buccinum undatum*) and red whelk (*Neptunea antiqua)* have almost the

same distribution as netted dog whelk, but occurs on greater depths (10-1000 m) and are recommended as an indicator species when monitoring shipping lanes. Mud snail (*Peringia ulvae*) is widespread in the North Sea and the Baltic Sea, and is a suitable alternative indicator for TBT effects in these areas (Shulte-Oehlmann *et al.* 1997; Gercken and Sordyl 2007). Common periwinkle (*Littorina littorea*) can be used for monitoring in shallow sub-tidal areas. However, for monitoring purposes, the intersex phenomenon is regarded as a less sensitive parameter when considering TBT-levels compared to imposex (Minchin *et al.* 1997; OSPAR 2008). Intersex should therefore only be used as an effect parameter for assessment of point sources like harbours and if the other recommended species displaying imposex are missing from the area. High degrees of development of both imposex and intersex in dog whelk and common periwinkle have significance beyond the individual organism level, in that they have been associated with changes in the reproductive capacity of populations through lack of juveniles, poor recruitment, local extinction etc. The occurrence of imposex in common whelk, red whelk and netted dog whelk has also been linked with decreases in populations but whether this is a direct consequence of imposex is unclear.

The choice of indicator species will ultimately depend on which species that is present in the area. It should be common, to ensure the collection of 50 individuals per station, and represent the most sensitive species in case more than one species occur in the area in question. In coastal areas this means that: *dog whelk* > *netted dog whelk* > *mud snail* > *common periwinkle* and in offshore areas: *red whelk* > *common whelk*. The same species should also be used as indicator species the following years in order to evaluate the temporal development.

2.2 Time and area

Imposex in dog whelk (*Nucella lapillus*) has been widely used as an indicator of TBT contamination and is an extremely sensitive indicator, sterilisation of females occur at concentrations of <1 ng/ L (Gibbs et al. 1988; Stroben et al. 1995). Due to the sensitivity for TBT and the resulting sterilisation, it has become threatened in many areas. Dog whelk is absent from the Baltic Sea, as well as the Kattegat and unusual in Skagerrak and southern parts of the North Sea, leading to the requirement of other effect monitoring species in these areas. Suitability as monitoring species depends on the distribution and the sensitivity for TBT. What kind of area (coastal or offshore) being studied is also of importance when choosing indicator species. Netted dog whelk (*Nassarius nitidus*) is common in Skagerrak, Kattegat, the Belt Sea and the Sound, on depths between 1 -15 m (Stroben et al 1992; Hansen 2012) and is recommended as indicator species for coastal areas and point sources. Common whelk (*Buccinum undatum*) and red whelk (*Neptunea antiqua)* have almost the same distribution as netted dog whelk, but occurs on greater depths (10-1000 m) and are recommended as an indicator species when monitoring shipping lanes. Mud snail (*Peringia ulvae*) is widespread in the North Sea and the Baltic Sea, and is a suitable alternative indicator for TBT effects in these areas (Shulte-Oehlmann *et al.* 1997; Gercken and Sordyl 2007). Common periwinkle (*Littorina littorea*) can be used for monitoring in shallow sub-tidal areas. However, for monitoring purposes, the intersex phenomenon is regarded as a less sensitive parameter when considering TBT-levels compared to imposex (Minchin *et al.* 1997; OSPAR 2008). Intersex should therefore only be used as an effect parameter for assessment of point sources like harbours and if the other recommended species displaying imposex are missing from the area. High degrees of development of both imposex and intersex in dog whelk and common periwinkle have significance beyond the individual organism level, in that they have been associated with changes in the reproductive capacity of populations through lack of juveniles, poor recruitment, local extinction etc. The occurrence of imposex in common whelk, red whelk and netted dog whelk has also been linked with decreases in populations but whether this is a direct consequence of imposex is unclear.

The choice of indicator species will ultimately depend on which species that is present in the area. It should be common, to ensure the collection of 50 individuals per station, and represent the most sensitive species in case more than one species occur in the area in question. In coastal areas this means that: *dog whelk* > *netted dog whelk* > *mud snail* > *common periwinkle* and in offshore areas: *red whelk* > *common whelk*. The

same species should also be used as indicator species the following years in order to evaluate the temporal development.

2.3 Monitoring procedure

2.3.1 Monitoring strategy

Broad-scale surveys describing the intensity (i.e. frequency and severity) of TBT impacts in coastal waters would allow comparisons to be made between various stretches of coast and assessments of the potential for TBT-affected species to recover.

2.3.2 Sampling method(s) and equipment

Various sampling methods can be used, depending on the species and depth. Methods used are manual picking, collection by diver, traps, trawl hauls, frame scrapes and hand net (Figure 1, Table 1).

Netted dog whelk, dog whelk and common periwinkle in the intertidal zone can be collected by manual picking. Sampling with baited traps is suitable for netted dog whelk, common whelk and red whelk. Traps with a five mm mesh size are baited with crab, mussel or fish meat. It is recommended that the bait is placed in a fine net to prevent crabs and fish from reaching the meat. Time for deployment of traps ranges from two hours (netted dog whelk) to two-five days (common whelk and red whelk). When collecting common whelk, it is recommended to use static gear (traps). In areas where this is not possible (e.g. within the shipping lanes) a beam trawl with a two cm mesh size can be towed parallel to the lanes, but with added uncertainty as to sampling position. Red whelk can be collected using the same methods. A frame scrape with a 50 cm width is suitable for capturing netted dog whelk. Collection of mud snail is performed by dragging a hand net with a fine mesh along the bottom. The sample is sorted by hand in the field. To simplify the sorting, this is performed on a white surface. Snails are picked out with tweezers. A selection of 60-70 snails of uniform size from each station is brought back to the lab. If species determination is not possible in the field, all snails are kept in order to be determined in the dissecting microscope.

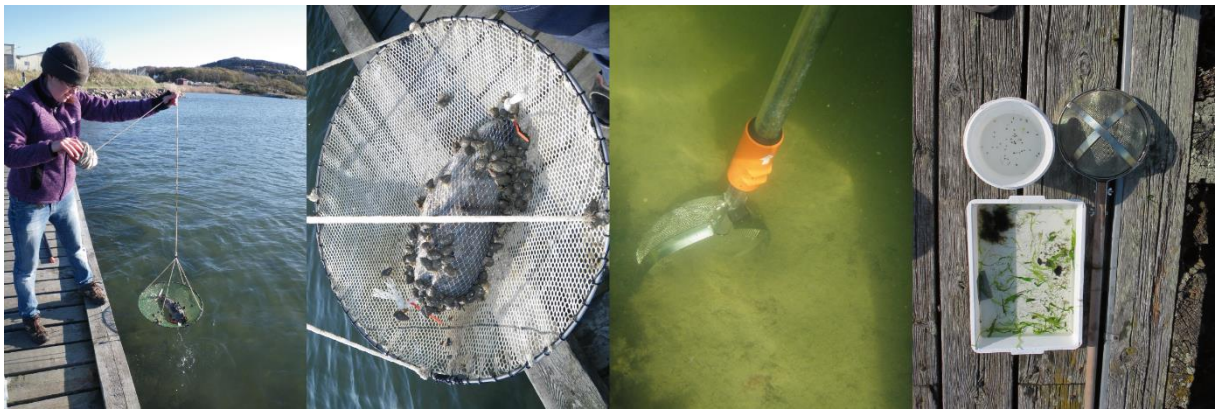

Figure 1. Some sampling methods and equipment. From left to right: collection of netted dog whelk with trap, trap with bait and netted dog whelks, dragging a hand net over the bottom, sorting mud snails. Photos Marina Magnusson

All animals should be analysed within seven days from capture why it is, depending on number of stations, advised that field collection and analysis in the lab is alternated, it is advised to bring some water from the sampling locations in order to change the water the snails are kept in. Also, consider the regrowth of the population and do not take all the snails captured. A selection of 60-70 equally sized snails from each station is made, and the rest returned to the place of capture. Examinations of the larger offshore species common whelk and red whelk can also be done on samples that have been stored frozen.

