Feeding response to fish feed diets in *Ciona intestinalis*; implications for IMTA

Masters of Science in Aquaculture Biology
Department of Biology, University of Bergen

Magnus Petersen

May 2016

Petersen, S. M. (2016) Feeding response to fish feed diets in *Ciona intestinalis*; implications for IMTA. MSc thesis.
University of Bergen.

Bergen, March 2016

Photo: Per-Otto Johansen

COPYRIGHT
© Sverre Magnus Petersen
The publication may be freely cited where the source is acknowledged

AVAILABILITY
Open

PUBLICATION TYPE
Digital document (pdf)

INTERNAL SUPERVISOR(S)
Thorolf Magnesen, Christofer Troedsson

KEY WORDS
- Sea squirt, Tunicata, ascidian,
- Fish feed, cultivation, filter feeding
- Dissolved, particulate discharge
- Integrated-multi Trophic Aquaculture (IMTA)
- Filter feeding, individual, retention, clearance rate
- Filtration, Clearance rate, Retention Efficiency
- Norway, Bergen, Espegrend

NØKKELORD
- *Ciona intestinalis*, sekkedyr, oppdrett,
- Filtrering, integrert multitrofisk havbruk
- Norge, Bergen, Espegrend

CONTACT DETAILS

Magnus Petersen
Thormøhlensgt. 53 A/B
5020 Bergen
Norway
Phone: +47 95005535
Mail: magnus.petersen@gmail.com

Thorolf Magnesen (Supervisor)
Thormøhlensgt. 53 A/B
5020 Bergen
Norway
Phone: +47 95927754
Mail: thorolf.magnesen@uib.no

Christofer Troedsson (Supervisor)
Thormøhlensgt. 55
5020 Bergen
Norway
Phone: +47 41215576
Mail: Christofer.Troedsson@uni.no

Abstract

Norway is the largest salmon producer in the world. The associated discharge with continued growth from salmon production has vitalised interest in developing integrated multi trophic aquaculture (IMTA) in order to mitigate environmental effects, while producing additional commercial species.

The solitary filter feeding ascidian, *Ciona intestinalis* ability to ingest and filter fish feed was determined using qPCR and two common filter feeding methods. To establish whether *Ciona* actually ingested fish feed, a bioassay detecting soy RuBisCO in fish feed, was conducted on extracted DNA from intestines in animals given fish feed, algae and seston. We found that fish feed was removed from the water column. In the group fed algae and fish feed, 10/10 animals ingested fish feed, while the group given solely fish feed, 7/10 animals ingested fish feed, we argue that the difference between the two treatments might be regulated by squirting provoked by large particles in fish feed.

The reported filtering capacity of *Ciona intestinalis* varies considerably in the literature. Therefore we developed our own individual flow through system with special care to avoid methodological constrictions. Furthermore we used two common indirect approaches; the flow through and clearance methods, with individuals and groups of ten animals, respectively. The experimental animals were fed diets with varying concentrations of fish feed and results from the two methods were compared. Our data indicate that animals can clear mixtures of fish feed and seston at a rate of 50-60 mL/min (3-3.6 L/h) per g^{-1} dry weight, unless the concentrations are too high (>40.000 particles/mL). Clearance rates did not differ between both methods and were within values from previous studies. The response to elevated particles concentrations is characteristic of a Holling type I functional response. However at intermediate concentrations, clearance rates were initially reduced, but gradually increased over time in individuals, while this was not found when measuring multiple animals, suggesting non-synchronous inter-individual variation in feeding responses.

The ability to retain particles differed between the two methods, but differences disappeared when non-feeding individuals were excluded. Our data were within reported values, and animals completely retained particles over 2μm (100%), while clearing particles down to 1.5μm with around 75% efficiency.

Lastly our data suggests that *Ciona* can be used as an efficient bio-filtering organism in salmon driven IMTA, and would likely extract more available particulate discharge than the commonly used Blue Mussel.

Contents

ABSTRACT 3
CONTENTS 4
FOREWORDS 5
1 INTRODUCTION 6
1.1 Background and potential use of discharge 6
1.2 Biology 10
1.3 Filter feeding in Ciona intestinalis 11
1.4 Methodological issues in previous filtration studies 12
1.5 Aim of study 14
2 MATERIALS AND METHODS 15
2.1 Preparation and equipment 15
2.1 Experiment one; cohort feeding study using the clearance method 17
2.2 Experiment two; fish feed detection using qPCR 19
2.3 Experiment three; individual feeding study using the flow through method 22
2.5 Formulas 25
2.6 Statistical analysis 26
3 RESULTS 28
3.0 Sampling error from the electronic particle counter 28
3.1 Experiment one; cohort experiment using the clearance method 29
3.2 Experiment two; fish feed detection from intestines 33
3.3 Experiment three; individual experiment using the flow through method 36
4 DISCUSSION 44
4.1 Ciona intestinalis does ingest fish feed 44
4.2 Methodology for RE and CR estimation 46
4.3 Retention efficiency 47
4.4 Effect of particle concentration on feeding 51
4.5 Effect of time on feeding 52
4.6 Variation in clearance rates 53
4.7 Implications for integrated multi-trophic aquaculture 55
4.8 Potential for nutrient assimilation on a regional scale 56
4.9 Conclusions and future research 58
REFERENCES 59
APPENDICES 68
APPENDIX A. CERTIFICATE OF FISH FEED USED IN ALL EXPERIMENTS 68
APPENDIX B. qPCR CYCLE SETUP 69
APPENDIX C. SEQUENCES OF PRIMERS AND PROBE USED IN qPCR 69
APPENDIX D. PROTOCOL FOR DNA EXTRACTION 70
APPENDIX E. ANIMALS USED IN INDIVIDUAL EXPERIMENT 71
APPENDIX F. ANIMALS USED IN THE COHORT EXPERIEMENT 71
APPENDIX G. PARTICLE DISTRIBUTION IN THE INDIVIDUAL STUDY 72
APPENDIX H. PARTICLE CONCENTRATION IN THE FISH FEED DETECTION STUDY 72
APPENDIX I. VOLUME OF PARTICLES FED IN THE INDIVIDUAL STUDY 73
APPENDIX J. UNSTANDARDISED RE IN FEEDING EXPERIMENTS 74
APPENDIX K. SETTLING OF PARTICLES IN THE COHORT STUDY 75

Forewords

I would like to thank Thorolf Magnesen and Christofer Troedsson for helpful supervision during the entire process of my master`s thesis. Also a special thanks to Mette Hordnes and Thomas Sørlie for support and good company at the Espegrend Marine station. Thanks to Helen Graham for useful discussions and Samuel Rastrick for feedback. I am especially grateful to Tore Strohmeier for valuable advice and technical support. I would also like to show my appreciation to Jessica Ray and Katrine Skaar for the invaluable support and help in the use of molecular methods in the laboratory. Furthermore, thanks to Heikki Savolainen and Julie Skadal for help in allocating equipment and technical assistance. You have all played an important part in my research, and I could not have done it without you.

1 Introduction

1.1 Background and potential use of discharge

Global aquaculture production is growing every year and production exceeded 66.6 million tonnes in 2012, while capture fisheries amounted to 91.3 million tonnes (FAO, 2014). However, as the global population is projected to surpass 9 billion in 2050, the Food and Agricultural Organization of the United Nations (FAO) has estimated that food production must increase by 70%, in order to meet the increase in demand (FAO, 2009; Ytrestøyl et al., 2015). Simultaneously most of the food is expected to derive from the aquaculture sector (Béné et al., 2015; FAO, 2014; HLPE, 2014).

In Norway, production of Atlantic salmon and rainbow trout exceeded 1.3 million metric tonnes in 2014 and used around of 1,6 million tonnes of fish feed (Fiskeridirektoratet, 2016). This makes Norway the current largest producer of salmon in the world, and production increased by 30% between 2010 and 2013 (FAO, 2014; Ytrestøyl et al., 2015). With the rapid development of aquaculture along the coast, some have voiced concerns on the nutrient discharge from the production (Carroll et al., 2003; Kutti et al., 2007b; Wang et al., 2012).

Discharge of nutrients can lead to eutrophication, algal blooms and in high concentrations; anoxia with successive regime shifts that can reduce biodiversity and alter the ecosystem (Diaz and Rosenberg, 1995).

In Norway fjords become oligotrophic during summer and the release of effluents can increase primary production (Handå et al., 2012b; Strohmeier et al., 2015; Svåsand et al., 2015). It was estimated by Jørgensen (1996) that 25% to 50% of the organic N, C and P produced by phytoplankton sinks to the benthos and undergo microbial breakdown that often consumes oxygen and leads to deterioration of benthic ecosystems (Carroll et al., 2003; Cloern, 2001; Islam, 2005; Kalantzi and Karakassis, 2006; Kutti et al., 2007b; Meyer-Reil and Köster, 2000; Wang et al., 2012).

Wang et al. (2013) estimated that in a typical Norwegian salmon farm (with a 3% feed loss) about 62% of fed carbon (C), 57% of fed nitrogen (N) and 76% of fed phosphorus (P) were lost into the environment. Around 40% of feed C was respired as CO_2, while 39% of feed N and 24% of feed P were excreted as dissolved inorganic nitrogen and phosphorus, respectively. About 19% of feed C, 15% of feed N and 44% of feed P were released as particles. The results are consistent with other studies, although there is variations due to temperature, feeding regime and fish weight (Olsen et al., 2008; Wang et al., 2012).

Considering that the total annual fish feed use in Norway was 1,6 million tonnes in 2015 (Fiskeridirektoratet, 2016) and that Olafsen et al. (2012) estimated an annual increase in fish feed use to around 3,6 million tonnes by 2030; the potential to extract and recirculate discharged nutrients is high, and will increase.

In 2012 the three largest feed companies, responsible for supplying 90% of the feed used in Norway, reported a use of 15,000 tonnes of P, and a loss of 71% as estimated by Ytrestøyl et al. (2015) amounts to 10,700 tonnes, while estimations from Wang et al. (2012) would amount to 11,400 tonnes (76%) of released P. In comparison, runoff from sewage and agriculture was estimated to a total of 1,250 and 943 tonnes in the same year (Miljødirektoratet, 2014).

The potential to extract is especially relevant in the context of P depletion, which is expected to limit agricultural and fertiliser production in 40-90 years (Cordell et al., 2009). Global food production is heavily reliant on phosphorus fertiliser, as it is the limiting soil nutrient in large areas, such as Africa, Australia, Brazil and India (Cordell et al., 2011; Schroder et al., 2011). Today, most phosphorus used in agriculture is mined from accessible phosphate rock, and become concentrated fertilizer (Schroder et al., 2011). When used in agriculture, excess fertiliser become runoff and is transported through watercourses and finally end up highly diluted in the ocean (Cordell et al., 2009; Schroder et al., 2011). In 2010 the agriculture sector accounted for 90% of the total P use (Schroder et al., 2011). The loss of highly concentrated phosphate resources been characterised as unsustainable and the FAO has highlighted the need to recycle and reuse P terrestrially (Cordell et al., 2009; Cordell et al., 2011; Schroder et al., 2011).

Integrated multi-trophic aquaculture (IMTA) is a technique designed to utilize waste from the culture of one species as feed for another, often across trophic levels (Chopin et al., 2001; Jansen et al., 2015; Neori et al., 2004). In Norway, IMTA has mainly been concerned with the sugar kelp (*Saccharina latissima*) to extract dissolved inorganics and Blue Mussels (*Mytilus edulis*) to capture organic particles from salmon farming (Handå et al., 2012b; Handå et al., 2013; Jansen et al., 2015; Wang et al., 2013).

In a study by Handå et al. (2013) to investigate the feasibility of sugar kelp in a Norwegian IMTA system, they found an increase in growth within 200m of the salmon farm, but a seasonal mismatch was discovered between the salmon production cycle and the kelp`s life cycle (Handå et al., 2013). In the typical salmon production cycle, increased feeding and biomass in late summer increases the release of nutrients, while in sugar kelp an increase of temperature and epiphyte growth often leads to total mortality (Handå et al., 2013). Therefore direct assimilation of dissolved inorganic nutrients is lowest when most needed. Both the measured and simulated ammonium levels in the study by Handå et al. (2013) quickly reduced in concentration close to the fish farm, suggesting a rapid dilution and extraction by phytoplankton (Pitta et al., 2009).

The ability for filter feeders to directly capture particles from a fish farm is dependent on local conditions such as; tide, current and depth. Nonetheless, most particles sediment directly under the production site, while small particles are carried with the current (Cranford et al., 2013; Jansen et al., 2015). Furthermore culturing of extractive organisms in near proximity to salmon farms culture will physically constrict current flow in itself, hence the possibility to intercept particles is reduced with increasing biomass (Cranford et al., 2013). Therefore only a small proportion of the particulate discharge can be captured directly (Cranford et al., 2013; Jansen et al., 2015).

In study by Handå et al. (2012b) on mussels cultivated close to a Norwegian salmon farm, they found that mussels captured particles directly, but did not significantly increase in length over a year (Handå et al., 2012b). Nonetheless there was an increase in growth during summer compared to the reference, suggesting the utilisation of plankton from increased productivity by dissolved discharge from the salmon farm (Handå et al., 2012b).

There is evidence which suggests that dissolved nutrients from aquaculture discharge is quickly extracted by phytoplankton (Pitta et al., 2009).

Suspension feeders could consume these organisms, thereby indirectly capturing the released dissolved nutrients (Erga et al., 2012; Ledda et al., 2014; McClimans et al., 2010; Pitta et al., 2009; Strohmeier et al., 2015). Aquaculture production in Norway intensifies during late summer, with increased feeding and biomass, thus releasing more nutrients (Erga et al., 2012; Frette et al., 2004; Handå et al., 2013). Simultaneously most Norwegian fjords become oligotrophic during summer because of spring blooms exacerbating nutrients (Erga and Heimdal, 1984; Frette et al., 2004; Jansen et al., 2011). An increase of nutrient concentrations during this interval leads to increased primary production, hence more prey for suspension feeders (Aure et al., 2007; Erga et al., 2012; Frette et al., 2004). Therefore some believe that in Norway focus should be shifted from extracting particulates directly, typically in a close proximity to the production site, to utilising the resultant productivity from discharge release in an area or ecosystem perspective (Jansen et al., 2015).

An ecosystem approach of nutrient recycling with filter feeders has been extensively studied, mainly with the Blue Mussels (Delegrange et al., 2015; Filgueira et al., 2014; Filgueira et al., 2010; Handå et al., 2012b; Jansen et al., 2011; Lindahl et al., 2005; McClimans et al., 2010; Petersen et al., 2014; Strohmeier et al., 2015; Troell et al., 2009; van Broekhoven et al., 2015). Multiple studies of controlled upwelling in oligotrophic fjords to increase primary production have shown potential for extracting dissolved nutrients (Aure et al., 2007; Filgueira et al., 2010; McClimans et al., 2010; Strohmeier et al., 2015). For example Aure et al. (2007) estimated a 2-3 fold increase in photosynthetic activity when mixed with nutrient rich deep water, and a separate later study found increased dry flesh weight in mussels (Strohmeier et al., 2015). This increased primary production allows for extraction and recirculating of nutrients that otherwise might be difficult to utilise (Filgueira et al., 2014; Filgueira et al., 2015).

Other studies have focused more on the potential to avoid eutrophication effects, by extracting plankton resulting from nutrient laden water (Holthuis et al., 2015; Jansen et al., 2011; Lindahl et al., 2005; Loo and Petersen, 2013; Nielsen, 2014; Petersen et al., 2014; Sebastiano et al., 2015; Strohmeier et al., 2015). On the Swedish west coast, investigations on the culturing of mussels to mitigate eutrophication have found an efficient nutrient recycling capacity. For example, Lindahl et al. (2005) estimated that roughly 140–180 tonnes of Blue Mussels were produced in 12–18 months, removing 1.4–1.8 tonnes of N and 80–100 kg of P. However mussels cultured in the presence of harmful or toxic effluents may cause the mussels to become inedible, thus reducing cost-effectiveness (Lindahl, 2011; McClimans et al., 2010; Nielsen, 2014). Mussel biomass was instead proposed to facilitate biogas production, but mussel shells represents a problem in biogas production as they are not suitable for wet digestion and can cause blockages in pipes and damage sensitive parts of the production system (Aldentun, 2013; Nkemka and Murto, 2013).

Investigations on the potential to avoid eutrophication have not only been conducted for the Blue Mussel in Sweden, but also on the similarly efficient filter feeder, the solitary ascidian *Ciona intestinalis* (Linnaeus, 1767). Being a suspension feeder *C. intestinalis* can directly extract particulates in the water and indirectly consume primary production resulting from the release of dissolved nutrients. Compared to the Blue Mussel, *C. intestinalis* is composed of soft tissue

and has proven suitable for biogas production (Norén et al., 2012). In Lysekil municipality on the Swedish west coast, there is currently a culturing effort of *C. intestinalis* as a means to clean effluents from the municipality. Animals are harvested for biogas production and subsequent fertiliser use (Lindahl et al., 2005; Loo and Petersen, 2013; Norén et al., 2012). It has been estimated that the potential energy production for an area of 30km^2 cultivated with *C. intestinalis* produces 1.900 GWh/yr (Norén, 2015). In addition fertilisers based on the resultant waste from biogas production, is being used (Norén, 2015).

In Norway research and development on the use of *C. intestinalis* as a new marine biomass is explored by the University of Bergen and Uni Research. Both fish feed production and the use of cellulose are being pursued (Høgøy et al., 2015; Troedsson et al., 2015).

The potential to cultivate *C. intestinalis* is likely large as it is found circumglobally and has a high reproductive potential (Carver et al., 2006; Dybern, 1965; Troedsson et al., 2015). Troedsson et al. (2013) estimated that sub-sea farming of tunicates can provide at least 250 tones of dry weight per hectare of ocean surface per year, which is a significantly higher yield than most land crops. In addition it is expected to have minimal environmental impact in the area (Loo and Petersen, 2013; Norén et al., 2012; Troedsson et al., 2013). The resulting biomass can be used as fish feed, fertiliser, pharmaceutical products and for biogas production (Høgøy et al., 2015; Loo and Petersen, 2013; Troedsson et al., 2015).

1.2 Biology

1.2.1 Taxonomy and defining characteristics

Table 1 Taxonomy

Kingdom:	Animalia
Phylum:	Chordata
Subphylum:	Tunicata
Class:	Ascidiacea
Order:	Enterogona
Suborder:	Phlebobranchia
Family:	Cionidae
Genus:	*Ciona*
Species:	*C. intestinalis*

Ciona intestinalis (Linnaeus, 1767) is a solitary Ascidian belonging to a large group of diverse benthic and planktonic filter feeders, which are both colonial and solitary (Table 1). In the subphylum Tunicata there are around 2,150 described species. The unifying characteristics placing tunicates within the chordates are the presence of a dorsal nerve chord, gill slits, notochord and post anal tail. With the exception of Appendicularians, all these characteristics occur only in the juvenile tad-pole stage. In addition the tunicate, a rubbery type sheath made of a type of cellulose, surrounds the animal and has given the subphylum its name (Fiala-Médioni, 1978b; Petersen, 2007; Randløv and Riisgård, 1979; Ruppert et al., 2004). These characteristics and the relative ease of culturing, rapid growth rate and high fecundity have made *C. intestinalis* an interesting model species for chordate evolution (Cirino et al., 2002; Dehal et al., 2002; Dishaw et al., 2014; Satoh, 2002).

The Ascidiacea class contains most of the tunicates, which are primarily sessile, benthic filter-feeders, found in shallow waters (<200m). This class usually have a filter-feeding organ (branchial basket) which anatomy is used to distinguish the members in the suborders (Petersen, 2007; Ruppert et al., 2004).

1.3 Filter feeding in *Ciona intestinalis*

Suspension feeders play a pivotal role in marine ecosystems, both by top-down grazing on primary producers and seston (particles in the water column) and indirectly by bottom-up regulation of nutrients. In exerting this regulation, suspension feeders have often been described as ecosystem engineers (Dame, 2011; Dame et al., 2001; Gili and Coma, 1998; Rimondino et al., 2015; van Broekhoven et al., 2015; Wright and Jones, 2006).

Outer circular smooth muscles control the siphon openings and regulate current and flow. Within the inhalant siphon buccal tentacles perturb in order to avoid ingesting large and harmful particles. Once inside the pharynx, particles are removed from the water by passing a filter made of mucus and produced by an organ called the endostyle. Flood and Fiala-Médioni (1981) determined that this mucus filter consisted of filaments intertwined longitudinal and transverse, creating a rectangular mesh size of 0.4x 0.7μm. The results corroborate with retention efficiencies found in several studies, and entails that free-living bacteria can be retained (Petersen and Svane, 2002; Randløv and Riisgård, 1979; Riisgård, 1988).

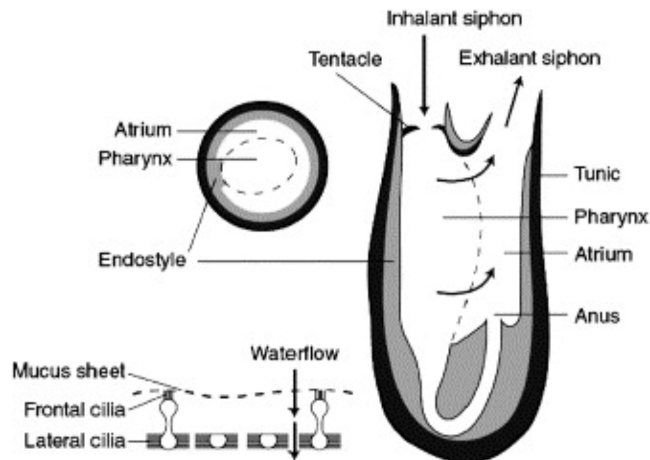

Figure 1 Schematic representation of the anatomy in a suspension feeding ascidian from (Petersen *et al.*, 2007)

Particles that are larger than the mesh opening become trapped in the mucus sheet, which is then transported towards the Oesophagus while rolled to a food string and ingested. The mucus entrapped particles then reaches the stomach and travels through the digestive system. The anus releases faecal pellets, which are carried by the flow out through the exhalant siphon. The testes and ovary have separate canals and also release gametes through the atrial siphon (Petersen, 2007; Ruppert et al., 2004).

The pharyngeal basket is covered in perforated openings (stigmata), which are lined with beating cilia that are responsible for generating flow. This flow is temperature dependent and increases with temperature until around 21°C, where it rapidly decreases (Petersen, 2007; Riisgård and Larsen, 2010; Ruppert et al., 2004). The viscosity, not biological regulation appears to regulate cilia beating and thus flow (Larsen and Riisgård, 2009). Large particles, high particles concentrations and disturbance can cause the animals to "squirt", which is used to regulate feeding (Armsworthy et al., 2001; Petersen et al., 1999). Waste material and overloading in the pharyngeal basket causes the animals to cease ciliary beating, close the stigmatal openings and contracting the body both longitudinally and circularly (Petersen, 2007). The response creates a quick and powerful " flush" of the pharyngeal basket removing waste through the oral siphon (Day, 1919). After squirting siphons are gradually reopened and a lag-phase between 20-140 min until feeding continues at similar rates (Petersen, 2007).

1.4 Methodological issues in previous filtration studies

Central to measuring filtration capacity in filter feeders is the accurate determination of retention efficiency (RE) and clearance rate (CR), which measure the efficiency of cleared particles cleared at different sizes and the volume of particles cleared respectively (Coughlan, 1969; Cranford et al., 2011; Filgueira et al., 2006; Riisgård, 2001). In order to calculate CR, one must first ensure that the particles sizes used are completely retained (Coughlan, 1969), which is commonly achieved by examining a size range where particles are assumed to be completely retained, often between 1 and 10µm for mussels and *C. intestinalis* (Jørgensen et al., 1984a; Petersen and Riisgård, 1992). The particle size with the highest RE is set to 100% and proportionally scaled to the remaining particle sizes. When this is done on individual animals, inter-individual variations are reduced and the mean represents the cohort RE response (Møhlenberg and Riisgård, 1978; Randløv and Riisgård, 1979; Strohmeier et al., 2012).

In the reviews by Carver et al. (2006) and Petersen (2007), both authors point to the variability in filtration estimations for *C. intestinalis,* even in similar temperatures (Table 2). High concentrations of particles, disturbance, methodological errors and pre-treatment are claimed to cause the discrepancies (Carver et al., 2006; Petersen, 2007; Petersen and Riisgård, 1992).

For example Petersen and Riisgård (1992) found that clearance rates were over three times higher than previous estimates than findings from Fiala-Médioni (1974), the discrepancy was explained by that previous studies might have had a high algal concentration, inhibiting feeding.

Studies on filtration of suspension feeders in general have according to Riisgård (2001) had problems with erroneous use of methods, leading to incorrect results. Especially the flow-through method, which requires that certain conditions are satisfied in order for the CR estimations to be valid (Filgueira et al., 2006; Riisgård, 2001). For accurate clearance rates, food passing the chamber must be completely available to the animal, chamber geometry should minimise recirculation and food should be completely retained by the animal (Filgueira et al., 2006; Petersen, 2007; Riisgård, 2001). When these criteria are met, particles that are 100% retained, represent the actual clearance rate (Coughlan, 1969; Riisgård, 2001).

