TBT-induced imposex related to age and length in *Buccinum undatum* at two localities in Balsfjorden.

by

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ABSTRACT

Background: The organotin compound tributyltin (TBT), used as an antifouling agent in ship hull paint, is known to cause imposex in several species of marine gastropods. Imposex is the imposition of male sexual characteristics on females, and can lead to female sterility. The common whelk, *Buccinum undatum*, is a common species in Norway and has received attention for its export potential. Because *B. undatum* varies geographically in reproduction and morphology it is important to study the species before exploiting the populations. No work has been done to assess imposex in the common whelk in Norway, and little is known about the species in Norway altogether.

Methods: *Buccinum undatum* was caught at Berg and Balsnes in Balsfjord, Troms county, Northern Norway, for comparative studies of imposex and morphology. Shell length and male penis length were measured, and females were checked for imposex. Age was read by counting striae on the backside of the operculum. Sediment samples were taken at each location for analysis of TBT contents, and snails with and without imposex were also analyzed for TBT.

Results: There was a significant difference in shell length between Berg and Balsnes. The whelks at Berg seemed to be longer than at Balsnes. There were no differences in incidence of imposex between the localities, nor between length groups within each locality. There seemed to be an increase in percentage of imposex with increasing age at Balsnes. No female whelks had imposex severe enough to cause sterility. TBT consentration was below detection limit in the sediments at both locations, while it was detected at low concentrations in females both with and without imposex.

Conclusions: *Buccinum undatum* display morphological differences between close localities. TBT contamination at Berg and Balsnes is low and has low impacts on the whelk populations with respect to imposex. Imposex has no impact on reproductive abilities of the common whelk at Berg and Balsnes.

Key words: *Buccinum undatum*, tributyltin (TBT), imposex, morphology.

1 INTRODUCTION

The common whelk (*Buccinum undatum* L.) has long been an important commercial species. In northern Europe, it has mostly been used as bait or food for other commercial species, but in southern Europe and in East Asia it is considered a delicacy. In Norway, *B. undatum* has lately received much attention for its export potential to East Asia. It is a common species along the Norwegian coast, and the price was around 7 NOK per kilo in 2008 (Tviberg 2008). Several surveys have been conducted with the intention of mapping the exploitable population. However, few studies have focused on the biology of the species (Juliussen 2007) and (to the knowledge of the author) nobody has studied the species response to pollution in Norway. If *B. undatum* is to be exploited as a food source, knowledge of the species biology and management of the population is important to avoid overexploitation and other threats to the species.

1.1 Imposex and TBT

Organotin compounds have long been known to be very toxic, and to cause disturbances in the sexual development of several benthic species, especially molluscs. The organotin compound tributyltin (TBT) is known to negatively affect more than 100 species of snail in several countries (Walday *et al.* 1997). Female whelks react to the compound by developing a state called imposex.

The term imposex was first used by Smith (1971) to describe the imposition of male sexual characteristics in female snails, that is the female snail develops a penis and a vas deferens (sperm duct). The phenomenon was first observed in the dog whelk *Nucella lappilus* (L.) (Blaber 1970) and it is irreversible when first developed (Mensink 1999). In serious cases imposex can cause sterility in the female snail as the developing vas deferens might block the pallial oviduct and hinder the female snail from being inseminated and lay eggs, and therefore threaten local populations (Gibbs and Bryan 1986).

Imposex has been linked to TBT-exposure (Smith 1981). Experiments show that TBT works as an antiestrogen (Petrović *et al.* 2001), disrupting a cytochrome P-450 dependent aromatase that is involved in the break down of androgens. This leads to increased levels of testosterone in the female snail which leads to imposex development (Bettin *et al*. 1996). The contens of cytochrome P-450, which is also involved in the metabolism of TBT to less toxic compounds, is low in molluscs. This limits their ability to break down TBT, and therefore probably makes them sensitive to TBT-exposure (Lee 1991).

In Arcachon Bay in France TBT was discovered to be the reason for low oyster catches in the 1980s. Oyster larvae show nearly 100% mortality rates when exposed to TBT concentrations higher than 0.1 μg/l and adult individuals get shell thickening which renders them unsalable (Alzieu 1991).

TBT have been used as an antifouling agent in paint for ship hulls since 1960 (Clark *et al.* 1988), and it leaks slowly into the marine systems. TBT is hydrophobic and binds to particles when released into the water. It therefore sinks down to the bottom and is buried in the sediment. The degradation of TBT in water and sediment varies greatly dependent on the physical conditions in which it is released, biological life in the area and the TBT concentration. Lee (1989) reported a half life of 2 days in fine-grained sediments, and 13 days in sandy sediment. In anaerobic conditions TBT can persist in the sediment for more than 2 years (internet reference 1). TBT contamination can have biological effects at concentrations close to instrumental detection limit (Evans and Nicholson 2000). In some areas 100% of female *N. lappilus* was found with imposex because of TBT and other imposex inducing contaminants.

TBT use in boats less than 25 m long was banned in France in 1982 and several other countries followed (Evans *et al.* 1995). In Norway these regulations have been in use since 1989. In 2003 new use of TBT became illegal, and boat hulls had to be repainted with TBT-free paint within 2008 (internet reference 2).

Imposex in *N. lapillus* has been monitored in Norway since 1984, and it is the only species examined for imposex in Norway. Since the ban of new use of TBT, levels of imposex have shown a downward trend (Green *et al.* 2008).

1.2 Biology of *Buccinum undatum*

Buccinum undatum, is a boreal species common in the North Atlantic Ocean (Dakin 1912; ten Hallers-Tjabbes *et al.* 1996). It is found in the sublitoral zone down to 600 m (Pain 1979), and is benthic dwelling all its life with no planktonic stages (Himmelman and Hamel 1993). It is a predatory snail, but feeds on carcasses whenever possible (Dakin 1912). The common whelk itself is preyed upon by several species of fish, crabs and echinoderms (Thomas and Himmelman 1987).

Morphologically *B. undatum* varies greatly in different areas. Some populations are so different from each other that the whelks' morphology can be identified to the specific locality they come from within larger areas (Juliussen 2007). *Buccinum undatum* can reach a size up to 150 mm and live up to 13 years (Gunnarsson and Einarsson 1995). Size for sexual maturity is found to be 50-70 mm in males and 70-80 mm in females, but this may vary between localities (Martel *et al.* 1986). Reproduction is annual and time of the year for reproduction varies geographically. In Skagerrak eggs are laid in October until December (Valentinsson 2002), while in the Gulf of St. Lawrence in Canada the eggs are laid in June-August (Himmelman and Hamel 1993).

Imposex in *B. undatum* was first reported from the North Sea in 1991 in areas with high shipping traffic (Mensink 1999). In 1995 as much as 96% of the population on the Eastern Scheldt had imposex (Mensink *et al.* 1997).

Mensink (1999) exposed *B. undatum* to TBT *in ovo,* in juvenile, adolescent and adult stages. He reported that TBT did not affect individuals *in ovo,* and adult females only accumulated TBT and metabolites, and did not develop imposex. Juveniles and adolescent individuals did not respond to a TBT concentration of 10 ng/l, but when increasing the dosage to 100 ng/l all whelks showed development of male sexual characteristics. Some of the juveniles exposed to 1 μg/l even developed double penises. All of the individuals hatched in this dosage died. All juveniles exposed to TBT showed significantly reduced growth compared to the control group. Imposex did not affect reproduction.

1.3 Purpose of the study

The following work examines the occurrence of TBT-related imposex in the common whelk at two locations, Berg and Balsnes, in Balsfjorden, northern Norway. Imposex is also related to length and age of the whelks.

2 MATERIALS AND METHODS

2.1 Field

On September 14th-15th 2006, using the research vessel, "F/F Hyas", of the University of Tromsø, traps were set at four locations around Tromsø: Berg, Balsnes, Eidkjosen and Skattøra (figure 2.1 a).

Eidkjosen is a small harbour where currents are slow and sedimentation is high. The sediments are severely polluted with TBT (Olsson 2002). Skattøra is an industrial area and presumably the waters and sediment outside it are highly polluted.

