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Growth of the Polychaete *Hediste diversicolor* (O. F. Müller, 1776) fed on Smolt Sludge and Biogas Residues

Evaluation of RNA/DNA Ratio as growth
indicator

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Trondheim, May 2018. Fredrik Hofseth Berntsen

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Abstract

The last decades have seen decimation of several fish stocks, leading to increased governmental restrictions on the worlds wild fish catch. In contrast, the consumption demand of marine food has increased greatly. To meet the increasing demand, and simultaneously reduce the pressure on wild fish stocks, a strategy is to use integrated multi-trophic aquaculture systems (IMTA) to recycle nutritional waste into new marine biomass.

The objective of the present study was to examine if the polychaete *Hediste diversicolor* could grow when fed diets consisting of waste streams from Atlantic salmon smolts (*Salmo salar*) and residues from biogas production. It was also a goal to study, to which extent their nutritional condition, growth and survival rate was affected by the diets. To test this, the polychaetes were subjected to different food regimes over a 30-day cultivation trial with a following RNA/DNA analysis.

The results indicated that *H. diversicolor* was able to utilize both food sources to promote growth in biomass, when grown under suitable densities and conditions for the species. There was however a significant difference in the growth rates between the diets; *H. diversicolor* fed on commercial fish feed, showed a significantly higher growth rate compared to the groups fed on the smolt and residue diets. The nutritional condition of *H. divericolor* was examined by use of an RNA/DNA analysis. The results showed a significant difference in this ratio between two of the diets, but no correlation was observed with growth rates of polychaetes. A starvation experiment was conducted to investigate the effect of starvation on the RNA/DNA ratio, and a weak correlation was found between the growth rates and observed RNA/DNA ratios in this experiment. For both experiments, no significant differences in survival rates were found.

Conclusively, *H. diversicolor* have shown to be capable to utilize the nutrients in smolt waste streams and biogas residue to promote growth. The result of low correlation between RNA/DNA ratios and growth rates, may suggest that the current method was not properly calibrated for growth determination of *H. diversicolor*. Further trials are needed to get an accurate way of measuring RNA/DNA ratio for *H. diversicolor*, and to verify if this method indeed is suitable for growth determination of polychaetes.

Sammendrag

De siste tiår har bevitnet ødeleggelser av flere fiskebestander, noe som har ført til en innføring av strengere fiskekvoter verden over. Til tross for dette har etterspørselen etter maritim mat hatt en voldsom økning de siste tiårene. For å møte den økte etterspørselen, og samtidig redusere trykket på ville fiskebestander, kan en strategi være å benytte integrerte multi-trofiske akvakultursystemer (IMTA) for å resirkulere næringsrikt avfall til ny marin biomasse.

Hensikten med denne studien var å undersøke hvorvidt børstemarken *Hediste diversicolor* kunne vokse når den ble fôret med avfall fra et landbasert smoltanlegg, samt biogass-avfall. For å teste dette ble det gjennomført et 30-dagers foringseksperiment, med påfølgende RNA/DNA analyse.

Resultatene fra denne studien indikerte at *H. diversicolor* var i stand til å benytte næringsstoffene i begge diettene til vekst i biomasse når den ble avlet frem under passende tetthet og forhold for arten. Det ble funnet en signifikant forskjell i vekstraten; *H. diversicolor* fôret på kommersielt fiskefôr viste en signifikant høyere vekstrate i forhold til de øvrige diettene. Som et redskap ble RNA/DNA forholdet benyttet for å kartlegge veksten til *H. diversicolor*. Resultatene viste en signifikant forskjell mellom to av diettene, men resultatene korrelerte ikke mot de observerte vekstratene. Et sulteforsøk ble utført med hensikt å kartlegge effekten av sult på RNA/DNA forholdet hvor en svak korrelasjon ble observert. Begge forsøkene viste ingen signifikante forskjeller i overlevelsesrate mellom diettene.

For å oppsummere, så har *H. diversicolor* vist seg å være kapabel til å bruke næringsstoffene i smolt- og biogass-avfall til vekst. RNA/DNA resultatene viste lav korrelasjon med vekstrate, noe som kan indikere at metoden brukt i denne studien ikke er tilpasset for å vise vekst for *H. diversicolor*. Videre testing trengs for å få dannet en presis måte å måle RNA/DNA forholdet for *H. diversicolor*, og for å se om det i hele tatt er mulig å benytte denne metoden for å indikere vekst hos børstemarker.

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Abbreviations

AM	Amino acid
ANOVA	Analysis of variance
C	Carbon
DM	Dry matter
DNA	Deoxyribonucleic acid
dsDNA	Double-stranded DNA
DW	Dry weight
FAO	Food and Agriculture Organization of the United Nations
FCR	Economic feed conversion ratio
FF	Fish feed – Lerøy Belsvik
IMTA	Integrated multitrophic aquaculture
LAR	Larviva-Biomar®
L/D	Light to darkness
N	Nitrogen
P	Phosphorus
R	Biogas residue – Scandinavian biogas
r	Person's correlation coefficient
RAS	Recirculation aquaculture system
RNA	Ribonucleic acid
SGR	Significant growth rate
SS	Smolt sludge – Lerøy Belsvik
STEB	Reaction Stop Buffer
Tris EDTA	Trisaminomethane Ethylenediaminetetraacetic acid
WW	Wet weight

1 Introduction

In the past century the increased size of fishing fleets and increase in efficiency of modern fishing equipment has decimated fish stocks previously regarded as infinite (Nakken, 2008, p. 37-38). The total catch of the worlds wild stock fisheries reached a peak in 1989, with a catch of approximately 90 million metric tonnes of fish. The year after, most of the commercial stocks had a great decline indicating that fishing on these levels would be non-sustainable (Food and Agriculture Organization of the United Nations, 2016, p. 13). However, this catch does not even come close to meeting the worlds demand for fish. The world supply of fish for human consumption has grown at a rate of 3.2 % in the period 1961-2013, resulting in 20 kg fish per capita in 2015 (FAO, 2016, p.71).

To reach this demand without decimating the wild fish stocks, alternatives must be used. The use of aquaculture is a promising possibility. The last few decades have seen a steady increase in aquaculture fish production for human consumption, as presented in Figure 1.1. Aquaculture surmounted to approximately 50% of the fish caught for human consumption in 2013-2015, and this contribution is thought to reach 57% in 2025 (FAO, 2016, p. 179).
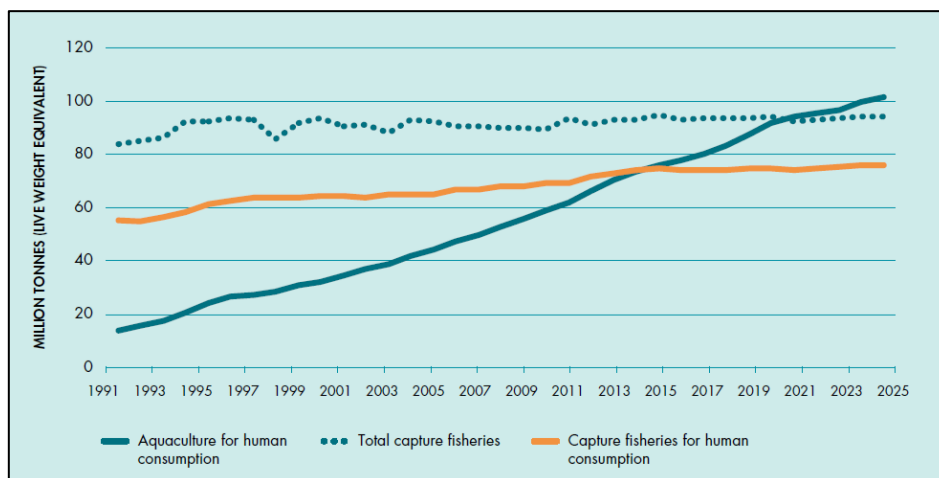
Figure 1.1. Relative contribution of aquaculture and capture fisheries in catch of fish for human consumption. The dotted line indicates the total capture fisheries catch, including fish catch not used for human consumption. The y-axis represents catch of fish in million tonnes (live weight). The x-axis represents years (FAO, 2016, p. 175).

Feeding of aquaculture fish involve the use of marine resources (Ytrestøyl, Aas & Åsgård, 2015) and a subsequent release of organic and inorganic waste to the environment (Wang, Olsen, Reitan & Olsen, 2012). Due to an increasing demand, the price of fish meal and fish oil

has risen the last decade and is not expected to return to prior levels (FAO, 2016, p. 82). Fish feed is therefore regarded as one of the most limiting factors to aquaculture production and further sustainable aquaculture expansion (FAO, 2016, p. 25; Nielsen et al., 2017).

To reduce the amount of feed used, more research is done both on the efficiency of the feed, and the possible reuse of waste as new marine biomass. Several studies have been conducted to investigate if polychaetes could be used to utilize the otherwise lost nutrients. They all indicate that polychaetes could potentially recycle the nutrients into new marine biomass, thus reducing the fishing pressure on wild fish stocks and reducing the amount of effluents (Batista et al., 2003[b]; Nesto, Simonini, Prevedelli, & Da Ros, 2012; Santos et al., 2016; Seekamp, 2017).

1.1 Waste composition and treatment

The aquaculture industry is under constant scrutiny from environmental groups because of the apparent negative environmental effects due to release of wastewater (Doupe, Alder, & Lymberyl, 1999). The wastewater consists mainly of uneaten fish food and fish faeces, and its composition is directly related to the composition and quantity of the feed fed to the species (Siddiqui, 2003). The feed is typically rich in carbon, phosphorous, and nitrogen (Axler et al., 1996). As much as 62 % nitrogen, and 70 % carbon and phosphorus in feed input were lost into the environment at sea cage sites in 2009 in Norway (Wang et al., 2012), as presented in Figure 1.2.

When these nutrient-rich waste streams reach the environment, the environmental issues occur. Principal wastewater issues are; hyper-nutrification, eutrophication resulting in algal blooms, oxygen depletion, and destruction of benthic environments. Because of these negative effects on the environment, enormous pressure is exerted from environmental institutions worldwide on aquaculture operations to effectively clean the wastewater and waste before it reaches the environment (Siddiqui, 2003). This is a very costly process. Therefore, environmental management poses as a restraint for aquaculture growth. However, with proper, sustainable management, the negative effects of aquaculture could be minimized. Thereby, reducing social and ecological conflicts and providing greater long term economic security (Doupe et al., 1999).
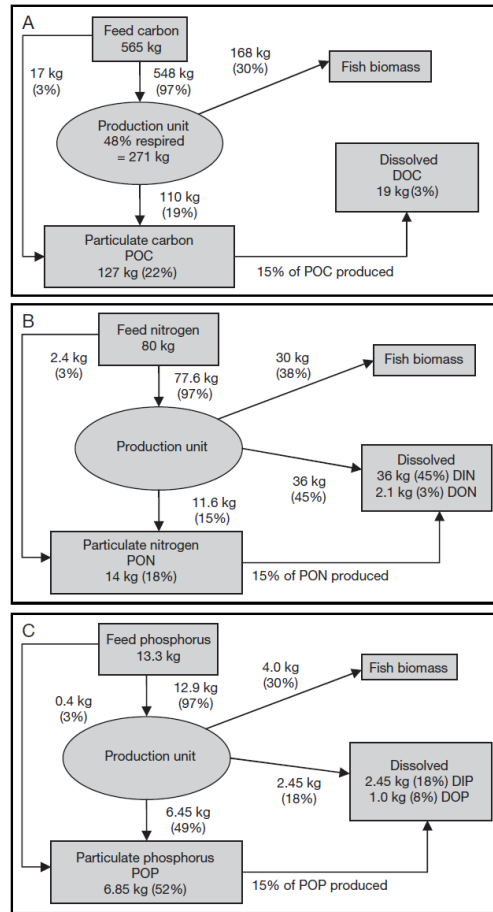
Figure 1.2. Annual nutrient fluxes (kg [t produced × yr]⁻¹) and components (kg t⁻¹ produced) for Norwegian salmon farming in 2009: (A) C, (B) N and (C) P (Wang et al., 2012).

If efforts are made on retaining the waste within the system, the waste-streams could for instance be used as fertilizer in agriculture, nourishment within aquaponics, as feed for algae, or as microorganism cultures (Lekang, 2013, p. 118). The use of integrated multitrophic aquaculture (IMTA) to reduce loss of nutrients is also an intriguing possibility.

IMTA could be described as; the practise where an organism uses the by-products (waste) of another as fertilizer, food, and/or energy (Chopin and Robinson, 2004; Chopin, 2006). The idea being to use a co-culture with species in different trophic and nutritional levels where nutrient loss from one species is nutrient input to another (Chopin and Robinson, 2004; Chopin, 2006, Reid et al., 2008). Drivers for IMTA could be found at several levels in the production cycle. It could contribute to additional revenues from producing additional crops and may contribute to a higher reuse of otherwise lost nutrients (Troell et al., 2009). IMTA could in other words enable the farmer to expand production despite limitations on nutrient emissions proposed by

environmental regulations. IMTA is the only practical remediation approach offering additional revenues in additional commercial crops, compared with other bio mitigation approaches (Troell et al., 2009). Upon establishing an IMTA system it is important to consider which species are most adapted to live in the habitat/culture unit. To ensure successful growth and economic value farmers should use; local species, species that complement each other on different trophic levels, species proven to grow under current conditions and species who have an established market or could potentially create a new market (Barrington, Chopin & Robinson, 2009).

1.2 Utilization of polychaetes

Presently, polychaetes are mainly used in the niche market of angler fishing. They have high market value due to high demand. In western Europe the natural supply of polychaetes does not meet this demand and harvesting activities in the natural environment could potentially create a non-sustainable situation with detrimental effects on the environment. For this reason, non-indigenous species of polychaetes imported from Asia and USA now constitute the greater marked in Southern Europe. Intensive cultivation of polychaetes could ease the pressure on the natural populations and reduce the import of non-indigenous species. In addition to being used as angler bait, polychaetes could be used as feed/feed supplement to finfish- or crustacean-operations (Olive, 1999).

Polychaetes form a major component of the natural diets of marine finfish and larger crustacea. Also, their use as angler bait provides proof of their attractiveness to fish. As an alternative to fish meal and fish oil, the potential usage of polychaetes is great. There can also be found strong evidence that polychaetes could be used as a protein and lipid source in fish and crustacean diets (Olive, 1999). Several studies indicate that the ragworms composition reflect their diets marine constituents (García-Alonso, Müller, & Hardege, 2008; Santos et al, 2016; Seekamp, 2017). This indicating that they can utilize the nutrients in their diet to enhance their fitness (Granada, Sousa, Lopes & Lemos, 2015).

In a study conducted by Seekamp (2017) comparing the biochemical composition of *Hediste diversicolor* fed with waste from a smolt rearing facility and fish feed, no significant difference was found. This implies that the use of *H. diversicolor* to treat smolt waste could give new marine biomass and reduce the pressure on wild fish stocks (Seekamp, 2017).

Nesto et al. (2012) studied juveniles of the polychaete species *H. diversicolor* cultivated at different densities and fed with commercial fish feed with varying protein content and one seaweed diet. The results indicated high growth rates even at medium densities. Based on these results, Nesto et al (2012) suggested that *H.diversicolor* would be suitable for commercial exploitation within indoor farming systems.

