OVERCOMING THE SEASONAL VARIATIONS IN FITNESS OF THE AMPHIPOD *COROPHIUM VOLUTATOR* AS AN ENVIRONMENTAL TOXICOLOGY TEST SPECIES USING LABORATORY CULTURED SPECIMENS

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I hereby declare that this thesis is my own original work. This work has not been previously submitted for any other degree at the Robert Gordon University or any other university. All external sources of information have been referenced and duly acknowledged.

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Abstract

The marine amphipod *Corophium volutator* is an important source of food for many fish and wading birds, making it an important species in an estuarine environment and a relevant toxicological test species. Historical data has indicated that seasonal variations in the fitness of *C. volutator* may make toxicity testing during summer months impossible. *C. volutator* were cultured in the laboratory to determine if variations in the fitness of wild *C. volutator* could be overcome with the use of cultured specimens. Adult and neonate *C. volutator* were cultured separately under different feeding regimes with *Tetraselmis chuii* and *Rhodomonas reticulate* as food sources. Cultures were maintained at a salinity of 30-35 ppt, with a temperature of 15 °C ± 2, dissolved oxygen was maintained at ≥80% saturation. Assessments of growth rates, time until sexual maturity, and numbers of offspring produced were made from these trials. During this study, a growth rate of 0.0648 mm ± 0.0185 mm per day was determined. The preferred feeding regime was a combination of *T. chuii* and *R. reticulate.* The average number of offspring produced per *C. volutator* over 132 days of culturing was 8.6. Experiments indicate that *C. volutator* may be cultured under laboratory conditions, however, further work is required to increase the reproductive output of cultured *C. volutator.*

Key Words: *Corophium volutator,* Laboratory culture, Reproduction, Seasonal variation, Toxicity testing, Growth rate

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List of abbreviations

Cd	Cadmium
LC_{50}	Lethal concentration, 50%
OSPAR	Oslo and Paris Commissions
PPT	Parts Per Thousand
TBT	Tributyltin
UV	Ultraviolet

Chapter 1. Introduction

1.1 *Corophium volutator*

C. volutator is an amphipod of the Corophiidae family and is often referred to as a mud shrimp (Neal and Avant, 2006). *C. volutator* lives in muddy estuary sediments, where it occupies U-shaped burrows (Fish, 2011). *C. volutator* has a long slender body which is dorsally flattened and clearly segmented (Fish, 2011). The head is small in comparison to the rest of the body. On the head there are two pairs of forward pointing antennae; the second pair are a distinguishing feature of *C. volutator* and are long and thick, particularly in males (Neal and Avant, 2006; Fish, 2011). There are seven pairs of segmented legs with the top segment of each being typically small and separate from that of the next segment (Fish, 2011).

1.2 Predation and importance in the ecosystem

C. volutator is an extremely important food source for many predators; they are a direct link between primary producers (e.g algae), and predators (e.g. wading birds and fish). Birds such as redshank (*Tringa tottanus*), dunlin (*Calidris alpine*) and shelduck (*Tadorna tadorna*) (Hughes, 1988), as well as invertebrates such as the brown shrimp (*Crangon crangon*) (Cattrijsse *et al.*, 1997), and fish such as sand goby (*Pomatoschistus minutus*) (Jaquet and Raffaelli, 1989) are some of the known predators of *C. volutator*. Generally male *C. volutator* are active on the sediment surface with females being much less active, for this reason it is hypothesised that they are more vulnerable to predators (Wilson and Parker, 1996). This results in a much higher number of females than males in a given population and this trend has been observed without exception (Wilson and Parker, 1996).

1.3 Distribution and abundance

C. volutator are widely distributed along the North Atlantic coastlines. They are particularly prevalent in the Bay of Fundy, on the Atlantic coast of North America (Murdoch *et al*., 1986). The sub-species *C. volutator orentialis* is also found in Japan (Omori and Tanaka, 1998). Densities of *C. volutator* vary considerably and have been known to occur in extremely high densities, often over 20,000 individuals/m² (De Backer *et al*., 2010) with up to 100,000 individuals/m² in the North Atlantic (Hughes, 1988). They can also occur in entirely marine sediments. The benthic sediments of the Baltic Sea, 50 meters below sea level have been found to support *C. volutator* (Peters and Ahlf, 2005), however, little is known about the *C. volutator* in these environments possibly due to the difficulties in researching these habitats.

1.4 Reproduction

C. volutator only reproduce sexually. The number of broods is generally between 1 - 2 broods per year (Peer *et al.*, 1986; Neal and Avant, 2006). Northerly estuaries such as the Baltic Sea and the Ythan estuary in the north east of Scotland have just one brood per year (Wilson and Parker, 1996). Southerly study areas such as Dovey Estuary (Wales) have two per year (Peer *et al.*, 1986; Neal and Avant, 2006). In areas where *C. volutator* produce two broods per year, the first generation occurs in the spring, this generation reaches sexual maturity by late summer, after the release of offspring this generation dies. The second generation is hatched by late summer and survives the winter to reproduce early the following summer (Peer *et al*., 1986; Drolet *et al*., 2013). The number of offspring produced from one brood ranges between 11 and 100 and is

determined by the size of the female (Neal and Avant, 2006). Females are only receptive for mating directly after moulting (Neal and Avant, 2006). Females moult more frequently than males and predominately at spring tides (McCurdy *et al*., 2000). This short window in which mating can take place results in strong competition between males for receptive females (Forbes *et al.*, 1996; Neal and Avant, 2006). Males walk along the sediment surface led by chemical signals released by receptive females (Krång and Baden, 2004). These chemical signals are yet to be identified. Some swimming has been observed on tides and is most likely related to mating, generally males searching for females, however, this behaviour is not frequent and is not considered integral to reproduction (Hughes, 1988).

1.5 Factors affecting the reproduction of *C. volutator*

There are many biotic and abiotic factors which may influence *C. volutators* ability to reproduce in the laboratory. These factors may inevitably differentiate laboratory cultured specimens from wild harvested specimens. These factors include:

- Sex ratio
- Temperature
- Salinity
- Light
- Tidal rhythm
- Food source
- Parasitic infections

1.5.1 Seasonal variations in sex ratio

The sex of *C. volutator* can be determined by a number of methods including; examining for the presence of a penial papillae in males, and oostegites in females (Gratto 1979) , or examination of the second antenna which is larger in adult males compared to adult females (Boates 1980). A method for determining the sex of juvenile *C. volutator* based on morphology involves examination of small spines on the first article of the first antenna. Under a microscope spines, differ in size and shape in male and females (Schneidesr *et al.,* 1994). The changing sex ratio of *C. volutator* through the seasons has been well documented and is attributed to the natural life cycle of *C. volutator* (Peer *et al*., 1986; Drolet *et al*., 2013). Studies have shown that *C. volutator* populations are dominated by females. This trend appears to be the rule, however, the extent to which females dominate the population varies dramatically throughout the year. In the Gulf of Gdafisk, Poland, *C. volutator* populations in the month of August were shown to consist of as low as approximately 40% females (Figure 1) (Dobrzycka and Szaniawska, 1995). In July, the population reached a maximum of 84% females (Dobrzycka and Szaniawska, 1995). Research carried out by Schneidesr *et al.,* (1994) suggests that sex bias predation does not influence the sex ratio of *C. volutator* as previously thought. Schneidesr *et al.,* (1994) found that an average of 20% of neonates contained in the brood pouch were male, however, this study assumes that juveniles can be sexed by morphology.

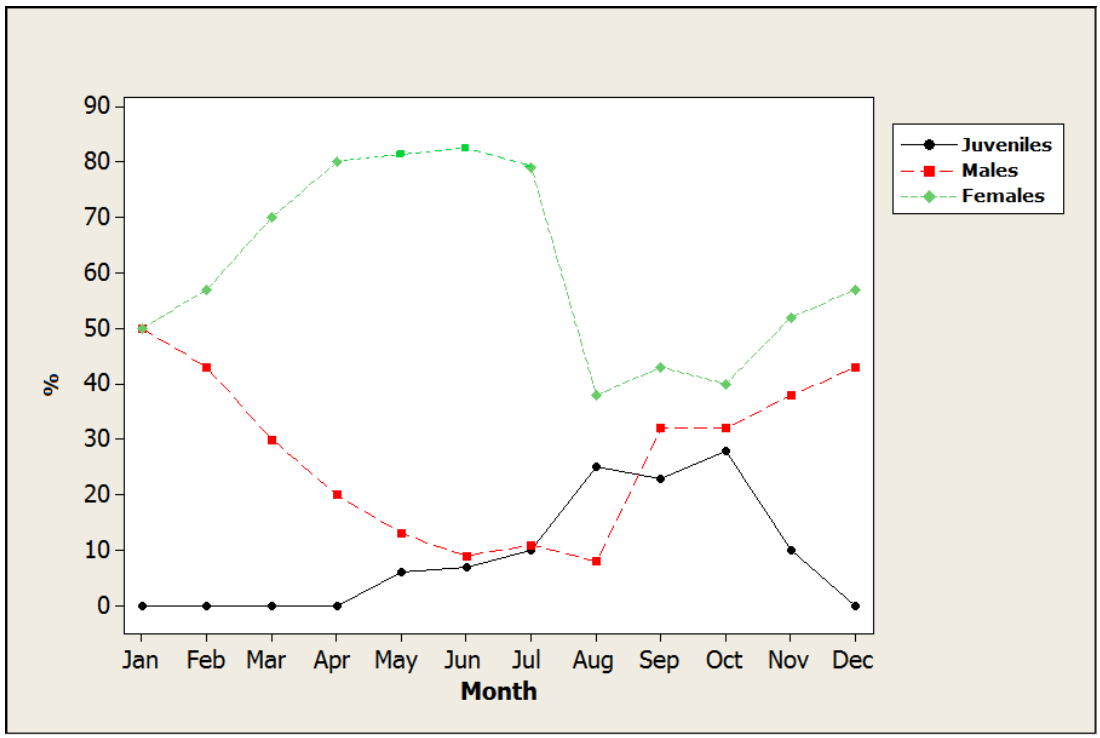

Figure 1, *C. volutator* population dynamic throughout the year in the Gulf of Gdarisk, Poland (adapted from Dobrzycka and Szaniawska, 1995).

1.5.2 Temperature

As an estuarine species *C. volutator* are adapted to the temperature fluctuations associated with estuarine environments. The temperature of shallow estuarine water is influenced by solar heating and ambient air temperature (Prandle, 2009). *C. volutator* have been proven to be resistant to the thermal stresses, with no correlation between cold snaps (i.e. freezing conditions) and increased mortalities (Drolet*., et al.,* 2013). Although temperature fluctuations are a predominant feature of estuarine environments, it has been shown that *C. volutator* are capable of reproducing at constant temperatures (Peters and Ahlf, 2005). Temperature influences the number of broods carried in one season with warmer areas producing two broods and cooler areas producing only one (Wilson and Parker 1996). Although excessive temperatures have been shown to retard reproductive output, when the reproductive output of *C. volutator* was measured in laboratory conditions under a range of temperatures 15 °C, 19 °C and 23 °C, the highest number of *C. volutator* was produced under 15 °C (Peters and Ahlf, 2005). Temperature has also been shown to have a major influence on growth. Under laboratory conditions, impaired growth rates were observed at temperatures of 5 °C (0.0048 mm/day) and 10 °C (0.019 mm/day) with higher growth rates observed at temperatures of 15 °C (0.069 mm/day) and 25 °C (0.067 mm/day) (Kater *et al.*, 2008). Virtually no difference in the growth rate was observed between the latter temperatures (Kater *et al.*, 2008). The highest growth rate observed in wild populations was 0.08 mm per day (Möller and Rosenberg, 1982). A growth rate of 0.07 mm per day was found in culturing trials held at 15 °C by Peters and

Ahlf, (2005) suggesting that this temperature is conducive with a high growth rate.

