Project no. 032556

GRRAS

Towards Elimination of Growth Retardation in Marine Recirculating Aquaculture Systems for Turbot

Cooperative research (CRAFT)

Deliverable 2: Report on the production of GIF by turbot, the effects on growth performance of turbot of local GIF production within RAS and the presence of GIF at commercial farm level

Due date of deliverable: October 2008
Actual submission date: November 2008
Start date of project: 1/11/2006
Duration: 2 years

Organisation name of lead contractor for this deliverable
Wageningen IMARES

Project co-funded by the European Commission within the Sixth Framework Programme (2002-		
Dissemination Level		
PU	Public	
PP	Restricted to other programme participants (including the Commission Services)	
RE	Restricted to a group specified by the consortium (including the Commission Services)	
CO	Confidential, only for members of the consortium (including the Commission Services)	X

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Report number C115/08

Institute for Marine Resources and Ecosystem Studies

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Location IJmuiden

Client: Commission of the European Communities
Research Directorate-General
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Publication Date: December 2008

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BTW nr. NL 811383696B04.

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Summary

Literature review (Task 2.1)
The literature review revealed that in general fishes use a wide variety of chemical compounds for communication. Although chemical communication has not been established in turbot or any other flatfish species, it seems unlikely that turbot do not use chemical communication. First of all many fish species employ chemical signals, both freshwater and saltwater, from diverse families and genera and researchers suggest that chemical communication is common to all fish. Secondly it seems unlikely that many aspects of turbot behavior can take place without chemical communication.
Assuming that turbot use chemical signals to communicate with conspecifics, and these chemicals accumulate continuously in RAS, all this "noise" is likely to have negative effects on fish performance. For example, pheromone-mediated reproductive processes are known to result in behavioral and biochemical changes triggered by the detection of the pheromone. Therefore, continuous accumulation of these chemicals may induce biochemical and behavioral changes at inopportune times, thereby causing disrupted chemoreception. The same applies to accumulation of other chemicals, such as alarm pheromones and possibly others.
Responses to pheromone cues at inappropriate times are believed to be energetically costly and can detract from feeding activities. Therefore, the effect of potentially continuous (high) concentrations of chemical signals in RAS, irrespective of the 'meaning' of these signals, should be considered as being involved in causing growth retardation in RAS.

GIF production by turbot (Task 2.1)
We wanted to demonstrate GIF production in turbot following a methodology that proved to be successful for other fish species. Our approach included induction of GIF production under crowded conditions in GIF production tanks, collection of GIF from the water by activated carbon, extraction of GIF from the activated carbon, preparation of test solutions from the extract and testing the growth inhibiting properties of the test solutions in bio-assays involving early life stages (ELS) and juveniles of turbot. Preliminary work on bio-assays based on ELS led to unexplainable results and we choose to continue our work with juvenile growth trails, which offered the advantage of having growth as direct read-out parameter. Three experiments were performed. Preliminary LC-MS analysis of extracts showed that the composition of GIF extracts was different from control extracts, but no compounds were indentified or quantified.
Experiments consisted of rearing of experimental fish in test solutions prepared from GIF extracts in a range of concentrations and included controls. Experimental systems were operated as static renewal systems. Growth and water quality were measured. The first experiment suggested GIF presence in the test solutions but the experiment suffered also from high mortalities making the results unreliable. This presence of GIF could not be reproduced in the other two trials which both showed no effect of test solutions on growth performance.
From these results we can not conclude that turbot do not produced GIF. The lack of growth response to the test solutions can be attributed to several factors following the experimental procedures. It is possible that turbot indeed do not produce GIF. It is also possible that turbot do not produce GIF under the conditions in the GIF production tank. As husbandry conditions in GIF production tank mimicked commercial conditions and when this did not result in GIF production, GIF production is unlikely to occur under commercial conditions and to be of importance in commercial turbot culture. Indeed no effects of culture systems on growth were observed. Another explanation is that GIF were not retained on the activated carbon or not successfully extracted from the carbon. This would be in contrast to research on zebra fish where this methodology was successfully applied. In addition preliminary LC-MS analysis showed that GIF extracts differed from control extracts. The wide range of concentrations tested excluded in our opinion the sensitivity of the experimental animals to be outside this range. Experimental fishes were possibly not sensitive to the test solutions at all, but this would, again, be in contrast with earlier work on zebra fishes. Experimental set ups were improved for each following experiment while the first experiment suggested GIF activity of the test solutions, excluding in our opinion confounding factors responsible for masking effects of treatments. In conclusion: GIF production of turbot could not be demonstrated.

GIF transfer between tanks (Task 2.3)
In a RAS, locally produced GIF may eventually reach all tanks since water is centrally treated and redistributed to all tanks in the system. As a result, turbot that don't produce GIF (as for their size or husbandry conditions) but share a RAS with GIF producing conspecifics, may still be exposed to GIF and their growth may be affected despite the fact that stocks are physically separated in different tanks. In order to understand better the potential effect of GIF production inside RAS, the effects of stocking different size classes of turbot in one RAS were studied. We investigated the effect of rearing water that was previously in contact with conspecifics on growth of juvenile turbot. A series of three experiments was performed, addressing the effect of size of the conspecifics and the effect of water treatment.

For Experiment 1 and 2 the experimental set up consisted of three tanks (50 L) receiving effluent water from a tank stocked with large (Exp. 1) or small (Exp. 2) turbot (referred to as GIF production tanks) and three tanks receiving borehole water (Control). TAN and oxygen levels were equalized between treatments and solids were removed from the GIF tank effluent prior to use. The set up of Experiment 3 was the same as Experiment 1 but included a third treatment in which the water coming from the GIF production tank was led through a bed of containing 13.5 kg activated carbon (NORIT GAC 830 plus) before flowing into three replicate experimental tanks. Each experimental tank was stocked with 10 juvenile turbot, which were allowed to acclimatize to the experimental conditions. Experiment 1 lasted for 49 days, Experiment 2 for 90 days and Experiment 3 for 45 days. All experiments were performed at France Turbot, Noirmoutier, France.

We hypothesized that growth of juvenile turbot is affected when their rearing water was previously in contact with conspecifics and that this effect is due to the excretion of growth inhibiting factors (GIF) by these conspecifics to the water. Experiment 1 indeed showed a large effect on specific growth rate (SGR) of water that originated from a tank stocked with large turbot at commercial density. This result was however not reproduced in Experiment 2 and 3. Growth performance in the Control treatment of Experiment 1 and all treatments in Experiment 3 was within normal ranges given the size of the fish and the water temperature. In Experiment 2, growth performance was poor in both treatments, indicating that growth was possibly affected by other factors, overruling the effect of the experimental treatments on growth. We consider this a more likely explanation for the lack of effects of treatments than the size of the turbot in the GIF production tank. Experiment 1 and 3 showed a significantly larger increase of the coefficient of variation (CV) during the experimental periods among the fish receiving water from the GIF production tank. This is in agreement with earlier findings for *Prochilodus lineatus*, which showed an increase size variability due to exposure to chemical factors released by conspecifics. No effect on growth rate was observed for this species, which is in agreement with our findings in Experiment 3. In Experiment 1 the larger increase of CV of the fish that received the GIF production tank effluent was possible also caused by the higher initial CV and not the treatment only. A very interesting finding of Experiment 3 is that the large increase in CV as a result of exposure to water that was previously in contact with conspecifics, seems to be counteracted by treating the water with activated carbon: the GIF+activated carbon treatment yielded the same low increase in CV during the experiments as the bore hole water treatment. This suggests that the effect on CV is caused by chemical factors originating from the GIF production tank which are effectively retained on the activated carbon. Based on thresholds for growth, water quality differences were judged to have no effects on growth in all experiments.

In conclusion: from Experiment 1 and 3 it is clear that preconditioning of rearing water by large turbot can affect the growth of juvenile turbot. In Experiment 1 we observed an increased size variation and a reduced SGR due to preconditioning of the rearing water. The observed effects are possibly due to dissolved substances originating from the GIF production tank. Possibly chemicals released by the large turbot, as the effect on size variation of juvenile turbot was effectively counteracted by pretreatment with activated carbon. The absence of an effect on growth in Experiment 2 is likely attributable to confounding factors overruling experimental treatments, as reflected in the overall poor growth.

The presence of GIF at farms (Task 2.4)
The effect of culture water originating from a commercial turbot farm applying a recirculation system (farm water) on growth of juvenile turbot was tested in a semi-static renewal system. Treatments consisted of a range of farm water dilutions, farm water pre-treated with activated carbon and a control. Farm water originated from Groente- en Viskwekerij Cornelisse BV, Stavenisse, The Netherlands and was taken from a densely stocked tank. Nitrite and nitrate levels were equalized among treatments.

The initial individual mean weight was equal for all treatments. No effect of farm water nor an effect of activated carbon treatment of farm water on growth performance was observed: final weight, feed conversion rate and specific growth rate were equal for all treatments and no significant relation between SGR and farm water content of the test solutions was found. The initial coefficient of variation was equal for all treatments and increased during the experiment. No effect of treatments on the coefficient of variation (CV) was observed: the final coefficient of variation and the increase in coefficient of variation was equal for all treatments. Water temperature, oxygen level and nitrite nitrogen level were equal in all experimental treatments. Differences were observed for TAN, nitrate, salinity and pH, but considered to be of no effect on treatments. In conclusion the experiment did not show any growth inhibiting properties of farm water originating from a commercial turbot farm using a recirculation system. However, treatment effects may have been obscured by suboptimal experimental conditions for growth.

General discussion and conclusion

We hypothesized that growth retardation of turbot in recirculating aquaculture systems (RAS) is caused by the production of growth inhibiting factors (GIF) by turbot and the subsequent accumulation of these GIF in the culture water. The literature review revealed that it is likely that turbot excrete chemical signals for communication purposes, which are likely to affect growth when accumulating in RAS. However, we were unable to demonstrate GIF production by turbot, despite using a methodology that proved to be successful in other fish species. Also for water originating from a commercial turbot farm using RAS growth inhibiting properties could not be demonstrated. On the other hand growth and size variation of juvenile turbot was found to be affected when fish were reared in the effluent water of a tank containing large turbot, and the effect on size variation was counteracted by pre-treatment of the water by activated carbon. These findings demonstrate that growth of juvenile turbot is affected by dissolved substances originating from larger conspecifics. In conclusion: turbot produce compounds that affect the growth of conspecifics. The importance of these GIF in commercial turbot aquaculture in RAS remain unclear.

General introduction

One of the working hypotheses of this project is that growth retardation of turbot cultured in recirculating aquaculture systems (RAS) is caused by the accumulation of growth inhibiting factors (GIF) produced by the turbot themselves in the culture water. The production of GIF has been demonstrated for a few fish species. These species were stocked in aquaria in crowded conditions. The water was then extracted using activated carbon and extracts were used to prepare test solutions for early life stage bio-assays, demonstrating the presence of growth inhibiting factors. Production of GIF by turbot has never been investigated.

Our first goal was to demonstrate the production of growth inhibiting factors by turbot following the methodology of Yurl and Perlmutter (1970). A series of successive experiments was performed. Extracts from turbot culture water were tested for GIF presence in early life stage tests using eggs and larvae and small scale growth trials with juveniles (task 2.1).

Our second goal was to demonstrate the transfer of GIF between tanks. Four experiments involving the integration of experimental tanks in a farm scale setting were performed (task 2.3).

Our third goal was to demonstrate the presence of GIF at commercial farms. Two juvenile growth trials were performed (task 2.4).

1 Literature review potential growth inhibiting factors (GIF) produced

1.1 Introduction

In this project it is hypothesized that the problem of growth retardation of turbot in RAS is caused by accumulation of growth inhibiting factors (GIF), produced by the turbot itself and/or bacteria associated to the biological water treatment. Because these GIF haven't been identified yet a literature survey was performed to provide an overview of chemical substances produced and excreted by fish that might have GIF activity.

1.2 Materials and methods

We considered two categories of potential GIF for which separate sub questions were defined.

1. **Metabolites and endocrine substances**
   - *Which metabolites and endocrine substances are produced and released by fish?*
   - *Which of these substances could possibly accumulate in RAS?*
   - *What circumstances trigger or modify their release?*
   - *Which of these substances can be taken up by fish?*
   - *What effects do these substances have on fish?*
   - *Is it possible that a substance for internal regulation of processes in fish (endocrine substance) has (negative) effects on other fish in RAS through accumulation?*

2. **Substances released for the purpose of chemical communication with other fish (pheromones**)
   - *What information is exchanged between fish through chemical communication and under which circumstances does this take place?*
   - *Which fish species are known to use chemical communication?*
   - *Which chemical substances are used?*
   - *Which of these substances could possibly accumulate in RAS?*
   - *Which pheromones are known to affect growth?*
   - *Is it possible that other pheromones have negative side-effects such as growth retardation in high concentrations?*
   - *Is it possible that the situation in RAS (high fish density, recirculation of water) leads to growth retardation through disturbance of fish chemical communication?*

Information was collected by searching relevant databases available via the Wageningen University library. Searched databases include Aquatic sciences and fisheries abstracts (ASFA), Biological abstracts (BIOSIS) and CAB abstracts.

1.3 Results

1.3.1 Fish metabolites

In aquaculture production systems the major metabolic waste products of fish cause changes in the concentrations of ammonia, nitrate, other nitrogenous products, carbon dioxide, solids, phosphate and organic materials. In addition these products cause secondary effects resulting in changes of pH, oxygen demand and bacterial composition (Clark *et al.*, 1985). In the following paragraphs, these products and their effects on fish will be discussed briefly.

Ammonia
The nitrogenous waste product ammonia is excreted through the gills as un-ionized ammonia and as urea. The unionized form can be toxic to fish and repressive to growth and can even be lethal at high concentrations. Acceptable concentrations depend on several factors, for example water pH and sensitivity of fish species (Colt, 2005; Clark *et al.*, 1985; Person-Le Ruyet *et al.*, 2003). The NOEC (no-observable-effect concentration) range for growth has been found to be 0.05-0.20 mg/L NH_3-N in 0.3-100 g turbot (Person-Le Ruyet *et al.*, 2003). TAN (total ammonia nitrogen, sum of the ionized and unionized form) concentrations are safe to growth of turbot juveniles below 2-3 mg/L (Boeuf *et al.*, 1999) and depress growth at levels over 5-10 mg/L (Boeuf *et al.*, 1999).

Nitrite and Nitrate
Accumulation of nitrite and nitrate is mainly the result of biological oxidation of ammonia by nitrifying microorganisms. Both these substance are toxic to fish and toxicity depends on several factors. To avoid negative effects on (growth of) fish, concentrations of these substances should be kept within an acceptable range (Colt, 2005; Clark *et al.*, 1985). Although no specific information is available on save levels for turbot, it is believed that in general, nitrite-nitrogen concentrations should not exceed 10 mg L^{-1} for long periods of time and in most cases should remain below 1 mg/L (Losordo *et al.*, 1998). Studies of the effects of nitrates have shown that aquatic species can tolerate extremely high levels (>200 mg/L) of nitrate-nitrogen in production systems (Losordo *et al.*, 1998).

Carbon dioxide
CO_2, a waste-product of respiration, is excreted through gills (Clark *et al.*, 1985). Elevated CO_2 concentrations in water are not highly toxic to fish, as long as sufficient dissolved oxygen is present (Losordo *et al.*, 1998). However, growth reduction appears to be linearly related to carbon dioxide concentration (Colt, 2005) and CO_2 concentration affects the toxicity level of ammonia (Colt, 2005). Little information is available on safe CO_2 levels, but it is assumed that for most species, free carbon dioxide concentrations in culture water should be maintained at less than 20 mg/L to maintain good growth conditions (Losordo *et al.*, 1998).