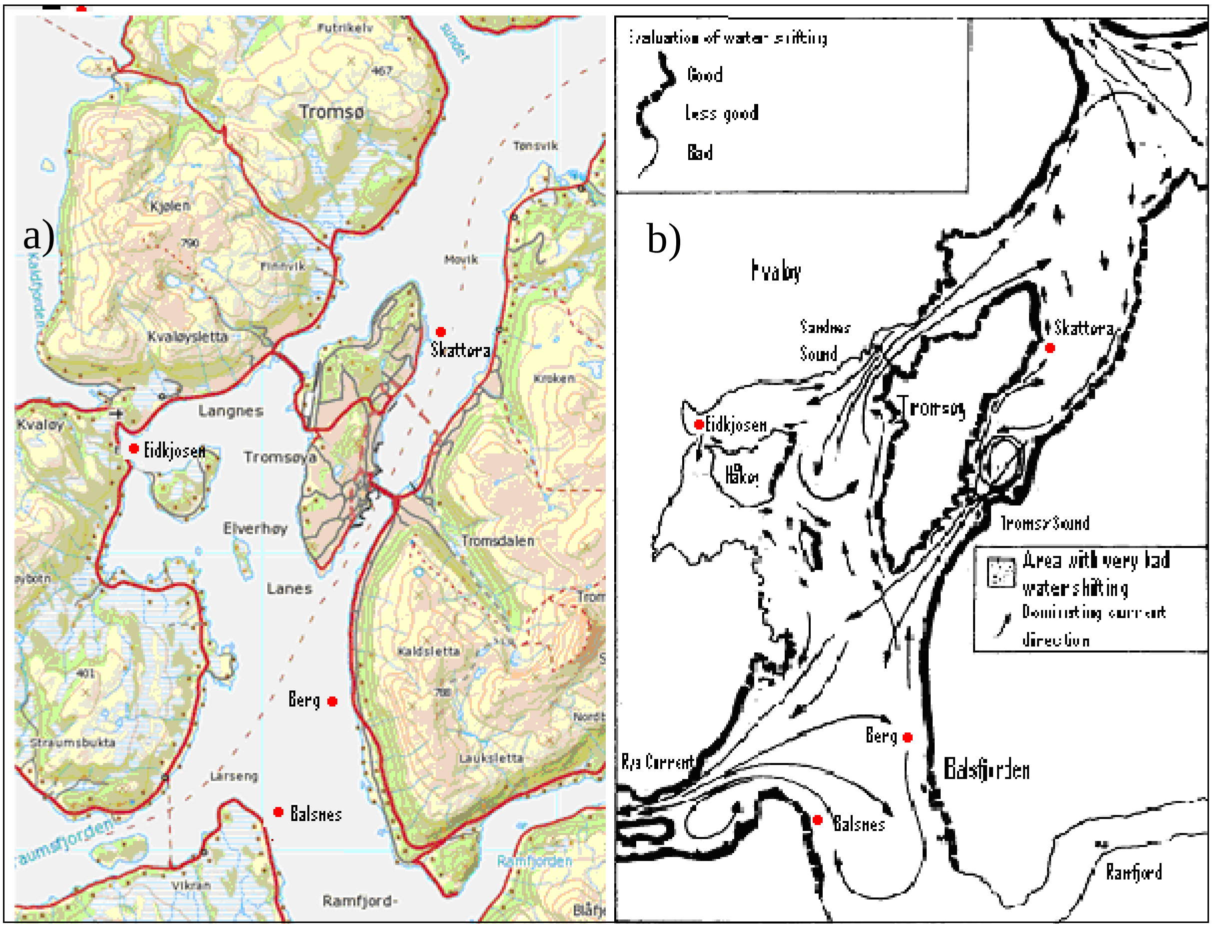

Figure 2.1 a) The sampling areas (modified from Fiskeridirektoratet. no). b) The current system in the Tromsø area (Oug *et al*. 1985).

Berg (69° 35.05' N; 018° 55.9' E) and Balsnes (69° 33.32' N; 018° 51.34' E) were chosen as presumably low polluted areas, characterized by strong currents and good water exchange (figure 2.1 b). Balsnes is opposite Berg, 4.3 km across the fjord that has a maximum depth of about 80 m (figure 2.2). The bottom at Berg mainly consists of gravel and small rocks. Balsnes lies in a back eddy of the main stream and the bottom is characterized by shell sand and gravels.

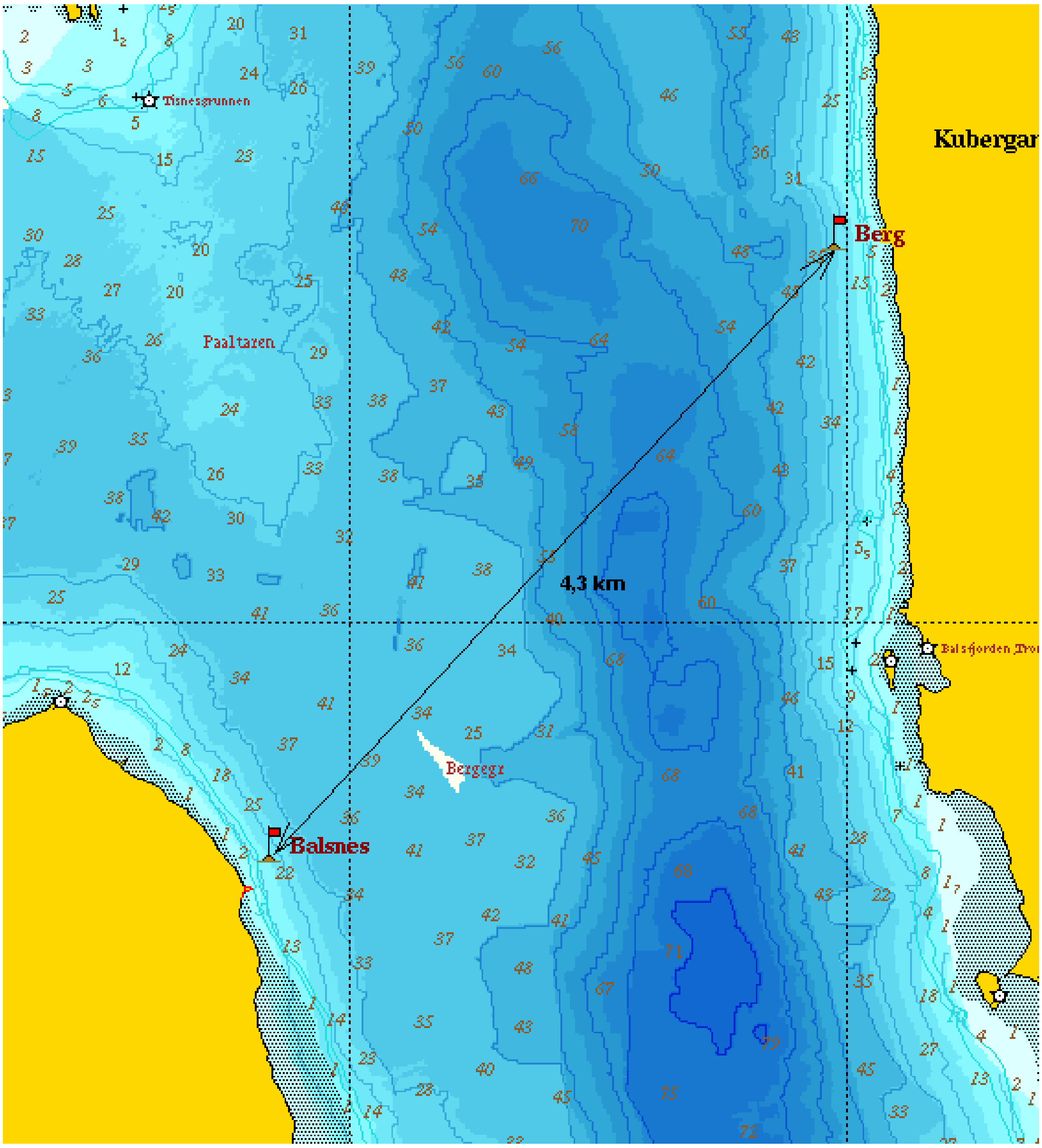

Figure 2.2 Depth profile and distance between the sampling areas (map from OLEX).

Welsh snail pots were used for catching *B. undatum* (figure 2.3 a). They were cylinder shaped, made in black plastic with a net stretched over the top. The diameters of the traps were 33 cm at the top and 40 cm at the bottom. There were drilled holes in the bottom to let water seep through when setting the traps. These holes were 25 mm in diameter and small whelks could escape through them, making the Welsh pot selective for larger snails.

Figure 2.3 a) Welsh snail pot. b) Snail pot used for catching small snails.

Each pot was baited with large pieces of frozen herring and set at 30 m depth with 10 m between them for 24 hours before hauling. When hauled, the snails in each trap were carefully taken out, to avoid breaking off parts of the snail shell. The number of snails in each trap was noted (table 6.1). The snails were put into labelled buckets filled with sea water to keep the snails alive during transport.

10 traps were set at Berg and 10 at Balsnes. At Berg 1027 individuals of *B. undatum* were caught at Berg together with 7 individuals of *Neptunea antiqua*. At Balsnes 577 individuals of *B. undatum* were caught at Balsnes together with 5 *N. antiqua*.

5 traps were set at Eidkjosen and 5 at Skattøra. At Eidkjosen only 5 *B. undatum* were caught. At Skattøra all the bait in the traps had been eaten, possibly by hagfish (*Myxine glutinosa*), and only 6 *B. undatum* and 2 *N. antiqua* were caught here. Because of the low catches these two areas were excluded in the material and it was decided to run analysis only on the catches from Berg and Balsnes.

On the 30th of May 2008 new samples were taken at Berg and Balsnes. *Buccinum undatum* was caught for analysis of TBT content. Sediment samples from the upper 3 cm were taken with a van Veen grab, for analysis of total organic carbon (TOC), grain size and TBT content.

The sediment samples had to be taken carefully to avoid stirring the sediments too much. The water above the sediment in the grab had to be clear for the sample to be accepted for analysis.

In an attempt to catch smaller whelks than those caught in 2006, five special pots were set in between the five Welsh snail pots. They were cone shaped, made in white plastic with 1 cm holes in the bottom, and a top and bottom diameter of 24 cm and 50 cm (figure 2.1 b). Unfortunately these pots were completely ineffective, and no snails were caught with them. The Welsh pots caught 111 snails at Berg and 124 snails at Balsnes.

2.2 Laboratory work

During sample processing, the whelks were kept in separate tanks for each sample location. Each tank had constant supply of unfiltered sea water from 22 m depth, 250 m off the shore of Tromsø. The snails were not fed whilst in the tank.

In 2006 an unknown number of snails from both localities were cannibalized by the other snails while in captivity. These snails were not studied further. Of the catch at Berg snails were randomly taken out to be used in a different study, not to be given back, and snails in length 4.3-5 cm were sorted out to be used alive in an educational experiment. These last snails died in the experiment and were given back frozen. The freezing partly destroyed the inner soft parts of the snail, making them difficult to extract whole from the shell. These snails were not processed further in this study. Thus, comparing the length distribution of snails shorter than 5 cm within the two areas was not possible, as the results would be skewed. As the snails were taken out with only length as sorting parameter, this would not have any effect on the sex distribution within the area, although the age distribution could be slightly affected.