1.3 *Hediste diversicolor*

The common ragworm, *Hediste diversicolor* (O.F. Müller, 1779), commonly referred to as *Nereis diversicolor*, is a polychaete widely distributed in estuarine and lagoon habitats in the North Atlantic Ocean, from North Africa to the North of Europe (Mettam, 1979, 1981). *H. diversicolor*, as depictured in Figure 1.3, inhabits the uppermost 20 cm of mud or muddy sand sediment where it lives in burrows with depth up to ~ 15 cm (Budd, 2008). Increasing burrow depth is found with increasing body size (Esselink & Zwarts, 1989). Size increases with declining latitude (Scaps, 2002), with a maximum length of approximately 20 cm, with a commercial size of 10 cm, which corresponds to 0.5 g (Nesto et al., 2012).

Kingdom	Animalia
Phylum	Annelida
Class	Polychaeta
Order	Phyllodocida
Family	Nereididae
Genus	*Hediste*
Species	*Hediste diversicolor*

Figure 1.3. H. diversicolor (Photo: Marianne Uhre, 2017).

The polychaetes live in populations of varying size. Field studies conducted in the Odenese fjord in Denmark showed a varying density from ~300 to 3000 individuals per square meter (Vedel & Riisgård, 1993). Reduced growth is reported with densities above 1000 individuals per m^2 suggesting negative influence from intraspecific competition (Nesto, 2012). Populations of *H. diversicolor* usually use 2-3 years to reach spawning state (Olive & Garwood, 1981). Upon reaching maturity *H. diversicolor* changes colour from red-brown to light green for

mature males, and deep green for mature females. Spawning occurs when the temperature reaches 5-11°C, following a period of low temperatures, synchronized by full or new moon (Scaps, 2002).

This polychaete species shows high physical tolerance to changing environmental conditions such as temperature, salinity and hypoxia (Kristensen, 1983; Ozoh & Jones, 1990). It can withstand changing salinity levels from freshwater (~0 ‰) to 70 ‰, however they cannot reproduce when levels are under ~5 ‰ due to restrictions in larvae development (Scaps, 2002).

In addition to its high stress tolerance, it has a changing feeding behaviour which allows it to obtain nourishment as a carnivore, herbivore, suspensivore, and detritivore (Bradshaw et al., 1990; Riisgård, 1991; Nielsen, Eriksen, Iversen & Riisgård, 1995). *H. diversicolor* collects food using two main tactics; either by using its jaws to catch food, or by using mucus. When catching food using its jaws, it actively crawls on top of the substrate to search for food and ingests it immediately. Using mucus, it secrets two mucus-strings alongside its body which traps food particles. When retracting into its burrow, it pulls with it the trapped particles which are thereby ingested (Scaps, 2002). Their method of choice varies throughout the year. Through field studies, Vedel et al. (1994) found that *H. diversicolor* filter feeds (passively uses mucus to trap phytoplankton) 50-100% of the time in May-August when there is an abundance of food, while in early spring and autumn that method accounts only for 5-20% (Vedel, Andersen & Riisgaard, 1994).

The most important growth factor is food availability (Scaps, 2002). Feeding experiments using high protein feed, has given especially high growth rates (Nesto et al., 2012; Santos et al., 2016; Batista et al., 2003[b]). After food availability, temperature is the most important growth factor. Optimum growth has been reported at temperatures around 19 °C. For this reason, bigger worms are often found at lower latitudes (Scaps, 2002).

Biomass growth of *H. diversicolor* have typically been measured comparing initial and final wet weights after a given amount of days (Batista et al., 2003[b]; Nesto et al., 2012; Santos et al., 2016; Seekamp, 2017). This method is time consuming and full of potential errors. As an alternative, the measuring of RNA/DNA ratios to indicate growth could be used. Successful trials using this method have been conducted studying larval fish growth (Malzahn, Clemmesen & Rosenthal, 2003; Dahlhoff, 2004; Yandi & Altinok, 2015), making this an intriguing method for growth determination of *H. diversicolor.*

1.4 RNA/DNA ratio

RNA-DNA ratio is the most widely-used biochemical index for measuring the recent growth of marine organisms. The method assumes that the DNA amount in an organism is constant under changing environmental situations, whereas the RNA amount varies with age, life-stage, size, and varying environmental conditions. RNA is directly involved in protein synthesis. Under suboptimal conditions the organism synthesises less protein. Thus, organisms in good nutritional condition tend to have higher RNA/DNA ratio compared to organisms under stress (Chícharo & Chícharo, 2008).

Several studies on fish larvae RNA/DNA ratios have found the ratio to increase with excessive feeding, and subsequently decrease with starvation trials (Yandi & Altinok, 2015; Dahlhoff, 2004; Malzahn et al., 2003). An example of this is presented in Figure 1.4 (Yandi & Altinok, 2015).
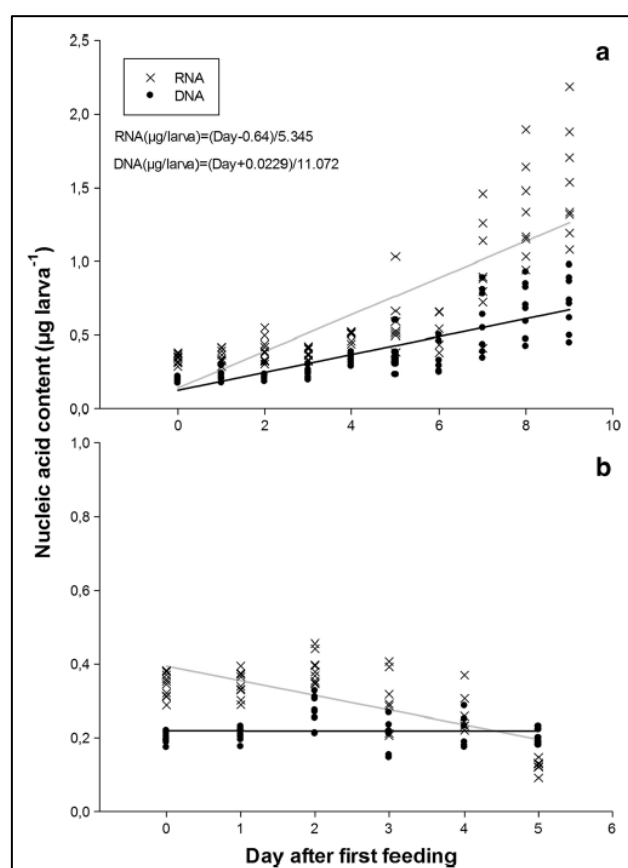
Figure 1.4. Changes of total RNA and DNA quantity of fed control (a) and unfed control (b) of T. mediterraneus larvae (Yandi & Altinok, 2015).

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Even if a relationship as described above is representable for many species it is important to note that exceptions has been found, indicating that it is not necessary the case for all species (Chícharo & Chícharo, 2008). Few, if any, prior studies have been conducted using RNA/DNA ratio to indicate polychaete growth.

1.5 Aim and hypotheses of the study

The present study was part of the "COMPLETE research-project" at SINTEF Ocean and NTNU. The overall aim of the current study was to evaluate if polychaetes could utilize the nutrients in waste streams from land-based aquaculture facilities and biogas residues to promote growth. The assessment was based on the following two sub-objectives:

(1) The investigation of how different waste streams affect polychaete growth- and survival rate.
(2) Evaluation of growth of polychaetes using RNA/DNA ratio analysis.

A thirty-day cultivation experiment has been conducted to test the following hypotheses:

(1) Smolt sludge and biogas residue will be a good nourishment for growth of *H. diversicolor*.
(2) The RNA/DNA ratio will reflect the growth data.

2 Materials and Methods

2.1 Overview

An overview of the work process in this study is presented in Figure 2.1

Polychaete sampling I

October

Cultivation experiment I

October

Polychaete sampling II

February

Cultivation experiment II

February

Analyses of
RNA/DNA ratios

April
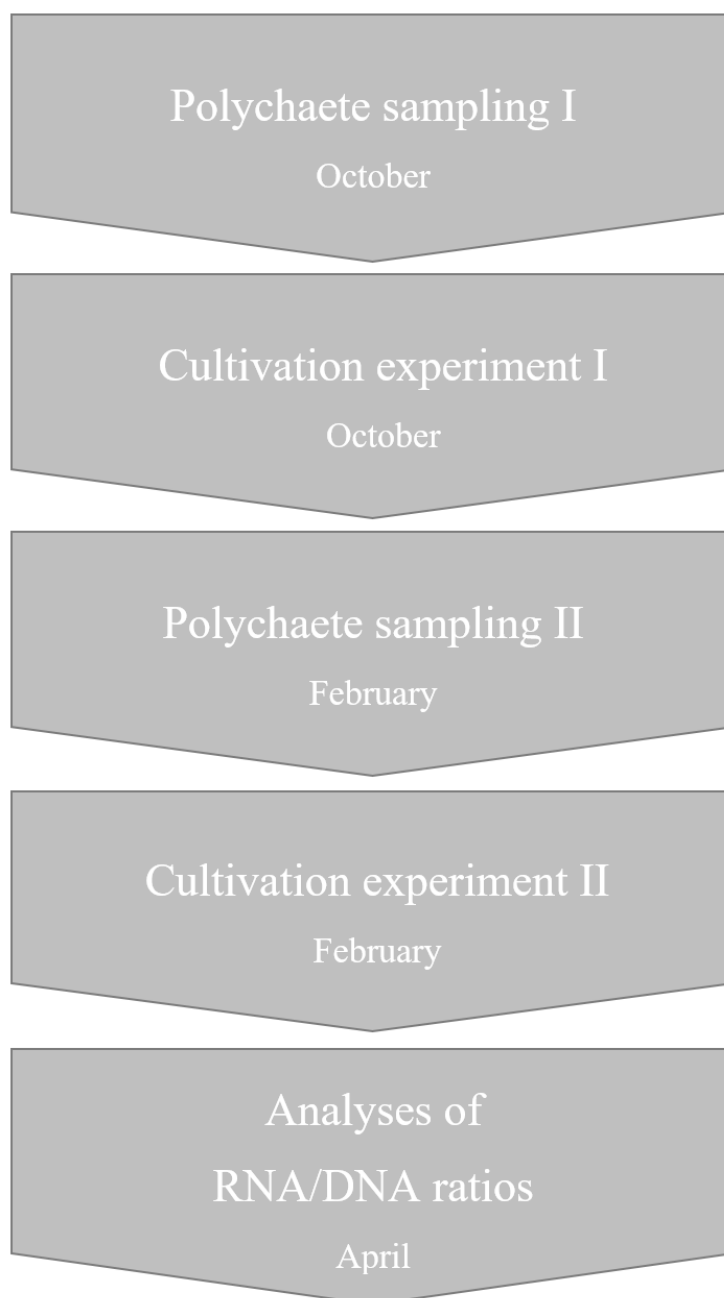
Figure 2.1. Overview of the practical-work prosses in this thesis. Polychaetes were sampled in the field on two separate occasions. The sampled polychaetes were transported to the laboratory at SINTEF Sealab where cultivation experiments were carried out. Polychaetes harvested from the experiments were frozen and later used in RNA/DNA ratio analysis.

2.2 Collection of polychaetes

2.2.1 Location for sampling

The polychaetes were collected from their natural environment, Leangenbukta (63.439069 N, 10.473618 E), on two separate occasions, shown in Figure 2.2.
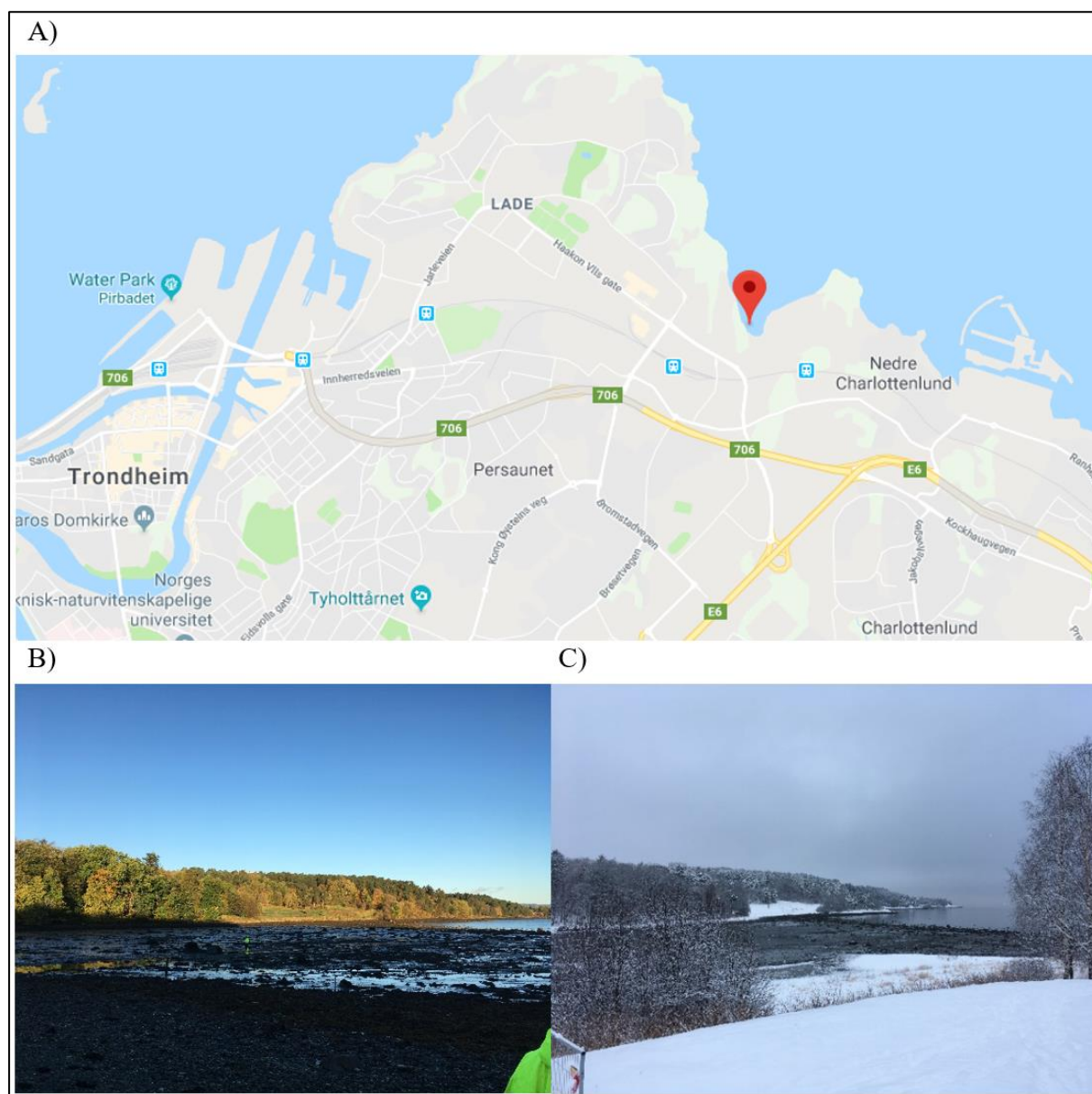
Figure 2.2. A) Leangenbukta (Google maps, 2018). B) Leangenbukta the 10th of October (Photo: Fredrik Berntsen, 2017). C) Leangenbukta the 22nd of February (Photo: Fredrik Berntsen, 2018)

2.2.2 Polychaete sampling I

The first sampling was done at low tide the 10th of October 2017. Five people participated in the collection using shovels and pitchforks to dig up the polychaetes which was situated 10-30 cm in the muddy substrate. The water temperature was approximately 8 ℃. The polychaetes were separated from the sediment using our hands and stored in buckets containing damp newspaper and some local sediment to simulate their natural environment. After the collection, the polychaetes were transported to the laboratory at SINTEF Sealab. A total of approximately 1600 polychaetes were sampled for use in "cultivation experiment I".