Phosphate
Phosphate can accumulate in water as a result of excretion through kidneys and possibly also through leaching from faeces and food (Colt, 2005). Little information is available on effects of phosphate on fish growth and health and on safe levels of this substance.

Solid wastes
Solid wastes originate principally from fragmentation of faeces and can be divided in four categories: Settleable, suspended, floatable and dissolved (Clark *et al.*, 1985; Colt, 2005; Losordo *et al.*, 1998). The first two are of major concern to RAS. If not removed, suspended solids can significantly limit the amount of fish that can be grown in the system and can irritate the gills of the fish. Furthermore, buildup of fine solids has been related to disease outbreaks (Colt, 2005).

1.3.2 Endocrine substances

In this section some of the major fish hormones are briefly discussed. Because it would be beyond the scope of the research question of this survey to give a full description of all fish hormones, only the major and primary effects of some important hormones involved in the regulation of growth, reproduction and stress are discussed, particularly with respect to circumstances of cultured fish. It should be noted that hormone systems in fish are interrelated meaning that hormones involved in stress regulation also affect growth and reproduction, and reproduction hormones play a large role in growth as well and vice versa. Special attention will be paid to steroid hormones, as the release of these hormones and effects of accumulation in water on fish have been documented relatively well.

Hormones involved in the regulation of growth
A variety of hormones have been shown affect fish growth through regulation of a wide range of processes such as food intake, ingestion, adsorption, assimilation, metabolism and excretion (Blanton and Specker, 2007; Zohar, 1989; Matty, 1986).
The major hormones involved in the regulation of growth are:

- Gonadotrophin-Releasing Hormone (GnRH)
- Growth Hormone Releasing Hormone (GHRH)

- Growth hormone (GH)
- Insulin
- Thyrotropin Releasing Hormone (TRH)
- Thyroid Stimulating Hormone (TSH)
- Somatotropin Releasing Inhibiting Factor (SRIF)
- Somatomedins
- Thyroid hormones (T3 and T4)
- Steroid hormones

Some of these are directly involved in growth regulation (e.g. growth hormone, insulin, thyroid hormones and steroid hormones) while others are indirectly involved through their role in the regulation of levels of other hormones.

Growth hormone (GH)

Growth hormone is a pituitary peptide hormone that is known to enhance growth by stimulating appetite and improving food conversion rate in a variety of fish species (Zohar, 1989; Matty, 1986; Pickering, 1993). However, this might not be the case in all fish species, because increased physiological levels of growth hormone have also been observed in combination with decreased food intake and growth rate (in goldfish) (Matty, 1986).

Thyroid hormones

The thyroid hormones triodothyronine (T3) and thyroxine (T4) are amino acid derivatives and assist in the control of osmotic regulation, metabolism, somatic growth, development, maturation, post hatching metamorphosis, skin pigmentation and behavior (Blanton and Specker, 2007; Matty, 1986; Mathiessen, 2003). Examinations of the effects of exposure of fish to these hormones in water have shown various results. In a variety of fish species, thyroid hormones taken up from water improve performances and survival rates (Zohar, 1989; Matty, 1986). However, adverse effects have also been reported. In an experiment with minnow for example, exposure to T3 significantly decreased growth rate (Blanton and Specker, 2007).

Hormones involved in the regulation of reproduction

The major hormones involved in the regulation of reproduction are:

- Gonadotrophin-Releasing Hormone (GnRH)
- Gonadotrophins
- Catecholamines
- Steroids
- Thyroid hormones (T3 and T4)

Gonadotropin-Releasing Hormone (GnRH)

GnRH is a peptide hormone, secreted by the hypothalamus and involved in the production and maturation of gametes. Several disruptions of reproduction processes which occur in many farmed fish, such as a lack of oocyte maturation, ovulation, and spawning, have been attributes to a failure of GnRH function (Zohar, 1989).

Steroids

There is strong evidence that fish release all kinds of steroids into the water. Male and female goldfish for example, are known to release a wide range of steroids in free, glucuronidated and sulphated forms (Scott and Ellis, 2006). Furthermore, there is reason to believe that other fish also release large quantities of many hormonal products into the water (Sorenson *et al.*, 1998).

The mode of excretion differs for free and metabolized forms of steroids. Metabolized (sulphated or glucuronidated) steroids are excreted into the water via the urine and bile, while free steroids are thought to be excreted by passive 'leakage' across the gills (Scott and Ellis, 2006). Besides the evidence that fish release steroids into the water, it is also well known that they take up steroids from the surrounding water. It has been shown that very short periods of exposure of fish to steroids in water are sufficient to induce major effects (Zohar, 1989). For example, juvenile male turbot have been proven to be susceptible to hormonal imbalance as a consequence of short-term exposure (15 days) to a synthetic estrogen (Labadie and Budzinski, 2006). Most reported effects are stimulating, including increased growth, improved feed conversion (Matty, 1986) and accelerated gonadal development (Zohar, 1989). However, in a variety of flatfish species, lipophilic contaminants in water (which act as anti-estrogens or estrogen-mimics) have been shown to induce precocious sexual maturation, retarded

gonad development, reduced egg weight and reduced overall spawning success (Mathiessen, 2003). Furthermore, long-term exposure of fish to steroids dissolved in water has been proposed to cause changes in skin coloration (Zohar, 1989).
These contrasting observed effects may be explained by the conclusion of many other researches that growth promoting effects of steroids are reduced or even reversed when high doses or longer treatments are applied, and that they also vary according to sexual maturity of treated animals (Le Gac *et al.*, 1993). This explanation is supported by the natural mechanism that enables internally released reproductive steroids to accelerate growth during early stages of sexual maturation of fish, while further elevation of reproductive steroid titres during later stages of sexual maturation reduces growth, to direct resources to gonad development (Pickering, 1993; Okuzawa, 2003).
Next to the effects on endocrine regulation in fish, water released steroids are known to play an important role in chemical communication among fish. This pheromone function of steroids is discussed below.

Hormones involved in the stress response

Stress
Potential stressors to cultured fish are handling, crowding, social domination by other fish and (changes in) external conditions (for example low oxygen levels). Most stressors induce a neuro-endocrine cascade in fish, involving an immediate release of catecholamines and activation of the hypothalamic-pituitary-interrenal axis (HPI) (Schreck *et al.*, 2001; Pickering, 1993). The stress response is adaptive in terms of keeping the animal alive but can be maladaptive in terms of performing other necessary life functions.
Situations of long-term stress (which can exist in aquaculture) are known to affect life span, fecundity and the immune system in a negative way. Furthermore, decrease of growth due to stressful conditions has been documented for many species (Pickering, 1993; Schreck *et al.*, 2001), including turbot (Stefánsson *et al.*, 2002).
Hormones involved in the stress response include catecholamines, corticotrophin releasing factor (CRF), corticotropic hormone (CTH) and glucocorticoids (cortisol).

Catecholamines
The catecholamines adrenaline, nor-adrenaline and dopamine are secreted by the adrenal gland directly in response to stressors (acute stress response) and assist in responding to the immediate threat by modifying vascular and respiratory systems and by mobilizing carbohydrate reserves (Pickering, 1993). These hormones/ neurotransmitters could have short-term growth-suppressing effects but there is little evidence that levels of these substances are ever chronically elevated in fish (Pickering, 1993).

Cortisol
Cortisol is the predominant stress steroid (glucocorticoid) in teleost fish that is released by the adrenal gland into the blood when the HPI-axis is stimulated by a stressor. It has a variety of functions, including regulation of energy-mobilizing and iono-osmoregulatory processes (Pickering, 1993; Schreck *et al.*, 2001).
In cases of acute stress, cortisol levels are normally elevated for only a few hours, while in cases of chronic stress (for example in the case of continuous social domination by a conspecific), cortisol levels may be elevated for many weeks. Catabolic effects of this prolonged cortisol elevation are responsible, to some extent, for growth suppression (Pickering, 1993). In some cases of chronic stress though, such as overcrowding, cortisol levels of brown trout, *Salmo trutta* have been shown to return to basal values (Pickering, 1993). However, even if cortisol level return to basal values in chronically stressed fish, growth rates continue to be suppressed (Pickering, 1993), as growth is the result of many processes regulated by many interacting hormones.
Corticoids, like other steroids, are released into the water in the free form primarily via the gills or in conjugated forms via urine or bile (Scott and Ellis, 2006). They have also been suggested to be excreted through feces, but measurements of cortisol metabolites in feces have shown little success (Scott and Ellis, 2006). Since dissolved sex steroids in water are known to be able to induce major effects in fish through interference with endocrine function (see above) it is very likely that dissolved cortisol and/or other corticoids may affect fish in a similar way.

1.3.3 Substances released for chemical communication

Pheromones

A pheromone can be defined as *"an odour or mixture of odorous substances, released by an individual (the sender) and evoking in conspecifics (the receivers) adaptive, specific, and species-typical response(s), the expression of which need not require prior experience or learning."* (Sorenson and Stacey, 2004) In this section substances are reviewed that can be distinguished from 'normal' metabolic products in the sense that they are discrete chemical signals that have evolved as a component of the fish communication system.

Although pheromonal communication of invertebrates has been investigated intensively and is understood quite well to date, relatively little is known about fish pheromones (and of vertebrate pheromones in general). Since communication between individuals of a species of fish by chemical agents was first demonstrated in 1932 (Solomon, 1976), only a handful of fish pheromones have been isolated and chemically identified (Sorenson and Stacey, 2004).

In spite of the lack of information concerning the chemical identity of pheromones, it has become evident that these substances play an important and possibly universal role in fish communication. Chemical communication has been demonstrated in at least ten teleost families with freshwater species, including Petromyzontidae, Cyprinidae, Catostomidae, Cobitidae, Ictaluridae, Clariidae, Gasterosteidae, Salmonidae, Cottidae and Gobiidae(Corkum and Belanger, 2007). Recent studies document the importance of chemical communication in catadromous species (e.g., European eels, family Anguillidae) (Corkum and Belanger, 2007) and a saltwater species (white seabream, family Sparidae) (Olivotto *et al.*, 2002). Furthermore, chemical communication has been suggested in many aspects of fish behavior and development.

Pheromones reviewed in the following paragraphs have been subdivided into separate categories based on their function (if known), circumstances of release and/or effects on other fish:

- Reproductive pheromones
  - Male reproductive pheromones
  - Female reproductive pheromones
- Pheromones involved in non-reproductive aggregation
- Alarm pheromones
- Stress/disturbance pheromones
- Pheromones involved in beneficial conditioning of water
- Crowding factor

Reproductive pheromones

In fish, as in other vertebrates, reproductive behavior is synchronized with gonad development in the individual by hormones, while reproductive activities between individuals are synchronized by pheromones. These reproductive pheromones are known to be commonly used by fish and play a role in many aspects of reproduction, such as gender recognition (Corkum and Belanger, 2007), aggregations prior to reproduction (Corkum and Belanger, 2007), pair formation (Solomon, 1976), reproductive behavior (Liley, 1982; Stacey, 1983) and synchronization of gamete maturation and/or spawning. (Stacey, 2003; Scott *et al.*, 2005; Sorensen, 1992).

Chemical identity and fish species

It is well established that the reproductive pheromones of many fish are water released reproductive hormones, or products of these hormones (Sorenson *et al.*, 1998; Stacey, 2003; Sorensen, 1992). Many fish detect two to six different hormonal products at nanomolar concentrations (Sorenson *et al.*, 1998) and the major fish reproductive hormones (steroids and prostaglandins) and their metabolites have been shown to have pheromonal activity in a number of diverse fish species, including the round goby (*Neogobius melaostomus*), a loach (*Misgurnus anguillicaudatus*), several cyprinids (goldfish, *Carassius auratus*; common and crucian carps, *Cyprinus carpio* and *Cyprinus carassius*; tinfoil barbs, *Puntius spp.*), the african catfish (*Clarius gariepinus*) and salmonids (atlantic salmon, *Salmo salar*; arctic char, *Salvelinus alpinus*) (Stacey, 2003). Additionally, underwater EOG (electro-olfactogram) studies have revealed that the olfactory systems of many other fish species (from families including Siluriformes, Characiformes and Perciformes) are acutely and specifically sensitive to hormonal compounds (hormones and their precursors and/or derivatives) (Stacey, 2003; Sorenson and Stacey, 2004; Corkum and Belanger, 2007), suggesting a pheromone function of these compounds in these and possibly many other related species as well.

Surveys of EOG sensitivity of various fishes have also revealed that reproductive pheromones are often species specific (Sorensen, 1992; Sorenson *et al.*, 1998). This can be explained by the fact that reproductive pheromones often don't comprise a single hormone or hormonal product but are frequently mixtures. The overall composition of the blend and the ability of conspecifics to discern it determine the species specificity of the pheromone. Closely related sympatric species tend to use the same pheromone components, but in different ratios. It has also been proposed that species specific mixtures might employ non-hormonal components or specialized hormonal derivatives associated with novel metabolic enzymes (Sorenson *et al.*, 1998). Furthermore, some fish species detect only conjugated steroids while others detect only non-conjugated steroids suggesting specialization associated with urinary and non-urinary release (Stacey, 1983).

The reproductive pheromone system of goldfish

Reproductive pheromones have been studied most intensively in goldfish and salmonids, especially Atlantic salmon (*Salmo salar*). Although these species have very different life histories and reproductive habits, they do seem to employ at least some of the same components of reproductive pheromones (Sorenson *et al.*, 1998). Because the goldfish reproductive pheromone system is best understood, it will be used here to illustrate several aspects of both female and male reproductive pheromones. Details of this system may only be relevant to related species with similar reproductive systems, but it has been proposed that multifunctional pheromones like that of goldfish are present in other species as well (Stacey, 2003). Some substances involved, for example 17α,20ß-dihydroxy-4-pregnene-3-one (17,20 ß -P), are known to act as a pheromone in many species of fish (Kolodziej et al., 2003).

Female reproductive pheromones

(Pre)ovulatory female goldfish produce and release into the water large quantities of at least several dozen gonadal steroids and their derivatives, the compostion of which changes over time (Sorenson et al., 1998). The best understood substances among these are preovulatory steroids and postovulatory prostaglandins, which act on male physiology, behavior and mating success (Sorenson et al., 1998; Stacey, 1983; Stacey, 2003; Sorensen, 1992).

Preovulatory pheromone is a mixture of androstenedione (AD), 17α,20ß-dihydroxy-4-pregnene-3-one (17,20 ß -P) and 17,20 ß -P-20 ß -sulfate (17,20 ß -P-S) These components induce distinct responses at picomolar concentrations. 17,20 ß -P for example induces swimming and searching behavior in males and both 17,20 ß -P and 17,20 ß -P-S affect milt volume, thereby increasing sperm release and sperm motility and stimulating male competitive behavior (Sorenson *et al.*, 1998; Stacey, 1983; Stacey, 2003; Sorensen, 1992).

Next to its effects on males, 17,20 ß -P also affects other females. Exposure of females to this component of the pheromone increases occurrence of ovulation, thereby offering a mechanism for females to synchronize ovulation (Stacey, 2003).

After ovulation, the postovulatory pheromone, prostaglandin F2α (PGF2α), is released. This pheromone triggers courtship and also milt increase in males (Stacey, 2003).