To find the right method for sedation 5 snails were tried sedated first with freshwater, which took more than 24 hours, and then magnesium chloride which took up to 10 hours, so these sedation methods were abandoned. Eventually, 96% ethanol was used which killed the snails within minutes, also slightly hardening their soft parts making them easy to get whole out of the shell. The 96% ethanol was reused for several buckets of snails.

After death each snail was numbered and the shell length measured with a slide calliper from the apex to the end of the columella (figure 2.4). The calliper connected to a computer so the length of the snail was saved automatically into an Excel data sheet.

Figure 2.4 Length measure of *Buccinum undatum* from the apex to the end of the columella.

After measuring, the snail shell was carefully broken with a hammer so the soft parts of the snail could be taken intact out of the shell. The operculum was carefully separated with a scalpel, and put in a tagged plastic bag for later age determination.

For sex determination it was normally enough to look for a penis behind the right tentacle of the snail (figure 2.5 and 2.6). But penis size changes throughout the reproductive cycle, and especially for small individuals it can be difficult to distinguish males from females with imposex. Using a microscope one can see that the vas deferens (the sperm duct) in male whelks has windings in the seminal vesicle over the digestive gland before it continues to the testis, whereas the seminal vesicle in females is a basically straight line. This sign was used for exact sex determination. Because of treatment with 96% ethanol the muscles and penis of the whelks contracted a little, making it difficult to measure the exact length of the penis. Penis length in males was therefore measured with a ruler down to closest millimetre after stretching the penis a little. Females were examined for imposex.

There are three commonly used measures for imposex intensity: The relative penis length, the Mensink sequence and the vas deferens sequence (all described in Strand 2003).

The relative penis length is based on the size of a penis homologue in females. It was difficult to apply for this study because few female whelks had a penis homologue long enough to measure. If a penis homologue was appearing as a small bud it was set to 1 mm. If it was possible to lift the penis slightly with a pincer it was measured down to closes half millimetre. The Mensink sequence is based on the development of the penis homolouge (figure 6.1), and was applied. However, some females were found with a vas deferens but no penis homolouge, so the Mensink sequence should be interpreted with care.

For this study the vas deferens sequence (VDS) and the vas deferens sequence index (VDSI) was chosen (Strand 2003) (figure 2.7) as the main measure for imposex. This is the most robust measure because it takes both the developing vas deferens and the penis homologue into account (Walday *et al.* 1997). Only the number of the VDS is used when calculating VDSI. It is calculated as the sum of VDS divided by the total number of females (n_F):

$$\mathbf{VDSI = \Sigma\ VDS / n_{F}.}$$

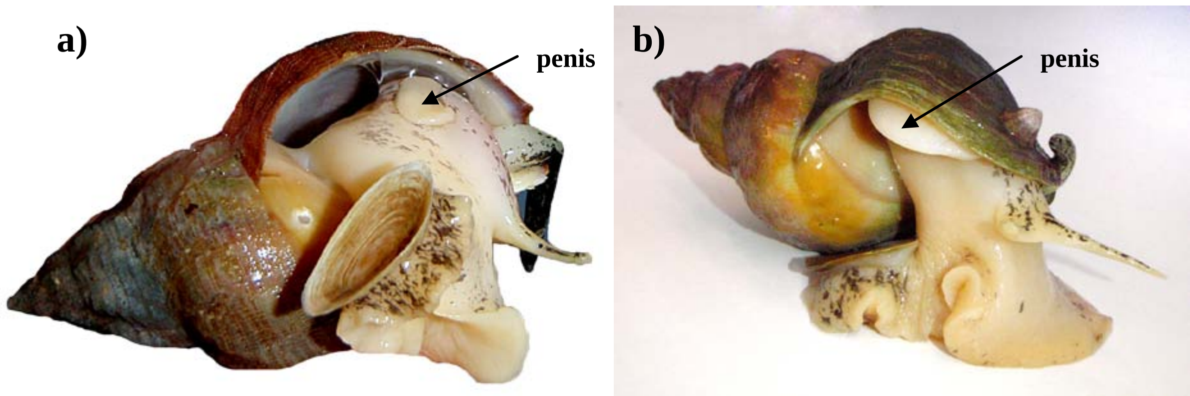

Figure 2.5 Males of *Buccinum undatum*. a) A non-reproductive male about 50 mm in length with a small penis (arrow) seen underneath the upper part of the shell. b) A reproductive male about 70 mm in length with part of its 50 mm long penis (arrow) protruding from underneath the shell.

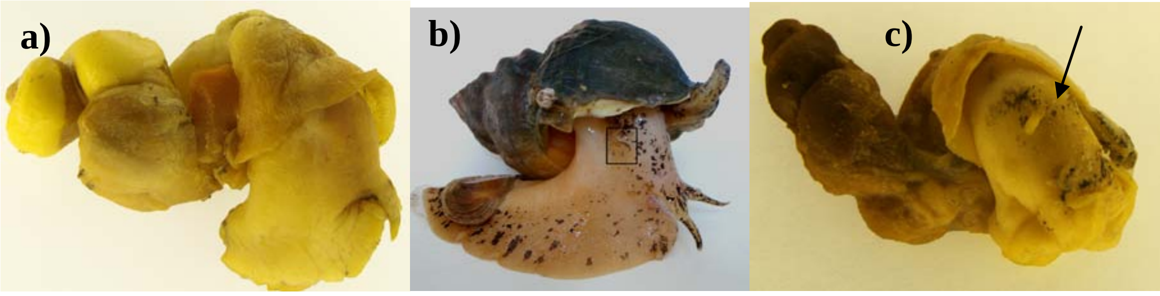

Figure 2.6 Females of *Buccinum undatum*. a) A healthy female with its shell removed. b) A female with vas deferens sequence stadium 1b seen in the rectangle. c) A female with imposex in vas deferens sequence stadium 3a, and its shell removed. The arrow points to the 2 mm long penis homologue.

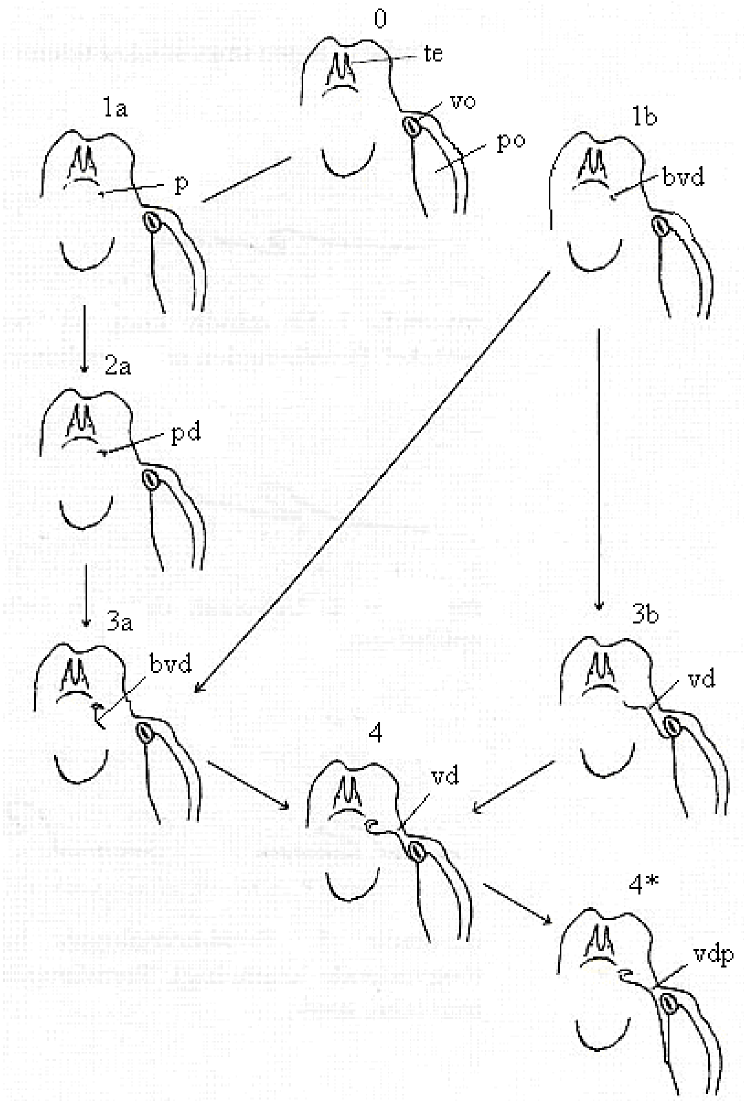

Figure 2.7 The vas deferens sequence as used for *Buccinum undatum*. 0 = no imposex, 1, 2, 3and 4 is increasing vas deferens (sperm duct). The letter **a** indicates the presence of a penis homologue and b indicates no penis homologue. Abbreviations: **te** = tentacle, **vo** = vaginal opening, **po** = pallial oviduct, **bvd** = beginning vas deferens, **p** = penis without penis duct, **pd** = penis with penis duct, **vd** = vas deferens, **vdp** = vas deferens running past the vaginal opening and in under the pallial oviduct (Adapted from Strand 2004).

2.3 TBT analysis

Because of limited economy only two female snails from each location were sampled for TBT content analysis, one with imposex and one without. The snails were taken out of the tank and exposed to air for a few minutes so the snail would crawl out of the shell. This made it possible to identify the males from the females because of the quite large male penis protruding behind the right tentacle. Individuals thought to be imposexed females had a very small visible penis, barely 2 mm in length. The whelks not used for TBT analysis were set free.

