BEHAVIOURAL TEST OF *LUMBRICULUS VARIEGATUS* IN TBT SPIKED AND FIELD CONTAMINATED SEDIMENTS

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Abstract

TBT (Tributyltin) has been used as antifouling agents in paints for ships before being banned due to its harmful effects to marine organisms. It mainly is associated with imposex phenomenon in female dogwhelks and shell thickening in oysters. Although having been banned for several years, TBT are still persistent in nature due to longer half-life ranging for few decades under anaerobic conditions. Most studies concerning TBT have been done with higher trophic level organisms and very few have been done with benthic organisms.

In this study, the effect of TBT on sediment dwelling oligochaete *Lumbriculus variegatus* was studied. Two different types of tests were performed for this study. In traditional toxicity tests, feeding, growth and mortality over exposure period of 28 days were studied where as in behavioural tests, selection or avoidance of contaminated sediment by the organisms during 48 hour exposure was studied. The endpoints from the two tests were compared. TBT was spiked into different sediments (artificial and natural) at different concentrations according to sediment dry weight.

Growth and reproduction of *L. variegatus* was significantly high in the artificial sediment at lower concentration of TBT and no mortality was seen at lower concentration. However in the highest concentration (35000 µg/Kg), 100% mortality was seen. In the Lake Höytiäinen sediment, reduced growth of the organisms was observed with low reproduction rate but no mortality in lower concentration of TBT. In highest concentration, mortality rate was high correspondingly to the artificial sediment.

In behavioural test, *L. variegatus* showed some avoidance to the contaminated sediment in artificial sediment however in natural sediment the avoidance was not significant. It might have been due to the solvent used for TBT. From the study, it is clear that high concentration of TBT is toxic to benthic organisms but they can thrive in lower concentration given that there is enough food sources present in the sediment. Different species of organisms should be tested as reaction of different species is different to chemicals. For behavioural test a highly sensitive organism is to be used for best result. Also, the solvent used in the sediment should be dried out or at least used in minimal amount so that it does not alter with the behaviour of the organism. With improvement and development in avoidance behaviour test, it can be used as first screening tool in evaluating sediment contamination and help to develop risk assessment that can be used before the acute contamination takes place.

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1 INTRODUCTION

1.1 Background on TBT

Organotin compounds are those in which at least one compound bond between tin and hydrocarbon is present (Ingham *et al.*, 1960). The first organotin was prepared by Sir Edward Frankland over 150 years ago. Frankland synthesized diethylin-diiodide in 1849 and later in 1859 prepared tetraethyltin (Van der kerk, 1975 cited in Hoch 2001). Since then many investigations have been done as a result of which there are more than 800 known organotins in the World today. Organotin compounds are mostly of anthropogenic origin. In nature, tetramethyltin can be formed by methylation in estuarine sediments by abiotic and biological pathways (Guard *et al.*, 1981). Organotin were not used for any commercial applications for about a century (Champ & Seligman, 1996). But it all changed with the expansion of the plastic industry, particularly the production of Polyvinyl Chloride (PVC) during 1940s (Blunden *et al.*, 1984 cited in Champ & Seligman 1996). The PVC polymers were unstable under the influence of heat and light which was later prevented by the addition of organotin derivatives (Yngve, 1940 cited in Hoch 2001). Since then organotin compounds have been used in other industrial commodities as well. And with the discovery of biocidal property in late 1950s, they are used as toxic ingredient in timber preservatives (Hoch, 2001).

Amongst the organotin compounds main focus is given to Tributyltin (TBT) pollution in water and sediment due to its high toxic effect to the aquatic ecosystem even at very low concentrations (Chagot *et al.*, 1990). TBT is a synthetic organotin and its general formula is $(CH_3CH_2CH_2CH_2)_3Sn\text{-}X$, where X is an anion such as Chloride or a covalently bonded functional group (Figure 1).

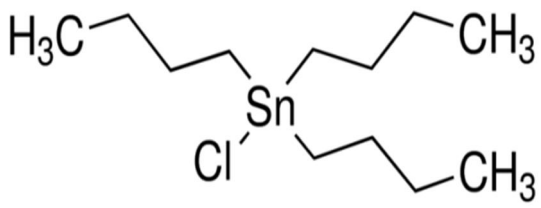

Figure 1 Chemical structure of Tributyltin Chloride (Sigma Aldrich)

TBT has been widely used as antifouling agents in the paints on ships and small vessels as well as in aquaculture facilities since 1960s (Murai *et al.*, 2005). The unwanted growth of

barnacles, seaweeds, algae and other marine organisms on a boat's bottom immersed in water is known as fouling. It has been the mariner's curse since the humans have made their first sail (Du *et al.*, 2014). The fouling creates roughness on the vessel hull reducing the vessel speed per unit energy consumption. An increase of 10 µm of the average vessel hull can increase the fuel consumption by about 0.3-1% (Champ & Seligman, 1996). To reduce the cost of running the vessel, antifouling paints are used in the vessel hulls. In the beginning, copper based (Cu_2O) antifouling paints were used but they become ineffective within a year. So, much longer effective biocides were required (Champ & Bleil, 1988). TBT was first used in antifouling paints in Europe in the late 1950s and soon became popular as a very effective antifouling agent worldwide (Clark *et al.*, 1988 cited in Du *et al.* 2014). TBT based antifouling paints works efficiently by killing the fouling organisms like barnacles, algae and mussels and lasts much longer than the copper based antifouling paints. TBT is also used as wood preservative against insects, fungi and bacteria (Bennett, 1996).

However, adverse effect of TBT on non-target (non-fouling organisms) molluscs was found in 1986 (UK DoE, 1986). During 1980s, two most clear effects of TBT were the thickening of oyster shell and imposex in female dogwhelks, a marine mollusc (Waldock & Thain, 1983; Gibbs & Bryan, 1986; de Mora & Pelletier, 1997; Hoch 2001). Abnormal shell growth were observed in *Crassostrea gigas* by the oyster farmers from the East coast of England and in Arcachon Bay, Atlantic coast of France during late 1970s (Key *et al.*, 1976; Alzieu *et al.*, 1980 cited in Silva *et al.* in 2014). In addition to the anomalies in shell growth, the number of oyster larvae settling on hard surface was very little in some areas of Arcachon Bay. This suggested the toxic effect of TBT in the early stages which was going to effect the oyster population over time (His & Robert, 1983; Alzieu, 1986). A serious effect of TBT was reported in common dogwhelk (*Nucella lapillus*) in Southern England. A penis-like outgrowth occurred in female sexual organs of gastropod *N. lapillus* (Blaber, 1970). This deformation of development of male sex organ in female gastropods is referred to as imposex (Gibbs & Bryan, 1996). Imposex affects the reproduction eventually leading to the decline in the population of dogwhelk.

TBT have high toxic effect to aquatic life even at low nanomolar aqueous concentration (Hoch, 2001). It can cause impairment to low level organisms at concentration as low as 1 ng TBT L^{-1} and to higher level organisms at concentration as low as 1 µg TBT L^{-1} (Gibbs & Bryan, 1996). For short term exposures, lethal effects of TBT can be seen at 0.6 µg L^{-1} in copepods (WHO, 1990). Toxicity of TBT to marine fish varies from 1.5 to 36 µg L^{-1} in short

term exposure. TBT affects the growth of bacterium *Legionella pneumophila* at concentration between 0.5 and 1.1 ng L^{-1} and it turns bactericidal above 1.1 ng L^{-1}(Sorraco & Pope, 1983). EC_{50} value for fresh water algae was reported at 42 µg TBTO L^{-1} for 96-hour exposure period (RIVM, 1989) and for marine algae *Skeletonema* was reported 0.33 µg TBTO L^{-1} for 72- h exposure period (Thain, 1983). In 1985, His & Robert found out that TBT acetate reduces the growth of *Crassostrea gigas* larvae and caused death within 10 days at concentration of 0.05 µg L^{-1} (WHO, 1990). TBT has toxic effect to the aquatic organisms in higher trophic level as well. The 96-h LC50 of TBTO for marine fish ranges from 1.5 to 36 µg L^{-1} whereas for the freshwater fish the range is from 13 to 240 µg L^{-1} (Alabaster, 1969; Foster, 1981; Thain, 1983; Bushong *et al.*, 1988; RIVM, 1989).

Studies related to TBT pollution are mainly focused on the area having high boat activity such as boat yards, harbours and dry docks. The major source of TBT-contamination is emission of TBT from antifouling paints which contaminates the water and sediments of marinas, lakes and coastal area. TBT enters to these systems through leaching from the paints, scrapping off of the paints and cleaning activity in the dock (Du *et al.*, 2014). High concentrations of TBT can be detected in area far from the coastal regions as well (Hardy & Cleary, 1992). The solubility of TBT compounds depend upon the factors such as pH, temperature, oxidation-reduction potential, concentration and composition of dissolved organic matter (Clark *et al.*, 1988). Solubility of TBTO was reported to be 750 µg L^{-1} at pH 6.6, 31 000 µg L^{-1} at pH 8.1 and 30 000 µg L^{-1} at pH 2.6 (Maguire *et al.*, 1983 cited in Clark *et al.* in 1988).

Due to low solubility and lipophilic nature of TBT, large proportion of TBT is found adsorbed into the clay fraction of particulate matter. Between 60-90% of TBT is adsorbed in the water column according to laboratory studies and field measurement (Randall & Weber, 1986). Adsorption of TBT depends on different factors such as salinity, types of exchangeable cations, pH-value, temperature, amount of suspended particles and presence of dissolved organic matter (Batley, 1996). Once it is adsorbed, the decrease of TBT concentration takes place mainly by degradation. Degradation of TBT involves progressive debutylation from the Sn cation.

$$R_3SnX \longrightarrow R_2SnX_2 \longrightarrow RSnX_3 \longrightarrow SnX_4$$

In the environment, the removal of the organic groups can be done by various mechanisms such as physico-chemical mechanisms (hydrolysis and photodegradation) and biological mechanisms (degradation by microorganisms and metabolism by higher organisms).

Photodegradation of TBT by Ultraviolet (UV) light is theoretically possible and is the fastest route of degradation in water. The UV light with a wavelength of 290 nm consists energy of approximately 300 kJ mol^{-1} which is enough to break the Sn-C bond that requires energy in range of 190-220 kJ mol^{-1} (Skinner, 1964). But as the transmittance of UV light decreases with the depth in water column, photolysis occurs only in the upper few centimetres and not in the greater depth and sediments (Maguire *et al.*, 1983). Photolysis due to sunlight is slow and has half-life of more than 89 days (Maguire *et al.*, 1985). In chemical degradation, the Sn-C bond can be attacked by both nucleophile and electrophile reagents. Mineral acid, carboxylic acids are capable of causing such degradation. Half-life for this type of degradation has been found to range from 1 minute to 115 days (Maguire *et al.*, 1983).