Male reproductive pheromones

Male goldfish adjust milt volume in response to male cues, but evidence that such responses are mediated with pheromones is preliminary (Stacey, 2003). Male pheromones released in milt are known to play a role in species that engage synchronous mass spawning, for example herring (Stacey, 1983). Furthermore, it has been suggested that in species in which males defend potential nest-sites, pheromones may serve to attract ovulated females to the nest site (Stacey, 1983) and mark the territory (Liley, 1982), whereas in species that form persistent pair bonds and defend small territories, pheromonal stimulation of the female may be important in maximizing reproductive output (Liley, 1982).

Plasticity of olfactory sensitivity to pheromones

Testosterone has been found to be a potent odorant in Atlantic salmon, *Salmo salar,* but sensitivity of male appeared for a limited period during October only and only after pre-exposure to the urine of ovulated females. Furthermore, immature fish did not respond at any time (Hara, 1994). For male and female platy-fish, *Xiphophorus maculates*, maturation of the olfactory system, responsible for perception of pheromones, has been proven to occur at a specific stage of sexual maturation (Schreibman *et al.*, 1984). It is unknown if the olfactory sensitivity to reproductive pheromones commonly appears only during certain periods coinciding with certain endocrine processes and if this plasticity of sensitivity occurs for other types of pheromones as well.

Pheromones involved in non-reproductive aggregation

Some fish are known to have the ability to determine relatedness of conspecifics through odor recognition. These "familial" odors can play a role in several aspects of the fish social system, including parent-young recognition, dominance hierarchies, schooling/shoaling and migration(Liley, 1982; Sorenson and Stacey, 2004; Solomon, 1976).

Several suggestions have been made about the source of the odor. Skin mucus appears to be the major source (Liley, 1982) but it seems likely that more secretions are involved (Sorenson and Stacey, 2004). Urine has been implicated as a potential source in salmonids (Sorenson and Stacey, 2004) and several species of Ictalurus (Liley, 1982). In the latter, seminal vesicles and urophysis have been proposed to play a role in secretion as well (Liley, 1982).

Species recognition

For many fish species, conspecific odor has been demonstrated to promote aggregation/shoaling. Although little research has been done to identify the odor, suggestions have been made about the involvement of bile acids and L-amino acids (Sorenson and Stacey, 2004).

Kin and individual recognition

The identity of the individual, kin-specific odor that plays a role in for example parent-young recognition and the establishment of dominance hierarchies is unknown. Evidence exists though that in North American Ictalurid catfish at least some components of the odor are L-amino acids and it seems likely that complex mixtures are involved (Sorenson and Stacey, 2004).

Conspecific size recognition

The pintado catfish, *Pseudoplatystoma coruscans,* has recently been proven to be able to recognize conspecific size by chemical cues related to size. Although the chemical agent involved is unknown, it has been suggested that other size-related traits may be identified instead of size. As different motivational internal states may be chemically communicated among conspecifics in fish, the different reactions observed in an experiment towards water preconditioned with small and water preconditioned with large pintados may be an assessment of such internal states, which in turn may be related to size (Ciaquinto and Volpato, 2005).

Migration

It is known for at least a few species of migratory fish that localization of feeding/and or spawning habitat involves tracking of conspecific odor. Therefore, migratory attractants might be regarded as a special type of aggregation pheromone. Much research on this subject has been directed to the homing migration of salmonids (Liley, 1982; Solomon, 1976). Migrating adults of these fish are attracted to streams containing conspecifics and are able to discriminate between different populations on the basis of odor (Liley, 1982). These odors are thought to be released by skin mucus (Liley, 1982).

Migratory pheromones have been characterized in about a dozen of fish species and appear to be composed largely of bile acids (Sorenson *et al.*, 1998). For one intensively investigated migratory species for example, the sea lamprey, some key components of the pheromone are petromyzonamine disulphate (PADS), petromyzosterol disulphate (PSDS) and petromyzonal sulfate (PS) (Corkum and Belanger, 2007).

Alarm pheromones

Many species of fish employ specific pheromones to warn conspecifics in threatening situations. These "alarm pheromones" have been demonstrated in several species of darter, goby, cyprinodontiforms and many others, but have especially been investigated intensively in fishes of the super order Ostariophysi (Jordao and Volpato, 2000).

The Ostariophysan alarm system, or "Schreckstoff system", consists of distinctive epidermal club cells; alarm substance cells, which contain the alarm pheromone, called "Schreckstoff". This substance is released into the water if cells are broken as a result of injury of the fish, evoking behavioral changes; the "fright reaction" in other fish that may reduce their vulnerability to predation. This response has been observed in the vast majority of Ostariophysan species tested (Jordao and Volpato, 2000).

Some researchers who examined the biochemical nature of Schreckstoff, have concluded that the active component is probably hypoxanthine-3(N)-oxide. In contrast, others concluded that alarm substance is likely to be a complex of an active compound with a protein, and that the active component is not a pterin (Jordao and Volpato, 2000). The alarm pheromone of a non Ostariophysan species, *Corynopoma riisei* has been suggested to be a mucopolysaccharide (Liley, 1982).

Alarm pheromones show less species specificity than other investigated fish pheromones (reproductive pheromones for example). For the Ostariophysan Schreckstoff as well as for the alarm pheromones of other species, there is substantial cross-reaction between species and even genera. (Jan and Smith, 1992) However, the intensity of the response to alarm substance in the receiver is related to the phylogenetic proximity of the releasing species (Liley, 1982).

Stress/disturbance pheromone

Chemical communication is known to play an important role in transfer of warning information in fish. Most of the studies on this subject report chemicals released from damaged skin. However, "disturbance pheromones", chemicals released by stressed, but not injured, animals, are also known to exist in many aquatic organisms (Jordao and Volpato, 2000). Although these substances have scarcely been studied in fish, a few experiments have proven their existence.

An experiment with Iowa darters for example, has revealed that these fish release chemicals that induce alert posture in conspecifics when visually stimulated by an artificial model of a predator. These chemicals have been defined as "alerting pheromones" (Jordao and Volpato, 2000).

Individuals of pacus, *Piaractus mesopotamicus*, have also been shown to be able to chemically warn conspecifics without being injured. When these fish visually detect a predator they show altered behavior and release chemicals that induce conspecifics to adopt a similar behavioral response. It has been suggested that at least two chemicals might be involved, one of them possibly an alerting pheromone (Jordao and Volpato, 2000).

Finally, a recent study with white sea-bream, *Diplodus sargus*, revealed the presence of chemical communication between stressed fish. Stress in these marine fish was not induced by perception of a seriously threatening situation, such as the presence of a predator, but was caused by high stocking density (Olivotto *et al.*, 2002). From the results of this study it seems reasonable to suggest that, at least for some fish species, chemical communication might take place in situations of stress in general, whether acute or chronic and regardless of the identity of the stressor.

Little is known about the chemical structure of the signal involved. It has been proposed though for Iowa darter, pacu and crayfish that a nitrogenous waste product, possibly ammonium, might be a component of the disturbance pheromone (Jordao and Volpato, 2000).

Pheromones involved in the beneficial conditioning of water

Under certain conditions, homotypically conditioned water, water in which fish of the same species have been present, can promote rather than retard growth, at least of the species of fish which have been experimentally tested (Livengood, 1937; Shaw, 1932). Much of this work has been done with common goldfish, *Carassius auratus* (Livengood, 1937). The effect is only positive if the period of conditioning is short (Rose, 1960).

It is known that fish introduce food particles and other organic matter into water and the nutritional value of the food particles is known to be beneficial to growth of other fish (Livengood, 1937). The beneficial effect of homotypically conditioned water may also be explained as being a result of removal of toxic substances (Shaw, 1932). However, it has been established that the presence of food particles and the removal of toxic influences alone can not explain the growth-promoting effect of homotypically conditioned water. Furthermore, a growth promoting substance has been extracted from the skin of goldfish, which is known to be effective in very low concentrations (Livengood, 1937).

Although the function of the growth promoting substance is unknown, it might be involved in species aggregation and therefore be (regarded as) an aggregation pheromone. Because homotypically conditioned water only has a beneficial effect on growth when conditioning period was short, while the effect is negative after a longer period or when conditioning was done by many fish (crowded conditions) it has been suggested that both effects (beneficial conditioning and crowding effect) might be the result of the same chemical agent at different concentrations (Solomon, 1976).

Crowding factor

The existence of the crowding phenomenon in fish, that involves overall decrease in growth rate or differential growth rates and adversely affected performance under crowded conditions in spite of an abundance of food, has been recognized for a considerable time. The major role of chemical communication plays in this crowding effect has been established relatively recent. Under natural conditions, levels of the "growth inhibiting factor" or "crowding factor" below levels causing retarded growth and reproduction may serve to disperse fish and prevent exhaustion of local resources, spread of infectious diseases, inbreeding and other negative consequences of overcrowding (Solomon, 1976; Kamps and Neill, 1999).

To date, chemical agents excreted by fish are known to affect many processes under crowded conditions, including growth, development, reproduction, and mortality. These effects will be discussed in the following paragraphs.

Decrease in growth rate of individuals

Density can influence growth through exploitation, competition and behavioral or chemical interference. The involvement of chemical interference through a specialized chemical agent in growth retardation has been proven for several fish species (Solomon, 1976; Yu and Perlmutter, 1970; Rodriguez-Munoz *et al.*, 2003; Lutnesky and Adkins, 2003).

For the sea lamprey for example, the existence of a waterborne agent excreted by conspecifics that reduces larval growth has been proposed, as research has shown that sea lamprey larvae exposed to water preconditioned at low densities grow faster than those subjected to water preconditioned at high or intermediate densities (Rodriguez-Munoz *et al.*, 2003).

The effect can be age specific as was observed in zebrafish, for which a growth inhibiting factor has been extracted from fish holding water. This factor was proven to cause growth retardation in juvenile fish, while no significant effect was seen in post-larval stage fish (Yu and Perlmutter, 1970).

Furthermore, the role of growth inhibiting factors produced by fish in the control of fish growth is possibly very important as these substances have been proposed to be more important limiting factors than ammonia (Yu and Perlmutter, 1970).

Increase of differential growth rates

Many experiments with growth inhibiting factors have shown that smaller fish are generally more affected by the substance than larger fish ((Rose, 1959a; Solomon, 1976; Barbossa and Volpato, 2007; Rose, 1959b). This leads to increased differences in growth rate or heterogeneous growth under crowded conditions, causing even greater size differences.

Study of chemical modulation of heterogeneous growth in *Prochilodus lineatus* has shown that in groups of fish that received water in which a conspecific had been held, a higher frequency of smaller individuals, and consequently a lower frequency of larger individuals was observed (Barbossa and Volpato, 2007).

Several explanations have been proposed about how growth inhibiting factors contribute to heterogeneous growth. Volpato *et al* (1989) suggested that chemicals are in contact with all fish, but growth is suppressed only in some individuals (Barbossa and Volpato, 2007). It has been proposed for tadpoles (in which the crowding effect has been intensively investigated) that large and small tadpoles produce similar amount of crowding factor per unit of body weight, but the small individuals are more sensitive to its effect (Rose, 1960). Furthermore, rapidly growing individuals of this animal have been proven to produce a larger amount of the inhibiting factor than slowly growing ones, thereby exerting a larger growth inhibiting effect on others (Rose, 1960).

Retardation of embryonic development

In zebrafish, the same factor that retards growth of juveniles also retards embryonic development. Exposure to the extracted substance caused a significantly lower hatching percentage of embryos as compared to embryos that were not exposed to the substance (Yu and Perlmutter, 1970).

Depression of heartbeat in embryos and newly hatched fry

The extracted growth inhibiting factor of zebrafish slowed down the heart beat of 48h zebrafish embryos from an average of 125 to an average of 80 beats per minute (Yu and Perlmutter, 1970). A similar heart-rate effect has been observed in newly hatched goldfish fry exposed to extracts of crowded water (Francis *et al.*, 1974).

Inhibition of sexual maturation

It is well documented that sexual maturation in several species of poeciliid fishes and in females of the wrasse *Pseudolabrus celidotus,* may be delayed by social factors (Sohn, 1977; Borowsky, 1987). In these fish species, maturity is inhibited when an individual is not dominant, as determined by its relative size, at a time near the genetically determined age of maturation. Therefore, the larger, dominant fish of these species mature at younger age and smaller size than the smaller, subordinate ones. Social inhibition of maturation is known to be correlated with agonistic behavior, but other potential social stressors, such as pheromones, have also been suggested to contribute to the phenomenon (Borowsky, 1987; Lutnesky and Adkins, 2003).

Decrease of female gonadal size

In an experiment with mosquito fish, *Gambusia affinis,* it was observed that females of this species showed reduced growth and smaller ovary size when reared in water previously containing two adult females. No effect of the female-conditioned water was seen in males. It was suggested that the chemical presence of adult females may inhibit the reproductive effort of smaller females through stunting their growth (Lutnesky and Adkins, 2003).

Inhibition of spawning

A so-called reproduction control factor, excreted by fish under crowded conditions, has been shown to prevent spawning (Solomon, 1976).

Decreased number of offspring

In guppy, *Poecilia reticulate*, the production of offspring varies inversely with the concentration of adults. Furthermore, the number of off spring eaten by adults increases with the density of the adult population. This form of population control has been suggested to be regulated by a specific waterborne product (Rose, 1959a).

Effects on mortality

In a number of fish species, a chemical agent secreted by fish under crowded conditions is known to increase mortality (Solomon, 1976; Yu and Perlmutter, 1970; Lutnesky and Adkins, 2003). The extracted crowding factor of zebrafish (mentioned earlier for its effect on growth and embryonic development) has been shown to cause increased mortality in post larval and adult zebrafish (Yu and Perlmutter, 1970). The reduction of growth and ovary size in females of mosquito fish reared in female-conditioned water was also accompanied by a significantly higher mortality rate (Lutnesky and Adkins, 2003). Increased mortality in white cloud mountain fish and bleeker as a consequence of accumulating crowding factor has been explained by the fact that smaller fish stopped eating while larger fish didn't and continued to grow (Solomon, 1976). Finally, a study of *Tilapia mossambica* in high density culture has revealed that a chemical agent causes anaphylactic shock and death in conspecifics once a certain density threshold is exceeded. For this species it was concluded that the agent was a high-molecular weight substance present in the fish's slime layer (Kamps and Neill, 1999).

Chemical structure

To date, very little is known about the chemical identity of the substance(s) that cause(s) the effects named above. It is not even known if the same chemical agent is responsible for all effects or if more than one substance is involved. It has been proven though, that growth inhibiting factors produced by zebrafish, blue gourami and goldfish can successfully be extracted from water with activated carbon and methylchloroform (Francis *et al.*, 1974; Yu and Perlmutter, 1970). It has also been proposed that crowding factor of goldfish may be a chemical similar to phthalate esters or a complex between a natural phthalate and other lipids (Francis *et al.*, 1974). The suggestion about GIF being a lipid or lipid-like material has been made by other investigators as well (Roales, 1981). Finally, it is very likely that the chemical agent is not identical in all fish species that employ such a substance, for species specificity of the crowding factor was observed in many species for which this has been investigated (Francis *et al.*, 1974; Rose, 1959a; Yu and Perlmutter, 1970).

