Master's thesis

NTNU
Norwegian University of Science and Technology
Faculty of Natural Sciences
Department of Biology

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Bioremediation of aquaculture sludge by cultivation of *Hediste diversicolor* (O.F. Müller, 1776)

Master's thesis in MSc Ocean Resources
Supervisor: Kjell Inge Reitan
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Sammendrag

Oppdrett av Atlantisk laks (*Salmo salar*) produserer store mengder organisk partikulært avfall. I motsetning til åpne havmerder, samles det partikulære avfallet som slam ved landbaserte oppdrettsanlegg. Bioremediering av dette næringsrike restavfallet fra oppdrettsnæringen gjennom kultivering av lavtrofiske arter i Integrert Multi-Trofisk Akvakultur (IMTA) har blitt foreslått som en potensiell løsning på avfallsproblematikken og ressursutnyttelsen i oppdrettsnæringen.

Ved å fôre grupper av børstemarken *Hediste diversicolor* (O.F. Müller, 1776) i en kultiveringsrigg på en gradient av ulike mengder slam fra enten smolt (S) eller post-smolt (PS) anlegg (5-40% av børstemarkens totale nitrogeninnhold fôret daglig), var målet med denne studien å undersøke det bioremedierende potensialet til arten. Under kultiveringsforsøket ble det etablert en positiv korrelasjon mellom fôrmengde og den gjennomsnittlige individuelle spesifikke vekstraten (%SGR) til børstemarken, hvor individene som mottok de høyeste fôringsnivåene oppnådde en %SGR sammenlignbar med resultater fra tidligere forsøk på samme art. Det ble ikke observert en stagnering av vekstrate ved høyere fôringsnivåer, noe som tyder på at *H. diversicolor* kan oppnå høyere %SGR ved større fôringsintensitet enn hva som ble prøvd under dette eksperimentet, selv om betydelige variasjoner mellom replikanter av samme fôringsnivå gjorde det vanskelig å identifisere trender og signifikante forskjeller. Ved kjemiske analyser av børstemarken før og etter fôringsforsøket samt av de to slamtypene ble effektiviteten av den biologiske resirkulasjonen av karbon (C), nitrogen (N) og fosfor (P) fra slammet under forsøket bestemt basert på standardiserte metoder for bestemmelse av tørrstoff (DM), C, N, P og total organisk material (TOM). Det gjennomsnittlige innholdet av C, N og P i *H. diversicolor* i alle fôringsnivå økte under fôringseksperimentet, hvor det laveste fôringsnivået resulterte i det laveste opptaket. Hvor stor andel av tilgjengelig C, N og P fra fôret som ble tatt opp og omdannet til ny børstemark biomasse igjennom vekst varierte stort mellom replikanter, hvorav P var næringsstoffet undersøkt som ble dårligst utnyttet, tilsvarende bare 1% av den totale mengden P fôret. Det høye P-innholdet i slammet sammenlignet med i *H. diversicolor* er den sannsynlige forklaringen på den lave utnyttelsen. Både C og N i slammet ble mer effektivt utnyttet.

Det konkluderes med at både S og PS slam virker å være godt egnet som fôrkilde for *H. diversicolor*, da hverken vekst eller næringsutnyttelse påvirkes signifikant på tross av de kjemiske forskjellene i innholdet av de to slamtypene. Men den lave omgjøringen av C, N og P fra slammet og til ny børstemark biomasse bør tas i betraktningen ved bruk av *H. diversicolor* som en bioremedierende art, og en kombinasjon av næringsresirkulerende prosesser kan være aktuelt. Eksperimentelle studier hvor *H. diversicolor* fôres med større mengde slam og med flere replikanter bør utføres for å videre fastslå artens bioremedierende potensial.

Abstract

The farming of Atlantic salmon (*Salmo salar*) produces large quantities of particulate organic waste. Unlike open-cage systems, in land-based systems, this organic waste is collected as sludge via filtration processes, and bioremediation through Integrated Multi-Trophic Aquaculture (IMTA) has been proposed to utilize these nutrient rich discharges, by cultivating species that feed on this waste.

The objective of the present study was to assess the bioremediation potential of cultivating the deposit feeding polychaete *H. diversicolor* (O.F. Müller, 1776) on Atlantic salmon aquaculture sludge. Groups of polychaetes were fed with a gradient of increasing amounts of both smolt (S) and post-smolt (PS) sludge (5-40% of the polychaetes total nitrogen content fed daily). A positive correlation between specific growth rates (%SGR) and amount of feed supplied (% N) was found, and mean individual %SGR of polychaetes receiving the higher feeding levels were comparable to growth rates found in previous studies. No stagnation of growth rates was observed with increased feeding intensity, indicating that polychaetes were able to tolerate even higher feeding intensities than tested in the present study, although high variations between mean %SGR of tank replicates made it hard to identify significant differences and clear patterns.

By chemical analysis of the polychaetes pre and post feeding and of the sludge offered, the efficiencies of the biological recirculation of carbon (C), nitrogen (N) and phosphorus (P) were determined based on standardized methods for determination of dry matter (DM), C, N, P and total organic matter (TOM). The polychaetes were found to increase their C, N and P content during the feeding period in all sludge feeding levels , with the lowest mean increase registered in the lowest feeding level. The efficiency in converting C, N and P content in the sludge into new polychaetes biomass by growth varied considerably, with P being the poorest utilized nutrient only accounting for 1% of the P supplied to tanks incorporated into new polychaete tissue. This can be explained by the high contents of P in aquaculture sludge compared to the nutritional needs of the polychaetes. The conversion of N and C was more efficient.

It is concluded that both S and PS sludge appears to be well suited as a sole feed source for *H. diversicolor*, with neither growth nor conversion efficiencies significantly affected by the differences in the chemical content between the two sludge types. However, the low conversion efficiencies, especially of P, should be taken into consideration when applying them as a bioremediation species, as they are not likely to utilize all of the available nutrients, thus possibly making a combination of sludge treatment processes favourable. Experimental studies feeding polychaetes on even higher feeding levels of sludge with higher replicate numbers than tested in the present study should be performed to determine the optimal feeding intensity for growth and nutrient recycling.

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Abbreviations

%N	Amount of polychaetes total nitrogen content fed every day [%]
C	Carbon
$C_{CONV.}$	Amount of C in sludge converted into new C in polychaetes [%]
DM	Dry matter
DO	Dissolved oxygen
Feeding level A	Feeding level receiving nitrogen equivalent to 5 %N
Feeding level B	Feeding level receiving nitrogen equivalent to 10 %N
Feeding level C	Feeding level receiving nitrogen equivalent to 20 %N
Feeding level D	Feeding level receiving nitrogen equivalent to 40 %N
IMTA	Integrated Multi-Trophic Aquaculture
IW	Initial worms
N	Nitrogen
$N_{CONV.}$	Amount of N in sludge converted into new N in polychaetes [%]
P	Phosphorus
$P_{CONV.}$	Amount of P in sludge converted into new P in polychaetes [%]
PS	Post-smolt
RAS	Recirculating Aquaculture Systems
S	Smolt
SD	Standard deviation
TOM	Total organic matter
WW	Wet weight

1. Introduction

1.1. Challenges of Atlantic salmon production

Fisheries have traditionally been the main source of fish for consumption, but due to overexploitation of commercial fish stocks the yield has stagnated over the last decades. However, the global consumption of fish is increasing as a result of a rapidly growing aquaculture industry, which in 2018 accounted for over half of the total fish production for human consumption (FAO, 2020). With its long history and strong ties to the sea and harvesting of marine resources, Norway is a traditionally strong fisheries nation. From its early beginnings in the 1970s, the country has also experienced a rapidly expanding aquaculture industry (Paisley et al., 2010), and was in 2018 the leading producer of farmed Atlantic salmon (*Salmo salar*) with a global production share of 55.3% (Iversen et al., 2020).

Aquaculture production of Atlantic salmon is technologically advanced compared to cultivation of most other finfish species, and the high consumer demand and regional limitations of production has made it a profitable aquaculture species on the global market (FAO, 2020). Atlantic salmon is, along with Rainbow trout (*Oncorhynchus mykiss*), the most important aquaculture species in Norway, but the growth in the sector has levelled off since 2014, mainly due to salmon lice infestation problematics and environmental concerns (Olafsen et al., 2012; Olaussen, 2018). For a continued growth of the production of salmon and aquaculture as a whole to satisfy the increasing demand of protein and marine food resources from a growing global population, several problems concerned with the sustainability and environmental effects of the industry have to be solved (Kutti et al., 2007; Olafsen et al., 2012; Torrissen et al., 2013; Lekang et al., 2016; Aas et al., 2019; Song et al., 2019).

Achieving a sustainable production includes minimizing the input of resources, energy use, waste discharge and environmental impacts while maximizing the production efficiency (Bartley et al., 2007; Aas et al., 2019). Production of this high-trophic species demands lipid and protein rich feed sources, and the inclusion of fish meal and fish oil from pelagic fisheries in salmon feed raises sustainability concerns as it increases the demand of other fish stocks while using marine protein otherwise edible for humans (Naylor et al., 2000; Deutsch et al., 2007). From 90% in 1990, the inclusion rate of fish meal and fish oil in salmon feed has been lowered to 18% and 11%, respectively, mainly by substituting the marine ingredients with plant-based materials (Ytrestøyl et al., 2015). However, plant-based substitutes have been known to cause adverse health effects, lowered feed efficiency and growth rate in salmon (Francis et al., 2001; Gatlin et al., 2007; Aas et al., 2019), and several other substitutes are being evaluated such as animal by-products (Linton, 2014), unicellular bacteria and organisms (Wei et al., 2021), microalgae

(Sørensen et al., 2016), insects (Tacon, 2002) and marine ingredients from lower trophic levels (Linton, 2014).

1.2. Nutrient discharge

Along with the high resource requirements, the cultivation of Atlantic salmon produces substantial amounts of organic and biogenic wastes, such as organic particulate and dissolved matter and inorganic nutrients, rich in carbon (C) and the important macronutrients nitrogen (N) and phosphorous (P) (Olsen and Olsen, 2008; Wang et al., 2012).

Faeces, uneaten feed and dead fish all contribute to the organic waste production of intensively cultivated Atlantic salmon. Although a feed conversion rate (FCR) of 1.1 in adult salmon is far more efficient than other traditional husbandry animals such as cattle and pigs (Fry et al., 2018), some of the feed administered to the fish will still be lost to the surrounding environment. This feed loss is dependent on several factors such as life stage, operation and feed source, but models suggest that <5% of the amount fed to the salmon remains uneaten and is lost in open-cage salmon aquaculture (Cromey and Black, 2005; Reid et al., 2009, Wang et al., 2012).

Of the feed that is eaten, a fraction of non-digested and unassimilated food will end up as faeces, and has been calculated to approximately 15% (Reid et al., 2009) and 19% (Wang et al., 2012) of the feed consumed by grow-out salmon. The fate of these organic particles depends on whether the aquaculture facility is land-based or at sea in open cages. In land-based facilities the particles are accumulated and filtrated out as sludge along with other organic substances such as feed spill and bacteria from biofilters and biofilms in RAS systems (Piedrahita, 2003; Lomnes et al., 2019). In open-cage net pens, larger particles sink to the bottom and settle on the sea floor below the facility while smaller particles stay in suspension and travel greater distances with the moving water masses (Olsen and Olsen, 2008; Wang et al., 2012).

In addition to the organic particulate and dissolved matter, a large part of the nutrients released by salmon farming comes in the form of dissolved inorganic metabolic by-products such as CO_2 from respiration and NH_4^+ and PO_4^{3-} through excretion (Olsen and Olsen, 2008; Wang et al., 2012).

When waste discharge from aquaculture facilities is released directly to the environment, it causes a nutrient influx into the aquatic and benthic ecosystems, potentially altering its functioning and species abundance (Olsen and Olsen, 2008, Wang et al., 2012). Inorganic nitrogen is generally the limiting nutrient of algal growth in coastal ecosystems (Howarth and Marino, 2006; Noroi et al., 2011), and when freely released to the water body it stimulates phytoplankton, macroalgae and bacteria growth with readily bioavailable nutrients (Olsen and Olsen, 2008; Wang et al., 2012). Dissolved organic nutrients remains suspended over a longer time period, providing phytoplankton and bacteria with favourable growth conditions (Olsen and Olsen, 2008). This increasing amount of N and P in the water column has the potential to produce harmful algal blooms and eutrophication events, leading to alterations of the marine ecosystem and oxygen depletion (Howarth and Marino, 2006; Olsen and Olsen, 2008).

Small particulate organic matter stays suspended in the water column and is spread widely where it acts as a food source for smaller fish, bacteria and filter feeding zooplankton and mussels (Troell et al., 2009). The discharge of larger-sized particulate organic matter sinks to the seabed quite readily, causing severe impacts on benthic communities in close proximity to sea cages (Carvajalino-Fernández et al., 2020). The organic matter that reaches the sediments is eaten by detritus-feeding animals (Wang et al., 2012) and changes the characteristics of the sediment and the benthos communities (Hargrave et al., 1997). Some of the uneaten feed pellets can be eaten by fish aggregating around the farms (Dempster et al., 2005).

1.3. Bioremediation and IMTA

The discharge of important macronutrients such as nitrogen (N) and phosphorous (P) from aquaculture sites are, as we have seen, if not collected or otherwise utilized, released to its surroundings, wasting valuable resources. N is a limiting nutrient in most aquatic ecosystems (Howarth and Marino, 2006), while P is essential element for every living organism without any possible available replacement (Elser and Bennett, 2011; li et al., 2018b; Yogev et al., 2020). The mining of phosphate minerals is the main source of P used on a global scale (li et al., 2018a; li et al., 2018b), with the reserves expected to be depleted within the next 100 years being categorized as a non-renewable resource (Wu et al., 2016; li et al., 2018b). If released directly to the environment or otherwise poorly recapturing and utilizing nutrients in the sludge, essential resources in food production for human consumption is lost, with a high influx of N and P potentially disrupting marine and benthic ecosystems (Olsen and Olsen, 2008).

Aquaculture of Atlantic salmon in Norway and other Western countries is commercially run as a mono-culture, where aquaculture facilities produce one single species (Chopin et al., 2001). For centuries, Asian countries such as China have practiced a polyculture approach, where the waste of one species becomes feed for others (Chan, 1993). The increasing concern of the environmental effects of intensive aquaculture production have led to a growing interest for the co-cultivation of aquaculture species belonging to different trophic layers, to mitigate negative effects and maximize the utilization of resources put into the system (Chopin et al., 2001; Troell et al., 2009; Ellis and Tiller, 2019). Bioremediation of compounds involves the usage of organisms that are able to utilize or degrade organic wastes or material, and by using organic waste streams from salmon aquaculture, additional new biomass can be cultivated, creating value while reducing the resource wastage and avoiding the negative environmental effects of nutrient loadings to the surroundings.

Integrated Multitrophic-Aquaculture (IMTA) builds on the concept of the old Asian techniques, where non-competing species from different trophic levels are cultivated in a synergistic relationship, utilizing the waste of one species as a feed source for another. (Chopin et al., 2001; , Troell et al., 2009; Ellis and Tiller, 2019). The wastes produced by a fed species such as salmon, provides an excellent food source for several extractive feeders, suspension feeders, filter-feeders and deposit-feeders (Holdt and Edwards, 2014; Ellis and Tiller, 2019), many of which are also of high commercial value. To minimalize possible negative environmental impacts of salmon farms and maximize the nutrient utilization, ideally, both inorganic and organic nutrients, dissolved and particulate, should be taken up. As an example, seaweed extracts inorganic nutrients from surrounding water masses, and the high discharge of dissolved inorganic nitrogen from salmon aquaculture along with the commercial value of seaweed makes the co-cultivation of salmon and seaweed a promising prospect (Wang et al., 2012). In addition, suspension feeders such as mussels are able to extract organic nutrients released to the water masses and could therefore also be considered for usage in IMTA systems (Cranford et al., 2013; Irisarri et al., 2015).

The waste stream of larger particulate organic matter from salmon farming serves as a potential feed source for deposit feeding animals. In open-cage systems, cultivation of detritus- and faeces-consuming animals below or close to salmon sea farms have been suggested to prevent the accumulation of organic matter waste in the sediments (Buschmann et al., 2008), thus utilizing the nutrient rich waste. Although the majority of the particulate organic waste is produced in the sea based open-cage aquaculture of Atlantic salmon, and the effects of it are more severe due to the direct discharge to the surroundings, particulate wastes are also a topic of concern at land-based aquaculture facilities (Aas and Åsgård, 2017, Lomnes et al., 2019). In these production systems, faeces, feed spill and other organic particles are filtrated and collected as sludge, and the processing and disposal of this nutrient rich substance is often problematic due to the technological, time and space-consuming processes involved in de-watering and preservation (Aas and Åsgård, 2017). In proposed land-based IMTA systems, the deposit feeders would feed on the waste streams removed from the tanks (Buschmann et al., 2008). Several species have been suggested for removal of organic particulate matter in this type of IMTA system (Israel et al., 2019; Wang et al., 2019b; Baltadakis et al., 2020; Nederlof et al., 2020), one of them being polychaetes.