2.2.3 Polychaete sampling II

The polychaetes were collected at low tide the 22nd of February, two people participated in the collection using the same method as for "polychaete sampling I". The water temperature was approximately 2 ℃. A total of 160 polychaetes were sampled for use in "cultivation experiment II".

2.3 Cultivation system

The cultivation system was a "X-Hab" (Pentair Aquatic Eco-system; MBK Installations) recirculation aquaculture system (RAS), shown in Figure 2.3.

The system was constructed as a fully automated RAS, with several water filtering units, including a biofilter (moving bed bioreactor), mechanical filters (cartridge filter and filter pads), and a chemical carbon filter. To further increase the biosecurity the incoming water was sterilized using UV-sterilizer. The water quality was continuously measured by a Pentair measuring system. The water exchange was set to 5% per day. Incoming water was pumped from 70 metres depth from the Trondheim fjord, and preheated to ~17 ℃ before entering the tanks. The cultivation system consisted of 20 polystyrene tanks (LxHxW = 473x260x178 mm, total volume = 16 L). A light regime of L/D = 16/8h, was run throughout the experiments.
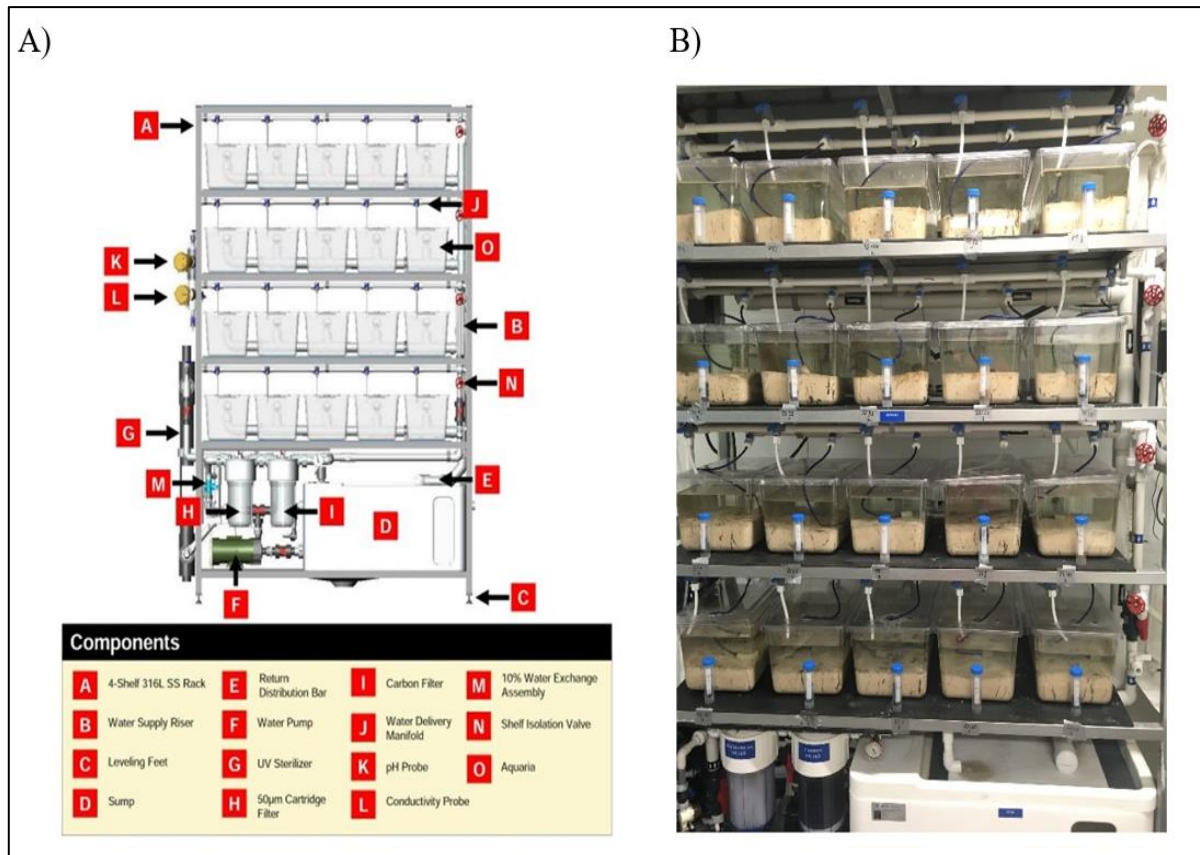
Figure 2.3. A) Schematic overview of the RAS-model used in this experiment (MBKI, 2018). B) The operational system 19th of October 2017 (Photo: Fredrik Berntsen, 2017).

2.4 Cultivation experiments

A total of two cultivation experiments were conducted, cultivation experiment- I and II. In both cases the polychaetes were prepared for the experiment in the following way:

The sampled polychaetes from "polychaete sampling I and II", were stored in trays filled with seawater (pumped from 70 metre depth) where they were kept for approximately two hours. Sediment residue was removed, and the worms were sorted. The smaller, dead, damaged or overmatured (green in colour) individuals were discarded, whilst the remaining polychaetes were counted and placed in clean containers in specific bulk sizes. Upon reaching the desired number in one container, a new container was used. Polychaetes were taken out of the container, water was wiped off using paper, and they were weighed in bulks and sorted into rearing tanks. The preparation process is presented in Figure 2.4.
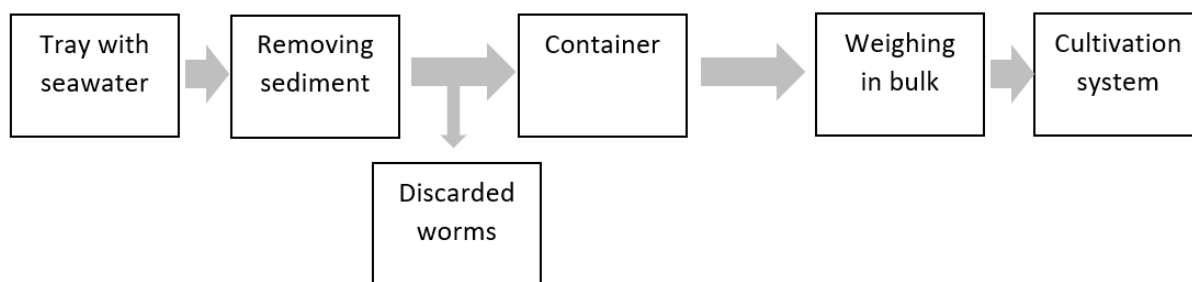
Figure 2.4. Cleaning and sorting process of polychaetes.

After the experiments the surviving polychaetes were taken out of the tanks and weighed as previously described. Portions of the cultivated polychaetes got frozen (-18 °C) immediately for later use in the RNA/DNA ratio analysis.

2.4.1 Cultivation experiment I – 30-day trial

In the cultivation system, there were 20 pre-prepared tanks filled with an 8 cm deep chamotte layer and roughly 8 litres of seawater (water column = 6.5 cm). The polychaetes were added to the tanks. After adding a bulk of polychaetes to the first tank, a new bulk of polychaetes were weighed and supplied to the next tank until reaching equal number in each tank. Further additions of polychaetes in bulks were done to each tank, making the time of placement for the polychaetes in the different tanks as uniform as possible.

In this experiment a total of 1600 polychaetes were collected. Due to damage, varying size and maturation, 1400 were used. On that basis, 70 polychaetes (1400/20) were cultivated in each tank in the RAS, added to the tanks in the following bulk sizes: 20-30-20. The density of worms in the tanks was approximately 850 individuals per m^2 of bottom substrate.

The polychaetes were fed immediately from one of five different diets presented in Table 2.1. The polychaetes were fed every three to six days with approximately 15 grams (WW) of feed deposited to each tank, or approximately 1.5 grams of fish feed pellets due to lower water content. The diets consisted of either smolt sludge (SS), biogas-residue (R), or fish feed pellets (FF), in different combinations. The FF diet served as positive control, based on good results from prior experiments.

Table 2.1. The diets, presented in abbreviations, and the correlating composition ratios and number of replications.

Diet	Diet composition	Replicates
FF	100% FF	4
SS 100	100% SS	4
SS 66	66% SS and 33% R	4
SS 33	33% SS and 66% R	4
SS 0	100% R	4

The fish feed and smolt sludge was delivered from Lerøy Belsvik, whilst the biogas residue was delivered from Scandinavian biogas. The smolt sludge went through a 5-minute centrifugation – KR22i centrifuge (Jouan SA/Thermo Fisher Scientific, USA), at 4500 rpm before use. All diets were stored in a fridge (4 °C) between feedings. The diets were analysed by personnel at SINTEF Ocean using an elemental analyser – ESC 4010 (Costech Analytical Technologies, Inc., USA). The SS and R had different water content (%) and C/DW ratio, but similar N/DW ratio. The diet composition was sorted by adding a certain amount of SS (WW) and R (WW) until meeting the desired ratio. Example for SS 33; 266 g of SS and 133 g of R was added, making it ~33% SS and ~66% R. The N-, C-, amino acid (AM)- and water content in the different diets are presented in Table 2.2, along with the C/N ratio.

The distribution of the different diets was randomized, assigning each tank (1-20) with a random diet, presented in Figure 2.5. This to eliminate eventual benefits due to location in the RAS. There were four replicates of each diet to increase the validity of the experiment.

Table 2.2. Water, C, N and amino acid content in FF, SS and R. The superscripts denote significant differences within each column. a and b indicate a significant difference of $p < 0.01$. c indicates a significant difference of $p < 0.05$

Feed	Water (%)	C (ugC/mgDW)	N (ugN/mgDW)	AM (% DW)	C/N ratio
FF	7.9 ± 1.3^a	449.2 ± 29.3^a	94.7 ± 0.5^a	41.1 ± 2.2^b	4.75 ± 0.33^a
SS	77.2 ± 0.2^b	409.9 ± 2.8^a	49.7 ± 1.4^b	19.0 ± 0.2^a	7.58 ± 0.16^b
R	78.2 ± 0.4^c	380.5 ± 6.9^b	50.2 ± 0.3^b	19.0 ± 0.2^a	8.26 ± 0.22^c

FF	FF	SS 100	SS 66	FF
1	2	2	1	3
SS 0	SS 33	SS 33	SS 100	SS 33
2	1	2	3	4
SS 0	SS 66	FF	SS 0	SS 100
4	4	4	1	4
SS 66	SS 33	SS 0	SS 100	SS 66
2	3	3	1	3

Figure 2.5. Overview of the diets in the different tanks in the RAS. The boxes indicate their real position in the RAS (Figure 2.3). The diet abbreviations are found in Table 2.1, whilst the numbers indicate replication.

To get an even feed distribution over the chamotte substrate, the circulation in the tanks were stopped and the feed was poured manually into the tank after mixing it with water in small beakers. After 30 days, the polychaetes were harvested and weighed, whereby samples of 4-5 worms were frozen (-18 °C) for later RNA/DNA analyses.

2.4.2 Cultivation experiment II – Starvation trial

To further test the use of RNA/DNA as a method for growth determination of polychaetes, an additional experiment was run with the objective of examining the effect of negative growth on the RNA/DNA ratio. In this experiment, 108 polychaetes were used (polychaete sampling II). These were cultivated in 15 polystyrene containers (LxHxW = 124x110x85 mm, total volume = 1 L) filled with seawater and chamotte (7.5 cm). The containers resided in four of the 20 tanks in the cultivation system, as illustrated in Figure 2.6.

C3		3c	3b		2c	2b		1c	1b
C2									
C1		3d	3a		2d	2a		1d	1a
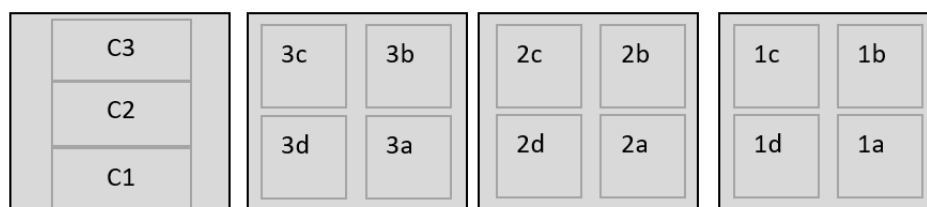
Figure 2.6. Setup of different containers in the tanks, as seen from above. The letters indicate the amount of days the polychaetes were reared before harvest: a = 3, b = 6, c = 9 and d = 12 days. The numbers indicate replication. C1-C3 are control replicates.

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Each container housed five polychaetes, the exception being the control-containers housing 16 polychaetes each. The worm density in each starvation container was 474 individuals m^{-2} and was 1518 individuals m^{-2} for each control container. The cultivation procedure varied for each experiment. The starved polychaetes received no treatment and were harvested after three, six, nine or twelve days. The fed polychaetes received a surplus (0.1 g DW) of fish feed pellets (FF) every day. Roughly four polychaetes were taken from each container after three, six, nine and twelve days, by doing the following: The containers were taken out of the tanks, some polychaete-containing chamotte was removed and put into a tray. From there, the desired number of polychaetes were taken out, and the remaining chamotte were put back into the container to ensure no loss of individuals in the tray. The containers were then placed back into the tanks.

For each harvest, both starvation and control, the worms were weighed and put into 15 ml beakers. The beakers got labelled with container letter and replication number, and frozen (-18 ℃) for later RNA/DNA analysis.

2.5 Analyses

2.5.1 Water quality analyses

The water quality in cultivation experiment I was measured approximately every two days, from 16th of October to 8th of November, using a YSI Pro DSS multiparameter water quality meter (YSI Incorporated, USA). The following parameters were measured;

    A: Dissolved oxygen (%)

    B: pH

    C: Temperature (℃)

    D: Salinity (‰)

A reading of ammonia was also conducted, but it was almost no traces of ammonia due to this experiment running in a RAS with effective filters transforming and absorbing the ammonia.

The pH, temperature (℃) and salinity (‰) in cultivation experiment II were controlled and measured every two days, from the 22nd of February to the 6th of March, using the built in Pentair measuring system.

2.5.2 Change in biomass, specific growth rate and survival

A   Survival

The polychaetes survival (%) was calculated by subtracting the dead individuals (*di*) from the initial population (*ip*) and divided by the initial population (*ip*) presented in equation 1.

$$(1) \quad Survival\ (\%) = \left(\frac{(ip-di)}{ip}\right) * 100$$

B   Change in biomass

Biomass (WW) was measured using a VWR Collection Science Education scale (SE 622, VWR International, USA) before and after the experiments. The change in biomass was found using equation 2.

$$(2) \quad Change\ in\ biomass\ (\%)\ = \left(\frac{(W_f-W_0)}{W_0}\right) * 100$$

Where W_0 represents the initial wet weight (g), W_f represents the final wet weight (g).

C   Specific growth rate

The specific growth rate (SGR) was calculated according to Winberg (1971), presented in equation 3.

$$(3) \quad \mu = (\ln W_0 - \ln W_t) * t^{-1}$$

μ is the specific growth rate (d^{-1}), *t* is the number of days cultivated, W_0 is the initial wet weight and W_t is measured wet weight after *t* days.