1.5.3 Salinity

C. volutator are adapted to fluctuating salinities and can tolerate exposure to freshwater and full seawater, however, in areas with salinities consistently less than 5 ppt, *C. volutator* are found in reduced numbers (Mc Lusky, 1967). Salinity is an important factor in the survival of *C. volutator* in estuarine environments. The ability of *C. volutator* to withstand high temperatures is dependent on salinity, with the highest tolerance to temperature being observed at 30 ppt (Mills and Fish, 1980). A field study has shown that the highest growth rate occurs at salinities of approximately 15 ppt, however, growth rates were only slightly lower at salinities of approximately 4 ppt and 31 ppt suggesting that salinity is not a major influence on the growth rate of *C. volutator* (Mc Lusky, 1967). Salinity is known to have an impact on the ability of *C. volutator* to moult and lay eggs, the lowest recorded salinity at which female *C. volutator* moulted and laid eggs was 20 ppt (Mills and Fish, 1980). Although fluctuating salinities are a predominant feature of estuaries, it has been demonstrated that *C. volutator* can reproduce at constant salinities of 30 ppt in laboratory conditions (Peters and Ahlf, 2005).

1.5.4 Lighting

The role light plays in the life cycle of *C. volutator* and the impact on the reproductive output of *C. volutator* is poorly understood. A 16 hour light and 8 hour darkness regime was successfully used in experiments

carried out by Peters and Ahlf, (2005).

1.5.5 Tidal rhythm

As an estuarine dwelling species *C. volutator* are adapted to tidal systems. Behavioural observations such as surface activity may be linked to mating, migration (Fish and Mills, 1979) or parasitic infections (Damsgaard *et al*., 2005). *C. volutator* have shown a preference for surface activity during submersion by high tide and little surface activity during emersion (De Backer *et al*., 2010). Laboratory culturing has been carried out successfully in the absence of a tidal rhythm indicating that emersion is not necessary for *C. volutator* life cycle (Peters and Ahlf, 2005).

1.5.6 Parasitic infections

C. volutator may become infected with parasites such as the microphallid trematodes *Maritrema subdolum* and *Microphallus claviforrnis*. These parasites have complex life cycles involving the mud snail *Hydrobia* sp, *Corophium* sp and with various wading birds as the final hosts (Damsgaard *et al.,* 2005). Parasites infect the gills of *Corophium sp* leading to anaemia and results in difficulty obtaining oxygen. This is linked to increased surface activity of the parasitised *C. volutator* (Damsgaard *et al.,* 2005).

1.6 Environmental toxicology testing using *C. volutator*

C. volutator is the preferred test species used in the Oslo and Paris Commissions (OSPAR 2006) "sediment reworker test". This test is used to determine the chemical toxicity of substances used in the North Atlantic oil industry for regulatory purposes. In 1995, OSPAR developed a "Harmonised Offshore Chemical Notification Format" as part of a system for reducing the harmful substances/preparations which were entering the marine environment. To monitor these chemicals, four protocols were ring tested and developed:

- Growth inhibition test using the marine algae *Skeletonema costatum*
- Acute toxicity test using the marine copepod *Acartia tonsa*
- A sediment bioassay using an amphipod *Corophium* sp
- Protocol for a fish acute toxicity test

The "Sediment bioassay using an amphipod *Corophium* sp" test has been designed to determine the toxicity of substances which are fated for marine seabeds, i.e., sinking test substances. The objective of this test is to determine the LC_{50} of a test substance, i.e., the "Lethal Concentration" which kills 50% of the specimens. The test is run over a 10 day period using adult *C. volutator* (specimens which are over 5 mm in length, excluding antennae). Specimens are exposed to test chemicals via spiked sediment. At least two replicates of 20 *C. volutator* are exposed per concentration. At the end of the test, the number of surviving and dead *C. volutator* are counted. Although this is a chronic test it does not take into account key life stages (Scarlett *et al.,* 2007).

A 72 hour acute test is also used for whole sediment toxicity tests (Chapman *et al*., 1992) although this test is not used for regulatory purposes. The acute toxicity test using *C. volutator* was commonly used prior to the OSPAR 1995 regulation. It was the most useful whole sediment toxicity test for general assessment in Europe (Chapman *et al*., 1992), however, since the OSPAR 1995 regulations it is less routinely carried out. Currently there are no guidelines for toxicity testing which require cultured *C. volutator*, instead *C. volutator* are harvested from wild populations.

Although *C. volutator* is not commonly cultured in the laboratory, Peters and Ahlf (2005) demonstrated that cultures can be produced under laboratory conditions year round with a high success rate, reproduction took place in 98% of batches in their study.

1.6.1 Collection of wild *C. volutator* for toxicity testing

The OSPAR (1995) "A Sediment Bioassay using an Amphipod *Corophium* sp" does set out guidelines for the collection of *C. volutator* for testing. These guidelines state that the *C. volutator* shall be collected from an inter tidal zone (mud or muddy sand). The *C. volutator* are collected by removal of the top 5 cm of sediment and laid in trays for sieving on site or in a laboratory later. This OSPAR (1995) protocol recognises that difficulties may be experienced when collecting as a result of varying populations, densities of 50,000 m^2 can occur, however, this may fall to below 1,000 m^2 in winter (OSPAR protocol 1995).

1.6.2 Variations in the lipid levels of *C. volutator* and other amphipods

A number of benthic amphipods experience seasonal variations in their lipid levels including *C. volutator, Gammarus oceanicus, Monoporeia affinis* and *Pontoporeia femorata* (Dobrzycka and Szaniawska, 1995: Clarke *et al*., 1985: Lehtonen, 2004). Dobrzycka and Szaniawska (1995) assessed the lipid levels of *C. volutator* using modified calorimetric microbomb (Phillipson type), using a mixture of chloroform, methanol and water to extract the lipids, and found that lipid level of *C. volutator* populations fluctuate dramatically throughout the year.

Lehtonen, (2004) observed seasonal variations in lipid levels of the benthic amphipod *Monoporeia affinis* at a near shore station, with a decline in lipid levels toward late autumn and gravid females displaying the lowest lipid levels. The benthic amphipod *Gammarus oceanicus,* rapidity accumulates lipids in spring followed by the utilisation of these resources in the production of offspring (Clarke *et al*., 1985).

In *C. volutator ,* as in the afore mentioned amphipods, these fluctuating lipid levels have been linked to reproductive stresses, with the lowest lipid levels being observed post reproduction (Dobrzycka and Szaniawska, 1995). In the Gulf of Gdafisk, Poland, it was observed that the highest lipid levels were generally found in winter and spring, the lowest lipid levels generally found in late winter and summer, coinciding with the release of offspring (Figure 2) (Dobrzycka and Szaniawska, 1995).

In the freshwater amphipod *Gammarus minus* "fatter" specimens ("estimated as fat content and residuals of body mass regressed against body length") were found to be fitter than specimens with less fat reserves, where brood size was a measure of fitness (Glazier 2002).

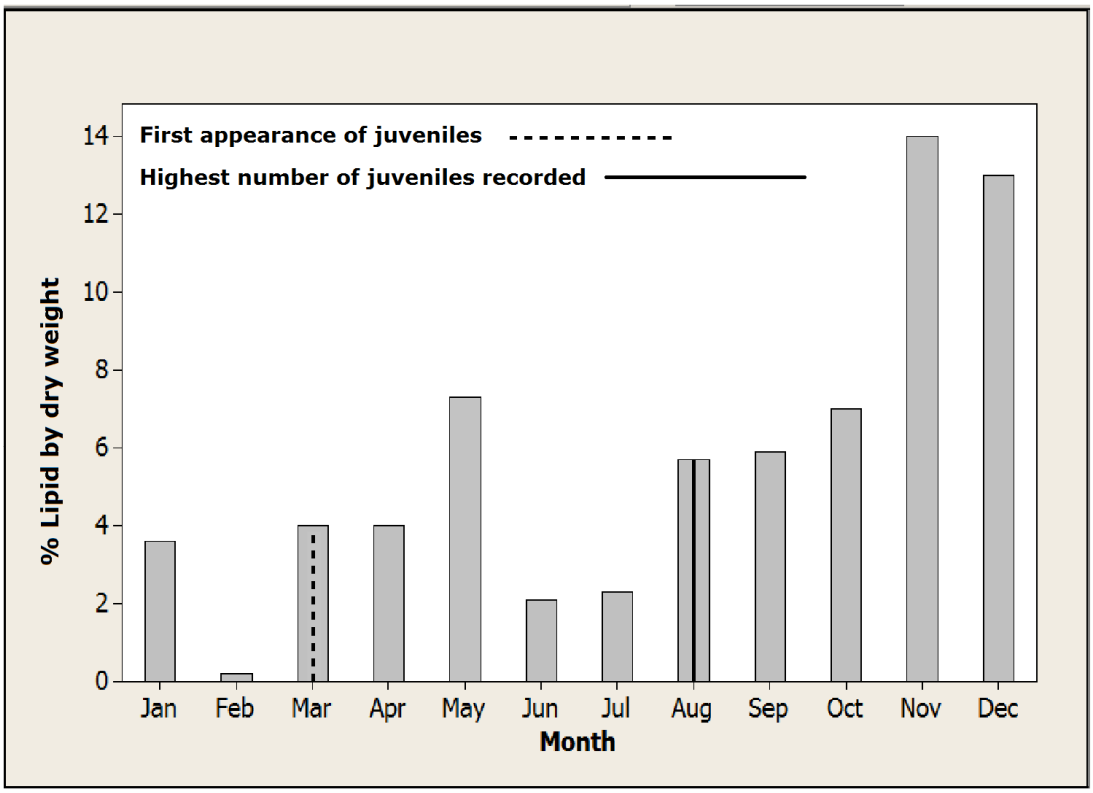

Figure 2, Lipid levels between October 1991 and September 1992 in the Gulf of Gdansk at Swarzewro, Poland (adapted from Dobrzycka and Szaniawska, 1995).

1.6.3 Seasonal variations in the sensitivity of *C. volutator* to the toxicant cadmium

It has been observed that seasonal changes influence the tolerance of *C. volutator* to the toxicant cadmium, in a 72-hour acute water phase test, a clear pattern of fluctuating LC_{50} values were found over a 7 year period where 80 reference chemical tests were carried out (Kater *et al*., 2000). *C. volutator* were most sensitive to the toxicant cadmium over the months of July, August and September (Figure 3) (Kater *et al*., 2000). After September, a clear increase in tolerance to cadmium was observed followed by a slight decline in tolerance during the month of February. Kater *et al*., (2000) suggest a link between the seasonal changes in sensitivity of *C. volutator* to cadmium and the moulting cycle.