1.4. Polychaetes *Hediste diversicolor*

The polychaete species *Hediste diversicolor* was first described by O.F Müller in 1776, belonging to the family Nerididae. Inhabiting shallow marine and brackish waters in the North Temperate Zone on both sides of the Atlantic, it plays a pivotal role in ecosystems of estuaries across Europe (Scaps, 2002; Bischoff et al., 2009). Tolerating high variations in both temperature (Wolff, 1973), salinity (Wolff, 1973) and available oxygen (Kristensen, 1983), it's an adaptable species well suited for the life in estuaries and littoral zones (Scaps, 2002), and by digging burrows in the sediment it creates a refuge from its many predators (Esselink and Zwarts, 1989). The species is able to switch between different feeding tactics dependent on several factors such as type and abundance of food and the presence of predators (Riisgård, 1991; Fidalgo e Costa et al., 2006). It is both omnivorous and predatory, switching between filter-feeding, foraging for prey and collection of organic material along the substrate (Esselink and Zwarts, 1989). As demonstrated by Riisgård (1991) and again by Vedel and Riisgard (1993), when phytoplankton is abundant in the water masses the polychaetes are filter-feeders, trapping suspended food particles in a secreted mucus net. They also forage actively for prey species or detritus and organic material on the substrate surface (Roenn et al., 1988), a tactic exposing them to predators and therefore often most important in the absence of its many predatory species (Scaps, 2002; Fidalgo e Costa et al., 2006).

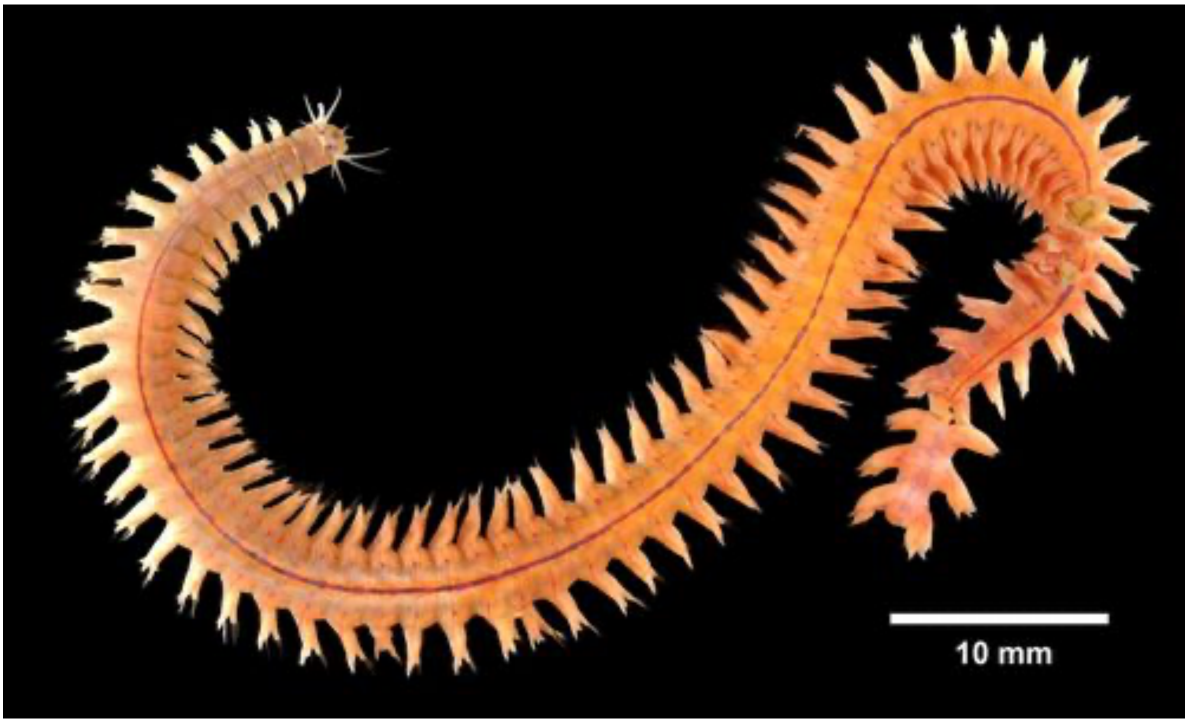


Figure 1: *Adult individual of Hediste diversicolor (Lazo-Wasem., 2016)*

The occurrence of polychaetes in surface sediments beneath fish farms and recent cultivation studies done with *H. diversicolor* suggest that this species is able to feed on organic particulate waste from finfish aquaculture (Heilskov and Holmer, 2001; Brown et al., 2011; Pajand et al., 2017; Yousefi-Garakouei et al., 2019; Wang et al., 2019b). Cultivation of *H. diversicolor* on waste streams from land-based and sea-based aquaculture of Atlantic salmon enables bioremediation of nutrient discharges, utilizing these important resources and producing valuable biomass, and the addition of *H.diversicolor* as an extractive species below open sea cages or on sludge waste streams from land-based salmon farms in an IMTA system enables the recapture of nutrients from particulate waste (Honda and Kikuchi, 2002; Granada et al., 2016; Nederlof et al., 2020). The polychaete species is also of commercial interest, being a popular bait used for recreational fisheries, and its biochemical composition makes it a promising candidate as a low trophic marine supplement in aquaculture feeds as well (Bischoff et al., 2009; Wang et al., 2019b).

1.5 Aims and hypotheses

The overall aim of this thesis was to assess the bioremediation potential of *Hediste diversicolor* feeding on salmon aquaculture sludge from land-based systems. Groups of *H. diversicolor* were fed with a gradient of increasing amounts of sludge from either a smolt or post-smolt facility, respectively, and the main goal was divided in the following sub-goals:

- Determining the optimal feed concentration for maximal growth and the saturation point where the organisms no longer increase in biomass with increased feeding intensity.
- Investigating the efficiency of the biological recirculation of C, N and P offered to the polychaetes.

Based on the experiments, the potential for cultivation and the bioremediation services offered by the species by utilizing organic waste from aquaculture facilities was estimated and discussed in the context of IMTA. The evaluation of the bioremediation effects of *H. diversicolor* were based on standardized methods for analysis of dry matter, total organic matter, carbon, nitrogen, and phosphorous of polychaetes before being fed on aquaculture sludge and at the end of the feeding experiment, along with the chemical content of the diets.

The hypothesis tested were the following:

1. *H. diversicolor* is able to feed on and utilize aquaculture sludge to gain biomass
2. Growth of *H. diversicolor* increases with the amount of sludge fed up to a certain point
3. *H. diversicolor* bioremediates nutrient emission from aquaculture facilities by taking up and utilizing carbon, nitrogen and phosphorous from salmon sludge.
4. There will be no significant difference in the bioremediation capability of polychaetes fed on either smolt or post-smolt sludge.

This thesis was connected to the RCN funded projects “Cultivation of Polychaeta as raw material for feed (POLYCHAETE)” (Project number: 280836) and “Nutrients in a Circular Bioeconomy: Barriers and Opportunities for Mineral Phosphorous Independence in Norway” (Project number: 268338).

2. Materials and methods

2.1 Growth experiment

2.1.1 Collection of polychaetes and sludge

Individuals of *H. diversicolor* were collected in the wild for use in this experiment. A total of three sampling trips were made by car from Brattørkaia to collecting sites near Spongdal (63°21'20.27"N, 10°9'58.77"E) and Buvika (63°18'34.08"N, 10°10'31.39"E), two villages in close proximity to Trondheim, Norway. During low tides, shovels were used to dig ~20cm down into a substrate mixture of sand and clay in the intertidal zone of beaches, picking out individuals of *H.diveriscolor* by hand and collecting them in trays filled with substrate, seaweed and seawater (picture 2). The digging was done on clayous beaches close to small streams delivering an influx of fresh water along Trondheimsfjorden. Collecting hours were limited due to the tides, as the only appropriate time to dig in the intertidal zone was close to and during low tides. Two trips to a beach near Buvika and one trip to a beach near Spongdal resulted in a total of ~1100 individuals of *H. diversicolor*. No discrimination of size or fitness was made during the collection.

After collection, the polychaetes were transported to SINTEF SeaLab at Brattørkaia, Trondheim and put in holding tanks before the start of the experiment. The holding tanks were connected to a flow through system with a minimum of 100% water exchange every 24th hour and polychaetes were fed common salmon feed once a week for approximately a month.

Picture 2*: Collection of H. diversicolor during low tide from a substrate mixture of sand and clay in the intertidal zone of beaches close to Buvika and Spongdal, Trondheimsfjorden. Photo: August R. Nymoen.*

The two different feed resources used in this experiment – smolt and post-smolt sludge, were collected from land-based salmon farming facilities in Trøndelag and Nordland. Sludge originating from salmon smolt production was collected from Lerøy Belsvik by car, while post-smolt sludge was flown in from LetSea AS. Both sludge types were centrifuged for 5 minutes at 3000 rpm (Heraeus Multifuge X3R, Thermo Scientific) to reduce water content and stored in smaller plastic bags in a freezer for later use as feed for the polychaetes.

2.1.2 Experimental setup

During the experiment, *H. diversicolor* was fed specific amounts of either smolt or post-smolt sludge in a 30-day feeding period to investigate the bioremediation efficiency, growth rates and biochemical composition of the polychaetes. In addition, a total of 15 randomly picked individuals of polychaetes were taken out of the holding tanks, put in seawater to empty their guts, flushed with freshwater, weighed, dried and then frozen prior to the start of the experiment as baseline values and reference point, referred to as Initial Worms (IW) throughout this thesis.

Two Pentair XR3 cultivation systems (Aquatic habitats, Pentair plc, USA) were used (figure 2) – recirculating rigs consisting of 20 tanks (16L) each which enabled monitoring of the abiotic factors temperature, salinity, oxygen saturation and pH. Each tank was filled with ~10cm of sediment ("sandkassesand" (sandbox sand) from Coop Obs Bygg and Byggern) and pre-heated seawater from Trondheimsfjorden with a minimum of 100% water exchange per day. Table 1 describes the set values of temperature [°C], dissolved oxygen [%], salinity [ppt] and pH, measured every third day by YSI ProDSS Multiparameter Water Quality Meter (YSI Incorporated, USA) throughout the feeding period.

***Table 1**: Set values of abiotic parameters during the feeding period.*

Parameter	Unit	Set value	Reference
Temperature	ºC	16.5	(Sandmann, 2019)
pH		7.85	(Wang et al., 2019b)
Salinity	ppt	35	(Wang et al., 2019b)
Dissolved Oxygen	%	100	(Wang et al., 2019b)
Light regime	Light:dark	16:8	(Olive, 1999; Wang et al., 2019b)

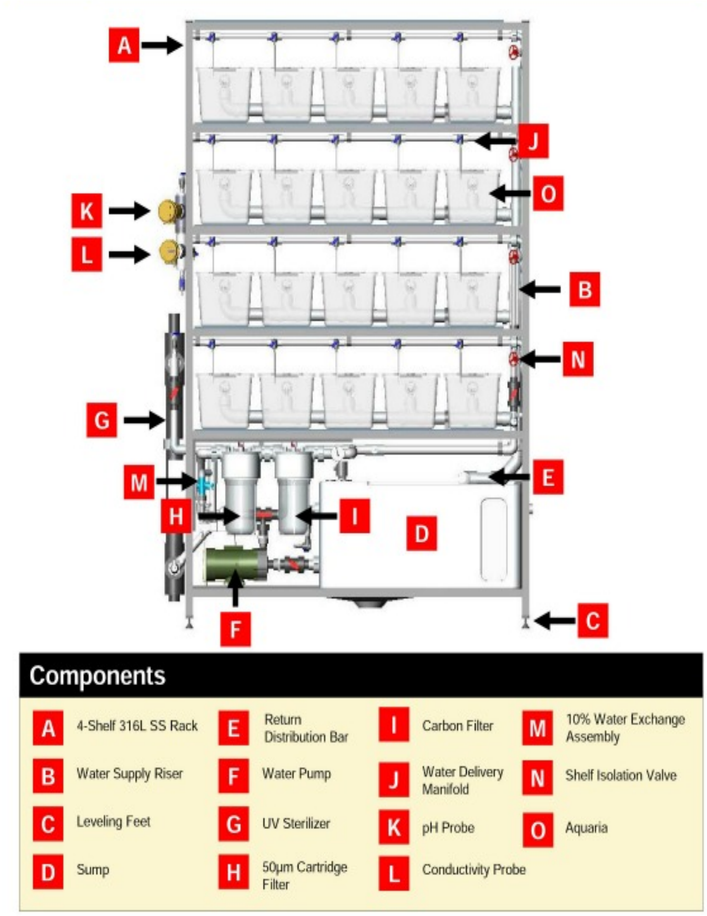
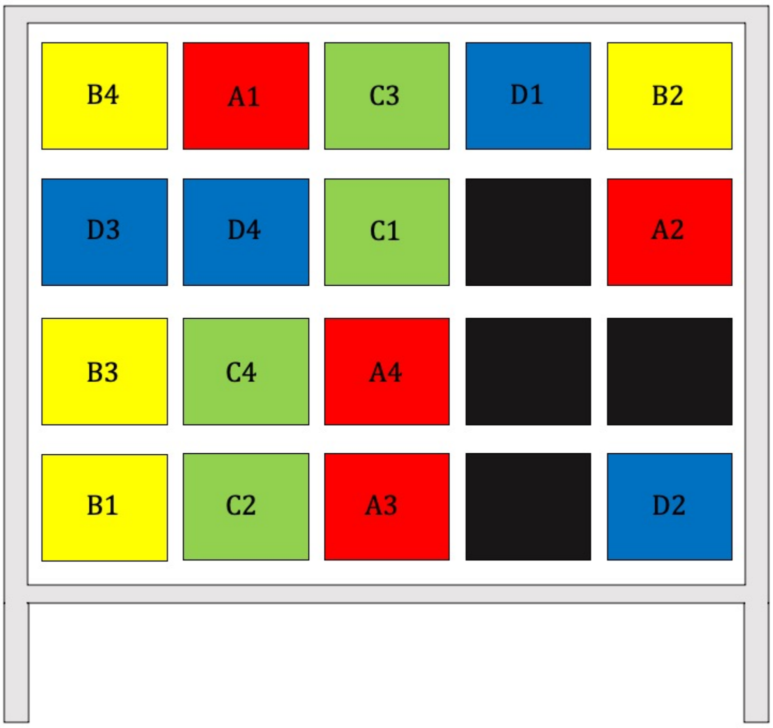


Figure 2: *Illustration of one of the two Pentair XR3 cultivation systems used in the 30-day feeding experiment. Source: mbki.com (MBK Installations Ltd, 2020)*

2.1.3 Feeding regime

For each diet of smolt (S) or post-smolt (PS) sludge, there were four different feeding levels of increasing amounts of sludge fed to the polychaetes. These feeding levels were based on nitrogen content of the diet (%N) of the total nitrogen content in the polychaetes that were fed every day. Feeding level A received a nitrogen level equivalent to 5% of the polychaetes' total nitrogen content on a daily basis, B 10%, C 20% and D 40%. There were four replicate tanks for each feeding level in the cultivation rigs.

Two diets of sludge each comprising four different feeding levels with four replicate tanks of each feeding level amounted to a total of 32 tanks placed in two separate Pentair rigs: one for each diet (figure 3). The placement of each replicate in the Pentair rigs was randomized (=RAND() formula in Microsoft Excel version 16.48) to minimize potential differences in abiotic factors between feeding levels.



B4	A1	C3	D1	B2
D3	D4	C1	⬛	A2
B3	C4	A4	⬛	⬛
B1	C2	A3	⬛	D2

Figure 3*: Placement of tanks in the Pentair XR3 cultivation system. Feeding levels (A-D) colour coded with a total of four replicates (1-4) of each feeding level (empty tanks in the rigs marked as black). Placement in rig the same for both smolt and post-smolt diets, randomized by =RAND() command in Microsoft Excel version 16.48. Illustration: Bjørn Kristensen*

Throughout this paper, diets will be referred to as S (smolt) or PS (post-smolt) depending on the type of sludge administered, and feeding levels as A, B, C or D depending on the %N fed. Feeding level replicates are numerated 1-4.