2.5.3 RNA/DNA ratio

When measuring the amount of RNA and DNA in an organism, fluorometric determination is often used (Calderone et al., 2001). In the present study a Qubit 3 fluorometer (© Thermo Fisher Scientific Inc., 2014) was used to measure the fluorometric emission by the RNA and DNA molecules in the sampled polychaetes. Fluorescence is a short-lived emission which occur when the molecule is exited and moving back to ground state (Skoog, West, Holler & Crouch, 2004). Competing with fluorescence, nonradiative relaxation can occur whilst exciting the molecules. Nonradiative relaxation converts the energy into heat in the solvent contributing to a reduction of energy in the fluorescence compared to the initial absorbance energy, as shown in Figure 2.7 (So & Dong, 2001; Skoog et al., 2004).
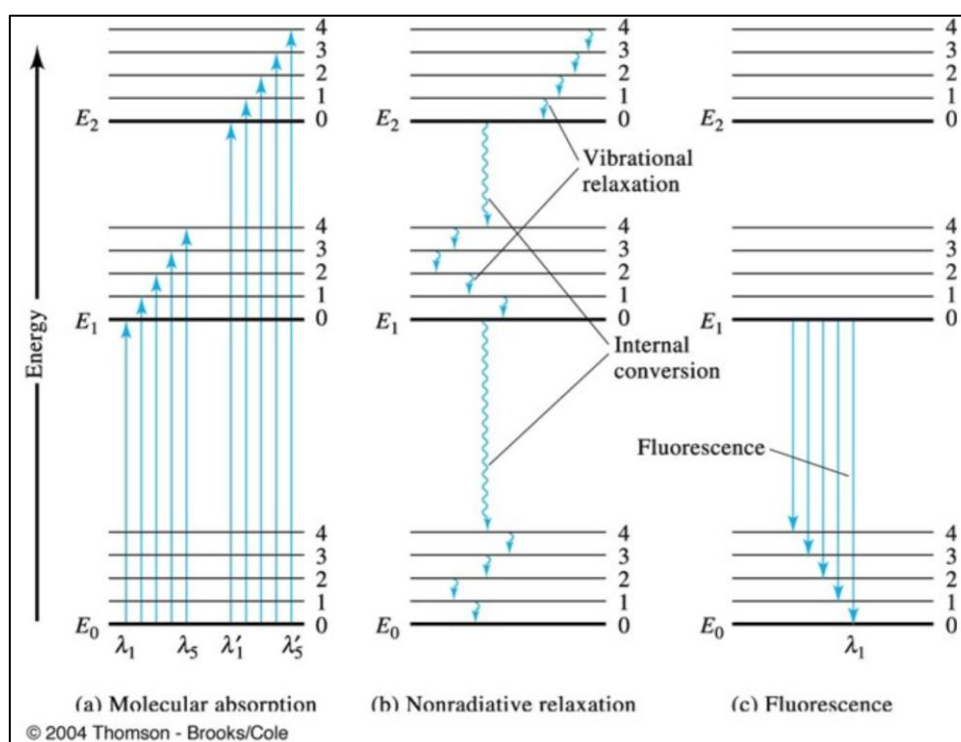
Figure 2.7. Energy-level diagram. E0 is the lowest energy level, E1 is a higher energy level. λ represents the wavelengths, a longer line represents more energy, hence shorter wavelengths. The figure shows; (a) absorption of incident radiation, (b) nonreactive relaxation, and (c) fluorescence emission by a molecular species (Skoog et al., 2004).

Each molecule emits fluorescence within a specific fluorometric spectra (Skoog et al., 2004). Using the Qubit system, dsDNA molecules were excited when irradiated with blue light resulting in less energetic green fluorescence, whilst RNA molecules were excited when irradiated by red light resulting in fluorescence within the far-red light spectra. To amplify the fluorometric signal from the RNA and DNA molecules, fluorescent dyes were added in accordance to supplier information. Prior to the RNA and DNA concentration measurements the polychaete samples were prepared with major modifications in accordance to Caldarone et al. (2001) in a seven-step protocol presented in Table 2.3.

Table 2.3. Stepwise protocol for Qubit RNA and DNA assay.

Step	Procedure
1	The polychaetes were freeze dried at -80 °C.
2	They got crushed into powder (homogenized) in Eppendorf tubes (2 ml) using glass beads in a Precellys 24 (Bertin Technologies) shaking machine running for 2x20 seconds at 5000 rpm.
3	1-2 mg[*] from each of the powdered samples was weighed into Eppendorf tubes (1,5 ml).
4	The samples along with a steel ball, 100 µl lysis buffer (1% sarcosil Tris-EDTA – STEB) and 100 µl Tris-EDTA were transferred to round bottom Eppendorf tubes (2 ml).
5	Homogenization was run in a TissueLyser II (©Qiagen) for two minutes.
6	300 µl Tris EDTA (total of 4x volume of lysis buffer) was added to each tube. The tubes got centrifuged at 16 000 g, 4 °C for 15 minutes.
7	10 µl of the lysis (supernatant) from each sample was used for Qubit RNA or dsDNA assays, using the BR (broad range) kits according to supplier information.

[*]The polychaete powder was weighed using a microgram balance (Mettler-Toledo, USA).

2.5.4 Statistics

In the present study Microsoft Excel 2016 was used for raw data input and Sigma plot 14.0 was used to treat the raw data and make graphs. In Sigma plot, the data was treated running a one-way ANOVA with sensitivities $p < 0.01$ and $p < 0.05$. To compare the samples a Bonferroni t-test ($p < 0.01$ and $p < 0.05$) was run for pairwise comparison of the different data.

The Bonferroni t-test is a simple and general method, with certain requirements. To use the Bonferroni t-test, the samples should have similar sample sizes and variances, otherwise the test may fail to locate all of the significant differences (Day & Quinn, 1989). The Bonferroni t-test was designed for pairwise comparisons and comparisons of means, provided a fixed number of comparisons to be made in advance (Day & Quinn, 1989).

Further comparisons of the results were conducted by correlation analyses. A strong correlation, signifying a strong dependence on each other, are found with Pearson's correlation coefficient (*r*-value) close to ±1. A value close to zero signify a weak or no relationship. *r*-values range from; $1 > r > -1$.

3 Results

3.1 Cultivation experiment I – 30-day cultivation trial

3.1.1 Water quality analysis

Water quality parameters are presented in Table 3.1. The dissolved oxygen and pH measurements differ significantly ($p < 0.05$) for the control (FF) tanks versus the others. The temperature measurements differ significantly in the tanks containing SS 100 and SS 66 in comparison to the control (FF) with the respective p values being $p < 0.01$ and $p < 0.05$. There were no significant differences in the salinity between the different dietary treatments.

*Table 3.1. Dissolved O_2 (%), pH value, Temperature (℃) and Salinity (‰) values for the different dietary treatments (mean ± SD, n = 40). Superscripts denote significant difference ($p < 0.05$) within the column. b is significantly different from a. ab does not differ from neither a nor b. * denote significant difference of $p < 0.01$.*

Diet	Dissolved O_2 (%)	pH	Temperature (℃)	Salinity (‰)
FF	93.5 ± 4.4^b	7.6 ± 0.1^b	16.8 ± 0.2^b	34.6 ± 0.4^a
SS 100	96.6 ± 1.7^a	7.7 ± 0.1^a	16.7 ± 0.2^a	34.6 ± 0.3^a
SS 66	97.0 ± 1.4^a	7.7 ± 0.1^a	$16.7 \pm 0.1^{a*}$	34.6 ± 0.3^a
SS 33	96.5 ± 2.2^a	7.7 ± 0.1^a	16.7 ± 0.2^{ab}	34.6 ± 0.3^a
SS 0	97.4 ± 1.3^a	7.7 ± 0.1^a	16.7 ± 0.1^{ab}	34.4 ± 0.7^a

3.1.2 Survival rate

There was no significant difference ($p > 0.05$) in the survival rate of *H. diversicolor* between the polychaete groups fed different diets (Table A.1 in Appendix I). The survival rates, with corresponding standard errors are presented in Figure 3.1.
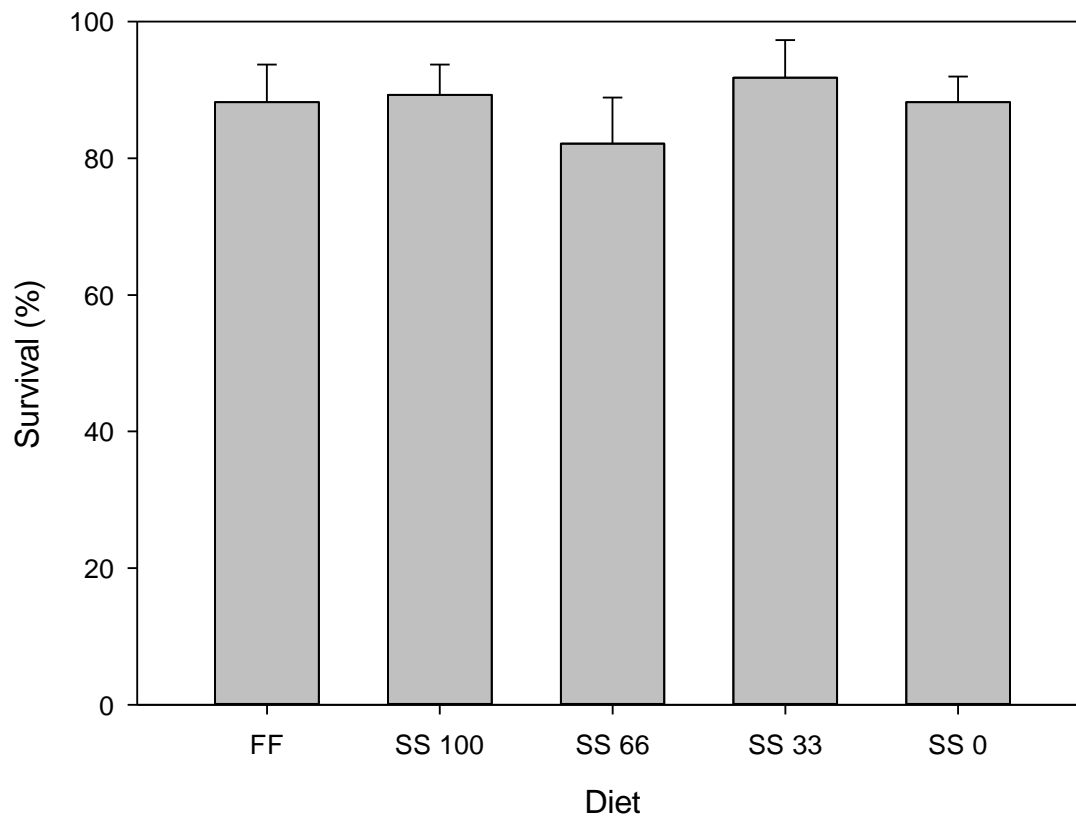
Figure 3.1. The survival rate when fed the different diets (mean ± SD, n = 4). The x-axis represents the different dietary treatments. The y-axis represents the survival rate (%).

3.1.3 Change in biomass

A significant difference ($p < 0.01$) was found in wet weight (g) when comparing initial- and final weights of the polychaetes after the 30-day trial for all groups (Table A.2, Appendix I). There was no significant difference between the dietary treatments in the initial weighing, but a significant difference of $p < 0.05$ was found between the polychaetes fed on the fish feed diet and the others on the final weighing. A comparison of the individual initial and final weights (g) for the respective diets are presented in Figure 3.2.
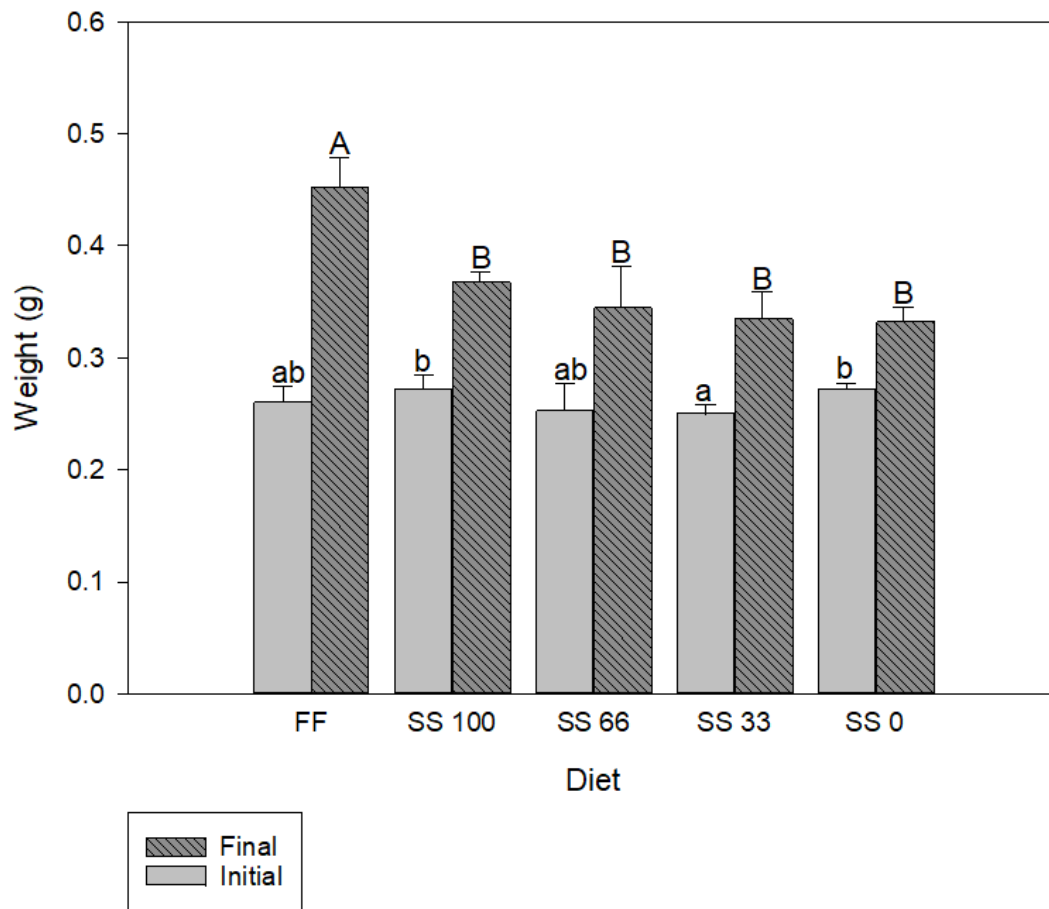
Figure 3.2. The initial and final weight (g) of individual polychaetes (mean ± SD, n = 4) cultivated for 30 days. The x-axis represents the different dietary treatments. The y-axis represents the weight (g). Superscripts in capital letters are significantly different (p < 0.01) from the lowercase letters. The different capital letters indicate significant difference (p < 0.01) between the final weights, while different lowercase letters indicate significant difference between the initial weights (p < 0.05).