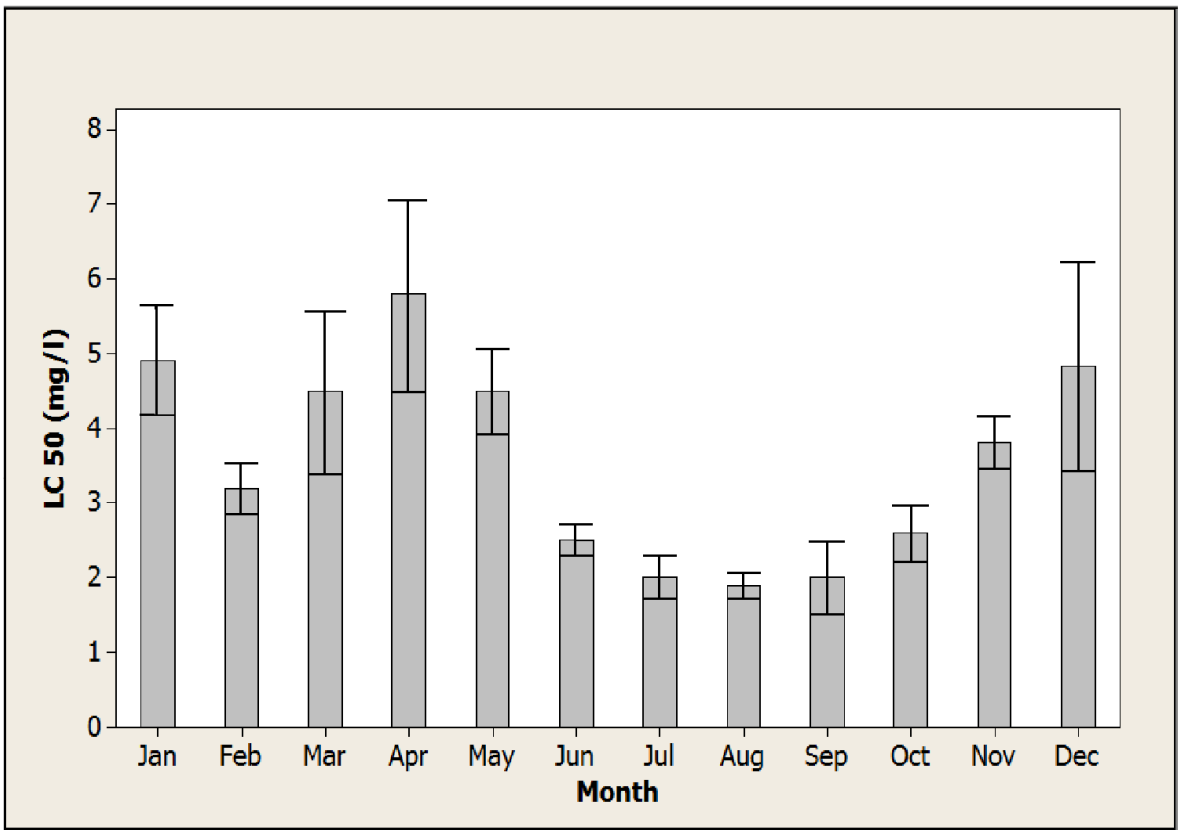

Figure 3, Average cadmium LC_{50} with standard error for each month (1991 - 1998) as found in the Eastern Schldt Bay, Netherlands (adapted from Kater *et al*., 2000).

1.6.4 Varying lipid levels in amphipods and toxicity testing

Meador (1993) has found a direct correlation between the decreased lipid level of the amphipod *Rhepoxynius abronius* Barnard (Phoxacephalidae) and *Eohaustorius estuarius* Bosworth (Haustoriidae) and increasing sensitivity to the toxicants tributyltin (TBT) and cadmium (Cd). In this study, Meador (1993) found that when wild harvested *R. abronius* and *E. estuarius* were held in the laboratory for several weeks, a two to three fold decrease in lipid levels occurred which correlated with an increase in sensitivity to tributyltin and cadmium.

1.7 Laboratory cultured *C. volutator* as a toxicological test specimen

Peters and Ahlf (2005) found that field collected *C. volutator* were more sensitive than laboratory cultured specimens, when compared in 72-hour toxicity tests with the reference substance ammonium chloride [following the Standard Operating Procedure of Schipper *et al*., (1999)]. The ammonium chloride LC_{50} value for laboratory bred amphipods was 85.18 mg/l (95% confidence interval 73.53–98.66 mg/l). The LC_{50} value for the field collected amphipods was 40.29 mg/l (95% confidence interval 23.66–68.63 mg/l). Similar findings were also found using the amphipod *Corophium multisetosum* "Cadmium test indicated high temporal variability in the LC_{50} values of field amphipods (2.40–6.55 mg L^{-1})" with laboratory cultured specimens having a cadmium LC_{50} within this range 5.81 mg L^{-1} (Menchaca *et al.,* 2010).

1.8 Triggers for the release of offspring

To culture *C. volutator* in the laboratory, the environmental conditions which trigger the release of offspring and allow populations to thrive must be understood. Unfavourable environmental conditions, i.e., low temperatures, are thought to be associated with a prioritisation on lipid storage in *C. volutator* (Dobrzycka and Szaniawska 1995). An increase in the lipid level along with biological transformations in the females occurs just before the release of offspring (Dobrzycka and Szaniawska 1995).

1.9 The diet of *C. volutator*

C. volutator can feed on a variety of organic matter, consuming particles 4 - 63 µm in diameter; bacteria, algae and detritus (Neal and Avant, 2006). *C. volutator* have two methods of feeding; (1) searching the sediment surface for detritus and micro-organisms, then using the second antennae to scrape selected matter into the burrow. Once in the burrow, they use the pleopode to pass the gathered material over the mouth parts, this is called "selective deposit feeding", (2) by filtering particles from the water column when the burrows are submerged by the tide, this method appears to have equal levels of efficiency (Gerdol and Hughes, 1994: Fish, 2011). It is difficult to quantify which method of feeding is predominant or what type of food is most important in the diet of *C. volutator*. With the use of radio labelled diatoms it has been found that *C. volutator* consume approximately 4000 small diatoms in one hour (Gerdol and Hughes, 1993). In a study *Navicula salinicola* has been identified as an appropriate food source for *C. volutator* (Peters and Ahlf, 2005), although the quantity of diatoms required to sustain the *C. volutator* was not quantified in this study. A comparison between *C.*

volutator fed on a diet of diatoms and those fed detritus, derived from *Spartin* sp grass, found detritus to be an inferior food source which aids survival whereas diatoms support the rapid growth of *C. voutator* during summer months (Stuart *et al.,* 1985). A combination of aquarium food which is available commercially has also been proven to support the growth and reproduction of *C. volutator* (Scarlett *et al.,* 2007).

1.10 *Tetraselmis chuii* and *Rhinomonas reticulate*

The genus *Tetraselmis* is an important one in the aquaculture industry and has been used as a food source for fish, shrimp and shellfish (Da Costa and De Franca 1998: Shannon *et al.,* 2007). *Tetraselmis chuii* has proven a successful food source for the amphipod *Caprella grandimana* when used in combination with one diatom, *Phaeodactylum tricornutum* (Baeza-Rojano *et al.,* 2011). *T. chuii* can reach high cell densities of approximately 6.06 $x10^5$ cells mL^{-1} when cultured in F/2 media (Lopez-elias *et al.,* 2011). The algae *R. reticulate* is commonly cultured in laboratories as a food source for *Acartia tonsa,* which is used in environmental toxicology testing (Zhang *et al*., 2013) and for use as a food source for *Mytilus edulis* (Leonardos and Lucas, 2000).

Chapter 2. Aims and objectives

2.1 Aims and objectives of the study

The objective of this study is to determine if the seasonal variations in the fitness of *C. volutator* can be overcome by using laboratory cultured specimens.

The aims of the study are to:

- Identify the timeframe in which *C. volutator* specimens in the Bay of Suckquoy, Orkney, are unfit for toxicity testing based on control batch mortalities

- Determine if *C. volutator* can be cultured in a simple laboratory system, removed from tidal influences, dramatic fluctuations in temperature and salinity and in the absence of a light/dark regime

- Determine suitable food for culturing *C. volutator* which can be easily produced in the laboratory

- Identify an appropriate timeframe in which *C. volutator* should be cultured to provide adult specimens during periods of failing wild *C. volutator* fitness

Chapter 3. Experimental settings, material and methods

3.1 Methods

3.1.1 Trials and data collected

Three sets of data were collected for this study;

1. Culturing of *C. volutator* neonates
2. Culturing of adult *C. volutator*
3. Collection of data on wild harvested *C. volutator*

The culture of *C. volutator* neonates and adults were run concurrently over a period of 132 days.

3.2 Trial 1. Culture of *C. volutator* neonates

This trial was conducted to determine:

- The time required for a neonates *C. volutator* to reach adulthood
- The preferred feeding regime of *C. volutator*
- The number of offspring produced under a variety of feeding regimes.

3.3 Trial 2. Culture of adult *C. volutator*

Was conducted to determine:

- If *C. volutator* can be cultured in a simple laboratory system, removed from tidal influences, dramatic fluctuations in salinity and temperature and in the absence of a light/dark regime
- The preferred feeding regime of *C. volutator*

3.4 Experimental conditions used in the culturing of *C. volutator* adults and neonates

Set environmental conditions were maintained in all tests, a temperature of 15 °C ± 2 °C, salinity of 30 - 35 ppt and a dissolved oxygen level of ≥60% saturation (maintained using aeration in Trial 2, Culture of adult *C. volutator*). All cultures were kept in darkness. The temperature was maintained by means of an air conditioning unit. The treated seawater (see 3.8) with a salinity of 36 ppt was reduced to 30 ppt before use by means of dilution with deionised water.

3.5 Collection and holding conditions of test specimens and sediment

Records for the collection of wild harvested *C. volutator* for the purpose of toxicity testing in began 2009. *C. volutator* was separated from the sediment with the use of 600 µm sieves. *C. volutator* were then placed in three 30 litre buckets with water from their estuary and transported to the laboratory. Adult *C. volutator* were then placed in aerated holding tank with 30 - 100 litres of saline water and detritus (recovered from sieving the sediment) (Figure 4). The holding tank was maintained at a temperature of 15 ± 2 °C. The salinity of the holding water was slowly increased from the initial salinity, to 30 ppt over a period of 8 days. The sediment was prepared by sieving with a 600 µm sieve and freezing at -20 °C for 48 hours to destroy any potential competitive species present in the sediment. The initial *C. volutator* and sediment used for culturing of all *C. volutator* was collected from the Bay of Suckquoy, Orkney on the 26th of April 2011.

3.6 Sediment characteristics

Previous analysis carried out by Opus Maxim Ltd. has found the sediment at the Bay of Suckquoy, Orkney to have the following characteristics: Particle size of the sediment was well-sorted, fine sand with a silt/clay content of 31.6% by weight; median particle diameter was 100 μm; the organic material content was estimated from weight loss on ignition to be 2%.

3.7 Historical data on control tank mortalities, 2009 - 2010

Immediately after harvesting wild *C. volutator* in the estuary, technicians recorded some simple pieces of data as well as observations. The *C. volutator* were collected and placed in water from the estuary (collection took place during low tide, therefore the water collected was mainly derived from a river flowing through the estuary). The parameters salinity and temperature were recorded. The *C. volutator* were then brought back to the laboratory and placed in a tank where they were maintained. The data collected on *C. volutator* between 2009 – 2012 was collected by various technicians.

3.8 Seawater collection and treatment

Seawater was pumped from Scapa Flow, Orkney and passed through a sand bed at the laboratory and then treated with UV light, the seawater had a salinity of 36 ppt. Seawater used in the culturing of algae was further filtered using a 1 μm filter. All seawater used in the preparation of algae seeding cultures was also autoclaved before use.

3.9 Sizing and measuring of *C. volutator*

C. volutator were sized based on body length. *C. volutator* were separated into three categories by size; adults ≥5 mm, juveniles 2 - 4 mm and neonates <2 mm. *C. volutator* were placed in a sieve and gently washed to one side of the sieve. Sizing was then carried out by visual comparison using a ruler which was placed next to specimens.