Table 2 Discrepancy in body dry weight specific (excluding tunic) clearance rates for *C. intestinalis* reported from different sources, adapted from Petersen (2007).

Temperature (°C)	Clearance rate (mL min^{-1} g^{-1})	Source
?	72-82[a]	Jørgensen (1949)
12	27[a]	Goldberg et al. (1951)
16	1.6-63[a]	Kustin et al. (1974)
15	58	Fiala-Médioni (1974)
15	72	Fiala-Médioni (1978b)
10	47[a]-128	Randløv and Riisgård (1979)
15	80	Robbins (1983)
15	118[a]- 198	Petersen and Riisgård (1992)
13-17	140	Petersen and Svane (2002)

(a): Weight specific clearance calculated on total dry weight.

According to Riisgård (2001) the biggest obstacles have in previous studies have been a result of suboptimal chamber geometry and incorrect flow, that causes CR dependence of flow (Fig. 2). In order to account for these pitfalls, Filgueira et al. (2006) offered a flow-through validation method. The geometry of the chamber, should ideally direct the flow towards the inhalant, whilst guiding the flow away from the exhalant. Secondly the flow rate should be high enough for the clearance rate of the animal to be independent of the flow. A plot between CR and flow easily reveals at which the point the CR deviates from the flow (Filgueira et al., 2006; Larsen and Riisgård, 2012; Petersen and Riisgård, 1992; Riisgård, 2001).

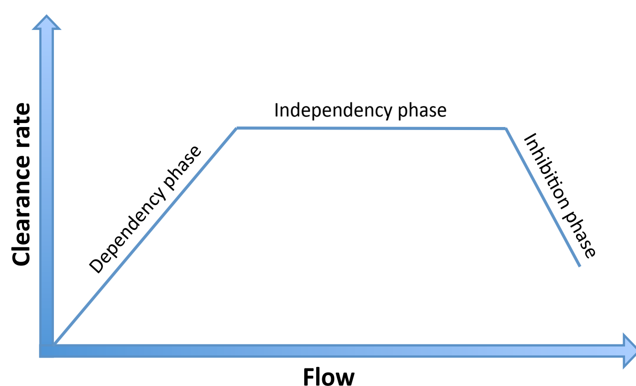

Figure 2 Performance of an optimal geometry chamber used to determine CR in filter feeders. In the dependency phase CR is proportional to flow, as the flow increases CR measurements will reach a plateau, and become independent of flow, only these measurements in the independency phase represent actual filtering capacity. Lastly the inhibition phase is reached when physical stress inhibits feeding.

Another cause for variation of results is studies with multiple individuals in the same chambers. *C. intestinalis* can squirt and eject particles, which is likely to happen when individuals are in close proximity. Squirting leads to a temporary increase in particle concentration, thus masking "true" clearance rates and retention efficiency. Similarly incorrect measurements can be obtained when inhalant and exhalant flows are allowed to mix prior to analysis (Filgueira et al., 2006; Riisgård, 2001).

Furthermore most studies on *C. intestinalis* have used algal cells of a narrow particle size (Carver et al., 2006; Fiala-Médioni, 1978a; Goldberg et al., 1951; Joly et al., 2007; Jørgensen et al., 1984b; Kustin et al., 1974; Petersen, 2007; Petersen and Riisgård, 1992; Petersen and Svane, 2002; Randløv and Riisgård, 1979; Robbins, 1984; Sigsgaard et al., 2003; Zhang and Fang, 2000). A wider range of particles sizes could reveal currently unknown feeding responses. To date, no studies have quantified individual filtration of *C. intestinalis* with a flow-through method that has undergone a flow-validation.

1.5 Aim of study

Our primary goal was to investigate whether *C. intestinalis* ingested fish feed, and to describe the filtration response over time when subjected to different fish feed concentrations. The secondary goal was to develop a method for accurate filtration measurements in individual animals and assess its potential as a bio filter in a IMTA system.

To accomplish our primary goal, we will analyse *C. intestinalis* intestine, given fish feed, algae and seston with a qPCR bioassay in order to establish that fish feed is ingested. For investigating feeding responses over time, we employ two "indirect" methods, based on the rate particles are cleared from a volume of water in a given time. First we obtain filtration data with the use of the clearance method on multiple animals. To reach our second goal we develop a flow-through method on chambers fitted with individual animals, to account for possible confounding factors, as well as inter-individual feeding responses. With measurements from an electronic particle counter, we analyse the filtration data from both methods, in order to compare differences in feeding responses and to validate our setup.

Our goals lead to the implementation of these three experiments;

1. Experiment one, cohort feeding using the clearance method. Where groups of ten animals are fed fish feed with different concentrations to establish mean cohort response.
1. Experiment two, fish feed detection study using quantitative polymerase chain reaction methods (qPCR). Where groups of *C. intestinalis* are fed mixtures of fish feed, algae and seston, subsequently dissected and intestine samples were analysed.
3. Experiment three, individual feeding using the flow through method. Individuals are subjected to different flows and fish feed concentrations to characterise individual feeding response and validate the experimental chamber.

Our hypothesis is that *C. intestinalis* will eat fish feed similarly to natural seston and algae. We expected that clearance rates will be lowered in response to high concentrations, and that time will not influence feeding. Retention efficiency is expected to be within previously reported values, and particles over 2μm should be completely retained. Furthermore we expect that *C. intestinalis* will be able to function as an extractive particulate bio filter in an IMTA system.

2 Materials and methods

The measure of variance referred to as "±" is in this text standard deviation, unless specified otherwise.

2.1 Preparation and equipment

2.1.1 Collected animals and pre-treatment

All *Ciona intestinalis* used in this study were collected from Scalpro AS outside Bergen (60°31'05.5"N, 4°54'19.4"E). The animals settled on PVC plates attached to ropes hanging on a long line according to Troedsson & co-workers (Troedsson et al., 2011), which were deployed in April 2015. The animals were collected either individually by hand, or by collection of single plates for all experiments conducted here. After harvesting, the animals were placed in a race-way system with natural unfiltered seawater, ambient temperature and salinity. The animals were collected and transported (60min) in plastic containers to Espegrend Marine Biological Station Espelandsvegen 232, Blomsterdalen, Norway (60°16'10.6"N 5°13'22.5"E). The animals were acclimatized in a wet-lab facility with a flow of unfiltered natural seawater from 40m.

2.1.2 Particle counter

In order to determine clearance rates, retention efficiency and particle characteristics; water samples were analysed for particles with an electronic sampler, Pamas GmbH field laser particle counter (Model S4031GO). The counter measured technical triplicates for every sample, which was set for 10mL. The counts from the control chambers were compared to the counts from the experimental (*C. intestinalis)* chambers. 32 size channels were selected from 1-55µm; 1, 1.50, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 30, 35, 40, 45, 50, 55µm. However only particles between 1-10µm were used to calculate filtration.

2.1.3 Weight

The wet weight and length of collected individuals were determined with a measuring scale (KERN KB 3600-2n) and a 30cm ruler before and after each experiment. Because *C. intestinalis* "squirts" out water from the buccal chamber when stimulated, the length was measured before wet weight, in order to allow most of the water to be released. At the end of each experiment, individuals were frozen (-20°) for further analysis. Dry weight and ash-free dry weight (DW and AFDW, respectively) was measured for the animals in the individual experiment. The dry weight was determined by placing the frozen individuals in a heating cabinet (Fermarks type TS 8024™) at 60°C for three days. Dried samples were measured using measuring scale (Sartorius scale type ME235 Genius™) and directly transferred to an incinerator (Naberthern IFM 3201), which was set to 500°C as maximum temperature in order to determine organic content. The samples were then transferred to a desiccator containing silica gel until they reached ambient temperature, and were reweighed. The amount of fish feed added to each experiment was determined using a measuring scale (Mettler AC88 Delta Range®).

2.1.4 Fish feed concentrate protocol

The fish feed used in this study was Spirit Ørret 300 from Skretting (4.5 mm pellets), and the product certificate is supplied in Appendix A. To prepare the concentration prior to each feeding experiment, a protocol was developed to ensure a homogenous solution of fish feed in the experimental container (Table 3). Commercial fish feed pellets were weighed and ground by a mortar and blended in an OBH Nordica 6656 blender together with seawater.

Table 3 Preparation steps for fish feed concentrate used in the feeding experiments, ensuring that a homogenous mixture of fish feed was available to the animals.

1. Add fish feed (g) to 100mL seawater (1min)
2. Mix in blender (30s)
3. Wait 5 min
4. 30s blending
5. Wait 5min
6. 30s mix
7. Add the concentrate

2.1 Experiment one; cohort feeding study using the clearance method

2.1.1 Experimental setup

In order to secure the animals orientation in the experimental tank and allow them to filter without physical disturbance, the animals were glued with cyanoacrylate (Super Glue precision®, Loctite) to small pieces of Velcro, which were fastened to a corresponding line at the bottom of a holding tank for acclimation (30.06.15). The animals were then transferred to the experimental tanks overnight (02.07.15) and were given unfiltered seawater at 3.3l/min.

Four identical aquaria (84L) were filled with 10 *C. intestinalis* each. The multiple clearance rate experiment was conducted by the clearance method, essentially as described by Riisgård (2001), but food was added in one batch and incubated for 4 hours. Furthermore the exponential clearance method is expected to account for recirculation (Coughlan, 1969). In order to use all our measurements, and discover changes over time, we calculated RE and CR at 30min intervals, rather then relying on two measurements: the initial concentration and final concentration (Cranford et al., in press).

Between sampling days, animals received unfiltered seawater by flow-through. Tanks #1-3 were triplicates, receiving the same amount of fish feed, while Tank #4 did not receive any fish feed and served as a control. All the tanks were fitted with aeration stones on the bottom to keep the food suspended in the tank as well as oxygenation for *Ciona*. Seawater temperature remained relatively constant 10 ± 0,1°C, but increased slightly during the experiment (+1°C). Salinity was stable at 31 ± 0,05 ppt. Although the individuals used during these experiments came from the same cohort, there was some variation in size (10,5 ± 3,86cm long and had a wet weight of 33,2 ± 22,1g; see Appendix F).

The samples were taken in the middle of the water column, close to the aeration bubbling, since particles could stick to the surface lipid layer and therefore generate elevated numbers. Samples were collected in cylindrical plastic containers (300mL), and were homogenized prior to analysis (turned upside down a few times). Following, the analysis, the containers were rinsed with freshwater and prepared for the next sampling.

In order to describe feeding responses between very high and very low particle concentrations, the experimental groups were given a concentration of fish feed that increased exponentially each sampling day. The fish feed amount, given to the three tanks (T1-3) were; 0.001, 0.01, 0.1, 1 and 10g (Table 4). Before the experiment began, faecal pellets and sediment was removed from the bottom of the aquaria, and animals were left undisturbed for at least 60 minutes.

Table 4 Overview of experimental conditions and fish feed amount tested during the sampling dates in the cohort study.

Date/time	Temperature (°C)	Salinity (ppt)	Fish feed amount (g)
03.07 – 08:45 – 19:45	10.1	30.9	0.001
04.07 – 15:30 – 01:15	10.1	31.0	0.01
05.07 – 09:30 – 18:40	10.2	30.9	0.1
06.07 – 10:25 – 22:30	10.4	30.8	1.0
07.07 – 10:20 – 21:45	10.3	30.8	10.0

Each feeding experiment was run for a total of eight hours and water samples were collected every 30 minutes. After four hours the animals were transferred to a temporary holding aquaria with flow through. In order to

account for sedimentation, an identical experiment was rerun without animals after the initial experiment with animals. In between, the tanks were "flushed" at least 30 minutes with unfiltered seawater to remove excess sediment from the previous trial, before they were refilled. The measurements were during the data analysis corrected for sedimentation by estimating the proportion of particles settled, as opposed to cleared by the animals. This proportion was then removed from the measured end count in the experimental treatments.

Before the next experiment, the health status of each individual animal was assessed. If an animal appeared to not feed and react to stimuli, they were replaced before the next experiment. In order to assure a homogenous mixture of the fish feed, the tank was thoroughly mixed for 30 min before the animals we added.

2.2 Experiment two; fish feed detection using qPCR

The experiment was conducted in order to determine whether all animals were eating fish feed, and if the addition of algae influenced ingestion. It also served as a validation of methods, by confirming whether fish feed were detectable in intestinal samples from *C. intestinalis,* and not in unfiltered seawater and several algal species.

2.2.1 Experimental setup

The animals were transferred to Espegrend 18.03.2015, were they were given unfiltered seawater 3,1 ± 1,1L/min, at 7 ± 0,3°C, and salinity at 30,4 ± 0,2 ppt during the experimental period. 40 animals were selected roughly equal in size (average length 13.4 ± 2.28cm and wet weight 57.5 ± 17.4g) and equally divided into four identical cubic aquaria (v=51L) representing four separate treatments (Fig. 3). Each tank was fitted with an aeration stone on the bottom, located in the opposite corner from the runoff. The experiment followed the clearance method, essentially as described by Riisgård (2001), but without sustaining the particle concentration.

First a sample was taken, to measure the background particle concentration. Soon after, the experiment started when the water was shut and flow-through seized. After the flow was terminated, the respective treatments were added (Table 6). The fish feed concentrate was prepared as previously described in section 2.1.4. Samples were taken in the middle of the water column every 30 minutes for four hours per treatment. Directly after each sampling day, the animals were removed and the tank was flushed, emptied and refilled for the following day.

Table 5 Overview of tank and respective diet given once every sampling date in the fish feed detection study.

Tank #	Concentration
1. Control	Seawater
2. Algae	15.000cells/mL
3. Algae + fish feed	7.500cells/mL + 1g fish feed
4. Fish feed	2g fish feed

Tank #1 was only given unfiltered seawater from a 40 m inlet at Espegrend and acted as a control in the experiment. Tank #2 was fed an algal solution (0,14L estimated to yield 15.000 cells/mL), Tank #3 was fed a mixture of algae (7.500 cells/L) and 1 g fish feed and finally, Tank #4 was fed 2 g fish feed (Table 6). The animals in each treatment received their respective feed once per day, from 20-25.03, also with the use of the clearance method.

The microalgae solution consisted of a mixture of primarily four species of algae; the single celled algae *Isochrysis sp.* (T-iso strain CCAP 927/14), *Pavlova lutheri* (CCAP 931/1), *Chaetoceros muelleri* (CCAP 1010/3) and *Skeletonema costatum* (CCAP 1077/5) at a ratio of 3:2:3:1. Furthermore small quantities (1%) *of Chaetoceros calcitrans f pumilum* (CCAP 1010/11), *Tetraselmis suecica* (CCAP 66/4) *and Rhodomonas sp.*. The algal solution was produced from a continuous algal production unit (Seasalter Systems) at Scalpro AS, producing algal concentrations at 2.103 cells μL^{-1}. The culture was held in a climate-regulated room, at 14°C and in a 12/12 photoperiod from artificial light armoires during the experiment.

Figure 3 Picture of the experimental setup in the fish feed detection study. Water samples were collected directly in the aquarium near aeration.

2.2.2 Sampling

Dissection sampling was carried out 26.03.15 after the *C. intestinalis* had been feeding for 5 days. The animals were weighed and measured for length. Prior to dissection they were rinsed with freshwater to avoid contamination of the feed during the dissection. Roughly 1 cm of the intestine was dissected from adjacent to the end of the stomach, for all the 40 animals. The intestine samples weighed an average of 0.281 ± 0.098g. Sampling video available at (https://drive.google.com/file/d/0B-A4xvw9SMaIbVBkRXdBeWFaZ2s/view?usp=sharing).

In order to homogenize the dissected intestine samples, they were placed in a preheated (56°C) 1.5mL Eppendorf tube with 180µL of lysis buffer (ATL, QIAGEN®). 20µL of proteinase K was added and the sample was shaken, left over night at 56°C and then frozen (-20°C) for further analysis. In order to disintegrate the control fish feed pellet, we added 10mM tris-buffer (pH8).

2.2.3 DNA extraction

Extraction of genomic DNA was conducted using the DNeasy® Blood & Tissue Kit from QIAGEN, essentially according to manufacturers recommendations (Appendix D), with a slight modification to maximise DNA yield by repeating step 6 (adding AE buffer and centrifuging at 8000 rpm for 1 minute). Both the extraction and TaqMan detection is nearly identical to Ray et al. (2016).

Frozen samples in ATL buffer and Proteinase K were liquefied on ice and heated to 56°C. Samples were then vortexed for 2-3s. Next, RNAse (4µL) was added to each sample (QIAGEN kit, 20mg mL^{-1}), incubated at room temperature for two minutes, and vortexed for 15s. Thereafter, 400µL of 1:1 solution with AL buffer (QIAGEN kit) and 96% ethanol were added and vortexed briefly. Elution after washing according to the manufacturers protocol (Appendix D), was conducted by adding 2 x 100µL 56°C elution buffer (QIAGEN kit). Following DNA extraction, three aliquots (33µl) were prepared and frozen in separate freezers; -80, -20 and -20°C awaiting qPCR analysis.

Before further analysis, the genomic DNA quantity and quality was assessed using a spectrophotometer NanoDrop ND 1000 (Thermo Scientific,

Nanodrop1000®) and a fluorometer (ThermoFisher Scientific, Qubit® 3.0). This DNA extraction was chosen because of high reproducibility and efficiency in previous studies (Nejstgaard et al., 2008; Ray et al., 2016; Troedsson et al., 2009).

2.2.4 TaqMan detection of soy RuBisCO

The probe and primer information is supplied in Appendix C. The analysis was accomplished with a previously developed DNA RuBisCO primer for Soy (Sanden et al., 2011). The probe used in this study was labelled with the fluorescent reporter dye 6-carboxyfluroescin (6-FAM) in the 5´-end and with the Black Hole Quencher-1 at the 3´-end, and was cleaned with HPLC. The primers were cleaned by desalting and were purchased freeze-dried from Sigma-Aldrich.

In preparation for the qPCR, a dilution of 1:10 DNA/Buffer was considered suitable. All analyses were done in triplicates. Samples were prepared for qPCR with 4µL of template, diluted with 1µL primer (soyRubisco forward), 1µl (soyRubisco reverse), 0.5 µl probe, 1µl BSA (Bovine Serum Albumin), 2.5µl H_2O and 10µL SssoAdvanced™ (Universal Probes Supermix, Bio-Rad) in each well.

The prepared samples were inserted into a CFX96 Real-Time System (Bio-Rad), and run through the cycle protocol found in Appendix B.

Molecular methods are described in further detail in Sanden et al. (2011) and Ray et al. (2016). Furthermore this assay was chosen, as previous use in sediment analysis close to fish farming showed a high specificity for Soy RuBisCO (per. comm. Skaar, 2016).

2.3 Experiment three; individual feeding study using the flow through method

2.3.1 Development of the experimental setup

As the qPCR soy-detection results suggested that not all animals were ingesting fish feed, we developed a system to detect inter-individual feeding. In order to construct a flow-through chamber which gives accurate measurements, it is necessary to ensure that the food given was available to the animal and homogeneously mixed (Riisgård, 2001). In addition it is crucial to minimise recirculation in the experimental chambers (Filgueira et al., 2006). To accomplish this, a series of trials with dye and milk were conducted, by characterising the hydrodynamics of the chamber. The trials resulted in a number of improvements, which lead to a continuous flow-through system for individual animals.

Firstly, we modified soda-bottles, which gave a conical shaped chamber, giving us a circular flow and ensuring that particles were accessible to the animal. Secondly we oriented the animals upside down, which contributed to sustain their position and avoid contact with internal surfaces during high flows. Such physical stimuli could otherwise lead to squirting and reduced feeding. Furthermore, the animals orientation also contributed in avoiding recirculation, as the incoming flow passes the inhalant siphon before it passes the exhalant; thus the water exiting the animal is transported away from the inhalant, thereby avoiding the possibility for recirculation by exhalant water (Fig. 4A). Further on we noticed that tubes stuck directly in to the chamber gave a concentrated flow through the centre of the chamber. By using a Eppendorf tube (1.5mL), fixed at the end of the inserted tube in the chamber, and melting holes in a helix shape, incoming water was forced out horizontally, and gave a properly mixed flow through the chamber (Fig. 4B). After testing this system, we noticed that some animals had closed siphons for prolonged periods, and found that minute bubbles were intermittently transported from the overhead tanks aeration to the chambers and interfering feeding. By tilting the chambers, bubbles passed along the chamber walls and bypassed the animals, and resulted in resumed feeding (Fig. 4C). Month long incubations and particle measurements in the system followed, with no noticeable difference in mortality compared to other animals from the same

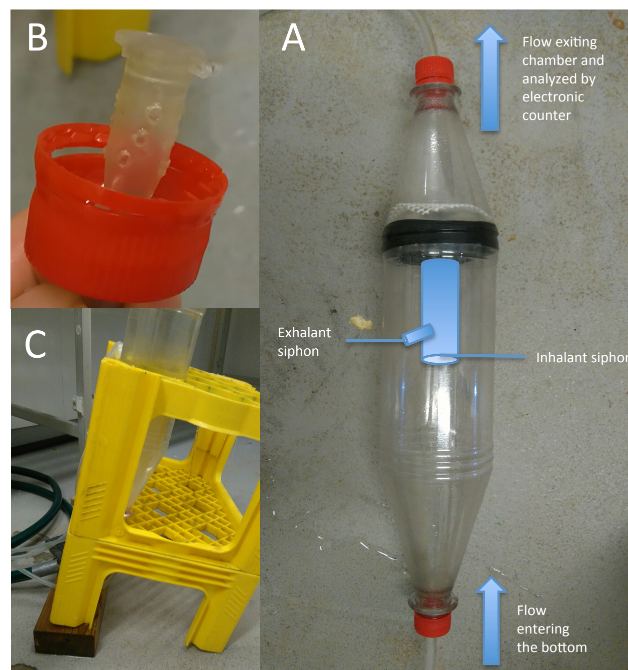

Figure 4 Pictures of the development of the individual chambers. A depicts the flow through the chamber with the orientation of the individual reducing the possibility of recirculation. B shows the modified Eppendorf tube (1.5mL), which acted as a diffusor and contributed to homogeneously mix the fish feed solution throughout the chamber. C depicts the slight tilt the individual chambers placed in a rack, to avoid bubbles, which intermittently could disturb feeding.

cohort. Furthermore milk and dye tests revealed that animals were feeding, and later confirmed by measurements from the electronic particle counter. Therefore we are convinced that the system provides accurate measurements by circumventing common constrictions in the flow through method, we are also confident from long incubations with low mortality, that the system is not suboptimal for the animals.

2.3.2 Experimental setup

The individual experiment was conducted with the indirect flow-through method (Coughlan, 1969; Filgueira et al., 2006; Riisgård, 2001), compared to the clearance method, the flow-through method delivers an immediate response in filtering, and was used in 93 % of 133 peer-reviewed studies on bivalve filtration reviewed by Cranford et al. (2011). Essentially by measuring the difference in particle concentration between chambers with, and without animals. We operated ten cylindrical 2L chambers; three did not contain animals and served as controls, as well as to account for any chamber effect, while the remaining seven contained individual *Ciona* (Fig. 5). The seven animals were on average 10,2 ± 1cm long and had a wet weight of 14,49 ± 2,05g (see Appendix E). Each chamber was fitted with an individual valve, which was used to control the flow for each animal. The water exiting the chamber was measured for particle counts and size distribution by the electronic counter.

The animals were placed in the chambers a day prior to experimental start, and subjected to unfiltered seawater for 20 hours at a flow of 400mL/min. The pressure was kept constant during the different flows by constantly overflowing the overhead tank (Fig. 5A). Background samples of the unfiltered seawater (i.e. no fish feed added), were analysed for particle counts 60-120 min prior to the addition of fish feed.

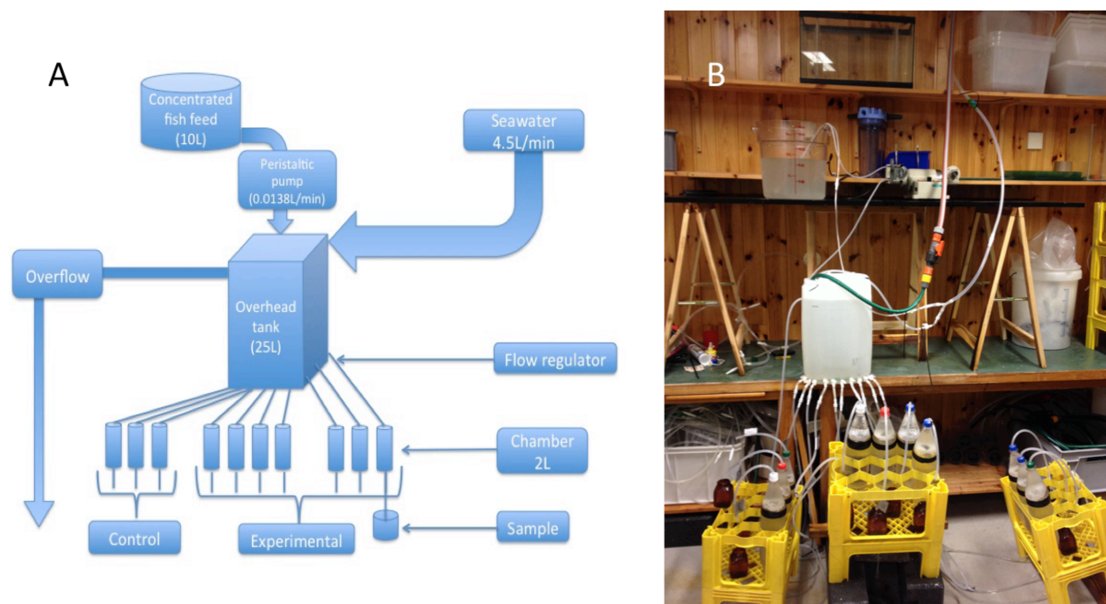

Figure 5 Graphical representation (A) and picture, of the experimental setup (B). Unfiltered seawater was mixed with concentrated fish feed (concentration dependent on experiment) in the overhead tank. In order to avoid pressure changes and thus flow in the chambers, seawater pressure was set to always overflow (4.5L/min) from the overhead tank. The flow of the mixed fish feed and seawater was then individually adjusted with a plastic valve for every chamber. Both the overhead and concentrated fish feed tank were aerated from the bottom.