The amount of feed (sludge) administered to tanks [g day^{-1}] was calculated based on the wet weight of the polychaetes in the tanks at the start of the feeding period. Feeding amounts for each tank were calculated by Equation 1.

$$\text{Amount fed} = \frac{(\mathrm{WW_p} * \mathrm{DM_p} * \mathrm{N_p}) * \mathrm{FL}}{\mathrm{N_s} * \mathrm{DM_s}} \qquad \text{(Equation 1)}$$

With *Amount fed* = amount of sludge given to tank [g day^{-1}]
WW_p = pooled wet weight of all polychaetes in the tank [g]
DM_p = dry matter content of of the worms [%]
N_p = nitrogen content of polychaetes dry matter [%]
FL = feeding level assigned to tank [% N]
N_s = nitrogen content of dry matter of the sludge [%]
$\mathrm{DM_s}$ = dry matter content of the sludge [%]

Proximate dry matter (20%) and nitrogen (9.5%) content in *H. diversicolor* and nitrogen content in sludge (3.95%) used for calculation of feeding amounts were obtained from previous studies by Wang et al. (2019). Initial wet weights of polychaetes [g] in each tank were measured as described in section 2.1.4, and the dry matter content of both S and PS sludge was determined by weighing out approximately 5 grams of sludge (Mettler Toledo, XA204DR) with an accuracy of four decimals, drying them in a oven at 60°C (Termaks) for two days before measuring the weight again.

2.1.4 Feeding period

The feeding period lasted for 30 days, individuals of *H. diversicolor* were separated into different tanks of two Pentair XR3 cultivation systems and fed the different feeding levels of either S or PS sludge diets.

Before the start of the feeding period, polychaetes were moved from the holding tanks and placed into seawater for one hour to empty their guts (Seekamp, 2017). Afterwards, individuals were randomly picked, and any adherent water removed; wet weights at the start of the experiment was recorded in grams with two decimals (VWR, Avantor®, 611-2602) before the polychaetes were assigned to a tank in the experimental rig. A total of 15 individuals were put into each tank at the beginning of the feeding period.

H. diversicolor were fed according to their assigned diet and feeding level every second day at approximately the same time. Sludge was taken out of the freezer, thawed, and weighed out by an analytical scale (Mettler Toledo, XA204DR) into plastic tubes and administered by emptying the tubes into the tanks and flushing them with tank water to ensure there were no leftovers. Water flow in the system was stopped for one hour after feeding to avoid suspended feed particles from leaving the system.

Once a week, the tanks were drained of water, flushed and refilled using a siphon to clean the tanks. Especially in the tanks receiving higher feeding levels, there were observations of sludge and particulate matter accumulation and biofilm formations on the sediment (picture 2).

Due to bacterial growth, one of the tanks receiving feeding level D of the S diet (replicate D4) was emptied and eliminated after day 11 of the experiment. Individuals found in the tank were recorded, weighed, frozen and the data and biomass was included in the growth experiment and chemical analysis. Black stains were also observed in the sediments, most common in the sediment of tanks receiving high feeding levels, likely to be caused by the presence of hydrogen sulphide (H_2S) (picture 3) (Murphy et al., 1999, He et al., 2017).

***Picture 2**: Sludge accumulation and biofilm formations observed during the feeding experiment, almost exclusively found in tanks receiving high feeding levels, but occasionally also in lower feeding levels. Picture: Bjørn Kristensen*

***Picture 3**: Black stains observed in sediments, more commonly observed in tanks receiving high feeding levels, indicating the presence of hydrogen sulphide (H_2S). Picture: Bjørn Kristensen*

After 30 days, at the end of the experiment, the tanks were emptied of water and the remaining polychaetes were sampled by carefully digging through the sediment, picking out individuals by hand. Polychaetes were placed in buckets of seawater for an hour to empty their guts, before being rinsed in freshwater. Adherent water was removed and wet weights were recorded – polychaetes from the same replicate (tank) were pooled together when weighed (VWR, Avantor®, 611-2602) before individually placed in Eppendorf tubes, flushed with nitrogen, and stored in a -80°C freezer.

2.1.5 Biomass and growth

Pooled wet weights (WW) of *H. diversicolor* were measured in grams with two decimals for each replicate tank before the start of the feeding period and again after the 30 day feeding period using an analytical scale (VWR, Avantor®, ECN 611-2602). Mean biomass gain was calculated for individual polychaetes in the replicate tanks following equation 2:

$$Biomass\ gain = \frac{WW_{final}}{n_{final}} - \frac{WW_{intial}}{n_{initial}} \qquad \text{(Equation 2)}$$

With *Biomass gain* = mean biomass gain of individual polychaetes in the tank [g].
WW_{final} = pooled biomass WW at the end of the feeding period [g]
n_{final} = n worms in tank at the end of the feeding period
$WW_{initial}$ = pooled biomass WW at the beginning of the feeding period [g]
$n_{initial}$ = n worms in tank at the beginning of the feeding period

The average specific growth rate SGR [day^{-1}] of individual polychaetes in each tank was calculated by equation 3 (Jørgensen, 1990):

$$\mathrm{SGR} = \frac{ln(iWW_{final}) - \ln(\mathrm{i}WW_{initial})}{t} \qquad \text{(Equation 3)}$$

With SGR = average specific growth rate of individual polychaetes [day^{-1}]
iWW_{final} = Average individual biomass WW measured at the end of the feeding period [g]
$iWW_{initial}$ = Average individual biomass WW before the beginning of the feeding period [g]
t = duration of the experiment [days]

Average percentage specific growth rate (SGR) [% day^{-1}] of polychaetes in each tank was calculated using equation 4:

$$\%\mathrm{SGR} = 100 * (\exp(SGR) - 1) \qquad \text{(Equation 4)}$$

With *%SGR* = average percentage specific growth rate of individuals in tank [% day^{-1}]
SGR = specific growth rate [day^{-1}]

The correlation between the real % of N fed at each given feeding level and polychaete %SGR was determined by linear regression. The real %N fed was calculated by equation

1, substituting DM and nitrogen content values of polychaetes and nitrogen content of sludge from Wang et al. (2019) with values determined in section 2.2.1. and 2.2.2.

2.1.6 Survival

Survival in each replicate was measured by equation 5 and expressed in percentage. Each replicate had a total of 15 individual polychaetes in the tank at the beginning of the feeding period, and the number of polychaetes surviving the 30-day period were determined as the number of individuals recovered from the sediments at the end of the experiment. Only whole worms that were found when digging through the substrate by hand were accounted for.

$$Survival = \frac{n_{final}}{n_{initial}} * 100\% \qquad \text{(Equation 5)}$$

With $Survival$ = percentage survival of worms placed in tank [%]
$n_{initial}$ = n worms in tank the beginning of the feeding period
n_{final} = n worms in tank at the end of the feeding period

2.2 Chemical analysis

2.2.1. Overview of analyses conducted

The dry matter (DM) content of IW, polychaetes fed on S and PS diets and the two sludge types were determined, and the DM samples were used for later analysis performed in this thesis.

To determine the bioremediation efficiency of *H. diversicolor*, analysis of carbon (C), nitrogen (N) and phosphorus (P) content of the diets and the polychaetes were done. The nutrient conversion budget was calculated as the percentage of the C, N and P fed to the polychaete that were taken up and converted into new polychaete biomass.

The ash content was analysed to determine the total organic matter composition of the samples. Analysis of total carbohydrate content was attempted, but the results from this analysis were discarded due to experimental errors.

2.2.2 Dry matter

Polychaetes were freeze-dried (Labconco, Freeezone) individually before pooling all polychaetes originating from the same replicate tank and weighing the pooled dry weight (Mettler Toledo, XA204DR) in gram with four decimals. Dry matter content (DM) is given in mg gWW^{-1} (equation 6).

$$\text{DM content}_{pooled} = \left(\frac{DW_{pooled}}{WW_{pooled}}\right) * 1000 \qquad \text{(Equation 6)}$$

With $DM\ content_{pooled}$ = Dry matter per gram wet weight [mg gWW^{-1}]
WW_{pooled} = Pooled wet weight of all individuals from the same replicate [g]
DW_{pooled} = Pooled dry weight of all individuals from the same replicate [g]

Pooling of individuals from the same tank was done in order to have enough DM sample to test for biochemical composition. After weighing, the biomass from each replicate was mortared by use of a mortar and pestle to a powder-like substance to homogenize the sample, although some chitin pieces originating from the exoskeleton of polychaetes were impossible to grind completely (picture 4).

Picture 4*: Homogenized powder of dry matter content of all polychaetes from the same replicate (tank) in the feeding trial. Visible fragments likely to be chitin from exoskeleton. Photo: Bjørn Kristensen*

2.2.3. Carbon and Nitrogen

Carbon (C) and Nitrogen (N) content was determined by use of gas chromatography for polychaetes fed different feeding levels of S and PS diets, IW and the two sludge types.

Approximately 500 µg (1000 µg for sludge) of freeze-dried ground samples were weighed (Mettler Toledo UMT2) and put in tin cups which were sealed and stored in a freezer before moved to a desiccator 24h prior to the analysis. The analysis was conducted by NTNU Department of Biology (Elin Bjørndal Njåstad) using a Vario El cube elemental analyser (Elementar). Tin capsules containing samples reacted with oxygen before combustion at 1800 ºC, converting the samples into measurable gaseous components and the elemental peaks compared to a known standard of Acetanilid (Elementar, 2019). C and N content converted to and expressed in mg gDM^{-1}.

2.2.4. Phosphorus

Phosphorus determination of dry matter samples was done by photospectrometral methods based on Koroleff (1976). Dry matter samples (~500 µg) were weighed into plastic vials (Mettler Toledo UMT2) and the following reagents were added: distilled water (10 ml), sulfuric acid (0.1 ml) and potassium persulfate (2 ml). Samples were diluted with distilled water (1:3 polychaete samples, 1:10 sludge samples) before being autoclaved for 30 minutes at 1.1 bar, 120°C and phosphorous content measured by the NS-EN ISO 6878 method using an autoanalyzer photospectrometer (Flow Solution IV, O.I Analytical). Phosphorus content expressed as mg gDM^{-1}.

2.2.5. Elemental ratio

The elemental ratios C:N, C:P and N:P in the DM of IW, polychaetes fed S or PS sludge and the two sludge types were calculated by equation 6 based on the C, N and P contents previously measured.

$$Elemental\ ratio = \frac{element\ X\ [mg\ gDM^{-1}]}{element\ Y\ [mg\ gDM^{-1}]} \qquad \text{(Equation 6)}$$

With *Elemental ratio* = the elemental ratio of the DM content of the sample
Element X = the given element in concern (C, N or P) [mg gDM^{-1}]
Element Y = the given element in concern (C, N or P) [mg gDM^{-1}]

2.2.5. Total organic matter

Ash content was determined by weighing (Mettler Toledo ME 104) samples into glass vials, placing them in a muffle furnace at 500°C for six hours and weighing them again to measure the amount of ash left after combustion. Total organic matter (TOM) represents the amount of DM lost during the combustion, expressed in mg gDM^{-1} (equation 7).

$$TOM = 1000 - \frac{W_{ash}}{W_{DM}} \qquad \text{(Equation 7)}$$

With TOM = total organic matter content of dry matter [mg gDM^{-1}]
W_{ash} = weight of sample after combustion [mg]
W_{DM} = weight of dry matter sample before combustion [g]

One sample from each replicate of *H. diversicolor* fed different feeding levels of S and PS sludge and IW was analysed. No analysis replicates were done due to limited amount of sample needed to achieve measurable amounts of ash content left after combustion. Three samples were taken from the two feeding sludge types to analyse its TOM content.

2.2.7. Conversion of C, N and P

The percentage of C, N and P fed *H. diversicolor* through sludge that was taken up by the polychaetes and converted into new C, N and P in the biomass by growth was calculated and expressed in percentage conversion of the feed. Calculations were based on the content of C, N and P in polychaetes before the start of the feeding period (IW), after the feeding period and of the diets offered (S and PS sludge) during the feeding period. To account for mortality in replicates, the conversion of C, N and P were calculated for the mean individual in each tank following equation 8.

$$Conversion = \frac{Contents_{after} - Contents_{before}}{Contents_{fed}} * 100\% \qquad \text{(Equation 8)}$$

With $Conversion$ = Mean percentage of C, N or P fed to individual *H. diversicolor* taken up and converted into new C, N and P in biomass [%]
$Contents_{after}$ = contents of C, N or P [mg] of the WW in the average individual of each replicate at the end of the feeding period.
$Contents_{before}$ = contents of C, N or P [mg] of the WW in the average individual of each replicate before the start of the feeding period, based on mean wet weight of polychaetes in the tank and elemental content of IW.
$Contents_{fed}$ = contents of C, N or P in the diets fed to the mean individual of each replicate [mg].

2.3. Statistics

Calculations of growth data (biomass gain, feeding amounts, %SGR, survival), chemical composition (DM content, TOM content, C, N and P content and ratio) as well as nutrient gain and conversion were all calculated with use of Microsoft Excel version 16.48. For statistical analysis, IBM SPSS Statistics Version 27 was used when investigating significant differences between feeding levels, diets and sludge types, as well as correlation between feeding levels and %SGR.

When investigating significant differences between polychaetes fed different feeding levels of the same diet, between similar feeding levels of different diets and comparing feeding levels with IW, the replicate basis used was the individual tanks of each feeding level. Each feeding level was fed to a total of four tanks containing polychaetes, with growth and chemical analysis done on a pooled sample of the worms from each tank, resulting in each feeding level having a $n = 4$. 95% confidence intervals and a significance level of $p < 0.05$ were used for all statistical analysis conducted.

Significant differences were investigated between *H. diversicolor* receiving different feeding levels of the S diet, and between feeding levels of the PS diet. Polychaetes from feeding levels of both diets was also compared to IW. This was done on calculations of growth (%SGR, survival) and of chemical analysis (DM, C, N, P , elemental ratios and gain, TOM and conversion efficiency). When the assumptions of normally distributed data, homogeneity of variance and no outliers were met, one-way ANOVA was used to determine if there were any significant differences between feeding levels of the same diet and between feeding levels and IW. Shapiro-Wilk's test of normality was used to determine if groups were normally distributed, Levene's test was performed to determine the homogeneity of variance and potential outliers were detected by visual inspection of boxplots. If there were significant differences between populations, a Tukey Post-Hoc test was used to determine where the difference laid. When the assumption of homogeneity of variance was violated, Welch's ANOVA was used to investigate significant differences, and Games-Howell Post Hoc test to determine between which feeding levels and/or IW the difference was significant. When dealing with non-normally distributed data, the non-parametric Kruskal-Wallis H test was used to determine differences between medians, when the distributions were equally shaped assessed by visual inspection of boxplots.

Independent sample t-test was used to identify significant difference of growth data and chemical composition between polychaetes fed on similar feeding levels but of different diets when assumptions were met. Normality of data, homogeneity of variance and outliers were checked by Shapiro-Wilk's test, Levene's test and visual inspection of boxplot, respectively. If the data was non-normally distributed, Mann-Whitney U test was used to compare mean ranks or medians, dependent on the shape of distributions.

Significant differences in initial and final WW of polychaetes within each feeding level was determined using dependent sample t-test when assumptions of normality and no outliers were met.

Significant differences in chemical content between the mean of the two sludge types administered was investigated using independent t-tests when assumptions were met (n=4). When dealing with non-normality of data, Mann-Whitney U test was used to compare the medians or mean ranks of both sludge types.

2.4. Cooperation

The cultivation experiment, along with the collection of wild individuals of *H.diversicolor* and sludge were done in cooperation with Thomas Hagby Dahl, a fellow MSc student at the Ocean Resources study program at NTNU, and co-supervisor Inka Anglade, PhD candidate at NTNU Department of Biology.

Determination of carbohydrate contents in both *H.diversicolor* and aquaculture sludge were attempted for use in the thesis of Thomas Hagby Dahl, and the method shown in Appendix 2, but the results were discarded and not used due to methodological uncertainties and non-coherent data.

3. Results

3.1. Cultivation period

3.1.1. Biomass growth

Mean individual biomass of polychaetes measured as wet weight (WW) in grams before the start and at the end of the feeding period for *H. diversicolor* fed different feeding levels of either smolt (S) or post-smolt (PS) sludge diets are shown in figure 4 and 5, respectively. The initial average WW of individuals used in this experiment was 0.25±0.05g and did not differ significantly between any of the feeding levels or diets (one-way ANOVA).

Mean individual polychaetes in all feeding levels of the S diet, except A, recorded an increase in WW during the feeding period, although the only significant difference between initial and final WW were found in feeding level C (paired-sample t-test). For polychaetes fed on the PS diet, all feeding levels recorded a mean increase in individual WW, with significant changes found in feeding level B, C and D (paired-sample t-test). Growth measurements for each tank replicate in the cultivation experiment is shown in Appendix 2, including pooled and mean individual WW.