1.4 Implications for aquaculture in RAS

1.4.1 Metabolites

Levels of the metabolic waste products described above in RAS are very similar to those in flow-through systems. Culture water in RAS is treated to remove waste solids, oxidize ammonia and nitrite-nitrogen, remove carbon dioxide and aerate or oxygenate the water before returning it to fish tanks to keep concentrations of these compounds within appropriate ranges for optimal fish growth (Colt, 2005). However, it is known that concentrations of nitrite, nitrate, CO2 and suspended solids in RAS can exceed concentrations of these substances in flow-through systems (Clark *et al.*, 1985). It has also been proposed that the potential impacts of fine solids are one of the greatest uncertainties in high intensity reuse systems (Clark *et al.*, 1985).

However, in a one year trial comparing flow-through systems with RAS, an observed slower growth of seabass in RAS could not be explained by differences in any of the parameters named above alone

(Colt, 2005). It therefore seems very unlikely that the problem of growth retardation is caused (solely) by potential (small) differences in concentrations of any of these major metabolic waste products of fish.

1.4.2 Hormones and hormonal products

It seems very likely that high stocking densities in fish culture may lead to unnaturally high concentrations of dissolved hormones and hormonal products in culture water. Furthermore, excretion rates of some hormones may especially be higher in cultured fish, for example stress hormones produced as a result of chronic stress. Effects of these substances might be more pronounced in RAS as compared to flow through systems because water purification in RAS doesn't remove all organic substances (Anonymous, 2005). So unless a substance is stable for only a very short period and broken down rapidly, it will accumulate in RAS at a level at which removal via water replacement and degradation is in equilibrium with production.
Many excreted hormones are peptides, which are rapidly degraded (Shore and Shemesh, 2003). However, steroid hormones have been suggested to be chemically very stable. For example, the average half life of naturally released cortisol has been shown to be 16 hours (Scott and Ellis, 2006). Furthermore, steroid hormones are excreted in the free (active) form or as conjugates, which very readily degrade to the free form (Scott and Ellis, 2006; Shore and Shemesh, 2003).
It is therefore possible that accumulation of hormonal compounds takes place in RAS at higher levels compared to flow through systems, at least for the most stable compounds such as steroids. This might have serious consequences for cultured fish because steroids dissolved in water are possibly capable of interfering with any of the internal signaling systems in fish in which they play a role (reproductive, immune, and central nervous system). Endocrine interference may especially be likely to occur if transfer of hormones takes place between groups of fish of different age categories within a system. Such transfer has been demonstrated to occur in RAS in an experiment with trout, where injections of testosterone in sexually immature trout elevated plasma-testosterone levels in fish not injected with testosterone but housed in the same recirculation system (Budworth and Senger, 1993).

1.4.3 Pheromones

It is clear that fish use a wide variety of chemical compounds for communication. When produced under aquaculture conditions, the accumulation of chemical signals in the rearing water in RAS would be a potentially very important difference when compared to flow through systems.
Chemical communication has not been established in turbot or any other flatfish species. However, many chemical signals have been shown to be employed by many fish species, both freshwater and saltwater, from diverse families and genera. Furthermore it has been suggested by many researchers that chemical communication might be common to all fish. For many aspect of turbot behavior it is unlikely that these can take place without chemical communication.
As for the lack of information on the identity of most chemical signals and the low concentrations at which chemical signals are effective, the presence and effects of chemical signals in RAS cannot be addressed with direct measurements but requires indirectly methods.

1.4.4 Crowding factor

Although it is unknown if turbot produce crowding factor(s), such a substance has been demonstrated for many fish species and would be an obvious explanation for growth retardation of fish in high density RAS if accumulation takes place.
It has often been observed that the effect of this substance decreases in the presence of unrelated species, since these may remove the species or genus specific inhibitors, by uptake and/or metabolisation (Rose, 1959a; Rose, 1960). It might therefore be interesting to investigate if turbot culture may benefit from combining it with culture of unrelated species in the same culture system.

1.4.5 Pheromones involved in non-reproductive aggregation

Turbot are known to form spawning schools in nature, with complex structures reflected in specific male-female ratios and ratios of fishes of various age categories (Stankus, 2003) and it is highly likely that chemical communication is involved. Furthermore it is known that size hierarchies are established

by turbot in culture (Irwin *et al.*, 1999; Stefánsson *et al.*, 2002). The establishment of social hierarchies in cultured fish is unfavorable in all aquaculture systems, as it has been proven that subordinate individuals experience chronic stress, leading to growth suppression, as a result of continuous social domination by conspecifics. This phenomenon has been demonstrated to occur in turbot (Irwin *et al.*, 1999; Stefánsson *et al.*, 2002). The effect has been proven to be reinforced in some fish species by regular grading and restocking in aquaculture to maintain populations of roughly equal sized individuals for management reasons, since new hierarchies have to be established (Thorpe, 2004).
Although it is unknown if chemical communication is involved in the establishment of these social structures in turbot, this has been observed in many other fish species and might cause problems in RAS, as in these systems chemical cues of large and dominant individuals could accumulate and affect fish in the whole system. Therefore the possibility that individual specific chemical signals related to social hierarchies are involved in causing growth retardation of turbot in RAS should not be ruled out.

1.4.6 Stress/disturbance pheromones

Very little is known about pheromones excreted by stressed or 'disturbed' fish. The existence has been demonstrated though in a saltwater fish species, and if such a pheromone is excreted by turbot, this could be problematic in aquaculture, especially in RAS where this substance could possibly accumulate in high concentrations. Stress is known to be disadvantageous to growth, reproduction and the immune system, therefore it would be very undesirable that a few stressed individuals in an aquaculture system could possibly induce the same state in all fish in that system.

1.4.7 Possibility of disrupted chemoreception

If turbot use chemical signals to communicate with conspecifics, and if many of these signals accumulate continuously in RAS, it seems likely that this has negative effects. For example, pheromone-mediated reproductive processes are known to result in behavioral and biochemical changes triggered by detection of the pheromone. Therefore, continuous accumulation of these chemical signals may induce biochemical and behavioral changes at inopportune times, thereby causing disrupted chemoreception. The same applies to accumulation of other chemical signals, such as alarm pheromones and possibly many others.
Although documentation is currently lacking, responses to pheromone cues at inappropriate times are believed to be energetically costly and can detract from feeding activities in reuse systems (Kolodziej *et al.*, 2003). Therefore, the effect of potentially continuous (high) concentrations of chemical signals in RAS, irrespective of the 'meaning' of these signals, should also be considered to be possibly involved in causing growth retardation in RAS.

1.5 Conclusions

The metabolic waste products nitrite, nitrate, CO_2 and solids might contribute to the problem of growth retardation by direct inhibition of growth but may more likely be indirectly involved in causing the problem by increasing the fish's susceptibility to other stressors, as concentrations of these metabolites deviate only slightly from concentrations in flow-through systems.

Excreted (metabolites of) endocrine substances might be involved in causing growth retardation through disruption of the fish's endocrine system. Of major concern may be glucocorticoids (cortisol), because of their role in the stress response, which is known to be suppressive to growth, and sex steroids, because of their crucial role in growth and maturational processes. However, it seems likely that many hormones are involved, provided that the chemical properties of these compounds allow them to accumulate in RAS. If hormonal compounds are involved in growth retardation, then the generally observed occurrence of growth retardation from a certain age of fish may be related to changes in the sensitivity of the fish to exogenous hormones, as a result of endocrine changes related to the maturational/gonadal stage of the fish at this moment.

Several substances involved in chemical communication among fish might contribute to growth retardation in RAS, provided that these substances are excreted by turbot, which has not been proven to date. Crowding factors, individual specific pheromones involved in establishment of social hierarchies

and regulation of population structures and stress/disturbance pheromones may directly affect fish in a way that leads to growth suppression. Furthermore, potentially continuous (high) concentrations of chemical signals in RAS, irrespective of the 'meaning' of these signals, may cause disrupted chemoreception, which could be energetically costly and lead to reduced food intake. If pheromone compounds are involved in the causation of growth retardation, then the generally observed occurrence of growth retardation from a certain age of fish may be related to changes in the sensitivity to pheromones occurring at this life stage, coinciding with certain endocrine processes (related to puberty/maturation).

Growth retardation is possibly the result of many factors that affect fish growth and could therefore be a combined effect of accumulation of all the suggested substances named above, probably in combination with effects of high stocking density in culture systems. Although this survey focused on substances produced by turbot only, substances originating from other sources, for example micro-organisms and food, might be involved in causing the problem as well. With respect to the age of first occurrence of growth retardation, this age can be suggested to be related to the endocrinal stage of fish as described above, but may also reflect the point (fish age or concentration of substances) at which adverse affects of accumulating substances are no longer outweighed by positive effects of the optimized constant conditions (for example temperature and oxygen level) in RAS.

2 The production of growth inhibiting factors by turbot (Task 2.1)

2.1 Preliminary early life stage test and juvenile growth trails with Dover sole

Introduction
An experiment was set up to develop the methodology for demonstration of GIF production by turbot. The activities consisted of inducing the production of GIF, extraction of GIF from the water, preparation of test solutions from the GIF extracts and testing these test solutions for GIF activity in two types of bio assays. For practical reasons the development of the methodologies were performed with Dover sole instead of turbot as all life stages of this fish are readily available at our facilities, while turbot would require imports from France. Once the methodologies were developed successfully, the activities would be repeated for turbot in order to meet the objectives of this project.

Materials and methods

GIF production and extraction
To induce the production of GIF 15 Dover sole with an average weight of 82g were stocked in a glass tank of 40L with a bottom area of 0.06 m^2. This resulted in a stocking density of 20.5 kg/m^2, which is considered to be a high stocking density for this species. The high stocking density was expected to induce GIF production. The fish were not fed and the stagnant water was aerated to provide sufficient oxygen levels. An identical tank without fish was installed as a control.
After 14 days the water was collected from the tanks and stored in glass containers at 4°C prior to GIF extraction.
During the GIF extraction the water from the fish tank and the control tank were treated identically. Prior to GIF extraction the water was filtered to remove solids. The water was then led simultaneously over 10 Solid Phase Extraction (SPE) cartridges with 5000mg C18 HC (Alltech) at a flow rate 8 ml/min/cartridge.
The control extract was eluted with 50 ml methanol/cartridge, whereas the fish tank extract was eluted with 50 ml methanol, 50 ml acetonitrile and 20 ml hexane per cartridge, as methanol alone was observed to result in poor elution. The solvents were evaporated under vacuum at 35°C and the residue was blown to dryness by N_2. The resulting residue was then dissolved in 250 ml demineralized water.

Early life stage test
Early life stage tests were performed with GIF and Control extracts to demonstrate GIF presence. Treatments consisted of three concentrations of the GIF extract and three concentrations of the control extract. For both the concentrations were 10, 1, 0.1 times the final (but unknown) concentration in the GIF production and the Control tank (assuming 100% recovery during extraction). The true concentrations were unknown as the GIF concentration in the GIF production tank was unknown. Test solutions were prepared from the GIF and Control extracts in artificial seawater. A Control treatment consisted of artificial seawater without addition of any extract (not to be confused with the Control extract and therefore referred to as Seawater). The total number of treatments was seven.
For each of the treatments 24 eggs were incubated individually in two well plates (NUNC) with 12 wells of 4ml per plate. Embryonic development was monitored during 8 days at 16°C. As indicators for development served survival rate, hatching rate, eye pigmentation, heart beat rate and mouth opening.

Juvenile growth trial
Treatments consisted of five concentrations of GIF extract and Control extract. For both the concentrations were 3, 1.5, 1, 0.5 and 0.1 times the final (but unknown) concentration in the GIF production and the Control tank (assuming 100% recovery during extraction). The true concentrations were unknown as the GIF concentration in the GIF production tank was unknown. Test solutions were prepared from the GIF and Control extracts in artificial seawater. The same GIF extract as in the ELS with Dover sole was used in this trail. A Control treatment consisted of artificial seawater without addition of any extract (not to be confused with the Control extract and therefore referred to as Seawater). The total number of treatments was eleven, with 3 replicates per tank, resulting in a total of 33 tanks. Each tank was stocked with 10 Dover sole juveniles (7-8mg) and filled with 400ml test

solution. 50% of the test solution was replaced daily to maintain good water quality. During seven days all tanks were fed with artemia nauplii in excess, twice daily. At day 8 the total wet and dry biomass was determined for each tank.

Results

Early life stage test
No effect of treatment could be observed on hatching rate (Fig. 3.1) and eye pigmentation (Fig.3.2). Significant differences in survival existed between treatments but the relation with the treatments is unclear (Fig. 3.3). No data for heart beat rate and mouth opening could be collected as systematic observation of these parameters failed.

Juvenile growth trial
No significant differences in final wet weight were found (Fig. 3.4).

Discussion
The results are inconclusive. The GIF production by Dover sole could not be demonstrated in the larval assay and the growth assay, but it is unclear why exactly. There are several possibilities: Dover sole do not produce GIF at all, Dover sole do not produce GIF under the experimental conditions or GIF were not extracted from the water.
In both assays the results from the Control extracts were not different from the Seawater controls, meaning that the GIF extraction procedure does not affect growth and development in the assays.
It is concluded that further development of the early life stage and growth assays is required.

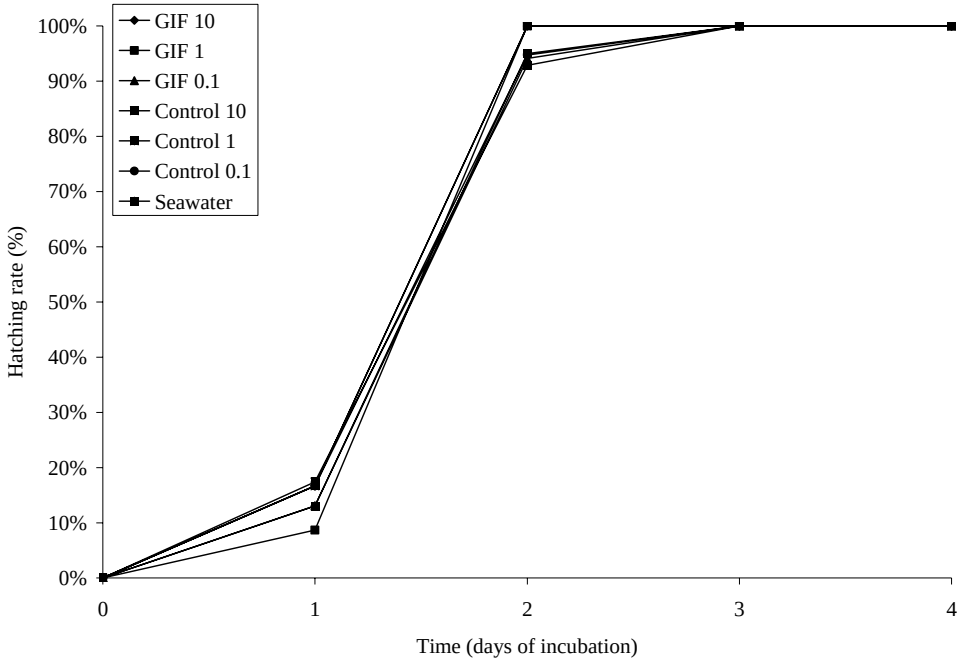

Fig 3.1 Hatching rate of Dover sole over time for the experimental treatments

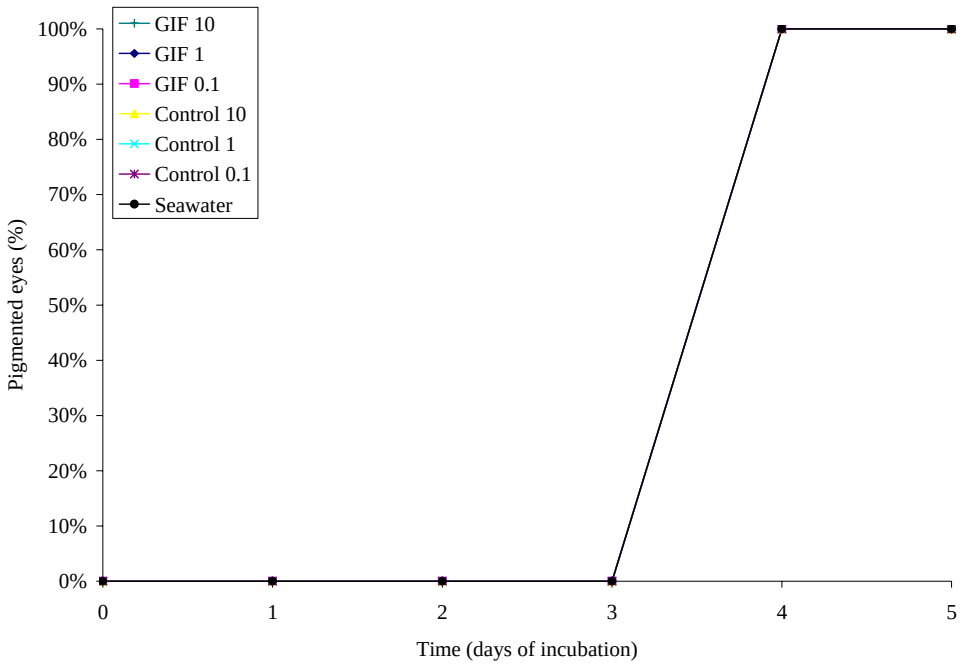

Fig. 3.2 Eye pigmentation of Dover sole over time for the experimental treatments