The whelks taken out were put in a freezer for half an hour to sedate the snails as much as possible. They were measured and examined for imposex as described above, packed in marked plastic bags and frozen. The snails were packed in ice packaging to keep them frozen along with the sediment samples, before it was sent to analysis. Analysis was taken care of by Unilab Analyse AS.

TBT content in whelks were found using method DIN 19744. The samples were prepared with tetramethylammonium hydroxide (TMAH). TBT was extracted with heksane, and derivated. Quantification limits for TBT was 0.3-0.4 μg/kg wet weight.

Content of TBT and metabolites in the sediment samples were found using method DIN 19744. TBT was extracted with methanol or heksane, and cleaned with alumina. Derivation was carried out using natriumtetraethyl borat ($NaBEt_4$). Detection limit for TBT and metabolites were 1 μg/kg dry sediment.

Grain size in the sediments was found using method K01-540/1-6101. Total organic carbon was found using method ISO 10694, EN 13137. Detection and quantification was carried out by coulometric analysis, and quantification limits were 0.01% dry sediment.

2.4 Age determination

Each operculum was carefully cleansed for all remnants of muscle tissue. Because the opercula had been frozen, most muscular tissue came off easily without having to scrape it off. The opercula that was dried and difficult to clean, were put for 5-10 minutes in clean water so the muscle tissue could soften and be removed easily.

For colouring the opercula they were put in 2-5 ml of a strong solution of methylene blue for about ½ -1 minute. The solution could be reused for about 10 opercula before it became too weak and had to be changed.

The colouring made striae clearly visible on the back side of the operculum, that is the side connected with the muscular tissue of the snail. The striae were examined in a microscope and counted with the inner ring (the central node) as the first and the ring before the outer edge as the last (figure 2.8).

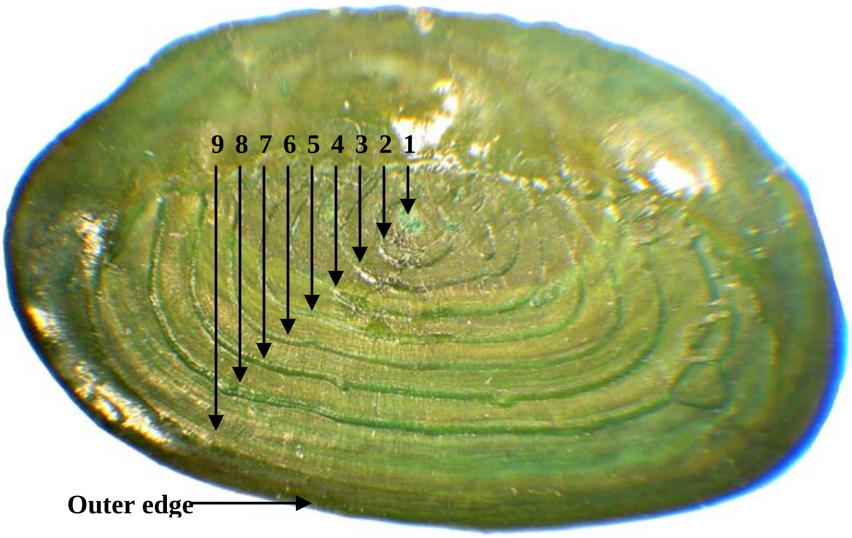

Figure 2.8 Back side of an operculum colored with methylene blue, from a nine year old *Buccinum undatum*.

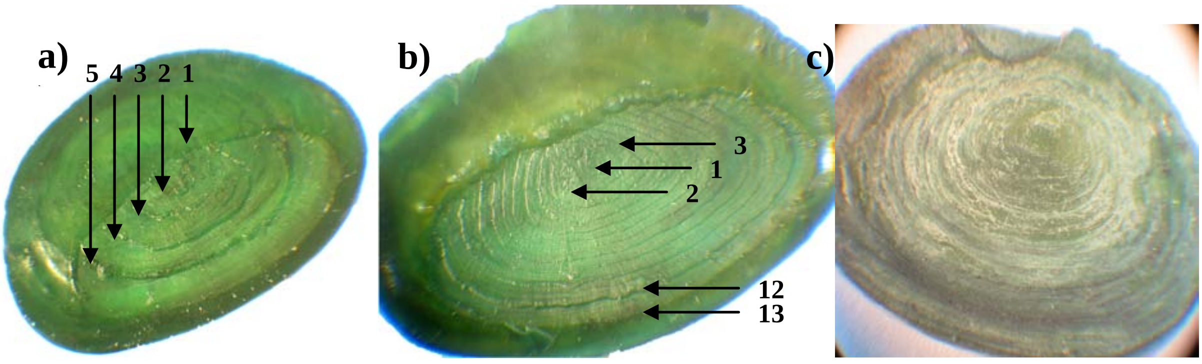

Figure 2.9 Operculae of *Buccinum undatum* colored with methylene blue. a) Operculum of a five year old whelk. b) Opercumul of a thirteen year old whelk. c) Front side of an operculum.

2.5 Data processing and statistics

Only snails caught in 2006 were included in the analysis. The snails caught in 2008 were only processed to find individuals suitable for TBT content analysis and was not meant to be part of the original data set.

All data were entered into Excel 2003, where basic calculations also were performed. Graphics and statistics have been conducted in Mystat: A student version of Systat.

Two-way ANOVA (ANalysis Of Variance) and Chi-square tests were used for demographic data and comparison between measurements from Berg and Balsnes. A log-linear model (Quinn and Keough 2002) was used to find the factors that decided imposex.

3 RESULTS

3.1 Population structure

After completing the laboratory work the material consisted of 1163 whelks from the 2006 catch. All were length measured. From Berg three snails were age read but not sex determined, and 13 snails were sex determined but not age read because of missing or destroyed opercula. Only one snail from Balsnes was age read but not sex determined. Because of these missing readings not all figures are based on the exact same number of snails. Further information on the original catch and data is given in table 6.1.

The length frequency distribution of *B. undatum* caught at Berg and Balsnes shows that the number of small snails at Berg was lower than at Balsnes (figure 3.1).

The length distribution was unimodal at both localities. The mode at Berg was at 65 mm and at 55 mm at Balsnes. There were no significant differences in length between sexes at Balsnes ($\chi^2 = 6.60$, df = 12, P = 0.883). This was also the case at Berg even when only snails above 50 mm were selected, to account for the sorting out of small snails ($\chi^2 = 6.23$, df = 12, P = 0.904).

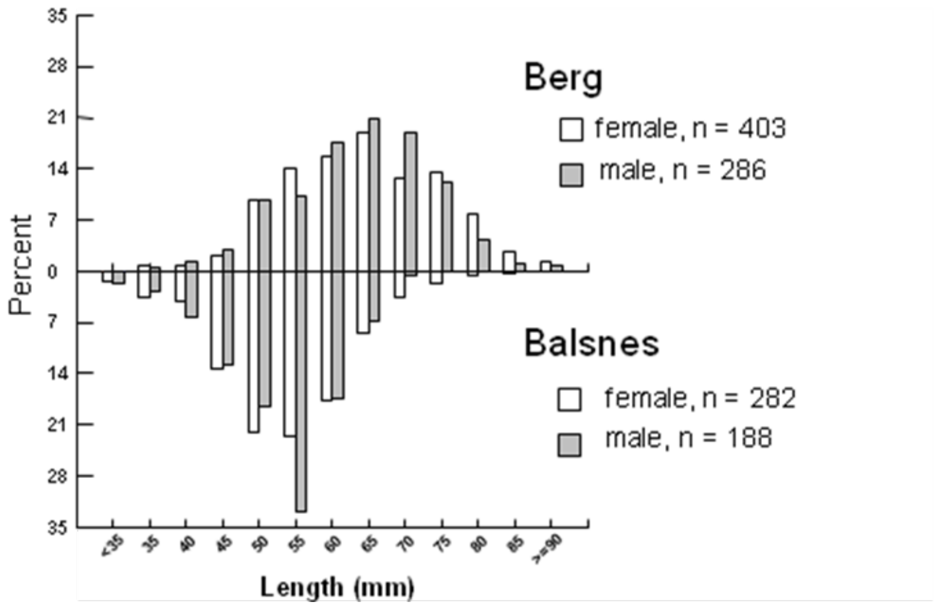

Figure 3.1 Bar chart illustrating the length distribution of *Buccinum undatum* caught at Berg and Balsnes given in percent of males and females from each locality.

The age distribution (figure 3.2) was unimodal and similar between both localities. At Balsnes there was no difference in age distribution between the sexes, confirmed by a chi-square test ($\chi^2 = 1.66$, df = 10, P = 0.998). At Berg the chi-square test showed a slightly significant difference in age distribution between male and female ($\chi^2 = 23.5$, df = 10, P = 0.009). There seemed to be a mode around 6 years at both Berg and Balsnes, but the median age was 7 years at Berg and 6 years at Balsnes for both sexes. There was a decline in the number of whelks older than these ages at both localities.

Figure 3.2 Bar chart illustrating the age distribution of *Buccinum undatum* caught at Berg and Balsnes given in percent of males and females from each locality.

Length increased with age at both localities for both sexes (figure 3.3). A two-way ANOVA based on length data for females (4-10 years old) and males (4-9 years old) clearly shows that the size at age between Berg and Balsnes are unequal (females: $F_{1,648} = 64.8$, $P < 0.001$; males: $F_{1,438} = 66.7$, P = < 0.001). This was also the case when the sorting out of snails at Berg was taken into account by testing for length data on females 6-10 years old ($F_{1,533} = 41.7$, $P < 0.001$) and males 6-9 years old ($F_{1,325} = 60.6$, $P < 0.001$).