Table 1. Species, collection areas, collection methods and number of individuals to be collected from each station/ area in the monitoring programme.

Species	Collection area	Collection method	Number per station
Dog whelk (*Nucella lapillus*)	Coastal areas and point sources. Intertidal zone.	Manual collection on large rocks in the intertidal zone.	40*
Netted dog whelk (*Nassarus nitidus*)	Coastal areas and point sources. 1-10 m depth.	Baited traps with mussel/fish meat as bait (2 hours). Small scrape or diver.	40*
Common whelk (*Buccinum undatum*)	Major shipping lanes in offshore areas. Soft bottom and reefs. 15-100 m depth.	Baited traps with mussel/fish meat as bait (2-5 days). Beam trawl, diver.	100*
Red whelk (*Neptunea antiqua*)	Major shipping lanes in offshore areas. Soft bottom and reefs. 15-100 m depth.	Baited traps with mussel/fish meat as bait (2-5 days). Beam trawl, diver.	40*
Mud snail (*Peringia ulvae*)	Coastal areas and point sources. 1-10 m depth.	Dragging with hand net or sediment grab.	50**
Common periwinkle (*Littorina litttorea*)	Point sources (harbours, piers along docks). Intertidal zone.	Manual collection on large rocks or stones in the intertidal zone.	40*

*Recommended number, of which ~50% are female

**50 individuals are used in Swedish monitoring program

Modified from Strand 2013.

2.3.3. Sample handling and analysis

The gastropods are kept and transported alive under cool conditions. They can be kept alive for a week when kept at 5°C in seawater with air supply. Animals should be analysed as soon as possible but no later than seven days after sampling. If, for example for logistical reasons, it is necessary to preserve samples prior to analysis, specimens may be frozen. However, freezing will affect penis length measurements and data should be corrected to make them comparable to data from live specimens. The identification of imposex and intersex stages should be unaffected by freezing. Frozen gastropods are thawed at room temperature before analysis. It is important to note that storage in alcohol or formaldehyde may not take place before determination of imposex is completed.

Before the live gastropods are examined, they are anesthetized in a 7% $MgCl_2$-solution in order to obtain maximum relaxation of muscles. Time for anaesthesia depends on the species and the individual size, 1-2 minutes for mud snail, 20-30 minutes for dog whelk, netted dog whelk and common periwinkle and 2-3 hours for common whelk and red whelk. Animals should not be kept in the anaesthesia solution too long, since this can make the analysis difficult. Before breaking the shell, shell height should be measured to the nearest 0.01 mm with a digital calliper. The shells are cracked with pliers or a vice, and the soft parts are taken out with tweezers. Further studies take place under a dissecting microscope (Figure 2). Specimens parasitized by trematodes or other endoparasites should be excluded from the analysis.

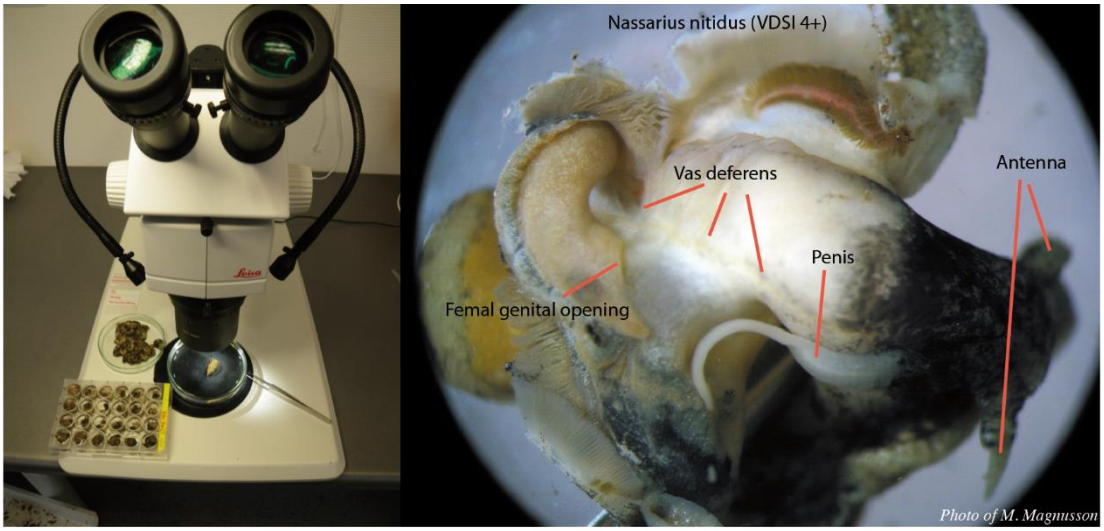

Figure 2. Analysis of imposex in netted dog whelk. Left: dissecton microscope with anesthetized animals in 24 well plate. Right: Female netted dog whelk with vas deferens sequence index 4.

2.3.4. Determination of imposex

2.3.4.1 Age determination

The age of the gastropods is of significant importance when the intensity of imposex is valued, partly in relation to the present TBT-level, partly in comparison between geographical and temporal observations. E.g. common whelk can be older than 15 years, and as it seems like imposex primarily develops during the first years of life (Mensink *et al.* 1997), it can be discussed whether a given intensity of imposex is due to an earlier or the present TBT-level. Age in gastropods can be assessed in three ways – from sexual maturity, size (shell height), and in some cases the number of growth rings on the operculum. Only adult gastropods within a defined size range (dependant on species and the geographical area) are used for calculating the parameter values.

2.3.4.2 Sexual maturity

Only adult (sexually mature) gastropods should be included in the data set. Sexual maturity in males and females are assessed as either adult or juvenile depending on how well developed the gonad is. In gastropods assessed as adults, the gonad (ovary or testis) is distinctly yellow or orange-red and clearly separate from the digestive gland. In subadult gastropods the gonad will not be visible. If the gastropods are infected with trematodes, the reproductive organs can be significantly affected.

2.3.4.3 Shell height

Shell height is a good approximation of age, and a size range is selected for each species. Considering the common whelk it is recommended that the size range in the respective areas is chosen based on the growth rings in the operculum. Note that the height of the shells is also dependant on, among other things, the salinity in the area. Geographical variations should thus be considered, when the size range is determined. Shell height is measured with 0.1 mm certainty with a digital calliper.

Size ranges for the different species:

- Common periwinkle 15-30 mm
- Common whelk 50-100 mm, 3-15 growth rings
- Dog whelk 25-35 mm
- Mud snail 3-6 mm
- Netted dog whelk 15-25 mm

- Red whelk 60-120 mm, 3-15 growth rings

2.3.4.4 Growth rings on operculum

In common whelk and red whelk it is recommended to count the growth rings on the operculum after it is pulled off, in one piece, from the foot. In some cases it is easiest to study the imprints left on the foot after the operculum has been pulled off. The number of growth rings is a good measurement of age, since they generally represent yearly growth. However, only about 50 % of the individuals show clearly separated growth rings (Kideys 1996). For common whelk, preferably gastropods with ages from 3-10 years (i.e. 3-15 growth rings) should be included in the material. The growth rings are therefore compared to the shell height, and a size range based on shell height is selected based on the collective material from an area. Number of growth rings is not so easily counted in the other gastropod species.