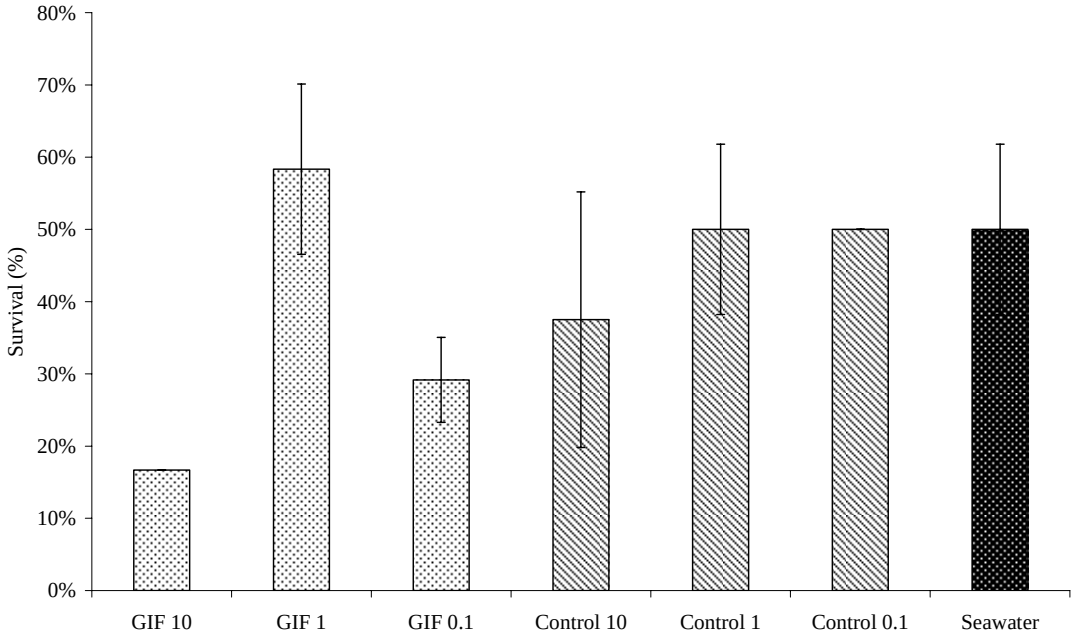

Fig 3.3 Mean (n = 3) survival rate at the end (day 8) of the bio-assay for the different treatments

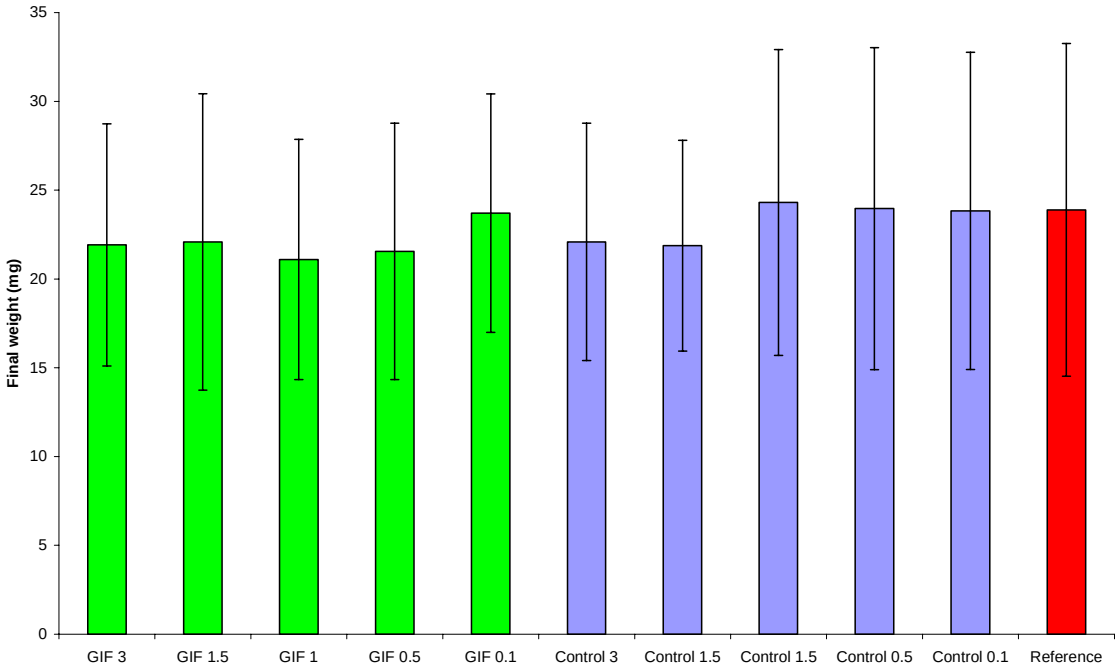

Fig 3.4. Final average wet weight (mg) in the biogrowth assay for the treatments.

2.2 Demonstration of GIF production in turbot by juvenile growth trails

2.2.1 Introduction

Following the difficulties associated to the early life stage tests (ELS) to demonstrate the production of growth inhibiting factors by Dover sole it was decided to use juvenile growth trails to demonstrate GIF activity of test solutions rather than early life stage tests. Juvenile growth trails offer the advantage of having growth as direct read out parameter, as opposed to the ELS in which the read out parameters are indirect indicators for growth retardation.
Three juvenile growth trails, hereafter referred to Experiments 1 to 3, were performed in the period November 2007 to April 2008. Based on the results and experience gained during the previous experiment, the set up of the following experiment was further optimized.
All experiments involved the collection of GIF on activated carbon from the outflow of a tank densely stocked with turbot, followed by GIF extraction. The GIF extracts were used to prepare test solutions, which were tested for growth inhibiting properties in the juvenile growth trials.

Experiment 1

2.2.2 Materials and Methods Experiment 1

General set up
The effluent of a tank stocked with turbot under commercial densities and expected to produce GIF, was led over activated carbon. The activated carbon was extracted with chloroform, which was then evaporated. The extract was dissolved in demineralised water and diluted into test solutions. The effect of the test solutions on growth of turbot juveniles was assessed in a static renewal test.

Collection and extraction of GIF
GIF production was induced by stocking turbot at commercial density (Table 3.1), at France Turbot SAS, Noirmoutier, France. The water flow over this tank was not recirculated to exclude biological filters as possible source of GIF.
After removal of solids by sedimentation, the outflow (2.3m^3/hr) of this tank was led over a column with 1.5kg activated carbon (NORIT GAC 830 plus) for 643 hours. Activated carbon was stored at -70°C prior to extraction.

Table 3.1 Characteristics of the GIF production tank.

Number of fish		190
Mean weight	(g)	900
Total biomass	(kg)	171
Fish density	(kg/m^2)	34
Flow rate	(m^3/hr)	2.3
Tank volume	(m^3)	5.3
Tank bottom area	(m^2)	2.8

A subsample of the well mixed activated carbon (dry weight 535g) was extracted by accelerated solvent extraction (ASE). The activated carbon was distributed evenly over 11 cells, which were flushed twice with 50ml chloroform at 23°C, yielding a total volume of 1.1L chloroform. Chloroform was evaporated in a rotary evaporator at 24°C and 190-218 mbar, after which the residue was transferred into a 500ml volumetric flask and redissolved in 500 ml demineralized water. A control extract was prepared by extraction of untreated activated carbon following the same procedure. Extracts were stored in glass bottles at 4°C.

Preparation of test solutions
The preparation of test solutions with GIF extract was hampered by the unknown concentration of GIF in the GIF production tank, the unknown recovery rate of GIF from the water to the activated carbon and the unknown recovery rate of GIF from the activated carbon to the extraction fluid. The GIF concentration in the extract was calculated assuming a combined recovery rate of GIF from water to carbon and from carbon to extraction fluid of 10% (Table 3.2). Based on the GIF concentration in the

extract, three test solutions (A, B, C) were prepared in artificial seawater with a dilution factor of 0.1 and GIF concentrations expressed as fractions (15, 150 and 1500 times) of the GIF concentration in the GIF production tank (Table 3.2). Artificial seawater prepared from demineralized water and sea salt (Instant ocean) without GIF extract served as control test solution (D). A second control (E) was based on the extraction of untreated activated carbon to assess possible (combined) effects of the extraction procedure on fish response to the test solutions. Table 3.3 gives an overview of the test solution composition. A total volume of 7.5L was prepared for each test solution in two batches: at the start and at midterm of the experimental period and stored in 5L glass bottles.

Table 3.2 Calculation of the relative GIF concentration in the extract.

GIF collection	Unit	Value	Remark
GIF concentration		X	Unknown
Amount of carbon installed	(kg)	1.5	Measured
Flow rate	(L/h)	2272	Measured
Exposure time	(hr)	643	Measured
Recovery GIF water to carbon*	(%)	10	Estimated
GIF extraction			
Recovery GIF from carbon*	(%)	100	Estimated
Amount of carbon extracted	(g)	535	Measured
Extract volume	(ml)	500	Measured
GIF concentration in extract		~100,000 X	Calculated

*) The estimated recovery of GIF from farm water to carbon and from carbon to extraction fluid, yields a combined recovery from farm water to extraction fluid of 10%

Table 3.3 Composition of test solutions. X refers to the GIF concentration in the GIF production tank

Test solutions		A	B	C	D	E
GIF concentration		15X	150X	1500X	0	0
GIF extract content	(ml/L)	0.15	1.5	15	0	0
Control extract content	(ml/L)	0	0	0	0	15
Artificial seawater content	(ml/L)	1000	998.5	985	1000	985

Set up

Each of fifteen polyethylene test chambers (10 x 10 x 12.5 cm) was filled with 500 ml of a randomly assigned test solution, resulting in three test chambers for each of the five test solutions. Hundred and sixty five juvenile turbot of 21 days post hatch (France Turbot, Noirmoutier, France) were randomly divided over the fifteen tanks and placed in a temperature controlled room with a photoperiod of 16L:8D. Fishes were allowed to acclimatize to the experimental conditions for 5 days. During the acclimatization and experimental period, the experimental system was operated as a semi-static renewal system: 50% of the total test solution volume was replaced once daily in the morning. During draining of the water, uneaten feed and feces were removed with disposable pipets. Each jar was equipped with an aeration tube to secure sufficient supply of oxygen.

All tanks were supplied with newly hatched *Artemia* nauplii twice daily at a density of approximately 7-15 *Artemia*/ml. Dissolved oxygen concentration (Oxyguard Handy Gamma) was measured in all units on Day 4. Temperature and pH (WTW pH 315i) and salinity (WTW Cond 315i) were measured on Days 1 to 5. Total ammonia nitrogen and nitrite nitrogen concentration (Merck cuvette tests) were measured on Days 3 and 5 in one replicate of each treatment.

Qualitative analysis of GIF and Control extract

A qualitative LC-MS analysis of the GIF extract and the Control extract was performed by the University of Swansea, Wales. For details on the analysis see Deliverable 3.

Data collection & statistics

Mortalities were recorded and removed daily. At day 8 of the experimental period fishes were killed by adding a few drops of phenoxyethanol to the test chambers. Individual final wet weight was measured (Mettler Toledo AT261 Delta range) after rinsing in demineralized water and removal of excess water

with paper tissues. Standard length was measured using a binocular with a graduated ocular. Total dry weight per test chamber was determined after forty-eight hours at 105°C.
Final wet and dry weight, final standard length and final survival and dissolved oxygen levels were tested for significant differences among all treatments and between GIF and Control treatments by One-way ANOVA. Temperature, pH and salinity were tested for significant differences among treatments by Repeated measurements ANOVA. GenStat 10.1 was used for all statistical tests.

2.2.3 Results Experiment 1

Fish performance
The small size of the juveniles at the start of the experiment did not allow for weighing, hence the initial weight per treatment was not measured and growth cannot be calculated.
When considering all individual experimental treatments., the control (D) and extract control (E) solutions showed a trend towards a significantly higher final weight than test solution B ($P<0.10$, Table 3.4). The control (D) and extract control (E) solutions showed a trend towards a significantly higher standard length than test solutions A and B, but not C ($P<0.10$, Table 3.4). No differences in final dry weight, coefficient of variation (CV) and survival rate were observed among the treatments (Table 3.4).
When grouping GIF treatments (ABC) and Control treatments (DE) and comparing their performances, a significantly lower final wet weight, dry weight and standard length were observed (Tables 3.4).

Water quality
Temperature, pH and dissolved oxygen concentration were equal among the treatments (Table 3.5) and were within the normal ranges for survival of turbot juveniles.
Salinity was lower in the Control extract test solution (E) (Table 3.5). Total ammonia levels range from 0.45 to 0.90 mg N/L and nitrite levels range from 0.02 to 0.04 mg N/L. Due to lack of measurements across the replicates it is not possible to test the differences among the treatments in ammonia and nitrite levels for significance.