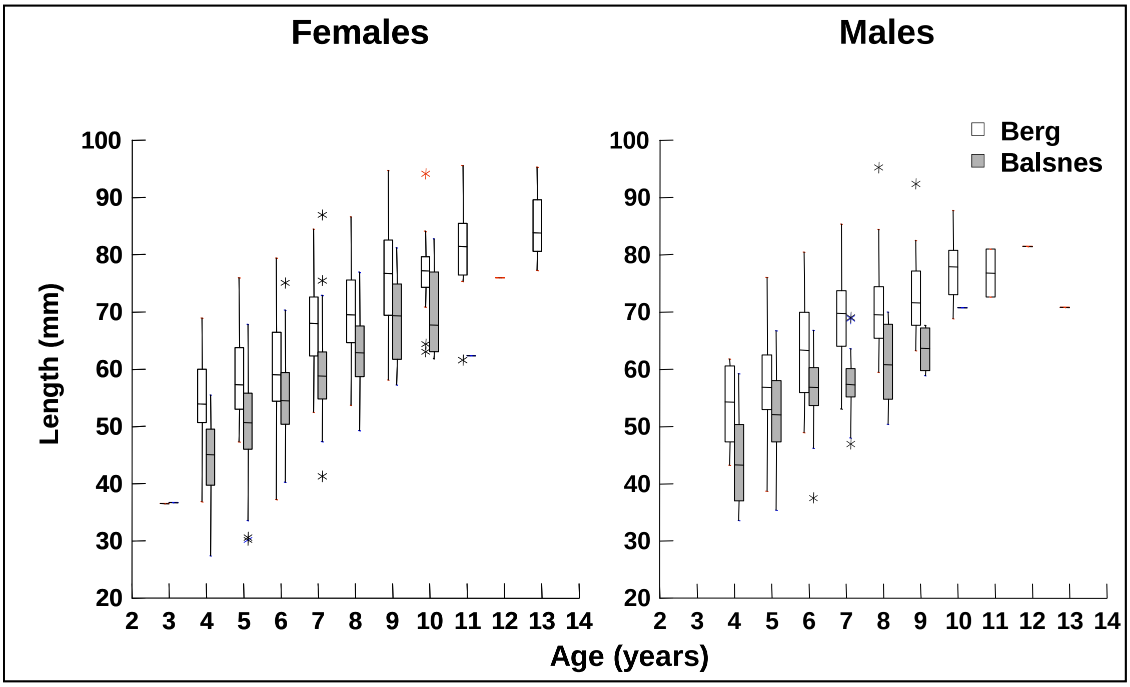

Figure 3.3 Box plot illustrating length-at-age relationship of male and female *Buccinum undatum* at Berg and Balsnes. * represents outliers.

Male penis length increased with both shell length (figure 3.4 a) and age (figure 3.4 b) at both localities. A two-way ANOVA for penis length at age 4-9 years, showed significant different between localities ($F_{1,437} = 4.92$, $P = 0.027$). At the same shell lengths penis length at Balsnes was longer than at Berg (figure 3.4).

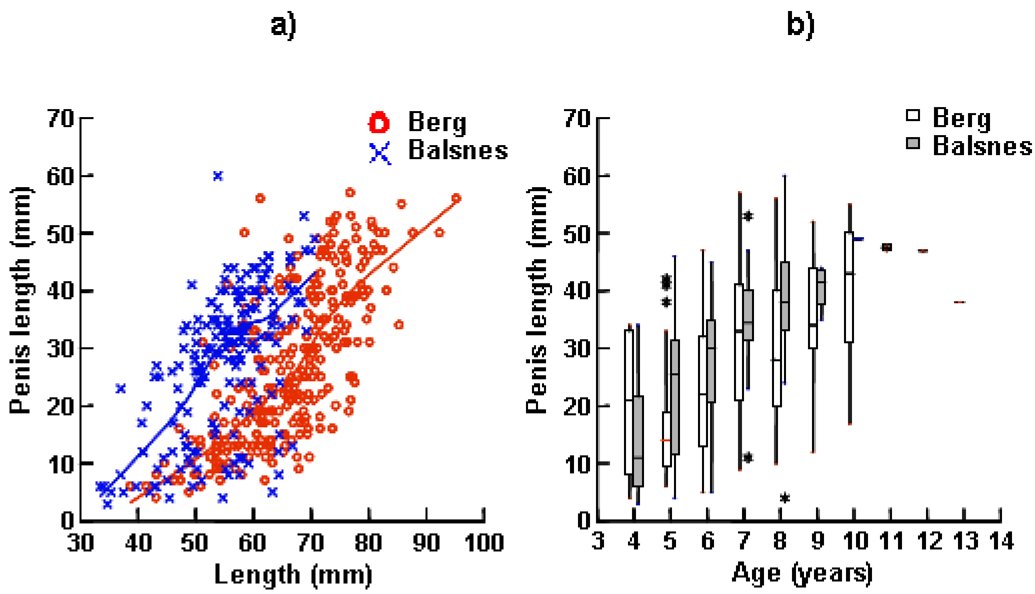

Figure 3.4 a) Scatter plot illustrating the relationship between male penis length at shell length. b) Box plot illustrating male penis length at age in *Buccinum undatum* at Berg and Balsnes. * represents outliers.

3.2 Imposex

Imposex was found in 23.1 % of the females from Berg, and in 21.6 % of the females from Balsnes. There were no significant differences between localities ($\chi^2 = 0.2$, df = 1, P = 0.66). At Berg imposex was found at all ages except 4 and 12 years, whereas at Balsnes imposex was not detected at ages 3, 4, 11, 12 and 13 years. There seems to be an increase in imposexed females with increasing length and age, but this varies somewhat between localities (figure 3.5). A summary of imposex data is given in table 6.2.

The results from a log-linear model explaining imposex for length groups and age groups have been plotted together with the observed values in figure 3.5. For length groups the simplest model was length group + locality + length group * locality ($\chi^2 = 14.824$, df = 13, P = 0.318). The interaction between length group and locality was most important for explaining the degree of imposex, followed by length group. Locality was of less importance in the model (table 6.3).

The results from a log-linear analysis explaining imposex for age groups found the simplest model to be age + locality + age * locality ($\chi^2 = 6.540$, df = 11, P = 0.835). Age was the most important variable for explaining the degree of imposex, followed by age * locality. As for the length data, locality was of less importance in the model (table 6.4.)

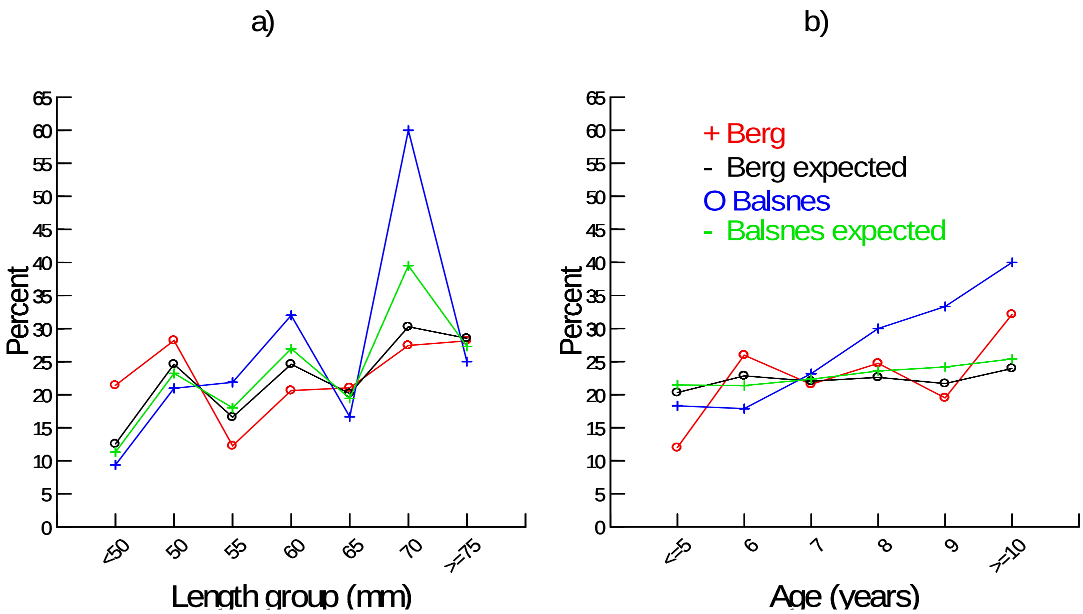

Figure 3.5 Line graph illustrating female *Buccinum undatum* with imposex as percent of total number of females at: a) Length group. b) Age. Legend applies for both figures.

The vas deferens sequence with age was very similar between Berg and Balsnes (figure 3.6).

There were also very few differences in development between ages. At all ages most whelks with imposex had a vas deferens stadium at 1a, although there were a few snails with higher stadiums at ages 6, 7 and 8 years. The Mensink sequence gave the same results as the vas deferens sequence with most imposexed females in stages 1 and 2 (figure 6.2).