Biodegradation of TBT is the most important mechanism for TBT degradation in water and sediments which have half-life ranging from several days to weeks in water and from several days to several months in sediment (Maguire *et al.*, 1983; Clark *et al.*, 1988; de Mora *et al.*, 1989; Stang *et al.*, 1992). According to studies in metabolism of TBT, some species of bacteria, algae and fungi possess capability to degrade TBT and debutylate TBT to less toxic dibutyltin, monobutyltin and finally inorganic tin (Barug, 1981; Maguire *et al.*, 1983). Biodegradation kinetics depends upon environmental factors such as light, temperature, pH, nature of the microflora and also on the concentration of TBT being lower than the lethal for the microorganism. Under the anaerobic conditions, biodegradation still exists and is more rapid than under aerobic condition (Maguire & Tkacz, 1985). Maguire *et al.* (1986) reported that under aerobic condition the half-life of TBT varies between 4 to 5 months whereas under anaerobic condition it is under 1.5 months. However in sediment, degradation was found to be a slow process. In aerobic layers, the half-life was between 4 and 5 months whereas in deeper anaerobic condition half-life of TBT was not obtained for more than 500 days (Thain and Waldock, 1989).

Due to the lipophilic character, TBT are persistent in the environment and contribute to bioaccumulation in living organisms. Most studies are concerned about the uptake of TBT by aquatic organisms due to its high toxicity to several organisms including important sea food resources such as molluscs (bivalve), crustaceans and fish. Bivalves are able to accumulate

upto >5 µg g^{-1} of TBT whereas Crustaceans and fish accumulate much lower amounts of TBT (Laughlin, 1996). Pacific oyster *Crassostrea gigas* exposed for 22 days to TBTO concentration of 0.15 µg L^{-1} and 1.25 µg L^{-1} had bioconcentration factor of 6000 and 2000 respectively (Waldock *et al.*, 1983). Tsud *et al.* (1987) reported that *Cyprinus carpia*, a type of carp, which was exposed for 14 days to TBTO concentration varying between 1.8 and 2.4 ng L^{-1} had bioconcentration factor of 1000. Recent studies have found the accumulation of TBT by higher trophic aquatic organisms such as marine mammals and birds as well through the food chain (Iwata *et al.*, 1995). But still there are very few reports related to the contamination in higher trophic levels. Human exposure to TBT is inevitable as well. TBT can enter human body by ingestion of sea food such as mussels, fish from contaminated water bodies and also through direct contact with contaminated water and sediments. There have not been much studies dealing with contamination of humans and the toxic effect of TBT in humans (Hoch, 2001).

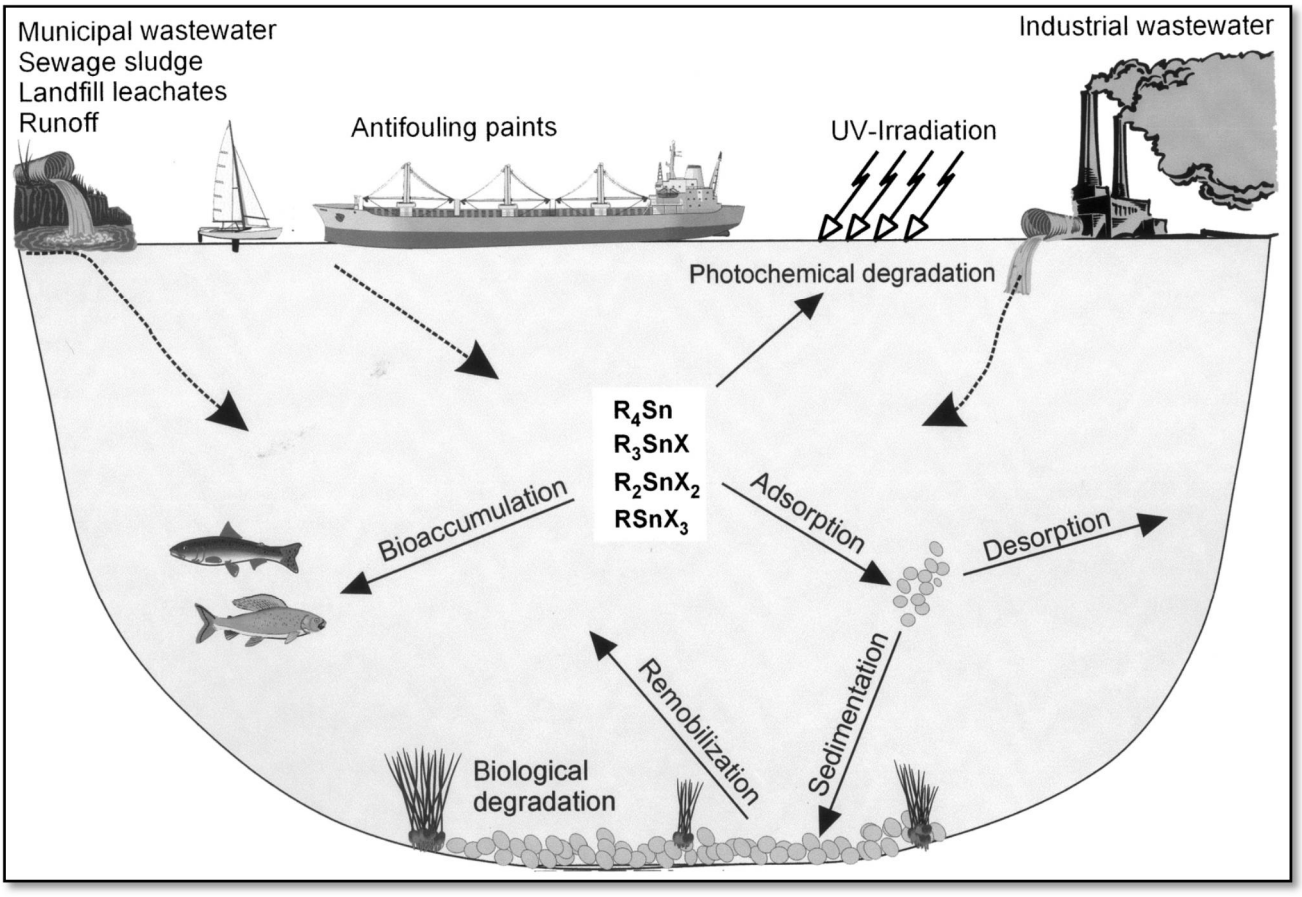

Figure 2 Distribution and fate of Organotin compounds and their general routes into aquatic environment (Hoch, 2001)

1.2 TBT in the sediments

The soils and sediments works as a trap for most of the contaminants that are found in the aquatic ecosystems. The sediments have been recognized as the primary environmental sink for TBT in marine and estuarine systems. TBT has relatively low solubility in water (10^{-4} gmol L^{-1}) and readily partitions out of the water column (Maguire *et al.*, 1983). Due to the high hydrophobicity of TBT, they concentrate more on the organic phase of the environment and get easily adsorbed onto the suspended particulate matter. The deposition of the suspended particulate matters leads to the TBT scavenging in sediments. The concentration of TBT is relatively higher in sediments than those in the water.

Although the application of TBT in antifouling paints for the vessels <25 metres in length is banned in most of the countries (IMO, 2001; EU, 2003), the question remains as how much of this contaminant have been accumulated in the sediment during last few decades. The half-life of TBT in sediment ranges in terms of year than days or weeks in the water column. A study of the sediments from Arcachon Bay by Astruc *et al.* (1989) reported the high concentration of TBT even after it was deposited 8-15 years ago suggesting the half-life of TBT to be measured in years or even decades. The slow degradation of TBT in sediments suggests its persistence in the aquatic environment even after the input from the external source has been ceased. Slow release of the TBT into the overlaying water may occur. There is always a risk of possible contamination of the aquatic environment from resuspended sediments. Activities such as dredging, swirling, movement of organisms on the sediment or other natural disturbances such as storms may cause mobilization of sediments and resuspending it in the water column.

TBT are easily taken up by aquatic organisms due to its hydrophobic nature. TBT contained in sediments enters the biota through the uptake of TBT by the benthic organisms. These benthic organisms feed on the sediment particles which contains high amount of TBT. Beside the benthic organisms, sediment dwelling organisms also have high risk of accumulating TBT. Once TBT enters the benthic organisms, then they are transferred to the higher trophic level through the food webs. Some marine organisms accumulate significant amount of TBT. Biomagnification of TBT takes place as we pass up the food chain. TBT can enter human through the sea-foods from contaminated sites but the toxic effects have not been documented till now. Also the high sensitivity of bivalves to TBT causes early mortality which minimizes the risk of TBT contamination in humans (Laughlin & Linden, 1987).

1.3 Behavioural tests

Nowadays, the soil has been contaminated with different chemical compounds leading to groundwater contamination and biomagnification of such compounds through food web (Hund-Rinke *et al.*, 2002). The contaminants alter the physicochemical conditions of the soil which affects the organisms living in the soil. Therefore only the determination of the chemical content in soil is not enough for the evaluation of ecological risk posed by the contaminated soil (Loureiro *et al.*, 2005). Ecotoxicological test systems along with chemical analyses detect not only the total pollutant and their metabolites in the soil but also the bioavailability, mobility and uptake of pollutant by organisms (Hund-Rinke *et al.*, 2002). Developments of an integrated ecosystem based assessment to define the ecological quality are being carried out on the basis of physico-chemical properties, biological abundance and diversity and/or chemical characters (Borja *et al.*, 2008). These assessment tools should be able to give the early warning about the exposure in order to stop the environmental degradation.

In soil ecotoxicology, acute and chronic standardized tests are often used for toxicity of chemical (Lokke & van Gestel, 1998). At present, Environmental risk assessment for regulations of a chemical are done by comparing the exposure level to no-effect levels. According to European risk assessment guidelines (EC, 2003); the acceptability of a chemical compound is based on the ratio between the predicted environmental concentration (PEC) and the predicted no-effect concentration (PNEC). Environmental risk assessment initially was more focused on the simple and clear endpoints, lethality and survival (LC50 that represents the lethal concentration to 50% of the population after a specified exposure time). Objections have been raised against the NOEC (Laskowski, 1995) and other regression-based statistics such as LC50 and EC*x* (Kooijman, 1996). The results from these statistics are not sufficient as they do not cover the whole data from the toxicity test and use only the end results from the prescribed exposure time. A process-based analysis was developed to cope with the limitations of the statistical analyses of ecotoxicological tests. This process-based analysis dealt with the sub-lethal effects and the relation between feeding, growth, development and reproduction (Jager *et al.*, 2006). Process-based analysis fosters extrapolation between species (e.g., from laboratory species to related field species of interest), between chemicals and from single-species test results to population consequences (Kooijman, 2000; Kooijman *et al.*, 2004). But most of the process-based analysis tests are chronic tests which are long and laborious (e.g., the earthworm reproduction tests lasts for 56 days; ISO, 1998a). A rapid and

sublethal avoidance behaviour test is being developed using earthworms that can be used as a quick and sensitive screening tool (Natal da Luz *et al.*, 2004).