Qualitative analysis of GIF and Control extract
The chromatogram (Fig. 3.5) resulting from LC-MS analysis of the GIF extract (F2) and Control extract (C2) shows that extracts have a different composition

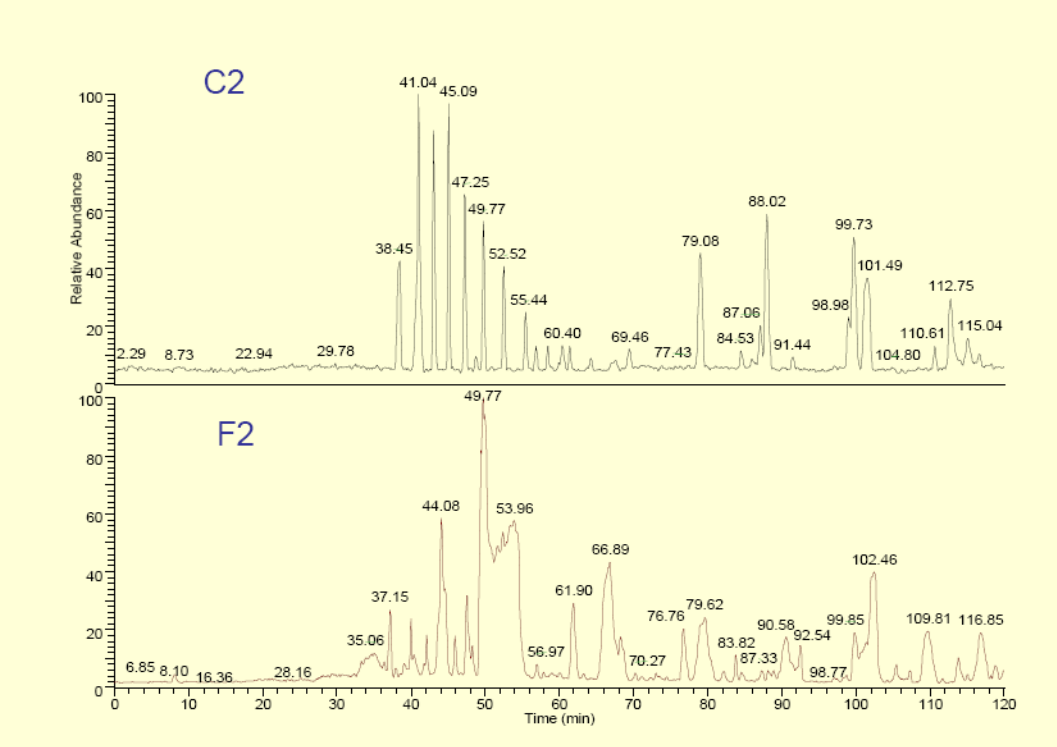

Fig. 3.5 Chromatogram resulting from LC-MS analysis of the GIF extract (F2) and the Control extract (C2).

Table 3.4. Mean values (SD) (n = 3) per experimental treatment for final wet weight, final dry weight, final standard length and survival. Values with different letters across rows are significantly different (ANOVA, P < 0.10).

		Test solutions					ANOVA all treatments		ANOVA GIF vs. Control (ABC vs. DE)
Parameter	Unit	A	B	C	D	E	P-value	LSD 10%	P-value
Final wet weight	(mg)	21.3 (4.2)[ab]	20.4 (5.1)[a]	21.2 (6.7)[ab]	28.7 (5.8)[b]	31.7 (4.9)[b]	0.09	0.008	0.003
Final dry weight	(mg)	3.95 (0.9)	3.59 (1.0)	4.09 (1.6)	5.42 (1.3)	6.03 (0.9)	0.12	-	0.006
Final standard length	(mm)	9.24 (0.60)[a]	9.23 (0.52)[a]	9.94 (1.17)[ab]	10.83 (1.06)[b]	11.07 (0.60)[b]	0.06	1.2	0.003
CV	(%)	55.3 (19.9)	58.7 (31.2)	34.1(32.6)	24.8 (21.6)	40.7 (29.7)	0.55	-	0.25
Survival	(%)	39.4 (5.2)	30.3 (13.9)	21.2 (13.9)	21.2 (10.5)	30.3 (13.9)	0.37	-	0.51

Table 3.5 Mean values (SD) per experimental treatment for temperature (n=14), pH (n=13), oxygen concentration (n=3), salinity (n=14), total ammonia nitrogen (n=2) and nitrite nitrogen (n=2). Values with different letters across rows are significantly different (Repeated measurements ANOVA, P < 0.05)

Water quality parameter		Test solutions						
	Unit	A	B	C	D	E	P-value	LSD 5%
Temperature	(°C)	16.7 (0.08)	16.7 (0.07)	16.6 (0.09)	16.7 (0.04)	16.7 (0.11)	0.78	-
pH		7.86 (0.02)	7.88 (0.06)	7.86 (0.10)	7.87 (0.03)	7.77 (0.03)	0.22	-
Oxygen	(mg/L)	6.1 (0.3)	6.3 (0.2)	6.5 (0.3)	6.1 (0.3)	6.1 (0.1)	0.20	-
Salinity	(‰)	33.7 (0.03)[a]	33.7 (0.02)[a]	33.7 (0.03)[a]	33.7 (0.05)[a]	33.3 (0.11)[b]	<0.001	0.08
Ammonia	(mg N/L)	0.90 (0.04)	0.76 (0.01)	0.59 (0.14)	0.71 (0.28)	0.45 (0.06)	-	-
Nitrite	(mg N/L)	0.04 (0.01)	0.04 (0.01)	0.05 (0.01)	0.03 (0.00)	0.02 (0.02)	-	-

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Experiment 2

2.2.4 Materials and methods Experiment 2

The previous juvenile growth trial (Experiment 1) was inconclusive due to confounding factors (see Discussion) in the experiment. The current experiment (experiment 2) was performed to repeat experiment 1 in an improved set up.

Collection and extraction of GIF
A subsample of 538g of the same activated carbon installed at France Turbot to collect GIF from fish water as in Experiment 1 was extracted to prepare test solutions for the current experiment.
The extraction of the activated carbon was the same as described for Experiment 1.

Preparation of test solutions
Test solutions were prepared from the GIF extract and the Control extract in the same as described for Experiment 1.

Set up
The set up of the current experiment was an improved set up as used for Experiment 1. Improvements related to water quality included: increased volume of the test chambers from 500 to 700 ml, increased replacement of the test solution per test chamber from 50 to 64% and increased frequency of test solution replacement from once to twice daily. Further the initial weight of the experimental fish was determined to enable measurement of growth and *Artemia* was replaced with dry feed.
Each of fifteen polyethylene test chambers (10 x 10 x 12.5 cm) was filled with 700 ml of a randomly assigned test solution, resulting in three test chambers for each of the five test solutions. Hundred and fifty juvenile turbot of 329 (14) mg (mean + SD) and 37 days post hatch (France Turbot, Noirmoutier, France) were randomly divided over the fifteen tanks and placed in a temperature controlled room with a photoperiod of 16L:8D. Fishes were allowed to acclimatize to the experimental conditions for 4 days. During the acclimatization and experimental period, the experimental system was operated as a semi-static renewal system: 64% of the total test solution volume was replaced in the morning before feeding and in the afternoon after the last feeding. During draining of the water, uneaten feed and feces were removed with disposable pipets. Each jar was equipped with an aeration tube to secure sufficient supply of oxygen.
All test chambers were supplied dry feed (Bernaqua Caviar) at a feeding level of 5%/d. Daily amounts of feed per test chamber calculated assuming an SGR of 5%/d and an FCR of 1. Daily feed loads were supplied in five portions supplied at 9.00, 11.00, 13.00, 15.00 and 17.00..

Data collection & statistics
Mortalities were recorded and removed daily. One day before the start of the experimental period experimental fish were weighed as a group per test chamber. For each test chamber the fish were transferred to a glass beaker. The test chamber was refilled with 700ml of test solution and placed on the balance (Mettler PE 3000) and tarred to zero. Fish were scooped from the glass beaker two by two using a small tea sieve and transferred to the test chamber on the balance after removal of excess water by placing the sieve on dry paper tissue. The total weight of 10 fish per test chamber was recorded.
At day 11 of the experimental period fishes were killed by adding a few drops of phenoxyethanol to the test chambers. Individual final wet weight was measured (Mettler Toledo AT261 Delta range) after rinsing in demineralized water and removal of excess water with paper tissues. Standard length was measured using a binocular with a graduated ocular. Total dry weight per test chamber after forty-eight hours at 105°C.
Initial and final wet and final dry weight, final standard length and final survival and dissolved oxygen levels were tested for significant differences among all treatments and between GIF (ABC) and Control (DE) treatments by One-way ANOVA. Temperature, pH and salinity were tested for significant differences among treatments by Repeated measurements ANOVA. GenStat 10.1 was used for all statistical tests.

Table 3.6. Mean values (SD) (n = 3) per experimental treatment for initial and final wet weight, final dry weight, SGR, final standard length and final coefficient of variation (CVt). P-values result from One way ANOVA.

		Test solutions					All treatments	ABC vs. DE
Parameter	Unit	A	B	C	D	E	P-value	P-value
Initial wet weight	(mg)	325 (16)	333 (22)	328 (19)	331 (10)	328 (16)	0.99	0.95
Final wet weight	(mg)	409 (31)	401 (27)	385 (32)	400 (18)	386 (13)	0.74	0.66
Final dry weight	(mg)	73.8 (5.8)	69.8 (6.3)	69.2 (5.3)	72.1 (3.8)	68.8 (2.3)	0.70	0.85
SGR	(%/d)	2.27 (0.3)	1.87 (0.1)	1.59 (0.5)	1.88 (0.3)	1.64 (0.6)	0.28	0.50
Final standard length	(mm)	24.9 (0.4)	24.6 (0.5)	24.6 (0.5)	25.0 (0.3)	24.6 (0.4)	0.60	0.66
CVt	(%)	25.7 (4.7)	21.1 (4.4)	18.9 (2.3)	22.1 (9.2)	24.1 (3.6)	0.60	0.68

Table 3.7 Mean (n=3) (SD) values per treatment as measured at Day 9 of the experimental period for temperature, pH, dissolved oxygen concentration, salinity and total ammonia nitrogen (TAN). P-values result from One way ANOVA.

Water quality parameter		Test solutions					
	Unit	A	B	C	D	E	P-value
Temperature	(°C)	16.6 (0.0)	16.6 (0.1)	16.5 (0.1)	16.5 (0.1)	16.5 (0.1)	0.31
pH		7.98 (0.06)	8.09 (0.06)	8.05 (0.05)	7.96 (0.10)	8.06 (0.02)	0.17
Oxygen	(mg/L)	7.3 (0.1)	7.1 (0.2)	7.1 (0.2)	6.8 (0.5)	7.1 (0.4)	0.33
Salinity	(‰)	33.0 (0.1)	33.0 (0.1)	33.1 (0.2)	33.0 (0.1)	33.0 (0.1)	0.78
TAN	(mg N/L)	5.1 (0.9)	5.5 (0.8)	4.9 (0.5)	5.1 (0.5)	5.1 (0.7)	0.88

2.2.5 Results Experiment 2

Fish performance
Initial wet weight was equal among all treatments (Table 3.6). No effect of test solutions was observed on final wet weight, final dry weight, final standard length and specific growth rate (SGR) among treatments and between grouped treatments GIF (ABC) vs Control (DE). SGR ranged from 1.59 to 2.27 %/d.

Water quality
No differences in water quality parameters were observed among the treatments (Table 3.7).

Experiment 3

2.2.6 Materials and methods Experiment 3

Given the results of the Experiment 2 which were inconclusive concerning the production of GIF by turbot, the experiment was repeated in Experiment 3.

Collection and extraction of GIF
A new GIF sample was collected for Experiment 3. GIF production was induced by stocking turbot at commercial density (Table 3.8), at France Turbot SAS, Noirmoutier, France. The water flow over this tank was not recirculated to exclude biological filters as possible source of GIF. After removal of solids by sedimentation, part of the outflow (0.3m^3/hr) of this tank was led over a column with 2.0 kg activated carbon (NORIT GAC 830 plus) for 28 days. At the same time bore hole water that also served as inflow of the GIF production tank was led over second column with 2.0 kg of activated carbon in a separate set up at a flow rate of 0.3 m^3/hr. After exposure, activated carbon was stored at -70°C prior to extraction.

Table 3.8 Characteristics of the GIF production tank.

Number of fish		156
Mean weight	(g)	1413
Total biomass	(kg)	220
Fish density	(kg/m^2)	79
Flow rate	(m^3/hr)	2.3
Tank volume	(m^3)	5.3
Tank bottom area	(m^2)	2.8

A subsample of well mixed activated carbon (dry weight 500g) that had been exposed to the outflow of the GIF production tank was extracted by accelerated solvent extraction (ASE). The activated carbon was distributed evenly over 11 cells, which were flushed twice with 50ml chloroform at 23°C, yielding a total volume of 1.1L chloroform. Chloroform was evaporated in a rotary evaporator at 24°C and 190-218 mbar, after which the residue was transferred into a 2000ml volumetric flask and redissolved in 2000 ml demineralized water.
Following the same procedure, a control extract was prepared by extraction of a well mixed subsample of the activated carbon that had been exposed to the bore hole water. All extracts were stored in glass bottles at 4°C.

Preparation of test solutions
The preparation of test solutions with GIF extract was hampered by the unknown concentration of GIF in the GIF production tank, the unknown recovery rate of GIF from the water to the activated carbon and the unknown recovery rate of GIF from the activated carbon to the extraction fluid. The GIF concentration in the extract was calculated assuming a combined recovery rate of GIF from water to carbon and from carbon to extraction fluid of 10% (Table 3.9). Based on the GIF concentration in the extract, three test solutions (A, B, C) were prepared in natural seawater (Oosterschelde, The Netherlands, filtered at 20µm and UV treated) with a dilution factor of 0.1 and GIF concentrations

expressed as fractions (1, 10 and 100 times) of the GIF concentration in the GIF production tank (Table 3.9). Natural seawater without GIF extract served as control test solution (D). A second control (E) was based on the extraction of activated carbon exposed to bore hole water to assess possible (combined) effects of the extraction procedure on fish response to the test solutions. Table 3.10 gives an overview of the test solution composition. A total volume of 58L was used each test solution, which prepared in four batches during the experimental period and stored in 20L glass bottles.

Table 3.9 Calculation of the relative GIF concentration in the extract.

GIF collection	Unit	Value	Remark
GIF concentration		X	Unknown
Amount of carbon installed	(kg)	2.0	Measured
Flow rate	(L/h)	300	Measured
Exposure time	(hr)	240	Measured
Recovery GIF water to carbon*	(%)	10	Estimated
GIF extraction			
Recovery GIF from carbon*	(%)	100	Estimated
Amount of carbon extracted	(g)	535	Measured
Extract volume	(ml)	2000	Measured
GIF concentration in extract	(mg/L)	~3600 X	Calculated

*) The estimated recovery of GIF from farm water to carbon and from carbon to extraction fluid, yields a combined recovery from farm water to extraction fluid of 10%

Table 3.10 Composition of test solution. X refers to the GIF concentration in the GIF production tank.

Test solutions		A	B	C	D	E
GIF concentration		1X	10X	100X	0	0
GIF extract content	(ml/L)	0.278	2.78	27.8	0	0
Control extract content	(ml/L)	0	0	0	0	27.8
Seawater content	(ml/L)	1000	997.2	972	1000	972

Set up

Hundred and fifty juvenile turbot of 28 days post hatch (France Turbot, Noirmoutier, France) were randomly divided over the fifteen test chambers filled with filtered and UV treated natural seawater and placed in a temperature controlled room with a photoperiod of 16L:8D. Fishes were allowed to acclimatize to the experimental conditions for 14 days. At the start of the experimental period each test chamber was filled with 700 ml of a randomly assigned test solution, resulting in three test chambers for each of the five test solutions. Due to mortalities during the acclimatization period, not all test chambers could be stocked with 10 experimental fish.