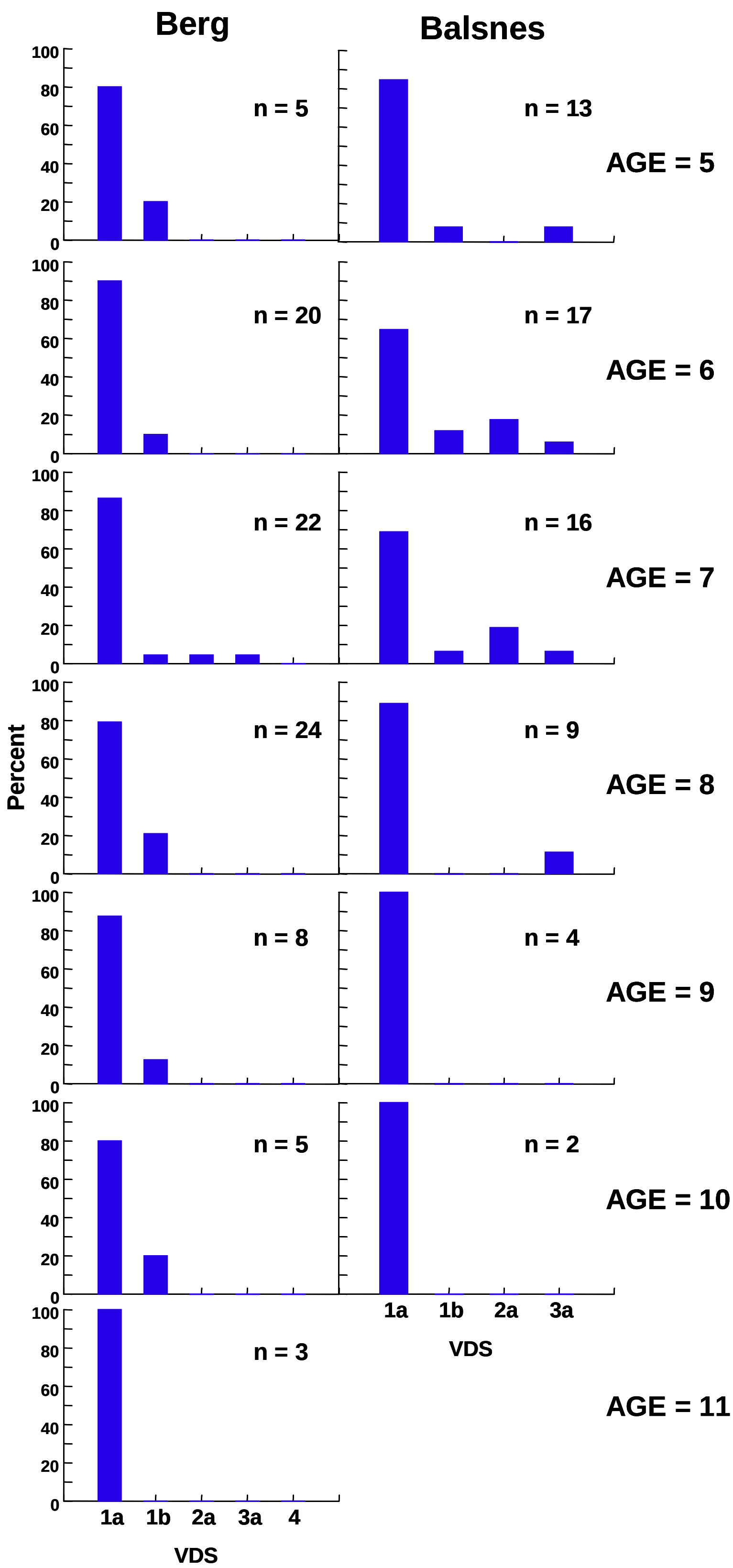

Figure 3.6 Distribution of the vas deferens sequence at age in years. Numbers of individuals at each age is given inside the graph as n. Ages 3, 4, 12 and 13 years has been removed from the figure since there were either none or only one individual in these groups.

The VDSI in length group <50 mm was higher at Berg than at Balsnes, while at length group 70 mm the VDSI was highest at Balsnes (figure 3.7 a). There were no differences in age specific VDSI between Berg and Balsnes (figure 3.7 b).

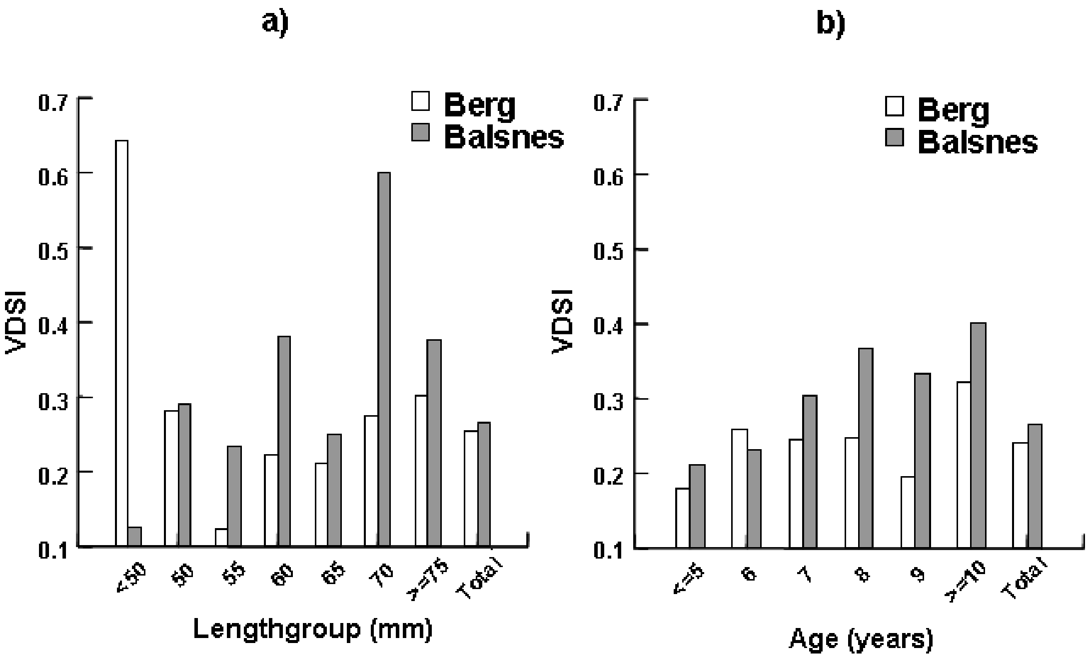

Figure 3.7 Bar chart of vas deferens stadium index (VDSI) and a) length, and b) age in *Buccinum undatum* at Berg and Balsnes.

There was no difference in length at age between the imposexed and the healthy females (figure 3.8). This was confirmed by a two-way ANOVA for individuals 5-11 years old at Berg ($F_{1,366} = 0.001$, $P = 0.975$), and individuals 5-10 years old at Balsnes ($F_{1,254} = 0.874$, $P = 0.351$)

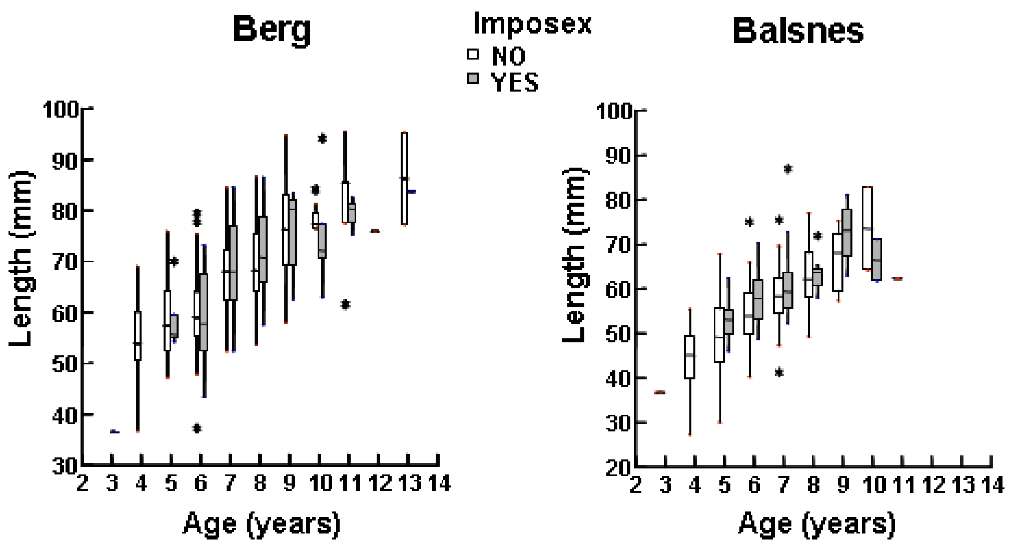

Figure 3.8 Box plot illustrating age-length relationship between female *Buccinum undatum* with and without imposex at Berg and Balsnes.

3.3 TBT content analysis

The two whelks from Balsnes had higher values of TBT and its metabolites, dibutyltin (DBT) and monobutyltin (MBT), than those from Berg (table 3.1).

Table 3.1 Results of TBT content analysis of *Buccinum undatum*. n=1 for both locations. For the complete table see table 6.4

ELEMENT	SAMPLE	Balsnes Imposex	Berg Imposex	Balsnes no imposex	Berg no imposex
Monobutyltinkation (MBT)	µg/kg	10	1	7	2
Dibutyltinkation (DBT)	µg/kg	9	5	11	4
Tributyltinkation (TBT)	µg/kg	13	<1.00	7	<1.00

Both sediment samples contained TBT and metabolites (table 3.2) below the detection limit, less than 1 µg/kg DS (dry sediment).