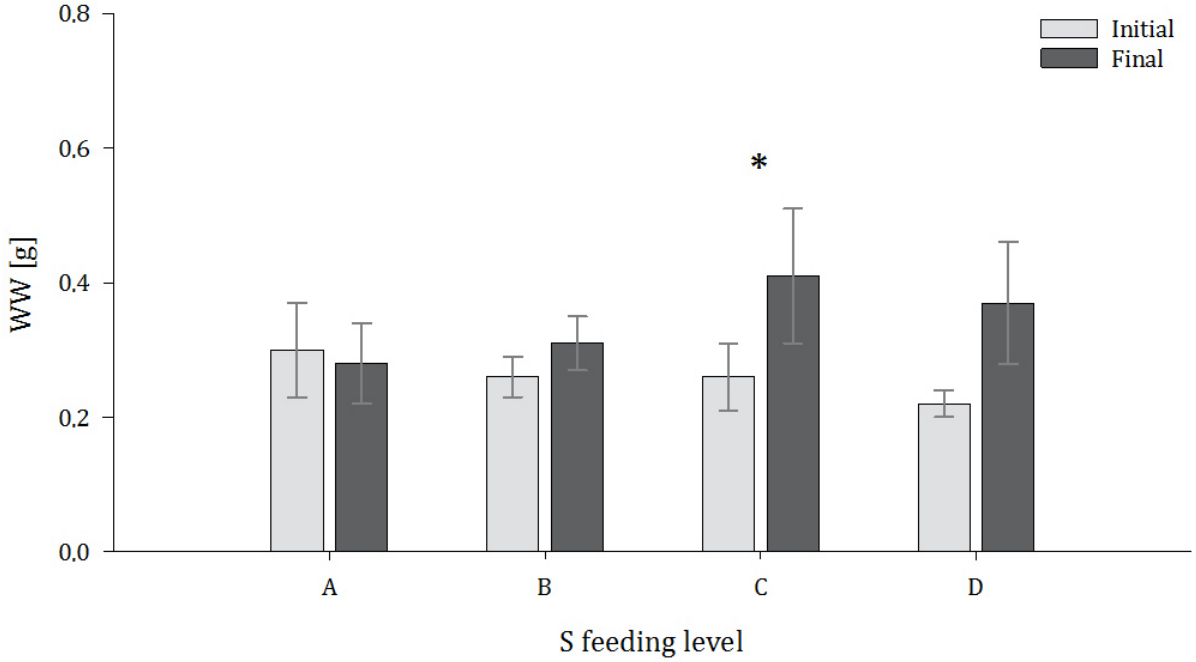


Figure 4: *Mean±SD of initial and final average individual WW [g] of polychaetes in 4 replicate tanks of feeding level A (5%N), B (10%N), C (20%N) and D (40%N) of the S diet. Significant differences between mean initial and final WW in each feeding level expressed as "*".*

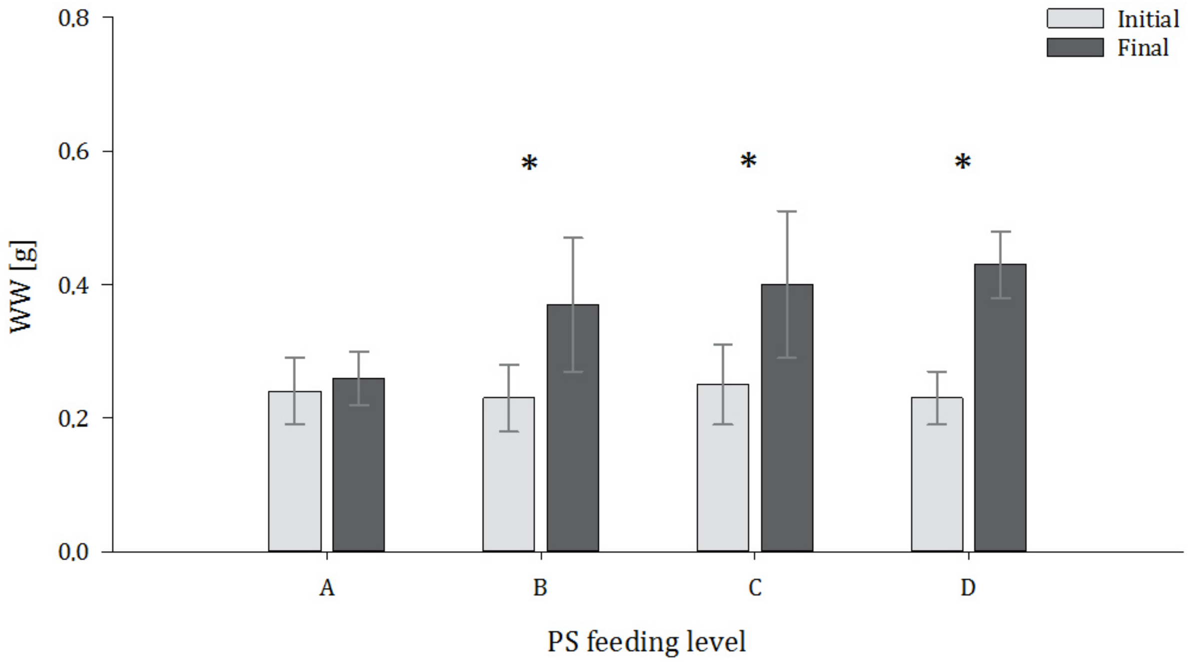


Figure 5: *Mean±SD of initial and final average individual WW [g] of polychaetes in 4 replicate tanks of feeding level A (5%N), B (10%N), C (20%N) and D (40%N) of the PS diet. Significant differences between mean initial and final WW in each feeding level expressed as "*".*

3.1.2 Percentage specific growth rate (%SGR)

During the 30-day feeding period, mean individual %SGR [% day^{-1}] of *H. diversicolor* in each feeding level was found to vary between -0.14-2.17 when fed on S diets, and 0.22-2.10 on PS diets, and the %SGR (mean±SD) of polychaetes in feeding levels of both diets is shown in figure 6. The only polychaetes to report a negative mean %SGR were in feeding level S-A (-0.14±0.36), while for both diets the highest mean were registered in feeding level D (2.17±1.34 and 2.10±0.91 fed S and PS sludge, respectively). When comparing mean %SGR between polychaetes in increasing feeding levels of the same diet, the only significant difference found was that in feeding level C the growth rate was significantly higher than A when fed on the S diet, while feeding level D where significantly higher than A when fed the PS diet (one-way ANOVA). By comparing equal feeding levels fed on different diets, %SGR of polychaetes were not found to be significantly different dependent on the sludge diet they received (independent-samples t-test).

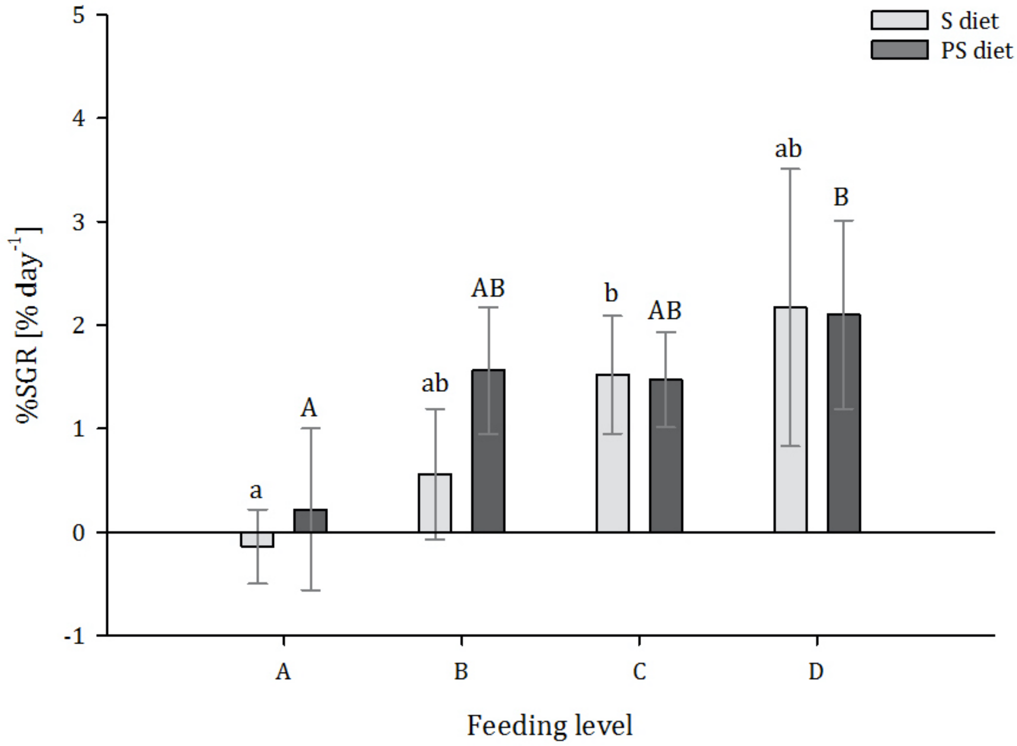


Figure 6: *Mean+SD of %SGR [% day^{-1}] in average individual polychaetes in 4 tank replicates of feeding level A (5%N), B (10%N), C (20%N) and D (40%N) of S and PS diets. Unequal superscripts between feeding levels of the same diet indicate significant difference.*

3.1.3 Linear regression of %SGR and real %N fed to the polychaetes

Figure 7 shows the linear relationship established between the actual amount of feed administered during the feeding period [%N day^{-1}] and the mean individual %SGR of *H.diversicolor.* Real %N supplied to each feeding level was calculated based on amounts [mg] of feed administered and chemical analysis of sludge and polychaetes after the end of the feeding period (section 3.1.5) and shows the actual percentage of N supplied to the tanks at each feeding level during the feeding period, which differed slightly from the amounts calculated before the start of the experiment.

By linear regression there was found a positive linear correlation between amounts of feed [%N] supplied at each feeding level and %SGR of polychaetes, with elevated growth rates as the feeding intensity increased and real % N fed explaining 52% of the variation in %SGR of polychaetes fed S sludge, and 32% when fed on PS sludge. Feeding levels were found to significantly predict the %SGR of polychaetes (one-way ANOVA), and by comparing the slopes of the regression lines for S and PS diets, no significant difference was found in the correlation of feeding amounts and growth rates between the two diets (hypothesis test).

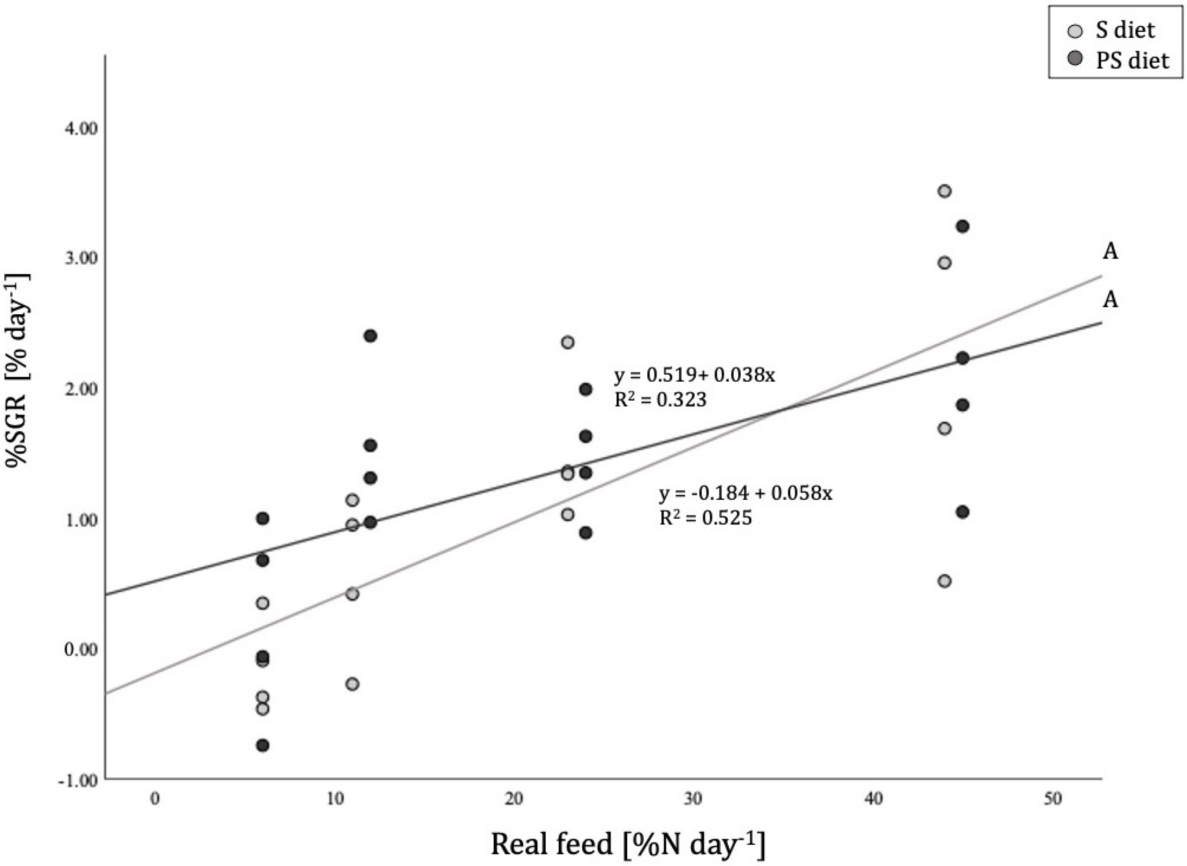


Figure 7: *Linear regression between the amount of feed supplied [%N day^{-1}] and mean individual %SGR [% day^{-1}] of polychaetes in the 4 replicate tanks of each feeding level receiving S or PS sludge and their regression equation and R^2-values. Equal superscripts indicate no significant difference between the slope of the regression line for S and PS.*

3.1.4. Survival

Mean survival rates [%] of polychaetes in each feeding level of both diets are shown in figure 8. With two tank replicates recording survival rates of 100% (D1 fed S and A2 fed PS) and the lowest rate recorded being 53% (D3 fed PS), the mean survival ranged between 80-90% for feeding levels across both diets, and the mortality was low for polychaetes fed on both S and PS sludge. By comparing survival rates in feeding levels of the same diet, there were found no significant difference in survival dependent on the amount of sludge they received (one-way ANOVA), or whether they were fed S or PS sludge (independent samples t-test).

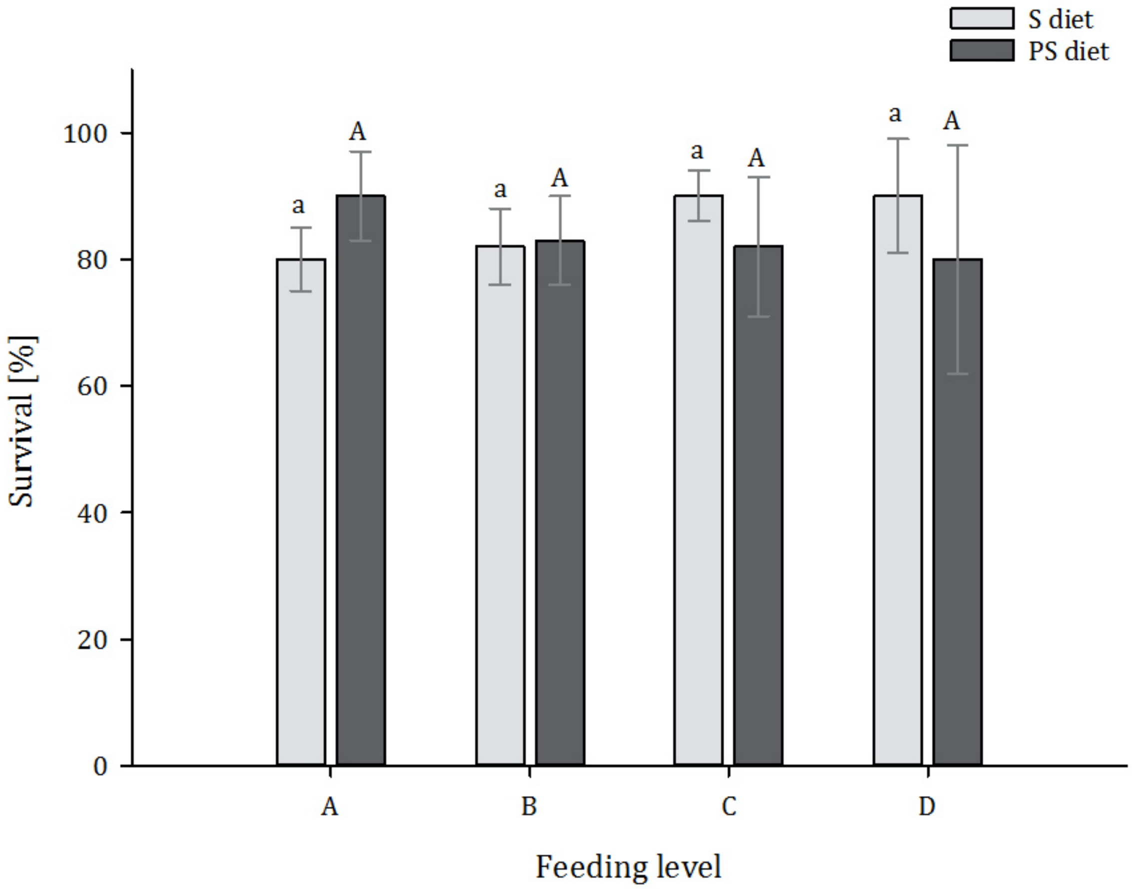


***Figure 8**: Survival [%] of H. diversicolor (Mean±SD of 4 replicate tanks) in feeding level A (5%N), B (10%N), C (20%N) and D(40%N) of S and PS diets, respectively. Unequal superscripts between feeding levels of the same diet indicate significant difference.*

3.1.5. Feeding amounts

Table 2 shows the amount of sludge fed to each tank, calculated by equation 1 and based on the amount of nitrogen administered to the polychaetes in each feeding level. Calculated amounts of sludge needed to supply nitrogen corresponding to feeding level requirements done prior to the experiment, along with the administered amounts (WW) of sludge and the actual %N fed during the experiment is shown. After analysing the DM and N content of polychaetes and the two sludge types (section 3.2.1 and 3.2.3, respectively), the actual amount of nitrogen [% N] fed to each tank was determined, and all feeding levels of both S and PS diets received 1-5 percentage higher %N from the feed than what was originally calculated.