Each sampling day a distinct quantity of fish feed was added in the concentrated fish feed tank, and constantly fed to the overhead tank by the peristaltic pump (Fig. 5A). This steady supply of fish feed concentrate was set to deplete simultaneously with the sampling, to ensure that all the fish feed was fed (around 10hours). We started with the highest fish feed concentration on the first sampling day, which decreased roughly exponentially (Table 6).

10 different flows were tested per fish feed amount (Table 5), in order to determine at which flow clearance rates became independent from flow (Riisgård, 2001). If the flow is too low the animals does not filter maximally, and clearance rates do not represent actual filtration (Filgueira et al., 2006; Riisgård, 2001). A protocol to assess optimal flow geometry was adapted from Filgueira et al. (2006), and samples which were not taken in the flow independent phase (Fig. 2) were omitted from the analysis.

Table 6 The flows tested, each flow was set for one hour to acclimatise the animal. Then a sample was taken and the flow was changed, testing all ten flows each sampling day. The order in which the flows were set, were randomized prior to start.

Flow (mL/min)
50
75
100
125
150
175
200
250
350
450

The flow rate was determined by timing the filling of a set volume of water exiting each chamber. All the chambers were subject to the same flow for 1 hour, when a sample was taken in a 50mL plastic beaker from each chamber. After sampling, the flow was adjusted for the next sample, and the order of the flows were randomized prior to sampling.

Table 7 Overview of experimental condition during the individual experiment. Fish feed concentration represents in this case the fish feed quantity undergone the concentrate protocol.

Date/time	Temperature °C	Salinity ppt	Fish feed added (g)
22.09 – 11:45 – 02:00	11.3	31.5	35
23.09 – 13:30 – 00:30	11.4	31.7	3.5
24.09 – 11:47 – 23:00	11.5	31.0	0.36
26.09 – 10:30 – 00:30	11.4	31.1	0.038
27.09 – 10:30 – 00:00	11.5	31.0	0.003

2.5 Formulas

When calculating ash free dry weight (AFDW), the ash weight remaining after incineration (inorganic) is subtracted from the dry weight measured prior to incineration;

$DW-AW$, Equation 1

where DW is Dry Weight and AW is Ash Weight.

Clearance rates of a chamber following the clearance method can be calculated as follows (Riisgård, 2001);

$CR_{Multi} = (V/_{n*t}) * \ln(C_{t0}/C_{t1})$, Equation 2

where CR_{multi} is clearance rate of an individual in a chamber without flow, V is the volume of the chamber, n is the number of animals, t the duration between measurements, C_{t0} is the concentration of particles at an initial time, while C_{t1} is the concentration of particles after a given time t. Used in cohort experiment.

From Coughlan (1969) the formula for retention efficiency of a filter-feeder in a chamber without flow is;

$RE_{Multi} = \ln(C_{t0}/C_{t1})$, Equation 3

RE_{multi} is the retention efficiency of the number of animals in the chamber, C_{t0} is the concentration at an initial time 0, while C_{t1} is the concentration after a given time t. Used to determine retention efficiency in the cohort experiment.

The clearance rate of an individual in a flow through chamber can be calculated with the following formula (Riisgård, 2001);

$CR_{Ind} = (1 - (PC_E / PC_C)) * F$, Equation 4

where the PC_e is the particle concentration exiting the experimental chamber, while PC_C is the particles concentration exiting the control chamber. measurements are non-standardised and F is the flow rate (mL/min).

The retention efficiency of a flow through chamber may be found by this formula (Strohmeier et al., 2012);

$RE_{Ind} = 1 - (PC_E / PC_C)$, Equation 5

where PC_C is mean particle count exiting the control chambers and PC_E is particle count exiting the experimental chamber.

The formula for predicting filtration rate found by Petersen and Riisgård (1992) in *C. intestinalis* with total dry weight (g) is;

$F = 118 (W_g)^{0.68} + 1.46 (T-15)$, Equation 6

where F is flow in mL/min, W_g is total dry weight in grams and T is temperature in °C.

Standardisation of clearance rate to g^{-1} total dry weight was adapted from the allometric equation from Filgueira et al. (2006) by changing mussel length to *C. intestinalis* dry weight.

$CR_{std} = CR_{exp} * (DW_{std}/DW_{exp})^{0.68}$, Equation 7

where CR_{std} is the standardised clearance rate, CR_{exp} is the clearance rate measured during the experiment, DW_{std} is the dry weight which is standardised to and DW_{exp} is the dry weight found in the study. The weight exponent is the same as found by Petersen and Riisgård (1992)

The volume of particles was calculated to a spherical shape from the diameter by the formula;

$V = 4/3\ \pi\ r^3$, Equation 8

where π is the mathematical constant relating the circumference to its diameter, r is the diameter.

2.6 Statistical analysis

All analysis was performed with the statistical software RStudio, version 0.99.486 (R Development Core Team 2013, http://www.r-project.org), and with Microsoft Excel (Mac 2011, Version 14.5.9). The significance level for all analyses was set to 0.05, and only significant statistics were reported. Where a significant main effect was detected, Tukey pairwise comparisons among levels were conducted using the "multcomp" package in R (Hothorn et al., 2008). Total particle counts were tested with a one-way ANOVA, and post-hoc Tukey, if a significant main effect was found. Results were presented with the R package "ggplot2" (Wickham, 2009).

2.6.1 Clearance rate

CR was standardised to 1g total dry weight and used as response variable. In addition an identical analysis was performed with power transformed data (Box-Cox) for normalisation of data (Box and Cox, 1964). The clearance rates (CR_{std}) with individual chambers for each animals was analysed by constructing a repeated measures ANOVA (LME) with individual chambers as random effects, from the "nlme" package in R (Pinheiro et al., 2015). In the cohort study, a similar model was used, but instead of individuals, replicate tanks were set as random effects. Visual inspections of residual plots did not reveal any obvious deviations from the homoscedasticity or normality. Variables and interactions were backwards selected, starting with the variables of interest and removing non-significant interactions or variables. In order to test for differences within treatments a post-hoc (Tukey HSD) test was applied. Negative clearance rates were set to zero. After obtaining the final model, an identical model without the temporal correlation term *corAR1* (Box et al., 2015) were tested against each other, and the model with the lowest AIC was chosen.

The formula specified in R was as follows for the multiple and individual model of standardised clearance rates;

cohort <- lme(cr48~feed, random=~+1|tank, cor=corAR1(), na.action=na.omit)

For the individual study;

individual <- lme(s.cr~feed+time+feed:time, random=~+1|individual)

Where s.cr is the response variable representing the standardised clearance rate in mL/min, time is minutes duration since fish feed was added, and feed is the amount of fish feed given to the animals. The argument "*na.action=na.omit*" specifies that NAs in the dataset are omitted from the analysis.

2.6.2 RE

The calculation of standardised retention efficiency (RE_{std}) was done similar to Strohmeier et al. (2012) and Riisgård (1988). The highest retention efficiency of the measured size classes were standardised to 100%, and the remaining size classes were proportionately scaled for each animal, and for each feed supplied. Likewise the multiple study was standardised for each tank of ten animals, and for each sampling day (feed). It is necessary to standardise for each treatment because both accuracy of measurements and RE is dependent on particle counts, and because RE is a relative measurement, comparison between feeds cannot be directly extrapolated otherwise.

Standardised retention efficiency (RE_{std}) was arcsin transformed in order to normalize the binomial data (Berg et al., 1996). For both the multiple clearance study and the individual flow-through, we performed a linear mixed effects analysis (LME) model with, from the "nlme" package (Pinheiro J. et al., 2016). Factors included in the model were found by backwards elimination; starting with the most advanced model and stepwise removing non-significant variables and interactions. Random effects were tank and chamber for the multiple and individual studies respectively. Visual inspections of residual plots did not reveal any obvious deviations from the homoscedasticity or normality. In order to test for differences within treatments a post-hoc (Tukey HSD) test was applied. After obtaining the final model, an identical model without the temporal correlation term *corAR1* (Box et al., 2015) were tested against each other, and the model with the lowest AIC was chosen.

The formula specified in R was as follows for the cohort and individual study was;

cohort<-lme(a.sf.re~time+feed+diameter+time:feed,random=~+1|tank, cor=corAR1()),

individual<-lme(a.sf.re~ time+ feed+diameter+time:feed, random=~+1|individual),

Where "a.sf.re" is the arcsine transformed standardised retention efficiency of particles. "time" is duration in minutes since the animals were given fish feed, "feed" is the amount of fish feed given in grams, and diameter is the particle diameters measured.

2.6.3 Volume estimates

Particles were assumed to be spherical when estimating volume. Therefore calculations resulted in the equivalent spherical diameter (ESD), of particles measured. Diameter size was set to the median value for each size-channel and then multiplied with counts. For example, the size channel 1.5-2 µm, a diameter value of 1.75 was multiplied with the counts found in the 1.5-2 µm size range.

3 Results

3.0 Sampling error from the electronic particle counter

In order to quantify the reliability of the electronic particle counter, the sampling error was assessed by calculating the coefficient of variation (CV), of the three technical replicates measured per sample. Thus the CV describes the variability within the same sample. The results indicated that the measurements became increasingly unreliable when measuring large particles, but dropped at high fish feed concentrations (Fig. 6). Suggesting that the accuracy is dependent on the amount of particles counted in each size bin. Hence large particles with few counts were heavily influenced by variability. For particles under 10µm the CV was below 10% for seston (fewest particles) (Fig. 6). Considering these results we chose to use particles that were not seriously influenced by variability (under 10% CV), and exclusively used particles under 10µm to calculate RE (1-10µm) and CR (4-8µm).

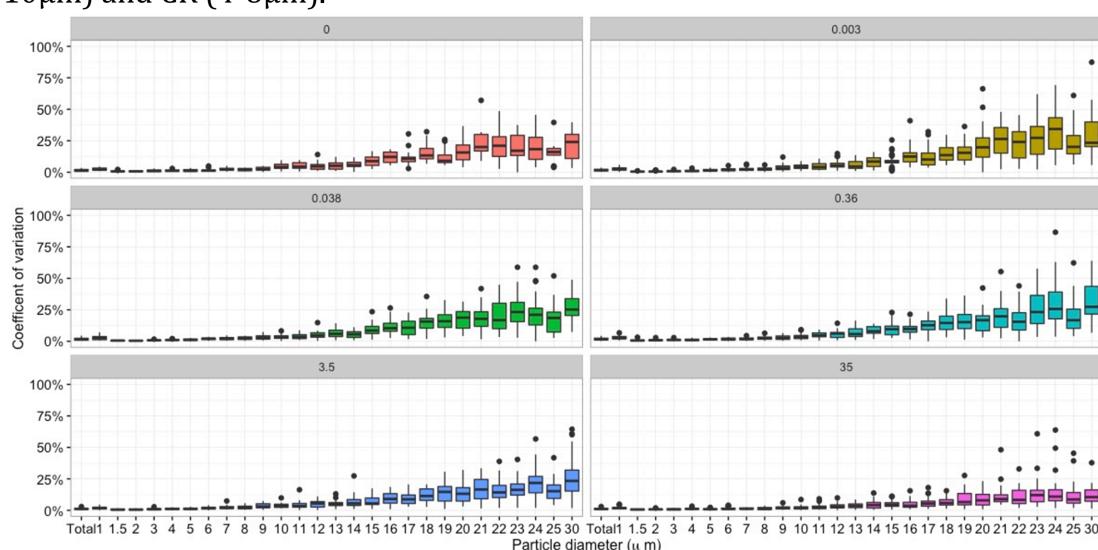

Figure 6 Boxplot of the percent sampling error (CV) between three subsamples (10mL) in the particle counter in particle size bins between 1-30µm and Total (1-200µm). The box plots are divided into the treatments given seston and different amount of fish feed. The particle counts were used to calculate RE between 1-10µm and CR from 4-8µm in the individual study.

3.1 Experiment one; cohort experiment using the clearance method

3.1.1 Background water and fed concentrations in the cohort study

In the cohort experiment, salinity remained stable at 31 ± 0.03ppt, while temperature was 10.2 ± 0.1°C during the five day experiment. As each treatment was conducted on separate dates, it was necessary to account for possible variation in particle concentration from the unfiltered seawater, consequently we characterised the initial concentrations prior to adding fish feed.

The particle counts in the background water varied between 15.000 to around 18.000 particles/mL during the five days. On day 3, the background water had a significantly higher total concentration than the other days (Tukey HSD, $p<0.0001$). Conversely the background on day 1 was significantly lower compared to the other days, except day 4 (Tukey HSD; $p<0.0001$). However total particle counts of background water on day 2, 4 and 5 did not differ significantly.

When fish feed was added, particle concentration significantly increased in a linear fashion on day 3 (0.1g), day 4 (1g) and day 5 (10g) (Tukey HSD, $p<0.0001$), with the highest particle counts when given 10g of fish feed (Fig. 7). As the concentration increased almost linearly, the background particle concentration probably had a negligible effect on total particle concentration during the cohort experiment (Fig. 7). Fish feed added on day 1 (0.001g) and 2 (0.01g) had no significant effect on particle concentration and did not significantly differ from background particle concentration (ANOVA, Tukey HSD).

Furthermore some particles larger than 2µm in diameter, may have disintegrated into smaller ones during the four-hour settling experiment, as there was an increase in percentage of small particles, while a reduction of larger ones (Appendix K). Nevertheless this was corrected for when calculating the RE and CR.

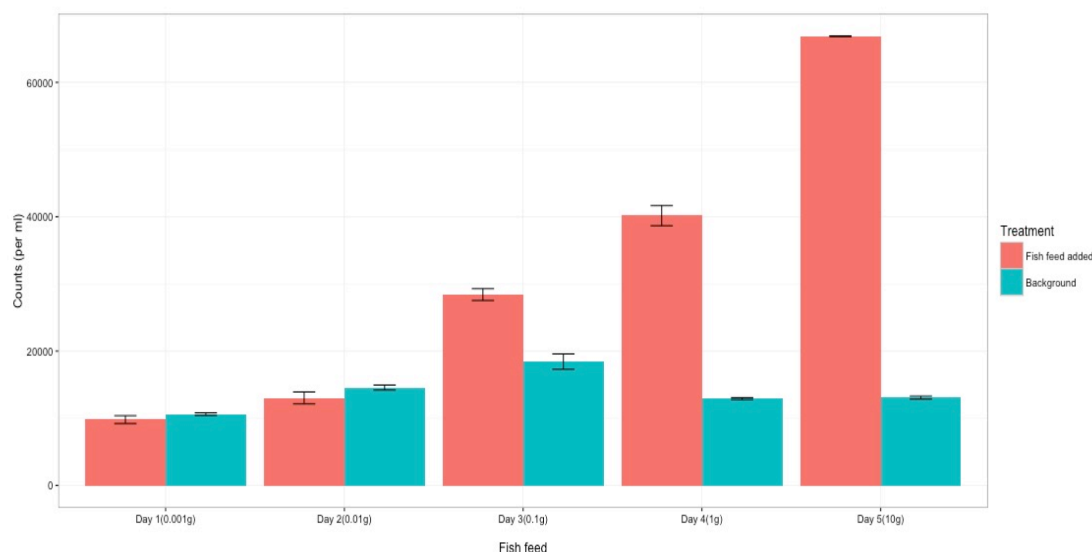

Figure 7 Mean particle count of all sizes (1-200µm) per mL prior to adding fish feed ("Background") and 30 minutes after adding fish feed ("Fish feed added") on all the five sampling dates during the cohort study. Error bars represent standard error.

3.1.2 Retention efficiency of multiple animals using the clearance method

In order to calculate clearance rates, it is necessary to base calculations on 100% retained particles. To ascertain how efficient *Ciona* is at retaining particles of different sizes, we measured the standardised retention efficiency (RE_{std}) for ten animals in three tanks over 4 hours for 5 days, given different amounts of fish feed. Particle retention quickly reached 100% in particles over 2μm (Fig. 8). The smallest range (1-1.5μm) varied in RE_{std} between 0-100%, but tended to be between 50-75% (Fig. 8). From the linear mixed effects model and the following Tukey HSD post-hoc test, particles from 2-10μm had a significantly higher RE_{std} than 1-1.5μm (Table 8). Retention efficiency in particles over 2μm did not differ significantly (ANOVA, Tukey HSD). Which suggests that particles over 3μm are effectively (100%) retained, and the limit for complete retention is between 2-3μm.

However the RE_{std} shape changed with the addition of fish feed (Fig. 8). The average percent particles removed in the tanks receiving 10 and 1g fish feed was between 0.5 and 8%, compared to 20, 17 and 17% for the tanks given 0.1, 0.01 and 0.001g of fish feed, respectively (Appendix J, Fig. J1). Meaning that in the highest treatment (10g), *Ciona* were barley feeding. For the treatments where no fish feed was added, or the amount added did not change the concentration (0, 0.001 and 0.01g), the RE_{std}, reached 100% for particles between 2-3μm (Fig. 8). Under the high concentration (1 and 10g), maximum RE_{std} was between particle sizes 5-7μm, and smaller particles were less retained (Fig. 8).

However particles between 1-1.5μm in diameter were completely retained on two occasions in the lowest concentrations (0 and 0.001g), probably due to the correction of sedimentation (Fig. 8). Upon analysing results to account for settling of fish feed (without animals), we found that the smallest size class (1-1.5μm) fluctuated inexplicably at distinct measurements and resulted in incorrect retention on these two occasions (see Appendix K).

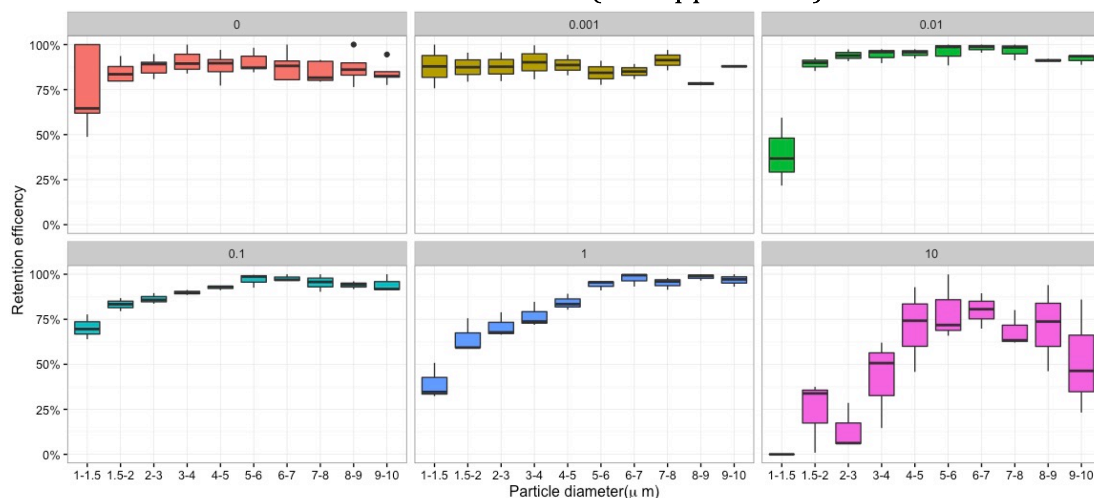

Figure 8 Box plot of standardised RE of all measurement in the cohort experiment between 1-10μm particles, given different fish feed amounts (grams) and seston ("0").

There was only one significant difference of RE_{std} between the fish feed treatments, with the 1g treatment having significantly higher RE than the 10g treatment (ANOVA, p=0.0136). Furthermore in the treatments receiving 1 and 10g fish feed a significant interaction was discovered between time:feed (ANOVA, p<0.039), and retention increased over time. However, overall RE_{std} was not significantly affected over time.

Table 8 Pairwise Tukey HSD post-hoc test of RE_{std} between particles in the 1-1.5μm size range and distinct sizes up to 10μm, calculated from all measurements throughout the cohort experiment.

Particle size	Vs. Particle size	Estimate	Std. Error	z value	P
1.5-2	1-1.5	4.474659	1.530055	2.925	0.0990
2-3	1-1.5	5.307938	1.526284	3.478	0.0176*
3-4	1-1.5	6.083052	1.526302	3.985	<0.01***
4-5	1-1.5	6.921183	1.526302	4.535	<0.01***
5-6	1-1.5	8.086893	1.526302	5.298	<0.01***
6-7	1-1.5	8.081040	1.526302	5.295	<0.01***
7-8	1-1.5	8.006433	1.526302	5.246	<0.01***
8-9	1-1.5	7.912600	1.526302	5.184	<0.01***
9-10	1-1.5	9.426255	1.526814	6.174	<0.01***

3.2.3 Clearance rates of multiple animals using the clearance method

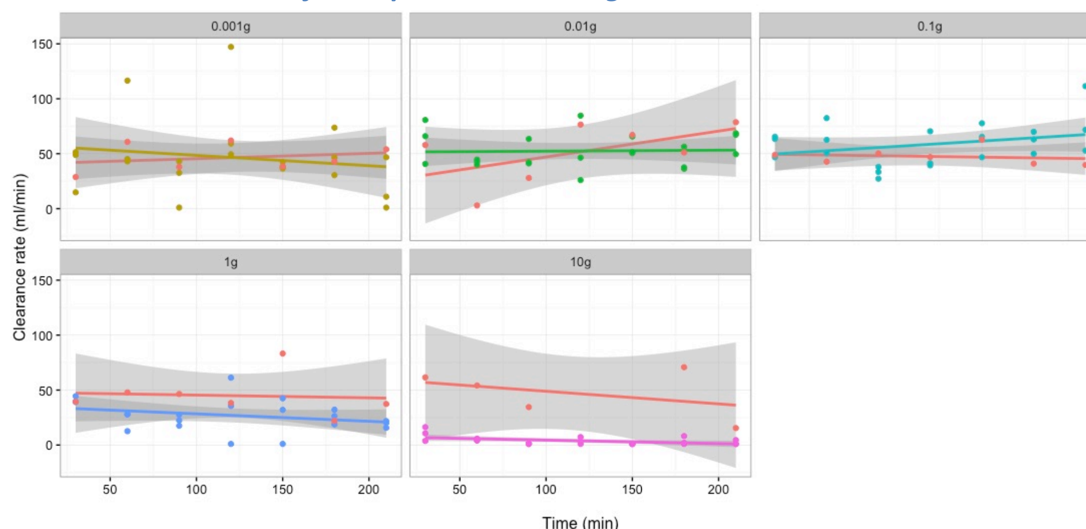

Figure 9 Clearance rates of particles 4-8μm per animal (n=10) given different amounts of fish feed over the duration of the experiment. The red line represents the control tank (unfiltered seawater). Lines are fitted from a linear regression model for comparison.

Clearance rates were calculated from all the measurements throughout the cohort experiment and based on the clearance of particle sizes between 4-8μm. Clearance rates did not change significantly over time during the 4 hours, despite declining particle concentrations from filtering animals (ANOVA, Tukey HSD). Average individual clearance rates varied around 50mL/min in the control treatment and fluctuated above 100mL/min on three occasions (Fig. 9). The mixed effects repeated measures ANOVA found that CR significantly declined in the treatments given 1 and 10g fish feed, though not when given 0.001, 0.01 and 0.1g, compared to the control (Table 9). Also, the animals receiving 0.1g fish feed had a significantly higher CR than the animals receiving 0.001g, but did not differ significantly from the control (Table 9).

Table 9 Pairwise Tukey HSD post-hoc test of mean CR calculated from the 4-8μm particle range between treatments.