Behavioural test is being acknowledged nowadays because of its higher sensitivity than the traditional LC50 (Hellou *et al.*, 2008). Behaviour is defined as the responses (action or reaction) coordinated by the organisms in conjunction with the internal or external stimuli. In behavioural test, the organisms are exposed to different conditions and the potential responses are reported as the behavioural endpoints. Behavioural endpoint consists of different activities that are ranked according to the time taken for the response or the relativity of the 'early warning'.

- E1: rapid response that would be expected as immediately protective
- E2: a sign of an impact that is less immediate than E1 and can progress further
- E3: behaviour after longer exposure with worse expected consequences

Table 1 show different available behavioural endpoints and the ranking of the behavioural responses observed in snails, *Ilyanassa obsolete* (Hellou *et al.*, 2009: Erskine *et al.*, 2010).

Table 1 Potential behavioural responses elicited by the exposure of a species to contaminants

Response	Example of ranking[a]
Avoidance/escape	E1
Balance, righting ability	E2
Burrowing	
Fear response	
Feeding	
Locomotion	E3
Mating, courtship response	
Memory learning	
Nesting, offspring protection	
Respiration	E3
Risk taking	

[a]Ranking illustrates the response of *I. obsolete* to harbour sediments.

An acute exposure to the contaminants has a relative change in the behaviour of the organisms which is more sensitive than survival. The results from the toxicity test combined with the behavioural endpoints can be helpful to identify the chemicals that need to be reduced to improve condition of the site. Behavioural tests are fast, easy to perform, noninvasive, cheap and have high ecological relevance (Hellou, 2011). However, behavioural

tests are not aimed to replace the conventional toxicity tests but can be used as 'early warning' signals for risk assessment (Yeardley *et al.*, 1996). Age and reproductive stage, seasonal variations and freshwater-marinewater organisms might affect the behavioural results. Therefore, the development of integrated behavioural tools along with chemical and toxicological aspects is essential for the management of sustainable ecosystems.

1.4 Purpose of the study

Most of the studies regarding the effect of TBT have been focused on organisms of higher trophic level and only handful of those regarding the lower level. This experiment is focused on studying the effect of TBT on sediment dwelling benthic organism. The main objective of this experiment is

1. To study the sensitivity of *Lumbriculus variegatus* to TBT contamination.
2. To compare the sensitivity of behavioural endpoints to the traditional toxicity endpoints (growth and reproduction)
3. To test the effect of sediment on effect of TBT
4. To study usefulness of behavioural endpoints in developing risk assessment of contaminated sediment

The behavioural endpoints can be more sensitive than the responses studied in traditional toxicity tests. Therefore, they might be used as the early warning signs of contamination. This study will give us new information about the sensitivity of *L. variegatus* and its applicability to develop risk assessment which could allow the counter reaction before the sediment is acutely contaminated.

2 MATERIALS AND METHODS

2.1 Test organisms

Sediment dwelling oligochaete *L. variegatus* was chosen as a test organism to study the behavioural endpoints and the chronic toxicity endpoints of the experiment. It has been widely used in sediment toxicity and bioaccumulation tests due to its representation as an ecological relevant component of freshwater ecosystem, suitability for assessing chronic

endpoints (growth, reproduction) and its sufficient biomass to assess bioaccumulation assays (Burton *et al*., 1992; Giesy & Hoke, 1989). It is exposed to the contaminants in the sediment via all important routes of concerns such as ingestion of the contaminated sediments and through surface epithelium (Phipps *et al.*, 1993). True sediment ingesting behaviour of *L. variegatus* makes them a good test organism for studying the toxicity and bioaccumulation of hydrophobic sediment-bound contaminants such as TBT (Leppänen & Kukkonen, 1998a).

A typical behaviour of *L. variegatus* is it buries into the sediment and then protrudes its tail into the overlaying water. It is mostly for gas exchange and also for excreting faecal pellets on top of the sediment. These behaviour results in bioturbation process through which the contaminants in the sediment are released back to the water column (Konovalov *et al.*, 2010). The faecal pellets can be used to measure the feeding rate of the organisms. Reproduction is usually by asexual fragmentation where a worm self-amputates into two or more body fragments. A new head, tail or both regenerates from each surviving fragment which eventually grows into a new worm.

The worms used in this experiment were from the University of Eastern Finland where it had been maintained in culture aquaria at 20±2 °C in a 16:8 light:dark cycle. Medium sized worms which did not show any signs of recent amputation were selected for both the behavioural and toxicity tests. The worms were then transferred into 100 ml beakers filled with 50 ml freshly prepared artificial water. The worms were allowed to acclimate to the lab conditions for overnight prior the experiment.

2.2 TBT and concentrations used

Tributyltin chloride (TBTC) used for this experiment was acquired from Sigma Aldrich. The molecular weight of TBTC was 325.51 g/mol with density of 1.207 g/mL at 25°C (96% purity). The molecular formula of TBTC is $[CH_3(CH_2)_3]_3SnCl$.

For the experiment, TBT concentrations ranging from 0.3 to 35 000 µg/Kg of sediment dry weight was used. As it was difficult to dispense the lower concentration of TBT with pipette, it was decided to make a stock solution out of the TBTC. Due to low solubility of TBT in water, TBTC was mixed with Ethanol (C_2H_5OH) to make the stock solutions. First, a stock solution of 5 µL with the ratio 1:100 of TBT and ethanol was prepared. Then, second stock solution of 5 µL with the ratio of 1:100 of the first stock solution and ethanol was prepared

and finally a third stock solution with the same ratio of 1:100 of second stock solution and ethanol was prepared. The density of the stock solution were 12.07 µg/µL, 0.1207 µg/µL and 1.207 ng/µL for the first, second and third stock solution respectively. The concentration was calculated according to the dry weight of the sediment per kilogram. The required amount of TBT solution to be spiked in the sediment was calculated using the process as described below.

The required weight of sediment was taken in a beaker. Dry weight of the weighed sediment was calculated. Then, it was multiplied by the required concentration of TBT. The result was then divided by the density of the TBT solution and the final result is the volume to be pipette from the stock solution to get the required concentration of TBT per kilogram dry weight of the sediment.

For example,

Required concentration (C) = 35 000 µg/Kg of sediment DW

Weight of the sediment (w) = 250 g = 0.25 Kg

Dry weight of the sediment (dw) = 16%

Density of the TBT solution (ρ) = 12.07 µg/µl

Then,

Total dry weight of sediment (DW) = w × dw = 0.25 Kg × 16/100 = 0.04 Kg

Weight of TBT required (W) = DW × C = 0.04 Kg × 35 000 µg/Kg = 1400 µg

Finally,

Required volume of TBT solution = W/ρ = 1400 µg/12.07 µg μl^{-1} =115.99 ~ 116 µl

Therefore, 116 µl of TBT solution from the stock solution is required to get TBT concentration of 35 000 µg/Kg of dry weight solution for 250 gram of sediment.

The volume of TBT required for other concentrations were also calculated in the same way. Table 2 shows the TBT concentrations, weight of the sediment, stock solution used and the volume of the solution calculated. Dry weight of the sediments used in the experiment was

52% for the artificial sediment and 16% for the Lake Höytiäinen sediment. The density of first, second and third stock solution is already mentioned above.

For the control beakers, they were spiked with appropriate volume of solvent (ethanol) only. The amount of ethanol was added according to the amount of ethanol in the stock solution for the highest concentration. It was added because to make the control sediment more similar to the TBT spiked sediments.

Table 2 Volume of TBT extracted from the stock solution

Concentrations (µg/Kg)	Weight of sediment(g)		Sediment dry weight		Stock Solution	Vol. of the solution (µl)	
	Artificial	Höytiäinen	Artificial	Höytiäinen		Artificial	Höytiäinen
35 000	250	250	130	40	first	377.11	116
3 000	450	450	234	72	first	58.18	18
300	450	450	234	72	second	581.83	180
30	450	450	234	72	second	58.18	18
3	450	450	234	72	third	581.83	180
0.3	450	450	234	72	third	58.18	18

2.3 Test sediments

Six natural sediments and one artificial sediment were used in this experiment including for the preliminary test. The natural sediment were obtained from the Finnish lakes Parkkimajärvi (P), Junttiselkä (J), Laakajärvi (L), Kirkkoselkä (K), Sysmäjärvi (S) and Höytiäinen (H) (Figure 3). Most of these lakes are highly contaminated with heavy metals due to mining activities. The heavy metals found in the sediments were nickel (Ni), zinc (Zn), copper (Cu) and arsenic (As) and some of the chemical concentration is shown in Table 3. Höytiäinen sediment was used as the reference sediment for studying the behaviour of *L. variegatus* in this study as it was clean compared to other sediment.

Table 3 Sediment concentration of Cu, Cr and As in mining-effected sediments

Lake	Cu (mg/Kg)	Cr (mg/Kg)	As (mg/Kg)
Junttiselkä	167	56	12.3
Laakajärvi	27	28	8.4
Kirkkoselkä	266	63.55	21.5
Sysmäjärvi	86	60	47.5

Parkkimajärvi	39	58	13.2
Höytiäinen (Ref.)	46.2	34.8	9.2

The artificial sediment was prepared in the lab according to the guidelines of OECD for the testing of chemicals (OECD 2007, Annex 4). The constituent of dry matter in the artificial sediment were 5% peat (particle size ≤ 0.5 mm), 75% quartz sand (Grain size ≤ 2mm, but 50% of the sand should be in the range of 50-200 µm) and 20% kaolinite clay. First of all, the peat is mixed with MQ- water and stirred well to prepare a suspension. The pH of the mixture is adjusted to 5.5±0.5 with $CaCO_3$. The suspension is then stirred for two days using an electric drill for stabilization of the pH. The pH is measured again and is adjusted to 6.0. Other constituents are added along with additional MQ-water and mixed to obtain homogenous sediment. The pH of the final mixture is adjusted between 6.5 and 7.5 with $CaCO_3$. A sample of final sediment mixture is taken and dried overnight at 105°C to measure

Figure 3 Locations of the natural sediments obtained for the experiment. Map by GTK Finland. Modified after Saarela et al. 2014

the dry weight. The dry weight of the sediment prepared for this experiment was 52%. TBT stock solution was then spiked to the artificial sediment and the reference sediment (Lake

Höytiäinen) as described in 2.2. As the artificial sediment lacks nutrient required for the benthic organisms, external food source is added to the sediment couple of days before the tests begins. In this experiment, urtica powder (ground, dried leaf matter from *Urtica sp.*) was added to the sediment at 0.5% of sediment dry weight content.