Based on the difference between the initial and final biomass, the total change (%) in wet weight was calculated, presented in Figure 3.3. A significant difference (p < 0.01) in the increase of biomass was found for the polychaetes fed the FF-diet compared with the other groups.
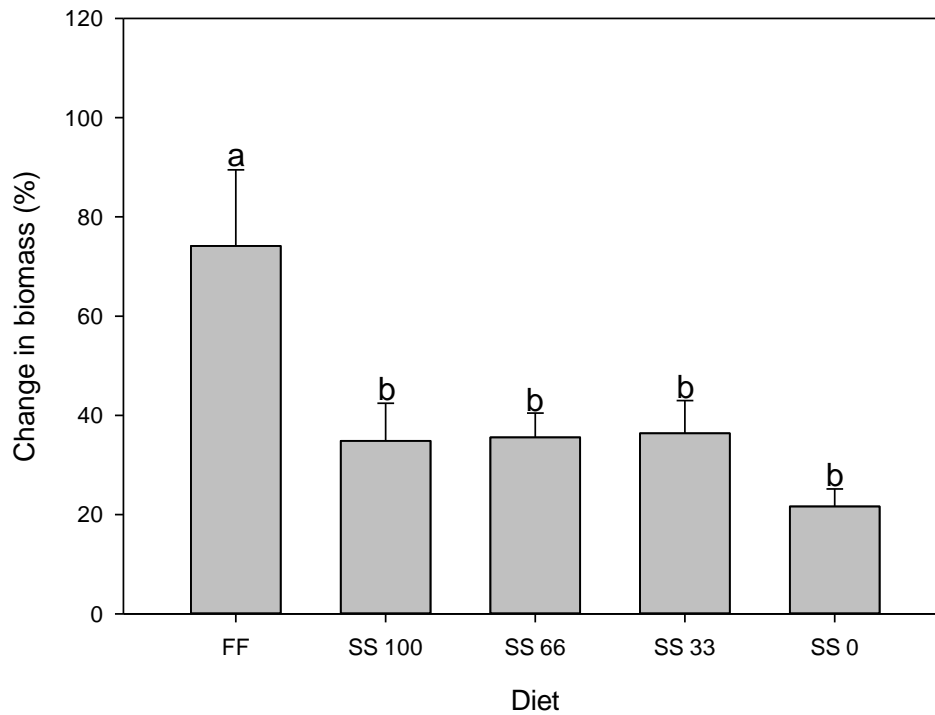
Figure 3.3. The change (%) in polychaete wet weight biomass (mean ± SD, n = 4), for the different dietary treatments. The x-axis represents the different dietary treatments. The y-axis represent change in biomass (%). Different superscripts denote insignificant differences (p < 0.01).

3.1.4 Specific growth rate

A significant difference of $p < 0.01$, was found when comparing the significant growth rates (SGR) for polychaetes fed on fish feed (FF) versus the polychaetes fed on the other diets. The SGR for the FF group was $1.8 \pm 0.3 \times 10^{-2}$ d^{-1} which was almost twice that of the others, spanning from $> 0.7 \pm 0.1 \times 10^{-2}$ d^{-1} to $< 1.0 \pm 0.2 \times 10^{-2}$ d^{-1}. No additional significant differences were found while comparing the other diets, presented in Figure 3.4.
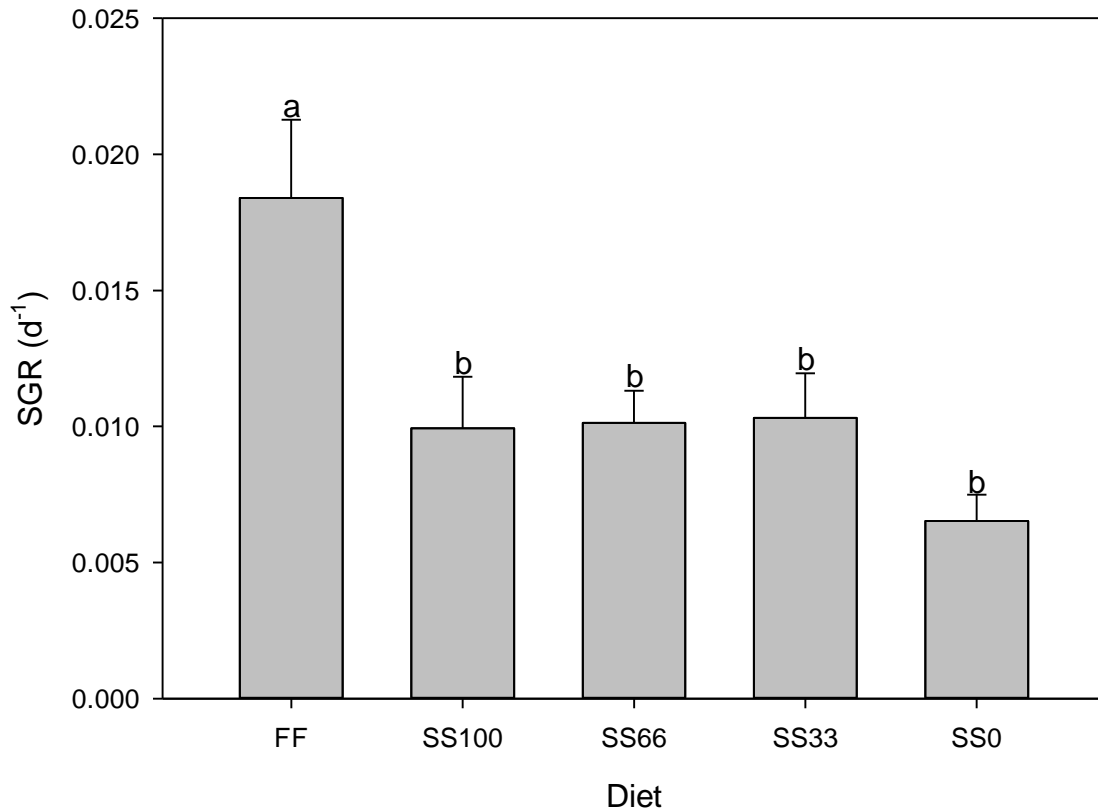
Figure 3.4. The specific growth rate (d^{-1}) (mean ± SD, n = 4) for the different dietary treatments. The x-axis represents the different dietary treatments. The y-axis represents the specific growth rate (d^{-1}). Different superscripts denote significant difference from the others (p < 0.01).

3.1.5 RNA/DNA ratio

The RNA and DNA concentrations in the polychaetes were divided by each other giving the RNA/DNA ratio for each dietary treatment and replica (Table A.3, Appendix I). The mean RNA/DNA values for each dietary treatment are presented in Figure 3.5. After comparing the groups, a significant difference of p < 0.05 was found between the polychaetes fed on SS 100 and the SS 33 diet. No other significant differences were found.
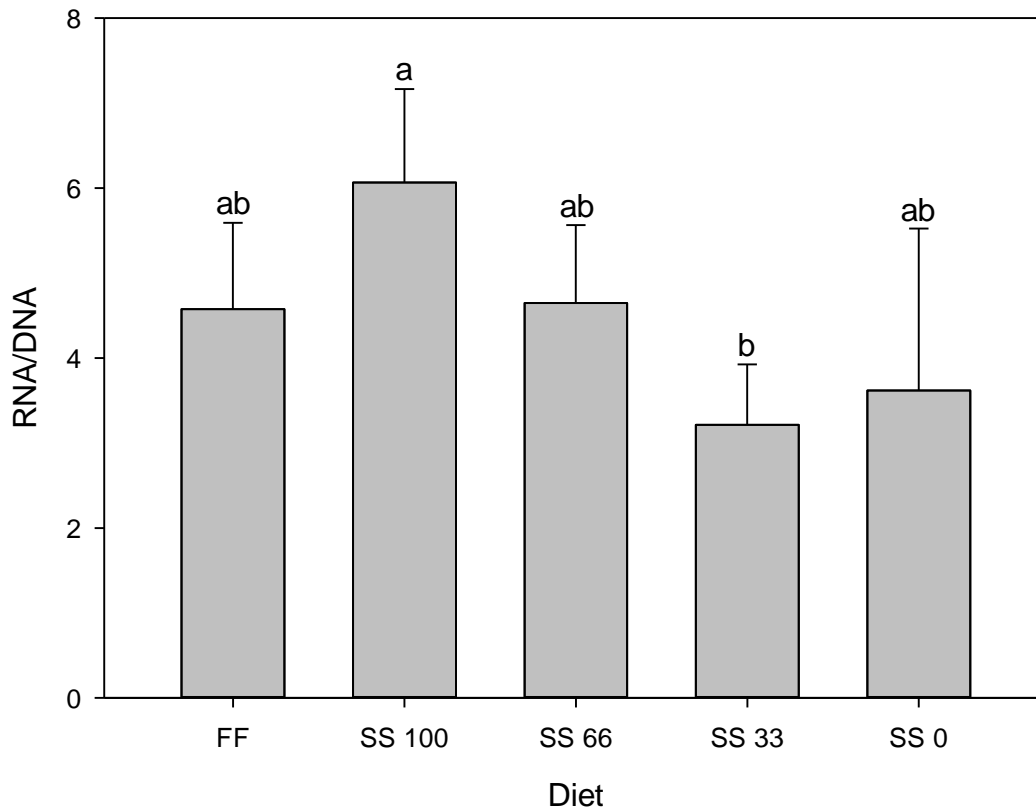
Figure 3.5. The RNA/DNA ratio (mean ± SD, n = 4) for the different dietary treatments. The x-axis represents the different dietary treatments. The y-axis represents the RNA/DNA ratio. Different superscripts denote significant differences (p < 0.05).

3.2 Cultivation experiment II – Starvation trial

3.2.1 Water quality analysis

The values for the measured water quality parameters were (mean ± SD, n = 9): A) pH was 8.3 ± 0.1, B) the temperature was 16.4 ± 0.3 °C, C) and the salinity was 40.9 ± 1.0 ‰.

3.2.2 Survival rate

The survival rate (mean ± SD) between the starvation groups varied from 92 ± 14 % to 83 ± 14 %. No significant differences (p > 0.05) were found whilst comparing the starvation groups, though a slight, yet insignificant decrease was observed towards the end of the experiment as shown in Figure 3.6.
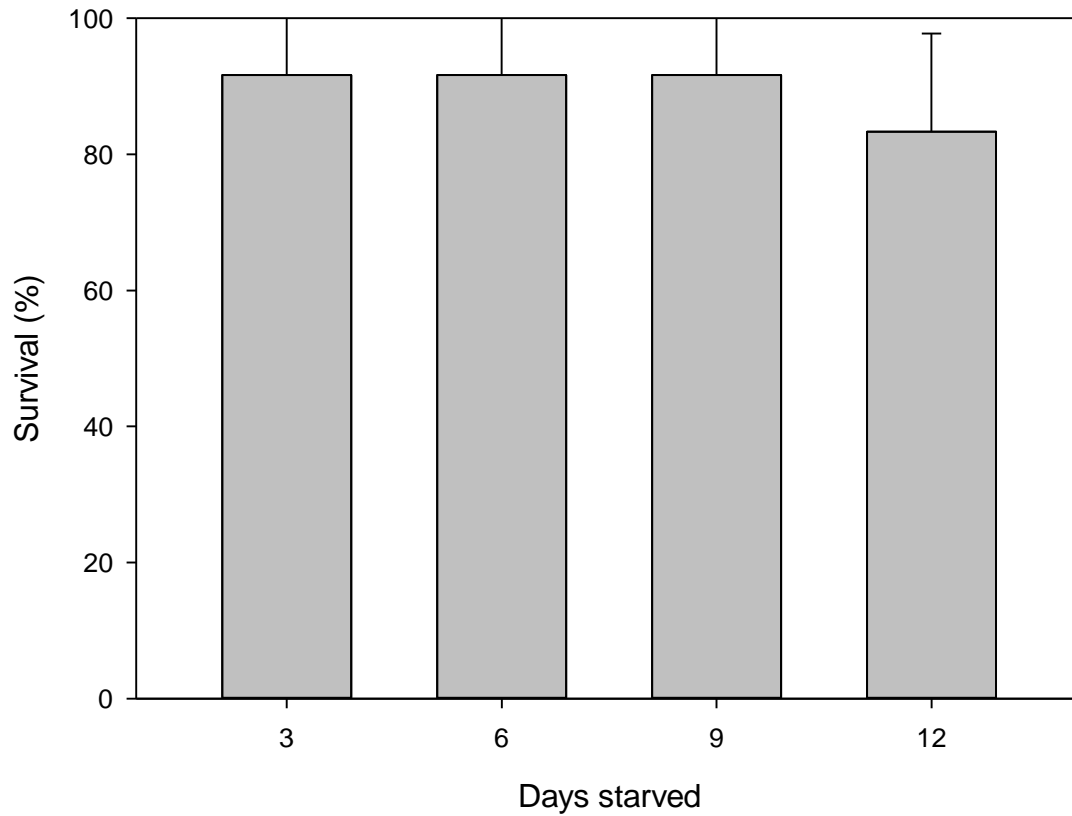
Figure 3.6. The survival rate (%) for the corresponding starvation duration (mean ± SD, n = 3). The x-axis represents the number of days starved. The y-axis represents the survival rate (%).

3.2.3 Change in biomass

When comparing the corresponding initial- and the final weights (g) of the starved polychaetes no significant difference ($p > 0.05$) were found (Table A.4, Appendix II). There were also no significant differences ($p < 0.05$) between the polychaetes starved for different durations. The initial- and final weights (g) of the starved polychaetes are presented in Figure 3.7
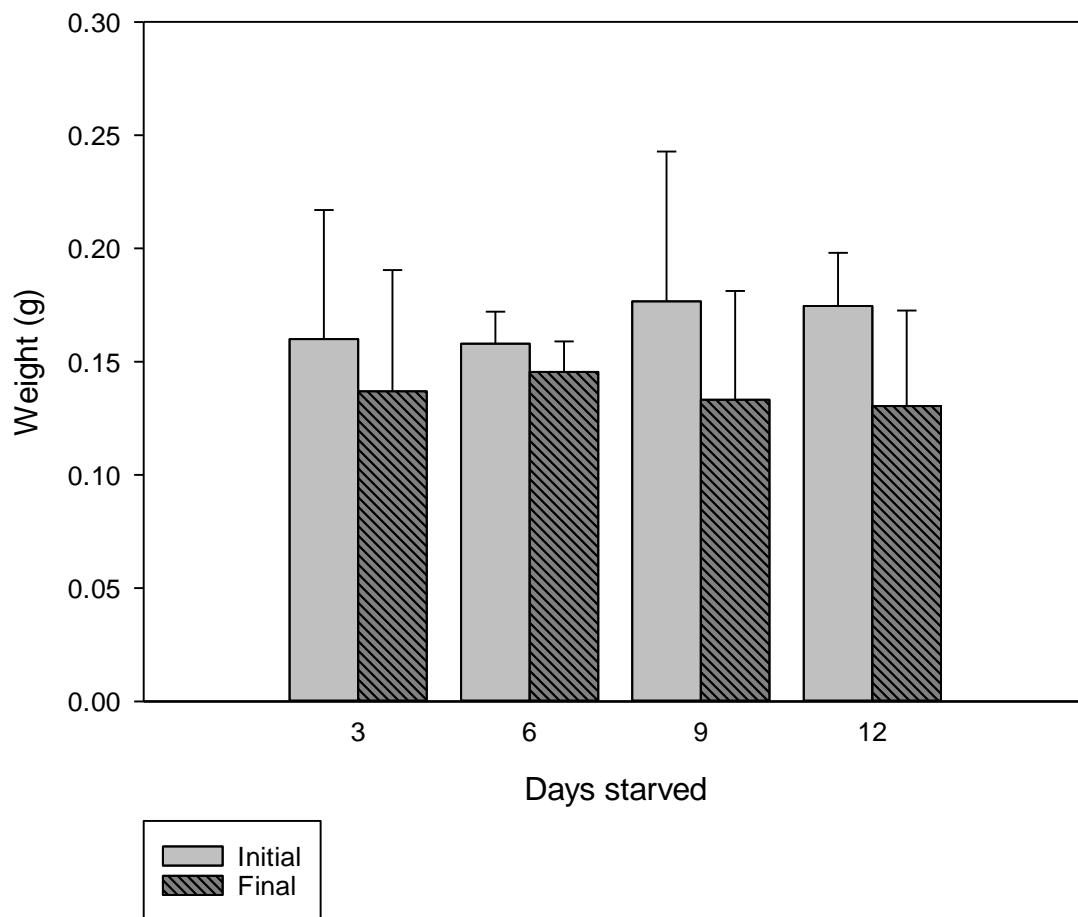
Figure 3.7. The initial- and final weights (g) for the starved polychaetes (mean ± SD, n = 3). The x-axis represents the number of days starved. The y-axis represents the mean individual weight (g).