Treatment	Vs. Treatment	Estimate	Std.Error	z value	P
0.001	0	-0.7508	1.4991	-0.501	0.99604
0.01	0	0.8653	1.4972	0.578	0.99226
0.1	0	2.6706	1.4972	1.784	0.46985
1	0	-5.6985	1.4972	-3.806	0.00203**
10	0	-14.9115	1.4991	-9.947	<0.001***
0.01	0.001	1.6161	1.0856	1.489	0.66599
0.1	0.001	3.4214	1.0693	3.200	0.01667*
1	0.001	-4.9477	1.0698	-4.625	<0.001***
10	0.001	-14.1607	1.0725	-13.204	<0.001***
0.1	0.01	1.8053	1.0828	1.667	0.54729
1	0.01	-6.5638	1.0666	-6.154	<0.001***
10	0.01	-15.7768	1.0698	-14.748	<0.001***
1	0.1	-8.3691	1.0828	-7.729	<0.001***
10	0.1	-17.5821	1.0693	-16.443	<0.001***
10	1	-9.2130	1.0856	-8.486	<0.001***

3.2 Experiment two; fish feed detection from intestines

3.2.1 Characterisation of the particles fed in the fish feed detection study

In the fish feed detection study, temperature was 7 ± 0,3°C and salinity 30,4 ± 0.2ppt. The total particle concentration in the treatment "Algae & fish feed" was roughly 60.000particles/mL, while the "Fish feed" treatment given 2g fish feed was around 45.000/mL (Appendix H). In the "Seawater" treatment, where animals only received unfiltered seawater (seston), the total particle count was 14.000/mL, which is considered the background particle concentration. Furthermore the treatment given algae reached 18.000/mL (Appendix H).

The particle distribution of algae and fish feed were different in both volume and counts (Fig. 10). When algae were added, there was an increase of a narrow size range 2.5-9µm, typical for algal species. In contrast to fish feed, were all the particle sizes increased, and the highest counts were in the smallest particle range (1-1.5µm) (Fig. 10). Generally, there was an almost exponential decline in particle counts with larger particles, and in both feeds, particle volume was dominated by large particles (>25µm), despite relatively few particles (Fig. 10). Thus fish feed differed from algae by higher concentration and volume of particles in practically all size ranges, compared to a concentrated increase in particles in the 2.5-9µm size range.

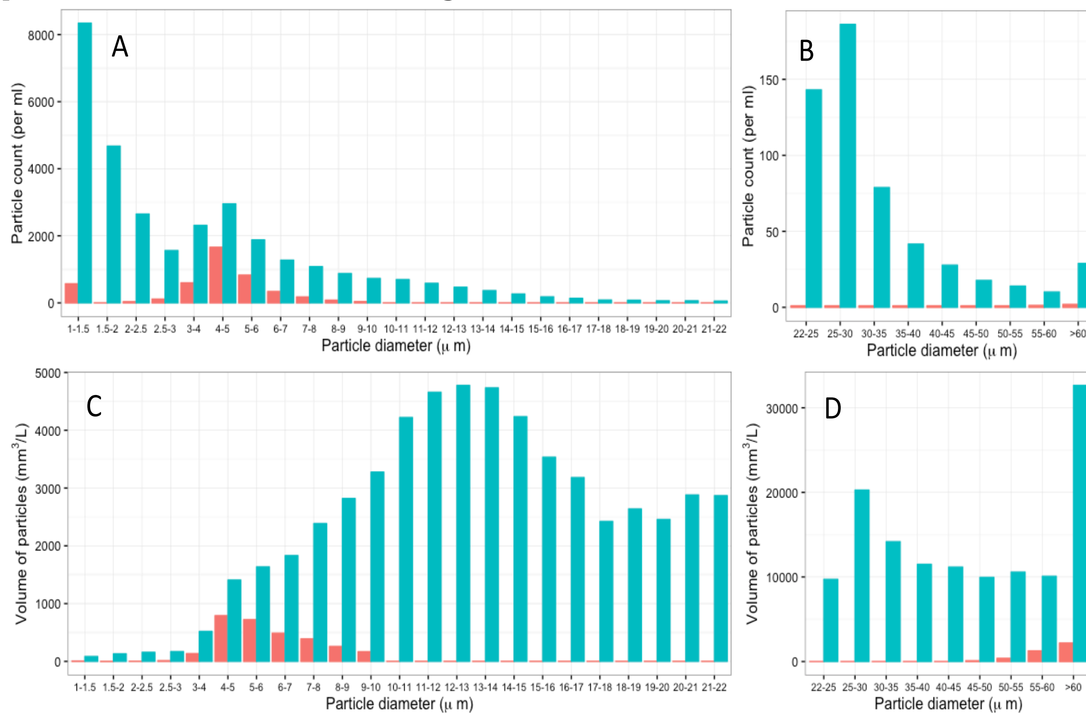

Figure 10 Added particle counts and particle volume of algae (estimated to 15.000 cells/mL) and fish feed (2g) delivered to the experimental tank, excluding the background. Red colour represents the algal particle concentration and volume, while the green bars represent fish feed particles. In order to accurately assess the added amounts, the initial background particle concentration was subtracted from the following measurement after 30min. Figure A and B depicts the counts of the fed diets for small and large particles respectively. While C and D measures the relative volume of these counts. Note the change in scale from between A, B, C and D.

3.2.2 qPCR results of individuals given fish feed and algae

In order to exclude the possibility to find false positives in the fish feed detection study, and to ensure that the method worked, an identical bioassay had been conducted on extracted DNA from *C. intestinalis* and the algal species used in the experiment (pers. comm. Skaar, 2015). A gel depiction of the products produced, revealed that neither the DNA from the algal species nor *C. intestinalis* produced any product with our bioassay, while the fish feed pellet did (Fig. 11).

Furthermore the extracted *C. intestinalis* DNA was used to quantify the amount present in the intestines by comparison with the known concentration of fish feed (positive control). By constructing a standard curve we established that the samples and positive control were linked linearly in our tested concentrations (Fig. 12)

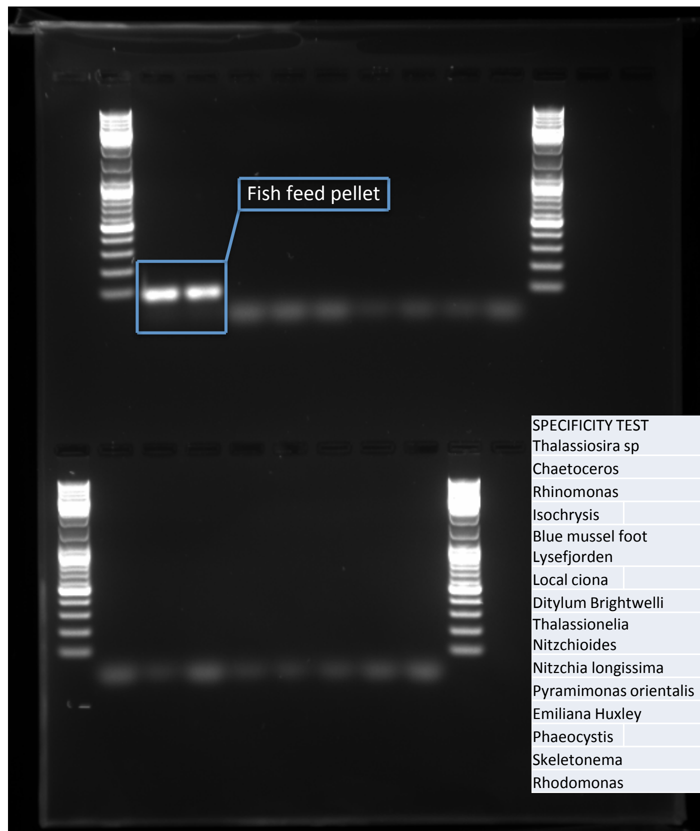

Figure 11 Gel depiction of the bioassay of different species used in the fish feed detection study, in order to exclude false positive results (RuBisCO product (71bp)). The species tested are included in the legend and encompass the algal species used in the algal treatment and *C. intestinalis*.

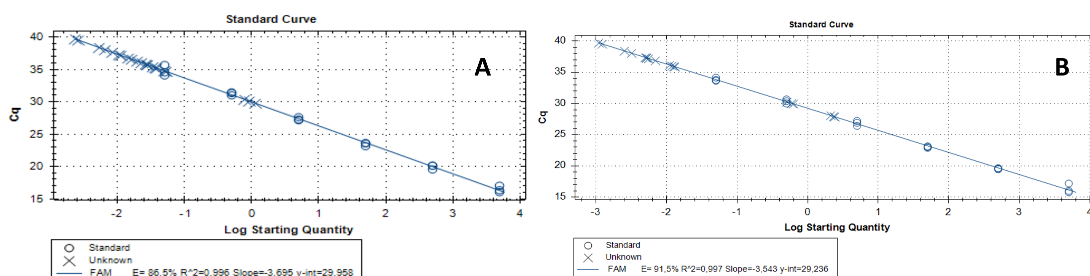

Figure 12 Standard curves used to quantify the PCR product. Circles represent positive control (fish feed pellet) at exponentially diluted concentrations, while crosses represent the samples that were detected in the qPCR assay. A and B represent the two experimental plates.

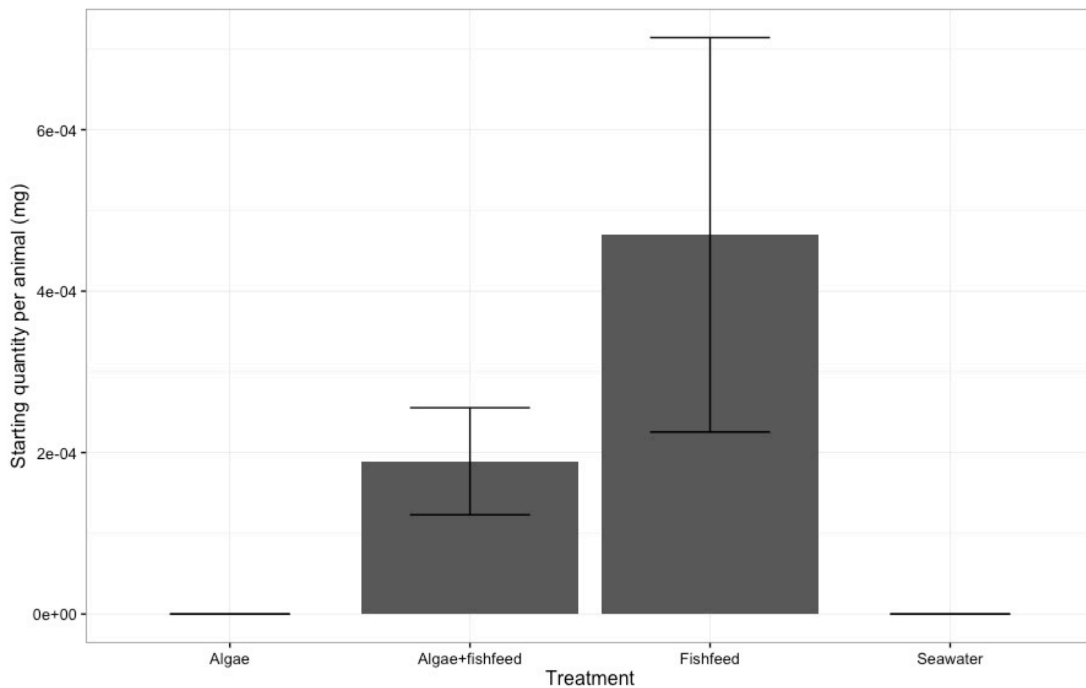

Figure 13 The back-calculated quantity of fish feed per animal intestine, in the different treatments. Algae+fishfeed reached detectable ranges for all individuals (n=10), while fish feed had higher quantity, but only 7/10 reached threshold values during the qPCR. Error bars represent standard error.

In order to establish that *Ciona* ingests fish feed and estimate the relative amount, we extracted DNA from intestine samples of ten individuals per treatment and performed qPCR on the soy RuBisCO gene, present in fish feed.

Only in individuals given fish feed was there a detectable signal over the threshold of 40 cycles (C_t), whilst individuals exclusively given algae and seston did not reach detectable levels in the qPCR. In the treatment "Algae + fish feed" which was fed a mixture of algae (15.000 algal cells/mL) and fish feed (1g), all the animals had detectable amounts of fish feed in intestines. Whereas the treatment "Fish feed", which received 2g fish feed, only seven of the ten intestine samples had detectable amounts of soy. As expected the "Fishfeed" group which received twice the fish feed to the "Algae + fishfeed" group, had about double the amount of fish feed in the intestines (Fig. 13).

3.3 Experiment three; individual experiment using the flow through method

As we found indications that not all animals were feeding in the fish feed detection study, and the cohort study concealed variations between individuals, we conducted a feeding experiment on individual animals.

3.3.1 Background water and fed concentrations in the individual flow-through study

The water temperature and salinity in the individual experiment remained stable at 11 ± 0.1°C and 31.3 ± 0.14ppt. The background particle concentration from the unfiltered seawater varied during the experiment (Fig. 14). These variations were noticed when beginning the individual study. The high particle count found on the first day prompted us to begin with the largest amount of fish feed, and move towards lower amounts during the five days. In contrast to the cohort study, where we began with the lowest fish feed concentrations, and increased daily.

The total background count was highest on day 1(35g) at around 45.000 particles/mL, and lowest on day 4 (0.003g) with around 27.000 particles /mL (Fig. 14). The sampling days differed significantly in background concentration (ANOVA, $p<0.0001$), except between day 1 (35g) and 2 (3.5g). Furthermore the background variation often matched the concentrations in the experimental diets, with the highest background during the highest fish feed concentrations (35g) and the lowest background during the lowest experimental diet (0.003g) (Fig. 14). An exception for this was 0.038g treatment experiencing higher background concentration than the 0.36g treatment (Fig. 14). It is recognized that background water during the individual study is roughly three times higher than during the multiple study (Fig. 7 and Fig. 14). The background particle distribution was dominated by small particles <2µm, but also had a slight peak in the 3-4µm size bin (Fig. 15).

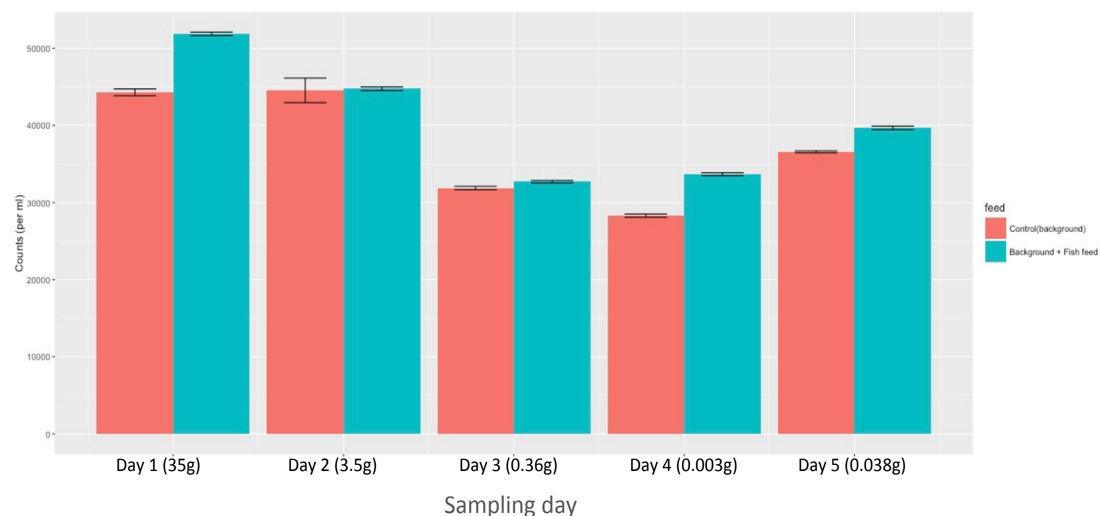

Figure 14 Mean total (1-200µm) particle concentration per mL of the background water for the sampling days (red bar) and total particle concentration when added fish feed (green bar). Error bars represent standard error.

This importance of this when calculating RE and CR is that the ratio of fish feed particles to background particles is not proportional, but it will not affect filtration measurements as they are measured relative to the controls, receiving the same particles. Furthermore the relative distribution of particles in the measured size ranges did not differ with increasing fish feed amount. Hence with increasing fish feed concentration, there was an increase in particle amount in each size class, but the size distribution remained the same (see Appendix G).

With the addition of fish feed, total particle counts significantly increased in all treatments (ANOVA, p<0.0001), except on day 2 (3.5g), as the background was only measured once during this day, which resulted in higher variability (Fig. 14). The addition of fish feed amount did not appear to increase linearly, suggesting that the background particle concentration affected the total particle concentration (Fig. 14). Unexpectedly the total particle counts were significantly lower when 0.36g of fish feed was added on day 3, than when 0.003g was added on day 4 (ANOVA, p=0.0431), due to the background variance (Fig. 14).

However the concentration measured in the three control chambers (no animals), receiving both fish feed and background particles did not statistically differ within each sampling day, including at low fish feed concentrations (0.003g) (ANOVA). For volume of the particles given throughout the experiment see Appendix I.

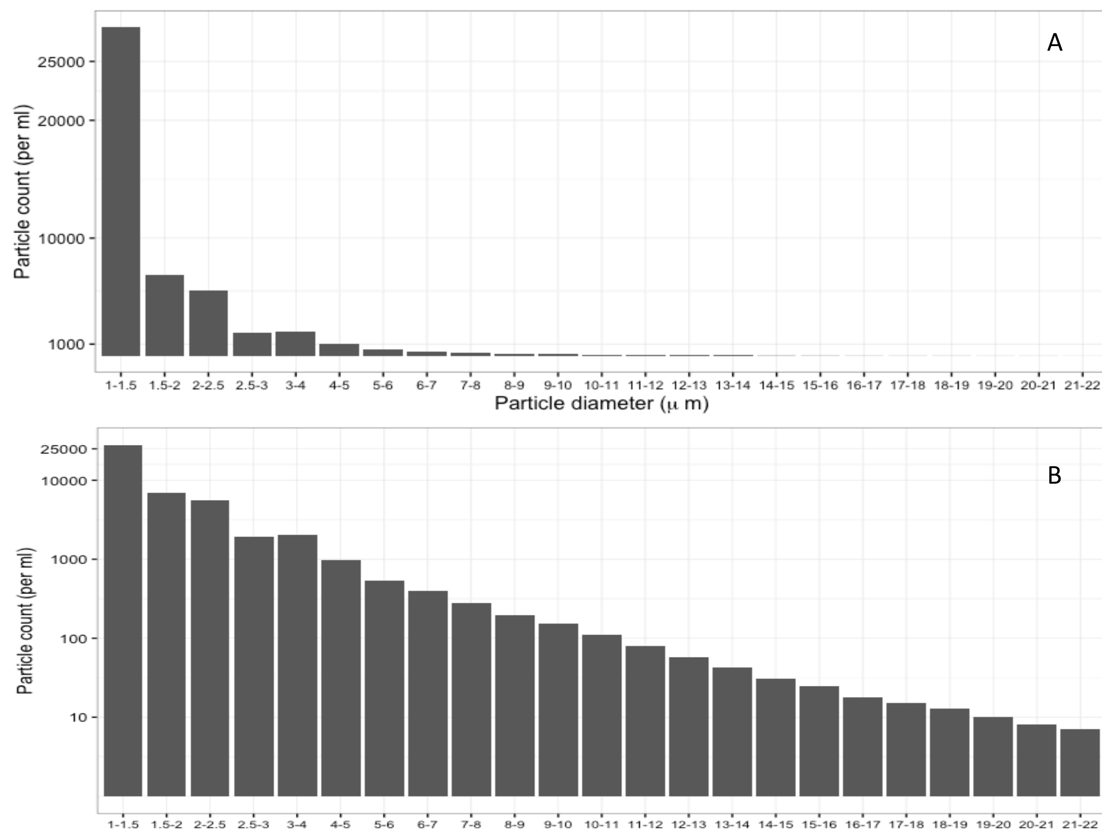

Figure 15 Average background particle concentration per mL of distinct sizes on the day with the highest count (day 1) (A), and on a logarithmic scale (B).

3.3.2 Chamber validation

When determining CR in individual chambers with flow-through, it is necessary to evaluate where the CR is independent of flow passing the individual chamber (see introduction section 1.4). We therefore tested ten flows between 50-450mL/min, in order to determine at which flow the CR plateaued (Fig. 16). The CR_{std} in the individual study appeared to reach maximum CR regardless of flows above 140 mL/min and sank when the flow exceed 220 mL/min, characteristic of the independency phase (Fig. 16). Although CR did not sharply decline in flows exceeding 220ml/min, which is typical in the inhibition phase, we decided to exclude higher flows, based on the gradual decline, and because large differences in flows can result in incorrect RE. Therefore the CR_{std} appears to have reached independence from flow between 140-220 mL/min, hence representing actual clearance rates. Consequently RE and CR measurements in the individual study are solely calculated from this flow range.

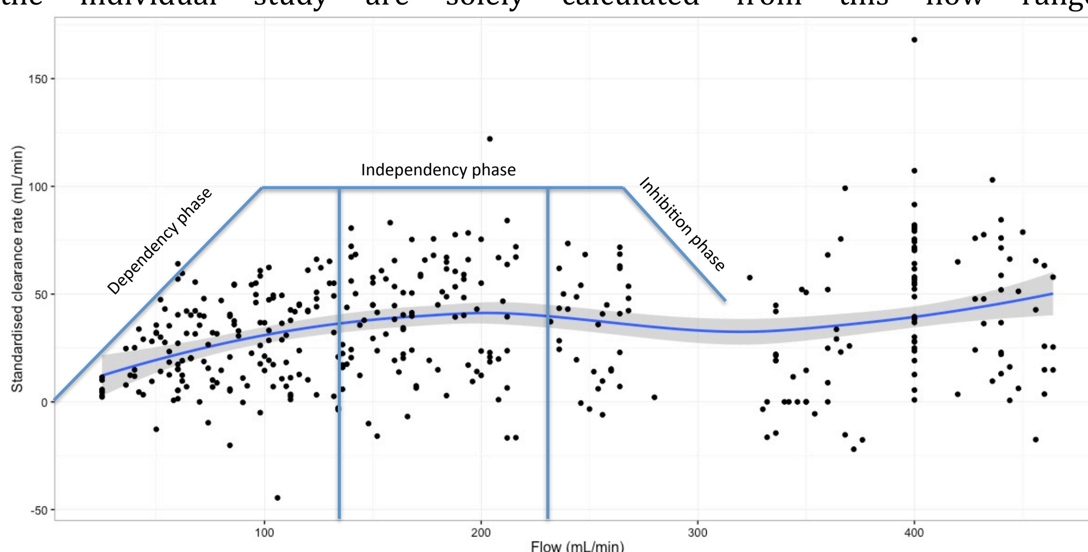

Figure 16 Standardised clearance rates based on 4-8µm particles over the tested flows (50-450mL/min) in individual chambers, all individuals and all the different treatments are included. Vertical lines mark the flow where CR is within the independency phase, and selected for use in the analysis of individual RE and CR.

3.3.3 Individual retention efficiency in experiment three

Retention efficiency for individual animals were tested in order to compare results with the cohort study, as well as to validate at what efficiency different sized particles were captured. Furthermore it is necessary to calculate CR from particles that are completely (100%) retained. Individual animals were standardised for each treatment (RE_{std}) and fitted into a linear mixed effects model.

From the analysis we found that RE_{std} increased with particle diameter in all treatments (LME; $p<0.0001$), and that the smallest particles (1-1.5µm) were significantly less retained than all particles over 2µm in diameter (LME; $p<0.0001$). The efficiency of retention in particles increased gradually and differed significantly between the different sizes (Fig. 17). In contrast to particle retention in the cohort experiment, were there was a sudden complete retention of particles over 3µm, complete retention in the individual study was in 9-10µm particles (Fig. 17 and Fig. 18). Furthermore particles in this size differed significantly in retention compared to all the smaller particle sizes (LME; $p<0.01474$), except the 8-9µm particles (Fig. 17). Nevertheless the smallest particles were completely retained (100%) in the control and in the lowest concentration (0.003g) on two occasions (Fig. 18).

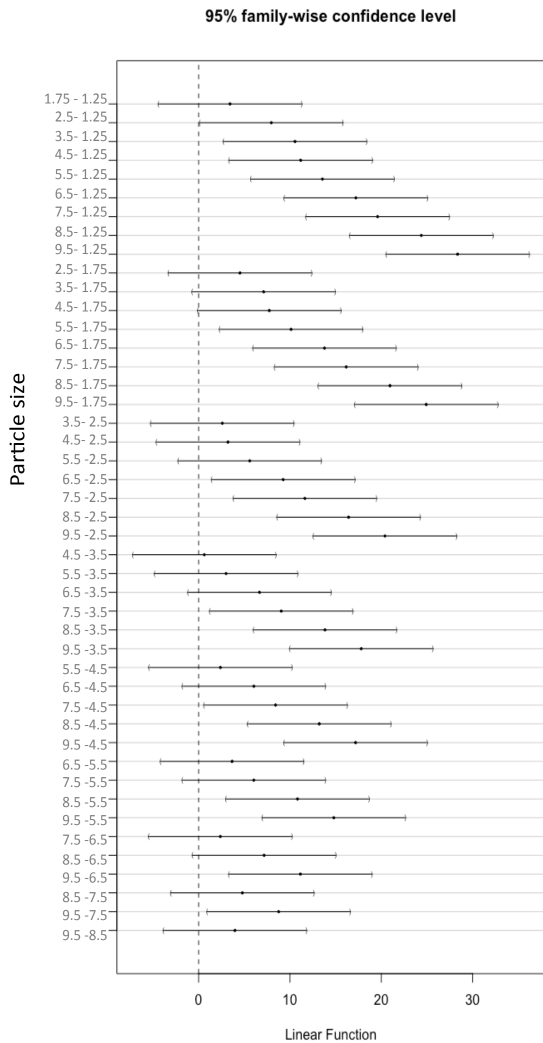

Figure 17 Tukey HSD pair-wise comparison of the arc-sinh transformed retention efficiency of all individuals in all treatments.