3.10 Measurement of oxygen, salinity and pH in culturing tanks, holding tanks and in the estuary

Dissolved oxygen, salinity and pH were measured using an oxygen meter (Jenway, 9200), salinity meter (CETI Digit-100 ATC) and a pH Meter (Jenway 3150). The measurement method was common to all parameters; the probe was placed in the tank, holding bucket or estuarine water, just above the bottom of the sediment surface or bottom of the container and stirred gently. When the meter readout stabilised, the value was recorded. Temperature was measured using the internal thermometer built into the dissolved oxygen meter. Calibration of parameter meters were carried out on days of use.

3.11 Trial 1. Culture of *C. volutator* neonates

C. volutator neonates were removed from the overlying water of the collected sediment by means of pipetting. Twenty neonates were placed into beakers (diameter 10 cm X 7 cm high) with a sediment depth of 2 cm (200 grams wet weight) and 300 ml of overlying water with a salinity of 30 ppt. Beakers were held in semi-static conditions with water changes carried out three times per week, (Monday, Wednesday and Friday) using treated seawater (1 µm filtered and UV treated) ≥90% of

the water was replaced during changeovers. Water changeovers were carried out using a siphon so as not to disturb the sediment or burrows. The parameters were checked throughout the trials; dissolved oxygen (% saturation), salinity (ppt) and temperature (°C). Parameters were checked before water changeovers were carried out. *C. volutator* was supplied with their respective food sources after water changes. Temperatures were maintained at 15 ± 2 °C. On day 132, the *C. volutator* were removed from the sediment using a 350 µm sieve. Three feeding regimes were used:

1. Two beakers supplied with *T. chuii* 2.4 X 10^6 cells per feeding increased to 3 X 10^6 after day 25

2. Two beakers supplied with *R. reticulate* 2.4 X 10^6 cells per feeding increased to 3 X 10^6 after day 25

3. Two beakers supplied with 1.2 X 10^6 cells of each algae *T. chuii* and *R. reticulate,* increasing to 1.5 X 10^6 cell after day 25.

4. One beaker was not supplied with any food source (control)

4.

3.12 Trial 2. Culture of adult *C. volutator*

Adult *C. volutator* were cultured over a period of 132 days. Changeovers were carried out three times per week (Monday, Wednesday, and Friday). One hundred adult *C. volutator* (≥ 5 mm in body length excluding antennae) were selected at random and placed into each tank. ≥90% of the water was replaced on days of changeovers. The parameters were checked throughout the trials; dissolved oxygen (% saturation), salinity (ppt) and temperature (°C). Parameters were checked before water changeovers were carried out. *C. volutator* were supplied with food 3 times per week after changeovers.

A total of five tanks were cultured under different feeding regimes:

1.Two tanks supplied with two litres of *T. chuii* cultures

2.Two tanks supplied with two litres of *R. reticulate*

3.One control tank which was not supplied with any food source

On day 132, the *C. volutator* were removed from the sediment using a 600 µm sieve.

3.13 Algae culturing

Algae culturing was carried out in a temperature controlled room at 20 ± 2 °C. The cultures were aerated with CO_2 enriched air. F/2 media was used to culture both *T. chuii* and *R. reticulate*. A total of seven litres of *T. chuii* and *R. reticulate* were cultured per week, six litres was used to feed the *C. volutator* adults and one litre flask used to supply the *C. volutator* neonates. The six litre flasks were seeded using 1000 ml of starter culture, which was checked for contamination by visual inspection under a microscope before use. F/2 media stock solutions were prepared every

six months and stored in the fridge at 2 - 5 °C, the media was prepared immediately before use, using 1 µm filtered and UV light treated seawater (36 ppt).

3.14 Algae feeding regime

Cell counts were carried out on both *T. chuii* and *R. reticulate* using a haemocytometer. *C. volutator* neonates were supplied with algae on Monday, Wednesday and Friday. The volume of algae harvested for the *C. volutator* neonates varied slightly depending on the concentration of the algae cultures. The adult *C. volutator* were supplied with two litres of algae culture.

3.15 Control chamber mortalities

C. volutator were harvested from the Bay of Suckquoy, Orkney for use in the 10 day sediment reworker test. Harvested *C. volutator* were held in accordance with the OSPAR (1995) guidelines. In accordance with these guidelines, 100 of the *C. volutator* were held in a control chamber (Figure 4). Batches with greater than 10% mortalities within 10 days were considered to be unfit for testing. This mortality rate is based on the OSPAR (1995) guidelines "Mortalities during holding should be acceptably low and if possible less than 10%."

3.16 Mortality rates of wild *C. volutator* 2009 - 2012

C. volutator were harvested from the Bay of Suckquoy, Orkney for use in commercial environmental toxicity testing from January 2009 until December 2012. The harvested *C. volutator* were held in 30 - 100 litres of estuarine water which was collected along with the *C. volutator*. When the holding water was found to have a salinity lower than 30 ppt, it was slowly adjusted to 30 ppt by means of dilution with seawater (36 ppt). The salinity was increased by no more than 3 ppt per day. The *C. volutator* were held for no more than 10 days before testing. A total of 100 adult *C. volutator* were held in a control chamber, (Figure 4) which consisted of a six inch diameter plastic pipe, with one end sealed off using 600 µm nylon mesh. This pipe was submerged upright with the mesh covered side facing down, this was held in place using a clamp. The mortalities in the control chamber were recorded up until testing. The number of *C. volutator* batches which were collected for testing but failed due to the high control mortalities (≥10%) was recorded along with the parameters of the estuarine water temperature and salinity.

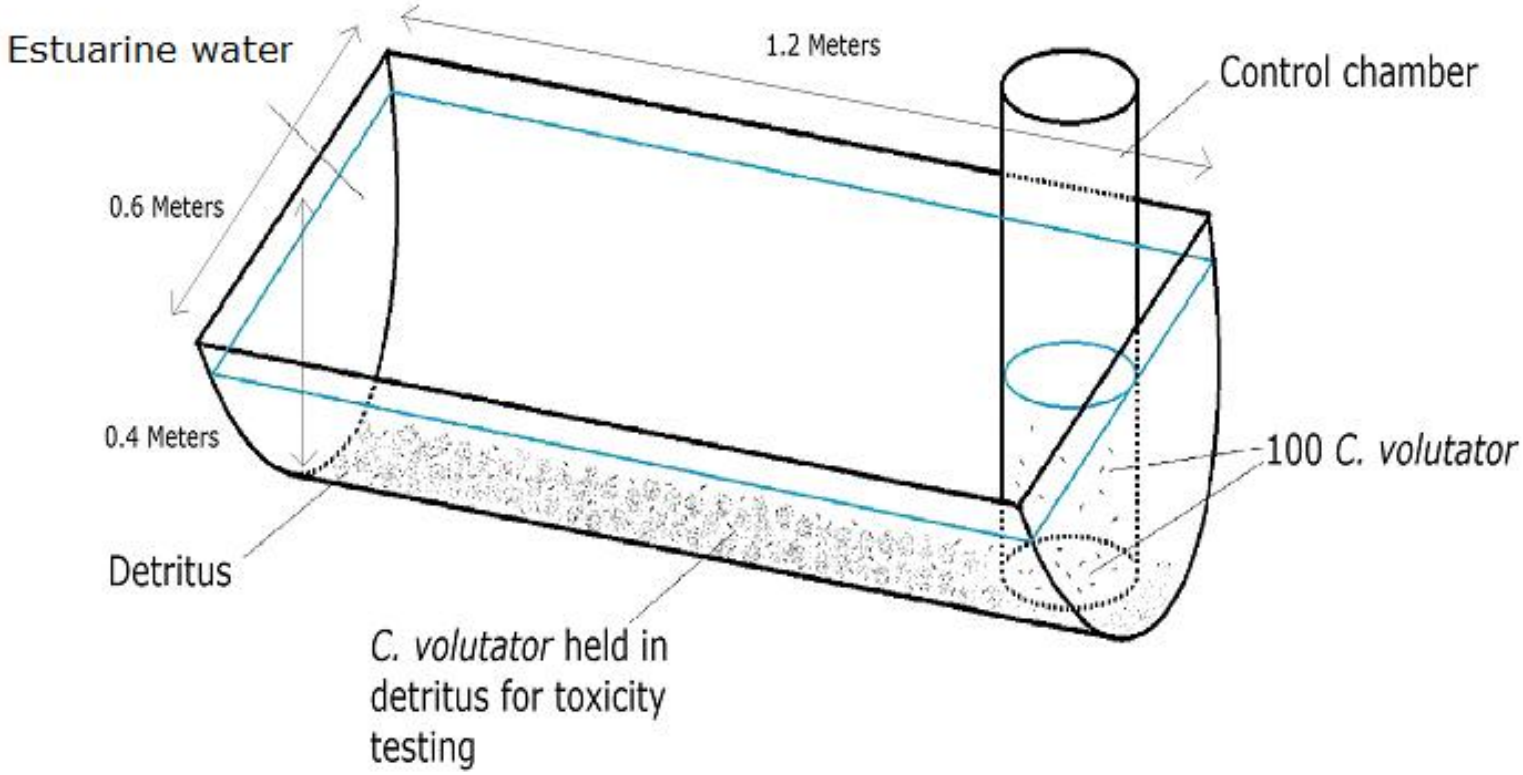

Figure 4, *C. volutator* holding tank and control chamber

3.17 Equipment and materials

- DO_2 Meter (Jenway, 9200) with temperature probe
- pH Meter (Jenway 3150) with temperature probe
- Salinity Meter (CETI Digit-100 ATC)
- 5 X 15L Glass tanks, 22 x 34 x 22.5 cm (width x length x height)
- Sediment
- Air-conditioning unit
- 1 X 600 µm sieve
- 1 X 350 µm sieve
- 10 X glass beakers
- 3 X 3 L algae culturing flask
- Improved neubauer haemocytometer
- 2 X 30 L plastic holding tanks
- 3 mm bore glass rods for aeration
- Air compressor - for aeration of algae cultures
- 10 mm bore silicone tube for syphoning

3.18 Statistical methods

Although beyond the scope of this study, statistical analysis would be useful to determine the statistical significance of the findings in Trial 1. Culture of *C. volutator* neonates and Trial 2. Culture of adult *C. volutator*. To carry out statistical analysis, more replicates would be required. Had more replicates been used in these trials, tests such as the ANOVA or the T- test could be used to test for statistical differences between the reproductive outputs of the various feeding regimes.

Chapter 4. Results

4.1 Control chamber failures and observations from 2009 - 2012

C. volutator were collected from the estuary and returned to the laboratory within 24 hours. A control chamber was set up to determine the percentage control mortalities over 10 days. Batches with less than 10% mortalities were considered fit for testing and those with ≥ 10% mortalities were considered unfit for testing under the validity criteria set out in the OSPAR (1995) guidelines, "Sediment Bioassay Using an Amphipod Corophium sp". Between January 2009 and December 2012, all failed *C. volutator* batches occurred between 4th of June and 27th of September each year with no failed batches occurring outside this of timeframe (Figure 5). No failed batches were recorded in the year 2009.