2.3.4.5 Parasite infection

The eventual presence of trematodes in the gonads and/or digestive gland should be noted, as gastropods infected with trematodes should be excluded from the data set and replaced by new individuals. The infection is visible as a grey film on the digestive gland or gonad, in some cases as a white or yellow spot on the digestive gland. Closer inspection reveals 1-3 mm long larvae in miracidium, redie or cercaria stage. About 10% of the common whelk population is expected to be infected with trematodes, but there are significant geographical variations (Køie 1969).

Note: Infection by Sporozoa or Tubellaria is common in gastropods, but these infections are ignored as they do not affect the reproductive organs (Køie 1969).

2.3.4.6 Sex determination

The gastropods are dioecious, and despite possible imposex characters there are obvious differences between adult females and males. Determination of sex in sub-adult individuals can be more difficult. The following general description of the female and male reproductive organs is based on common whelk. There are some minor differences related to species, but overall the placement of the individual organs is the same.

Note: It is very important that the sex of the gastropod is determined, i.e. the vaginal opening should be visible, to avoid juvenile males being analysed as females. Gastropods in which the sex cannot be determined with 100% certainty are discarded and replaced by new animals.

2.3.4.7 Female reproductive organs

The yellow-orange ovary is placed on top of the digestive gland in the last whorls. The oviduct connects the ovary with the pallial oviduct, which consist of albumin gland, spermatheca, egg capsule gland and bursa copulatrix with the vaginal opening. These organs are placed one after the other along the mantle cavity (Figure 3).

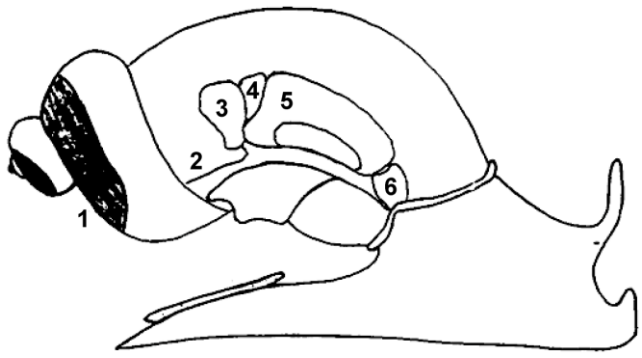

Figure 3. Female reproductive organs. 1) Ovary, 2) Oviduct, 3) Albumin gland, 4) Spermatheca, 5) Egg capsule gland, 6) Bursa copulatrix. From Strand 2004.

2.3.4.8 Male reproductive organs

The yellow-orange testis is, like the ovary in females, placed on top of the digestive gland in the last whorls. The vas deferens runs from the testis to the seminal vesicle, which is a coiled and folded part of the vas deferens located on the surface of the digestive gland. The vas deferens runs on through the prostate gland along the mantle cavity and from there along the surface of the upper part of the foot up to the penis. The penis is located above the right tentacle (Figure 4).

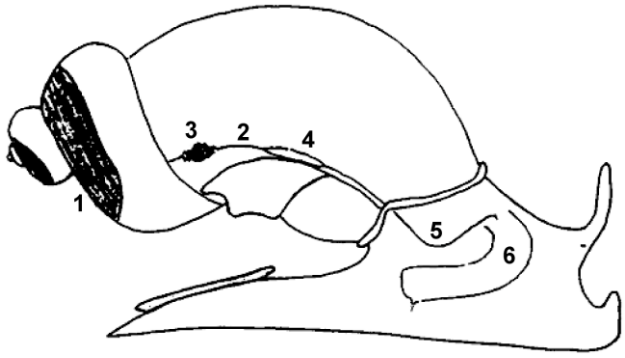

Figure 4. Male reproduction organs. 1) Testis, 2) Vas deferens, 3) Seminal vesicle, 4) Prostate gland, 5) Penis, 6) Penis duct. From Strand 2004.

2.3.4.9 Description and characterisation of imposex stages

The imposex phenomenon is not reversible and is defined as "a superimposition of male characters onto females" and consists in female gastropods primarily of a development of a pseudo-penis and/or vas deferens in the same places as in males, i.e. above the right tentacle. Development of imposex can be described either from penis length or by a classification scheme based on different development stages, which are characterised from how well developed pseudo-penis and vas deferens are (Figure 5). Far advanced stages can lead to sterilisation of females. This has been observed in dog whelk and mud snails, where the developed vas deferens grows over the vaginal opening, thereby preventing oviposition (Bryan *et al*. 1987). Similar sterile stages do not occur in netted dog whelk or common whelk (OSPAR 2008), although oviduct convolution also leading to sterility have been observed (Barroso *et al*., 2002; Strand 2013) as shown in Figure 6. The different ways to describe the development of imposex, and the corresponding parameter values which may be used to describe the intensity of imposex in a group of gastropods, are identified below. Scientific articles for further information regarding each species are presented in

Table 2.

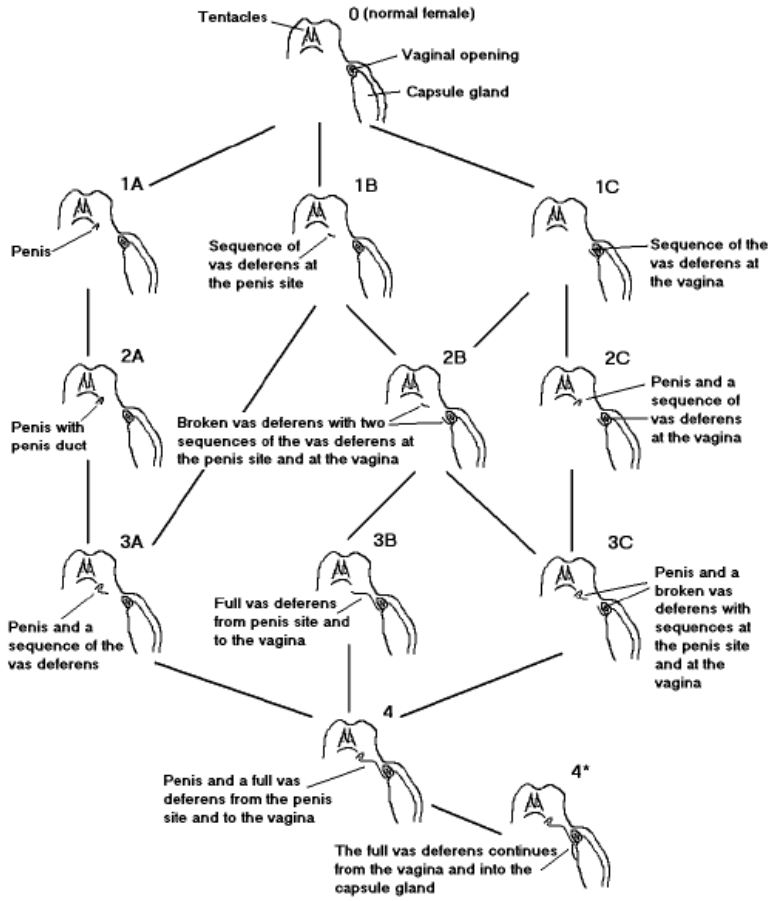

Figure 5. Classification of imposex. Imposex in marine gastropods can be classified after the Vas Deferens Sequence (VD) system, from which a VDS-index, describing the intensity of imposex in a population, can be calculated. The imposex stages 0-4 are developed in common whelk, red whelk and netted dog whelk. Dog whelk can develop tow more, sterile, stages 5-6. From Strand & Jacobsen 2002.

Figure 6. A further alternation of the female characters in form of a curled oviduct (convolution) blocking the transport of oocytes from the ovary to the capsule gland causing sterility in the red whelk (Neptunea antiqua) from the Belt Sea 2005.

(Photo: J. Strand).