Table 3.2 TBT and metabolites in sediment from Berg and Balsnes. Values are given as µg/kg DS (dry sediment). n =1 for both locations. For the complete table see table 6.4

ELEMENT	SAMPLE	Berg	Balsnes
Total Organic Carbon	% DS	<0.900	<1.10
Dried sediment	gram	64,7	66,1
Monobutyltinkation	µg/kg DS	<1.0	<1.0
Dibutyltinkation	µg/kg DS	<1.0	<1.0
Tributyltinkation	µg/kg DS	<1.0	<1.0

4 DISCUSSION

4.1 Population structure

Both length and age showed a unimodal distribution of *B. undatum* for both Berg and Balsnes. At Berg this is in part a result of sorting out snails up to 50 mm in length, but there were few small snails observed in the catch even before this sorting. The original catch was most likely also unimodal in length distribution. Since the snails were sorted out by length only, the sex distribution should not have been affected by this removal. In the Balsnes catch small whelks were few all along. The decline in whelks over 70 mm at Berg and 60 mm at Balsnes is most likely caused by natural mortality.

The absence of small snails in the catches is a common phenomenon in studies done on *B. undatum*. There might be several reasons for this. The Welsh snail pot is selective for larger snails, and small snails will escape through the bottom holes. Small individuals may be living in other depths and prefer other food sources than adults.

Shelmerdine *et al.* (2007) reported both unimodal and bimodal length distributions for *B. undatum*, and they suggested that a unimodal distribution was the result of high predation on small and young individuals up to a size where survival rate is higher. Therefore there will be few snails smaller than 4 cm and increasing numbers of individuals longer than 4 cm due to higher survival rates.

Twice as many whelks were caugt at Berg than at Balsnes with equal effort. Intuitively one would think that since there are obviously more snails at Berg than at Balsnes, the snails at Berg would be smaller due to intraspecific competition, as seen in many other species (Begon *et al*. 1990). The present study found the opposite: *B. undatum* was significantly longer in shell length at age at Berg than at Balsnes. This shows that intraspesific competition in *B. undatum* might be low at Berg despite possible higher densities of whelks. Other explanations for the observed length difference between the sites can be food availability, predation pressure and genetic differences (Weetman *et al.* 2006).

Whelks reared in laboratory conditions had maximum growth (measured as shell length) when fed on a combined diet of *Mytilus edulis*, cod and fish pellets, and slower growth when fed on *M. edulis* only (Nasution and Roberts 2004). If snails at Berg have access to a wider variety of food than those at Balsnes, then growth might be affected.

Thomas and Himmelman (1988) showed that *B. undatum* varied in morphology between areas with different predator species. Whelks found in areas with large crustaceans had thicker shells and longer shell length. Vulnerability for predation might decrease with increasing size. If there are predators present at Berg capable of eating large whelks, then size at survival is probably larger here than at Balsnes. Juliussen (2007) conducted a morphological study on *B. undatum* at Berg and Eines. Eines is located close to Balsnes on the same side of the fjord, so there is a reason to believe that populations of *B. undatum* in these two areas are similar in respect to physical and biological properties. Just like the present study, Juliussen (2007) found distinct morphological differences in *B. undatum* between Berg and Eines, using several more measures in the shell than shell length. Juliussen (2007) found that whelks at Berg were thicker and more robust in the shell structure than those at Eines. This supports the hypothesis of different predation pressure in accordance with the work of Thomas and Himmelman (1988).

A genetic difference between the two areas is also a possible explanation for the observed differences in shell length. Weetman *et al.* (2006) found that populations of *B. undatum* separated only by 1-2 km showed as much genetic differentiation as populations 20-200 km apart, suggesting local isolation of small populations. Berg and Balsnes are separated by a fjord 4.3 km across and up to 80 m deep. Because *B. undatum* has limited movement and no pelagic larvae, this is perhaps enough space to create separate populations.

The male penis length grew with age and length just like predicted, but it was bigger in snails at Balsnes than at Berg. This was unexpected as one would think that bigger snails had longer penises. It is evident here that “size does not always matter”. Penis length at maturity can be equal for males at 60 mm in length and males at 80 mm in length. It might be that *B. undatum* becomes sexually mature at earlier stages at Balsnes than at Berg. If predators at Berg include large whelks in their diet, then perhaps these whelks must invest more in shell growth before they become sexually mature, and therefore mature at larger sizes than at Balsnes. It is known that maturity length and age differs between areas, but no reports or evidence was found in the litterature to indicate that predation might affect reproductive age and length.

4.2 Imposex and TBT

According to Strand (2003) one should use only fertile whelks when monitoring imposex. This was not possible in the present study for several reasons. As mentioned, size at sexual maturity and time of reproduction during a year varies geographically, and between male and female. Length has in some cases been used as a measure of fertility, but because of the length difference found between Berg and Balsnes, this was not a reliable measure of fertility. Only one study has been conducted on sexual maturity in whelks in Balsfjorden, using male penis length as a measure of fertility (Juliussen 2007). This gave size at sexual maturity only for males, and since size at maturity may vary between males and females within an area, it did not help much when sexually mature females were sought.

As expected, low and similar percentages of imposex were found at Berg and Balsnes although Berg had about 2 percent more females with imposex than Balsnes. As the localities are influenced by the same water flow and therefore the same water pollution, there should be no differences in TBT concentrations in the water between the areas. Because *B. undatum* is bottom dwelling it is probably effected by TBT in the sediments and not in the water. The natural degradability of TBT varies greatly dependent on characteristics and bacterial life of the sediments it is disposed in (see Introduction). Balsnes had slightly higher total organic carbon values than Berg, perhaps because there is more bacterial life in the sediments, thus leading to better TBT degradability. Therefore there will be less TBT affecting *B. undatum* and fewer females developing imposex.

The log linear model showed that locality was an important variable in explaining imposex frequencies for both length and age, although the least important in the models. For length groups the most important variable was the interaction between length and locality followed by length alone. For age groups, age alone was the most important variable followed by the interaction between age and locality. This confirms that imposex development with length and age has to vary between the localities. There seemed to be a slight increase in imposex with increasing age and length at both localities, but this increase was best seen at Balsnes. Balsnes is located in a back eddy where finer sediments accumulate, and pollution might be higher here than at Berg where currents are stronger. Since imposex in *B. undatum* developes within the first 2 years after hatching and severity depends on TBT-concentration (Mensink 1999), it seems like TBT levels were higher 10 years ago when the oldest females were hatched. New reports show that TBT levels in the Tromsø area has decreased dramatically the past 10 years, although some areas close to harbours are still severely contaminated (Evenset *et al*. 2009).

The content of TBT and metabolites in the sediments were the same at both localities. They were also below detection limits, so the areas can be assumed fairly free of TBT according to the guidelines from the Norwegian pollution control authority (SFT) (Bakke *et al.* 2007). However, the low levels of TBT in sediments at Berg and Balsnes does not mean that TBT is not present in the biota. TBT or metabolites were found in all four snails examined in higher concentrations than in the sediment, and has most likely entered the whelks through their diet (Viglino *et al*. 2005). *Buccinum undatum* is known to feed upon *M. edulis* and pectinid bivalves (Taylor 1978). *Mytilus edulis* is found to contain high body burdens of TBT even with low levels in the surrounding water (Guolan and Young 1995) and the pectinid *Chlamys islandica* was found to have a TBT concentration of 2.7 ng/g in West Greenland (Strand and Asmund 2003). Both *M. edulis* and *C. islandica* are common species at Berg and Balsnes and available prey for *B. undatum*, and they may play a role in distributing TBT into the whelks.

Evans and Nicholson (2000) reported that severe TBT contamination was mostly local and that healthy populations of *N. lappilus* were found just a few kilometres from heavily contaminated ports. Sediments in the port of Tromsø was found to be highly contaminated by TBT (Evenset *et al.* 2008), where concentrations increased with depth in the sediments. There are no reports available that give a measure of the TBT contamination further out in Tromsøsundet. It is therefore difficult to say if the TBT at Berg and Balsnes comes from the port of Tromsø or if it comes from ship traffic in and around the areas.

When comparing VDS there were no difference between localities and ages. In a study on pseudohermaphroditism in 20 gastropod species, Fioroni *et al.* (1991) identified 7 stages of imposex in which four (1a, 2a, 3a, 5(sterile) was found in *B. undatum.* The present study found stages 1a, 1b, 2a and 3a, but none close to stage 5. This suggests that the populations of *B. undatum* at Berg and Balsnes are healthy and that imposex does not affect reproduction. This is most likely the case for the most of the Tromsø area, although *B. undatum* in areas with high TBT-pollution might be affected more severely.

It is also important to notice that TBT pollution may not be the only cause for imposex. It has also been indused by ethanol and copper in laboratory experiments on *N. lapillus,* and environmental stress caused imposex in *Lepsiella vinosa* (Evans and Nicholson 2000). It is not known if these factors also can cause imposex in *B. undatum*, but one should be careful in saying that all imposex in low polluted areas are induced by TBT when other factors can be involved.

According to Mensink (1999) TBT affected growth in *B. undatum* and therefore indirectly affected reproduction negatively. This was not found in the present study, probably because TBT levels have not been high enough to affect growth at Berg and Balsnes.

When looking at the VDSI it is evident that the index varies a lot more between length groups than between ages. This shows that using length as an age measure does no work very well. Although length increases with increasing age, it varies greatly within age groups both within and between localities. If length reflected the age in the whelks length and age groups would have shown almost the same pattern in the imposex distribution and VDSI. The question is if reading the opercula striae is a good indication of age. Age reading in gastropods is a difficult issue, but it is beyond the scope of this study to discuss it further.