2.4 Parameters tested

The main parameter tested in this experiment was the selection/avoidance behaviour of the *L. variegatus* over an exposure period of 48 hours. Avoidance of the organisms from the contaminated sediment implied that the sediment was not suitable for the worms to survive on. Before starting the experimental run, for each replicate, 8 worms of similar length were transferred to a 100 ml beaker for the worms to acclimate to the lab conditions. The worms were then put into the beaker and left for selecting the suitable sediment for next 48 hours. After the exposure time of 48 hours, the worms were sieved out from the sediment using a 200 µm sieve. The number of worms in the sediment was counted and noted down.

The growth and reproduction rate of *L. variegatus* over an exposure period of 28 days was also studied in this experiment. The growth was studied as the change in biomass of the worms in the sediment over the exposure period. Before starting each experimental run, when the test worms of similar length were being selected, extra 30 worms of similar length to the test worms were also separated. These extra worms were used to determine the initial biomass and were compared to the final biomass to calculate the change in biomass over the exposure period. The fresh worms were weighed for the measuring the wet weight (ww). The extra worms were dried overnight in the oven at 105°C to measure the dry weight (dw). The worms were sieved out of the sediments using a 200 µm sieve at the end of exposure period. The worms were transferred to beakers containing freshly prepared artificial water (approx. 50 ml) and left for 6 hours for depuration. This is to empty the guts of worms which might contain the sediments particles. The sediments otherwise will affect the final biomass altering the final result. After the depuration, all the worms from a single replicate beaker were weighed at same time to measure the wet weight (ww). The worms were dried overnight in the oven (105°C) and the dry weight (dw) of the worms was measured. All the biomass measurements were done using a microbalance (Sartorius 4503). The reproduction rate was calculated by comparing the number of individuals sieved out from each replicate to the initial number of worms added i.e. 10.

2.5 Experimental setup

In this study, two types of test (selection behaviour and toxicity) were performed. Each test had its own experimental setup. All experiment for the selection behaviour test was carried out in a rectangular glass boxes (70×100 mm). The box was divided into two equal compartments using a removable plastic split. A line representing the split was drawn on the outer side of the box (Figure 4). The control (clean) sediment was placed in one of the compartments and TBT spiked sediment on the other compartment. Same method was applied for both artificial and Höytiäinen sediment.

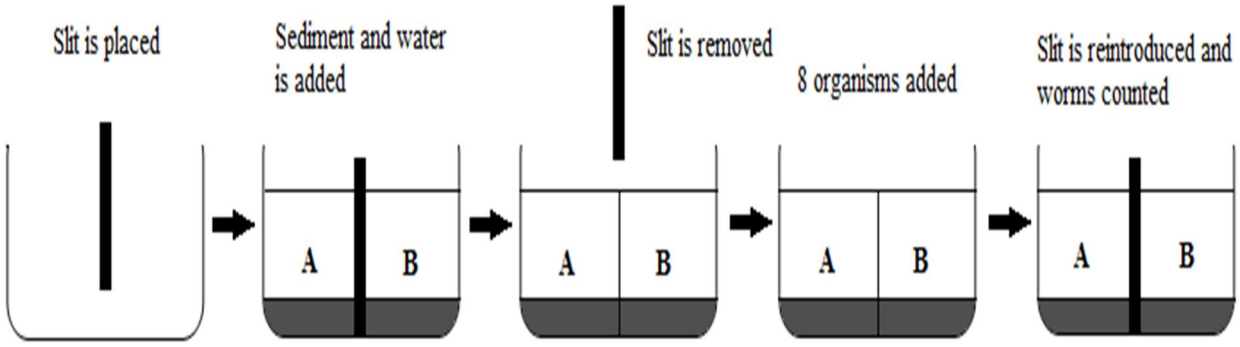

Figure 4 Scheme of the avoidance/selection behaviour response test setup

Approximately 50 g of sediment was poured on both compartments and same thickness of the sediment was maintained on both sides. The box was then filled with about 200 ml of artificial freshwater. (1 mMol hardness: $MgSO_4.7H_2O$, KCl, $CaCl_2.2H_2O$, $NaHCO_3$). The pH of artificial water was adjusted between 6 and 9 with HCl. Lake Höytiäinen sediment has the tendency to decrease the pH of the overlaying water with time therefore, the pH of artificial freshwater used in this sediment was adjusted close to 9. But artificial sediment tends to increase the pH of the overlaying water. Therefore the pH of the artificial freshwater used in artificial sediment was adjusted close to 6. The split was then removed and the system was left to settle overnight. At the start of the experimental run, 8 acclimatized *L. variegatus* were dropped to the sediment around the centre of the box. After the 48 hours test period, the split was reintroduced in the marked area and the worms were sieved using 200 µm sieve. Numbers of worms in each compartment were counted. In case worm/s was missing, it was presumed dead due to the contaminated sediment. TBT concentrations tested in behavioural test were 0.3, 3, 30, 300, 3000 and 35000 µg/Kg of sediment DW.

The toxicity tests were carried out in a 200 ml glass jars. The jars were filled with 50 gram of sediment and 150 ml of artificial freshwater was poured over the sediment. The jars were then closed with laboratory film and aerated with a glass Pasteur pipette reaching into the water. They were left overnight for settling down. Next day, 10 acclimatized *L. variegatus* individuals were dropped in each jars. The test room was kept at room temperature of 20±1 °C and a 16:8 h light:dark cycle was used (OECD 2008). The pH of the overlaying water and sediment, oxygen, temperature and ammonia concentration were measured in selected jars from each concentration. The pH, oxygen and temperature were measured at the beginning of experiment (day 0), mid of the experiment (day 14) and at the end of the experiment (day 28) whereas ammonia concentration was done only at the end of the experiment (day 28). Minimum standard for oxygen saturation was set at 40% and pH of overlaying water was at the range of 6-9. Even if one of the jars was outside of the range, water from the whole set was replaced with freshly prepared artificial freshwater.

Both the selection behaviour and toxicity tests had four replicates for each concentration of TBT. But due to limited sediment availability, 300 and 35 000 µg/Kg TBT concentrations in selection behaviour test for artificial sediment had 3 replicates each.

2.6 Preliminary experiment

Since the experiment was based on the behaviour of *L. variegatus*, in the preliminary test we decided to study the burrowing behaviour of the worms in different metal contaminated sediment. The six sediments used were from Finnish lakes Parkkimajärvi, Junttiselkä, Kirkkoselkä, Laakajärvi, Sysmäjärvi and Höytiäinen. The purpose of this test was to study the burrowing behaviour of the worm and select reference sediment for the main experiment. Only if *L. variegatus* burrows into the sediment then we can study the toxic effect of the chemicals on them.

In the test, 5 replicates for each of the natural sediment were prepared. In a 200 ml jar, 50 gram of sediment was poured and then 150 ml of artificial water was then added in the jar. An individual worm was dropped in the jar and the time taken by the worm to burrow into the sediment was noted down. The worm took less time to burrow into Lake Höytiäinen and Parkkimajärvi sediment compared to other sediments.

We also decided to test the selection behaviour of *L. variegatus* in these two sediments against Kirkkoselkä sediment, high metal contaminated sediment. 4 replicates containing Parkkimajärvi and Kirkkoselkä sediment and 4 replicates containing Höytiäinen and Kirkkoselkä sediment were prepared. 8 individuals of *L. variegatus* were dropped in each jar. After 48 h exposure period the worms were counted in all sediment.

2.7 Data processing

Data collected were put in Excel format at the beginning. One way analysis of variance (ANOVA) was used to study the effects of treatments. The graphs were created with GraphPad Prism 5 (GraphPad software).

3 RESULTS AND DISCUSSIONS

3.1 Preliminary test results

In the preliminary test, the burrowing time of *L. variegatus* was recorded. The burrowing time differed among the different metal contaminated sediments (Figure 5). *L. variegatus* took less time to burrow into Parkkimajärvi sediment than other sediments. The average time for *L. variegatus* to burrow in Parkkimajärvi sediment was 1min 15sec longest time being 2min. In other natural sediments average burrowing time recorded was 1 min 48 sec for Junttiselkä, 7 min 44 sec for Kirkkoselkä, 18 min 17 sec for Laakajärvi, 5 min 31 sec for Sysmäjärvi and 3 min 39 sec for Höytiäinen sediment. However in the artificial sediment, no burrowing activity was seen during the time-keeping period of 2 hours. Some of the worms were still laying in the surface of the artificial sediment even for a week. No burrowing activity in the artificial sediment might be due to high dry weight of the sediment which was 70%. In later tests, the dry weight was kept lower to 50% and the burrowing activities could be seen afterwards. The metal contamination in the sediment had less effect on the burrowing behaviour of *L. variegatus.* Highly contaminated sediment of Junttiselkä had nearly the same burrowing time as the clean sediment from Parkkimajärvi.

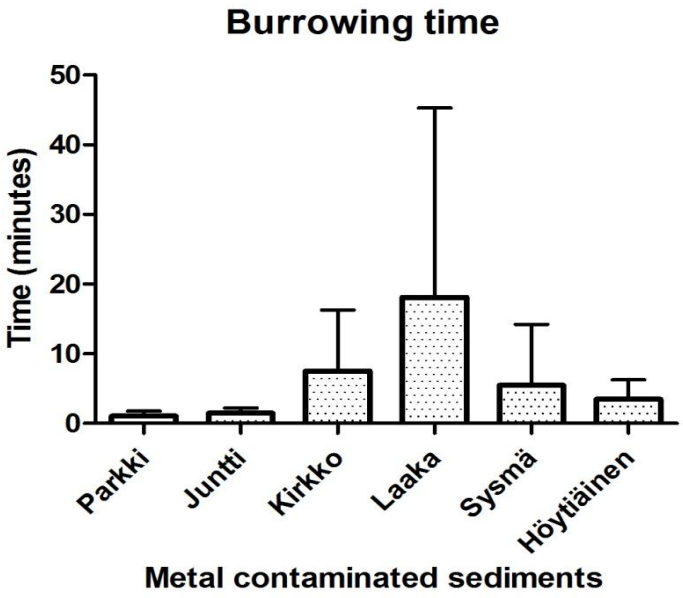

Figure 5 Burrowing time of *L. variegatus* in different metal contaminated sediments

The pH of the overlaying water and the sediment were measured before the worms were added and at the end of the tests i.e. day 7. The pH of the overlaying water should be between 6 and 9 for the toxicity tests. But there was problem keeping the pH value between neutral ranges of 6-9 in the most of the field-contaminated sediments. Acid mine drainage causes the oxidation of sulphides to sulphates. The digging activities of the affected system exposes these reduced sulphates to air leading to formation of sulphuric acid hence decreasing the pH of the system (Luoma and Rainbow, 2008).