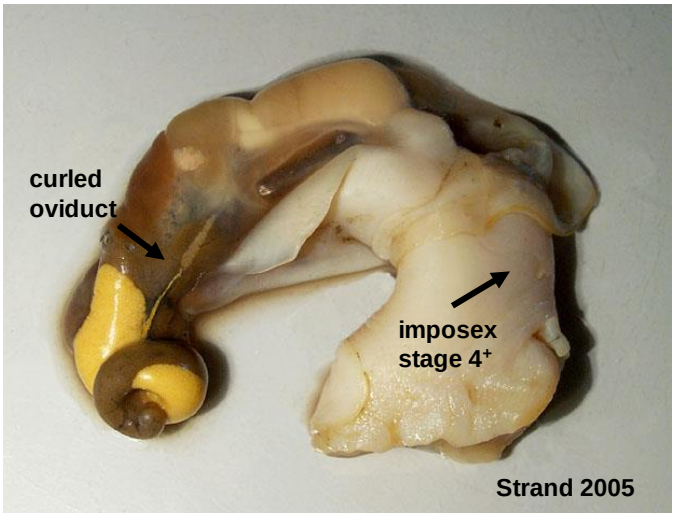

Table 2. Indicator species and additional scientific articles for further information regarding determination of imposex/intersex.

Species	Method for imposex/intersex determination described in:
Dog whelk (*Nucella lapillus*) and **Netted dog whelk** (*Nassarius nitidus*)	*Fioroni P., Oehlmann J. and Stroben E. 1991.* The pseudohermaphroditsm of prosobranchs; morphological aspects. Zoologischer Anzeiger 226(1), 1-26. *Gibbs, P.E., Pascoe, P.L. and Bryan, G.W., 1991*. Tributyltin-induced imposex in stenoglossan gastropods: pathological effects on the female reproductive system. Comp. Biochem. Physiol. 100C:23 1-235. *Stroben, E., Oehlmann J. and Fiorini P. 1992a.* The morphological expression of imposex in *Hinia reticulata* (Gastropoda: Buccinidae): a potential indicator of tributiltin pollution. Marine Biology 113: 625-636. *Stroben, E., Oehlmann, J. and Fioroni, P., 1992b. Hinia reticulata* and *Nucella lapillus*. Comparison of two gastropod tributyltin bioindicators. Mar. Biol. 114: 289-296. *Barroso C. M., Moreira M. H., Bebianno M. J. 2002*. Imposex, female sterility and organotin contamination of the prosobranch *Nassarius reticulatus* from the Portuguese coast. Mar Ecol Prog Ser 230: 127–135.
Common whelk (*Buccinum undatum*)	*Mensink B. P., van Hattum B., ten Hallers-Tjabbes C. C., Everaarts J. M., Kralt H., Vethaak A. D. and Boon J. P. 1997.* Tributyltin causes imposex in the common whelk, *Common whelk*. Mechanism and occurrence. – Nederlands Instituut voor Onderzoek der Zee, Rapport 6, Den Burg, Texel, the Netherlands. *Ten Hallers-Tjabbes, C.C., Kemp, J.F. and Boon, J.P., 1994.* Imposex in whelks *(Buccinum undatum)* from the open North Sea: Relation to shipping traffic intensities. Mar. Pollut. Bull. 28:311-313. *Strand J. and Jacobsen J. A. 2002.* Imposex in two sublittoral neogastropods from the Kattegat and Skagerrak: the common whelk *Buccinum undatum* and the red whelk *Neptunea antiqua*. Marine Ecology Progress Series, 244: 171-177.
Red whelk (*Neptunea antiqua*)	*Power, A. J. & Keegan B. F. (2001*). The significance of imposex levels and TBT contamination in the red whelk, *Neptunea antiqua* (L.) from the offshore Irish Sea. Marine Pollution Bulletin 42(9): 761-772. *Strand J. and Jacobsen J. A. 2002.* Imposex in two sublittoral neogastropods from the Kattegat and Skagerrak: the common whelk *Buccinum undatum* and the red whelk *Neptunea antiqua*. Marine Ecology Progress Series, 244: 171-177.
Mud snail (*Peringia ulvae*)	*Schulte-Oehlmann U., Oehlmann J., Fioroni P., and Bauer B. 1997.* Imposex and reproductive failure in Hydrobia ulvae (Gastropoda: Prosobranchia) Marine Biology (1997) 128: 257-266.
Common periwinkle (*Littorina littorea*)	*Bauer, B., Fiorini, P., Ide, I., Liebe, S., Oehlmann, J., Stroben, E. and Watermann, B. 1995.* TBT effects on the female genital system of *Littorina littorea,* possible indicator of tributyltin pollution. Hydrobiologia 309, 15-27.

2.3.4.10 Penis length

Penis length in both males and females is measured under a dissecting microscope with 0.1 mm accuracy. Penis length is measured from the root to the top. Both average penis length in females (FPL) and relative penis length index (RPLI) are calculated.

FPL = Σ penis length in females (mm) / total number of females (including females with stage 0)

RPLI = (FPL / MPL)*100

Note: If the intensity of imposex is determined in dog whelk it is recommended that the parameter relative penis size index (RPSI = 100% * (RPLI /100)^3) is calculated (OSPAR 2008).

2.3.4.11 Vas deferens sequence

Each vas deferens sequence (VDS) stage is described from how developed the pseudo-penis and vas deferens are. Vas deferens sequence index (VDSI) is calculated as the mean value of imposex stages in a group of gastropods.

VDSI = Σ imposex stage values of all examined females / number of females

A difference of about 1 unit between VDSI-values is considered significant (OSPAR 2008).

Stage 0: Normal female without any male characteristics.
Stage 1: a) A tiny penis without a penis duct, behind the right ocular tentacle.
b) No penis but a short, distal vas deferens tract behind the right ocular tentacle.
Stage 2: Penis with a closing or closed penis duct behind the right ocular tentacle.
Stage 3: a) Penis with penis duct continuing in an incomplete distal tract of the vas deferens that is growing out successively towards the vaginal opening.
b) Penis lacking; vas deferens running continuously from the right ocular tentacle over the bottom of the mantle cavity up to the vaginal opening.
Stage 4: Penis with a penis duct and a continuous vas deferens from the penis up to the vaginal opening. In stage 4+ the vas deferens passes the vaginal opening and runs into the ventral channel of the capsule gland.
Stage 5: *Peringia ulvae:* a sterile female without male sex characteristics, but with closed genital apertures. *Nucella lapillus:* a) The vagina and usually the bursa copulatrix are reduced and the vulva is absent. A more or less extended prostate gland can be found. b) The genital papilla is overgrown and the vaginal opening is occluded by proliferating vas deferens tissue, often forming 'nodules'. Beside these hyperplasic tissues, liquid-filled swellings under the mantle epithelium can sometimes be found. Both possibilities lead to reproductive failure and to sterility, because the egg capsules cannot be released
Stage 6: *Peringia ulvae:* a sterile female with closed genital apertures and male sex characteristics should be given the value 6x. To differentiate between imposex stage 1-4 of all females which exhibit closed genital apertures the types a, b, c and d are used. For example a virilised female of Stage 1 with closed female genital opening should be given the value 6a and a virilised female of Stage 4 with closed female genital opening should be given the value 6d.
Nucella lapillus: as in stage 5, the types a and b can be distinguished. Both represent the final stage of the female determined imposex with a still intact, purely female, ovary. The lumina of the capsule gland and its vestibulum are filled with an accumulation of abortive egg capsules, which are at first transparent and afterwards form an amorphous dark or black mass. This produces an intense swelling of the capsule gland and finally often a rupture, causing the death of the animal.

2.3.4.12 Penis classification Index

The penis classification index (PCI) are primarily based on how well developed the pseudo-penis is (Figure 7). This method should only be used when characterising imposex stages in common whelk. Characterisation of borderline cases between different stages is difficult and is based on the observer's experience. The PCI is calculated as the average of imposex stages in a group of gastropods. If vas deferens is present the value is increased with 0.5.