The mean change in biomass (%) for every third day starved was calculated using equation 2 and is presented in Figure 3.8.
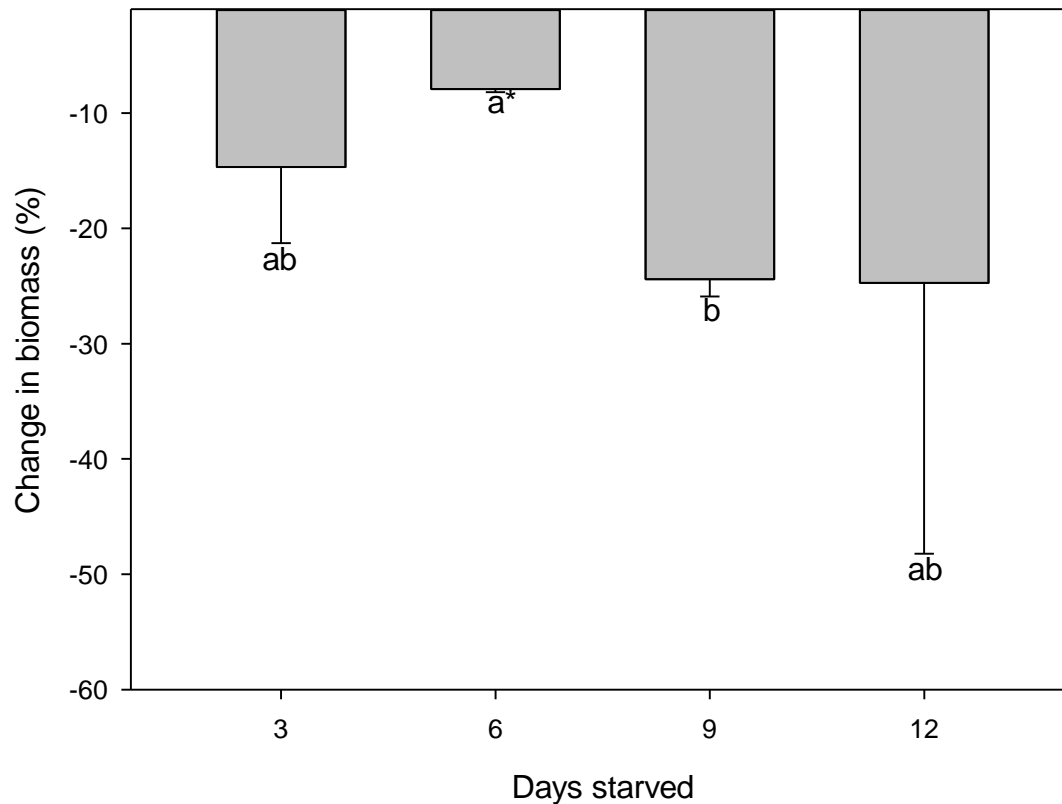
*Figure 3.8. The change (%) in biomass wet weight for the corresponding starvation duration (mean ±
SD, n = 3). The x-axis represents the number of days starved. The y-axis represents the change in
biomass (%). Different superscripts denote significant differences (p < 0.01). * only two replicas used.*

There was a significant difference (p > 0.01) in the change in biomass (%) between day six and
nine. Two replicas were used for the six-day starvation. No data on the weights for the control
groups were used.

3.2.4 Specific growth rate

Based on the difference between initial and final weights (Table A.4, Appendix II) using
equation 3, the specific growth rates for the starvation experiment was calculated. A significant
difference (p < 0.01) was found between the six- and nine-day starvation. The SGR compared
with the amount of days starved is presented in Figure 3.9.
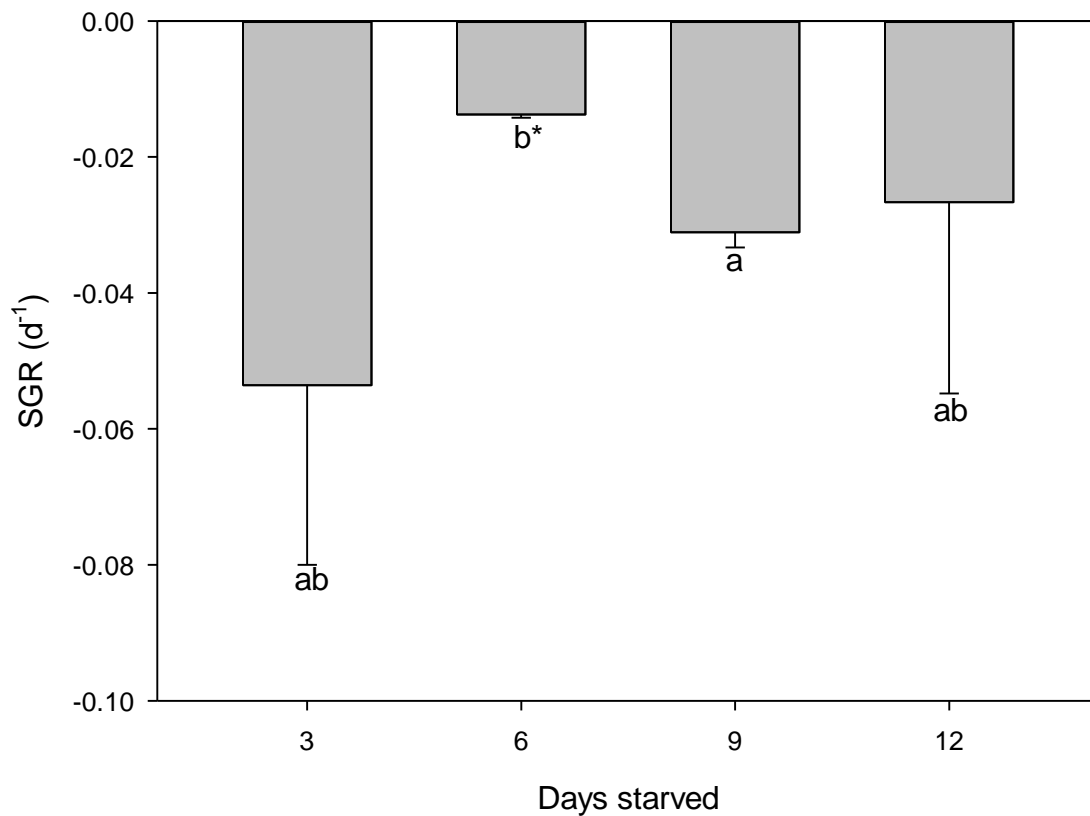
*Figure 3.9. The specific growth rate (d^{-1}) for the corresponding starvation duration (mean ± SD, n = 3). The x-axis represents the amount of days starved. The y-axis represents the specific growth rate. * two replicas used.*

3.2.5 RNA/DNA ratio

The RNA/DNA ratios for the starved groups and the control groups (Table A.5, Appendix II) are presented in Figure 3.9. A significant difference ($p < 0.05$) was found whilst comparing the RNA/DNA ratio between the three- and nine-day starvation. No other significant differences ($p > 0.05$) were found.
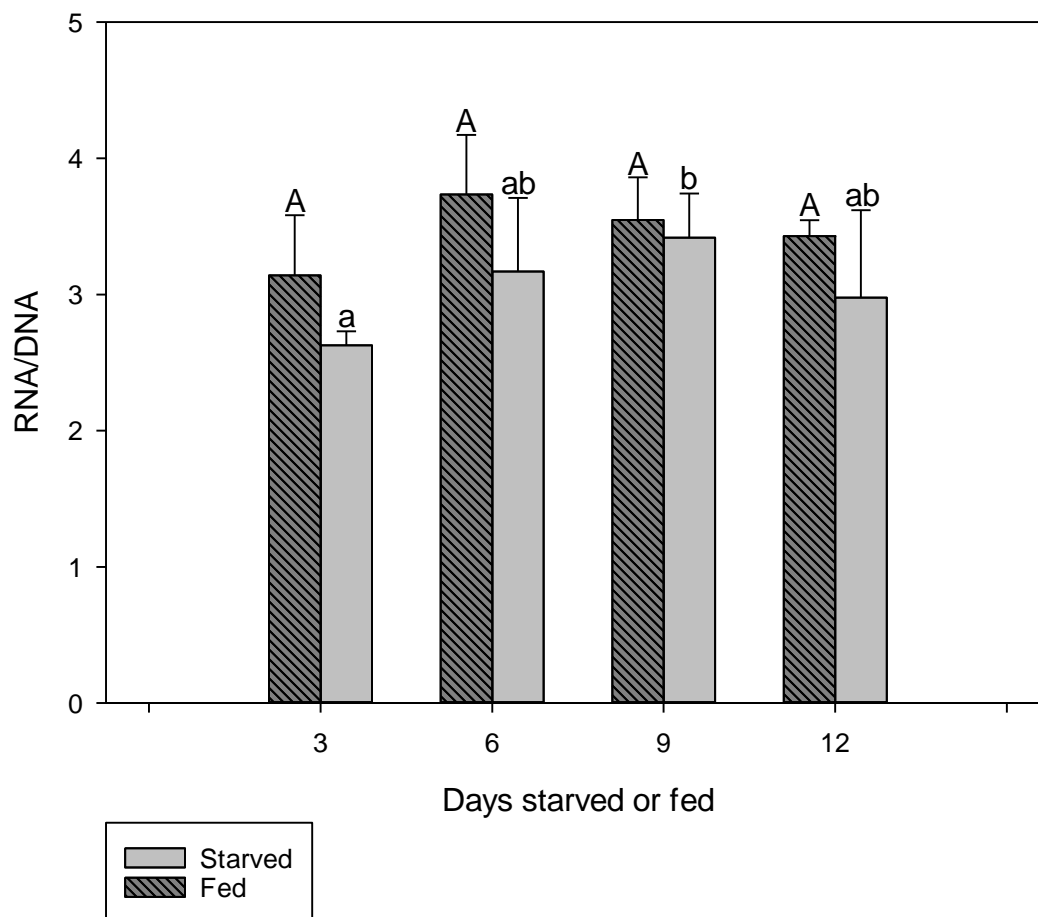
Figure 3.10. The RNA/DNA ratios (mean ± SD, n = 3) for starved and fed polychaetes for the corresponding starvation duration. The y-axis represents the RNA/DNA ratio. The x-axis represents the number of days starved or fed. Different lowercase superscripts denote significant difference (p < 0.05) between the starved polychaetes. Different capital superscripts denote significant difference (p < 0.05) between the fed polychaetes

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4 Discussion

In the present study, the effect of using different diets on the growth, survival and nutritional condition, on the polychaete *H. diversicolor*, was tested. The main objective was to study if the polychaete could utilize the nutrients in land-based aquaculture waste streams and biogas residue to promote growth, thus contributing to a higher reuse of otherwise lost nutrients by using the polychaetes as an alternative food source.

4.1 Survival rate

A   Cultivation experiment I

The survival rate in the experiment was between 82.1 ± 6.8 % and 91.8 ± 5.5 % (mean $\pm$ SD), and no significant differences was found in the number of surviving polychaetes for the different treatments. This suggests that these diets did not affect the survival rate differently in a major way over the 30-day period. The water parameters (Table 3.1) were well within the natural range of *H. diversicolor* (Scaps, 2002).

H. diversicolor changes feeding behaviour from filter feeder to carnivore dependent on the type of feed available (Vedel et al. 1994; Scaps, 2002). Based on previous cultivation experiments a calculated surplus of feed was given to each tank. Due to this surplus feeding, and the low density of polychaetes (850 individuals m^{-2}), it's not lightly that cannibalism or intraspecific competition have affected the results (Scaps, 2002). The polychaetes were counted manually after digging them out of the chamotte substrate. Loss of specimens in the residual chamotte on the final count and subsequent counting errors, is therefore considered a more likely reason for the observed mortality.

The survival rate in this experiment was lower than what was observed in other studies (survival > 95%) of *H.diversocolor* with similar setups (Nesto, et al., 2012; Santos et al., 2016; Batista et al., 2003[b]). In contrast to the studies mentioned above, the polychaetes used in this thesis were collected from their natural environment instead of using a cultivated brood-stock. Use of the sampled polychaetes from their natural environment may have included individuals with lower initial nutritional condition, compared to using cultivated brood-stock.

B   Cultivation experiment II

The survival rate in cultivation experiment II (83 ± 14 % to 92 ± 14 %) was similar as observed in cultivation experiment I. It could be expectable to find a decrease in the survival rate with increasing starvation time, as reported for larval fish starvation trials by Yandi & Altinok (2015). However, not as rapid, due to the polychaetes hardiness (Scaps, 2002). In a 65-day starvation study conducted by Batista et al. (2003[a]) a ~ 55% survival rate was reported for *N. diversicolor*, which suggests that the polychaetes high starvation tolerance. Cannibalism did mostly occur in experiments without sediments in which the worms resided, reducing the survival rate to ~10% (Batista et al., 2003[a]). Based on this, in combination with the current water and density conditions, close to no mortality was expected to find in this 12-day experiment. The observed reduction in survival rate was probably due to other reasons than the starvation itself.

The salinity in cultivation experiment II was measured to be quite high, 40.9 ± 1.0 ‰. Nevertheless, the water parameters were still within the reported comfortable range for H. diversicolor (Scaps, 2002). The salinity increased gradually to the presented value, starting from 35 ‰. It is assumed that this was due to evaporation within the half empty RAS system. This is not believed to have affect the polychaetes growth and survival rate in a negative manner (Neuhoff, 1979). On the other hand, the polychaetes resided within the substrate in small containers, and it is probable that the water conditions within the substrate may have been different than the levels measured in the RAS, making the water quality a possible source of error. In retrospect, it would have been more accurate to measure the interstitial water, or water close to the substrate for each container. A possible error, in addition to the inaccurate water quality measurements, could be found in that several spawning individuals in the present experiment were observed. This could subsequently have led to the immediate death of several male polychaetes (Dales, 1950).

4.2 Change in biomass and specific growth rate

A   Cultivation experiment I

A significant increase ($p < 0.05$) in biomass, from the initial- to the final weighting, for all dietary treatments was observed. The polychaetes fed on fish feed (FF) showed the highest increase of 74.16 ± 15.31 % which was significantly different ($p < 0.01$) compared to the sole smolt sludge diet (SS 100) and sole biogas residue diet (SS 0). They respectively showed an

increase of 34.87 ± 7.57 % and 21.65 ± 3.52. When comparing the specific growth rates of the polychaetes fed with the mentioned diets, a significant difference ($p < 0.01$) was found between the FF-diet and the others. The polychaetes fed with the FF-diet had an SGR of $1.8 \pm 0.3 \times 10^{-2}$ d^{-1}, which was almost twice that of polychaetes fed with a sole diet of smolt sludge or biogas residue. The polychaetes fed solely on smolt sludge (SS 100) and biogas residue (SS 0) had an SGR of $1.0 \pm 0.2 \times 10^{-2} d^{-1}$ and $0.7 \pm 0.1 \times 10^{-2} d^{-1}$, respectively. To investigate what's causing these differences, and if the observed growth rates could be considered as high for this species, comparisons with other studies growing *H. diversicolor*, were conducted.