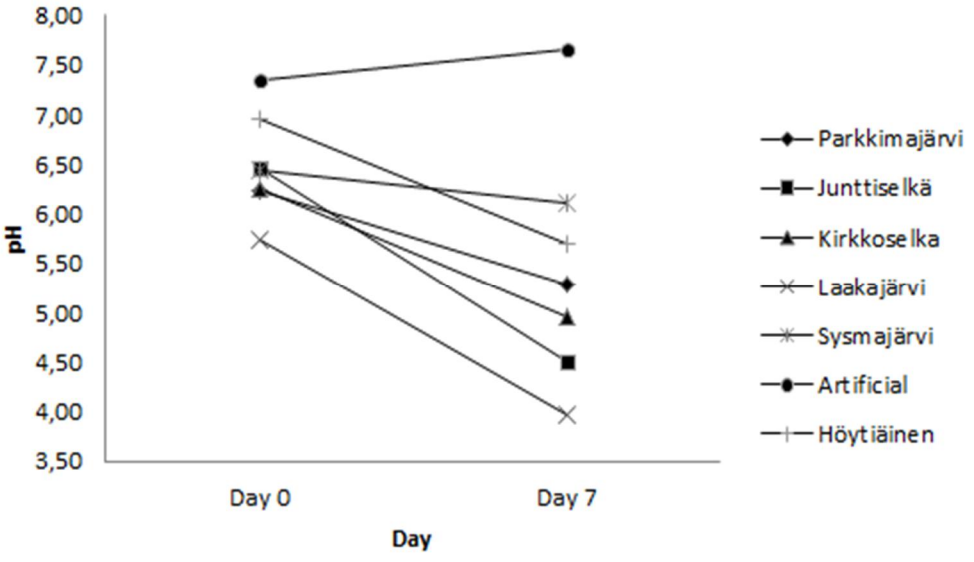

Figure 6 pH development over 7 days in mining affected sediments (Par, Jun, Kir, Laa,Sys), Clean reference sediment (Höy) and artificial sediment

The pH development over 7 days in the tested sediments can be seen in figure 6. The pH of overlaying-water in all natural, field-contaminated sediments before the test began was above 6 except for Laakajärvi (pH 5.75). But after day 7, pH of overlaying water in all sediments dropped below 6 except in Sysmäjärvi. Laakajärvi had the lowest pH with below 4 and pH in both Junttiselkä and Kirkkoselkä were below 5. This indicates release of sulphuric acid or acid generating sulphides from those sediments. Sediment pH was also below 6 in all of the natural sediments with exception in 2 Sysmäjärvi replicates. On the other hand, artificial sediment had pH within the neutral range. The pH of overlaying water in artificial sediment was 7.66 at day 7 and the sediment pH was 6.47.

From the results, we can already see that sediments from Junttiselkä, Kirkkoselkä and Laakajärvi were not suitable to carry out the toxicity tests (growth, reproduction and mortality) of 28 days using benthic organisms. Sysmäjärvi could be suitable according to the pH but due to high toxic effect of the sediment due to high contamination of heavy metals, it was not good to select it as the reference sediment for carrying out the experiment for the study. Sediment from Parkkimajärvi and Höytiäinen had pH which was suitable for carrying out the experiment.

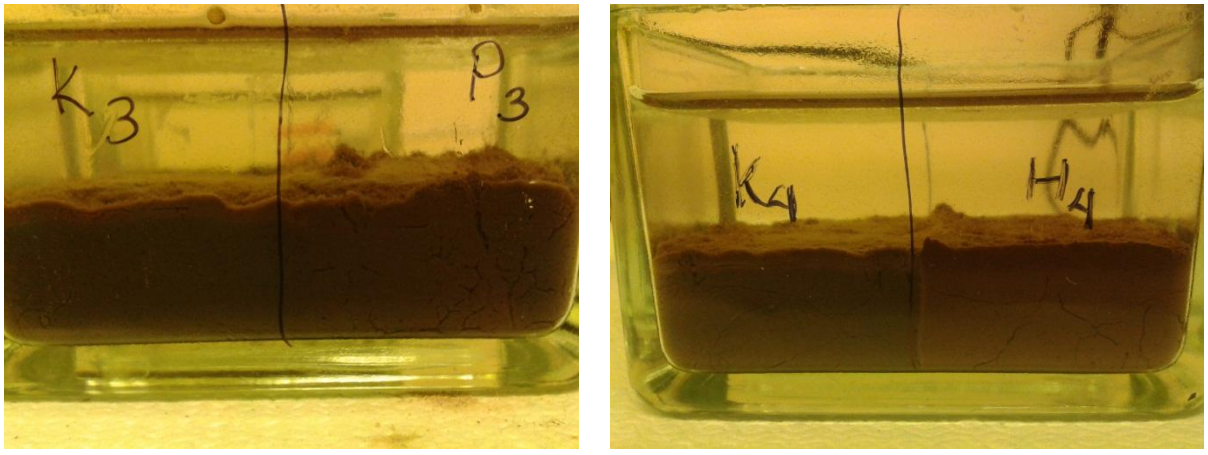

Figure 7 Selection behaviour of *L. variegatus* between Kirkkoselkä/Parkkimajärvi (left) and Kirkkoselkä/Höytiäinen (right)

In preliminary test, Avoidance behaviour tests of sediment between Parkkimajärvi and Kirkkoselkä & Höytiäinen and Kirkkoselkä were performed (Figure 7). *L. variegatus* showed clear avoidance of Kirkkoselkä sediment in both the cases. 100% avoidance of Kirkkoselkä was seen in the former case whereas only 15% of worms were found in Kirkkoselkä in the latter case. The avoidance may be due to high contamination of heavy metal in Kirkkoselkä sediment. Since there was clear avoidance shown by *L. variegatus* between highly contaminated sediment and less contaminated sediment, we concluded from the preliminary

test that avoidance behaviour could be used as end point in ecotoxicological tests. There was not much difference between Parkkimajärvi sediment and Höytiäinen sediment. Reference sediment was selected on the basis of preliminary test. Due to less contamination of Lake Höytiäinen than Parkkimajärvi and pH of Höytiäinen being more stable, Höytiäinen sediment was selected as the reference sediment for the study purpose.

3.2 TBT Toxicity test (Artificial sediment)

As there were very few literature reviews about the effects of TBT on benthic organisms and less was known about the concentration at which TBT was considered toxic to these organisms, we decided to carry out experiment for the concentration ranging from 0.3 to 3000 µg/Kg of sediment DW at first. But as there was not much significant difference in growth and reproduction between the different concentrations, a second experiment (Test 2) with the highest concentration 35 000 µg/Kg of sediment DW was carried out. Freshly prepared artificial sediment was used with no additional chemical except food source i.e. Urtica powder.

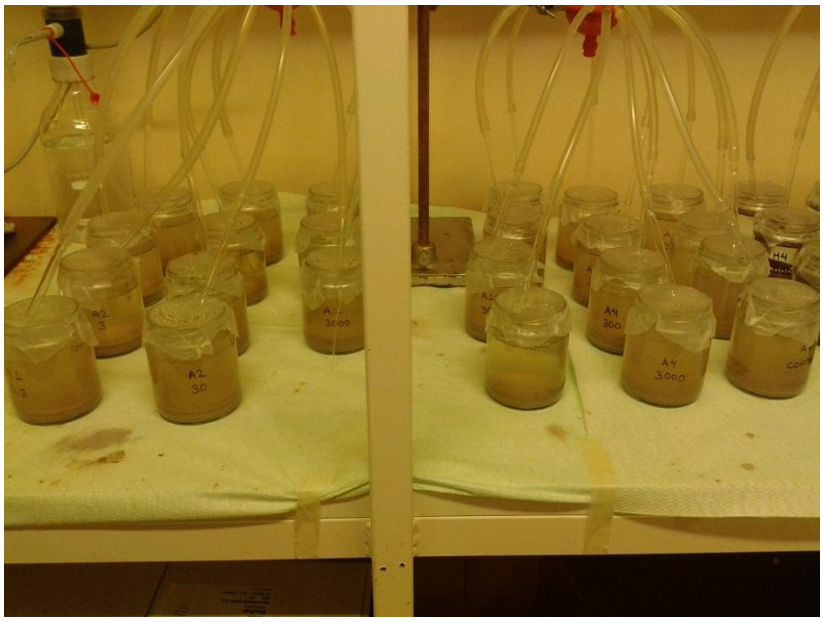

Figure 8 Experimental setup of TBT toxicity test in the artificial sediment

The initial individual biomass of the worms used in this experimental run was 1.264 ± 0.232 mg dw (8.201 ± 0.856 mg ww) for Test 1 and 0.658 ± 0.087 mg dw (4.14 ± 0.139 mg ww) for Test 2. No significant response by the test organism was seen for the growth and reproduction rate in all concentrations of Test 1 compared to Control 1 but in Test 2, both

control 2 and highest concentration (35 000 µg/Kg) showed significantly lowered growth rate and reproduction rate compared to Control 1 (One-Way ANOVA, $p < 0.05$) (Figure 9). There was not much variation between the concentrations except for the highest concentration. The organisms were able to grow (increase the biomass) significantly over the exposure time of 28 days. The biomass increased by 73.38 ± 7.43% in control 1 over the exposure period of 28 days. In 3 concentrations (0.3, 3 and 300), the growth was higher than that of control sediment 85.45 ± 15.18%, 85.80 ± 12.46% and 84.80 ± 2.27% respectively. However, in the highest concentration, no organisms were found in the sediment at the end of 28 days exposure period. Therefore, the organisms were presumed dead due to high toxicity of TBT. It meant 100% mortality if the organisms due to high concentration of TBT. Also in control 2, the growth was very low (15.38 ± 4.63%) compared to control 1. It might be due to the difference in lab temperature while performing Test 1 and Test 2. Test 1 was done during summer where the lab temperature was high affecting the sediment temperature (24 ± 1°C). Sediment temperature in Test 2 was however 20 ± 1°C which was the normal lab room temperature. The growth rate ranges from 15.38 ± 4.63% (Control 2) to 85.80 ± 12.46% (3 µg/Kg TBT concentration).