The analysis also revealed that, overall RE_{std} increased slightly over time (LME; $p<0.0001$). Furthermore, time influenced treatments differently and significantly increased RE_{std} at intermediate fish feed concentrations 0.36 (LME, $p=0.0041$) and 3.5g (LME, $p<0.0006$), but not in the in the lower (0.003 and 0.038g) and higher (35g) concentrations. Moreover RE_{std} declined with increasing fish feed amounts in all treatments (LME; $p<0.0001$).

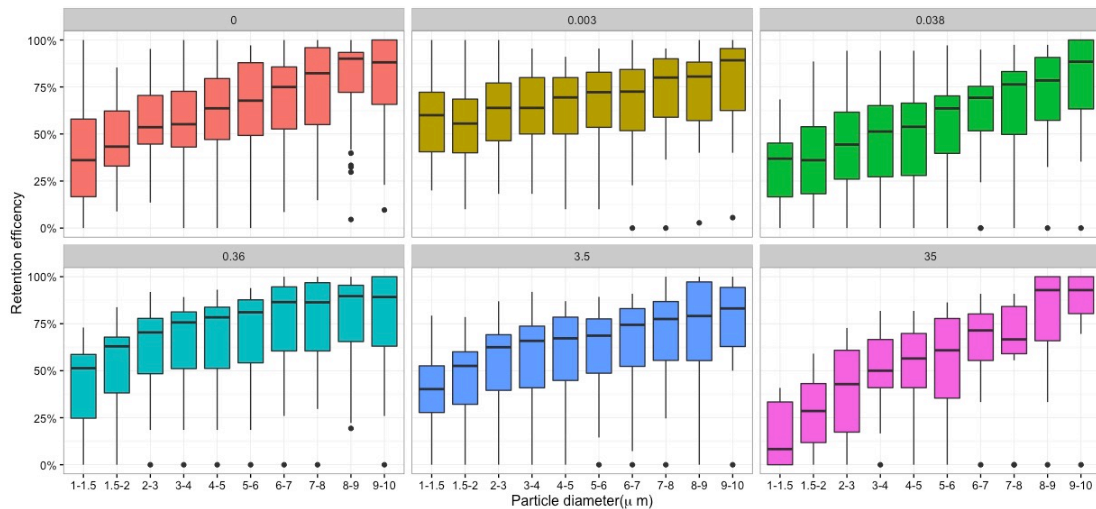

Figure 18 Box plot of individually and feed standardised RE between 1 and 10μm. The retention is calculated in all animals (n=7) given the experimental diets of fish feed (g), and seston ("0"). Calculated from all measurements throughout the individual experiment.

3.3.4 Clearance rates for individuals in experiment three

Standardised clearance rates (CR_{std}) were significantly affected by the addition of fish feed (LME; p=0.0008). With increasing fish feed, CR_{std} decreased, but this was not significant between treatments, however when the control was used for comparison, all the fish feed treatments had a significantly lower CR_{std} (LME; p=0.0003). Relative to the 0.003g treatment, the CR_{std} was on average lower by 18, 64, 27, 61 mL/min for animals given 0.038, 0.36, 3.5 and 35g of fish feed respectively (LME, ANOVA, p=0.0021).

Moreover, standardised clearance rates increased significantly over time, albeit not in the 0.003g treatment (LME; p=0.0056) (Fig.19). Additionally, time influenced treatments differently, for example we found a significant interaction between the 0.036g treatment and time, where CR_{std} increased significantly faster than the other treatments (LME; p=0.0025). The power transformed (Box-Cox) data found the same results, but were not included as the non-transformed data were more normally distributed.

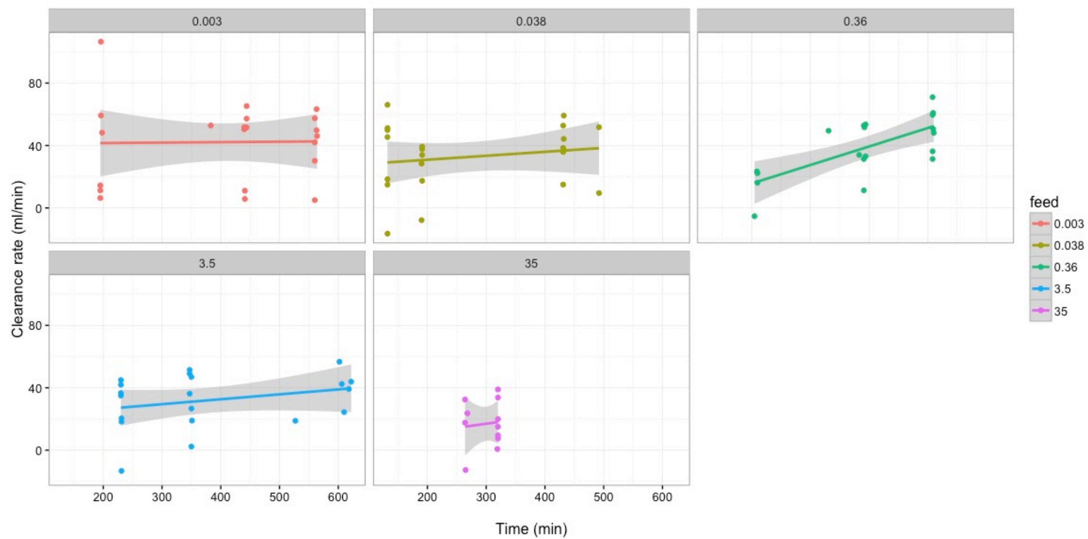

Figure 19 Standardised clearance rates for individuals (n=7) over time at different fish feed concentrations. Each point represents the CR of an individual. Regression lines were fitted from a linear model with corresponding 95% confidence intervals.

Additionally the average reduction (percent particles removed) when determining CR in flow-through system should not be to high, as the animal might then not filter maximally, and not to low, as it can approximate measuring error. In our highest concentrations the average clearance was 5%, and in our lowest it reached 19% (Table 10). This clearance is within recommended levels.

Table 10 Mean clearance in the treatments used to calculated CR and RE in the individual experiment

Treatment	Mean	SD
0,003	0,19	0,11
0,038	0,16	0,09
0,36	0,19	0,08
3,5	0,135	0,08
35	0,05	0,05

3.3.5 Variation in CR between individuals

Individual variations were accounted for in the statistical model, however variation in individual clearance was plotted for comparison (Fig. 20). Individuals 2 and 6 exhibited the highest clearance rates at all the concentrations, and individuals 1, 3 and 6 had relatively consistent clearance rates throughout the experiment.

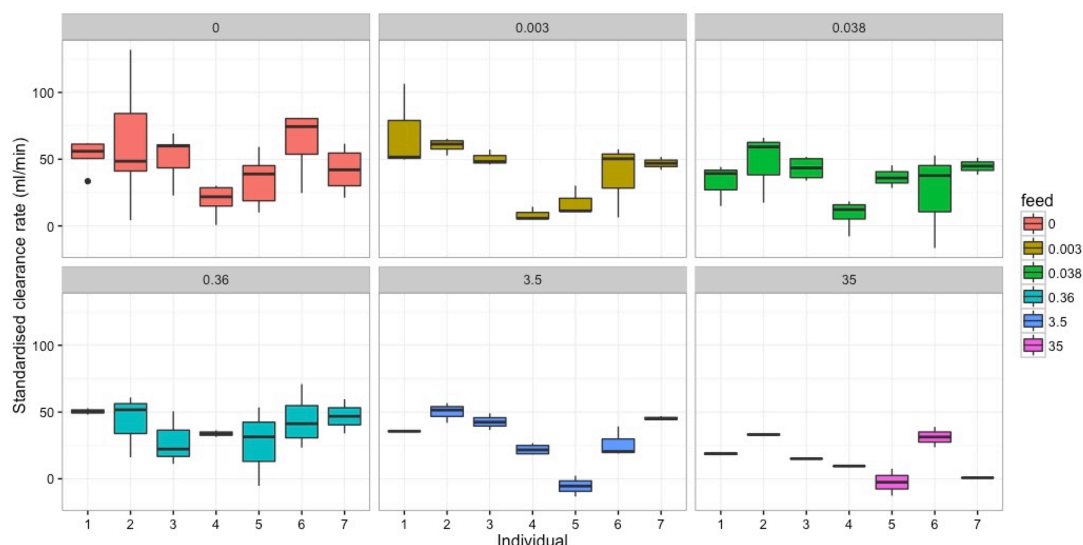

Figure 20 Box plot of the different CR_{std} in distinct individuals at the tested amounts of fish feed, used throughout the individual experiment. In addition individuals given no fish feed (during all the sampling days) are included for comparison.

In addition, none of the weight measures were significantly related to CR or RE in our model, which indicates that the animals were successfully selected to a similar size (Table 11).

Table 11 Relation between different weight measurements of the animals used in the individual feeding experiment.

Individual	Wet weight (g)	Length (cm)	Dry weight (g)	Ash-free dry weight (g)
1	12,57	9,5	0,81842	0,41143
2	13,82	9,5	0,70089	0,24558
3	13,43	10,5	0,66341	0,24025
4	14,11	9	0,69012	0,25295
5	15,24	10,5	0,71691	0,23297
6	18,76	12	0,97911	0,35990
7	13,49	10,5	0,66938	0,23635
Mean	**14,49**	**10,2**	**0,74832**	**0,28278**
SD	**2,05**	**1,0**	**0,11419**	**0,07213**

3.4 Predicted and methodological difference in CR calculation

The calculation of CR is highly dependant on the RE of the particles used, and particles should be completely retained calculate CR. However, some have included all particles sizes. To assess the relationship between standardisation clearance rate and retention efficiency, and to assess the congruence between the CR found in this study, with the predicted CR from the formula based on total DW, we compared the results in a boxplot (Fig. 21). The predictive formula is notably higher than the g^{-1} standardised CR, measured in this study, even after correction for temperature (Fig. 21). As expected the shape of the boxplot did not change after standardisation, and CR calculated from all particles were lower than CR based on completely retained particles (4-8μm) (Fig. 21).

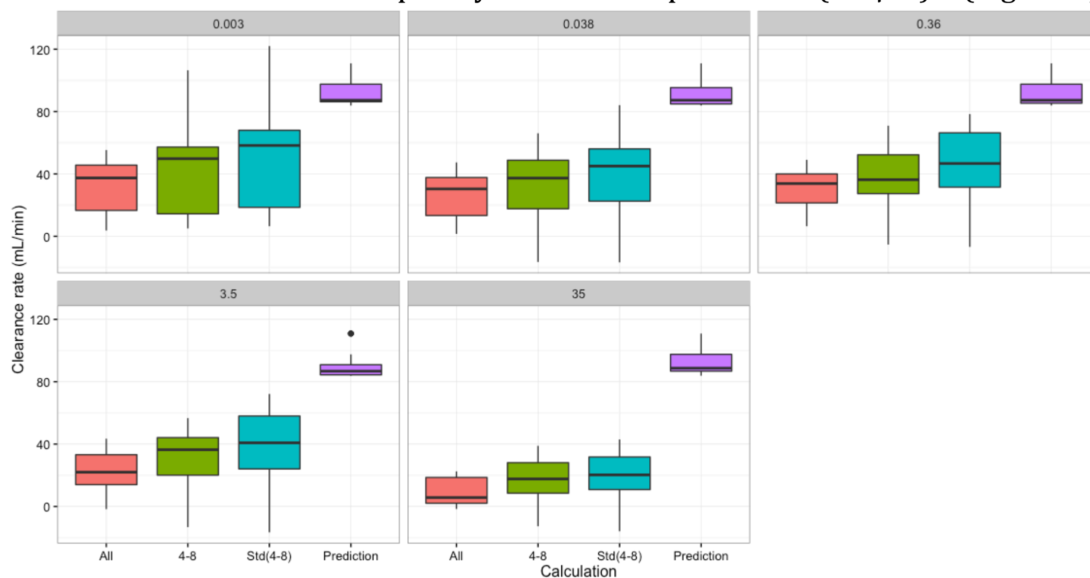

Figure 21 Box plot of the difference in CR calculation when including all particles, between 4-8μm, the g^{-1} DW standardised CR used in the present study and the predictive formula from Petersen et al. (1992).

4 Discussion

4.1 Ciona intestinalis does ingest fish feed

The results from the Taq-PCR assay targeting rubisco in soy using genomic DNA extracted from intestines of *C. intestinalis* supports the hypothesis that they can clear fish feed from the water column. The data suggests that a higher number of individuals ingested fish feed when algae were present, than when fed fish feed alone. Seven of the ten animals given 2g fish feed had detectable amounts in their intestines, while ten of ten animals given a mixture of algae and 1g fish feed had detectable amounts. As expected, the average amount of fish feed per individual was roughly double in the treatment given 2g, compared to the treatment given 1g of fish feed (Fig. 13).

Unlike algal cells however, fine particulate fish feed may have caused some of the DNA to become dissolved, where it could have been hydrolysed by marine prokaryotes in the unfiltered seawater (Paul et al., 1987). Therefore the back-calculated fish feed quantity is considered relative between treatments, rather than representing accurate total quantity.

The detection of seven intestines, rather then ten, over threshold values cannot be considered conclusive, as some animals might not be feeding or squirting in response to fish feed. Judging from the filtration experiments however, all animals seemed to be feeding at high concentration, albeit lower at high particle concentrations (Fig. 9 and Fig. 20), and animals were actively filtering and responding to stimulation prior to the experiment, excluding the possibility of dead animals.

Alternatively, filtration could be more efficient when algae are mixed with fish feed, over fish feed exclusively, and considering the presence of seston in the unfiltered water, an elevated ratio of algae to fish feed might be necessary to influence feeding on fish feed. This preferential feeding might be linked to *C. intestinalis* adaption to natural planktonic assemblages (Ruppert et al., 2004). There have been some indications of selective feeding in ascidians (Armsworthy et al., 2001; Jiang et al., 2008; Lesser et al., 1992). For example Lesser et al. (1992) found in a study utilizing flow cytometry and natural planktonic assemblages that *C. intestinalis* preferentially selected large phytoplankton (>16μm) over smaller (4-5μm). While Zhang and Fang (2000) found similar results, an apparent selection of 10 over 1.5μm particles. Furthermore particle selection is further supported by Jiang et al. (2008) who found that larger animals, retain large (5-20μm) over small (<5μm) particles. There is no physiological explanation for this fine-tuned particle selection, and Petersen (2007) disregards studies most studies which have found changing retention in ascidians and mentions methodological constrictions, such as recirculation and incorrect methods (discussed further in section 4.2 and 4.3.2), and point to that most experimental evidence show no difference in particles composition between suspended and ingested particles (Bingham and Walters, 1989; Klumpp, 1984; Randløv and Riisgård, 1979; Ribes et al., 1998; Robbins, 1983; Robbins, 1984). In addition both Robbins (1984) and Sigsgaard et al. (2003) concluded that *Ciona* showed no ability to distinguish between organic and inorganic particles. Therefore active selection is not believed to have influenced our results.

Nevertheless *Ciona* can eject contents in the branchial basket in response to disturbance, presence of large particles and in order to avoid overloading of the branchial basket under during high particle concentrations (Armsworthy et al., 2001; Day, 1919; Petersen et al., 1999; Sigsgaard et al., 2003).

Fish feed consist of a variety of ingredients, including large particles from fish such as bones, skin and plant material, likely capable of eliciting this response (Sørensen, 2012). Bingham and Walters (1989) observed that *Ciona* regularly eject large particles. Indeed, large particles were present in the fish feed we provided (Fig. 10), and with more fish feed, there would be more large particles, making it more likely to elicit a squirting response and subsequently reduce feeding (Jørgensen and Goldberg, 1953; Robbins, 1984; Sigsgaard et al., 2003).

Squirting is believed to be the principal regulator of feeding in ascidians, analogous to pseudofaeces production in *M. edulis* (Cranford et al., 2011; Petersen, 2007). Because squirting is related to high particle concentrations, it could also explain the deviation in detection of fish feed between the two treatments. Armsworthy et al. (2001) and Petersen and Svane (2002) observed that squirting increased in the ascidians *Halocynthis pyriformis* and *C. intestinalis,* given increasing concentrations of seston, sediment and algae.

The relatively high concentrations of fish feed given, could have induced squirting, and consequently inhibited feeding. Yet the total particle concentration was higher in the treatment were fish feed was detected in all ten animals (60,000/mL) given algae and fish feed, than in only seven animals given fish feed exclusively (45,000/mL). Therefore the increase of large particles due to the addition of more fish feed is considered the most likely explanation for the deviation in detection of fish feed in seven rather than ten individuals between the two treatments.

4.2 Methodology for RE and CR estimation

As pointed out by Strohmeier et al. (2012), it has been common practice in filtration studies in mussels and ascidians to assume that maximum retention is within a narrow particle range, often 1-10μm for mussels and *C. intestinalis* (Jørgensen et al., 1984b; Møhlenberg and Riisgård, 1978; Petersen and Svane, 2002; Randløv and Riisgård, 1979; Riisgård, 1988).

By using this common methodology, larger particles (>10μm) are omitted from the analysis, on the grounds that RE is a property of the mucus mesh and thus undergo a mechanical sieving process which is believed to completely retain particles which are unable to pass the mucus mesh (0.4 x 0.7μm) (Flood and Fiala-Médioni, 1981). Previous studies have determined that the particles over 2-3μm are completely retained (Jørgensen et al., 1984a; Randløv and Riisgård, 1979). For example in the study by Randløv and Riisgård (1979) particles from 3-10μm did not differ in retention efficiency. These particles were assumed to be 100% retained and used to compare the retention of particles smaller than 3μm with the relative different retention of 1μm (70%). In addition this size range is convenient since it includes typical microalgae species, often used in filtration studies (Armsworthy et al., 2001; Klumpp, 1984; Lesser et al., 1992; Petersen, 2007; Petersen and Svane, 2002; Petersen et al., 1999; Randløv and Riisgård, 1979).

Several studies have included a wider range of particle sizes (Armsworthy et al., 2001; Robbins, 1983; Strohmeier et al., 2012; Zhang and Fang, 2000). In the absence of uncertainty estimates these large particles are often compared to smaller particles. When few large particles are compared to smaller and more numerous particles, temporal variability will influence the particle counts and retention calculations, yielding artificially high or low efficiencies (Cranford et al., in press; Rosa et al., 2015). In addition negative efficiencies are regularly excluded from filter-feeding studies, resulting in a bias towards higher efficiencies of larger particles (Cranford et al., 2011; Randløv and Riisgård, 1979). Rosa et al. (2015) describes such a confounding factor as "mathematical happenstance" that can lead to statistical artefacts.

In order to evaluate the reliability of these commonly used methods, Cranford et al. (in press) determined with the use of the same particle counter and similar methodology as used in the current study, that particles above 11.25μm became too heavily influenced by temporal variability for seston and could not be included in the study. Based on these results and our own measured uncertainty at larger particle sizes (Fig. 6), we avoided possible inaccurate estimates by excluding particles over 10μm in diameter.

4.3 Retention efficiency

4.3.1 Retention in the cohort experiment

Because retention efficiency is the basis for calculating clearance rate, which again describes energy acquisition in most filter feeders, incorrect RE can therefore, in addition to result in erroneous energetic budgets for individual species, also translate to whole ecosystem and aquaculture carrying capacity models (Filgueira et al., 2014; Rosa et al., 2015; Strohmeier et al., 2012). As incorrect individual filtration is magnified immensely when converted to population level, accurate retention in filter feeding species is imperative for conservation, aquaculture and ecology (Bergström et al., 2015; Cranford et al., 2011; Dame, 2011; Filgueira et al., 2014; Filgueira et al., 2015; Strohmeier et al., 2012).

The retention in the cohort study exhibited a characteristic and expected particle retention (Fig. 8), with a sharp increase to complete retention for particles over 2μm, consistent with previous studies (Bingham and Walters, 1989; Cranford et al., in press; Klumpp, 1984; Petersen and Riisgård, 1992; Petersen and Svane, 2002; Petersen et al., 1999; Randløv and Riisgård, 1979; Ribes et al., 1998; Robbins, 1983; Robbins, 1984; Sigsgaard et al., 2003). Because *Ciona* captures particles using a mucus-net, which is not believed to be adjustable, active particle feeding selection should be absent. If so, we expected a clear shift in retention from particles 1-2μm to all particles over 2μm, which should not differ statistically (Jørgensen et al., 1984a; Petersen, 2007; Randløv and Riisgård, 1979; Ruppert et al., 2004).

However under the high particle concentrations when 1 and 10g of fish feed was added, the shape changed and variation increased (Fig. 8). Given the concurrent decline in clearance rates (Fig. 9), this is likely the result of feeding inhibition possibly by overloading of the branchial basket (Petersen et al., 1999; Sigsgaard et al., 2003). When feeding is reduced the measuring error and possible disintegration of particles gains importance and measurements become less accurate. Furthermore, the correction due to sedimentation will also gain in importance when feeding is inhibited, especially under high particle concentrations, as more particles sediment. We noted that large particles were reduced and small particles increased somewhat in the highest treatment (10g) during the settling trial, which were removed when we corrected prior to calculating RE and CR (see Appendix K). However, the settling correction may have become inaccurate over time in high concentrations, because the particle disintegration is almost certainly density dependent. When *Ciona* clears particles, less are subject to disintegration, which implies that, in the settling trial there were more small particles, which means that after correction there is a underestimation of small and a overestimation of large particles. Which is what we see in the results from the high concentration treatments, given 1 and 10g of fish feed (Fig. 8).

Furthermore the fact that retention was affected over time in the high concentrations (1 and 10g), lends support to a temporal disintegration of large particles in the high concentrations, thus retention estimated in these treatments cannot be considered accurate. Since time did not affect the lower concentrations (0.1, 0.01 and 0.001g), the particle disintegration seems to have played a

negligible role in these treatments, thus the retention is considered representative of actual retention.

Moreover the smallest particles (1-1.5µm) were sometimes completely retained (Fig. 17). Rather than a biological response this is likely due to technical limitations of the smallest size channel in the electronic particle counter (Strohmeier, 2016, pers.comm).

We concluded that the change in shape of retention efficiency in the high concentrations in the cohort study probably originated by a combination of reduced feeding, increased sedimentation, disintegration of large particles, and the subsequent RE standardisation of these measurements. These limitations underline the advantage of the individual flow-through method under high particle concentrations, as an adequate flow should render sedimentation negligible (Riisgård, 2001).

4.3.2 Retention in the individual experiment

In the individual experiment, standardised retention efficiency gradually increased with size, to maximum retention of 8-10µm particles (Fig. 17). We found that this gradual increase in retention was significant between the majority of the particle sizes (Fig. 16). These RE_{std} results differed from the cohort experiment, and we expected an abrupt in retention with size, rather than gradual as our data suggested (Carver et al., 2006; Locke and Carman, 2009; Petersen, 2007; Randløv and Riisgård, 1979).

In our case the different results in retention between the two experiments might have arisen from the difference of measuring multiple versus individual animals. The retention in the individual experiment was calculated from all the measurements, including animals that did not feed, hence revealing temporal inter-individual feeding. This has the implication that the average was lower in the individual than the cohort experiment, which may explain the statistical significant difference in particles over 3µm. Maximum retention (RE_{max}) may in this case be more representative of actual retention, as animals are then likely feeding. When non-feeding animals are standardised temporal variability gains importance and biases retention efficiencies towards larger particles (see section 4.2). A clear pattern emerges when comparing RE_{max}, with reported values of retention, which are often based on feeding animals under optimal conditions (Fiala-Médioni, 1978b; Jørgensen et al., 1984a; Randløv and Riisgård, 1979). Particles down to 1.5-2µm are retained with approximately 80% efficiency, and RE_{max} does not vary noticeably for particles over 2µm, which corresponds to the expected retention of a mechanical sieving process through the mucus mesh (Fig. 12) (Cranford et al., in press; Randløv and Riisgård, 1979). Again the smallest particles 1-1.5µm were completely retained in the individual experiment on two occasions, and were likely the results of technical limitations of the smallest size channel of the electronic particles counter (Strohmeier 2016, pers.comm).

Furthermore we found that the retention declined to 0-40% in small particles when exposed to the highest concentrations (Jørgensen et al., 1984a; Randløv and Riisgård, 1979). In this case, it was evident from the clearance rates that the animals reduced feeding (Fig. 12), likely due to gut fullness (Petersen et al., 1999) and as discussed above, retention calculated from these concentrations are not believed to be accurate.

Nevertheless variable RE, which is indicative of feeding selectivity, has been found in several flow-through studies in both ascidians and mussels, where there is no apparent physiological explanation for this ability (Armsworthy et al., 2001; Jiang et al., 2008; Rosa et al., 2015; Strohmeier et al., 2012; Zhang and Fang, 2000). Such findings have sparked a debate on the reliability and potential enhancement of commonly used methods in filtration studies (Cranford, 2001; Cranford et al., 2011; Filgueira et al., 2006; Larsen and Riisgård, 2012; Riisgård, 2001; Rosa et al., 2015).