Effects of TBT have mostly been directed towards the female gastropod looking for imposex or imposex-like stages. There is a possibility that also male *B. undatum* can be affected by TBT, perhaps in form of reduced growth or abnormalities caused by an unnaturally high level of androgens. TBT have been found to affect males of the golden apple snail (*Pomacea canaliculata*) in Taiwan, where males were found to have shorter penises in areas where imposex was common in females, than in areas with few imposexed females (Liu *et al.* 2006).

4.3 Conclusions

This study has confirmed that *B. undatum* populations close to each other can exhibit marked differences in morphology. The *B. undatum* populations in Balsfjorden are healthy with low levels of imposex, which in turn indicates low levels of TBT pollution. A slight decrease in imposex percentage with decreasing age at Balsnes might indicate that TBT levels are decreasing in the area, after new use of TBT became illegal in Norway in 2003. Further studies are needed to see if levels of imposex will continue to decrease in the future. It is necessary to do more extensive TBT analysis on snails of equal age. In future studies imposex in *B. undatum* can be examined without killing the snails, since female penis homologues were always shorter than male penises.

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6 APPENDIX

Table 6.1 Summary of original data

	Berg		Balsnes	
Total catch	1027		577	
Length meassured	692		471	
Sex determined	689		470	
Age read	679		471	
	Female	Male	Female	Male
Total	403	286	282	188
Age group				
<=5	50	41	71	76
6	77	84	95	60
7	102	78	69	33
8	97	41	30	14
9	41	19	12	4
>=10	28	18	5	1
Length group				
<50	14	13	64	44
50	39	28	62	35
55	57	29	64	62
60	63	50	50	33
65	76	60	24	13
70	51	54	10	1
>=75	103	52	8	0

Table 6.2 Summary of imposex in female *Buccinum undatum*,distribution in age and length groups at Berg and Balsnes.

	Berg		Balsnes	
	Imposex	No imposex	Imposex	No imposex
Total	89	306	61	221
Age group				
<=5	6	44	13	58
6	20	57	17	78
7	22	80	16	53
8	24	73	9	21
9	8	33	4	8
>=10	9	19	2	3
Length group				
<50	3	11	6	58
50	11	28	13	49
55	7	50	14	50
60	13	50	16	34
65	16	60	4	20
70	14	39	6	4
>=75	29	72	2	6

Tabel 6.3 Log linear test results for length groups.

Tests for Model Terms

Term Tested	**The Model without the Term**				**Removal of Term from Model**		
	ln(MLE)	Chi-square	df	p-value	Chi-square	df	p-value
Length group	-103,778	73,932	19	<0.001	58,776	6	<0.001
Imposex	-255,959	378,293	14	<0.001	363,137	1	<0.001
Locality	-88,745	43,866	14	<0.001	28,709	1	<0.001
Length group*Locality	-177,382	221,141	19	<0.001	205,984	6	<0.001

Tabel 6.4 Log linear test results for length groups.

Tests for Model Terms

Term Tested	**The Model without the Term**				**Removal of Term from Model**		
	ln(MLE)	Chi-square	df	p-value	Chi-square	df	p-value
Age	-170,962	224,643	16	<0.001	217,998	5	<0.001
Imposex	-243,630	369,978	12	<0.001	363,334	1	<0.001
Locality	-85,317	53,353	12	<0.001	46,709	1	<0.001
Age * Locality	-98,946	80,612	16	<0.001	73,968	5	<0.001

0

1

2

3

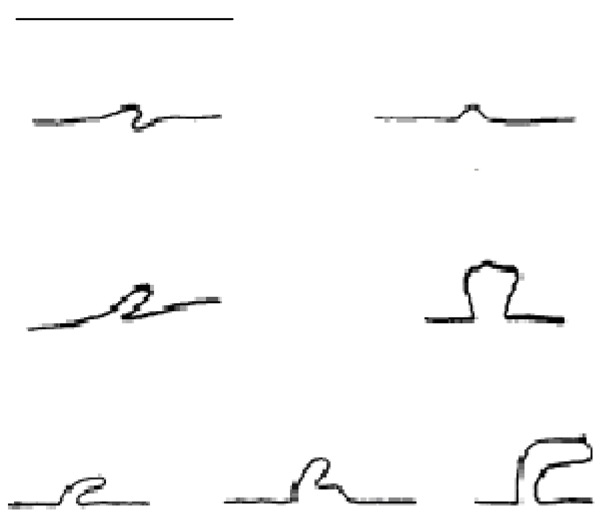

Figure 6.1 The Mensink sequence (MS). Stadium 0: No imposex characteristics is found in the female whelk.

Stadium 1: A bud smaller than 1mm in length can be seen above the right tentacle

Stadium 2: A small outgrowth that can be lifted from the epithelium is present.

Stadium 3: The penis homolouge is longer than 3mm and resembles the male penis in appearance.

(Adapted from Strand 2004)

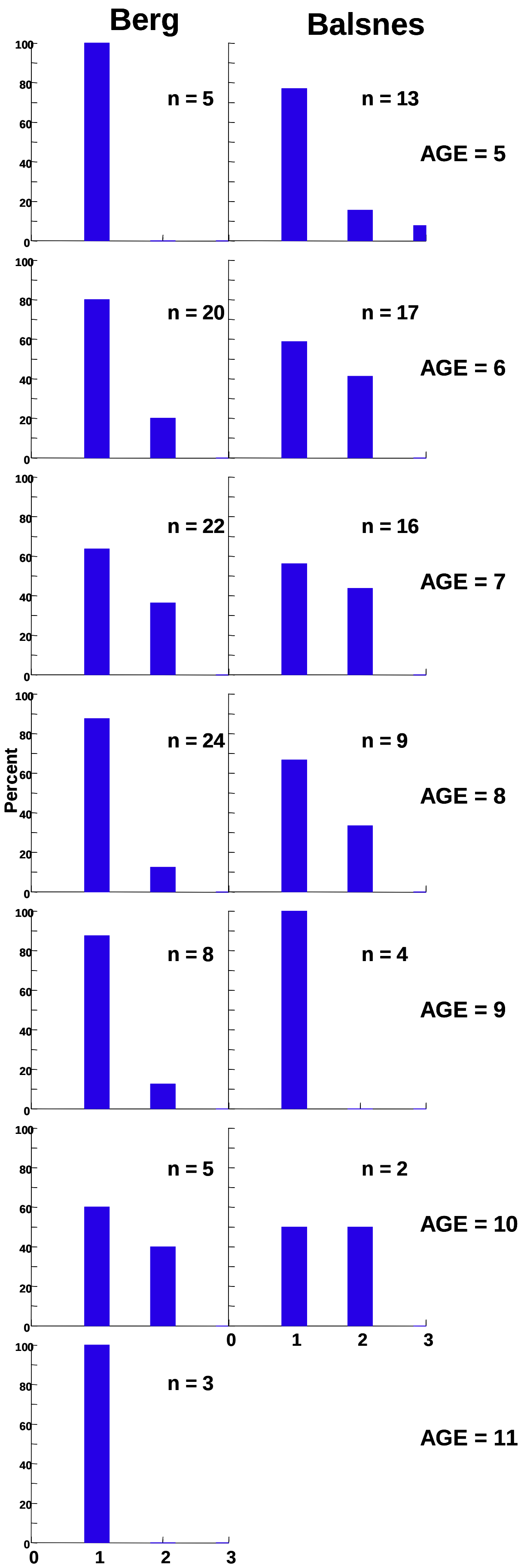

Figure 6.2 Female Buccinum undatum with imposex distributed in percentage per stadium in the Mensink sequence at ages. Numbers of individuals at each age is given inside the graph as n. Ages 3, 4, 12 and 13 has been removed from the figure since there were either none or only one individual in these groups.

Table 6.5 Results of TBT content analysis of *Buccinum undatum*. n=1 for both locations. For the complete table see table 6.4

ELEMENT	SAMPLE	Balsnes Imposex	Berg Imposex	Balsnes no imposex	Berg no imposex
Monobutyltinkation (MBT)	µg/kg	10	1	7	2
Dibutyltinkation (DBT)	µg/kg	9	5	11	4
Tributyltinkation (TBT)	µg/kg	13	<1.00	7	<1.00
Tetrabutyltinkation	µg/kg	<1.00	<1.00	<1.00	<1.00
Monooktyltinkation	µg/kg	<1.00	<1.00	<1.00	<1.00
Dioktyltinkation	µg/kg	<1.00	<1.00	<1.00	<1.00
Trisykloheksyltinkation	µg/kg	<1.00	<1.00	<1.00	<1.00
Monofenyltinkation	µg/kg	<1.00	<1.00	<1.00	<1.00
Difenyltinkation	µg/kg	<1.00	<1.00	<1.00	<1.00
Trifenyltinkation	µg/kg	<1.00	<1.00	<1.00	<1.00

Table 6.6 TBT and metabolites in sediment from Berg and Balsnes. Values are given as µg/kg DS (dry sediment). n =1 for both locations.

ELEMENT	SAMPLE	Berg	Balsnes
Total Organic Carbon	% DS	<0.900	<1.10
Dried sediment (gram)	%	64,7	66,1
Monobutyltinkation	µg/kg DS	<1.0	<1.0
Dibutyltinkation	µg/kg DS	<1.0	<1.0
Tributyltinkation	µg/kg DS	<1.0	<1.0
Tetrabutyltinkation	µg/kg DS	<1.0	<1.0
Monooktyltinkation	µg/kg DS	<1.0	<1.0
Dioktyltinkation	µg/kg DS	<1.0	<1.0
Trisykloheksyltinkation	µg/kg DS	<1.0	<1.0
Monofenyltinkation	µg/kg DS	<1.0	<1.0
Difenyltinkation	µg/kg DS	<1.0	<1.0
Trifenyltinkation	µg/kg DS	<1.0	<1.0