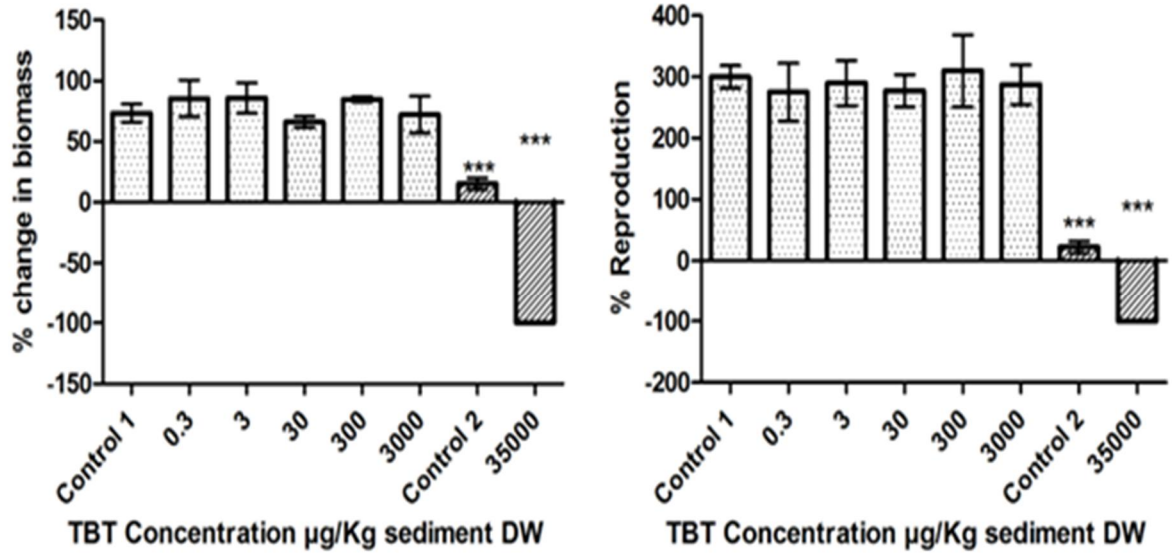

Figure 9 Growth % (change in biomass on dw) and reproduction (% increase in numbers) of *L. variegatus* in artificial sediment spiked with different concentrations of TBT for an exposure time of 28 days. *** Significant compared to Control 1 with $p < 0.05$

There is not much variation seen on the reproduction rate in concentration up to 3000 µg/Kg. Reproduction rate is high and the organisms tend to quadruple in some cases. The reproduction rate ranges from 275 ± 47.26% (0.3 µg/Kg TBT) to 310 ± 58.88% (300 µg/Kg

TBT). Reproduction in all but 300 is lower than Control 1 (300 ± 18.26%). Since all of organisms were dead in 35 000 µg/Kg TBT concentration, the mortality rate was calculated 100%. Also in control 2, the reproduction rate was much low (22.5 ± 9.57%) compared to control 1. The growth and reproduction rate tends to correlate with each other in all concentrations. The increase in growth is accompanied by increase in reproduction and vice versa. But an unusual pattern of increase and decrease in rates are seen between adjacent concentrations for both growth and reproduction. From this experimental setup, we can conclude that TBT tends to show less adverse effects on *L. variegatus* in lower doses. But high mortality rate can be seen in higher concentrations.

Although there is correlation between the growth rate and reproduction rate in different concentrations of TBT, still there is huge difference between the growth and reproduction rate in the same concentration. Two explanations can be suggested for this situation. In control 1 the reproduction is 300 ± 18.26% whereas the growth rate is just 73.38 ± 7.43%. It might be because the organisms went through multiple morphallaxis during the 28 days experimental period. After morphallaxis, the new-born individuals would stop feeding for 2-7 days depending upon the end (head or tail) hence stop the growth (Leppänen and Kukkonen 1998a). Leppänen and Kukkonen (1998a) stated that the reproduction depends upon the individual's biomass and minimum weight required for reproduction was 9 mg ww. High reproduction rate might be due to easily availability of nutrient source (urtica powder). Urtica powder was added just before commencing the experiment which gives it less time to be sorbed in the soil particles. High supply of food source mitigates the naturally occurring stress factors for *L. variegatus*. Due to this, the organisms tend to grow faster and are ready for morphallaxis earlier. But due to multiple morphallaxis and more number of individuals in a single jar, there is less food available at the end of the experiment. And also it is possible that when the worms were sieved, the worms might have undergone morphallaxis recently and had stopped the feeding.

But an alternative explanation could be theory of stress-induced reproduction in the organisms. This is just opposite of the first explanation. Here, unfavourable conditions causes stress in the organisms triggering reproduction before it has reached the size needed for normal morphallaxis. In such cases, reproduction occurs without any growth or even leads to reduction of biomass (Pakarinen *et al.,* 2011). In this experiment, temperature could have been the stress factor. In Test 1 where the temperature was 24 ± 1°C, the difference between growth and reproduction in control sediment is quite high (73.38 ± 7.43% for growth and 300

± 18.26 for reproduction). But in Test 2 when the temperature was 20 ± 1°C (OECD recommended temperature), the difference is very less (15.38 ± 4.63% for growth and 22.5 ± 9.57% for reproduction). Since there is not much indication of reduced growth in low concentrations, low TBT concentrations seem to have not much effect on *L. variegatus* in the artificial sediment. Furthermore, effects of TBT in the natural sediment must be tested before it can be assumed to have less toxic effects. The artificial sediment has reduced stress factors than natural sediment which might give a new explanation to the experiment.

3.3 TBT Toxicity test (Höytiäinen sediment)

An experiment was performed to determine the toxic effect of TBT on *L. variegatus* in the natural sediment along with the experiment with the artificial sediment. As there was not much effect of TBT on these organisms in low concentration, we were curious to see how TBT will perform in natural conditions. TBT was tested in the natural sediment from Lake Höytiäinen. The sediment was deprived of any added, high quality food source. The experiment was closer to natural conditions.

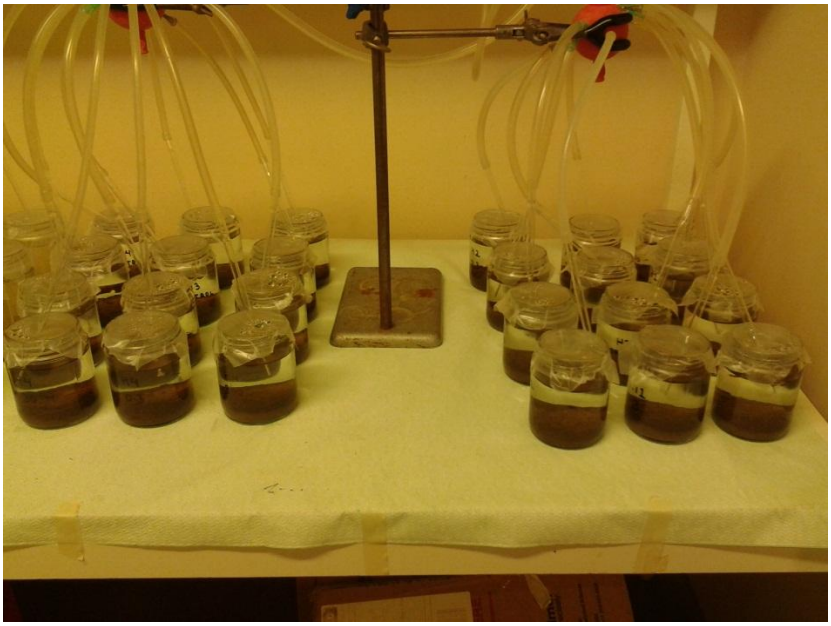

Figure 10 Experimental setup of TBT toxicity test in natural sediment

The initial individual biomass of the worms used in this experimental run was 1.037 ± 0.209 mg dw (5.922 ± 0.985 mg ww) for Test 1 and 0.658 ± 0.087 mg dw (4.14 ± 0.139 mg ww) for Test 2. The sediment seemed not to support the growth of *L. variegatus*. There was decrease in the total biomass in all the concentrations and both the controls (Figure 11). The biomass dropped by 24.78 ± 6.65% in control 1 and 4.1 ± 14.5% in Control 2 in accordance to

their respective initial biomass. Similar to that in the artificial sediment, the highest concentration (35000 µg/Kg TBT) showed significantly lowered growth rate compared to Control 1 (One-way ANOVA, $p < 0.05$). There was not much difference of change in biomass between the concentrations up to 3000 µg/Kg. Decrease in the biomass in the concentrations were 21 ± 7.02% , 20.33 ± 9.55%, 17.58 ± 4.14%, 29.23 ± 7.17%, 20.33 ± 10.81%, 95.98 ± 4.79% for TBT concentration of 0.3, 3, 30, 300, 3000 and 35000 respectively.

The reproduction rate in the natural sediment was very low compared to that in the artificial sediment. There was slight increase in the number of organisms in all of the concentrations in Test 1. The average number of organisms in control 1 increased by only 22.50 ± 12.58% over the exposure period of 28 days whereas it had even low increment of 7.5 ± 5% in control 2. 35000 µg/Kg TBT concentration had high mortality rate at 87.5 ± 15% over the exposure period of 28 days and was significant compared to control 1 (One-way ANOVA, $p < 0.05$). Reproduction rate in other concentrations were 17.5 ± 9.57%, 12.5 ± 15%, 17.5 ± 5%, 13.33 ± 15.28% and 22.50 ± 15% for TBT concentrations of 0.3, 3, 30, 300 and 3000 respectively. Similar to that in the artificial sediment, an unusual pattern of increase and decrease of growth and reproduction can be seen in adjacent concentrations in the natural sediment as well.