The growth of *H. diversicolor* may be affected by the amount of protein content in the feed (Nesto et al. 2012, Santos, et al. 2016). The C/N values in the diets were used suggesting the protein content in the feed in the present study. The C/N ratio was calculated by dividing the respective carbon and nitrogen content for the different diets (Table 2.2). The FF had a C/N ratio of 4.75 ± 0.33 (mean $\pm$ SD) which was significantly ($p < 0.01$) lower than the SS 0 (7.58 ± 0.16) and SS 100 (8.26 ± 0.22). The SS 0-diet had a significantly ($p < 0.05$) lower C/N ratio compared to the SS 100. When comparing the mean SGR, against mean C/N ratio between the FF, SS 100 and SS 0, a correlation value (*r*) of -0.89 was found. This correlation is presented in Figure 4.1. The negative *r*-value supports the assumption that high protein content in the feed affects SGR positively (Nesto et al, 2012; Santos et al, 2016), as increasing protein content will give a decreased C/N ratio. However, it is important to note that this *r*-value was based on very few data points and the standard deviations for the C/N data was excluded from calculation, which reduces the validity of the correlation.
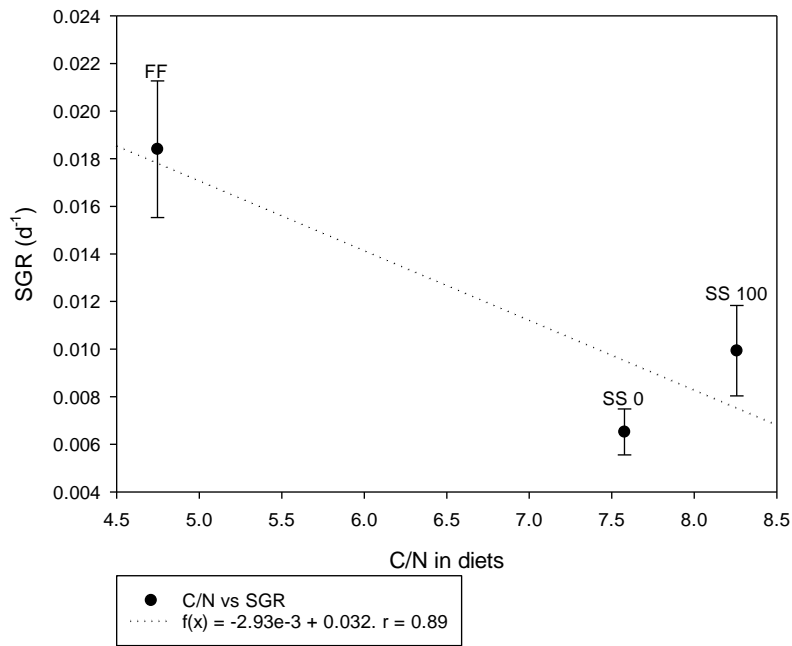
Figure 4.1. C/N (mean, n =3) in the FF, SS 100 and SS 0 diets versus the SGR (mean ± SD, n = 4) for the corresponding diets. The y-axis represents specific growth rate. The x-axis represents C/N in the diets. The dotted line represents the regression. The letters indicate the dietary treatment used.

When comparing the highest SGR found in the present study versus similar studies (Nesto et al., 2012; Santos et al., 2016) it becomes evident that the polychaetes in this study exhibited a lower SGR than was the case in the other studies. The highest SGR in the present study was $1.8 \pm 0.3 \times 10^{-2}$ d^{-1} which corresponds with an approximate increase of 1.8 % d^{-1}. Nesto et al. (2012) and Santos et al. (2016) reported respective growth rates of 6.3% and 6.5% d^{-1}. This despite the present study only lasting for half the duration of the others, which usually would report higher growth rates (Nesto et al., 2012; Santos et al., 2016). The differing growth rates could be explained combining several of the following factors:

Firstly, it could be fruitful to consider protein content. The protein content in fish feed used in the studies mentioned above differed (Nesto et al., 2012; Santos et al., 2016). Highest protein content was found in the LAR-diet (66%) in Nesto et al. (2012). Second highest was the Aquagold diet (46%) used in Santos et al. (2016), and third the FF (41% AM) in the present study. As previously discussed, the protein content in the feed seems to affect the growth rate greatly. Though this might contribute to the explanation for the difference in SGR in the present study versus the Nesto et al. (2012) study, it cannot explain the differing SGR between the

present study and the Santos et al. (2016) study due to similar protein content. Additional factors could be considered.

Secondly, genetic differences in northern and southern populations of *H. diversicolor* could play a part in explaining the different growth rates. As a theory for explaining the difference in the SGR between the diets used in the current study it holds little ground due to the polychaetes being collected in one location. As for comparison against the other studies of *H. diversicolor*, it might help to explain the observed differences in growth rates. The Nesto et al. (2012) study and the Santos et al. (2016) study were both conducted using *H. diversicolor* originating from southern Europe – Italy and Portugal, respectively. Several studies have shown that there are traceable genetic differences in different populations of *H. diversicolor* in Europe (Scaps, 2002; Breton, Dufresne, Desrosiers, & Blier, 2003). This is possibly due to limited gene flow resulting from short dispersal capabilities (Scaps, 2002). The southern European ragworms could have greater genetic abilities for growth compared with the Norwegian ones, considering that bigger worms are found at declining latitudes. However, the difference in size may also be caused by elevated temperatures increasing the worms metabolism (Scaps, 2002).

Thirdly, one could consider the age and size of the polychaetes. The growth rate of *H. diversicolor* is reduced with increasing age and size (Kristensen 1984; Tola, Masala and Piergallini, 2007, in Netso et al., 2012). While the Nesto et al. (2012) and Santos et al. (2016) studies used own brood stocks to produce juveniles for their experiment, the present study used wild caught polychaetes. A lower grade of age control could have led to a possible use of older individuals in the present study. When comparing the mean individual initial wet weights, the polychaetes in this study weighed (0.26 ± 0.02 g) significantly more than the other studies (0.025 ± 0.01 g and $< 0.18 \pm 0.04$ g). This suggest a higher number of mature individuals in the present study which in turn suggests a lower SGR (Tola et al., 2007, in Nesto et al, 2012; Kristensen, 1984). Based on this, it is therefore probable that an unevenness in the distribution of big and small polychaetes in the different cultivation tanks, could have contributed to the difference observed in the SGR in the current study. However, the results suggest that this was not as important for the growth in the present study, since the highest initial weights was found for the polychaetes reporting the lowest SGR (Figure 3.2).

The polychaete growth in the present study could also have been affected by the different characteristics of the diets. The FF-diet contained less water (Table 2.2) than the other diets, and was in pellet form, whilst the others were not. This could have given the polychaetes fed

the FF-diet more energy per particle ingested, subsequently leading to lower energy spent on feeding compared to the others (Brown, Eddy & Plaud, 2011).

One of the hypothesis in this thesis was that smolt sludge and biogas residue would be a good nourishment for growth of *H. diversicolor.* Despite the present study reporting lower growth rates than that reported by other studies of *H. diversicolor* (Nesto et al, 2012; Santos et al, 2016) the SGR was significantly higher compared with wild polychaetes living in North Atlantic waters. The SGR for the wild polychaetes was reported being 0.5×10^{-2} d^{-1} (Kristensen, 1984). The polychaetes fed on biogas residues and smolt sludge reported significantly higher values $> 0.7 \pm 0.1 \times 10^{-2}$ d^{-1}. This suggests that all the dietary treatments could function as good nourishment for the polychaete growth. Additionally, the results of the present and other studies of *H. diversicolor*, suggests that protein rich feeds may contribute to higher growth rates.

B   Cultivation experiment II

The starved polychaetes did not show any significant difference in biomass comparing the start weight and corresponding end weights. Nevertheless, the results suggested an insignificant (p > 0.05) decrease in biomass with increasing starvation time (Figure 3.7). The present study reported a decrease of 24.4 ± 1.51 % after nine days starvation. This is different, however slightly, to the value of 30.2 % as reported in a 10-day starvation trial of *H. diversicolor* conducted by Neuhoff (1979). There were some issues with the present study which could have reduced the accuracy of the results.

When measuring the difference between initial and final weight, one of the six-day replicas showed a weight increase of 30%, even as it starved. It was then concluded that this had to be a result of a measuring error. Therefore, these results were excluded from the sample when calculating mean growth, thus reducing the number of replicas for mean value calculation. In addition, the control weights could not be used to indicate growth in a satisfactory way due to flawed experiment setup. With the greatly varying sizes of the individuals, comparing the four worms taken out each sampling to a mean initial weight (n =16), would give very inaccurate results. Consequently, the results were excluded, making growth comparisons between starved and fed individuals impossible. In retrospect, the control setup should have been identical to the starvation sample setup which yielded usable data in this respect. Regarding the SGR for the starved worms, all durations reported negative values and a significant difference (p < 0.01) was observed between the polychaetes starved for six and nine days (Figure 3.9), which may

suggest an increasing loss of biomass with increasing starvation time. However, the same trend was not observed at day twelve, or day three for that matter. Further, only two replicas were used for the SGR at day six, which may reduce the validity of the data.

4.3 RNA/DNA ratio

A   Cultivation experiment I

One hypothesis in the present study was that the RNA/DNA ratios would reflect the observed biomass growth of the polychaetes. What the present study found, however, was that there was almost no correlation ($r = 0.08$) between RNA/DNA ratio and the SGR (Figure 4.2). On the other hand, several factors could have affected the results as; varying age composition, errors regarding the method used, and lack of material for comparison.

In the present study, polychaetes of different sizes were collected from their natural environment. No data on age or sex was recorded. A possible source of error when doing the RNA/DNA ratio measurements could be varying age composition in the samples (Chícharo & Chícharo, 2008).
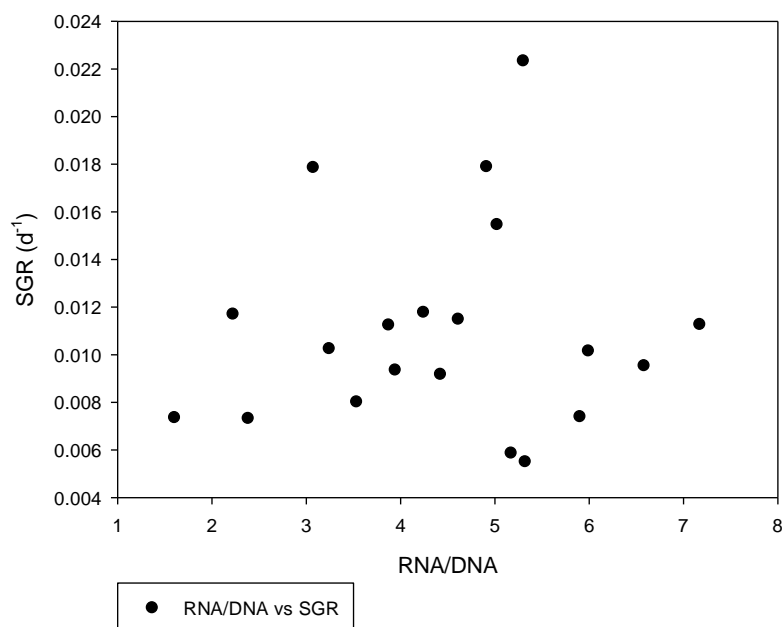

Figure 4.2. Specific growth rates (d^{-1}) for each dietary treatment versus the corresponding RNA/DNA ratios. The x-axis represents RNA/DNA ratio. The y-axis represents specific growth rate. A correlation (r) of 0.08 was found.

When studying fish larvae growth and corresponding RNA/DNA ratios, a trend of decrease in RNA/DNA ratio for larvae approaching late larval stage compared to the younger ones, was observed (Vinarge, Fonseca & Cabral, 2008), and indicated by other studies (Mathers, Houlian & Burren, 1994; Buckley, Calderone & Ong, 1999). As discussed earlier, the high mean start weight, compared to other studies measuring growth of *H. diversicolor* (Nesto et al., 2012; Santos et al., 2016), gives an indication that there's potentially many adult polychaetes present in the RNA/DNA samples. This could in turn contribute to a reduction in the observed RNA/DNA ratios (Chícharo & Chícharo, 2008). Contrary to this, the present study inconsistently reported higher RNA/DNA ratios with higher initial weight and lower RNA/DNA with lower initial weight (Figure 3.5). An explanation for this could be found when examining the number of worms used in the RNA/DNA analysis.

In the present study, 4-5 worms were taken out from the original treatment sample (n = 70) for RNA/DNA ratio measurement. It is a real possibility that the smaller ragworms could have been excluded from some samples and been over-represented in others. This could further have contributed to a lower RNA/DNA ratio for some samples, and a higher RNA/DNA ratio for others (Vinarge et al., 2008; Buckley et al., 1999; Mathers et al., 1994). To verify if this indeed was the case, the weight of each RNA/DNA sample could have been compared with their respective RNA/DNA ratios, making a calibration curve of weight versus RNA/DNA ratio. The age error could possibly have been reduced by doing so, but due to lacking weight measurements, no such comparisons were done.

Methodically; There are few, if any, prior studies using RNA/DNA ratios to determine recent growth rates of polychaetes. This begs the question: Are RNA/DNA ratios calibrated correctly for growth determination for these organisms? The results suggest no. RNA/DNA ratio correlate well with recent growth for most fish larvae, and for several molluscs and crustaceans (Frantiz, Grémare and Vétion, 1992). In some cases, however, RNA/DNA ratio could not be used as an index for growth, as was the case for the sea urchin *Paracentrotus lividus* (Frantiz et al., 1992) and for the crustacean *Carcinus maenas* (Houlihan, Waring, Mathers & Cray, 1990). Differences in the growth patterns of crustaceans versus fishes was used as a possible explanation to why the "no correlation" was found between the RNA/DNA ratio and growth (Houlihan et al, 1990). As an alternative method for measuring recent growth Houlian et al. (1990) proposed RNA/protein as a better tool to detect changes in growth rate due to its higher affinity to protein deposition. They argued that while growing, both protein breakdown and protein deposition occur. Therefore, RNA/protein gives a more accurate indication of recent

growth (Houlihan et al, 1990). Whether this applies to polychaetes is uncertain, however it is considered worth testing in a future study.

The present study used bulk weights in determination of the RNA/DNA ratio. Alternatively, individual weights could have been used. It is possible to use RNA/DNA ratio on a population level to measure growth, but it is more frequently used on organism level to measure individual growth (Chícharo & Chícharo, 2008). Using individual worms, and perhaps only the rear segments, could possibly make the results more precise. In fish larvae, the RNA/DNA ratio is reported to differ in body tissue (Olivar, Diaz & Chícharo, 2009). Adult polychaetes grow by adding segments from a growth zone right above the pygidium – the rear part of the animal (Seaver, Thamm & Hill, 2005). Using the whole animal, as done in this experiment, could have diluted the results. This because the most growth is expected to find place near the segment-adding area (Seaver et al. 2005).

Conclusively, using individual worms of similar age (preferably younger individuals), cultivating them separately, only using the rear segments and recording the sex, suggests a more accurate estimate of the RNA/DNA ratio. To suggest the nutritional condition of the worms a threshold value (larval protein growth = 0) indicating starvation is needed for the *H. diversicolor* (Robinson & Ware, 1988; Chícharo & Chícharo, 2008; Yandi & Altinok, 2015).