PCI = Σ imposex stage value of all examined females/number of females.

Imposex stage	seen from above	seen from the side
0		
No development of penis. Smooth epithelium at the site, where males have their penis.		
1		
Small knob at penis site indicating the development of a penis.		
2		
Small structure penis, which can wobble and be lifted up from the epithelium of the foot.		
3		
Penis is bent and with a shape which tends to look like a normal male penis.		

Figure 7. Characterisation of imposex stages according to the penis classification index (PCI). From Strand and Jacobsen 2002.

2.3.4.14 Other visible changes due to imposex

In advanced stages of imposex other visible changes to the reproductive organs might occur (Fiorini *et al.* 1991), for example a curling of the oviduct (convolution) and albumin gland, or a cleavage of the pseudo-penis so that it appears as a double penis.

Table 3. Recommended variables for imposex or intersex development in gastropods. From JAMP (OSPAR. 2008).

Imposex / Intersex variable	**Dog whelk** (*Nucella lapillus*)	**Common whelk** (*Buccinum undatum*)	**Red whelk** (*Neptunea antiqua*)	**Netted dog whelk** (*Nassarus nitidus*)	**Mud snail** (*Peringia ulvae*)	**Common periwinkle** (*Littorina litttorea*)
Percentage of females with imposex or intersex (%)	x	x	x	x	x	x
Vas deferens sequence index (VDSI)	x (0-6)	x (0-4)	x (0-4)	x (0-4)	x (0-6)	
Penis classification index (PCI)		x				
Relative penis size index (RPSI)	x			x		
Relative penis length index (RPLI)				x		
Intersex index						x (0-4)
Average length of prostate gland in females (FPrL)						x
Portion of sterile females	x		(x)			x
Average number of penial glands in males (PGI)						x

2.3.5 Determination of intersex in common periwinkle

The occurrence of intersex in common periwinkle can be used as an alternative to imposex when monitoring TBT in coastal areas. Intersex is, as imposex, a masculinisation of females, but they differ morphologically. Intersex is expressed as malformations of the female reproductive organs or as a complete conversion of the female reproductive organs to male organs.

For monitoring purposes, the intersex phenomenon is regarded as a less sensitive parameter when considering TBT-levels compared to imposex (Minchin et al 1997; OSPAR 2008; Strand 2013). Intersex should therefore only be used as an effect parameter if species displaying imposex are missing from the area.

2.3.5.1 Time and area

Monitoring areas are located in relation to a coastal point source of TBT, e.g. a harbour, marina or shipyard. At least three areas should be selected, they should be located on the same side of an expected source, e.g. at a distance of 0-0,05, 0.5-0.6, 2.5-10 km in order to map a possible gradient in contamination level between the highest effect level and the local background level. Note that the distances between station and TBT-source is smaller compared to the situation when netted dog whelk is used. Collection should take place in March-June.

2.3.5.2 Age determination

Age in common periwinkle is decided from sexual maturity and shell height. Only adult female common periwinkles (with a visible red ovary) within the size range 15-25 mm are used in the calculation of the parameter values. It is estimated that common periwinkle within this size range are not older than two years (OSPAR 2008). Shell height is measured within 0.1 mm certainty with a digital calliper.

2.3.5.3 Parasite infection

Periwinkles infected with trematodes should be excluded from the material, as such infections can affect the development of the reproduction organs (Lauckner 1980). Periwinkles with eroded shells, e.g. because of the spionid *Polydora ciliata* or barnacles should also be excluded. Especially old periwinkles can have the top of the shell completely eroded.

2.3.5.4 Sex determination

Common periwinkles are dioecious and despite possible intersex characters there are obvious differences between adult females and males (Figure 8). Determination of the sex in subadult individuals can be more difficult.

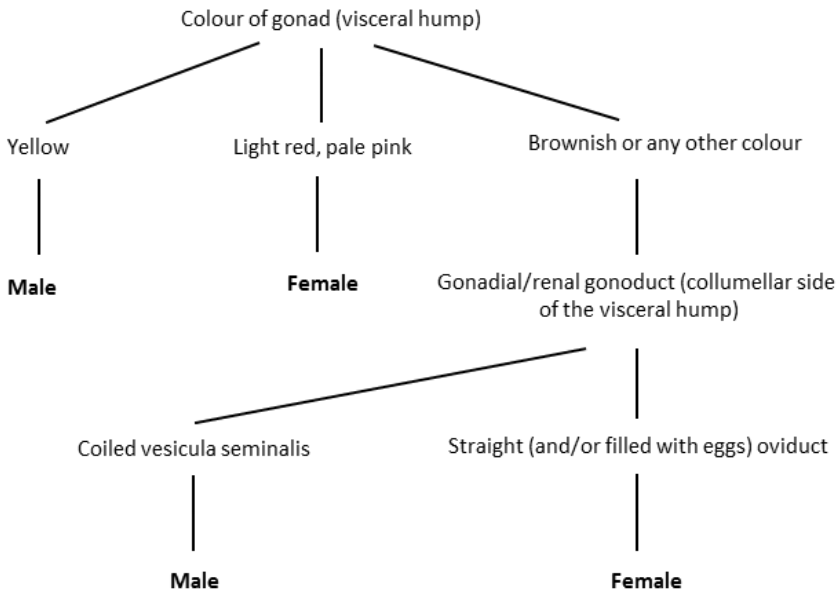

Figure 8. Schematic overview on how to distinguish between the sexes of common periwinkle. From JAMP (OSPAR 2008).

2.3.5.5 Female reproductive organs

The best characteristics are the pink to dark red ovary, placed alongside the digestive gland, and the straight (not curled) oviduct (often full of eggs), that connects the ovary with the pallial oviduct. The pallial oviduct, placed alongside the body cavity, consist of albumin gland, spermatheca, egg capsule gland, jelly gland, bursa copulatrix and the vaginal opening (Figure 9).

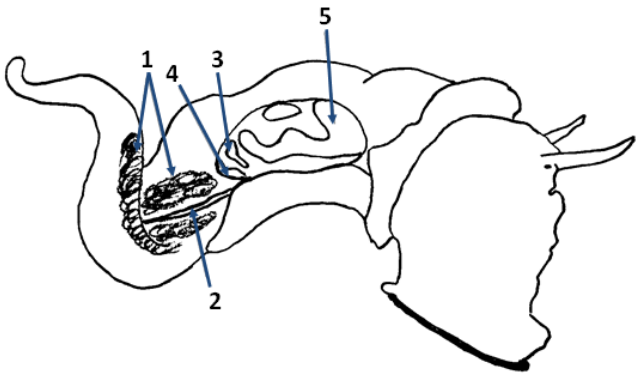

Figure 9. Female reproductive organs. 1) Ovary, 2) Oviduct, 3) Albumin gland, 4) Spermatheca, 5) Egg capsule gland. From Strand 2004.

2.3.5.6 Male reproductive organs

The most obvious characteristics are the yellow testis, placed together with the digestive gland, and the coiled seminal vesicle, that connects testis with the prostate gland. The prostate gland, placed along the body cavity, is cloven with a sperm groove that continues to the penis. The penis is placed close to the right tentacle. Penial glands are situated as buds on the outside of the penis (Figure 10).

Note: males can in some cases shed their penis during summer and autumn (Deutsch and Fioroni 1992).