***Table 2**: The actual amount of sludge fed [g] at each feeding point and in total during the experiment for each tank (feeding level replicate), based on calculated amounts needed to supply the polychaetes with the given amount of nitrogen content (%N) according to their feeding level. Differences in calculated amounts needed and what was actually fed is shown, along with the difference between calculated %N and real %N.*

		Calculated			Actual			Difference		
Diet	**Feeding level replicate**	**%N fed [Day^{-1}]**	**Amount fed [g feedingpoint^{-1}]**	**Total amount fed [g]**	**%N fed [day^{-1}]**	**Amount fed [g feedingpoint^{-1}]**	**Total amount fed [g]**	**%N fed [day^{-1}]**	**Amount fed [g feedingpoint^{-1}]**	**Total amount fed [g]**
Smolt	A1	5 %	1.237	18.554	6 %	1.230 ± 0.030	18.452	1 %	-0.007 ± 0.030	-0.102
Smolt	A2	5 %	1.150	17.257	6 %	1.152 ± 0.001	17.284	1 %	0.002 ± 0.001	0.027
Smolt	A3	5 %	1.050	15.743	6 %	1.048 ± 0.002	15.722	1 %	-0.001 ± 0.002	-0.021
Smolt	A4	5 %	1.718	25.777	6 %	1.717 ± 0.002	25.759	1 %	-0.001 ± 0.002	-0.018
Smolt	B1	10 %	2.111	31.659	11 %	2.111 ± 0.002	31.672	1 %	0.001 ± 0.002	0.013
Smolt	B2	10 %	2.462	36.936	11 %	2.463 ± 0.001	36.947	1 %	0.001 ± 0.002	0.012
Smolt	B3	10 %	1.961	29.410	11 %	1.962 ± 0.002	29.428	1 %	0.001 ± 0.002	0.018
Smolt	B4	10 %	2.382	35.725	11 %	2.382 ± 0.001	35.737	1 %	0.001 ± 0.001	0.012
Smolt	C1	20 %	4.221	63.318	23 %	4.222 ± 0.002	63.329	3 %	0.001 ± 0.002	0.010
Smolt	C2	20 %	3.414	51.208	23 %	3.414 ± 0.001	51.207	3 %	0.000 ± 0.001	-0.001
Smolt	C3	20 %	4.867	73.006	23 %	4.867 ± 0.002	73.004	3 %	0.000 ± 0.002	-0.002
Smolt	C4	20 %	5.259	78.888	23 %	5.258 ± 0.001	78.869	3 %	-0.001 ± 0.001	-0.019
Smolt	D1	40 %	6.943	104.146	44 %	6.944 ± 0.002	104.153	4 %	0.000 ± 0.002	0.006
Smolt	D2	40 %	8.373	125.598	44 %	8.373 ± 0.002	125.601	4 %	0.000 ± 0.002	0.002
Smolt	D3	40 %	7.981	119.716	44 %	7.983 ± 0.003	119.743	4 %	0.002 ± 0.003	0.026
Smolt	D4	40 %	7.658	45.949	44 %	7.657 ± 0.002	45.942	4 %	-0.001 ± 0.002	-0.007
Post-smolt	A1	5 %	0.570	8.548	6 %	0.568 ± 0.002	8.527	1 %	-0.001 ± 0.002	-0.021
Post-smolt	A2	5 %	0.631	9.465	6 %	0.634 ± 0.003	9.503	1 %	0.003 ± 0.003	0.038
Post-smolt	A3	5 %	0.759	11.388	6 %	0.759 ± 0.002	11.384	1 %	0.000 ± 0.002	-0.004
Post-smolt	A4	5 %	0.921	13.813	6 %	0.922 ± 0.003	13.829	1 %	0.001 ± 0.003	0.016
Post-smolt	B1	10 %	0.970	14.553	12 %	0.972 ± 0.002	14.579	2 %	0.002 ± 0.002	0.027
Post-smolt	B2	10 %	1.439	21.592	12 %	1.439 ± 0.003	21.581	2 %	-0.001 ± 0.003	-0.011
Post-smolt	B3	10 %	1.447	21.711	12 %	1.447 ± 0.002	21.699	2 %	-0.001 ± 0.002	-0.012
Post-smolt	B4	10 %	1.668	25.024	12 %	1.667 ± 0.003	25.008	2 %	-0.001 ± 0.003	-0.016
Post-smolt	C1	20 %	2.343	35.139	24 %	2.345 ± 0.002	35.171	4 %	0.002 ± 0.002	0.031
Post-smolt	C2	20 %	2.721	40.819	24 %	2.724 ± 0.002	40.857	4 %	0.003 ± 0.002	0.038
Post-smolt	C3	20 %	2.895	43.422	24 %	2.895 ± 0.003	43.422	4 %	0.000 ± 0.003	0.001
Post-smolt	C4	20 %	4.070	61.050	24 %	4.072 ± 0.002	61.078	4 %	0.002 ± 0.002	0.027
Post-smolt	D1	40 %	4.512	67.676	45 %	4.513 ± 0.001	67.701	5 %	0.002 ± 0.001	0.025
Post-smolt	D2	40 %	5.269	79.034	45 %	5.268 ± 0.002	79.017	5 %	-0.001 ± 0.002	-0.017
Post-smolt	D3	40 %	5.553	83.294	45 %	5.553 ± 0.002	83.301	5 %	0.000 ± 0.002	0.007
Post-smolt	D4	40 %	6.705	100.568	45 %	6.706 ± 0.003	100.584	5 %	0.001 ± 0.003	0.017

3.1.6. Abiotic factors

Temperature [°C], pH, salinity [ppt] and dissolved oxygen [%] were measured three times a week and mean±SD for all tanks at each measuring point in the rigs receiving either S or PS diets are shown in figure 9. Except for temperatures fluctuating between ~15.5-16.5 °C the first couple of measuring days and dissolved oxygen (DO) levels dropping to ~95 % towards the end of the feeding period, there were not observed large variations or deviations of set values between each measuring day, or between the two cultivation rigs.

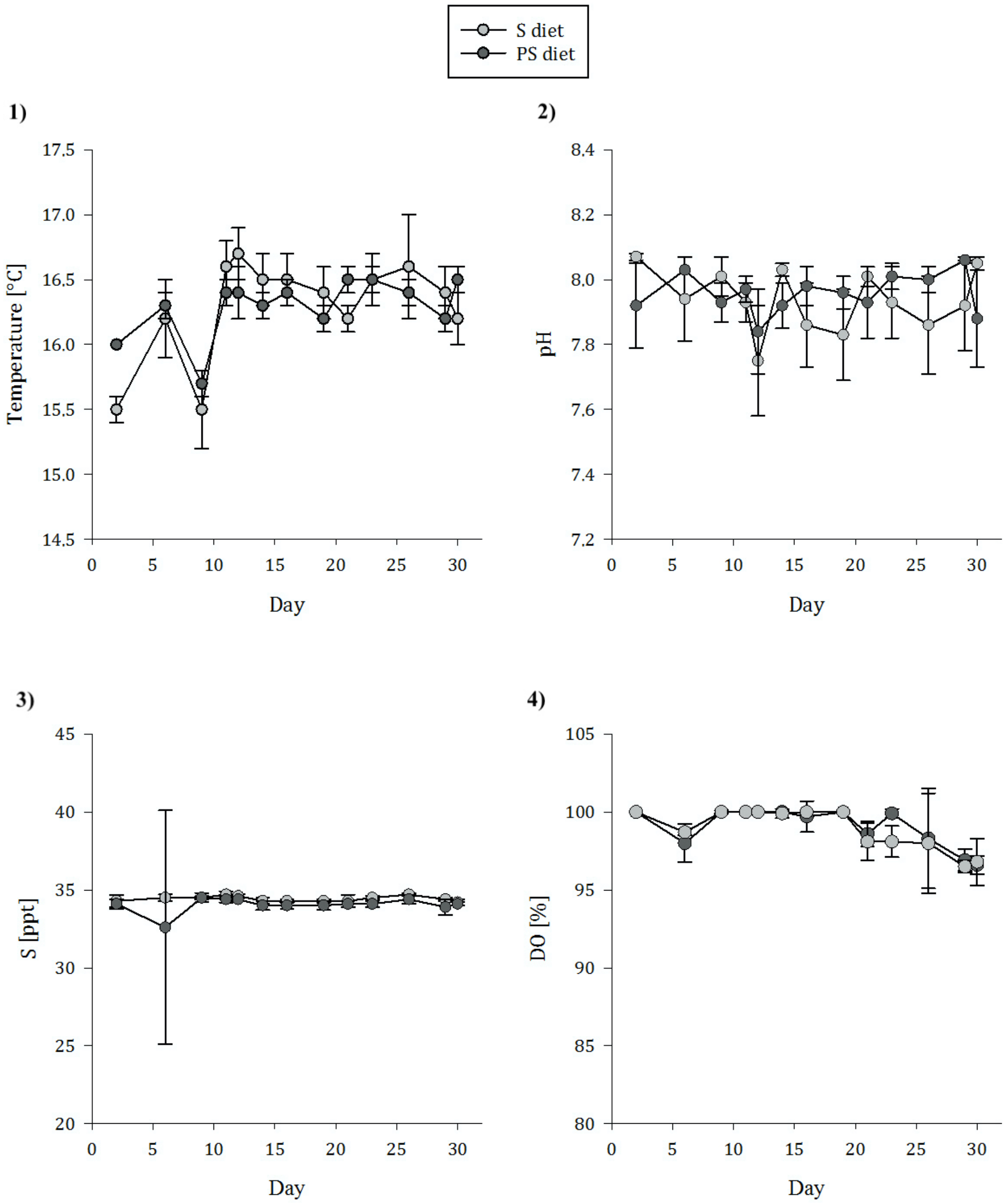


Figure 9: *The measured abiotic parameters 1) temperature [°C], 2) pH, 3) salinity [ppt] and 4) DO [%] in all tanks receiving either S or PS diets, respectively. Values expressed as mean±SD of all cultivation tanks in the given rig at measuring days.*

3.2 Chemical contents

3.2.1. Dry matter

Dry matter content (mean±SD) of IW, polychaetes from each feeding level of both diets and of the two sludge types are shown in table 3. No significant differences were found in DM content [mg gWW^{-1}] between polychaetes in feeding levels of the same diet, with mean DM contents ranging between 198-223 and 185-207 in feeding levels of S and PS sludge, respectively (one-way ANOVA and welch ANOVA). However, worms from feeding level D receiving S sludge were found to have a significantly higher DM content than IW (186) (Welch ANOVA). DM content did not seem to be significantly affected by the type of sludge administered to polychaetes (independent samples t-test). A significantly higher DM content was found in the sludge from the PS facility (216±2) compared to the S sludge (147±4) (independent sample t-test).

Table 3*: Mean±SD dry matter content (DM) of IW (n = 15), H. diversicolor in 4 replicate tanks of feeding level A (5%N), B (10%N), C (20%N) and D (40%N) of smolt (S) or post-smolt (PS) diets and the two sludge types (n = 6). Unequal superscripts within the same diet indicate significant difference between feeding levels, unequal numbers between sludge types.*

		DM content [mg gWW^{-1}]
IW		186^{aA} ±20
S	A	198^{ab} ±21
	B	200^{ab} ±21
	C	210^{ab} ±3
	D	223^{b} ±11
PS	A	185^{A} ±14
	B	179^{A} ±10
	C	205^{A} ±31
	D	207^{A} ±14
Sludge	S	147^{1} ±4
	PS	216^{2} ±2

3.2.2 Carbon content

Mean C content [mg gDM^{-1}] of polychaetes in IW and the four tank replicates of each feeding level receiving S sludge diet are shown in figure 10, and median C content in feeding levels of PS sludge in figure 11 (due to non-normality of data). No significant differences were found in the C content of polychaetes between any of the feeding levels fed S diets or when comparing them to IW (one-way ANOVA), with means ranging from 411.5-426.8. For polychaetes fed on PS diets, there were recorded increasing median values of C content with increasing feeding levels, ranging from 417.3-458.2, and with feeding level D being significantly higher than both IW and feeding level A (Kruskal-Wallis H test).

There were not observed any significant differences in polychaete C content dependent on the diet the polychaetes were fed, and due to non-normality of values in feeding level A fed PS sludge, table 4 shows the tests used to identify potential differences between means of similar feeding levels fed on different diets.

C content of the two sludge types were also analysed and means of S and PS sludge are shown in figure 12, with S sludge (424.0±3.5) being found to contain significantly higher mg C gDM^{-1} than PS sludge (363.0±2.9) (independent samples t-test).

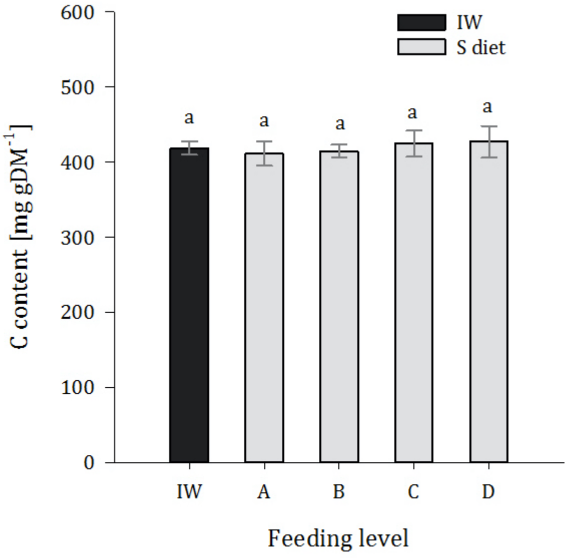


Figure 10: *Mean±SD carbon (C) content [mg g DM^{-1}] of IW (n=4) and polychaetes in the 4 tank replicates of feeding level A (5%N), B (10%N), C (20%N) and D (40%N) fed S diets. Equal superscripts indicate no significant difference between feeding levels or IW.*

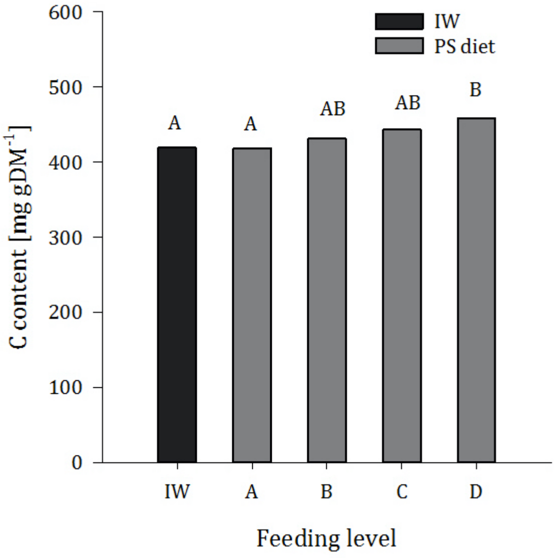


Figure 11: *Median carbon (C) content [mg g DM^{-1}] of IW (n=4) and polychaetes in the 4 tank replicates of feeding level A (5%N), B (10%N), C (20%N) and D (40%N) fed PS diets. Unequal superscripts indicate significant difference between feeding levels or IW.*

***Table 4**: Testing for significant difference between carbon (C) content [mg gDM^{-1}] of polychaetes fed similar feeding levels of S and PS diets (n = 4). Statistical test used, its parameters and values for both diets (S and PS) included, along with the result of the test (Sig. Difference).*

Feeding level	Test	Test parameter	S	PS	Sig. difference
A	Mann-Whitney U test	Mean rank	4.75	4.25	No
B	Independent samples t-test	Mean	414.5±8.6	425.3±19.8	No
C	Independent samples t-test	Mean	424.5±17.2	445.3±12.0	No
D	Independent samples t-test	Mean	426.8±21.1	458.1±6.1	No

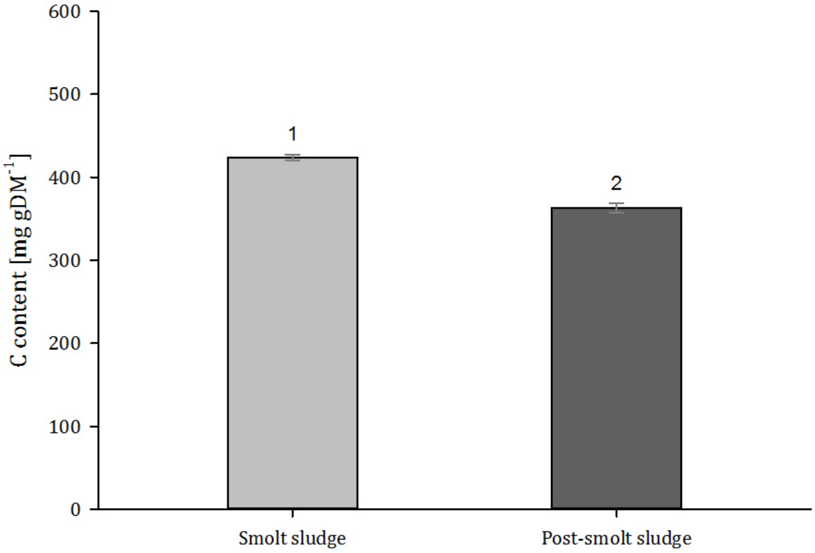


***Figure 12**: Mean±SD (n=4) carbon (C) content [mg gDM^{-1}] of the two sludge types fed to polychaetes. Unequal superscripts indicate significant difference in mean content.*

3.2.3. Nitrogen content

Mean N content [mg gDM^{-1}] of *H. diversicolor* fed different feeding levels of both S and PS sludge and IW is shown in figure 13. When fed on S diets, mean N content of polychaetes in different feeding levels ranged between 91.7-84.1, with feeding level C and D reporting significantly lower N content than feeding level A and IW (one-way ANOVA). There were not observed any significant difference in polychaete N content between feeding levels of PS sludge (one-way ANOVA), ranging between 90.9-85.8. Neither was there found any difference in N content dependent on the polychaetes receiving S or PS sludge diets (independent-samples t-test).