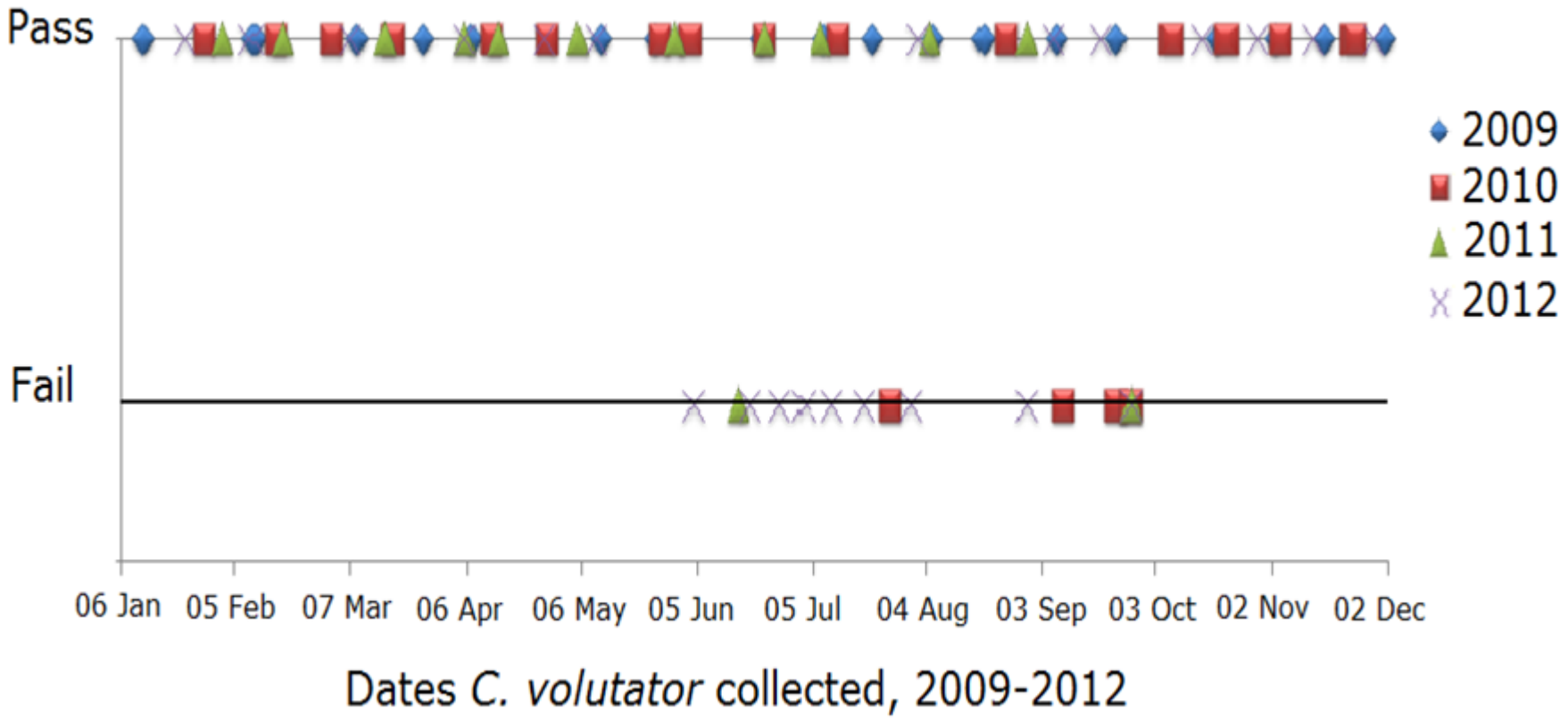

Figure 5, Wild harvested *C. volutator* batches which failed due to high mortalities (≥10%) from 2009 to 2012

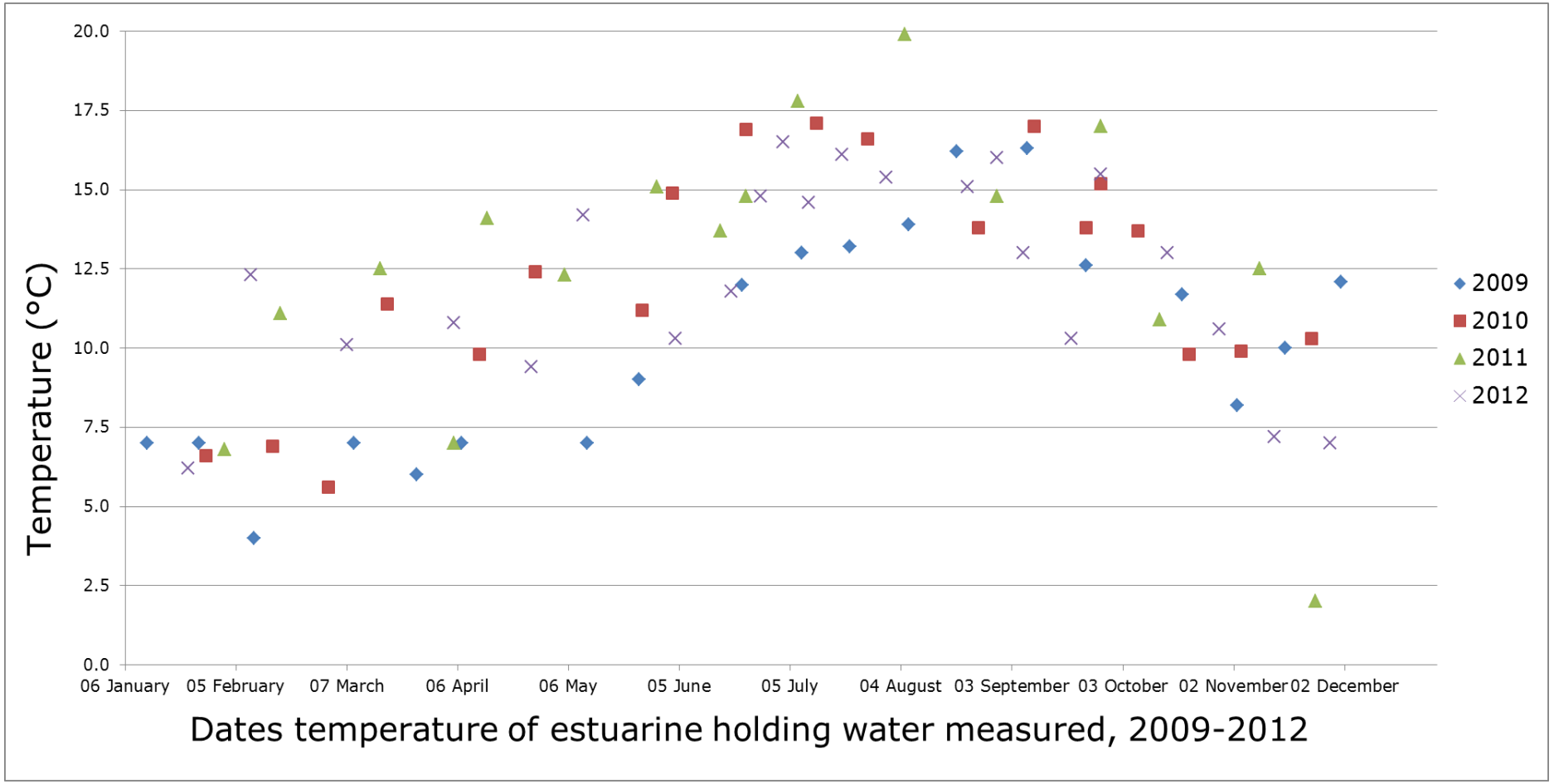

Figure 6, Temperature of estuarine water collected for holding and transporting *C. volutator* to the laboratory.

Years 2009 - 2012.

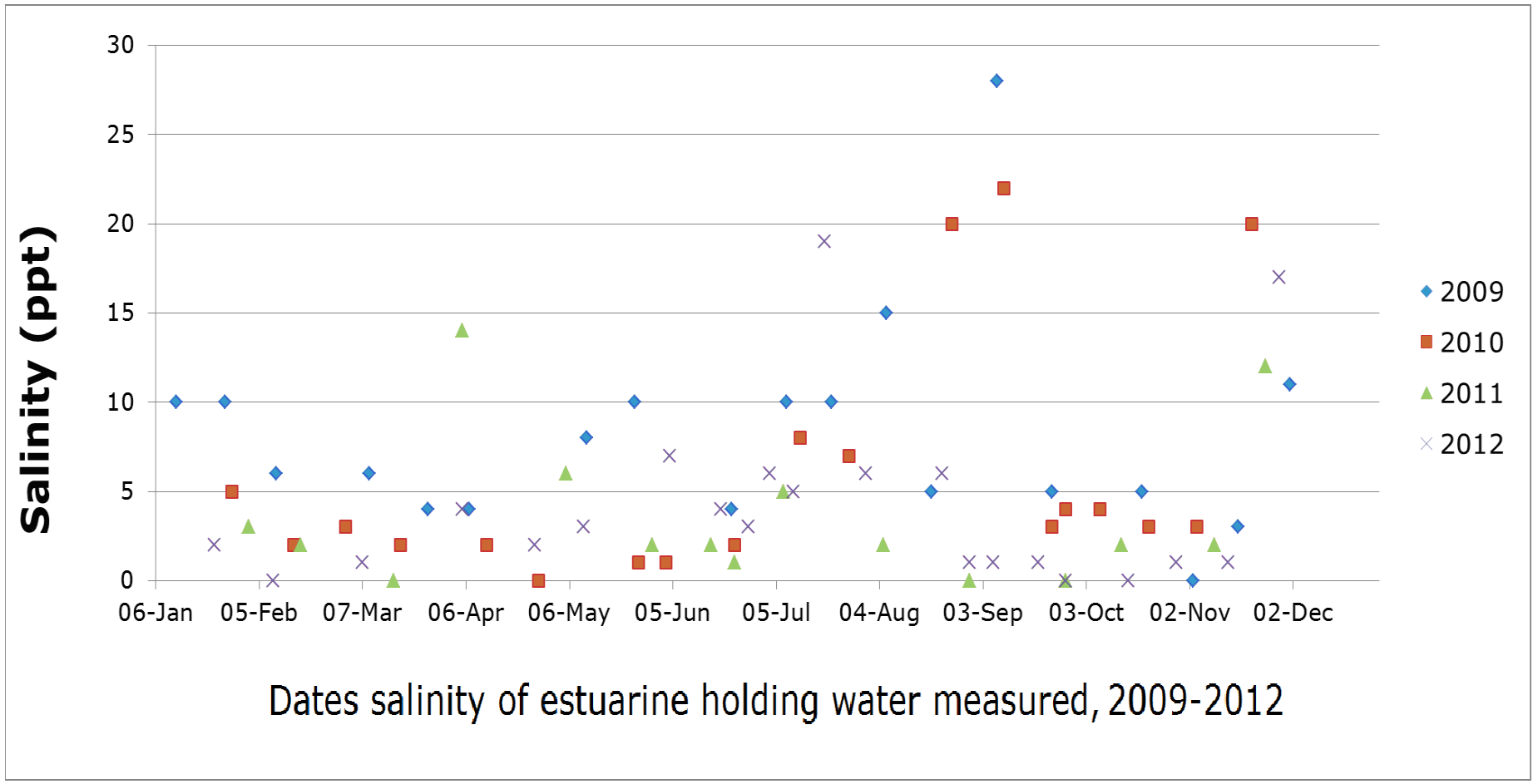

Figure 7, Salinity of estuarine water collected for holding and transporting *C. volutator* to the laboratory.

Years 2009 - 2012.