Strohmeier et al. (2012) investigated whether RE changed temporally and included large particles up to 35µm in Blue Mussels. Compared to *Ciona*, Blue Mussels have a more complex feeding apparatus, possibly providing a more plastic retention (Cranford et al., 2011; Strohmeier et al., 2012). Nevertheless, similarly to *Ciona*, *M. edulis* should have a relatively sudden increase to maximum retention above 4µm (Cranford et al., 2011; Møhlenberg and Riisgård, 1978; Rosa et al., 2015; Strohmeier et al., 2012). Results from Strohmeier et al. (2012) were similar to the individual study (results not included), in that large particles over 20µm were often maximally retained (100%), while particles that physiologically should be maximally retained (>4µm), were retained at a lower efficiency. Likewise Armsworthy et al. (2001) described a reduction of retention in particles over 5µm in the ascidian *Halocynthia pyriformis* when given high concentrations of sediment.

As seston and sediment concentrations decline near exponentially with increasing particle size, retention is calculated from widely different numbers, which increases the probability of statistical artefacts (Cranford et al., in press; Rosa et al., 2015; Strohmeier et al., 2012). Rosa et al. (2015) attempted to identify confounding factors and statistical artefacts, that might be responsible for variations in retention in Blue Mussels. By using spherical polystyrene beads in equal concentrations, delivered directly to the inhalant, with subsequent cross checking with three particles counting systems, they concluded that retention did not change and always remained at 100 % for particles over 4 µm (Rosa et al., 2015). They further suggested that results showing apparent physiological retention plasticity were not due to biological responses, but due to confounding factors such as non-spherical particles, the escape of motile cells and "mathematical happenstance" (Rosa et al., 2015). Strohmeier et al. (2012) considered improper use of particle counting instrumentation, method of data standardisation, disaggregation of flocculated particles and flow variations in the chamber, as possible sources of error when measuring retention in flow-through systems. On the other hand, Petersen (2007) disregards most studies which have found variation in RE and mentions methodological constrictions, such as recirculation and incorrect methods, and point to that most experimental evidence show no difference in particles composition between suspended and ingested particles (Bingham and Walters, 1989; Klumpp, 1984; Randløv and Riisgård, 1979; Ribes et al., 1998; Robbins, 1983; Robbins, 1984). In a recent study, Cranford et al. (in press) also critically evaluated particle retention in both the clearance and flow through methods, using *C. intestinalis* and *M. edulis,* with the same particles counter used in the current study. Although they applied seston, clay and algal cultures and not fish feed, the retention found was similar as to the individual study reported herein. Complete retention was between 8-11µm, where they deemed larger particles as inaccurate for determination, while

we found 8-10μm and similarly excluded particles over 10μm. Cranford et al. (in press) did find a similar gradual increase in retention and concluded that these results confirmed previous trials (Jørgensen et al., 1984a; Randløv and Riisgård, 1979).

We therefore conclude that the observed differences in RE between the two experiments presented here were likely caused by the inclusion of non-feeding individuals and thus inter-individual variability in feeding, and the subsequent standardisation these non-feeding animals, rather than selective particle feeding. When considering RE_{max} were animals are likely feeding, retention efficiencies are consistent with previously reported values (Cranford et al., in press; Jørgensen et al., 1984a; Klumpp, 1984; Petersen, 2007; Petersen and Riisgård, 1992; Petersen and Svane, 2002; Randløv and Riisgård, 1979; Sigsgaard et al., 2003).

4.4 Effect of particle concentration on feeding

The effect of adding fish feed changed the shape of RE_{std} and lowered clearance rates in both feeding experiments. Exposure to high particle concentrations, lead to a decline in feeding and effects became significant when given 1 and 3.5g and above for the multiple and individual experiments, respectively (Fig. 19). These results were expected, as *Ciona* reached a threshold of gut fullness, where further ingestion might have lead to overloading of the branchial basket and become harmful (Day, 1919; Petersen, 2007).

Ciona is adapted to natural seston in oligotrophic conditions, with maximum concentrations typically arising from spring blooms in the Norwegian coast (Knutsen, 2016; Ruppert et al., 2004). The maximum concentrations tested herein represent highly elevated concentrations compared to levels occurring in typical temperate coastal waters (Delegrange et al., 2015; Engel et al., 2008; Kiørboe et al., 1990; Kiørboe et al., 1994). Indeed Petersen and Riisgård (1992) reported that in algal densities around 20.000 cells per mL, feeding declined exponentially. The decline is caused by the cessation of stigmatal beating, possibly by nervous control of beat pattern (Bergles and Tamm, 1992; Petersen and Riisgård, 1992; Petersen et al., 1999). Furthermore Petersen et al. (1999) described that cilia beating decreased with rising algal concentrations, followed by increased squirting when concentration exceeded 20.000 cells/mL. Likewise Armsworthy et al. (2001) confirmed that squirting increased with high particle concentrations in the ascidian *Halocynthia pyriformis*.

In a similar study by Sigsgaard et al. (2003) found that filtration was reduced at high concentrations, but respiration remained high. They therefore concluded that this was a functional response to high particle concentrations to avoid harmful or limiting satiation, while retaining a constant ingestion rate.
This cessation of filtration has been characterised as a Holling type I functional response in several studies (Petersen and Riisgård, 1992; Petersen and Svane, 1995; Robbins, 1983), and resemble a Holling type I response, characterised by an almost linear particle clearance with increasing food concentration until the gut is completely filled, and feeding ceases or declines (Jeschke et al., 2004).

Because particle concentrations affect the reliability of measurements (Fig. 9), high concentrations may influence the accuracy of the clearance rates reported herein. The proportion of particles cleared is commonly used to assess the viability of flow-through experiments with filter feeders (Filgueira et al., 2006; Pascoe et al., 2009). When the percent particles cleared is too high the animal does not feed at maximum and if it is to low, the percentage removed approximates measuring error and become unreliable (Filgueira et al., 2006).

In the validation protocol for flow-through chambers, Filgueira et al. (2006) reported that measurements for Blue Mussels on seston would lose accuracy if the clearance (percent particles removed) is over 25% or less than 13%. Similarly Pascoe et al. (2009) reported a range between 5-30%, and recommended an average of 20% particles cleared, which gave accurate results for Blue Mussels with seston. In our highest concentrations the average clearance was 5%, and in our lowest 19% of particles were cleared (Table 10). It is therefore concluded that the percent of particles cleared are within recommended concentrations and measurements are consequently reliable.

4.5 Effect of time on feeding

Our measured standardised clearance rates in the individual experiment increased over time, except in the treatment with the lowest fish feed concentration (0.003g). Although significant, the time effect increased feeding only slightly (Fig. 19).

Other than short-term functional responses to increased particle concentrations, ascidians can change clearance rates according to temperature, as temperature limits cilia beating (Petersen and Svane, 2002; Petersen et al., 1999). Annual temporal variability has been described in the temperate ascidian *Halocynthia papillosa*, Ribes et al. (1998) reported that 55% of the variability could be described by the increase in temperature throughout a year. In our case temperature did not vary during the individual experiment, and cannot explain the increase in clearance. Temporal variability in mussels CR is associated with seasonality and short term responses in a matter of minutes and hours have been found (Cranford et al., 2011; Fréchette et al., 2016). Strohmeier et al. (2009) found short-term clearance rate responses in *Mytilus edulis* and *Pecten maximus* under relatively stable conditions. Fiala-Médioni (1978b) on the other hand, reported that pumping rates were constant and did not have any particular rhythm over 12 hours in *C. intestinalis* under stable conditions and constant feeding.

The interactions between treatment and time, found in both experiments support the previously discussed response to elevated particle concentrations, i.e. high particle concentrations reduced feeding. Still, time influenced treatments differently, and increased feeding at intermediate particle concentrations, while not at lowest and highest food treatments. This relationship was described in Petersen et al. (1999), in low concentrations the flow velocity exiting the atrial siphon was stable, apart from a few short squirts, but when the particle concentration increased, the exiting flow dropped and became much less stable.

Why the clearance rates were not affected by time in the cohort, but in the individual experiment, can be due to several reasons. The two methods employed subjects the animals to different conditions, in the cohort study the particle concentration changed from an initial maximum followed by a gradual decline, while the individual the particle concentration was sustained, representing more natural conditions (Cranford et al., in press). Furthermore the two experiments differed in duration, four hours in the cohort, while ten hours in the individual experiment, which might have concealed longer-term temporal effects. More importantly the temporal response would probably not have been detected unless it was synchronised between the animals in the cohort study, which might explain why such an effect has not been described previously (Carver et al., 2006; Petersen, 2007). Strohmeier et al. (2009) found that cessation and reduction in CR were not synchronous in bivalves (*M. edulis* and *P. maximus*), that exhibits similar functional responses as *Ciona* (Jeschke et al., 2004).

Compared to many other studies (Jørgensen and Goldberg, 1953; Petersen and Riisgård, 1992; Petersen and Svane, 2002), the clearance rates reported in the current study were measured in high particle concentrations, albeit depending on treatment (Armsworthy et al., 2001; Petersen et al., 1999; Robbins, 1984).

As such an effect has not been previously described in the literature (Carver et al., 2006; Petersen, 2007), we must be acutely aware of possible confounding influences, notably the high background concentration. Although total concentration did not statistically differ within each treatment, variation might have been sufficient to influence clearance rates when animals are feeding in high concentrations.

During the individual experiment, the concentration of background water varied sufficiently to consider the possibility that a autumn bloom occurred (Engel et al., 2008; Erga et al., 2012), hence background conditions in the individual study might represent the maximal concentrations most natural populations are exposed to (Ruppert et al., 2004). Although filtered water could have improved accuracy of measurements, it was not available, and on the other hand, natural seawater assemblages are more representative of conditions in the field. As the background concentration was high, animals may have been feeding at a reduced rate (Petersen et al., 1999), and if the background changed during the 10h experiment, this could have created a time dependency based on the background concentration. For example, if the background concentration decreased during the experiment, individuals may have increased clearance rates in response (Petersen and Riisgård, 1992; Sigsgaard et al., 2003). Indeed diel vertical migration and grazing of planktonic communities, could have reduced the particle concentration in the unfiltered seawater from 40m (Gibson et al., 2009; Klevjer et al., 2014). However no such indications were found in the control chambers within each sampling day, and there was no notable change in the particles spectrum either (Appendix G). Assuming constant and equal supply of fish feed, any background change should have been detected in the controls, yet there was no significant difference in particle concentration throughout each sampling day.

Therefore our results suggest a prolonged conditional response to elevated particle concentrations, where after an initial cessation of feeding and acclimation period, feeding gradually increases slightly at intermediate and high concentrations, but not at low concentrations.

4.6 Variation in clearance rates

Clearance rates are the primary means of determining energetics in filter feeders, and subsequently nutrient fluxes between ecosystems, therefore it is important to characterise temporal changes (Cranford et al., 2011). It has been a standard practice to omit zero or negative clearance rates in filtration studies (Jørgensen et al., 1984a; Møhlenberg and Riisgård, 1978; Petersen and Riisgård, 1992; Petersen and Svane, 2002). This will increase the precision of the mean response, but will cause an overestimation when extrapolating individual rates to the population level (Cranford et al., 2011). For example Petersen and Riisgård (1992) who found the predictive formula, excluded negative or zero measurements and only included high and stable clearance rates. Our standardised clearance rates were around half of the predictive formula (Fig. 21), possibly because we included all measurements over longer periods, making our results more representative of in situ feeding (Cranford et al., 2011).

However our results were within reported values from other studies (Table 2). Closest to our findings, are the total dry weight specific clearance rates

from Kustin et al. (1974) and Randløv and Riisgård (1979), who reported clearance at 63 and 47mL/min respectively, while found clearance rates between 55-60mL/min in our lowest concentrations in the individual experiment (Fig. 21). Moreover clearance rates found in the cohort study were around 50ml/min in the control group (Fig. 9). The wet weights and length measures of the animals used in the cohort study (Appendix F) are according to Carver et al. (2006), roughly equal to g^{-1} dry weight. Therefore clearance rates in the cohort study are more or less directly comparable to the clearance rates in the individual experiment. Differences between individuals did not vary consistently with any weight measures, but two individuals did have lower clearance rates consistently through the experiment (Fig. 20). Weight specific clearance rates are consistently used through the literature, and relationship between body size and clearance has been widely reported (Fiala-Médioni, 1974; 1978b; Jørgensen and Goldberg, 1953; Petersen and Riisgård, 1992; Randløv and Riisgård, 1979; Sigsgaard et al., 2003). The absence of finding this relationship in our individual study is likely the relatively small sample size and that individuals were of relatively equal size (Table 11).

We conclude that our clearance rates are consistent with previous findings, and are approximately equal between our two methods.

4.7 Implications for integrated multi-trophic aquaculture

4.5.1 Conventional IMTA and benefits over the Blue Mussel

The majority of discharge particles from aquaculture consist primarily of fish faeces and excess fish feed (Cranford et al., 2013; Ervik et al., 1997; Hansen et al., 2001; Kutti et al., 2007a; Neori et al., 2004; Wang et al., 2012; Wang et al., 2013). An attractive solution to reduce this discharge is to let other organism feed on these particles generating an added value with environmental benefits. Cranford et al. (2013) evaluated constraints facing the capture of particulate discharge from salmon farming using other organisms. Focusing on the Blue Mussel, they concluded that inability to intercept large particles due to sedimentation was the limiting factor. Local conditions vary in how many particles are accessible, but the majority of particulate discharge sediment relatively quickly, preventing direct uptake (Carroll et al., 2003; Irisarri et al., 2015; Jansen et al., 2015; Jansen et al., 2016; Kutti et al., 2007a; Wang et al., 2012). Nevertheless small particles may be intercepted, as these particles are less prone to gravitational sedimentation, hence they remain in suspension for longer and thereby increase the probability of interception by filter-feeders through horizontal transport by the current (Cranford et al., 2013; Jansen et al., 2015). However, small particles will not be efficiently retained by mussels and may therefore not be the optimal organism in salmon driven IMTA (Petersen, 2007; Randløv and Riisgård, 1979; Riisgård and Larsen, 2000). Indeed the retention efficiency rapidly diminishes for particles under 4μm in the Blue Mussels (Chopin et al., 2001; Jørgensen, 1990; Møhlenberg and Riisgård, 1978; Riisgård, 1988). *C. intestinalis* on the other hand retains particles down to 1-2μm with 70% efficiency (Carver et al., 2006; Cranford et al., in press; Daigle and Herbinger, 2009; Randløv and Riisgård, 1979). Furthermore Handå et al. (2012a) reported that 75% of fish faeces particles and around 85% of fish feed particles were in the size range between 2-5μm. Particle concentrations below 2μm might be even higher, although they may not contribute as much to the total biomass volume (Appendix I). Consequently a larger part of the discharge might be accessible to *C. intestinalis* and not as accessible for the Blue Mussel.

Our results suggest that the ratio of fish feed and algae will influence ingestion in *C. intestinalis*, however the data is uncertain. Handå et al. (2012a) conducted growth trials with Blue Mussels given mixtures of algae and fish feed, and another with algae and fish faeces. A significant, but small increase in shell length was found for mussels subjected to the fish feed treatment, while this was not found in the treatment receiving fish faeces (Handå et al., 2012a). Additionally mussels given only algae had the highest growth, followed by fish feed and algae, and lastly fish faeces and algae. The study suggests a lower nutritional content in fish faeces than in fish feed, possibly due digestion in the salmon (Handå et al., 2012a). Although the digestive systems are different between Blue Mussels and *C. intestinalis*, lower nutritional values will decrease the efficiency of digestion in *C. intestinalis* (Sigsgaard et al., 2003), and therefore also growth. It is currently unclear how the differential nutritional value between fish feed and faeces will influence life history parameters in *C. intestinalis*.

4.8 Potential for nutrient assimilation on a regional scale

An alternative to integrating filter feeders directly at production sites, is deployment in regions with intensive aquaculture, where they would assimilate discharged nutrients indirectly (Folke et al., 1994; Handå et al., 2012a; Lindahl, 2011; Pitta et al., 2009; Strohmeier et al., 2015; van Broekhoven et al., 2015). The region would then have a net zero discharge of nutrients. This has the advantage of also assimilating dissolved nutrients from primary production (Mente et al., 2006), and reducing the local impact of benthic communities directly under a possible salmon driven IMTA site by the filter feeders own faecal production (Cranford et al., 2013). However a disadvantage is the seasonality, which limits primary production, subsequently affecting prey availability (Cranford et al., 2013; Handå et al., 2012b; Jansen et al., 2015). For example, in Kertinge Nor a shallow costal inlet in Denmark, Petersen and Riisgård (1992) reported that the natural population of *C. intestinalis* filtered the equivalent volume the entire shallow fjord between one to ten days, depending on the season.

Lindahl et al. (2005) investigated the potential to extract nitrogen from runoff by the blue mussel on the Swedish west coast, in order to counteract coastal eutrophication. Their nutrient cycle model found a reduction of 20% of the particulate and dissolved nitrogen entering the Gullmar Fjord over 10 months (Lindahl et al., 2005).

4.5.3 Environmental effects of C. intestinalis culturing

Suspension feeders will provide nutrients to benthic habitats by faecal production (Wotton and Malmqvist, 2001). However studies from Sweden have shown a decline in benthic quality and biodiversity directly underneath *C. intestinalis* culturing sites (Loo and Petersen, 2013; Norén et al., 2012). This effect originates from faecal production and subsequent microbial breakdown, which consumes oxygen (Cloern, 2001; Folke et al., 1994; Meyer-Reil and Köster, 2000; Norén et al., 2012).

In Norway most fish farms have certified locations which are allowed to produce fish by assessing and monitoring the sites, while using ecological threshold values to determine what impact is acceptable, mostly based on the release of discharge (Ervik et al., 1997; Hansen et al., 2001). Norén et al. (2012) used some of these methods to determine the ecological effects of a *C. intestinalis* culture in an area that was shallow and had a weak current outside of Lysekil municipality in Sweden. Consequently the area represented a worst-case scenario. The results indicated that areas within a 30m radius were of "very bad" quality, with the bottom covered in sulphide-oxidizing (oxygen consuming) bacteria *Beggiatoa*. However 30m from the site, the bottom was characterised as "good" and by 100m "very good". Furthermore, the negative impact disappeared in 6 months (Norén et al., 2012). The effect of *C. intestinalis* culturing surrounding active aquaculture sites would have a limited environmental impact on the benthic habitat, depending on current and depth. However the culturing will presumably positively affect active production sites, because discharge intercepted and digested by *C. intestinalis* will contain less nutrients.

Still, in an IMTA system with benthic deposit feeders, this will constitute a more concentrated nutritional load, and possibly a higher yield of for example

sea cucumbers and polychaetes, as a result of sedimentation of faecal bound pellets (Barrington et al., 2009; Cubillo et al., 2016; Gili and Coma, 1998).

4.9 Conclusions and future research

Our data support our hypothesis, by showing that clearance rates were reduced by high particle concentrations and that retention was within previously reported values. However we found that feeding was affect over time, depending on particle concentration. In addition, our expectation of *Ciona* as a candidate for extractive use in an IMTA system, still remains. Our main results were:

1. Molecular results demonstrates that *Ciona* efficiently removes fish feed from the water column, and given the mixture of algae and fish feed, more animals ingested fish feed, than when given fish feed exclusively. We propose that this apparent feeding selection might be mediated by squirting, in response to large particles from fish feed.

2. We developed a flow through system for determining filtration in filter feeders, with special care to avoid methodological constraints. We believe the system provides robust data, is cheap and practical to construct. Therefore the system can be used for collecting data for many filter feeders.

3. The filtration data indicate that animals can clear mixtures of fish feed and seston at a rate of 50-60 mL/min (3-3.6 L/h) per g^{-1} dry weight, unless the concentrations are too high (>40.000 particles/mL).

4. Furthermore we discovered a previously undescribed temporal effect on clearance rates, which was dependent on particle concentration. Suggesting that *Ciona* might acclimatise to high particle concentration and increase clearance over time.

5. Measurements between two different commonly used methods in filtering studies gave similar results in clearance rates, but differed in retention by the inclusion and standardisation of non-feeding animals. However when excluding non-feeders, our values were within previously found in the literature. Particles over 2μm were 100% retained, while particles down between 1.5-2μm were captured at around 75% efficiency.

6. We underline the importance of including all data on feeding rather than exclusively "optimal", as small deviations are greatly amplified to the population level. Consequently filter feeders in marine ecosystem models might become inaccurate.

7. Our results provide support for the use *Ciona* as a bio-filtering organism in an IMTA system, as it can capture more of the available particles discharged from salmon farming, than the commonly used Blue Mussel. Yet, a regional approach might capture more discharge by indirectly including dissolved nutrients, but would be seasonally dependent.

Future studies should investigate how efficiently *C. intestinalis* digest fish faeces, and could be designed to study the life history effect as well as metabolic efficiency of the different food sources.

Furthermore future investigations should be weary of high particle concentrations when calculating retention efficiency and ideally construct an experiment with equal amounts of particles in each size class to reveal retention of large particles, which is possibly mediated by squirting in *Ciona*.

Additionally a follow up study on the temporal response to elevated particles should be confirmed, and if correct, tolerant individuals might prove more efficient bio filterers in high concentrations conditions.

References

Aldentun, Y., 2013. Biogas - new substrates from the sea. in: Aldentun, Y. (Ed.). The Regional Council in Kalmar county.

Armsworthy, S.L., MacDonald, B.A., Ward, J.E., 2001. Feeding activity, absorption efficiency and suspension feeding processes in the ascidian, *Halocynthia pyriformis* (Stolidobranchia: Ascidiacea): responses to variations in diet quantity and quality. Journal of Experimental Marine Biology and Ecology. 260, 41-69.

Aure, J., Strand, Ø., Erga, S.R., Strohmeier, T., 2007. Primary production enhancement by artificial upwelling in a western Norwegian fjord. Marine Ecology Progress Series. 352, 39-52.

Barrington, K., Chopin, T., Robinson, S., 2009. Integrated multi-trophic aquaculture (IMTA) in marine temperate waters. Integrated mariculture: a global review. FAO Fisheries and Aquaculture Technical Paper. 529, 7-46.

Béné, C., Barange, M., Subasinghe, R., Pinstrup-Andersen, P., Merino, G., Hemre, G., Williams, M., 2015. Feeding 9 billion by 2050 – Putting fish back on the menu. Food Sec. 7, 261-274.

Berg, D.J., Fisher, S.W., Landrum, P.F., 1996. Clearance and processing of algal particles by zebra mussels (*Dreissena polymorpha*). Journal of Great Lakes Research. 22, 779-788.

Bergles, D., Tamm, S., 1992. Control of cilia in the branchial basket of *Ciona intestinalis* (Ascidacea). The Biological Bulletin. 182, 382-390.

Bergström, P., Lindegarth, S., Lindegarth, M., 2015. Modeling and predicting the growth of the mussel, *Mytilus edulis*: implications for planning of aquaculture and eutrophication mitigation. Ecology and Evolution. 5, 5920-5933.

Bingham, B.L., Walters, L.J., 1989. Solitary ascidians as predators of invertebrate larvae: evidence from gut analyses and plankton samples. Journal of Experimental Marine Biology and Ecology. 131, 147-159.

Box, G.E., Cox, D.R., 1964. An analysis of transformations. Journal of the Royal Statistical Society. Series B (Methodological), 211-252.

Box, G.E., Jenkins, G.M., Reinsel, G.C., Ljung, G.M., 2015. Time series analysis: forecasting and control. John Wiley & Sons.

Carroll, M.L., Cochrane, S., Fieler, R., Velvin, R., White, P., 2003. Organic enrichment of sediments from salmon farming in Norway: environmental factors, management practices, and monitoring techniques. Aquaculture. 226, 165-180.

Carver, C.E., Mallet, A.L., Vercaemer, B., 2006. Biological synopsis of the solitary tunicate *Ciona intestinalis*. Bedford Institute of Oceanography Dartmouth, Nova Scotia, Fisheries and Oceans Canada.

Chopin, T., Buschmann, A.H., Halling, C., Troell, M., Kautsky, N., Neori, A., Kraemer, G.P., Zertuche‐González, J.A., Yarish, C., Neefus, C., 2001. Integrating seaweeds into marine aquaculture systems: a key toward sustainability. Journal of Phycology. 37, 975-986.

Cirino, P., Toscano, A., Caramiello, D., Macina, A., Miraglia, V., Monte, A., 2002. Laboratory culture of the ascidian Ciona intestinalis (L.): a model system for molecular developmental biology research. Mar. Mod. Elec. Rec.

Cloern, J.E., 2001. Our evolving conceptual model of the coastal eutrophication problem. Marine Ecology Progress Series. 210, 223-253.

Cordell, D., Drangert, J.O., White, S., 2009. The story of phosphorus: global food security and food for thought. Global environmental change. 19, 292-305.