B   Cultivation experiment II

When starving the polychaetes, as is for fish larvae, a decline in RNA/DNA ratio with increasing starvation time is expected (Gwak & Tanaka, 2001; Malzahn et al., 2003; Dahlhoff, 2004; Yandi & Altinok, 2015). The specific growth rate of the starved polychaetes was studied over three-, six-, nine- and twelve-day spans. Figure 4.4 presents the measured RNA/DNA values for the starved polychaetes against the corresponding SGR.
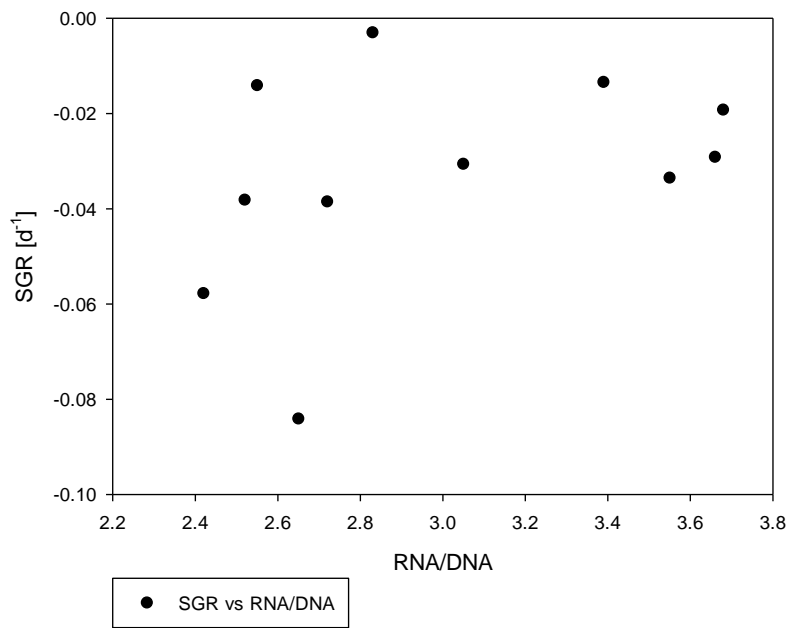
Figure 4.3. Specific growth rate for each starvation replica versus the corresponding RNA/DNA ratio. The y-axis represents the RNA/DNA ratio. The x-axis represents the specific growth rate. A correlation (r) of 0.38 was found.

An *r*-value of 0.38 suggests that there might be a relationship suggesting that lower RNA/DNA ratios correspond with lower SGR. However, the r-value is too weak to be conclusive.

To study how the RNA/DNA ratios changed with increasing starvation time a closer examination of the RNA and DNA concentrations were done. The concentrations are presented in Figure 4.5. When starving North Sea houting larvae (*Coregonus oxyrhinchus)* Malzahn et al. (2003) found a slight decrease of RNA concentration, and an increase in DNA concentration. In contrast to Malzahn et al. (2003), it seems that RNA concentration played the bigger role in affecting the RNA/DNA ratio in the current study (Figure 4.5). This may suggest that the polychaetes are affected differently in their RNA and DNA composition compare with fish larvae, which constitutes the major ground for comparison material. However, the RNA amount seems to play the bigger role in affecting the RNA/DNA ratio for Horse Mackerel larvae *T. mediterraneus* (Yandi & Altinok, 2015), making it difficult to conclude. Nevertheless, both these studies, Malzahn et al. (2003) and Yandi & Altinok (2015) did show negative RNA/DNA values for their starvation experiments, the present study did not. A possible explanation could be the induction of spawning.
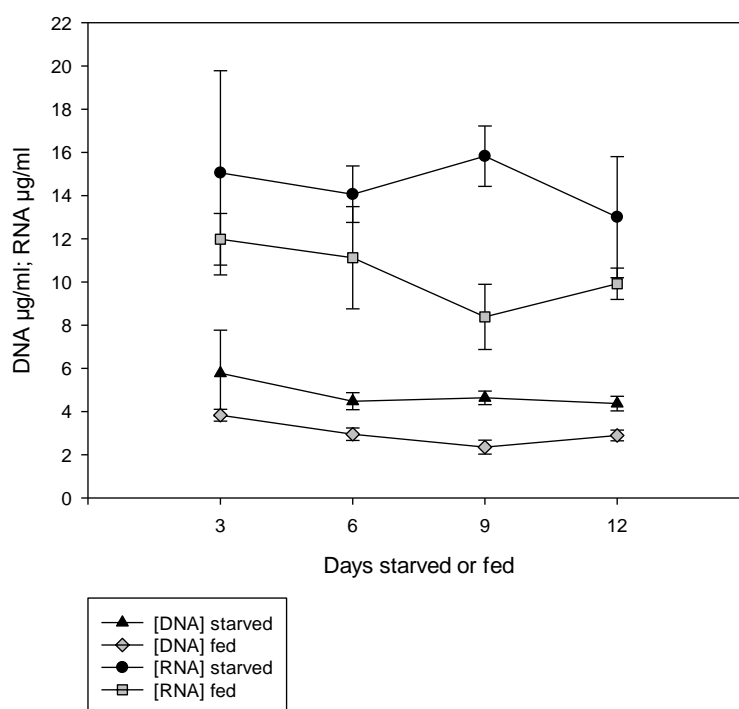
Figure 4.4. RNA and DNA concentrations (µg/ml) for the starved and fed polychaetes (mean ± SD, n =
3), adjusted for the sample weights (g) (Table A.6, Appendix II).

Spawning occurs when the temperature reaches 5-11°C, following a period of low temperatures (Scaps, 2002). When subjecting winter caught *H.diversicolor* to a light regime 16:8 (L/D), and temperatures of 16.5 °C we could have induced spawning. Even without food, the induced spawning could have led to an elevated RNA/DNA ratio. In a study conducted by Li et al. (2000) a significant increase in the RNA/DNA ratio in the ovaries of a starved groups of Pacific oyster (*Crassostrea gigas)* was found before spawning. *C.gigas* used mainly glycogenolysis as energy input in their gametogenesis and therefore increased their RNA without any feed input (Li, Osada & Mori, 2000). If *H.diversicolor* uses glycogenolysis as *C.gigas* in gametogenesis, this could help to explain the observed increase in RNA/DNA ratio in starved *H.diversicolor* as presented in Figure 3.9. Unfortunately, no literature was found on the matter regarding *H. diversicolor*.

While comparing growth of the mussel *Mytilus californianus* using RNA/DNA ratios, Dahlhoff (2004) found that the mussels grew more than the feed availability would indicate. Dahlhoff proposed that an increase in metabolic rate might have affected the results, with higher metabolism occurring at higher temperatures (Dahlhoff, 2004). In relation, Foley et al. (2016) argued that larval fish subjected to a rapid change in temperature, either before or after

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collection from their natural habitat, would exhibit higher RNA/DNA ratios than the ratio found for those not subjected to this temperature change (Foley, Bradly & Höök, 2016). The two mentioned studies used ectotherms, making it probable that the polychaetes, also ectothermic, in the current study would behave in a similar pattern. The polychaetes could have experienced a metabolic shock when moved from their natural environment (2 °C) to the cultivation tanks (16.5 °C). This could have led to an increase in the polychaetes RNA/DNA ratios (Figure 3.9). *H. diveriscolor* has reported optimum growth at 19 °C, therefore an increase in metabolism could be expected when increasing the temperature as done in this experiment (Scaps, 2002).

As expected, the control groups increased in RNA/DNA ratio over the first six days. Contrary to expectation, so did the starved polychaetes (Figure 3.9). In fact, no significant differences were found when comparing the RNA/DNA in the starved and fed polychaetes for the corresponding cultivation durations. However, when studying the later stages of the starvation experiment (day 9-12), there was a shift from increasing to decreasing RNA/DNA ratios, suggesting that the initial shock responsible for the increase in RNA/DNA ratio stalled. The results presented in Figure 4.5 suggests an insignificant increase on day 12 in RNA concentration for the fed polychaetes, while the starved polychaetes experienced a rapid, but still insignificant, decrease in their RNA concentration. This suggests that their energy reserves were used up, and with no food available, the starved polychaetes had a decreased RNA content whilst the fed polychaetes increased their RNA content. This gives further root to the assumption that the increase in the RNA/DNA ratios was based on a metabolic shock. However, due to short duration, it is difficult to confirm this trend.

Whether the RNA/DNA ratio found in cultivation experiments indicate good or poor nutritional condition is relying on the RNA/DNA threshold value indicating that the organisms are starving (Chícharo & Chícharo, 2008). The starvation threshold value, referred to as the "critical ratio", varies from species, size and temperature (Yandi & Altinok, 2015). There is however found a species-independent minimum RNA/DNA value of 1 necessary for survival (Clemmesen, 1994), but this value does not consider probable change in the ratio with increasing age (Chícharo & Chícharo, 2008). No prior studies using RNA/DNA ratio to characterize the nutritional condition of polychaetes were found. This gave the author little grounds for comparison, making it difficult to verify if the results in the present study indicate good or poor nutritional condition of polychaetes. However, considering the observed growth over the 30-day trial (Figure 3.4), it is unlikely that the polychaetes in cultivation experiment I were in poor nutritional condition.

5 Conclusive remarks and further study

The results of the present study indicate that *H. diversicolor* can successfully be grown on a sole diet of smolt sludge, biogas residue or a combination.

Significant differences were found when comparing the growth in biomass when feeding the polychaetes on different diets in cultivation experiment I. Feeding with commercial fish feed yielded the highest growth rates, which were significantly different from the other dietary treatments. No significant difference on polychaete growth was found when comparing the smolt sludge and biogas residue diets. Chosen cultivation density and water quality parameters were within the natural range for *H. diversicolor*, and the diets did not seem to affect the survival rate differently.

Based on studies conducted on the biochemical composition of *H. diversicolor* and the present study on growth rates, *H. diversicolor* seem applicable as a source for marine biomass when fed with waste stream from the land-based smolt aquaculture industry. A combination of high protein feed and smolt sludge, and or to a lesser extent biogas residue, might be preferable. A significant difference in the RNA/DNA in the combination diets was observed with an increasing amount of biogas residue. Contrary to expectations, the RNA/DNA ratio did not reflect the observed growth of *H. diversicolor* in the present study. Due to lacking scientific material on the matter, further testing is needed to prove whether the RNA/DNA ratios found in this study indicate good nutritional condition, and if the method itself is suitable to indicate growth of *H. diversicolor*.

For future studies, own brood-stocks producing juvenile polychaetes of the same age might be preferable to use in growth experiments. Determining growth using the RNA/DNA method requires a calibration to suit polychaetes. A critical value indicating starvation should be characterized for *H. diversicolor*. Regarding the polychaete harvest; sampling in the warmer periods could eliminate potential errors caused by spawning and metabolic differences. By using individuals of same size, at known sex ratios, and using only the rear segments, further errors could be reduced.

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Appendix I

Table A.1. Survival rates (%), initial and final (Mean ±SD) number (#) of polychaetes.

Diet	Mean survival (%)	Initial # individuals	Final # individuals
FF	88,2 ± 5.5	70	61.8 ± 3.9
SS100	88.9 ± 4.4	70	62.5 ± 3.1
SS66	82.1 ± 6.8	70	57.5 ± 4.7
SS33	91.8 ± 5.5	70	64.3 ± 3.9
SS0	88.2 ± 3.8	70	61.8 ± 2.6

Table A.2. Initial and final weights (g) for individual (mean ± SD, n = ~70), and their growth (%) (mean ± SD, n= 3). Superscripts denote significant differences (p < 0.05) within the column. b is significantly different from a. ab does not differ from neither a nor b.

Diet	Initial mean weight (g)	Final mean weight (g)	Mean growth (%)
FF	0.26 ± 0.01^{ab}	0.45 ± 0.03^{b}	74.16 ± 15.31^{b}
SS 100	0.27 ± 0.01^{b}	0.37 ± 0.01^{a}	34.87 ± 7.57^{a}
SS 66	0.25 ± 0.03^{ab}	0.34 ± 0.04^{a}	35.57 ± 4.88^{a}
SS 33	0.25 ± 0.03^{a}	0.34 ± 0.02^{a}	36.39 ± 6.60^{a}
SS 0	0.27 ± 0.03^{ab}	0.33 ± 0.01^{a}	21.65 ± 3.52^{a}

Table A.3. The RNA, DNA concentrations (µg/ml) and their RNA/DNA ratio (mean ± SD, n = 4). Superscripts denote significant differences (p < 0.05) within the column. b is significantly different from a. ab does not differ from neither a nor b.

Diet	Mean [RNA]	Mean [DNA]	Mean [RNA]/[DNA]
FF	22.15 ± 3.24^{a}	5.16 ± 1.94^{a}	4.58 ± 1.02^{ab}
SS 100	24.15 ± 7.13^{a}	3.98 ± 0.85^{a}	6.07 ± 1.10^{a}
SS 66	15.29 ± 6.94^{a}	3.20 ± 0.85^{a}	4.65 ± 0.92^{ab}
SS 33	14.55 ± 2.17^{a}	4.68 ± 1.05^{a}	3.21 ± 0.71^{b}
SS 0	18.90 ± 6.07^{a}	5.97 ± 2.41^{a}	3.62 ± 1.91^{ab}

Appendix II

Table A.4. Initial- and final weight(g) (mean ± SD, n = 3) with corresponding growth (%) compared with number of days starved.

Days starved	Initial mean weight (g)	Final mean weight (g)	Mean growth (%)
3	0.16 ± 0.06	0.14 ± 0.05	-14.67 ± 6.60
6	$0.16 \pm 0.01^*$	$0.15 \pm 0.01^*$	$-7.92 \pm 0.26^*$
9	0.18 ± 0.07	0.13 ± 0.05	-24.40 ± 1.51
12	0.17 ± 0.02	0.13 ± 0.04	-24.71 ± 23.50

*two replicas used due to possible measuring error.

Table A.5. A) The starvation values of RNA and DNA concentrations (µg/ml), and the corresponding RNA/DNA ratio (mean ± SD, n = 3). B) The control values of RNA and DNA concentrations (µg/ml), and the corresponding RNA/DNA ratio (mean ± SD, n = 3).

A	Days starved	Mean RNA ± SD	Mean DNA ± SD	Mean RNA/DNA
	3	28.1 ± 7.7	10.7 ± 2.9	2.6 ± 0.1
	6	19.5 ± 5.3	6.1 ± 0.8	3.2 ± 0.5
	9	24.9 ± 3.3	7.3 ± 0.3	3.42 ± 0.3
	12	22.9 ± 2.2	8.0 ± 2.1	3.0 ± 0.3
B	Days cultivated	Mean RNA ± SD	Mean DNA ± SD	Mean RNA/DNA
	3	20.4 ± 7.4	6.4 ± 2.0	3.1 ± 0.4
	6	17.9 ± 6.0	4.8 ± 1.6	3.7 ± 0.4
	9	10.2 ± 1.7	2.9 ± 0.3	3.6 ± 0.3
	12	13.7 ± 2.6	4.0 ± 0.7	3.4 ± 0.1

Table A.6. A total overview of the sample weights (mg), DNA and RNA concentrations (µg/ml) for the fed and starved polychaetes used in RNA/DNA analyses.

Duration (days)	Replica	Sample weight (µg)	[RNA] (µg/ml)	[DNA] (µg/ml)
		Starved		
	1	2.3	22.4	8.2
3	2	1.3	25.0	9.9
	3	2.2	36.8	13.9
	1	1.1	14.2	5.6
6	2	1.7	24.8	7.0
	3	1.3	19.4	5.7
	1	1.6	28.0	7.7
9	2	1.4	21.4	7.0
	3	1.7	25.2	7.1
	1	1.9	25.0	8.8
12	2	1.3	20.6	5.6
	3	2.3	23.0	9.5
		Fed		
	C1	1.7	21.4	6.0
3	C2	2.1	27.2	8.6
	C3	1.2	12.5	4.6
	C1	1.5	13.5	4.1
6	C2	2.2	24.8	6.6
	C3	1.2	15.5	3.7
	C1	1.2	10.4	3.1
9	C2	1.2	8.3	2.5
	C3	1.2	11.8	3.0
	C1	1.3	12.4	3.5
12	C2	1.2	12.0	3.6
	C3	1.6	16.7	4.8