4.2 Trial 1. Culture of *C. volutator* neonates

High variability was observed between duplicates in this trial, despite this some trends were observed. The majority of the twenty neonates cultured (<2 mm in total body length) were found to have reached adulthood by day 132 (Figure 8). Within this time, many of the initial *C. volutator* reached adulthood and produced offspring which were divided into juveniles and neonates depending on size. Upon sieving one of control beakers, three dead adult *C. volutator* were recovered. These adults did not reproduce as no other specimens were recovered from that beaker. The sediment in the control beakers was noticeably lighter in colour than that of the beakers supplied with algae. The parameters were recorded prior to the water change over in this trial. Temperature remained at 15°C ± 2°C in all of the beakers, salinity was between 30 – 35 ppt, the dissolved oxygen range was between 70% and 90% saturation. The pH of the beakers remained between 7.4 and 8.1.

- The highest reproductive output was found in the mixed algae food *T. chuii* and *R. reticulate*, followed by *C. volutator* supplied with *T. chuii*.
- *C. volutator* supplied with *R. reticulate* had the third highest reproductive output.
- The beakers supplied with *T. chuii* were found to have low numbers of *C. volutator* which reached adulthood by day 132.

- The controls beakers which were not supplied with any food source had the lowest survival rate and had no neonates surviving on day 132.

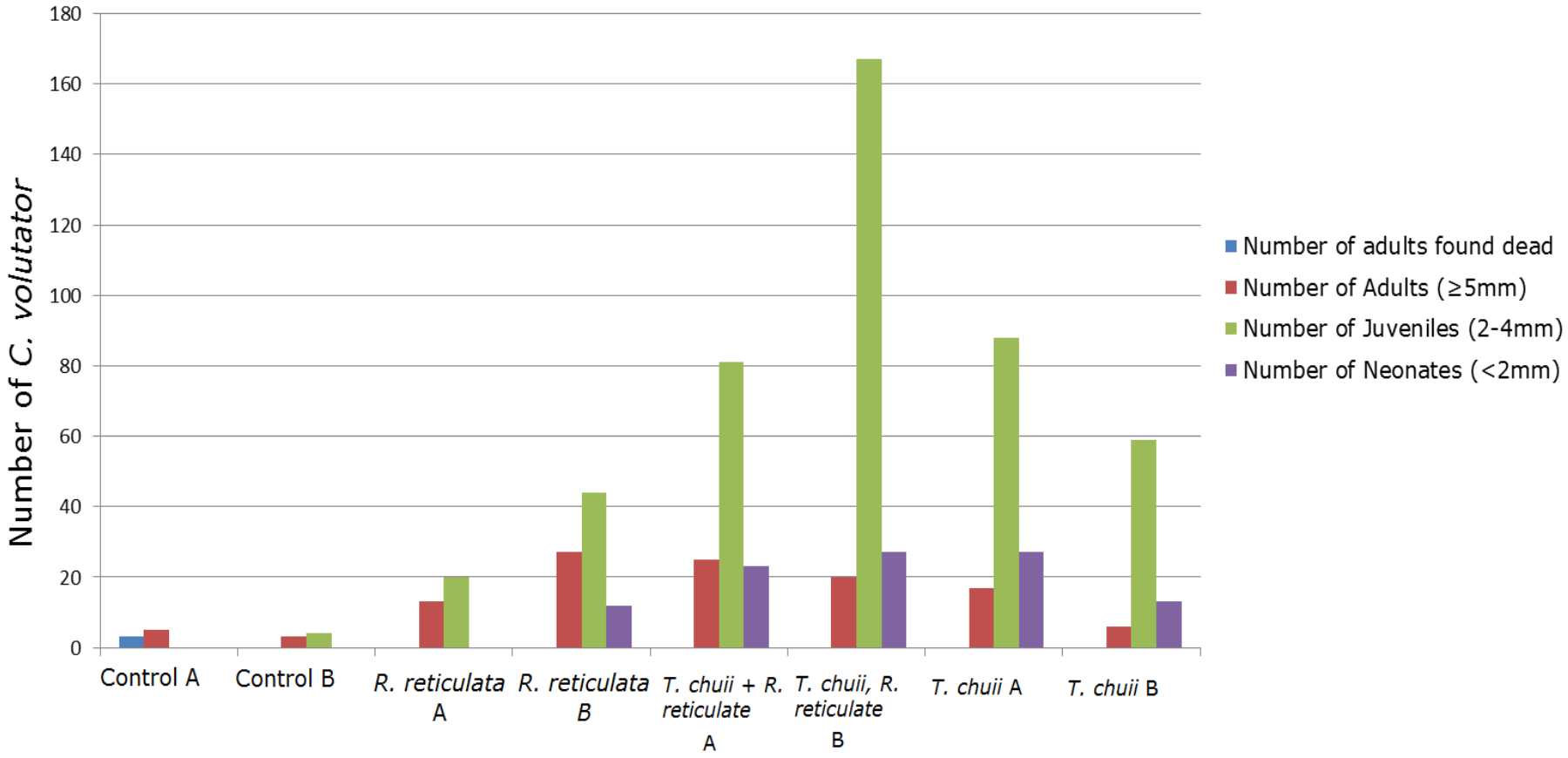

Figure 8, Number and size of *C. volutator* recovered after 134 days of culturing under different feeding regimes, each beaker was initiated with 20 neonates

4.3 Activity observed in Trial 1. Culture of *C. volutator* neonates

Neonates cultured in this trial were found to settle quickly and form visible burrows within 26 days (Figure 9). The numbers of males actively searching for receptive females was recorded three times per week, (observations coincided with days of changeovers, Monday, Wednesday and Friday) observations were made over a 30 minute period. The trial was initiated with *C. volutator* neonates (<2 mm) and cultured over a period of 132 days. The first *C. volutator* activity was observed on day 37 in the beaker supplied with *R. reticulate* (replicate A) (Figure 10). The highest number of *C. volutator* was observed on day 128, when 25 active *C. volutator* were recorded in the beaker supplied with *R. reticulate* replicate B.

Figure 9, Burrows observed in Trial 1, Culture of *C. volutator* neonates

The image shows the clear formation of *C. volutator* burrows and a juvenile *C. volutator* to the bottom right of the image. This picture was taken on day 40 of Trial 1, Culture of *C. volutator* neonates.

4.4 Growth rate observed in Trial 1. Culture of neonates

This trial was initiated with *C. volutator* neonates approximately 1.5 mm in body length excluding antenna and all under 2 mm in total body length. The *C. volutator* were not removed from the sediment for the purpose of measurement, as this may have resulted in stress and impair reproductive output or growth. The body lengths of *C. volutator* were recorded when observed on the sediment surface (see 3.9). On day 54, four *C. volutator* were observed on the sediment surface, these *C. volutator* all had a body length of 5.0 mm ±1 mm excluding antenna. These were measured by gently placing a small ruler beside the specimens. The body length of the initial *C. volutator* (1.5 mm) was subtracted from the observed length on the 54^{th} day (5mm ± 1 mm), therefore, the *C. volutator* grew a total of 3.5 mm ± 1 mm in 54 days or an average of 0.0648 mm ± 0.0185 mm per day. Similar growth rates were also observed earlier in the study when on day 37, a specimen was observed searching the sediment surface which was 4 mm ± 1 mm in body length, therefore, had a growth rate of 0.0676 mm ± 0.027 mm.

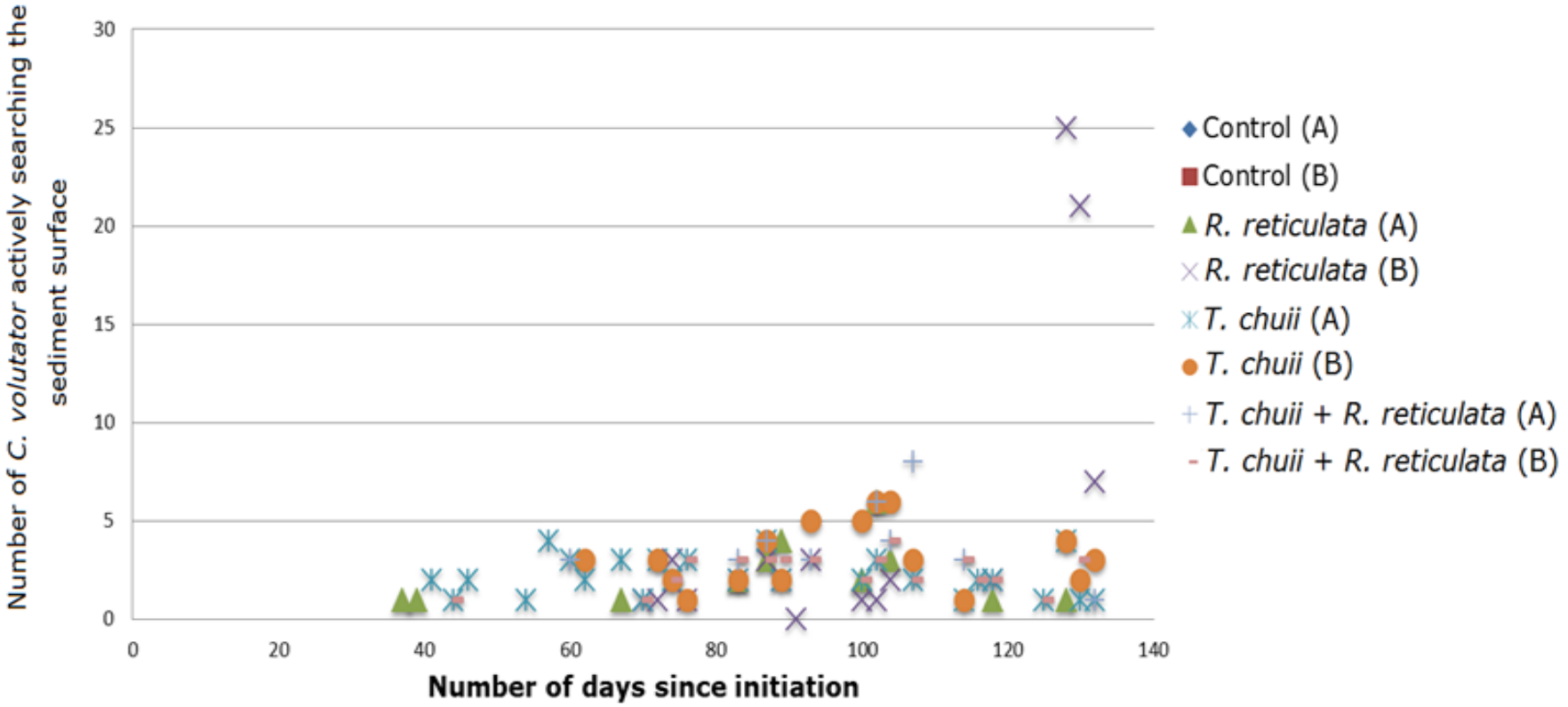

Figure 10, Surface activity of *C. volutator* observed in Trial 1, Culture of *C. volutator* neonates

4.5 Trial 2. Culture of adult *C. volutator*

One hundred adult *C. volutator* were cultured in tanks for a period of 132 days. On day 132, the tanks were sieved and the *C. volutator* were counted. Tanks containing 100 *C. volutator* were supplied with *T. chuii* or *R. reticulate* in duplicate. All tanks were found to be dominated with juvenile *C. volutator* (Figure 11). The highest number of *C. volutator* was found in the tank supplied with *T. chuii* replicate A, with a total of 1,976 of which 1,691 were juveniles. However, *T. chuii* replicate B had only 634 *C. volutator* of which 462 were juveniles. Tanks supplied with *R. reticulate* had lower number of *C. volutator* recovered, but were found to have neonates present. The parameters were recorded prior to the water change over in this trial. Temperature remained at 15°C ± 2°C in all of the beakers, salinity was between 30 – 35 ppt, the dissolved oxygen range was between 80% and 100% saturation. The pH of the beakers remained between 7.4 and 8.1.