When comparing the two sludge types (figure 14), the N content of S sludge (48.6±1.2) were found to be significantly higher than PS sludge (46.1±0.7), although the difference was small (independent samples T-test)

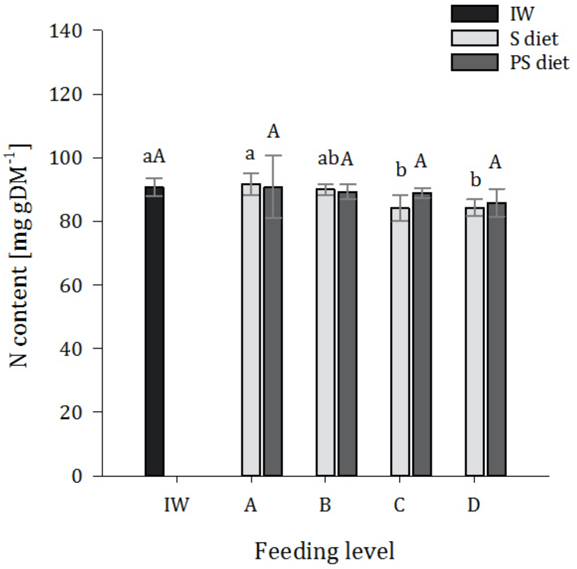


Figure 13*: Mean±SD nitrogen (N) content [mg gDM^{-1}] of polychaetes in 4 replicate tanks of feeding level A (5%N), B (10%N), C (20%N) and D (40%N) fed S or PS diets and IW (n=4). Unequal superscripts within the same diet indicate significant difference between feeding levels or IW.*

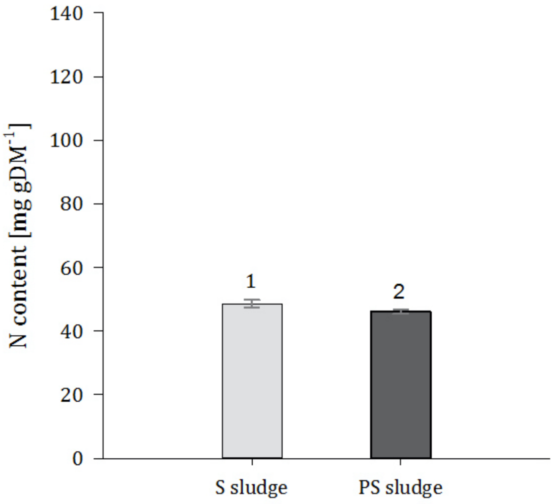


Figure 14*: Mean±SD (n=4) nitrogen (N) content [mg gDM^{-1}] of the two sludge types fed to polychaetes. Unequal superscripts indicate significant difference in mean content.*

3.2.4. Phosphorous content

Figure 15 shows the mean P content [mg g DM^{-1}] in polychaetes fed on feeding levels of S and PS diets, respectively, along with IW. Ranging between 8.1-9.5 and 8.1-8.5 fed S and PS diet, respectively, there was found no significant change in polychaete P content as the feeding levels increased within S (one-way ANOVA) or PS (Welch ANOVA) diets, nor were any levels different from IW (8.5). The P content was not affected by the diet they were fed (independent samples t-test).

The two different sludge types fed to *H. diversicolor* did not differ significantly in their P content (independent samples t-test), being 44.9±6.3 and 49.3±13.8 mg gDM^{-1} in S and PS sludge, respectively (figure 16).

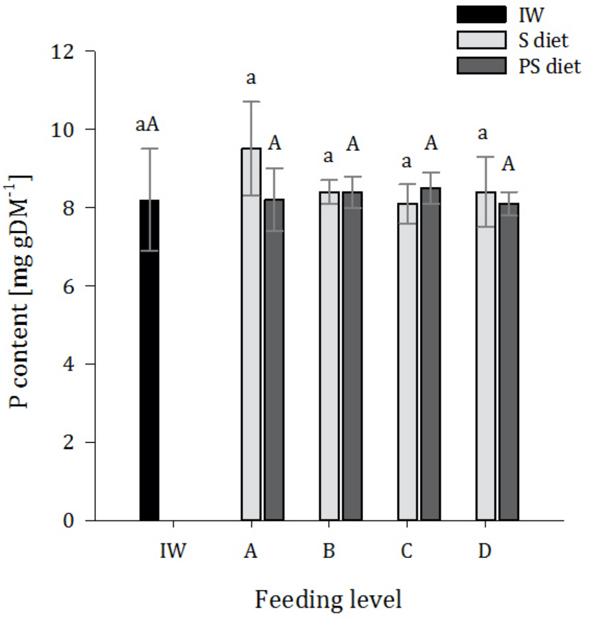


***Figure 15**: Mean±SD of phosphorous (P) content [mg gDM^{-1}] in H. diversicolor of 4 replicate tanks of feeding level A (5%), B (10%), C (20%) and D (40%) fed S or PS and IW (n=4). Equal superscripts within the same diet indicate no significant difference between feeding levels or IW.*

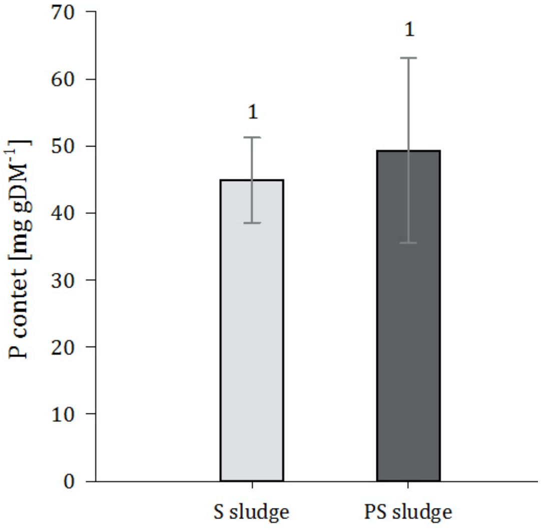


***Figure 16**: Mean(±SD) (n=4) of phosphorous content (P) [mg gDM^{-1}] of smolt and post-smolt sludge diets. Equal superscripts indicate non-significant difference.*

3.2.5. Elemental ratios

The elemental ratios (C:N, N:P and P:N) of the DM content of *H. diversicolor* in different feeding levels fed either S or PS diets are shown in table 5.

The only elemental ratio of the DM content of polychaetes that was found to differ based on feeding levels was C:N [mg C mg N^{-1}], which had an increasing ratio at higher feeding levels. Median C:N ratios ranged between 4.5-5.1 in polychaetes fed different feeding levels of the S diet, with feeding level C and D both found to have significantly higher ratios than A, B and IW (4.6) (Kruskal Wallis H test). Similarly, in polychaetes fed the PS diet, median C:N ratios ranged between 4.4-4.9 dependent on feeding level, with feeding level C and D having significantly higher ratios than A and IW (Kruskal Wallis H test). No differences in C:N ratios were found when comparing equal feeding levels of different diets (Mann-Whitney U test)

There were found no significant differences when comparing polychaete N:P ratios [mg N mg P^{-1}] between feeding levels of the same diet (one-way ANOVA), with means ranging between 9.8-10.7 and 10.5-11.1 when fed on S or PS sludge diets, respectively, or when comparing similar feeding levels of different diets (independent samples T-test).

Neither was there found any significant difference in the C:P ratio of polychaetes based on their feeding level (one-way ANOVA) or diet (independent samples T-test), ranging between 44.0-52.3 and 49.0-56.6 in polychaetes fed on feeding levels of S and PS sludge, respectively.

Elemental ratios of the two sludge types fed to polychaetes were also determined and are shown in table 6. The only elemental ratio found to differ significantly when comparing the two sludge types was C:N, with S sludge (8.7±0.2) having a significantly higher ratio than PS (7.9±0.0) (independent samples T-test). No differences were found in C:P (9.6±1.5 in S, 7.8±2.2, in PS) or N:P (1.1±0.2 in S, 1.0±0.3 in PS) ratios of the two sludges (independent samples T-test). It should be noted that both C:P and N:P ratios were considerably lower in the two sludges in comparison to the polychaetes.

Table 5*: C:N (median), C:P (mean±SD) and N:P (mean±SD) of IW (n=4) and polychaetes from 4 tank replicates of each feeding levels of either S or PS diets. Within each ratio, unequal superscript between feeding levels of the same diet and IW indicate a significant difference.*

Diet	Feeding level	C:N (Median*)	C:P (Mean±SD)	N:P (Mean±SD)
IW	-	4.6^{aA}	49.0^{aA} ±3.8	10.6^{aA} ±0.8
S	A	4.5^{a}	44.0^{a} ±4.7	9.8^{a} ±1.0
S	B	4.6^{a}	49.2^{a} ±2.4	10.7^{a} ±0.4
S	C	5.1^{b}	52.3^{a} ±3.4	10.4^{a} ±0.6
S	D	5.0^{b}	51.2^{a} ±4.3	10.1^{a} ±0.9
PS	A	4.4^{A}	49.0^{A} ±6.1	11.1^{A} ±1.3
PS	B	4.9^{AB}	52.3^{A} ±2.5	11.0^{A} ±0.3
PS	C	5.0^{B}	52.8^{A} ±4.0	10.5^{A} ±0.7
PS	D	5.4^{B}	56.6^{A} ±2.3	10.6^{A} ±0.5

* Comparison of medians due to non-normality of data

Table 6*: Mean±SD (n=4) of C:N, N:P and C:P ratios of the two sludge types fed to polychaetes. Unequal superscripts within each ratio indicate significant difference between the two sludge types.*

Sludge type	C:N (Mean±SD)	C:P (Mean±SD)	N:P (Mean±SD)
S	8.7^{a} ±0.2	9.6^{a} ±1.5	1.1^{a} ±0.2
PS	7.9^{b} ±0.0	7.8^{a} ±2.2	1.0^{a} ±0.3

3.2.5 Total organic matter

TOM contents [mg g DM^{-1}] of polychaetes fed on feeding levels of both S and PS diets are shown in table 17. Ranging between 812-817 in feeding levels of the S diet, and 807-871 in the PS diet, there were found no significant difference in the TOM content of polychaetes when comparing feeding levels of the same diets or with IW (one-way ANOVA). However, when comparing polychaetes from equal feeding levels of different diets, the TOM content in S-D was found to be significantly lower than the TOM content in PS-D (independent samples t-test).

When comparing the diets fed to the polychaetes, the TOM content of the S sludge (870±8) was found to be significantly higher than in the PS sludge (729±3), as visualized in figure 18.

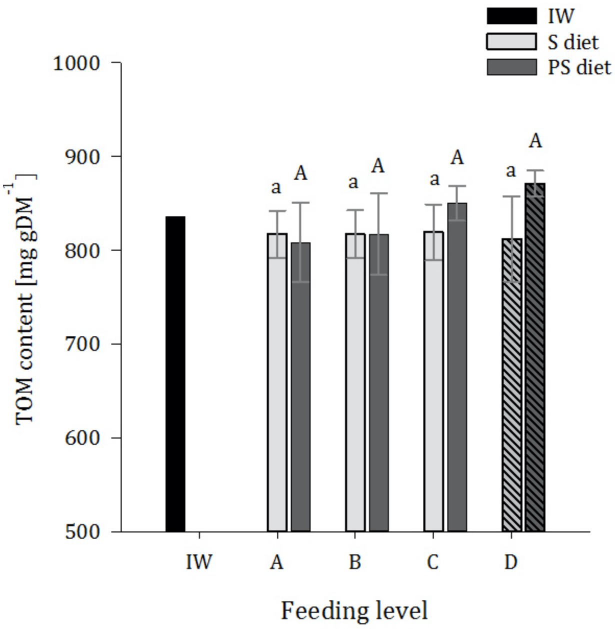


Figure 17: *Mean±SD total organic matter (TOM) content [mg gDM^{-1}] of H. diversicolor in 4 replicate tanks of feeding level A (5%), B (10%), C (20%) and D (40%) of S and PS diets, along with the TOM content in IW. Unequal superscripts within the same diet indicate significant difference between feeding levels, crosshatched bars significant difference between equal feeding level of different diets.*

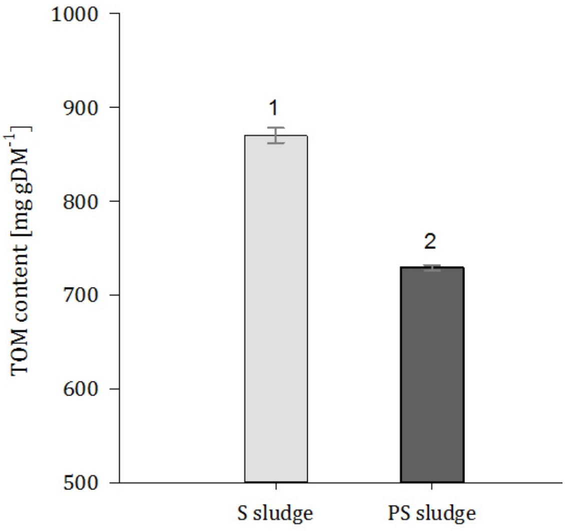


Figure 18: *Mean±SD (n=4) total organic matter (TOM) content [mg gDM^{-1}] in S and PS sludge. Unequal superscripts indicate significant difference.*

3.3. Utilization of C, N and P of the diets

3.3.1. Nutrient gain

The mean gain of C, N and P [mg] in the WW biomass of the average individual *H. diversicolor* from each feeding level during the cultivation period is shown in figure 19. There were not found any significant differences in the respective gain of C, N or P [mg] in polychaetes between feeding on S or PS sludge in any of the feeding levels (independent samples t-test).

In general, the increase of new C, N and P in the polychaete biomass was higher when receiving higher feeding levels of S or PS sludge diets. Feeding level A recorded low mean gains of all three nutrients, with a loss of C, N or P recorded in some of the replicate tanks during the experiment. Individuals gained more C in comparison to N, with P being the nutrient with the lowest gain during the feeding period.

A significant increase in individual C gain [mg] were found in polychaetes in feeding level C and D in comparison to A for both diets, while feeding level D of the PS diet also experienced a significantly higher gain than B (one-way ANOVA). For both polychaetes fed on S and PS diets, there were recorded a significantly higher increase of N in polychaetes from feeding level C and D during the feeding period compared to A (one-way ANOVA).

Regarding the increase of P, there were found to be significant differences between polychaetes receiving different feeding levels of the S diet (one-way ANOVA), but they were not identifiable by a Tukey post-hoc test. Polychaetes in the PS diet showed a significantly higher P gain in feeding level C and D in comparison to A.