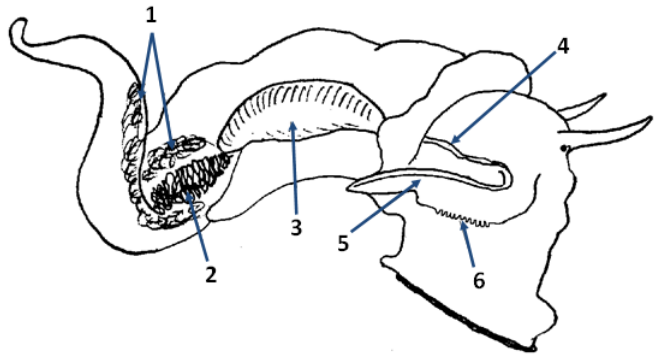

Figure 10. Male reproductive organs. 1) Testis, 2) Seminal vesicle, 3) Prostate gland, 4) Sperm groove, 5) Penis, 6) Penial glands on penis. From Strand 2004.

2.3.5.7 Description and characterisation of intersex stages

The intersex phenomenon in periwinkles is a gradual change of the female's pallial oviduct towards a masculine prostate gland and a development of penis and sperm groove. Development of intersex can either be described by a classification scheme based on the different stages of masculinisation of the pallial oviduct, or from the length of the possibly formed prostate gland (Figure 11). Possible intersex characters are easiest examined by first studying the pallial oviduct from the upper side of the mantle for a possible initial formation of a prostate gland. Thereafter the mantle cavity is cut open on the left side, in order to study any possible divisions of the pallial oviduct from the inside of the mantle. Three different parameter values can be used to describe the intensity of intersex in a group of common periwinkle; frequency (%), intersex index (ISI) and average length of prostate gland in females (FPrL). All three parameter values should be calculated. In addition, penial gland index (PGI) can be calculated for males as supplemental data. The penial glands are situated as buds on the outside of the penis.

2.3.5.8 Intersex index

The intersex phenomenon can be characterised from five intersex stages. The different stages are distinguished by how masculinised the pallial oviduct is. The intersex index (ISI), describing the intensity of intersex in a group of gastropods, can be calculated from the intersex stages. ISI is calculated as the average of intersex stages. ISI values larger than 1 indicates that a part of the females are sterilised due to intersex. Common periwinkles with intersex stages 2-4 are considered sterile (Bauer *et al*. 1995). Differences of 0.5 units between ISI-values are considered significant.

ISI = Σ intersex stage values of all examined females / number of females

Stage 0: Normal female without morphological changes.
Stage 1: The vaginal opening is enlarged with a smaller cut and the bursa copulatrix is split lengthwise.
Stage 2: The entire pallial oviduct is split ventrally, forming an open structure with the internal lobe of the female glands exposed to the mantle cavity.
Stage 3: Initial to fully developed prostate gland. An initial development of the prostate gland is easiest seen on the outside of the mantle as deformities of the pallial oviduct, visible as a white spot on the jelly gland and albumin gland. Alternatively, the pallial oviduct can be split along the whole length. Note that an initial development of a prostate gland can be present, even if the pallial oviduct is only split according to stage 1 and 2. The stage should never the less be characterised as stage 3.
Stage 4: A sperm groove runs from the formed prostate gland to a formed penis above the right tentacle, at the same place as in males. Stage 4 is present only in highly TBT-contaminated areas.

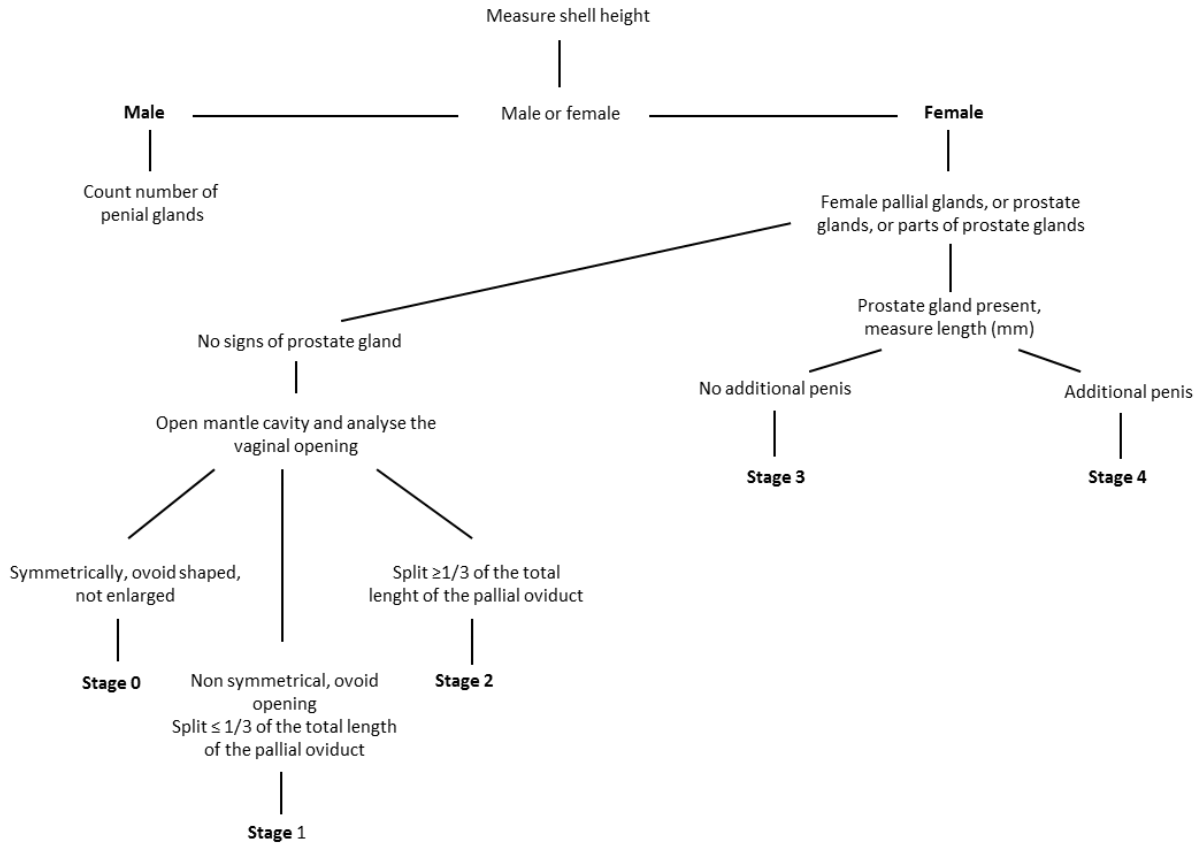

Figure 11. Schematic description of the intersex stages in common periwinkle. From OSPAR 2008.

2.3.5.9 Average length of prostate gland in females

The length of the possibly formed prostate gland is measured with 0.1 mm certainty under the dissecting microscope. The prostate gland is only present in the sterile intersex stages 3 and 4. The parameter value average length of prostate gland in females (FPrL) is calculated as the average length of the prostate gland in all females.

FPrL = Σ length of prostate gland in all examined females / number of females.

FPrL-values larger than 0 indicates that a certain proportion of the females are sterile.

2.3.5.10 Penial gland index

The number of penial glands in males is counted under the dissecting microscope. They become apparent if the penis is squeezed with tweezers, which makes the glands easier to count. In heavily TBT-contaminated areas the males have no or few penial glands, in low-contaminated areas the males have up to 25-40 penial glands (Bauer *et al*. 1997). A penial gland index (PGI), which is the average value of the number of penial glands in all examined male gastropods, can be calculated. Males that have shed their penis or where the penis is less developed should be excluded from the calculation and replaced by new individual.

PGI = Σ number of penial glands in all examined males / number of males.