During the acclimatization and experimental period, the experimental system was operated as a semi-static renewal system: 80% of the total test solution volume was replaced in the morning before feeding and in the afternoon after the last feeding, resulting in a overall water renewal rate of 160%/day. During draining of the water, uneaten feed and feces were removed with disposable pipets. Each jar was equipped with an aeration tube to secure sufficient supply of oxygen.
All test chambers were supplied dry feed (Bernaqua Caviar) at a feeding level of 5%/d. Daily amounts of feed per test chamber calculated assuming an SGR of 5%/d and an FCR of 1. Daily feed loads were supplied in five portions supplied at 9.00, 11.00, 13.00, 15.00 and 17.00.
Oxygen saturation and water temperature were measured in all test chambers on experimental days 1, 2, 5 to 9 and 12 to 16. pH (Hach Lange HQ 40D) was measured in all test chambers on experimental days 1and 2 and 5 to 9. Total ammonia nitrogen (TAN) was measured on experimental day 2 and 16.

Data collection & statistics

Mortalities were recorded and removed daily.
At day 1 (start) and day 16 (end) of the experimental period experimental fish were weighed as a group per test chamber. For thjs purpose fishes were transferred from each test chamber to a glass beaker. The test chamber was refilled with 700ml of randomly assigned test solution and placed on the balance

(Mettler Toledo AT261 Delta range) and tarred to zero. Fish were scooped from the glass beaker two by two using a small tea sieve and transferred to the test chamber on the balance after removal of excess water by placing the sieve on dry paper tissue. The total weight of all fish per test chamber and their number was recorded.
At day 16 the total wet weight and number per test chamber was determined following the same procedure, except for returning of fish to the test chamber.

Specific growth rate (SGR) was calculated based on initial and final average weight per test chamber. Initial and final wet weight, SGR and final survival were tested for significant differences among treatments by One-way ANOVA.
Average temperature, oxygen saturation and pH for the experimental period were calculated from the daily measurements and tested for significant differences among treatments by One-way ANOVA.
Initial and final TAN levels were tested for significant differences among treatments by One way ANOVA.
GenStat 10.1 was used for all statistical tests.

2.2.7 Results Experiment 3

Fish performance
Initial wet weight was equal among treatments (Table 3.11). Specific growth rate (SGR) ranged from 4.22 to 5.75%/d among treatments. No effect of test solutions on final weight and SGR was observed (Table 3.11).
Overall survival rate ranged from 63 to 93% among the treatments. Significant differences in survival rate were observed: the test solution with a GIF concentration of 10 (B) yielded a lower survival rate than the two control test solutions (D, E). As the survival rates in the other two test solutions A and C, both higher and lower in GIF than test solution B, are equal to the control test solutions, it is unlikely that the low survival in test solution B is indeed an effect of GIF content of the test solutions.

Water quality
Water temperature, pH, initial TAN and final TAN were equal among treatments (Table 3.12).

2.2.8 Discussion

The present experiments aimed to establish the production of growth inhibiting factors by turbot. Our methodology included induction of GIF production, collection of GIF from the water using activated carbon, extraction of GIF from the carbon followed by preparation of GIF test solutions and assessment of the growth inhibiting properties of the test solutions in a juvenile growth trail. This methodology was derived from Yu and Perlmutter (1970), who successfully demonstrated the production of crowding factors by the zebrafish (*Danio rerio*).

In Experiment 1 final weights showed differences ($P<0.10$) among treatments with a tendency towards higher final weights in the two control test solutions (D, E)) In addition when grouping GIF treatments and Control treatments, significant differences in final weight and length were found. All this indicates the presence of growth inhibitors in the test solutions. However we considered Experiment 1 as insufficient evidence for GIF production by turbot for a number of reasons. First of all the experiment suffered from a low survival of the experimental animals in all treatment groups. A survival rate of 80% in control treatments is generally used as a minimal requirement to accept the results of the bio-assay. Clearly the survival rate in the control treatments is lower in this experiment. In addition, the low survival rate resulted in low numbers of experimental fish per replicate and a high variation in individual weights within replicates (Table 3.4). Secondly, initial weights could not be measured. As a result growth could not be calculated and observed differences in final weight are possibly due to unknown differences in initial weight among treatments, despite the random distribution of experimental fish from a single stock. Although differences in the total ammonia and nitrite levels in the treatments could not be tested for significance the concentrations in control test solutions (D, E) are among the lowest measured levels (Table 3.5). The observed differences in final weight may therefore be attributable to the differences in ammonia and or nitrite levels in the treatments, although thresholds for growth are unknown for this life stage of turbot.

Given these confounding factors hampering final conclusions on the production of GIF by turbot based on Experiment 1, we decided to repeat this experiment with an improved set up. Improvements included the use of experimental fish of sufficient size to enable measurement of the initial weight, increased

Table 3.11. Mean values (SD) (n = 3) per experimental treatment for initial and final wet weight, SGR and survival. Values with different letters across rows are significantly different (ANOVA, P < 0.10).

		Test solutions					All treatments		ABC vs. DE
Parameter	Unit	A	P-value	P-value	D	E	P-value	LSD 10%	P-value
Initial wet weight	(mg)	119 (86)	96 (23)	104 (12)	119 (15)	103 (5)	0.23	-	
Final wet weight	(mg)	235 (48)	222 (15)	191 (24)	244 (14)	196 (39)	0.22	-	
SGR	(%/d)	4.46 (0.9)	5.75 (1.5)	4.05 (0.6)	4.81 (0.5)	4.22 (1.4)	0.36	-	
Survival	(%)	79 (18)[ab]	63 (14)[b]	93 (12)[a]	75 (5)[ab]	83(4)[a]	0.097	18.7	

Table 3.12 Mean (n=3) (SD) values per treatment for temperature, pH, dissolved oxygen concentration, salinity and total ammonia nitrogen (TAN). P-values result from One way ANOVA.

Water quality parameter		Test solutions					
	Unit	A	B	C	D	E	P-value
Temperature	(°C)	16.5 (0.1)	16.7 (0.1)	16.6 (0.1)	16.5 (0.1)	16.6 (0.1)	0.44
pH		7.89 (0.11)	7.72 (0.22)	7.82 (0.08)	7.86 (0.13)	7.89 (0.07)	0.62
Oxygen	(%)	150 (6)	144 (19)	147 (8)	156 (6)	148 (2)	0.68
Initial TAN	(mg N/L)	0.27 (0.01)	0.23 (0.08)	0.22 (0.06)	0.24 (0.06)	0.25 (0.08)	0.88
Final TAN	(mg N/L)	1.53 (0.26)	1.25 (0.38)	1.07 (0.34)	1.06 (0.23)	1.40 (0.28)	0.30

renewal rate and water volume of the test chambers to maintain a better water quality and the use of dry feed to yield better growth performance.
Despite these improvements the initial indication of the presence of GIF in the test solutions was not confirmed in the second experiment, which did not show differences in specific growth rate among the treatments (Table 3.6). The improved set up resulted in all measured water quality parameters being equal among the treatments (Table 3.7).
Experiment 3 was set up as a last attempt to demonstrate the production of GIF by turbot. The experimental set up was further improved by replacing the polyethylene test chambers by glass test chambers to exclude the possible inactivation of biologically active molecules in the test solution by adsorption to the walls of the test chambers. In addition test chambers were supplied with pure oxygen to prevent growth retardation due to suboptimal oxygen levels. Despite these improvements again no effects of test solutions on specific growth rate were observed (Table 3.11). As the survival rates in the test solutions A and C, both higher and lower in GIF than test solution B, are equal to the control test solutions, it is unlikely that the low survival in test solution B is indeed an effect of GIF content of the test solutions.
The goal of this series of experiments (Task 2.1) was to demonstrate the production of growth inhibiting factors (GIF) by turbot. Test solutions based on GIF collection and extraction showed no effects on specific growth rate of the experimental fish. It remains unclear whether this lack of response to the test solutions can be attributed to the absence of GIF production in turbot. The chain of events from the tank containing turbot of which GIF production was to be investigated leading to the actual tests in juvenile growth trails contains a number of potential bottlenecks for successful demonstration of GIF production that should be considered. The potential reasons for lack of response to the test solutions are reviewed in Table 3.12.

2.2.9 Conclusion

The production of growth inhibiting factors by turbot was not demonstrated.

Table 3.12 Overview of possible explanations for the lack response of experimental fish to the test solutions following the stepwise preparation of the test solutions.

Step	Explanation	Effect	Comment
1 GIF induction	Turbot do not produce GIF	No GIF in test solution	Husbandry conditions in GIF production tank mimicked commercial conditions: if no GIF production in experiment, GIF production is unlikely to occur under commercial conditions
2 GIF collection	GIF were not collected on activated carbon	No GIF in test solution	Crowding factors of zebrafish were successfully collected from water using activated carbon (Yu and Perlmutter, 1970).
3 GIF extraction	GIF were not extracted from activated carbon	No GIF in test solution	Crowding factors of zebrafish were successfully extracted from using activated carbon using methyl chloroform (Yu and Perlmutter, 1970).
4 GIF extraction	GIF were lost during the extraction process	No GIF in test solution	LC-MS analysis of the GIF extract showed that its composition differed from the Control extract, showing that something was collected from the water and successfully extracted from the activated carbon. It is however unknown whether these differences include GIF.
5 Preparation of test solutions	GIF concentrations in test solutions are too low	No response of experimental fish to test solutions	This is unlikely given the wide concentration range of the test solutions
6 Growth trial	Experimental fish are not sensitive to GIF in test solutions	No response of experimental fish to test solutions	Early life stages of zebrafish were shown to be sensitive to crowding factors produced by their adult conspecifcs (Yu and Perlmutter, 1970).
7 Growth trial	Confounding factors masked the effects of test solutions	No response of experimental fish to test solutions	This is unlikely as despite improvement of the experimental set up first results indicating GIF presence in the test solutions were not reproduced in the following experiments.

3 The effect of local production of growth inhibiting factors (Task 2.3)

3.1 Introduction

In this project we hypothesized that production and accumulation of growth inhibiting factors leads to growth retardation of turbot reared in recirculating aquaculture systems (RAS).
The production of growth inhibiting factors under crowded conditions has been demonstrated for zebrafish (Brachydanio rerio), blue gourami (*Trichogaster trichopterus*) (Yu and Perlmutter, 1970), sea lamprey (*Petromyzon Marinus*) larvae (Rodriguez-Munoz *et al.,* 2003) and mosquito fish (*Gambusia affinis*) (Lutnesky and Adkins, 2003). Homotypically conditioned water (water in which other fish of the same species have lived) was shown to promote rather than retard the growth of gold fish (*Carassius auratus*) (Allee *et al*., 1936 in Livengood, 1937). More recently exposure to chemical factors released by conspecifics was shown to increase growth variability of *Prochilodus lineatus* but no effect on growth was observed (Barbosa and Volpato, 2007). Chemical communication among stressed marine fish was demonstrated for white seabream *Diplodus sargus*), which showed elevated plasma cortisol levels when receiving water from a tank densely stocked with conspecifics (Olivotto *et al*., 2002).
Effects on growth rate as a result of exposure to chemical factors released by conspecifics due to crowding have not been demonstrated in turbot nor has this phenomenon been studied in relation to commercial aquaculture practice. In a RAS, in which several size classes of fish can be stocked within a single production system, locally produced GIF may eventually reach all tanks as for the central treatment and redistribution of water to the tanks in such a system. As a result, turbot that don't produce GIF (as for their size or husbandry conditions) but share a RAS with GIF producing conspecifics, may still be exposed to GIF and their growth may be affected. In order to understand better the potential effect of GIF production inside RAS, the effects of stocking different size classes of turbot in one RAS needs to be studied. Additionally, the results of this study provides insight in the production of GIF by turbot. We investigated the effect of rearing water that was previously in contact with conspecifics on growth of juvenile turbot. A series of four experiments was performed, addressing the effect of size of the conspecifics, the effect of water treatment and a commercial situation.

3.2 Experiments at France Turbot

3.2.1 Introduction

Three experiments were performed to investigate the transfer of growth inhibiting factors between tanks. All three experiments were based on connection of experimental tanks to the outflow of tanks stocked with turbot in commercial densities. These tanks, referred to as GIF production tanks, were all part of an experimental flow through system to exclude potential effects of water treatment on growth of experimental fish as would have been the case in recirculation systems. Experimental work was performed at France Turbot, Noirmoutier, France.

3.2.2 Materials and Methods

Experiment 1: the effect of large turbot on growth of juvenile turbot
The experimental set up consisted of three experimental tanks (50 L) receiving effluent water from a tank stocked with large turbot (referred to as GIF production tank)(Table 4.1) and three the same tanks receiving borehole water. This resulted in two treatments: exposure to water that was previously in contact with conspecifics (referred to as GIF) and exposure to water that was never in contact with conspecifics that served as control treatment (referred to as Borehole water), with three replicates for each treatment (Fig. 4.1).
A buffer tank was installed to control the borehole water supply to the Borehole water tanks. Ammoniumchloride was added to the borehole water to equalize total ammonia nitrogen (TAN) levels in all treatments, excluding it as a cause for growth differences between treatments. A sedimentation tank was installed between the outflow of the GIF production tank and the experimental GIF tanks to remove solids from the outflow of the GIF production tank before the water was used as inflow for the experimental tanks. Equal flow rates of approximately 52L/min

were installed for all experimental tanks as flow rate is known to affect growth of juvenile turbot (Schram *et al., in press*).
Each experimental tank was stocked with 10 juvenile turbot, which were allowed to acclimatize to the experimental conditions for 14 days. At the start of the experiment the average weight per tank ranged from 50.3 to 57.4g.
During weekdays fish were fed by hand three times per day at a level of 2%/BW/day (Le Gouessant, Turbot Label Rouge - Flot 4 mm). For each tank feed losses were determined by collecting and counting uneaten pellets each morning. During weekends fish were fed twice per day and uneaten pellets were not collected.
Experimental fish were obtained from a single batch produced at France Turbot (Noirmoutier, France) and were weighed individually at the start (day 1) and end (Day 41) of the experiment.

Experiment 2: the effect of small turbot on growth of juvenile turbot
The set up and treatments were the same as Experiment 1 expect for the lower average weight of the turbot in the GIF production tank (Table 4.1, Fig. 4.1) and the duration. Experiment 2 was run in parallel to Experiment 1 during 41 days, but was continued for another 49 days after termination of Experiment 1. At the start (day 1) and at the end (day 90) of the experiment all fish were weighed individually (Table 2). Experimental fish were obtained at the same time from the same batch as Experiment 1, produced at France Turbot, Noirmoutier, France.

Experiment 3: the effect of activated carbon on the effect of large turbot on growth of juvenile turbot
The set up of Experiment 3 was the same as Experiment 1 and 2 but included an third treatment in which the water coming from the GIF production tank was led through a bed of containing 13.5 kg activated carbon (NORIT GAC 830 plus) before flowing into three experimental tanks (Fig. 4.2). The small amounts of activated carbon washed out of the bed were replaced with new carbon on a daily basis.

Water quality
For Experiment 1 temperature (Hach Lange HQ 40D) and oxygen (Oxyguard) were measured daily and pH (Hach Lange HQ 40D). Total ammonia nitrogen (photometric) was measured five times and pH three times during the experimental period. Flow rates to the individual experimental tanks were measured six times (weekly).
For Experiment 2 temperature (Hach Lange HQ 40D) and oxygen (Oxyguard) were measured daily and pH (Hach Lange HQ 40D). Total ammonia nitrogen (photometric) was measured ten times and pH seven times during the experimental period. Flow rates to the individual experimental tanks were measured twelve times (weekly).
For Experiment 3 temperature (Hach Lange HQ 40D) and oxygen (Oxyguard) were measured daily and pH (Hach Lange HQ 40D). Total ammonia nitrogen (photometric) was measured five times and pH six times during the experimental period. Flow rates to the individual experimental tanks were measured five times (weekly).
All water quality measurements were done in the buffer tanks for each treatment, resulting in one measurement for the three replicates.