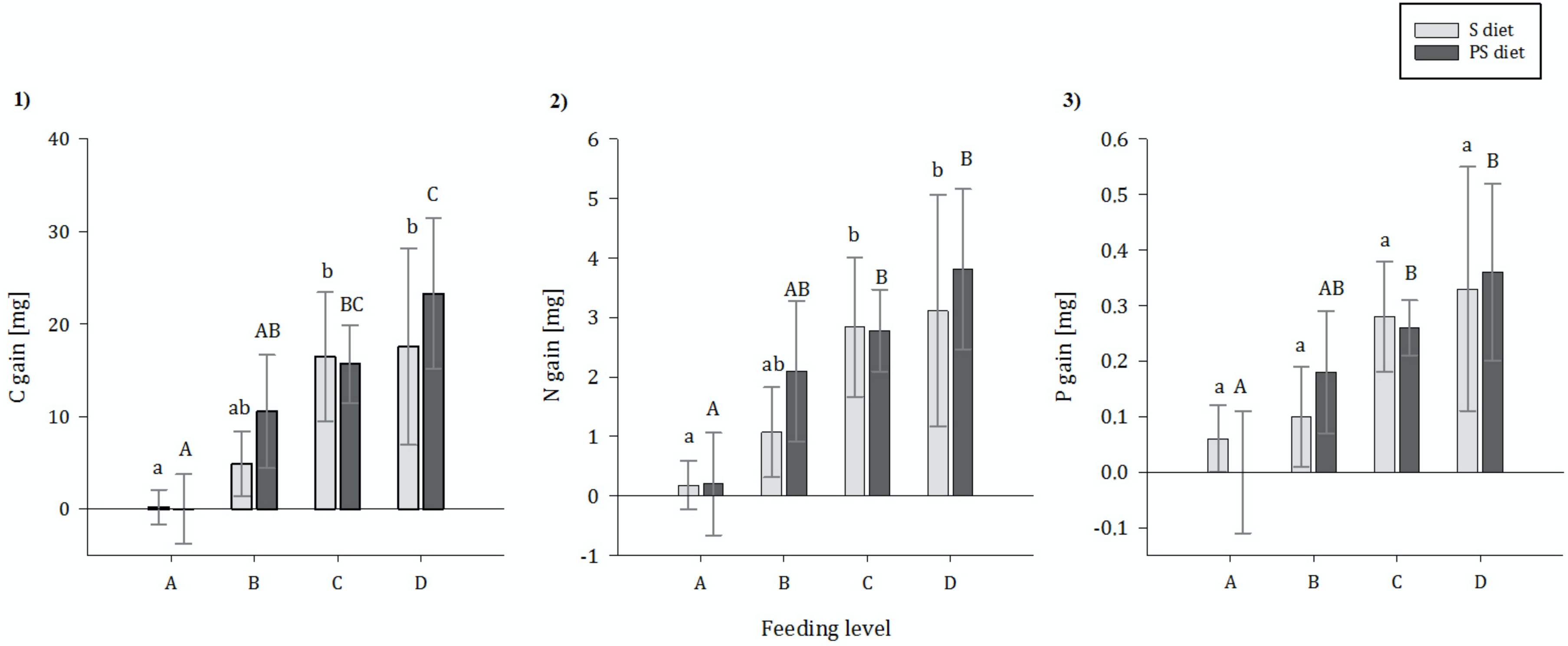


Figure 19*: Mean gain (mean±SD, n =4) of 1) C, 2) N and 3) P in the biomass of mean individual polychaetes [mg] in feeding level A (5%), B (10%), C (20%) and D (40%) of S and PS diets. Unequal superscripts indicating significant difference in mean gain of the given nutrient between feeding levels of the same diet.*

3.3.2 Conversion efficiency of available nutrient

To determine the bioremediation potential and efficiency of *H. diversicolor* feeding on sludge waste from a smolt and a post-smolt facility, respectively, their capability to take up and incorporate C, N and P from the sludge into new biomass was determined. The amount of C, N and P in the sludge given to the polychaetes that was utilized, taken up and converted into new C, N and P in biomass through growth is given as mean±SD values of the average individual polychaetes in each feeding level fed either S or PS. Although not providing information regarding their total uptake and removal of macronutrients from the sludge, it shows the proportion of the available C, N and P that is converted into new biomass. High variation was found in conversion efficiencies between the polychaetes in different tank replicates of the same feeding level.

The polychaetes' conversion efficiency of C from the sludge ($C_{conv.}$) is shown for each feeding level of both S and PS sludge in figure 20. When comparing mean $C_{conv.}$ [%] in feeding levels of the S diet, the only signficiant difference between feeding levels was that significantly more C where converted into polychaete biomass in feeding level C compared to A (one-way ANOVA) There were not found any significant differences in $C_{CONV.}$ between feeding levels of the PS diet (one-way ANOVA), nor when comparing equal feeding levels fed on either S or PS sludge (independent samples T-test)

Mean N conversion ($N_{conv.}$) from the sludge is shown in figure 21. No differences were found in $N_{conv.}$ [%] between mean individual polychaetes from any of the feeding levels receiving S or PS sludge (one-way ANOVA), or when comparing feeding levels of the different diets (independent samples T-test).

Figure 22 shows the mean P conversion ($P_{conv.}$) in feeding levels of S and PS diets, showing low levels of conversion of the available P compared to the other two nutrients. No significant difference or clear patterns of change between polychaetes' ability to remove P from the sludge and converting it into new P through growth [%] were found between any of the feeding levels of S or PS sludge (one-way ANOVA), or when comparing the two diets (independent samples T-test).

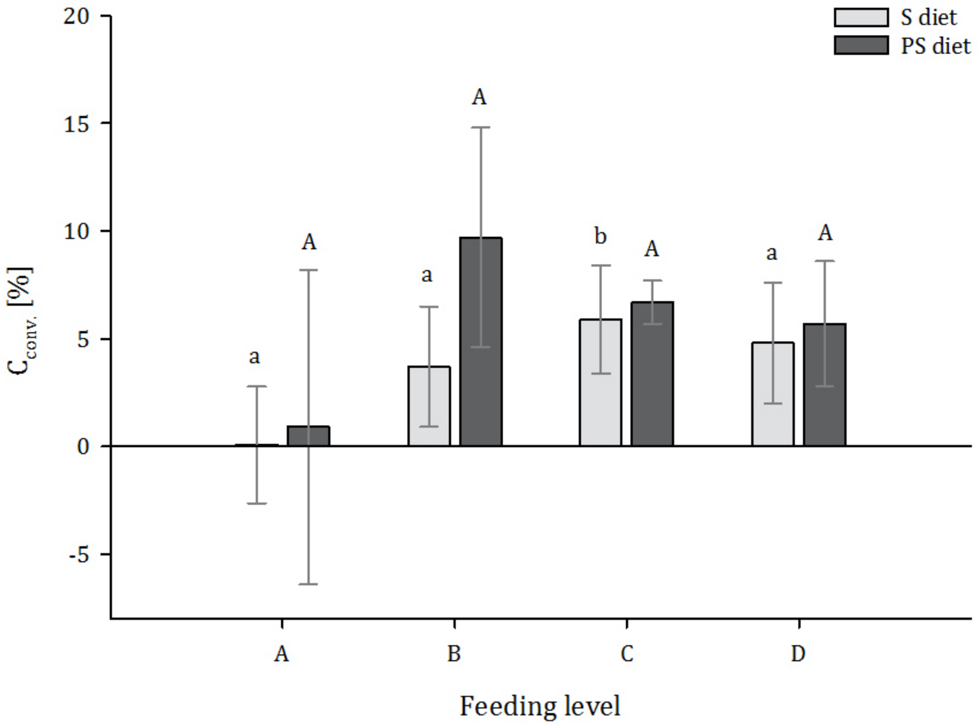


Figure 20: *Mean±SD (n=4) conversion of carbon from the feed [C_{conv}] by the average individual polychaetes fed on feeding level A (5%N), B (10%N), C (20%N) and D (40%N) of S and PS diets. Unequal superscripts within the same diet indicate significant difference between feeding levels.*

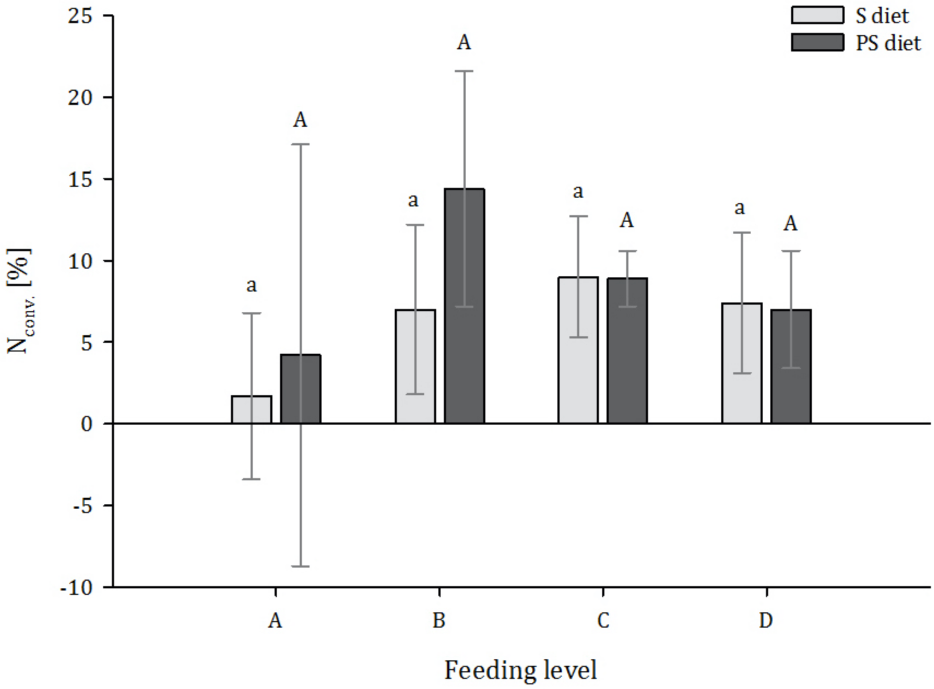


Figure 21: *Mean±SD (n=4) conversion of nitrogen from the feed [N_{conv}] by the average individual polychaetes fed on feeding level A (5%N), B (10%N), C (20%N) and D (40%N) of S and PS diets. Unequal superscripts within the same diet indicate significant difference between feeding levels.*

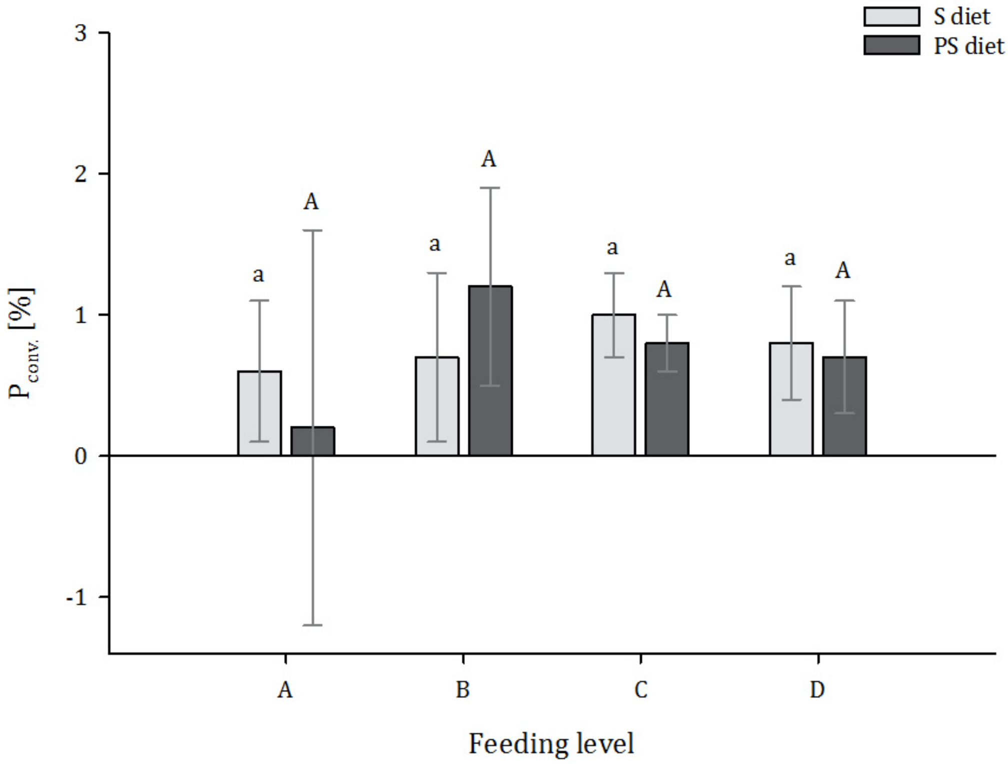


***Figure 22**: Mean±SD (n=4) conversion of phosphor from the feed [P_{conv}] by the average individual polychaetes fed on feeding level A (5%N), B (10%N), C (20%N) and D (40%N) of S and PS diets. Unequal superscripts within the same diet indicate significant difference between feeding levels.*

4. Discussion

4.1 Growth of H. diversicolor

Mean individual biomass gain and %SGR of polychaetes in all feeding levels of both diets except S – A were positive, indicating that *H. diversicolor* were able to utilize sludge originating from both smolt and post-smolt facilities to grow and increase its biomass. Feeding level A received the least amount of feed (sludge), and registered the lowest mean %SGR across both diets. The availability of food and energy were likely limited and the reason for the poor growth performance in feeding level A, although the survival rates remained high.

An earlier feeding experiment with *H. diversicolor* given fish feed, microalgae paste and Atlantic salmon aquaculture waste, reported individual %SGR of 2.54, 1.41 and 1.1 [% day^{-1}], respectively (Wang et al., 2019b), with specific growth rates believed to be low due to under-feeding, experimental design and maturation of polychaetes. The results of the present study show that polychaetes fed the highest feeding levels of S and PS sludge resulted in mean individual %SGR of 2.17±1.34 and 2.10±0.91, respectively, comparable to the growth rates of polychaetes fed on microalgae paste, mimicking their natural diet, and fish feed, a protein rich diet used in Wang et al., (2019). Studies on juvenile polychaetes of approximately 0.03 g reported a %SGR of *H. diversicolor* fed on aquaculture waste from beluga sturgeon *(Huso huso)* to 3.40 [% day^{-1}] (Pajand et al., 2017) , and between 3.06-2.18 [% day^{-1}] when fed on rainbow trout waste (*Oncorhynchus mykiss*) (Yousefi-Garakouei et al., 2019). Nesto et al. (2012) reported high growth rates of juvenile *H. diversicolor* which gradually decreased with increasing age and size of the individuals, and the size of the organisms used in the present study (0.25 g) is likely to cause lower growth rates as more energy is allocated to growth at younger life stages. The only studies found reporting growth rates towards 5-8 % day^{-1} were when cultivating juveniles of the species, mostly on protein rich diets such as commercial fish feed but also on vegetable products, and due to the size of the polychaetes used in the present study a growth rate similar to that in the juvenile size range was not expected (Batista et al., 2003; Fidalgo e Costa et al., 2006; Nesto et al., 2012).

Comparing the findings in the present study with previous literature is challenging, due to the great variations in composition and characteristics of sludge dependent on the fed species it originates from, its production stage, procedures, processing, feed quality and the feed spill (Lomnes et al., 2019). Sludge from RAS facilities, being a waste product, consists of undigested and uneaten feed, faeces and other biological components such as biofilm material (Lomnes et al., 2019). The amount of feed spill affects the nutrient composition of the sludge (Aas and Åsgård, 2017), and with early life stages of Atlantic salmon being efficient utilizers of the feed administered to them (Lomnes et al., 2019), it is likely to contain low amounts of uneaten feed. Feed spill will likely vary greatly between species and even between facilities (Aas and Åsgård, 2017), and with the great variation in sludge dependent on its origin, it makes it hard to compare with studies with sludge from other species such as beluga sturgeon and rainbow trout, or even with sludge originating from Atlantic salmon. With this in mind, we see that when cultivated on sludge with minimal processing originating from RAS-facilities of both smolt and post-smolt Atlantic Salmon in Norway, *H. diversicolor* were able to obtain individual growth rates comparable to when fed on feed of different origins, including other aquaculture wastes.

When food availability is the limiting factor for growth, organisms are likely to achieve increased growth rates with increasing amounts of feed made available. The amount of feed supplied in each feeding level were based on the assumption that the low N content in Atlantic salmon aquaculture sludge (Wang et al., 2019b) is likely to be the limiting nutrient for polychaete growth, supported by the reported elemental ratios and content of sludge and polychaetes in the present study as well as their recorded conversion efficiency of the available macronutrient. A positive linear relationship between %SGR and amount of N supplied to the polychaetes on a daily basis was established, with amount of N fed explaining 53% of the variation in %SGR when fed on S sludge, and 32% when fed PS sludge. These results indicate that the growth rates respond positively to increasing feeding intensities within the scope of this experiment, with the correlation not significantly different based on origin of the diet. The low replicate number makes the linear regression between growth and feeding amount sensitive to variations in %SGR between tank replicates, and might explain the relatively low percentage of variation explained, as the survival rates, densities and abiotic factors varied little between feeding levels over the course of the experiment.