2.3.6 Chemical analysis

Analysis of imposex is a relatively easy method for biological effect monitoring of organotin compounds. However, as effects seen in the gastropods are irreversible it is important to confirm by chemical analysis if the effects are caused by an ongoing exposure or are the result of an earlier exposure. The chemical analysis is of greater importance if the species lives for several years. Besides the biological variables, a number of chemical compounds are measured in the tissue: tributyltin (TBT), triphenyltin (TPhT) and their degradation products dibutyltin (DBT), monobutyltin (MBT) diphenyltin (DPhT) monophenyltin (MPhT).

With these variables the relation between organotins (TBT and TPhT) and their degradation products can be calculated.

After analysis of imposex, a collective sample of all the analysed females from each station is prepared. The sample is frozen until analysis. Chemical analysis of mud snail is not performed every year. Partly because the mud snail only lives for about two years (Schulte-Oehlmann *et al*. 1997), so imposex in this species is a result of an exposure within the last two years and partly because of its small size, approximately a thousand individuals are needed for the analysis.

Different analytic methods are used at different laboratories and the result can differ significantly. To ensure that the results are comparable it is important to use the same methods and laboratory over time. Low detection values and identical extraction and analysis methods are to be considered when changing laboratories.

There are several methods for determining organotins and specifying one or more is not appropriate. However, guidance on performance characteristics can be given as follows:

a) the method should be designed to measure TBT (rather than, for example, total tin or total organotin)
b) the concentration range of interest is around 0.005-1.6 mg/kg dry weight or 0.002-0.6 mg/kg wet weight
c) the detection limit should be 0.002 mg/kg dry weight (0.001mg/kg wet weight) or better
d) certified reference materials should be used as part of the quality assurance.

3. Data

3.1. Station description

The following observations are noted for each station: Date, position, depth, distance from closest major TBT-source (harbour or shipping lane), capture method, number of analysed gastropods (number of males / number of females).
When collecting gastropods by beam trawl, positions for start and stop of the haul and depth should be noted, as well as the width of the trawl and the mesh size.
Stations and possible TBT-sources within a distance of five km are noted.

3.2 Description of individual gastropods

For each examined gastropod the following biological support parameters are reported for each examined male and female gastropod:

- Species
- Shell height
- Sex
- Sexual maturity
- Age (for common whelk and red whelk)
- Presence of trematodes

In addition, the following individual data for imposex in gastropods are reported:

- Penis length in males and females
- Imposex stage (VDS) from 0-6 in females
- Imposex stage (PCI) from 0-3.5 in females (only relevant for common whelk)

In addition, the following individual data for intersex are reported for common periwinkle:

- Intersex stage (IS) from 0-4 in females
- Length of prostate gland in females
  Number of penial glands in males

At the station level, the following parameters, expressing the development stage of imposex or intersex in the examined populations are reported:

- Percent of females with imposex (%)

- Vas Deferens Sequence Index (VDSI) = Σ vds/number of females
- Penis Classification Index (PCI) = Σ pc/number of females
- Relative Penis Length Index (RPLI) = (FPL / MPL)*100
- Relative Penis Size Index (RPSI) = (FPL^3 / MPL^3)*100 (only relevant for dogwhelk)
- Proportion of sterile females (i.e. vds-stage >4 and females with coiled oviduct in gastropods or is>1 in common periwinkle)
- Intersex Index (IS) = Σ is / number of females common periwinkle
- Average length of prostate gland in female common periwinkle (FPrL)
- Average number of penial glands in males (PGI) in common periwinkle

3.3 Calculation of parameter values

The following parameter values describing the intensity of imposex in a group of gastropods is calculated: Frequency (%), penis length in females (FPL), relative penis length index (RPLI), vas deferens sequence index (VDSI), and penis classification index (PCI).

- Frequency: = 100%*n_I/n_F Σ
- FPL = Σ pl_I/n_F
- RPLI = 100%* Σ pl_I/n_F) / (Σ pl_M/n_M)
- VDSI = Σ vds/ n_F
- PCI = Σ pci/ n_F

The following parameter values describing the intensity of intersex in a group of female common periwinkles is calculated: Frequency (%), intersex index (ISI) and average length of prostate gland in females (FPrL). The penial gland index (PGI) is calculated for males.

- Frequency: = 100%*n_I/n_F Σ
- ISI = Σ ISI/n_F
- FPrL = Σ prl_I/n_F
- PGI = Σ pgi/ n_M

Where:
n_F is the total number of females
n_I is the number of females with imposex/intersex
n_M is the number of males
pl_I is the penis length in females with imposex
pl_M is the penis length in males
vds is the imposex stage from the vas deferens sequence
pci is the imposex stage from the penis classification index
prl_I is the length of the prostate gland in females with intersex (only relevant for common periwinkle)
pgi is the number of penial glands in individual males (only relevant for common periwinkle)

If the data material is of a size where parameter values can be calculated for sub-adult gastropods, this is recommended.

3.4 Data reporting and storage

For each station, the VDSI ±confidence interval, and the proportion of effected females are reported. In addition, the RPLI is calculated for each station, however, this index should be used carefully as penis length can vary over the year. Data is reported in table for each station and sampling occasion, or as diagram for all stations and years to show the temporal development.

4. Quality control

4.1 Quality control of methods

Sample containers should be clearly labelled to avoid mixing up samples. It is important that the traps/trawl/nets are emptied completely before being used at the next station. It can be difficult to remove all the snails in the traps as they can crawl into the bait, a practical measure is to keep the traps frozen between samplings.

All determinations should be carried out according to defined protocols by trained staff. Any deviations from the protocols should be recorded. Yearly experience of imposex analysis is of great importance when it comes to secure the result and to obtain a qualitative evaluation. Analysts should participate in workshops and calibration meetings when possible. Repeated measurement of penis length or prostate length in preserved specimens could be used to control biological measurements. This will control for differences in interpretation between analysts and for gross errors in microscope calibration.

4.2 Quality control of chemical analysis

The laboratory performing the chemical analysis should be accredited alternatively participate in inter calibration exercises to ensure a high quality of the analysis. Different methods are used at different laboratories and results can vary a lot. For comparable results from year to year it is important to, if possible, use the same methods and laboratory. When changing laboratory, the use of the same methods should be prioritised. TBT compounds can be purchased for the preparation of standard solutions. Reagents for the chemical analysis should be of appropriate quality to meet the needs of the determination. All determinations should be carried out by trained staff working to defined protocols. Any deviations from the protocols should be recorded. Normal care should be taken during chemical analysis to minimise contamination or loss of analyses and interference from other substances and to ensure accurate calibration of instruments.

4.3 Quality control of data and reporting

Currently, the quality of the data collected within the different national monitoring programs is assured on a national level. Each contracting party has its own quality assurance system within which all data used for common assessments of toxicological status has been considered.

Data is stored in national databases and also reported to ICES from where extractions are made for common assessments.

5. Contacts and references

5.1 Contact persons

Sweden:

Marina Magnusson
Marine Monitoring AB
Strandvägen 9
453 30 Lysekil
Sweden
Phone: +46702565551
E-mail: marina@marine-monitoring.se

Denmark:

Jakob Strand
Department of Bioscience - Marine Diversity and Experimental Ecology

Frederiksborgvej 399
4000 Roskilde
Denmark

Phone: +4587158654
E-mail: jak@bios.au.dk

5.2 References

Barroso C. M., Moreira M. H. and Bebianno M. J. 2002. Imposex, female sterility and organotin contamination of the prosobranch *Nassarius reticulatus* from the Portuguese coast. Marine Ecology Progress Series 230: 127–135.

Bauer B., Fioroni P., Schulte-Oehlman U., Oehlmann J. and Kalbfus W. 1997: The use of *Littorea littorina* for tributyltin (TBT) effect monitoring - Results from the German TBT survey 1994/1995 and laboratory experiments. Environmental Pollution 96(3): 299-309.