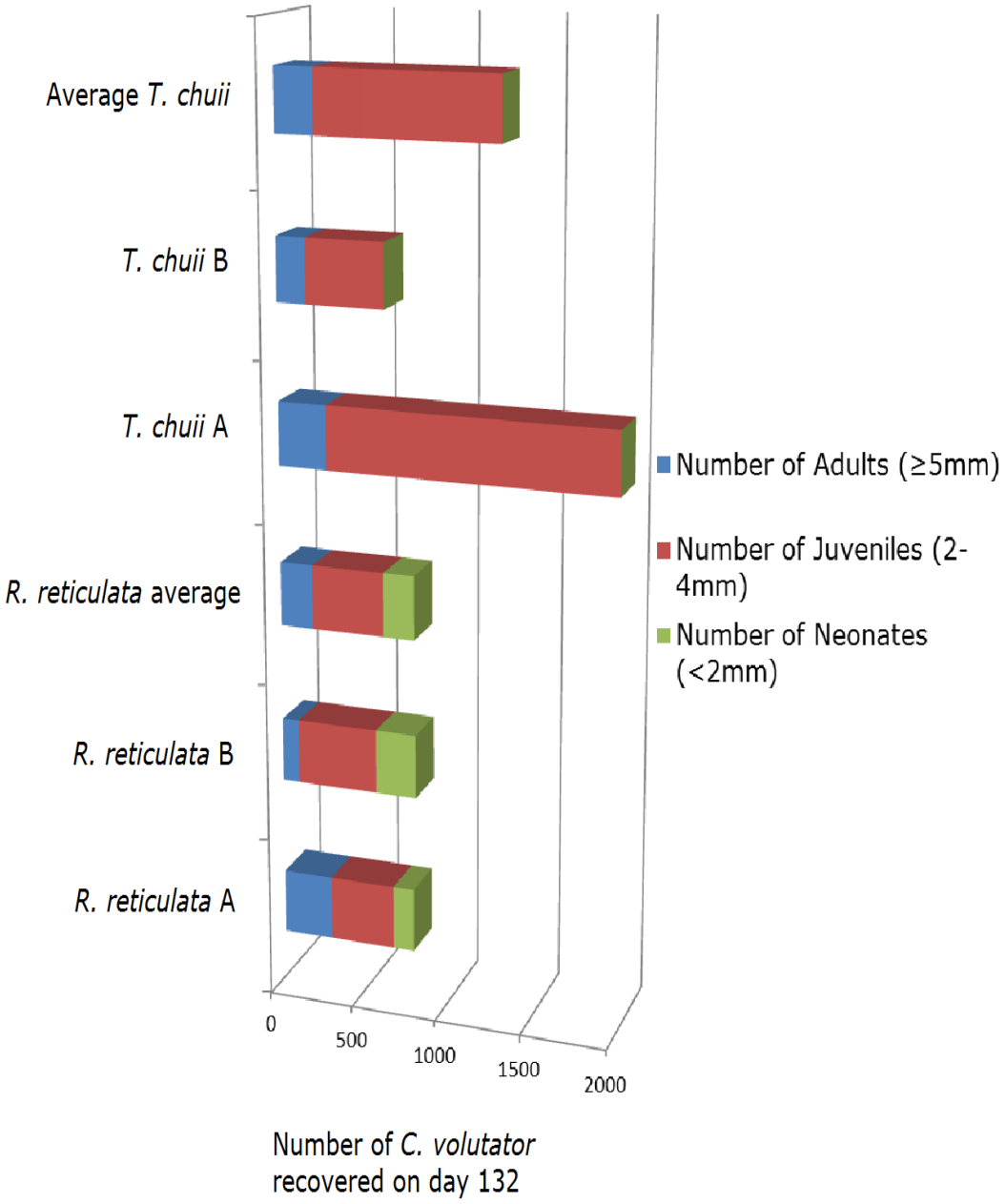

Figure 11, Number of *C. volutator* recovered on day 132 from Trial 2, Culture of adult *C. volutator*

Note: The control tank is not shown, no *C. volutator* were recovered from the control tank which was not supplied with algae.

Tank	Day 0 - 75	Day 76 - 118	Day 119 - 132
T. chuii A	10%	90%	0%
T. chuii B	14%	86%	0%
R. reticulate A	27%	55%	18%
R. reticulate B	0%	66%	33%

Table 1. Estimated percentage of total offspring released in each timeframe in Trial 2, Culture of adult *C. volutator*.

Note: Estimates are based on the size of individual *C. volutator* recovered on day 132 and the growth rate found in this study.

Chapter 5. Discussion

5.1 *C. volutator* control batch mortalities and seasonal variation in adult fitness

Analysis of historical data, relating to batch failures revealed a clear trend of declining fitness during the summer months. In this study, from 2009 - 2012, the number of *C. volutator* batches which failed due to high mortalities was variable (Figure 5). In 2009, none of the 18 *C. volutator* batches which were harvested for testing failed due to high mortalities, however, the years 2010, 2011 and 2012 all encountered batch failures. From the years 2009 - 2012, 45% of all *C. volutator* batches harvested between the 4th of June and 27th of September were unsuitable for toxicity testing due to high mortalities (≥10%). None of the failed batches had parameters which would indicate alternative issues, i.e, oxygen saturation below 80%, salinity in excessive of 36 ppt or pH values outside 7.5 – 8.4. It is possible that these failures are linked to the various seasonal changes in the *C. volutator,* which previous studies have found (Kater *et al*., 2000; Dobrzycka and Szaniawska, 1995).

5.2 Causes of declining *C. volutator* fitness during the summer months

No clear link was found between temperature and salinity (see Figure 6 and Figure 7) of the estuarine water collected and the declining fitness of *C. volutator* (Figure 5). Various population changes such as; changing sex ratio, fluctuation of lipid levels, release of offspring, and environmental changes such as; variations in food supply, daylight hours and temperature makes it difficult to separate each factor and determine which of these is most important in terms of *C. volutator* fitness as a

toxicological test species.

5.2.1 Seasonal variations in lipid levels of *C. volutator* and its implications on *C. volutator* as a toxicological test species

Literature reveals three relevant trends which may impact upon the validity of *C. volutator* as a test species during summer months:

- Decrease in the lipid levels
- Declining sensitivity to the toxicant cadmium
- An increased percentage of females and juveniles

A trend in the seasonal fluctuation of lipid levels in *C. volutator* populations are associated with the release of offspring with lower levels being observed post reproduction (Figure 2) (Dobrzycka and Szaniawska, 1995). The correlation Meador (1993) found between decreasing lipid levels (and therefore reduced fitness) of the amphipod *R. abronius* and *E. estuarius* and increasing sensitivity to the toxicants tributyltin (TBT) and cadmium (Cd), may be applicable to *C. volutator.* This would make any toxicological effect of chemicals tested on *C. volutator* more pronounced. However, this has not yet been determined in the laboratory. The trend observed in the Eastern Schldt Bay, Netherlands, by Kater *et al*., (2000), revealed increasing sensitivity to the toxicant cadmium during summer months (Figure 3), followed a remarkably similar trend to that found by Dobrzycka and Szaniawska, (1995) (Figure 2) in which lipid levels fell dramatically. Kater *et al*., 2000 observed the declining fitness of *C. volutator* as a toxicological test species during the months of February possibly as a result of the release of the first brood and again in June, July and August resulting from the second brood.

Dobrzycka and Szaniawska (1995) also found impoverished lipid levels during the months of February and again in June/July. The fragmented nature of these periods of decreased lipid levels is most likely due to the two broods produced by *C. volutator* during these months in warmer climates. Two factors linked to reproduction result in the decrease in lipid levels in the population; the first is the expenditure of lipids during the production of offspring, the second is the changing sex ratio and increasing percentage of juveniles in the population, which may have naturally lower lipid levels. *C. volutator* as other amphipods such as *Gammarus minus* (Glazier 2002), may become more sensitive to stresses caused by toxicants due to a reduction lipid levels.

Kater *et al*., (2000) mitigates the possibility of decreased lipid levels accounting for the increased sensitivity of *C. volutator* to cadmium by comparing laboratory held specimens with ones harvested from wild populations. Kater *et al*., (2000) collected specimens in December and maintained them in the laboratory by replacing the overlying seawater daily. After four to seven months, these were used in comparative toxicological studies with wild harvested specimens, using cadmium as a toxicant, comparison of toxicity results appear to follow similar trends. This may be due to an error in the methodology, the replacement of seawater, if collected from the estuary, may have supplied the *C. volutator* with a similar food supply to that of the wild *C. volutator* resulting in a similar lipid level and therefore a similar trend in cadmium LC_{50}. However Kater *et al*., (2000) does not specify the source of the replacement water. Kater *et al*., (2000) did not conduct analysis of lipid levels on wild harvested or laboratory cultured *C. volutator* before

comparative toxicity tests. Therefore, it is unclear whether or not the lipid levels varied greatly, thus, the correlation between the lipid levels of the *C. volutator* and declining fitness as a toxicological test species has not been confirmed or refuted, but may warrant further research considering the remarkably similar trends observed by Kater *et al*., (2000) and Dobrzycka and Szaniawska (1995).

Seasonal variations in lipid levels are linked to reproduction, however, there are other factors which are linked to reproduction such as moulting. Kater *et al*., (2000) suggests a link between the sensitivity of *C. volutator* and moulting, as *C. volutator* form a new carapace and calcify, they may also sequester cadmium into their carapace making them a more sensitive toxicological test specimen. This has not been proven in the laboratory.

Another possible factor resulting in the declining fitness of *C. volutator* is the changing sex ratio during the reproductive season and the natural life cycle of *C. volutator* (Figure 1). It is known that *C. volutator* have 1 - 2 broods per year, the first generation occurs in the spring, this generation reaches sexual maturity by late summer, after the release of offspring this generation dies in the summer months (Peer *et al.*, 1986; Neal and Avant, 2006). In the pre-reproduction period, there is a dramatic reduction in the percentage of males, this result from the high mortalities observed on completion of their "procreative function" (Dobrzycka and Szaniawska., 1995). It may be possible that adult *C. volutator* harvested during these periods of changing population dynamics, have high adult *C. volutator* mortalities, therefore resulting in declining *C. volutator* fitness as a toxicological test specimen as observed by Kater *et al*., (2000).

Indeed, considering the well documented *C. volutator* generations observed in spring and late summer, and the periods of increasing sensitivity to the toxicant cadmium i.e. February, July - September (Kater *et al*., 2000), pre-reproduction mortalities observed in male population and post reproduction mortalities may be a plausible explanation.

5.3 Possible causes of batch failures

The trends of declining lipid levels and changing populations dynamics may explain the batch failures observed in the 4th of June and 27th of September 2010 – 2012 (Figure 5). Diminished lipid levels may be particularly relevant as it is unlikely that *C. volutator* are capable of feeding when removed from the sediment as both feeding methods of *C. volutator* observed take place in their burrows (Gerdol and Hughes, 1994: Fish, 2011). In Trial 2, Culture of adult *C. volutator,* the detritus present in the sediment did not sustain adult *C. volutator* with none of the adults surviving until day 132. This indicates that *C. volutator* does not obtain sufficient sustenance from detritus to be maintained over a long period.