Cordell, D., Rosemarin, A., Schröder, J.J., Smit, A.L., 2011. Towards global phosphorus security: A systems framework for phosphorus recovery and reuse options. Chemosphere. 84, 747-758.
Coughlan, J., 1969. The estimation of filtering rate from the clearance of suspensions. Marine Biology. 2, 356-358.
Cranford, P., Strohmeier, T., Filgueira, R., Ø., S., in press. Potential methodological influences on the determination of particle retention efficency by suspension feeders; *Mytilus edulis* and *Ciona intestinalis*. Marine Ecology Progress Series.
Cranford, P.J., 2001. Evaluating the 'reliability' of filtration rate measurements in bivalves. Marine Ecology Progress Series. 215, 303-305.
Cranford, P.J., Ward, J.E., Shumway, S.E., 2011. Bivalve filter feeding: variability and limits of the aquaculture biofilter. Shellfish aquaculture and the environment, 81-124.
Cranford, P.J., Reid, G.K., Robinson, S.M., 2013. Open water integrated multi-trophic aquaculture: constraints on the effectiveness of mussels as an organic extractive component. Aquac Environ Interact. 4, 163-173.
Cubillo, A.M., Ferreira, J.G., Robinson, S.M.C., Pearce, C.M., Corner, R.A., Johansen, J., 2016. Role of deposit feeders in integrated multi-trophic aquaculture — A model analysis. Aquaculture. 453, 54-66.
Daigle, R.M., Herbinger, C.M., 2009. Ecological interactions between the vase tunicate (*Ciona intestinalis*) and the farmed blue mussel (*Mytilus edulis*) in Nova Scotia, Canada. Aquatic Invasions. 4, 177-187.
Dame, R.F., 2011. Ecology of marine bivalves: an ecosystem approach. CRC Press.
Dame, R.F., Bushek, D., Prins, T.C., 2001. Benthic Suspension Feeders as Determinants of Ecosystem Structure and Function in Shallow Coastal Waters. in: Reise, K. (Ed.), Ecological Comparisons of Sedimentary Shores. Springer Berlin Heidelberg, Berlin, Heidelberg, pp. 11-37.
Day, E.C., 1919. The physiology of the nervous system of the tunicate. J. exp. Zool. 28, 307-335.
Dehal, P., Satou, Y., Campbell, R.K., Chapman, J., Degnan, B., De Tomaso, A., Davidson, B., Di Gregorio, A., Gelpke, M., Goodstein, D.M., 2002. The draft genome of *Ciona intestinalis*: insights into chordate and vertebrate origins. Science. 298, 2157-2167.
Delegrange, A., Vincent, D., Duret, M., Amara, R., 2015. The use of mussels for mitigating the noxious effect of phytoplankton spring blooms on farmed fish. Aquacultural Engineering. 66, 52-61.
Diaz, R.J., Rosenberg, R., 1995. Marine benthic hypoxia: a review of its ecological effects and the behavioural responses of benthic macrofauna. Oceanography and marine biology. An annual review. 33, 245-203.
Dishaw, L.J., Flores-Torres, J., Lax, S., Gemayel, K., Leigh, B., Melillo, D., Mueller, M.G., Natale, L., Zucchetti, I., De Santis, R., Pinto, M.R., Litman, G.W., Gilbert, J.A., 2014. The Gut of Geographically Disparate *Ciona intestinalis* Harbors a Core Microbiota. PLoS ONE. 9, e93386.
Dybern, B.I., 1965. The life cycle of *Ciona intestinalis* (L.) *f. typica.* in relation to the environmental temperature. Oikos. 16, 109-131.
Engel, A., Schulz, K.G., Riebesell, U., Bellerby, R., Delille, B., Schartau, M., 2008. Effects of CO2 on particle size distribution and phytoplankton abundance during a mesocosm bloom experiment (PeECE II). Biogeosciences. 5, 509-521.
Erga, S.R., Heimdal, B.R., 1984. Ecological studies on the phytoplankton of Korsfjorden, western Norway. The dynamics of a spring bloom seen in relation to hydrographical conditions and light regime. Journal of Plankton Research. 6, 67-90.
Erga, S.R., Ssebiyonga, N., Frette, Ø., Hamre, B., Aure, J., Strand, Ø., Strohmeier, T., 2012. Dynamics of phytoplankton distribution and photosynthetic capacity in a western

Norwegian fjord during coastal upwelling: Effects on optical properties. Estuarine, Coastal and Shelf Science. 97, 91-103.
Ervik, A., Hansen, P.K., Aure, J., Stigebrandt, A., Johannessen, P., Jahnsen, T., 1997. Regulating the local environmental impact of intensive marine fish farming I. The concept of the MOM system (Modelling-Ongrowing fish farms-Monitoring). Aquaculture. 158, 85-94.
FAO, 2009. How to Feed the World in 2050, Rome: High-Level Expert Forum. UN, Rome.
FAO, 2014. The State of World Fisheries and Aquaculture. Food and Agriculture Organization of the United Nations, Rome.
Fiala-Médioni, A., 1974. Ethologie alimentaire d'invertébrés benthiques filtreurs (ascidies). II. Variations des taux de filtration et de digestion en fonction de l'espèce. Marine Biology. 28, 199-206.
Fiala-Médioni, A., 1978a. Filter-feeding ethology of benthic invertebrates (ascidians). V. Influence of temperature on pumping, filtration and digestion rates and rhythms in *Phallusia mamillata*. Marine Biology. 48, 251-259.
Fiala-Médioni, A., 1978b. Filter-feeding ethology of benthic invertebrates (ascidians). IV. Pumping rate, filtration rate, filtration efficiency. Marine Biology. 48, 243-249.
Filgueira, R., Labarta, U., Fernandez-Reiriz, M.J., 2006. Flow-through chamber method for clearance rate measurements in bivalves: design and validation of individual chambers and mesocosm. Limnology and Oceanography: Methods. 4, 284-292.
Filgueira, R., Grant, J., Strand, Ø., 2014. Implementation of marine spatial planning in shellfish aquaculture management: modeling studies in a Norwegian fjord. Ecological Applications. 24, 832-843.
Filgueira, R., Grant, J., Strand, Ø., Asplin, L., Aure, J., 2010. A simulation model of carrying capacity for mussel culture in a Norwegian fjord: Role of induced upwelling. Aquaculture. 308, 20-27.
Filgueira, R., Byron, C., Comeau, L., Costa-Pierce, B., Cranford, P.J., Ferreira, J., Grant, J., Guyondet, T., Jansen, H., Landry, T., 2015. An integrated ecosystem approach for assessing the potential role of cultivated bivalve shells as part of the carbon trading system. Marine Ecology Progress Series. 518, 281-287.
Fiskeridirektoratet, 2016. Biomassestatistikk. in: Næringsdepartementet (Ed.). Fiskeridirektoratet, Kyst og havbruksavdelingen, Tilsynsseksjonen, Online.
Flood, P.R., Fiala-Médioni, A., 1981. Ultrastructure and Histochemistry of the Food Trapping Mucous Film in Benthic Filter-Feeders (Ascidians). Acta Zoologica. 62, 53-65.
Folke, C., Kautsky, N., Troell, M., 1994. The Costs of Eutrophication from Salmon Farming: Implications for Policy. Journal of Environmental Management. 40, 173-182.
Fréchette, M., Urquiza, J.M., Daigle, G., Rioux-Gagnon, D., 2016. Clearance rate regulation in mussels: Adding the effect of seston level to a model of internal state-based regulation. Journal of Experimental Marine Biology and Ecology. 475, 1-10.
Frette, O., Erga, S.R., Hamre, B., Aure, J., Stamnes, J.J., 2004. Seasonal variability in inherent optical properties in a western Norwegian fjord. Sarsia. 89, 276-291.
Gibson, R., Atkinson, R., Gordon, J., 2009. Zooplankton diel vertical migration—a review of proximate control. Oceanography and marine biology: an annual review. 47, 77-110.
Gili, J.-M., Coma, R., 1998. Benthic suspension feeders: their paramount role in littoral marine food webs. Trends in Ecology & Evolution. 13, 316-321.
Goldberg, E.D., McBlair, W., Taylor, K.M., 1951. The uptake of vanadium by tunicates. The Biological Bulletin. 101, 84-94.
Handå, A., Ranheim, A., Olsen, A.J., Altin, D., Reitan, K.I., Olsen, Y., Reinertsen, H., 2012a. Incorporation of salmon fish feed and feces components in mussels (*Mytilus edulis*): Implications for integrated multi-trophic aquaculture in cool-temperate North Atlantic waters. Aquaculture. 370–371, 40-53.

Handå, A., Min, H., Wang, X., Broch, O.J., Reitan, K.I., Reinertsen, H., Olsen, Y., 2012b. Incorporation of fish feed and growth of blue mussels (*Mytilus edulis*) in close proximity to salmon (*Salmo salar*) aquaculture: Implications for integrated multi-trophic aquaculture in Norwegian coastal waters. Aquaculture. 356–357, 328-341.

Handå, A., Forbord, S., Wang, X., Broch, O.J., Dahle, S.W., Størseth, T.R., Reitan, K.I., Olsen, Y., Skjermo, J., 2013. Seasonal- and depth-dependent growth of cultivated kelp (*Saccharina latissima*) in close proximity to salmon (*Salmo salar*) aquaculture in Norway. Aquaculture. 414–415, 191-201.

Hansen, P.K., Ervik, A., Schaanning, M., Johannessen, P., Aure, J., Jahnsen, T., Stigebrandt, A., 2001. Regulating the local environmental impact of intensive, marine fish farming: II. The monitoring programme of the MOM system (Modelling–Ongrowing fish farms–Monitoring). Aquaculture. 194, 75-92.

HLPE, 2014. Sustainable fisheries and aquaculture for food security and nutrition. in: Organization, U.N.F.a.A. (Ed.), High Level Panel of Experts on Food Security and Nutrition of the Committee on World Food Security. FAO, Rome.

Høgøy, I., Troedsson, C., Magnesen, T., 2015. Tunicate ascidians; a new marin biomass. in: FORNY2020 (Ed.). UiB, Uni Research AS, BTO.

Holthuis, T.D., Bergström, P., Lindegarth, M., Lindegarth, S., 2015. Monitoring Recruitment Patterns of Mussels and Fouling Tunicates in Mariculture. Journal of Shellfish Research. 34, 1007-1018.

Hothorn, T., Bretz, F., Westfall, P., 2008. Simultaneous Inference in General Parametric Models. Biometrical Journal. 50 346-363.

Irisarri, J., Fernández-Reiriz, M.J., Labarta, U., Cranford, P.J., Robinson, S.M.C., 2015. Availability and utilization of waste fish feed by mussels *Mytilus edulis* in a commercial integrated multi-trophic aquaculture (IMTA) system: A multi-indicator assessment approach. Ecological Indicators. 48, 673-686.

Islam, M.S., 2005. Nitrogen and phosphorus budget in coastal and marine cage aquaculture and impacts of effluent loading on ecosystem: review and analysis towards model development. Marine Pollution Bulletin. 50, 48-61.

Jansen, H., Strand, Ø., Cranford, P., Handå, A., 2015. Multitrofisk Akvakultur: Hvor går utviklingen? Institute of Marine Research, Havforskningsrapporten 2015, pp. 44-46.

Jansen, H.M., Strand, O., Strohmeier, T., Krogness, C., Verdegem, M., Smaal, A., 2011. Seasonal variability in nutrient regeneration by mussel *Mytilus edulis* rope culture in oligotrophic systems. Marine Ecology Progress Series. 431, 137-149.

Jansen, H.M., Burg, S., Bolman, B., Jak, R.G., Kamermans, P., Poelman, M., Stuiver, M., 2016. The feasibility of offshore aquaculture and its potential for multi-use in the North Sea. Aquaculture International, 1-22.

Jeschke, J.M., Kopp, M., Tollrian, R., 2004. Consumer-food systems: why type I functional responses are exclusive to filter feeders. Biological Reviews. 79, 337-349.

Jiang, A.-l., Yu, Z., Cai, W.-g., Wang, C.-h., 2008. Feeding selectivity of the marine ascidian *Styela clava*. Aquaculture Research. 39, 1190-1197.

Joly, J.-S., Kano, S., Matsuoka, T., Auger, H., Hirayama, K., Satoh, N., Awazu, S., Legendre, L., Sasakura, Y., 2007. Culture of *Ciona intestinalis* in closed systems. Developmental Dynamics. 236, 1832-1840.

Jørgensen, B.B., 1996. Material Flux in the Sediment, Eutrophication in Coastal Marine Ecosystems. American Geophysical Union, pp. 115-135.

Jørgensen, C.B., 1949. Feeding-rates of sponges, lamellibranchs and ascidians. Nature. 163, 912.

Jørgensen, C.B., 1990. Bivalve filter feeding: hydrodynamics, bioenergetics, physiology and ecology. Olsen & Olsen.

Jørgensen, C.B., Goldberg, E.D., 1953. Particle filtration in some ascidians and lamellibranchs. The Biological Bulletin. 105, 477-489.
Jørgensen, C.B., Kiørbøe, T., Møhlenberg, F., Riisgård, H.U., 1984a. Ciliary and mucus-net filter feeding, with special reference to fluid mechanical characteristics. Mar. Ecol. Prog. 15, 283-292.
Jørgensen, C.B., Kiørboe, T., Møhlenberg, F., Riisgård, H.U., 1984b. Ciliary and mucus-net filter feeding, with special reference to fluid mechanical characteristics. Marine Ecology - Progress Series. 15, 283-292.
Kalantzi, I., Karakassis, I., 2006. Benthic impacts of fish farming: Meta-analysis of community and geochemical data. Marine Pollution Bulletin. 52, 484-493.
Kiørboe, T., Andersen, K.P., Dam, H.G., 1990. Coagulation efficiency and aggregate formation in marine phytoplankton. Marine Biology. 107, 235-245.
Kiørboe, T., Lundsgaard, C., Olesen, M., Hansen, J.L.S., 1994. Aggregation and sedimentation processes during a spring phytoplankton bloom: A field experiment to test coagulation theory. Journal of Marine Research. 52, 297-323.
Klevjer, T., Norheim, E., Aksnes, D., Strand, E., Knutsen, T., Melle, W., Wiebe, P., 2014. Zooplankton and micronekton vertical distribution and diel vertical migration behaviour in the Northern North Atlantic Ocean. in: ICES (Ed.), Online, pp. 3.
Klumpp, D., 1984. Nutritional ecology of the ascidian *Pyura stolonifera*: Influence of body size, food quantity and quality on filter-feeding, respiration, assimilation efficiency and energy balance. Marine ecology progress series. Oldendorf. 19, 269-284.
Knutsen, J.A., 2016. Havforskningsrapporten 2016, Fisken og havet. Institute of marine research, pp. 37-39.
Kustin, K., Ladd, K.V., McLeod, G.C., Toppen, D.L., 1974. Water transport rates of the tunicate *Ciona intestinalis*. The Biological Bulletin. 147, 608-617.
Kutti, T., Ervik, A., Hansen, P.K., 2007a. Effects of organic effluents from a salmon farm on a fjord system. I. Vertical export and dispersal processes. Aquaculture. 262, 367-381.
Kutti, T., Hansen, P.K., Ervik, A., Høisæter, T., Johannessen, P., 2007b. Effects of organic effluents from a salmon farm on a fjord system. II. Temporal and spatial patterns in infauna community composition. Aquaculture. 262, 355-366.
Larsen, P.S., Riisgård, H.U., 2009. Viscosity and not biological mechanisms often controls the effects of temperature on ciliary activity and swimming velocity of small aquatic organisms. Journal of Experimental Marine Biology and Ecology. 381, 67-73.
Larsen, P.S., Riisgård, H.U., 2012. Validation of the flow-through chamber (FTC) and steady-state (SS) methods for clearance rate measurements in bivalves. Biology open. 1, 6-11.
Ledda, F.D., Pronzato, R., Manconi, R., 2014. Mariculture for bacterial and organic waste removal: a field study of sponge filtering activity in experimental farming. Aquaculture Research. 45, 1389-1401.
Lesser, M.P., Shumway, S.E., Cucci, T., & , Smith, J., 1992. Impact of fouling organisms on mussel rope culture: interspecific competition for food among suspension-feeding invertebrates. Journal of Experimental Marine Biology and Ecology. 162, 91-102.
Lindahl, O., 2011. Mussel Farming as a Tool for Re‐Eutrophication of Coastal Waters: Experiences from Sweden. Shellfish aquaculture and the environment, 217-237.
Lindahl, O., Hart, R., Hernroth, B., Kollberg, S., Loo, L.-O., Olrog, L., Rehnstam-Holm, A.-S., Svensson, J., Svensson, S., Syversen, U., 2005. Improving Marine Water Quality by Mussel Farming: A Profitable Solution for Swedish Society. AMBIO: A Journal of the Human Environment. 34, 131-138.
Locke, A., Carman, M., 2009. Ecological interactions between the vase tunicate (*Ciona intestinalis*) and the farmed blue mussel (*Mytilus edulis*) in Nova Scotia, Canada. Aquatic Invasions. 4, 177-187.

Loo, L.-O., Petersen, J.K., 2013. Miljökonsekvenser av odling av tarmsjöpungen *Ciona intestinalis*, Online.
McClimans, T.A., Handå, A., Fredheim, A., Lien, E., Reitan, K.I., 2010. Controlled artificial upwelling in a fjord to stimulate non-toxic algae. Aquacultural Engineering. 42, 140-147.
Mente, E., Pierce, G.J., Santos, M.B., Neofitou, C., 2006. Effect of feed and feeding in the culture of salmonids on the marine aquatic environment: a synthesis for European aquaculture. Aquaculture International. 14, 499-522.
Meyer-Reil, L.-A., Köster, M., 2000. Eutrophication of Marine Waters: Effects on Benthic Microbial Communities. Marine Pollution Bulletin. 41, 255-263.
Miljødirektoratet, 2014. Tilførsler av næringssalter fra fiskeoppdrett. in: (NIVA), M. (Ed.). NIVA, Miljøstatus.
Møhlenberg, F., Riisgård, H.U., 1978. Efficiency of particle retention in 13 species of suspension feeding bivalves. Ophelia. 17, 239-246.
Nejstgaard, J.C., Frischer, M.E., Simonelli, P., Troedsson, C., Brakel, M., Adiyaman, F., Sazhin, A.F., Artigas, L.F., 2008. Quantitative PCR to estimate copepod feeding. Marine Biology. 153, 565-577.
Neori, A., Chopin, T., Troell, M., Buschmann, A.H., Kraemer, G.P., Halling, C., Shpigel, M., Yarish, C., 2004. Integrated aquaculture: rationale, evolution and state of the art emphasizing seaweed biofiltration in modern mariculture. Aquaculture. 231, 361-391.
Nielsen, P., 2014. Future challenges and possibilities for the Danish long-line production of blue mussels, *Mytilus edilus*. University of Copenhagen, Online.
Nkemka, V., Murto, M., 2013. Two-stage anaerobic dry digestion of blue mussel and reed. Renewable energy. 50, 359-364.
Norén, F., 2015. Förnyelsebar energi från ett rent hav. Marine Biogas AB, Online.
Norén, F., Norén, K., Magnusson, K., 2012. Slutrapport Ekologiska effekter av ascidieodling. Lysekils kommun, Online.
Olafsen, T., Winther, U., Olsen, Y., Skjermo, J., 2012. Value creation based on productive oceans in 2050. Det Kongelige Videnskabers Selskap (DKNVS) and Norges Tekniske Vitenskapsakademi., Online.
Olsen, L.M., Holmer, M., Olsen, Y., 2008. Perspectives of nutrient emission from fish aquaculture in coastal waters. The Fishery and Aquaculture Industry Research Fund, Online.
Pascoe, P., Parry, H., Hawkins, A., 2009. Observations on the measurement and interpretation of clearance rate variations in suspension-feeding bivalve shellfish. Aquatic Biology. 6, 181-190.
Paul, J.H., Jeffrey, W.H., DeFlaun, M.F., 1987. Dynamics of extracellular DNA in the marine environment. Applied and Environmental Microbiology. 53, 170-179.
Petersen, J.K., 2007. Ascidian suspension feeding. Journal of Experimental Marine Biology and Ecology. 342, 127-137.
Petersen, J.K., Riisgård, H.U., 1992. Filtration capacity of the ascidian *Ciona intestinalis* and its grazing impact in a shallow fjord. Marine Ecology-Progress Series. 88, 9-17.
Petersen, J.K., Svane, I., 1995. Larval dispersal in the ascidian *Ciona intestinalis* (L.). Evidence for a closed population. Journal of Experimental Marine Biology and Ecology. 186, 89-102.
Petersen, J.K., Svane, I., 2002. Filtration rate in seven Scandinavian ascidians: implications of the morphology of the gill sac. Marine Biology. 140, 397-402.
Petersen, J.K., Mayer, S., Knudsen, M.Å., 1999. Beat frequency of cilia in the branchial basket of the ascidian *Ciona intestinalis* in relation to temperature and algal cell concentration. . Marine Biology. 133, 185-192.

Petersen, J.K., Hasler, B., Timmermann, K., Nielsen, P., Tørring, D.B., Larsen, M.M., Holmer, M., 2014. Mussels as a tool for mitigation of nutrients in the marine environment. Marine Pollution Bulletin. 82, 137-143.
Pinheiro, J., Bates, D., DebRoy, S., Sarkar, D., R Core Team, 2015. nlme; Linear and Nonlinear Mixed Effects Models.
Pinheiro J., Bates D., DebRoy S., Sarkar D., 2016. Linear and Nonlinear Mixed Effects Models. R package version 3.1-124.
Pitta, P., Tsapakis, M., Apostolaki, E.T., Tsagaraki, T., Holmer, M., Karakassis, I., 2009. Ghost nutrients' from fish farms are transferred up the food web by phytoplankton grazers. Marine ecology progress series. 374, 1-6.
Randløv, A., Riisgård, H.U., 1979. Efficiency of Particle Retention and Filtration Rate in Four Species of Ascidians. Marine ecology progress series. 1, 55-59.
Ray, J.L., Skaar, K.S., Simonelli, P., Larsen, A., Troedsson, C., 2016. Molecular gut content analysis demonstrates that Calanus grazing on *Phaeocystis pouchetii* and *Skeletonema marinoi* is sensitive to bloom phase but not prey density.
Ribes, M., Coma, R., Gili, J.-M., 1998. Seasonal variation of in situ feeding rates by the temperate ascidian *Halocynthia papillosa*. Marine Ecology-Progress Series. 175, 201-213.
Riisgård, H.U., 1988. Efficiency of particle retention and filtration rate in 6 species of Northeast American bivalves. Marine Ecology Progress Series. 45, 217-223.
Riisgård, H.U., 2001. On measurement of filtration rates in bivalves: the stony road to reliable data: review and interpretation. Marine Ecology-Progress Series. 215, 307-310.
Riisgård, H.U., Larsen, P.S., 2000. Comparative ecophysiology of active zoobenthic filter feeding, essence of current knowledge. Journal of Sea Research. 44, 169-193.
Riisgård, H.U., Larsen, P.S., 2010. Particle capture mechanisms in suspension-feeding invertebrates. Marine Ecology Progress Series. 418, 255-293.
Rimondino, C., Torre, L., Sahade, R., Tatián, M., 2015. Sessile macro-epibiotic community of solitary ascidians, ecosystem engineers in soft substrates of Potter Cove, Antarctica. Polar Research. 34.
Robbins, I., 1983. The effects of body size, temperature, and suspension density on the filtration and ingestion of inorganic particulate suspensions by ascidians. Journal of Experimental Marine Biology and Ecology. 70, 65-78.
Robbins, I.J., 1984. The regulation of ingestion rate, at high suspended particulate concentrations, by some phleobranchiate ascidians. Journal of experimental marine biology and ecology. 82, 1-10.
Rosa, M., Ward, J.E., Ouvrard, M., Holohan, B.A., Pales Espinosa, E., Shumway, S.E., Allam, B., 2015. Examining the physiological plasticity of particle capture by the blue mussel, *Mytilus edulis* (L.): Confounding factors and potential artifacts with studies utilizing natural seston. Journal of Experimental Marine Biology and Ecology. 473, 207-217.
Ruppert, E., Fox, R., Barnes, R., 2004. Invertebrate zoology: a functional evolutionary approach Seventh ed. Brooks/Cole Publishing, Belmont, CA, USA.
Sanden, M., Johannessen, L., Berdal, K., Sissener, N., HEMRE, G.I., 2011. Uptake and clearance of dietary DNA in the intestine of Atlantic salmon (*Salmo salar L.*) fed conventional or genetically modified soybeans. Aquaculture Nutrition. 17, e750-e759.
Satoh, G., 2002. A Trajectory of Increasing Activity and the Elaboration of Chemosensory Modality: A New Perspective on Vertebrate Origins. Zoological Science 22, 613-626.
Schroder, J.J., Smit, A.L., Cordell, D., Rosemarin, A., 2011. Improved phosphorus use efficiency in agriculture: a key requirement for its sustainable use. Chemosphere. 84, 822-831.