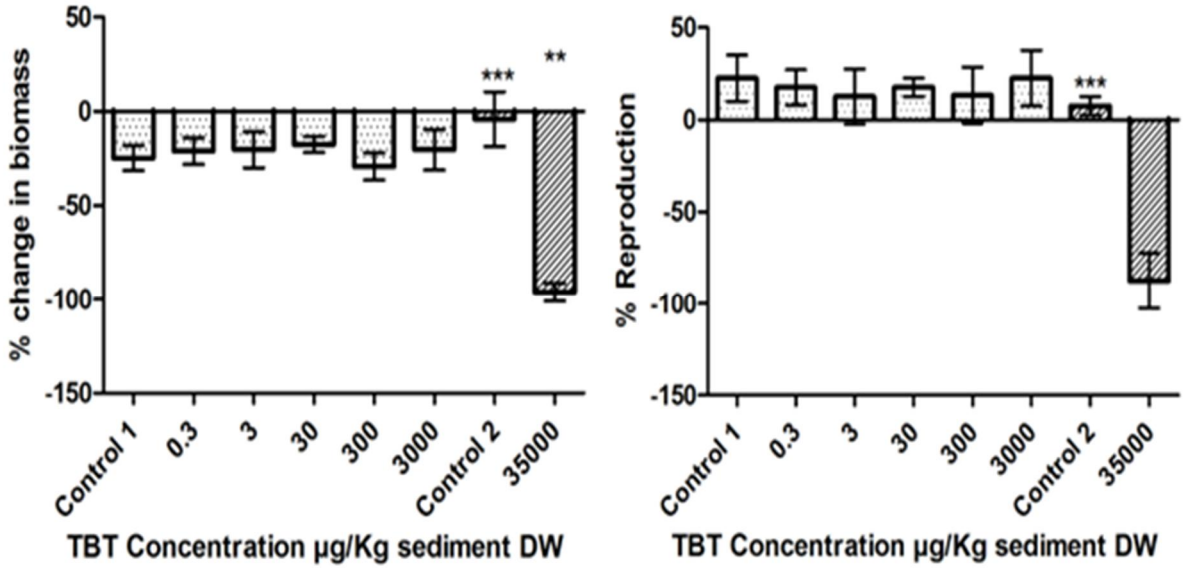

Figure 11 Growth % (Change in biomass of dw) and reproduction rate (% increase in numbers) of *L. variegatus* in natural, metal contaminated sediment (Höy) spiked with different concentrations of TBT for an exposure time of 28 days. ***significant compared

As the growth rate has been in negative for the natural sediment, we can assume that the organisms might have reduced the feeding habit during the exposure time. It might be, in

some cases, due to architomy being induced after which the worm stops feeding for certain time. But as the increase in number of organisms is way less than 100% in all the sediments, it can be indicated that not all the worms underwent reproduction. As we take the weight of all the worms from a single test system at once, an individual worm weight cannot be calculated. Some of the worms might be larger than others but still did not induce architomy. It all depends on the individual characteristics between the worms. Another possibility is that more individuals could have undergone reproduction but only few individuals survived at the end of the exposure time. Unlike artificial sediment where the reproduction rate was much higher, natural sediment lacks high supply of food source. These natural stresses could have affected the growth and reproduction of *L. variegatus* in the natural sediment.

There aren't many literatures discussing on the effect of TBT to the benthic community. Most of the published articles focus on oysters and higher trophic levels. Gildemeister (2006) studied the effect of TBT on *L. variegatus* over an exposure of 28-day in artificial sediment. The concentrations used were 2, 12, 58, 290, 1500, 7300 and 36500 µg/Kg TBT-Sn. Growth and reproduction rate were higher compared to the control and no worms were found in the 7300 and 36500 µg/Kg TBT-Sn treatment after 28 days. An EC_{50} of 1.12 mg/Kg TBT-Sn for number of worms and EC_{50} of 0.98 mg/Kg TBT-Sn for biomass were calculated using probit analysis.

The result from this tests show that low concentrations of TBT does not have much effect on growth and reproduction of *L. variegatus.* The long term effect might be severe even in low concentrations but for the exposure period of the test, growth and reproduction was higher than in clean sediment. But as the concentration goes higher more effect of TBT is seen and 100% mortality is seen in the highest concentrations. In the experiment performed by Gildemeister (2006), no worms were found in the 7300 µg/Kg TBT treatment. So, from these experiments it can be concluded that the toxic effect of TBT in short term experiment can be seen starting between 3000 and 7300 µg/Kg in artificial sediment. However in natural sediment, the effect of TBT starts to be seen from the lowest concentration. The decrease in the biomass of the organisms is seen in all of the concentrations including the control. Also the reproduction rate is low. The long term effect of TBT in natural sediment might be severe for benthic organisms even at low concentration. Lack of food source and other natural stresses might also be a cause beside the TBT in natural sediment as the control sediment had decreased growth rate similar to the TBT spiked sediment. As mentioned earlier, due to less information about effect of TBT on benthic organisms we cannot be certain about how much

role did the natural stresses play. More detailed studies in both natural as well as artificial sediment should be done about the effect of TBT on benthic organisms before a strong conclusion can be made.

3.4 Avoidance behaviour test

Besides the sediment toxicity tests, avoidance/selection behaviour test was also performed for *L. variegatus* in both the artificial and the natural (Höytiäinen) sediment. The worms were exposed to a box containing control and contaminated sediment for 48 hours and later sieved. Treatments used in the test ranged between 0.3 and 35000 µg/kg of Sediment DW. The results of the test can be studied with the help of figure 12 and figure 13. The worms tend to show some avoidance from the TBT contaminated sediment in all of the treatments. There was not much difference in avoidance percentage between the lowest and the highest concentration in the artificial sediment. The lowest avoidance was seen in 300 µg/Kg where 50% of organisms (average) were found in contaminated sediment and highest avoidance was seen in 35000 µg/Kg where more than 70% of the organisms were found in clean sediment. Percentage of organisms found in other treatments after the exposure period of 48 hour were 62.5% in 0.3, 63.5% in 3, 65.6% in 30 and 59.4% in 3000 µg/Kg of sediment dw. The control replicate had clean sediment on both sides and just 2 replicates were used due to less availability of the sediment. No mortality was seen during the exposure period in any of the treatments.

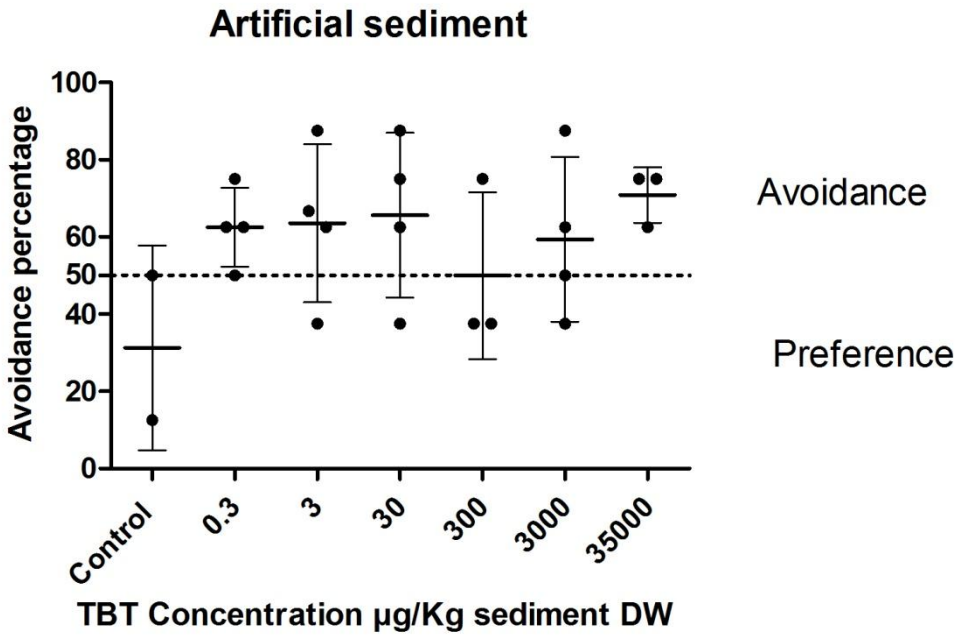

Figure 12 Percentage of the test-organism *L. variegatus* in different treatments of TBT spiked in artificial sediment after exposure time of 48 hour.

However in the natural sediment, avoidance by *L. variegatus* in the contaminated sediment was surprisingly quite low compared to that in the artificial sediment. Organisms preferred the contaminated sediment more than the control sediment except in the highest concentration. 4 replicates for each treatment were used. In 35000 µg/Kg TBT concentration, the avoidance percentage was just below 60%. Lowest avoidance was seen in 30 µg/Kg treatment where 15.63% of organisms (average) were found in contaminated sediment. Percentage of organisms found in other treatments in natural sediment after 48 hour exposure period were 18.75% in 0.3, 25% in 3, 29.17% in 300 and 18.75% in 3000 µg/Kg. Control sediment had clean sediment on both sides of the replicate. Mortality was seen in two replicates of the highest concentration where one organism in both the replicates was missing. They were presumed dead due to the toxic effect of the contaminants.

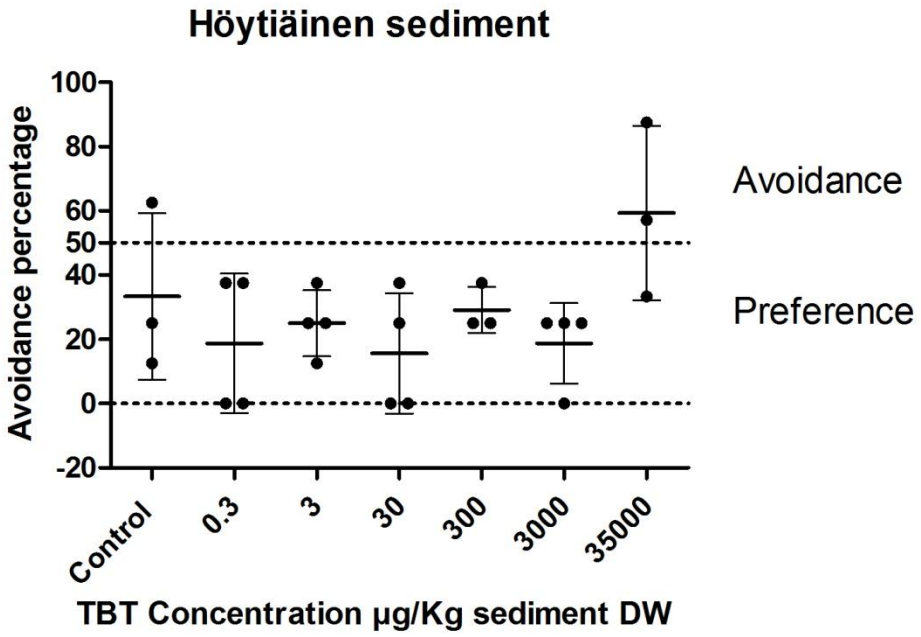

Figure 13 Percentage of the test-organism *L. variegatus* in different treatments of TBT spiked in natural (Höytiäinen) sediment after an exposure period of 48 hour

In both the natural and the artificial sediment, the control treatment had solvent-spiked sediment in both of its side. The distribution of worms is not average (50%) because of the fact that *L. variegatus* may have slight tendency to group together.

The unusual behaviour in the natural sediment might have been due to the ethanol spiked in the control sediment. Ethanol was used as solvent for TBT as described earlier in 2.2 and ethanol was also spiked to the control sediment to make it more similar to the TBT spiked sediment. As described in table 2, some treatments had more volume of the stock solution

than the other. 0.3, 30 and 3000 used less volume of the stock solution whereas 3, 300 and 35000 had more volume of it. So, there was less amount of ethanol in the sediment of the former group than that in the latter one. Also, when compared to the control sediment, there was less ethanol in the sediment of former group than the latter one. This difference in amount of ethanol could have played a role in the results from the behaviour test. The treatment where nearly equal volume of ethanol was present in control and contaminated sediment, the avoidance percentage from contaminated was more. And the treatment where there was less ethanol in contaminated sediment than control, the worm preferred contaminated sediment over control sediment.