The OSPAR (1995) "Sediment Bioassay Using an Amphipod Corophium sp" guidelines specify that *C. volutator* must not be held in the sediment but in a small amount of detritus. Both feeding methods available to *C. volutator* are carried out while burrowed in sediment (Gerdol and Hughes, 1994; Fish, 2011) making it unlikely that *C. volutator* obtain any substance while maintained in holding tanks. The inability to feed or access only minimal or poor quality food sources while in holding tanks may compound stress induced by low lipid levels already found upon

harvesting. Considering that ≥10% mortalities resulted in batch failures, and the changing population dynamic during summer months involving a dramatic decline in the adult population, it is not surprising that batch failures have only been observed during these months.

5.4 Overcoming seasonal variations in *C. volutator* fitness

Higher control failure rates result in commercial tests being postponed which may prove costly for commercial laboratories. To overcome this issue *C. volutator* may be cultured in the laboratory. Although the use of laboratory cultured *C. volutator* has not been specified by the OSPAR (1995) guidelines "Sediment Bioassay Using an Amphipod *Corophium* sp", cultured *C. volutator* have been proven to be comparable to wild harvested *C. volutator* in toxicity testing (Peters and Ahlf 2005). Provided that sufficient numbers of *C. volutator* can be achieved in the laboratory and the production of offspring can be managed so as not to encounter the same seasonal high mortality rates associated with wild populations, laboratory cultured *C. volutator* may prove to be preferable test specimens.

5.5 *C. volutator* culturing conditions

C. volutator have reproduced offspring in both Trial 1, Culture of neonates and Trial 2, Cultures of adult *C. volutator* in the absence of a tidal rhythm and a lighting regime (complete darkness) and with no apparent triggers to induce the onset of reproduction. The temperature of 15 ± 2 °C and salinity of 30 ppt has proven satisfactory for the culturing of *C. volutator.* The algae *R. reticulate* and *T. chuii* have been used successfully as a food source for *C. volutator* both individually and particularly when used as a mixed algal food source.

5.6 Growth rate as determined in Trial 1. Culture of neonates

The growth rate of 0.0648 mm ± 0.0185 mm per day observed is this study was similar to that found by Peters and Ahlf (2005) of 0.07 mm per day or 0.069 mm/day and 0.067 mm/day found by Kater *et al*., 2008). The growth rates in this study did not vary between the tests supplied with different algae.

5.7 Preferred feeding regime as determined in Trial 1. Culture of neonates

The number of offspring produced varied between replicates, although the preferred feeding regime in this study was that of the 50/50 combination of *T. chuii* and *R. reticulate* with an average of 171 *C. volutator* recovered at the end of the test (Figure 8). The second most productive feeding regime was *T. chuii* with an average of 105, the lowest output was recorded in the beakers supplied with *R. reticulate* with only 58 *C. volutator* recovered on day 132. *C. volutator* achieved a higher reproductive output on a mixed algae diet than on a mono specific algae diet. However the preferred feeding regime cannot be determined conclusively due to the high variability between replicates and the low number of replicates used. Therefore, further investigation is required to establish the optimum feeding regime.

5.8 Reproductive output in Trial 2, Culture of adult *C. volutator*

After a period of 132 days, the number of *C. volutator* recovered from the tanks supplied with *R. reticulate,* was 793 and 808 in replicates A and B, respectively (Figure 11). The number of *C. volutator* recovered from the *T. chuii,* was 1976 and 634 in replicates A and B, respectively. The vast difference between these replicates may be attributed to the

difference in brood sizes of individual females, i.e. 11 - 100 offspring depending on the size of the female (Neal and Avant, 2006). As the initial *C. volutator* were selected at random, it is also feasible that a higher female to male ratio may have contributed to a greater fecundity in replicate A of the tanks supplied with *T. chuii*. All tanks in the trial were dominated by a juvenile population (specimens < 5 mm in body length).

The aim of this trial was to determine if adult *C. volutator* could be produced for toxicity testing. It is clear that adult *C. volutator* can be produced in the laboratory, however, the culturing duration of 132 days in this trial was insufficient to provide adequate numbers of adults for testing. An estimated 15 - 40 extra days (based on the growth rate of 0.0648 mm ± 0.0185 mm per day as found in this study) would allow sufficient time for juvenile population to reach adulthood.

The approximate day on which the *C. volutator* released offspring was calculated by subtracting the time required for *C. volutator* to reach the specified size i.e. ≥57 days for adults (≥ 5 mm in body length) or juvenile or 14 - 42 days (2 - 4 mm in body length), from the total culturing time of 132 days. A small percentage of the population resulted from the release of offspring in the first 0 – 75 days (Table 1). The factors preventing *C. volutator* from producing a substantial number of offspring in this timeframe may include:

1) The time spent in holding tanks prior to use in the trial exerted stress on the *C. volutator* resulting in an extended acclimatisation period.

2) *C. volutator* may have been recovering after the release of offspring

prior to use in this trial.

The majority of offspring (55 - 90% depending on the food source and replicate) has been estimated to have been produced from day 78 to 118 from initiation, a period of only 40 days. This indicates a synchronised release of offspring. This occurred despite the lack of environmental triggers i.e. temperature fluctuations, salinity or a change in the lighting regime.

Observation bias may have occurred in Trial 2, Culture of adult *C. volutator*, due to the sieve size used. *C. volutator* neonates may not have been recorded in great numbers due to the use of a 600 µm sieve, the neonates which were less than 1 mm in length could possibly have passed through the sieve mesh by approaching antennae or abdomen first.

5.9 Number of broods observed in Trial 1. Culture of neonates

Within 132 days the initial *C. volutator* neonates had reached adulthood and had at least one brood which reached the juvenile stage (2 - 4 mm in body length). Under the optimum feeding regime all of the initial 20 neonates had reached the adult stage, however, specimens reaching adulthood under the other feeding regimes was variable. Under all feeding regimes *C. volutator* population was dominated with juveniles on day 132, with a small number of neonates also present in beakers, with the exception of *R. reticulate* replicate A. It is unclear if one or two broods were produced by any of the initial *C. volutator* in this trial. However, it is possible that the small neonate population present was the result of a second brood.

5.10 Total time required to obtain sufficient numbers of adult *C. volutator*

The majority of the population recovered in Trial 1, Culture *C. volutator* neonates and Trial 2, Culture of adult *C. volutator* were in the juvenile stage (2 - 4 mm), an estimated additional 29 days would be required for these specimens to reach the adult stage (assuming an average juvenile size of 3 mm and a growth rate of 0.0648 mm ± 0.0185 mm per day). This would make estimated total culturing time 161 days. However, considering that both trials had varying starting points (Trial 1, began with neonates and Trial 2, began with adults) and still had populations dominated by juveniles, it is likely that factors other than time are required to obtain a population of predominately adults. If the triggers which initiate the reproduction in *C. volutator* were understood they may be utilised to initiate reproduction and obtain adult *C. volutator* specimens within a shorter timeframe.

5.11 Laboratory cultured *C. volutator* as a toxicological test species

There are various factors which make laboratory cultured specimens preferable to wild harvested specimens; sampling difficulties, i.e., freezing conditions making collection of *C. volutator* impossible, cumulative thermal stresses resulting in decreased fitness, decrease in lipid levels, high levels of parasitic infection (microphallid trematodes) and/or pollution. These factors in theory may make field collected *C. volutator* a less reliable test species.

There are a number of factors which may result in differences between laboratory cultured *C. volutator* and wild harvested specimens.

- Laboratory cultured *C. volutator* are unlikely to have parasitic infections
- Lipid levels may differ in laboratory cultured and wild populations.

5.11.1 Parasitic infections

Parasitic infections may be present in wild harvested *C. volutator* specimens. Considering the multi host life cycle of the parasites microphallid trematodes *M. subdolum* and *M. claviforrnis* (Damsgaard *et al.,* 2005), theoretically it would be impossible for laboratory cultured specimens to carry such parasitic infections, provided that they were cultured in the laboratory for a number of generations resulting in the separation from the original brood stock. Parasitic infections may impact adversely on the sensitivity of *C. volutator* to toxicants as increased mortalities have been recorded in infected *C. volutator. C. volutator* are known to become anaemic with parasitic infections thereby resulting in an additional stress during toxicity testing. No research has been conducted on the sensitivity of parasitic infected *C. volutator* vs. non infected *C. volutator* to toxicants.

5.11.2 Comparable sex ratio of wild and harvested *C. volutator*

C. volutator females have been found to dominate wild populations, this was thought to result from the heavy predation of males during surface activity, however, Schneidesr *et al.,* (1994) has found that predation has little impact on the sex ratio of *C. volutator* populations. Therefore, the

absence of predation in laboratory cultured *C. volutator* should not result in a varying sex ratio of wild harvested and laboratory cultured *C. volutator.* In this study, the sex ratio was not determined due to time constraints; however, this data would have been invaluable. Had the sex ratio been determined for each of the culturing vessels in Trial 1, Culture of *C. volutator* neonates and Trial 2, Culture of adult *C. volutaor* the average number of offspring produced per female could have been determined. Comparing the average number of offspring per female produced in each trial would have provided a clearer picture on the preferable feeding regime, rather than comparing the number of offspring produced per culturing vessel or tank.

5.11.3 The sensitivity of laboratory cultured and wild harvested *C. volutator*

In order for laboratory cultured *C. volutator* to be a relevant test species it is important that the sensitivity of the cultured specimens is comparable to that of wild harvested *C. volutator.* Despite different biotic and abiotic factors in the life history of laboratory cultured and wild harvested *C. volutator*, studies have shown that laboratory cultured *C. volutator* are comparable to wild harvested specimens in toxicity tests (Kater *et al*., 2000; Peters and Ahlf, 2005).

Chapter 6. Future work

Further work is required to:

- Determine reproductive output (in numbers of offspring per female) and growth rates of *C. volutator* using different algal food sources and higher numbers of replicates for more conclusive results
- Investigate the triggers for reproduction in *C. volutator*, including tidal influence, light dark influences and lipid levels in females
- Carry out field research to determine the conditions conducive with high population densities of *C. volutator* in order to replicate these conditions in the laboratory
- Compare seasonal variation in the sensitivity of wild harvested *C. volutator* and laboratory cultured specimens, to a reference toxicant to determine if cultured *C. volutator* are preferable for use in toxicity testing
- Determine if a direct correlation exists between lower lipid levels and the sensitivity of *C. volutator* to toxicants

Chapter 7. Conclusion

In this study three of the four years examined had significant numbers of *C. volutator* batch failures during the summer months proving a requirement for laboratory cultured specimens. The feeding regime which resulted in the greatest number of offspring in this study was the mixed algae *T. chuii* and *R. reticulate* diet. This suggests that a mono-specific algal diet is not preferred by *C. volutator.* However, due to the low number of replicates and the lack of data on the sex ratio in these trials, statistically a preferable feeding regime cannot be determined. In both culturing of neonates in Trial 1, and adults in Trial 2, juveniles dominated the population on day 132, indicating a synchronised release of offspring (estimated at day 76 – 118, based on growth rate). This synchronised release of offspring occurred despite the different starting points of the trials, i.e., Trial 1, was initiated with neonates and Trial 2 initiated with adults, indicating a trigger for the release of offspring common to both trials. Based on the growth rates observed in this study, approximately 161 days of culturing would be required to obtain predominately adult specimens. However, a shorter culturing period may be attainable if the triggers for reproduction were understood and utilised. This study found that *C. volutator* does not require tidal influence, dark/light cycles or a fluctuating salinity which are present in their estuarine environment to reproduce. However, as this study has not evaluated the influence of these environmental factors on reproduction, their influence cannot be quantified, and it may be possible that the absence of these environmental influences decreases the fecundity of *C. volutator*. This may warrant further investigation.

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