Sebastiano, D., Levinton, J.S., Doall, M., Kamath, S., 2015. Using a Shellfish Harvest Strategy to Extract High Nitrogen Inputs in Urban and Suburban Coastal Bays: Practical and Economic Implications. Journal of Shellfish Research. 34, 573-583.
Sigsgaard, S., Petersen, J., Iversen, J., 2003. Relationship between specific dynamic action and food quality in the solitary ascidian *Ciona intestinalis*. Marine Biology. 143, 1143-1149.
Sørensen, M., 2012. A review of the effects of ingredient composition and processing conditions on the physical qualities of extruded high-energy fish feed as measured by prevailing methods. Aquaculture Nutrition. 18, 233-248.
Strohmeier, T., Strand, Ø., Cranford, P., 2009. Clearance rates of the great scallop (*Pecten maximus*) and blue mussel (*Mytilus edulis*) at low natural seston concentrations. Marine Biology. 156, 1781-1795.
Strohmeier, T., Strand, Ø., Alunno-Bruscia, M., Duinker, A., Cranford, P.J., 2012. Variability in particle retention efficiency by the mussel *Mytilus edulis*. Journal of Experimental Marine Biology and Ecology. 412, 96-102.
Strohmeier, T., Strand, Ø., Alunno-Bruscia, M., Duinker, A., Rosland, R., Aure, J., Erga, S.R., Naustvoll, L.J., Jansen, H.M., Cranford, P.J., 2015. Response of Mytilus edulis to enhanced phytoplankton availability by controlled upwelling in an oligotrophic fjord. Marine Ecology Progress Series. 518, 139-152.
Svåsand, T., Boxaspen, K., Bjørn, P., Taranger, G., 2015. Akvakultur - muligheter og begrensninger, Havforskningrapporten. IMR, Online, pp. 9-11.
Troedsson, C., Simonelli, P., Nagele, V., Nejstgaard, J.C., Frischer, M.E., 2009. Quantification of copepod gut content by differential length amplification quantitative PCR (dla-qPCR). Marine Biology. 156, 253-259.
Troedsson, C., Thompson, E., Li, J., Bouquet, J., Magnesen, T., Schander, C., 2011. Tunicol - A New Marine Biofuel, Onlines.
Troedsson, C., Thompson, E., Bouquet, J.M., Magnesen, T., Schander, C., Li, J., 2013. Tunicate extract for use in animal feeds, Online. Google Patents.
Troedsson, C., Thompson, E., Magnesen, T., Li, J., Bouquet, J.M., Høgøy, I., 2015. A new biomass for animal feed and cellulose applications. Tunichor, Online.
Troell, M., Joyce, A., Chopin, T., Neori, A., Buschmann, A.H., Fang, J.G., 2009. Ecological engineering in aquaculture—Potential for integrated multi-trophic aquaculture (IMTA) in marine offshore systems. Aquaculture. 297, 1-9.
van Broekhoven, W., Jansen, H., Verdegem, M., Struyf, E., Troost, K., Lindeboom, H., Smaal, A., 2015. Nutrient regeneration from feces and pseudofeces of mussel *Mytilus edulis* spat. Marine Ecology Progress Series. 534, 107-120.
Wang, X.X., Olsen, L.M., Reitan, K.I., Olsen, Y., 2012. Discharge of nutrient wastes from salmon farms: environmental effects, and potential for integrated multi-trophic aquaculture. Aquaculture Environment Interactions. 2, 267-283.
Wang, X.X., Andresen, K., Handa, A., Jensen, B., Reitan, K.I., Olsen, Y., 2013. Chemical composition and release rate of waste discharge from an Atlantic salmon farm with an evaluation of IMTA feasibility. Aquaculture Environment Interactions. 4, 147-162.
Wickham, H., 2009. ggplot2: Elegant Graphics for Data Analysis, Springer-Verlag New York.
Wotton, R.S., Malmqvist, B., 2001. Feces in Aquatic Ecosystems: Feeding animals transform organic matter into fecal pellets, which sink or are transported horizontally by currents; these fluxes relocate organic matter in aquatic ecosystems. BioScience. 51, 537-544.
Wright, J.P., Jones, C.G., 2006. The Concept of Organisms as Ecosystem Engineers Ten Years On: Progress, Limitations, and Challenges. BioScience. 56, 203-209.
Ytrestøyl, T., Aas, T.S., Åsgård, T., 2015. Utilisation of feed resources in production of Atlantic salmon (*Salmo salar*) in Norway. Aquaculture. 448, 365-374.

Zhang, J., Fang, J., 2000. Effect of temperature on feeding selectivity of *Styela clava* and *Ciona intestinalis*. Marine Fisheries Research. 22, 47-51.

Appendices

APPENDIX A. CERTIFICATE OF FISH FEED USED IN ALL EXPERIMENTS

PRODUKSJON SERTIFIKAT

Partinr	Produktnr	Navn
1361912	H51914	SPIRIT ØRRET300-40A 4,5 SE25KG

Betegnelse: Fullfôr til fisk

Bruksanvisning: www.skretting.no

Netto mengde: Se følgeseddel

Sammensetning:

Proteinkonsentrat av soya
Vegetabilsk olje av raps
Vitalt hvetegluten
Fiskeolje
Fiskemel
Hvete
Solsikkemel
Hestebønner, avskallet
Monoammoniumfosfat

Tilsetningsstoffer (pr kg):

Vitaminer

E 672 Vitamin A	3960	IU
E 671 Vitamin D3 (cholecalciferol)	1485	IU

Mikromineraler

E 1 Jern-Fe (jern(II)sulfat, monohydrat)	40	mg
E 2 Jod-I (Kaliumjodid)	0	mg
E 4 Kopper-Cu (Koppersulfat, pentahydrat)	5	mg
E 5 Mangan-Mn (Mangan(II)sulfat, monohydrat)	15	mg
E 6 Sink-Zn(sinksulfate, monohydrat)	99	mg

Fargestoffer inkludert pigmenter

E 161j Astaxanthin	40	mg

Analytiske bestanddeler:

Råprotein	40,3	%
Råfett	31,0	%
Råaske	4,7	%
Råtrevler	2,6	%
Fosfor	9,1	g/kg
Kalsium	5,3	g/kg
Natrium	2,0	g/kg

Holdbarhet: best før (dd.mm.åååå) 18.02.2015

Ansvarlig: Skretting AS, postboks 319 Sentrum, 4002 Stavanger

Registreringsnummer: αNO10050270

Patent

Sertifikat opprettet: 15. mai 2015

Figure A1 Product certificate of the fish feed used in all the experiments.

APPENDIX B. qPCR CYCLE SETUP

Table B1 qPCR cycle protocol for detection soy RuBisCO in fish feed (40 cycles)

Step	Temperature °C	Time (min)
1.	95,0	2:00
2.	95,0	0:10
3.	59,0	0:10
4. GOTO 2 (repeat cycle 39 times)	Repeat above cycle x39	Repeat above cycle x39
5.End		

APPENDIX C. SEQUENCES OF PRIMERS AND PROBE USED IN qPCR

Table C2 DNA sequences of the primers and probe used to detect soy RuBisCOin extracted DNA from *C. intestinalis* intestines.

	sRubisco
Forward primer	GGGCTTACCAGTCTTGATCG
Reverse primer	TGATTTTCTTCCCCAGCAAC
Probe	GGGCGATGCTACGGCCTTGA
PCR-fragment (bp)	71

APPENDIX D. PROTOCOL FOR DNA EXTRACTION

KSS 210910
Rev.: 230910
Rev.: 090211
Rev.: 110511

Purification of total DNA from animal tissues.

DNeasy Blood & Tissue kit, Qiagen.

Pre-treatment: 180 µl ATL, vortex.
20 µl proteinase K over night. Mix and incubate at 56 °C, with stirring.
The sample should not be gelatinous, only viscous. Freeze samples if not immediately continuing the protocol.

Thaw samples on ice. The protocol is performed at room temperature.

Before start gDNA extraction:
Heat samples at 56°C for 5 minutes.

Prepare a mix of buffer AL and 100% (absolute) ethanol for the number of samples you have. Prepare 200 µl of each per sample. For 25 samples: Prepare 5 ml from each.

gDNA extraction:

1. RNase A treatment: Add 4 µl **RNase A**. Mix (careful, no vortex). Incubate at room temperature for 2 minutes.
2. Vortex for 15 s. Add 400 µl **AL/Ethanol**. Vortex thoroughly to achieve homogenous solution.
3. Pipet the mixture (including precipitates) into the spin column placed in 2 ml-collection tube. Centrifuge at 6000 xg for 1 min (8000 rpm). Discard flow-through collection tube.
4. Place the spin column in a new tube (2 ml). Add 500 µl **AW1** buffer and centrifuge 1 min at 6000 xg (8000 rpm). Discard flow-through and collection tube.
5. Place the spin column in a new tube, add 500 µl buffer **AW2**, and centrifuge for 3 at 20 000 xg (14000 rpm). Discard flow-through and collection tube.
   Be careful to avoid contact with flow-through after centrifugation.
6. Place the spin column in a new tube (Eppendorf tube without the cap), pipette 10 buffer **AE** onto membrane. Incubate for 1 minute (RT). Elute by centrifugation at xg (8000 rpm) for 1 minute..
7. Mix sample (careful, no vortex), spin down. Prepare three aliquots of 33 µl each sample; freeze all three at -20°C.

Figure D1. Protocol for DNeasy® blood and tissue kit from QIAGEN® for DNA purification.

APPENDIX E. ANIMALS USED IN INDIVIDUAL EXPERIMENT

Table E1 Wet weight, dry weight, ash-free dry weight and length of animals used in the individual experiment.

Individual	Wet weight (g)	Length (cm)	Dry weight (g)	Ash-free dry weight (g)
1	12,57	9,5	0,81842	0,41143
2	13,82	9,5	0,70089	0,24558
3	13,43	10,5	0,66341	0,24025
4	14,11	9	0,69012	0,25295
5	15,24	10,5	0,71691	0,23297
6	18,76	12	0,97911	0,35990
7	13,49	10,5	0,66938	0,23635
Mean	**14,49**	**10,2**	**0,74832**	**0,28278**
SD	**2,05**	**1,0**	**0,11419**	**0,07213**

APPENDIX F. ANIMALS USED IN THE COHORT EXPERIEMENT

Table F1 Animals weight (g) and length (cm) in the cohort experiment

Tank 1 (Ind #)	Length (cm)	Weight (g)	Tank 2	Length (cm)	Weight (g)	Tank 3	Length (cm)	Weight (g)	Tank 4 (Control)	Length (cm)	Weight (g)
1	9,0	14,9	1	5,0	14,9	1	9,0	19,6	1	6,0	20,5
2	7,0	14,6	2	8,0	13,7	2	12,0	43,7	2	7,0	20,0
3	17,0	71,8	3	14,0	75,8	3	11,0	27,7	3	10,0	17,7
4	9,0	17,8	4	5,0	13,8	4	8,0	15,4	4	10,0	20,2
5	12,0	20,7	5	18,0	51,1	5	5,0	11,4	5	5,0	6,9
6	15,0	43,0	6	16,0	63,8	6	15,0	57,7	6	5,0	7,2
7	9,0	24,2	7	6,0	21,6	7	13,0	34,2	7	9,0	31,9
8	17,0	91,7	8	9,0	28,7	8	12,0	30,1	8	14,0	53,0
9	9,0	26,0	9	14,0	61,9	9	15,0	39,8	9	13,0	84,8
10	14,0	37,9	10	13,0	47,7	10	10,0	23,5	10	5,0	6,8
Mean	**11,8**	**36,3**		**10,8**	**39,3**		**11,0**	**30,3**		**8,4**	**26,9**
SD	**3,7**	**26,1**		**4,8**	**23,5**		**3,1**	**14,1**		**3,3**	**24,7**

APPENDIX G. PARTICLE DISTRIBUTION IN THE INDIVIDUAL STUDY

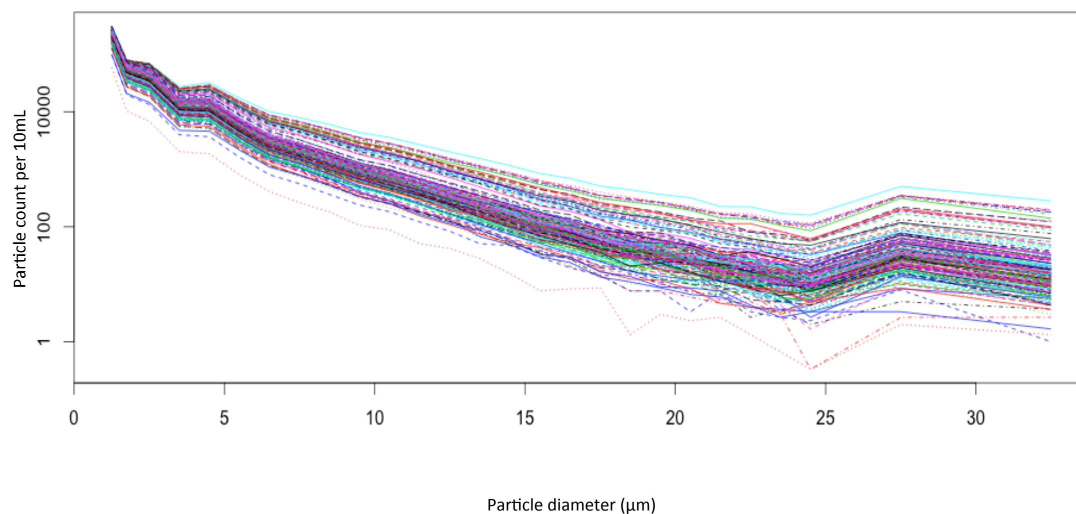

Figure G1 The distribution of counts in the different size classes for all concentrations in the individual study.

APPENDIX H. PARTICLE CONCENTRATION IN THE FISH FEED DETECTION STUDY

Table H1 Average total particle count in different treatments in the fish feed detection study.

Treatment	Mean total particle concentration/mL	SD
Algae	19124	301
Seawater	13852	13852
Algae + fishfeed	59907	62
Fishfeed	43460	1344

APPENDIX I. VOLUME OF PARTICLES FED IN THE INDIVIDUAL STUDY

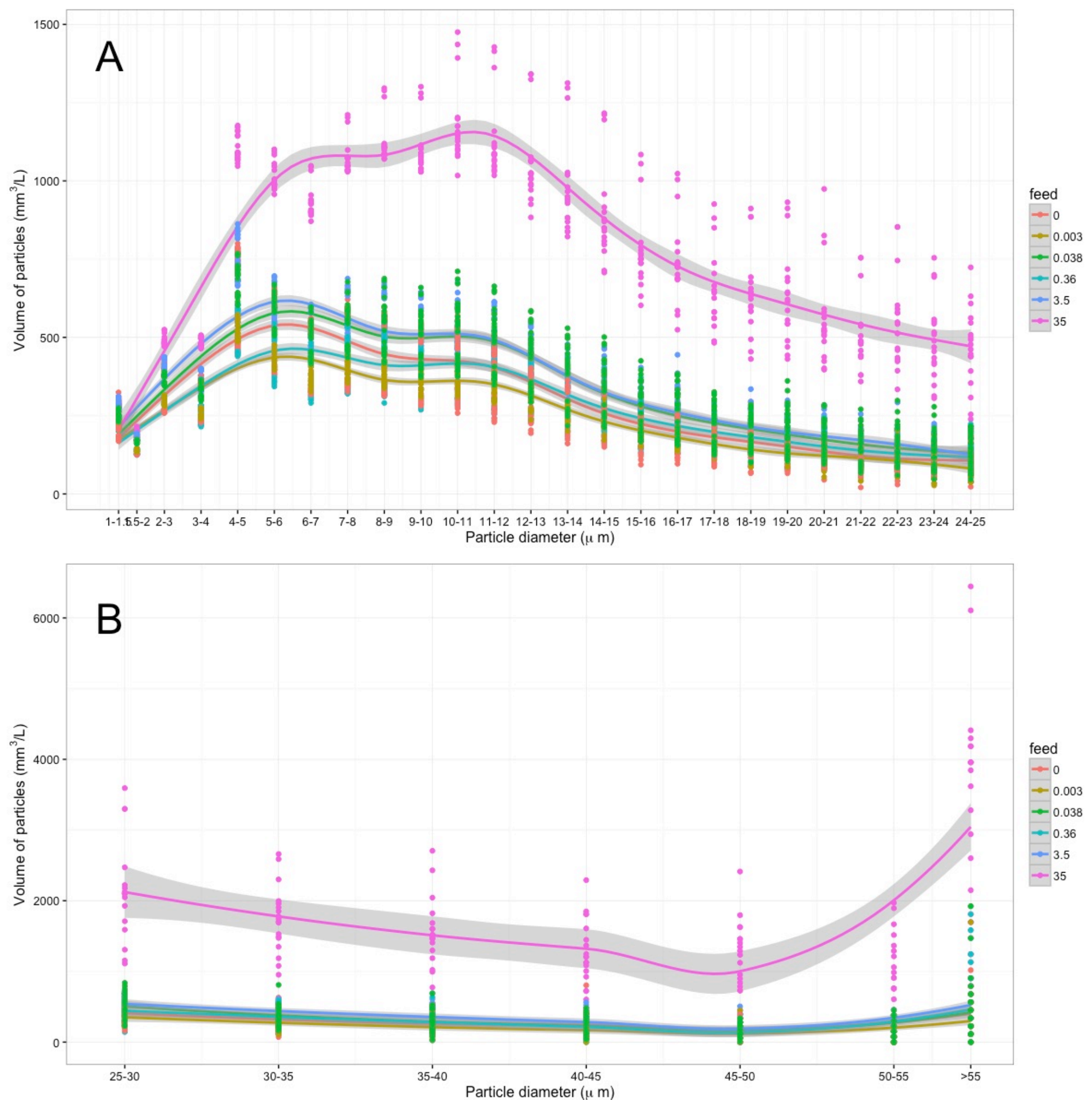

Figure I1 Volume estimation of particle at different diameters, given distinctive fish feed concentrations. Figure A depicts the volume of particles in a narrow range of particle sizes, while B is focused on a wide range. Regression lines fitted with a 95% confidence interval was included for comparative purposes. Dots represent a measurement when subjected to each fish feed concentration. Note the change in scale from A to B.

APPENDIX J. UNSTANDARDISED RE IN FEEDING EXPERIMENTS

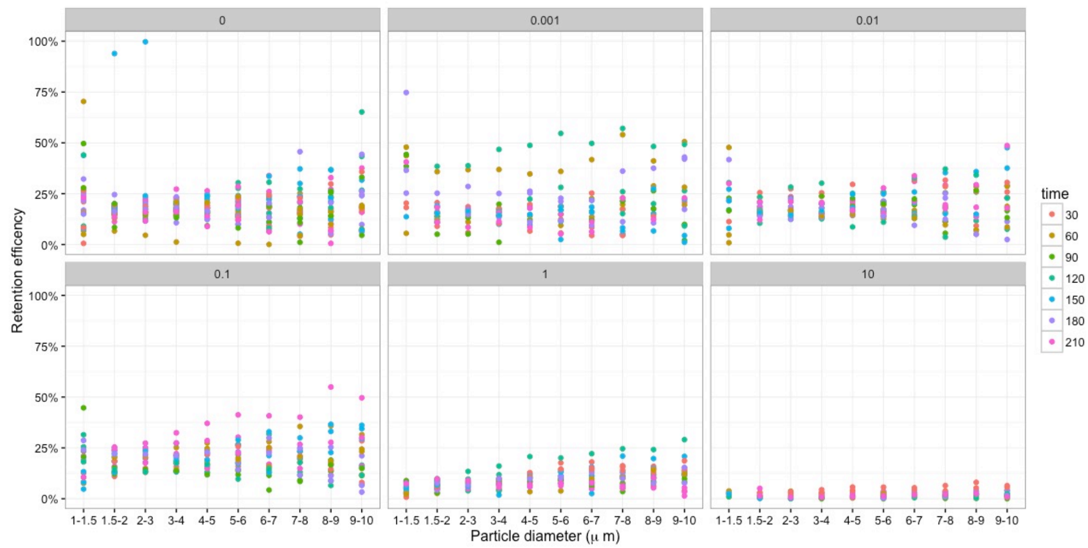

Figure J1 Unstandardised retention efficiency (clearance) of the different treatments in the cohort study.

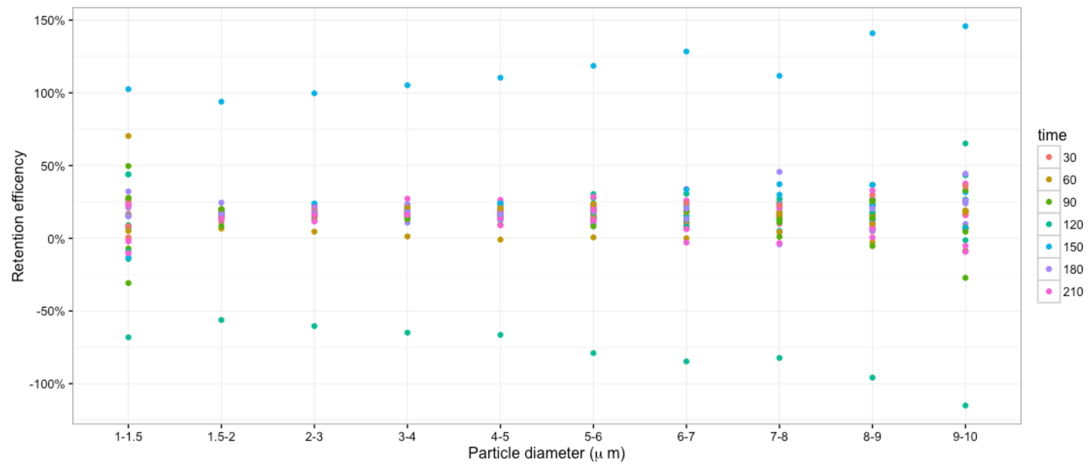

Figure J2 Unstandardised retention efficiency (clearance) of the treatment receiving unfiltered seawater from the cohort study.

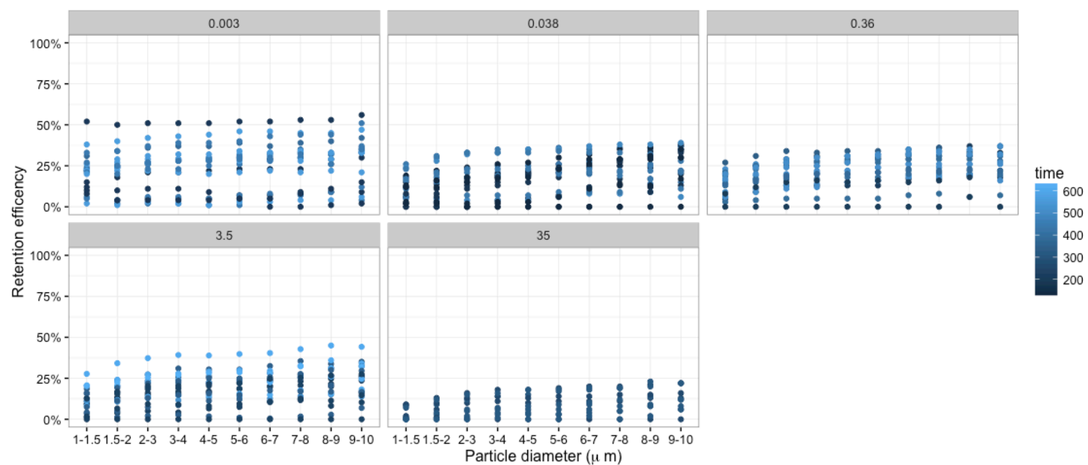

Figure J3 Unstandardised retention efficiency (clearance) of the different treatments in the individual study.

APPENDIX K. SETTLING OF PARTICLES IN THE COHORT STUDY

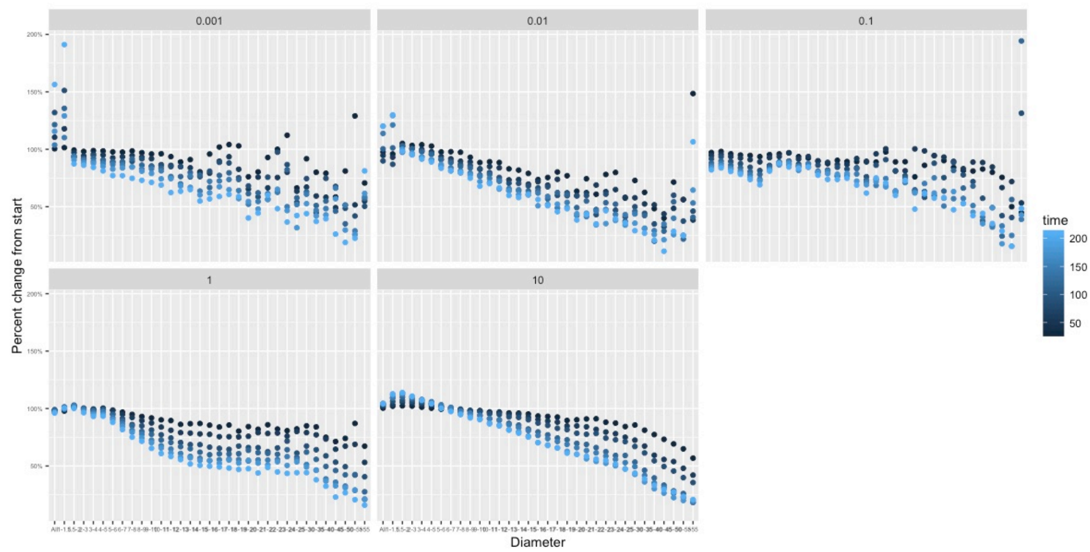

Figure K1 The sedimentation (no animals) of particles in distinct particle sizes over time, given the respective fish feed amounts from the cohort experiment.