With increased feeding intensities, eventually, a threshold where the growth of individuals no longer increases or correlates with increased feeding intensities will be reached due to the organisms behavioural or physiological limitations such as time spent on eating and uptake of nutrients or metabolic rates (Brown et al., 2004) . Despite the high feeding intensities in the highest feeding levels compared to the study by Wang et al. (2019) and the reported C and N requirement of other polychaete species (Honda and Kikuchi, 2002; Fang et al., 2016; Nederlof et al., 2020), the expected stagnation of %SGR was not observed when feeding polychaetes on a gradient of ~5-40% of their total N content supplied on a daily basis. Judging solely on the %SGR and assumption that food supply is the limiting factor for growth in the system, the findings imply that *H. diversicolor* would have been able to achieve higher growth rates if the food supply were higher, and that a feeding intensity of 40% was not sufficient to observe a stagnation in %SGR or determination of maximum growth rate. However, with high variations in mean individual %SGR in the replicate tanks of each feeding level, especially in the high feeding levels, and only four feeding levels each consisting of four tank replicates it makes it hard to establish clear patterns and significant differences in %SGR.

Together with the observations of organic particles accumulating on the surface sediments, most likely consisting of uneaten feed and polychaete faeces, there is a possibility that the highest feeding levels actually surpassed the maximum growth threshold and the polychaetes assigned these feeding levels were not able to utilize all the feed supplied to the system, but that this was not detected in the current study due to low replicate number and high variations. However, this is only speculative, as the feeding uptake and removal efficiency of the polychaetes were not measured in the present study, making it impossible to quantity the amount of feed eaten by polychaetes.

4.2 Conversion of macronutrients from sludge

As summarised by Nederlof et al. (2020), several approaches have been applied to define the bioremediation potential of a species, each with its own advantages and disadvantages. In the present study, the amount of C, N and P supplied to *H. diversicolor* through feed that were taken up and converted into C, N and P in new polychaete biomass by growth were used as a means of measuring their ability to recycle important nutrients from aquaculture waste, converting nutrient rich waste into a new product of commercial interest (thus giving a measure of the species' bioremediation potential).

The mean gain of C, N and P [mg] in individual polychaetes during the feeding period seemed to increase at higher feeding levels, independent of the diet, with P recording the lowest gain of the three nutrients. The amount of C, N and P gained during the feeding period shows that polychaetes are able to recycle and utilize the nutrients in both S and PS sludge to grow, but less so in the lowest feeding level. However, it does not provide information regarding their efficiency in converting these available nutrients supplied through the sludge, an important question to address when assessing their potential bioremediation effect.

The percentage of nutrients fed to the tanks that were taken up and converted into C, N and P in new polychaete biomass was dependent on the supply of nutrients and the species demand. Although a high variation in conversion efficiencies of C, N and P between tank replicates of each feeding level made it hard to identify significant differences in the conversion of nutrients compared to each other, a trend was seen where the proportion of available N and C taken up and converted into new polychaete tissue through growth was higher than P. Nutrient content of organisms compared to the nutrient content of food might give an approximate indication of the nutritional needs of the organism and the availability through food (Sterner and Schulz, 1998; Brown et al., 2004; Wagner et al., 2013). Together with previous studies done on the C and N requirements of other polychaete species (Nederlof et al., 2020), the gain of nutrients during the feeding period also reflects this need, as more C was gained by polychaetes compared to N, followed by P. By comparing the elemental ratios, both S and PS sludge contains high amounts of P and low amounts of N compared to polychaetes, with a N:P ratio 1.1±0.2 and 1.0±0.3 in the two sludge types, respectively. With a higher demand for the nutrient, polychaetes converted the available N more efficiently than P, where there were high amounts available and only a low demand. PS sludge consisted of significantly less C than S sludge, and a more efficient utilization and conversion of C in polychaetes fed on PS sludge might have been expected, but only a weak trend observed.

With mean conversion efficiencies [%] in feeding levels of S and PS sludge ranging between 0.07-9.80 for C, 1.7-14.4 for N and 0.2-1.2 for P, only a small fraction of the nutrients supplied to the cultivation units was converted into new C, N and P in polychaete biomass through growth. The conversion values only measures the percentage of macronutrients available which are taken up and allocated to growth specifically, whereas previous studies have reported that considerable amounts of the C and N taken up by the polychaete *P. aibuhitensis* are allocated to other metabolic processes, with the proportion allocated to growth varying considerably dependent on individual variation, feeding amounts, temperature and physiological state of the organism (Fang et al., 2016; Nederlof et al., 2020). This might also explain some of the variations observed between replicate tanks of the feeding levels, along with the low conversion efficiency of all nutrients in feeding level A as polychaetes likely allocated the nutrients taken up from the feed to metabolic processes necessary for survival and maintenance instead of growth. The amount of nutrients which are taken up and allocated to other metabolic processes other than growth were not measured in the present study, implying that the amount of macronutrients removed from the sludge and taken up in polychaetes is higher than the values reported.

Although *H. diversicolor* have reported high removal and uptake rates of C and N in previous studies (Pajand et al., 2017; Yousefi-Garakouei et al., 2019,), it is highly influenced by factors such as amounts and quality of feed supplied, species, density, life-stage and temperature (Honda and Kikuchi, 2002; Fang et al., 2017; Pajand et al., 2017; Gómez et al., 2019; Yousefi-Garakouei et al., 2019; Nederlof et al., 2020) , and the possibility that some of the feed that was administered to the cultivation tanks remained uneaten cannot be ruled out. If the removal efficiency and uptake of available nutrients was low, this would reduce the conversion efficiency of polychaetes as there would be nutrients in the system not utilized. No literature was found on the uptake and allocation of P in polychaetes fed on aquaculture waste, but the high content of P in the sludge compared to polychaetes is likely to explain the low conversion rates reported. The low densities of polychaetes in each cultivation tank during the experiment and high survival rates indicates that there was little territoriality and competition for feed resources, although the lower feeding levels reported lower growth rates. With high amounts of feed administered, and the feeding method previously criticized by Wang et al. (2019), the possibility that not all the feed was eaten, either due to over-feeding or unhomogenized distribution of feed resources, might explain the low utilization rates. However, if over-feeding was the case, it would likely also be reflected in a stagnation of %SGR and decreased conversion efficiency with increasing feeding intensities, which was not observed, and with no way of quantifying the feeding uptake or removal rate in the present study, this remains only speculative.

The high variations in conversion efficiencies of the three macronutrients between feeding replicates within each feeding level complicates the process of identifying trends and correlations. Only four feeding levels each consisting of four replicate tanks with 15 polychaetes makes the mean individual conversion rates highly sensitive to extreme values, and patterns of changes in conversion efficiencies with increased feeding levels might be more identifiable by an increasing sample size.

As expected based on the growth rates and nutrient gain in polychaetes, there were not observed any significant differences between conversion rates of any of the nutrients based on the origin of sludge, despite significant differences in the nutritional composition of S and PS sludge. By basing the amount of feed supplied to the polychaetes on the N content of the DM of both sludge types, the significantly lower DM content (147±4) in S sludge compared to PS (216±2), was taken into consideration. As the N content based on reported values in Wang et al. (2019) differed from the N content reported in present study with a significant difference between S (48.6±1.2) and PS (46.1±0.7), the actual amount of feed supplied to the polychaetes were higher than what was planned for and differed slightly between diets, but the differences were not large enough to significantly impact their nutritional conversion rates, gains or growth. The largest difference in the nutritional contents of the sludge was the C content, but the significantly lower C content of PS sludge did only result in a weak trend in higher conversion rates of C. Due to the significant differences in DM content, if the feeding amounts were based on wet weights, polychaetes would have to be fed a larger amount of sludge to receive the same amount of nutrients. But when basing them on the N content of the sludge, the polychaetes seemed to be able to obtain the same growth and nutritional gain on both S and PS sludge. However, the high variations in conversion rates in feeding levels of both diets should be taken into consideration also here, as it makes it hard to identify differences and trends.

4.3 Implications for IMTA and future studies

The use of RAS for land-based smolt production of Atlantic salmon has increased significantly in Norway in recent years, and there is a rising trend towards cultivation of post-smolts in land-based systems after smoltification to improve their survival and robustness after transfer to the sea (Dalsgaard et al., 2013). By demonstrating that polychaetes are able to gain biomass and nutrients when fed on aquaculture sludge, these organisms can be integrated to utilise waste products of both smolt and post-smolt RAS facilities, to recycle and incorporate nutrients from sludge into new polychaete biomass of commercial interest.

However, the amount of available nutrients fed to the polychaetes which was taken up and converted into new polychaete biomass by growth in the present study should be addressed. Even when considering that the polychaetes used had high initial weights in comparison to previous studies, only a small portion of the nutrients fed to the polychaetes ended up as new biomass. Especially the available P, which was found in high amounts in the sludge, was poorly utilized. Since P is considered a non-renewable resource, the goal should be to recycle as much of the P in sludge into new products of commercial interest as possible. A possible solution to the low conversion efficiency of available nutrients offered by *H. diversicolor* in the present study might be to combine the cultivation of polychaetes with other sludge treatments and applications, such as biogas (Wang et al., 2019a) or fertilizer (Yogev et al., 2020) production to fully recycle the nutrient rich sludge while still producing polychaete biomass.

Land-based IMTA systems with Atlantic salmon as the fed species and polychaetes as the organic matter exctractive species could take form as either coupled or decoupled systems (Nederlof et al., 2020), and polychaete assisted sand filters have been proposed as a coupled system alternative (Palmer, 2010; Marques et al., 2017; Jerónimo et al., 2020). In coupled systems, unlike decoupled systems, the sludge receives minimal of treatment and preservations, which have proven beneficial in previous studies on cultivation of polychaetes (Nederlof et al., 2020), but due to significantly lower DM contents of the PS sludge examined in the present study, *H. diversicolor* would need to be fed on higher amounts of post-smolt sludge (wet weight) in order to supply them with the same amount of N.

The further choice of strategy is likely to be dependent on the incentive; if the goal is to produce as much polychaete biomass on the waste products, maximizing their growth rates and nutrient uptake, the present study has shown both sludge types are suited. However, the optimal feeding concentrations supplied to the individual worms to maximize their growth were not determined and would need to be further evaluated in order to produce the highest amounts of biomass without surpassing the polychaetes ability to utilize the feed, maximising the production of polychaete biomass and recycling of nutrients from the sludge. This would prove beneficial for all three pillars of the sustainability of the aquaculture industry: improving the environmental sustainability by reducing nutrient wastage and environmental effects if wastes are released to the environment; economic sustainability through production of additional biomass without additional feeding; and social sustainability by positively affecting people’s perception of the industry. While the present study has determined that *H. diversicolor* are able to bioremediate smolt and post-smolt sludge, to fully determine the bioremediation potential of polychaetes on Atlantic salmon aquaculture waste, future studies should feed *H.* diversicolor on even higher amounts of sludge and with larger sample sizes to establish the feeding concentration for maximum growth, while providing information about the complete nutrient budgets for *H. diversicolor* and the removal rates of nutrients from the system.

6. Conclusion

The polychaete *H. diversicolor* was able to utilize both smolt and post-smolt sludge to both grow and convert nutrients from the sludge into new biomass, demonstrating the potential of cultivating the species on aquaculture sludge as the sole feed source to utilize and recycle the nutrient rich waste stream.

A positive correlation was found between the amount of feed administered (% N) and the %SGR of the individual polychaetes, and the growth rates of polychaetes fed the highest feeding levels (20 and 40 %N) were comparable to previous studies with this species. Despite the high feeding intensities, no stagnation in growth rates were observed, and consequently, the maximum growth of polychaetes fed on aquaculture sludge was not determined. Findings suggest that polychaetes are able to achieve even higher growth rates by increasing the feeding intensity beyond 40% of their total N content supplied on a daily basis, although high variations in %SGR between feeding level replicates made it difficult to identify trends and significant differences.

However, the efficiency in converting C, N and P content in the sludge into new polychaete biomass through growth varied considerably, with P being the poorest utilized nutrient only accounting for 1% of the P supplied to tanks incorporated into new polychaete tissue. Both sludge types (S and PS) contained high amounts of P compared to the elemental content of polychaetes, likely explaining the low conversion rate of available P. The conversion of N and C was more efficient, but high variations made it hard to identify clear patterns on how feeding amounts affected their conversion efficiency.

Comparing smolt sludge and post-smolt sludge as a diet for the polychaetes, both diets appears to be equally suited as a feed source, as growth, elemental composition and conversion efficiency did not differ between the polychaetes received the two different diets. However, due to the high P content of aquaculture sludge compared to the nutritional demand of polychaetes, integration of *H. diversicolor* as a deposit feeding species is likely not the sole solution in effectively utilizing the nutritional content of organic particulate waste streams. Combination of aquaculture sludge with other recycling elements might increase the efficiency of utilizing the N, P and C of the diets.

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Appendix 1 – Carbohydrate method

Determination of carbohydrate content in DM samples of both polychaetes and sludge were attempted, but the results were discharged due to non-coherent results. Different solutions to the problems were attempted, including removal of steps to eliminate the potential of non-homogenized samples, but the attempts were futile.

The method was based on Dubois et al. (1956), determining carbohydrate content in polychaetes and sludge by measuring the absorbance of samples in a spectrophotometer after reduction of carbohydrates by the phenol-sulfuric acid method, comparing them with a known standard of glucose. Dry matter samples (200 µg polychaetes, 100 µg sludge) where weighed (Mettler Toledo UMX2) in mg with four decimals into Chimex glass vials and diluted with 500 µl distilled water. 1 mL of phenol solution (3%) was added and the sample thoroughly mixed. After 20 minutes, 5 mL of concentrated sulfuric acid were added, and vials placed in an ice-bath before centrifuging for 10 minutes at 3000 rpm and 5 ºC (Heraeus Megafuge 16R). By using quarts cuvettes, the absorbance was read at 490 nm in a Cary 50 scan UV Spectrophotometer (Varian Inc.), and carbohydrate concentration in samples determined by comparing the absorbance to a standard curve of known glucose concentration.

Appendix 2 – Growth and survival data

***Table 7**: Measured initial and final biomass WW [g] of pooled and average polychaete from each replicate tank, along with the initial and final n (number of polychaetses). Surival rates [%] in each tank shown along with the average individual %SGR [% day-1] of polychaetes.*

Diet	Feeding level replicate	Initial WW [g]	$n_{initial}$	Average Initial WW [g]	Final WW [g]	n_{final}	Average Final WW [g]	Average individual %SGR [% day^{-1}]	Survival [%]
S	A1	4.29	15	0.29	3.81	12	0.32	0.35	80
S	A2	3.99	15	0.27	3.11	12	0.26	-0.09	80
S	A3	3.64	15	0.24	2.82	13	0.22	-0.37	87
S	A4	5.96	15	0.40	3.81	11	0.35	-0.46	73
S	B1	3.66	15	0.24	4.46	13	0.34	1.14	87
S	B2	4.27	15	0.28	4.20	13	0.32	0.42	87
S	B3	3.40	15	0.23	3.31	11	0.30	0.95	73
S	B4	4.13	15	0.28	3.05	12	0.25	-0.27	80
S	C1	3.66	15	0.24	6.36	13	0.49	2.35	87
S	C2	2.96	15	0.20	3.49	13	0.27	1.03	87
S	C3	4.22	15	0.28	5.91	14	0.42	1.36	93
S	C4	4.56	15	0.30	6.34	14	0.45	1.34	93
S	D1	3.01	15	0.20	7.23	15	0.48	2.96	100
S	D2	3.63	15	0.24	3.39	12	0.28	0.52	80
S	D3	3.46	15	0.23	4.96	13	0.38	1.69	87
S	D4	3.32	15	0.22	4.53	14	0.32	3.51	93
PS	A1	2.89	15	0.19	3.38	13	0.26	1.00	87
PS	A2	3.85	15	0.26	4.72	15	0.31	0.68	100
PS	A3	4.67	15	0.31	3.24	13	0.25	-0.74	87
PS	A4	3.20	15	0.21	2.72	13	0.21	-0.06	87
PS	B1	3.65	15	0.24	4.22	13	0.32	0.97	87
PS	B2	4.23	15	0.28	5.41	13	0.42	1.31	87
PS	B3	3.67	15	0.24	5.48	11	0.50	2.40	73
PS	B4	2.46	15	0.16	3.39	13	0.26	1.56	87
PS	C1	5.16	15	0.34	7.2	14	0.51	1.35	93
PS	C2	3.67	15	0.24	4.42	10	0.44	1.99	67
PS	C3	2.97	15	0.20	3.1	12	0.26	0.89	80
PS	C4	3.45	15	0.23	4.86	13	0.37	1.63	87
PS	D1	2.86	15	0.19	6.45	13	0.50	3.24	87
PS	D2	3.52	15	0.23	5.92	13	0.46	2.23	87
PS	D3	3.34	15	0.22	3.11	8	0.39	1.87	53
PS	D4	4.25	15	0.28	5.43	14	0.39	1.05	93

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