Bauer B., Fiorini P., Ide I., Liebe S., Oehlmann J., Stroben E. and Watermann B., 1995. TBT effects on the female genital system of *Littorina littorea,* possible indicator of tributyltin pollution. Hydrobiologia 309, 15-27.

Bryan G. W., Gibbs P. E. Burt G. R. and Hummerstone L. G. 1987: The Effects of Tributyltin (TBT) Accumulation on adult dog-whelks, *Nucella lapillus*: Long-term field and laboratory experiments. Journal of the Marine Biological Association of the United Kingdom 6:525-544.

Deutsch U. and Fioroni P.1992. The shedding of the penis in *Littorina littorea*: Some new aspects. Proceedings of the third International Symposium on Littorinid Biology, p. 309-311.

Fioroni P., Oehlmann J. and Stroben E. 1991: The pseudohermaphroditsm of prosobranchs; morphological aspects. Zoologischer Anzeiger 226(1), 1-26.

Gercken J. and Sordyl H. 2007. Biological effect monitoring in coastal waters off Mecklenburg-Western-Pomerania. Pilot study 1998-2003. Report on behalf of State Office of Environment, Nature Conservation and Geology Mecklenburg-Western Pomerania, 135 pp. (In German).

Gibbs P.E., Pascoe P.L. and Bryan G.W., 1991. Tributyltin-induced imposex in stenoglossan gastropods: pathological effects on the female reproductive system. Comparative Biochemistry and Physiology. 100C:23 1-235.

Gibbs P. E., Pascoe P. L. and Burt G. R., 1988. Sex change in the female dog-whelk, *Nucella lapillus*, induced by tributyltin from antifouling paints. Journal of the Marine Biological Association, UK Vol. 68, pp. 715–731.

Hansen J.W. (red.) 2012: Marine områder 2011. NOVANA. Aarhus Universitet, DCE – Nationalt Center for Miljø og Energi, 154 s. - Videnskabelig rapport fra DCE - Nationalt Center for Miljø og Energi nr. 34. http://www.dmu.dk/Pub/SR34.pdf (In Danish).

JAMP 1997: TBT-specific biological effect monitoring. – Joint Assessment Monitoring Programme, Technical annex 3. Revised 2003.

Kideys A. E. 1996. Determination of age and growth of *Buccinum undatum* L. (Gastropoda) off Douglas, Isle of Man. Helgoländer Meeresuntersuchungen 50: 353-368.

Køie M. 1969. On the endoparasites of *Buccinum undatum* L. with special reference to the trematodes. Ophelia 6: 251-279.

Lauckner G. 1980: Diseases of Mollusca. Gastropoda. In Kinne 0. (red.). Diseases of marine animals, Vol. I. John Wiley & Sons, NY, USA, pp. 311-424.

Magnusson M. 2015. Undersökningstyp: Biologisk effektövervakning av organiska tennföreningar. Version 1.1. 2015-04-23. Naturvårdsverket. (In Swedish)

Magnusson, M., Andersson, S., Bergkvist, J., & Granmo, Å., 2016: Biologiska effekter av organiska tennföreningar. Havet 2015 s. XX-XX. Havs och vattenmyndigheten. In press.

Magnusson, M., Cato, I. & Granmo, Å., 2012: Sekundär spridning och effekter av organiska tennföreningar från småbåtshamnar. Marine Monitoring AB, Rapport 2012:1, 26 sid. Lysekil. ISBN 978-91-86461-29-4

Magnusson, M. & Hilvarsson, A., 2011. "Effekter av TBT i Västra Götalands län 2011-en studie av förekomst av imosex", på uppdrag av Västra Götalands länsstyrelse ISBN 978-91-86461-25-6

Magnusson, M., Hilvarsson, A., Granmo, Å., 2011. "Förekomst av TBT i sediment från småbåtshamnar och dess effekt på nätsnäckor" R2012:3, Göteborgs stad, miljöförvaltningen, ISBN 1401-2448

Mensink B. P., van Hattum B., ten Hallers-Tjabbes C. C., Everaarts J. M., Kralt H., Vethaak A. D. and Boon J. P. 1997: Tributyltin causes imposex in the common whelk, *Buccinum undatum*. Mechanism and occurrence. Nederlands Instituut voor Onderzoek der Zee, Rapport 6, Den Burg, Texel, the Netherlands.

Minchin D., Bauer B., Oehlmann J., Schulte-Oehlmann U. and Duggan C. B. 1997. Biological indicators used to map organotin contamination from a fishing port, Killybergs, Ireland. Marine Pollution Bulletin 34(4): 235-243.

Oehlmann J., Stroben E., and Fioroni P. 1991. The morphological expression of imposex in *Nucella lapillus* (Linnaeus) (Gastropoda: Muricidae). Journal of Molluscan Studies, 57: 375-390.

OSPAR 2008. JAMP Guidelines for Contaminant-Specific Biological Effects. Technical Annex 3: TBT-specific biological effects monitoring. OSPAR Agreement 2008-09.

Power A.J. and Keegan B. F.(2001). The significance of imposex levels and TBT contamination in the red whelk, *Neptunea antiqua* (L.) from the offshore Irish Sea. Marine Pollution Bulletin 42(9): 761-772.

Schulte-Oehlmann U., Oehlmann J., Fioroni P., and Bauer B. 1997. Imposex and reproductive failure in *Hydrobia ulvae* (Gastropoda: Prosobranchia). Marine Biology (1997) 128: 257-266.

Strand J. and Jacobsen J. A. 2002. Imposex in two sublittoral neogastropods from the Kattegat and Skagerrak: the common whelk *Buccinum undatum* and the red whelk *Neptunea antiqua*. Marine Ecology Progress Series, 244: 171-177.

Strand J. 2004. Biologisk effektmonitering - imposex og intersex i havsnegle. In Andersen, Markager and Ærtebjerg (red). Tekniske anvisninger for marin overvågning (In Danish).

Strand J., Larsen M. M., Næs K., Cato I. and Dahllöf I. 2006. Tributyltin (TBT). Forekomst og effekter i Skagerrak. Uddevalla, Sweden: Rapport fra Forum Skagerrak II. 39 s. (In Danish). http://projektwebbar.lansstyrelsen.se/havmoterland/SiteCollectionDocuments/Publikationer/forum-skagerrak/tbt.pdf

Strand J. 2013. Imposex og intersex i havsnegle. Teknisk anvisning M27 til marin overvågning fra Det Marine Fagdatacenter, DCE Aarhus Universitet, 30pp (In Danish).

http://bios.au.dk/videnudveksling/til-myndigheder-og-saerligt-interesserede/fagdatacentre/fdcmarintny/gaeldendetekniskeanvisninger/#c236812

Stroben E., Oehlmann J. and Fiorini P. 1992a. The morphological expression of imposex in *Hinia reticulata* (Gastropoda: Buccinidae): a potential indicator of tributyltin pollution. Marine Biology 113: 625-636.

Stroben E., Oehlmann, J. and Fioroni, P. 1992b. *Hinia reticulata* and *Nucella lapillus*. Comparison of two gastropod tributyltin bioindicators. Marine Biology. 114: 289-296.

Stroben E. 1994. Imposex und weitere effekte von chronischer TBT intoxikation bei einigen mesogastropoden und bucciniden (Gastropoda, Prosobranchia). Cuvillier Verlag, Göttingen, Tyskland. (In German).

Stroben E., Schulte-Oehlman U., Fioroni P. and Oehlmann J. 1995: A comparative method for easy assessment of coastal TBT pollution by the degree of imposex in Prosobranch species. Haliotis 24:1-12.

Ten Hallers-Tjabbes C.C., Kemp J.F. and Boon J.P. 1994. Imposex in whelks *(Buccinum undatum)* from the open North Sea: Relation to shipping traffic intensities. Marine Pollution Bulletin. 28:311-313.