To see if the solvent has had any role in the selection behaviour of *L. variegatus*, we decided to carry out another selection behaviour test between clean sediment and solvent control. This test was performed for both the artificial and natural sediment. In the solvent control, same amount of ethanol was spiked as in the behavioural test. Nothing was added to the clean sediment. The procedure was same as before and identical worms were selected for the test. After exposure time of 48 hours, the worms were spiked out and the numbers of worms in both sediments were counted. In case of the artificial sediment, 78% of the worms (average) avoided the sediment spiked with solvent for clean sediment. Whereas, in the Höytiäinen sediment, the avoidance of solvent spiked sediment was 100%. No worms were found in the solvent control after the exposure period of 48 hours. The result from the test is given in figure 14 below.

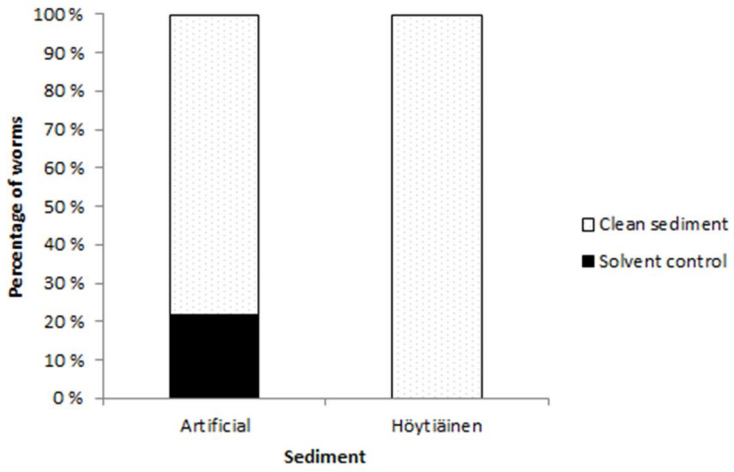

Figure 14 Percentage of worms found in solvent control vs clean sediment in artificial and natural sediment after exposure period of 48 hours

From the results of this test, it can be said that the solvent might have had some effect on the selection behaviour of *L. variegatus* in both the sediment. As the avoidance of the solvent

sediment by the organisms is much higher, the effect of ethanol cannot be ruled out. In the experiment with TBT, the sediment were spiked with TBT stock solution and kept in freezer for couple of weeks for the TBT to be adsorbed in the sediment properly. By this time some of the ethanol might have evaporated but still there might be enough ethanol present in the sediment. In the sediment which was spiked with less volume of stock solution, less ethanol is present and vice versa. This might have resulted in low avoidance of *L. variegatus* from sediment with less stock solution to clean sediment where higher level of ethanol was present. More detailed study on the effect of the solvent on *L. variegatus* should be done.

3.5 Effect on pH

The pH of the overlaying water and sediment were measured from one sample from each concentration for both the artificial and the natural sediment. According to OECD guidelines (2008), the pH of the overlaying water should be maintained between 6 and 9 throughout the exposure period. The pH of the stock solution of TBT used in this experiment was between 5.5 and 7. Though TBT does not have much impact on the pH of the test system but sediments used tend to change the pH of the overlaying water. The pH of overlaying water and sediment was measured before the start of the test run (day 0), at the mid (day 14) and at the end of the test run (day 28) to avoid reaching of values harmful to the test organisms. Random selection of a sample from each treatment was done.

The pH of overlaying water in artificial sediment had little change over the exposure period. There was slight increase in the pH by the end of the experiment. There was not much difference in pH values between the different treatments in the artificial sediment. The pH remained between 8 and 9 throughout the test run. On the other hand, the pH of overlaying water in the natural sediment decreased significantly over the exposure period. The pH in control sediment decreased from 8 at the beginning to just above 6 by the end of the experiment. The decrease was seen in the treatments as well. As seen in preliminary test, this was mostly due to the nature of Höytiäinen sediment. Low pH value was seen in two highest concentration compared to other treatments. In 35000 µg/Kg concentration, the pH value was measured below 6 at day 14 due to which the water of the whole system had to be changed. The measurement of pH done during day 0, day 14 and day 28 of the experiment is shown below in figure 15.

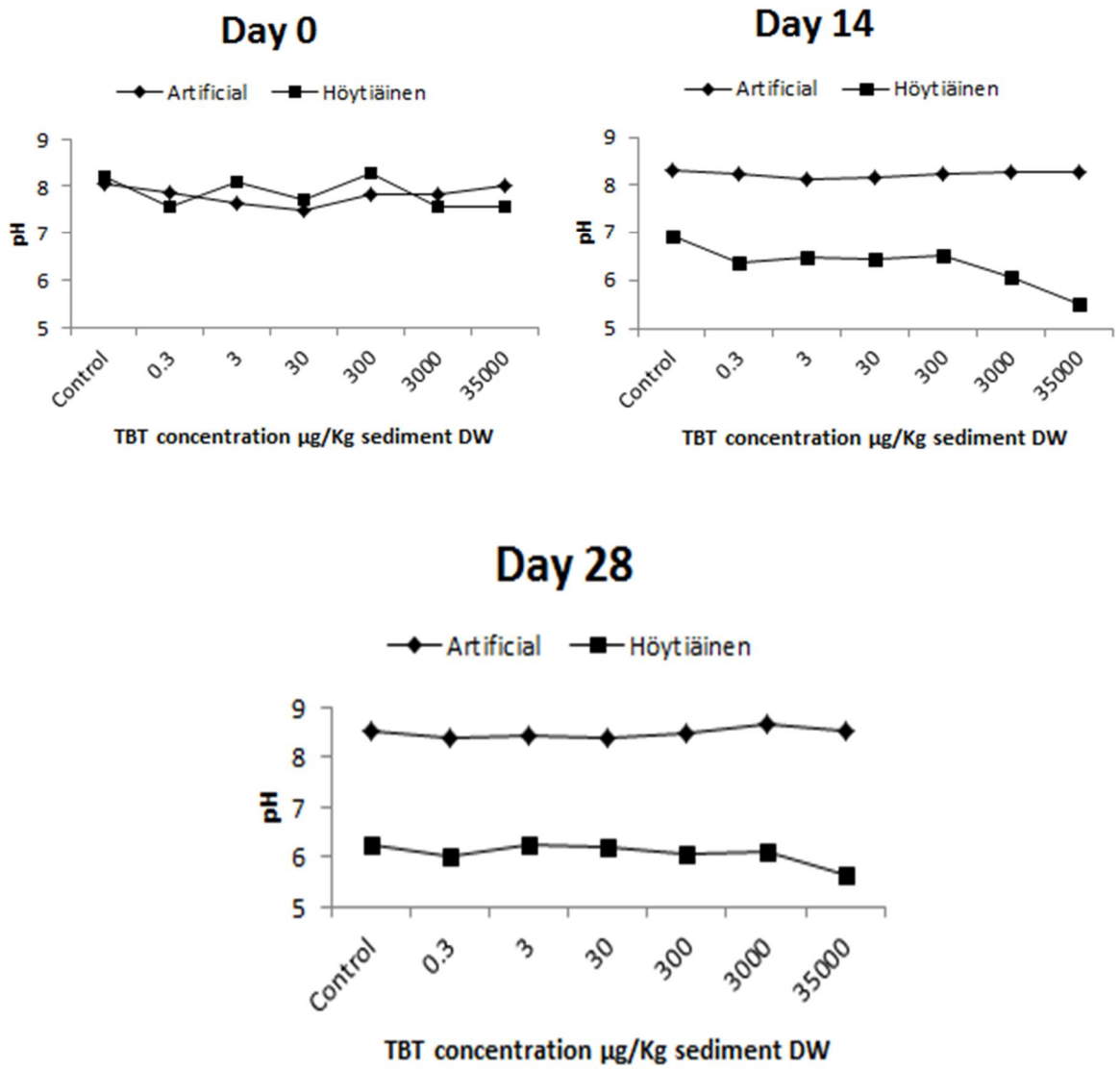

Figure 15 pH measurement of overlaying water of both the artificial and the natural sediment at day 0, day 14 and day 28 of the experiment

The pH of sediment and overlaying water influences the bioavailability of TBT. TBT at pH higher values are more bioavailable than those at lower pH-values (Fent, 1996; Looser *et al.*, 1998). Therefore, in toxicity test TBT was more bioavailable in the artificial sediment than in the natural sediment. Low K_{ow} value of TBT at lower pH than the higher pH means more TBT is found adsorbed in humic substance. Bioconcentration factor (BCF) of TBT was higher at pH 8 than in pH 5 (Looser *et al.*, 1998). But better understanding of effect of pH on bioavailability and its fate in water ecosystem has to be done in future experiments.

4 CONCLUSIONS

4.1 Toxicity tests

From the study, we can conclude that lower concentrations of TBT do not have much toxic effect on *L. variegatus* but above 3000 µg/Kg, TBT does show serious effect. In the artificial sediment, the growth of *L. variegatus* does not seem to be affected by low concentration of TBT when compared with the control sediment. 100% mortality is seen in the highest concentration. The pH of all the treatments is quiet close to the control and does not goes above 9 indicating not much effect of TBT on the pH of the sediment. However, the BCF is higher at higher pH meaning more TBT is accumulated by the organisms at higher pH.

However in the natural sediment, the growth of *L. variegatus* decreased in all the treatments of TBT. Though mortality was not seen in lower concentrations severe effect was observed in the highest concentration. Low pH reached during the study might be problematic for the worms. Further tests on other benthic organisms should be done as they might be more sensitive towards TBT than *L. variegatus.* Also, the difference in growth between the artificial and the natural sediment might be due to low external stress factor and easily available food source in artificial sediment. More study upon these factors has to be done in future investigations. Moreover, tests on TBT contaminated natural sediment should be done to study the effect on benthic organisms in real environmental scenario.

The solvent also might have effect in the results. In future studies for control, use of clean sediment and solvent control is to be done which can be combined to get control responses (pooled control) and the treatments should be compared with the pooled control (OECD, 2006a).

4.2 Behavioural test

In behavioural test, avoidance of TBT contaminated sediment by *L. variegatus* in the artificial sediment was clearly seen though in the natural sediment the avoidance was not significant. The result from the study suggests it might be due to solvent used. Therefore in future research minimal amount of solvent should be used.

Avoidance behaviour test can be regarded as valuable screening tool in evaluation of soil contamination. Results from behavioural tests can be used an early warning for sediment contamination and used to develop risk assessment tools that allows counter reaction before

the sediment are acutely toxic. Moreover, test involving different species should be used as different species react differently to the chemical stimulus. Improvement and development in the avoidance behaviour test means it can have advantage over other ecotoxicological tests for quantitative assessment of the contaminant bioavailability and toxicity.

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