Production parameters
Average initial and final weights per tank were used to calculate the specific growth rate (SGR) over the experimental period as follows:

$$SGR = (\ln(W_t) - \ln(W_1)) \times \frac{100}{t}$$

Where: SGR = Specific growth rate (% BW/d); W_t = Average weight at day 29 (g); W_1 = Average weight at day 1 (g); t = Number of days.

Feed losses per tank were estimated based on the amounts of pellets collected from each tank in the mornings of week days. Pellet weight (0.02g) was determined by bulk weighing and counting > 1000 pellets.

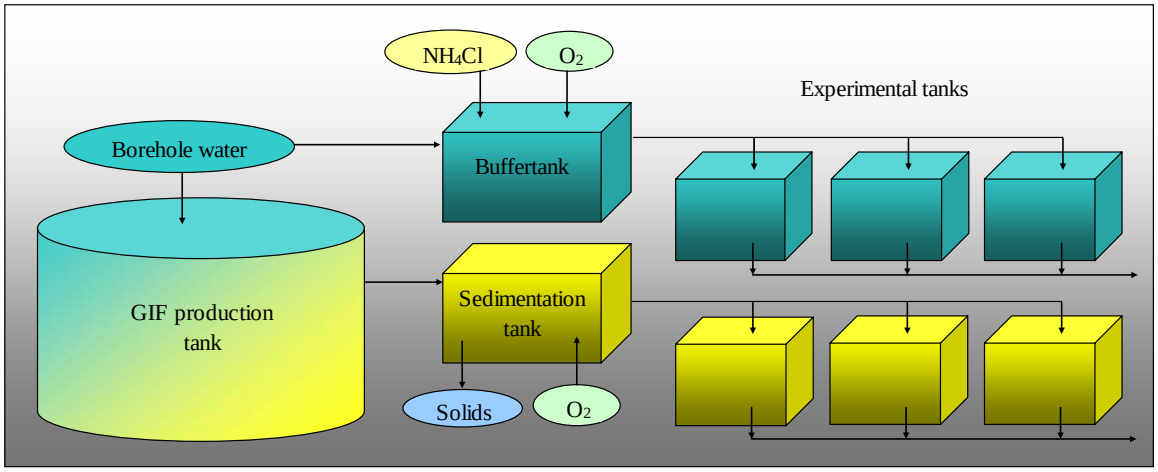

Fig 4.1. Schematic presentation of the experimental set up of Experiment 1 and 2 (not to scale).

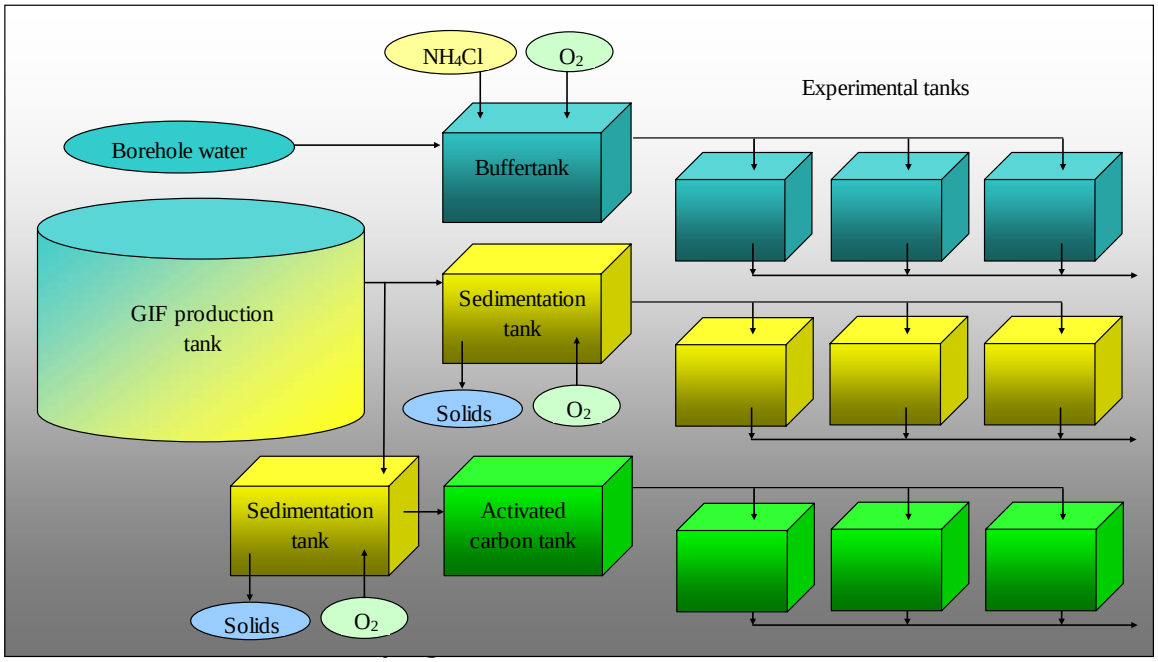

Fig. 4.2 Schematic presentation of the experimental set up of Experiment 3 (not to scale).

Total feed intake per tank was determined by subtracting the estimated feed losses and feed loads per tank, and used to calculate feed conversion rates (FCR) per tank as follows:

$$FCR = \frac{TFI}{(W_t - W_0)}$$

Where: FCR = Feed Conversion Rate (kg/kg), TFI = total feed intake (g) W_0 = total biomass at day 1 (g), W_t = total biomass at day t (g).

As a measure for size variation within tanks the coefficient of variation for weight (CV) was calculated for all tanks based on the initial and final mean weight and standard deviation per tank.

$$CV = \frac{\text{Standard deviation}}{\text{Mean weight}} * 100\%$$

The change in size variation during the experimental periods was determined as the difference between the initial and final coefficient of variation: ΔCV (%).

Statistics

Mean initial weight, final weight, specific growth rates (SGR), total feed intake, feed conversion rate (FCR), initial and final coefficient of variation and the increase in the coefficient of variation (Δ CV) were tested for significant differences among treatments using One-way ANOVA followed by least significant difference (LSD) post-hoc analysis. Flow rates were tested for significant differences using repeated measurements ANOVA followed by least significant difference (LSD) post-hoc analysis.

Table 4.1 Characteristics of the GIF production tanks

	Experiment 1	Experiment 2	Experiment 3
Water supply	Bore hole	Bore hole	Bore hole
Tank area (m^2)	5.3	5.3	5.3
# fish			
Start of experiment	113	201	102
End of experiment	105	201	91
Mean weight (g)			
Start of experiment	1839	999	2079
End of experiment	1927	1141	2063
Stocking density (kg/m^2)			
Start of experiment	39.2	37.9	40.0
End of experiment	39.3	43.3	35.4

3.2.3 Results of Experiment 1

Growth

Final mean weight (ANOVA, $P < 0.01$) was lower for the fish exposed to the water from the GIF production tank with large turbot compared to the borehole water (control) treatment (Table 4.2), while initial mean weight did not differ between treatments (ANOVA, $P = 0.39$). As a result the specific growth rate (SGR) of the fish exposed to the water from the GIF production tank was almost 50% lower than the control (ANOVA, $P = 0.017$, Table 4.2). Feed intake was equal between treatments (ANOVA, $P = 0.55$, Table 4.2) while feed conversion rate (FCR) showed a trend towards a higher FCR for the fish exposed to the water from the GIF production tank (ANOVA, $P = 0.058$, Table 4.2), suggesting that the observed difference in SGR was related to feed utilization rather than feed intake.

Initial size variation (expressed as coefficient of variation) showed a trend towards higher variation in the GIF treatment (Table 4.2, ANOVA, $P = 0.07$), while final size variation and the increase in size variation (ΔCV) was higher in the GIF treatment (Table 4.2, ANOVA, $P = 0.03$ and $P = 0.04$).

Water quality

Flow rates ranged from 50.2 to 53.6 L/min for the borehole water treatment and from 50.5 to 53.8 L/min for the GIF treatment. Overall mean flow rates were not different between treatments (One-way ANOVA, $P = 0.62$). In addition, no differences in flow rates occurred between the treatments on sampling days (repeated measurements ANOVA, P time*treatment = 0.77, Fig 4.3).

Water temperature ranged from 12.6 to 15.4 °C for the borehole water treatment and from 12.4 to 15.6 °C for the GIF treatment. Overall mean (SD) water temperature was 13.8 (0.8) °C for the bore hole water treatment and 13.7 (0.8) °C for the GIF treatment. Water temperature did not show large differences between the treatments during the course of the experiment (Fig. 4.4).

Oxygen saturation range and overall mean (SD) were 116 to 162 % and 140 (10.8) % for the borehole water treatment. For the GIF treatment oxygen saturation ranged from 115 to 202 % and the overall mean (SD) was 153 (22.4). Oxygen saturation was mostly higher in the GIF treatment, especially in the second half of the experimental period (Fig 4.5).

In the borehole water treatment pH ranged from 7.40 to 7.46 and from 7.31 to 7.39 in the GIF treatment and did not show large differences between the treatments during the course of the experiment (Fig 4.6).

Total ammonia nitrogen concentration range and overall mean (SD) concentration were 0.13 to 0.79 mg N/L and 0.31 (0.28) mg N/L in the borehole water treatment. For the GIF treatment total ammonia nitrogen concentration ranged from 0.29 to 0.46 mg N/L and the overall mean (SD) was 0.35 (0.06) mg N/L. During the first half of the experimental period TAN was lower in the bore hole water treatment whereas in the second half it was higher than in the GIF treatment (Fig. 4.7).

Table 4.2. Mean (SD) values per treatment for initial weight, final weight, SGR, total feed intake, FCR, initial and final coefficient of variation, the increase in the coefficient of variation (Δ CV) and survival rate. P-values result from One-way ANOVA (n=3), except for flow rate where P-value results from repeated measurements ANOVA (n = 18). Marked mean values marked are different from the Control at a 5% significance level (**) or 10% significance level (*).

		Treatments		
Parameter	Unit	Borehole water (Control)	GIF	P-value
Initial weight	(g)	55.8 (1.8)	53.6 (3.4)	0.39
Final weight	(g)	80.0 (3.9)	64.6 (3.6)**	0.01
SGR	(%/d)	0.88 (0.14)	0.45 (0.12)**	0.02
Total feed intake	(g)	189 (25)	178 (19)	0.55
FCR		0.79 (0.04)	1.73 (0.62)*	0.06
Initial CV	(%)	19 (3)	26 (4)*	0.07
Final CV	(%)	19 (5)	36 (8)**	0.03
ΔCV	(%)	2.3 (1)	10.4 (5)**	0.04
Survival	(%)	100 (0)	100 (0)	
Flow rate	(L/min)	51.2 (1.0)	51.6 (0.8)	0.62

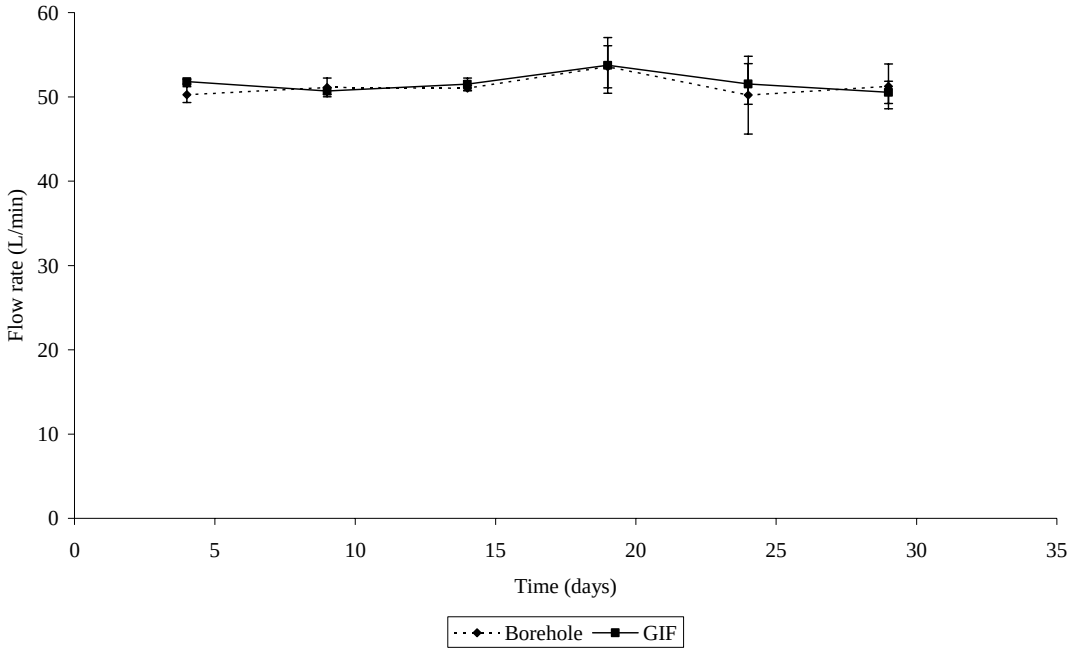

Fig 4.3. Mean flow rate (n = 3)(L/min) of the water supply to the treatments during the experimental period.

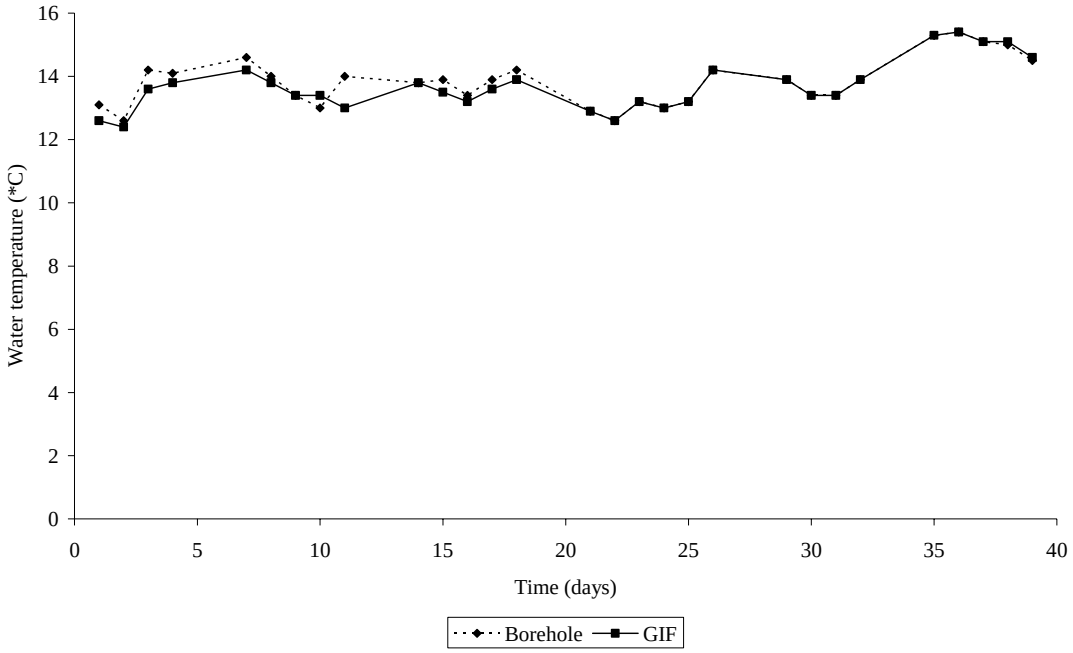

Fig 4.4. Water temperature (°C) in the treatments during the experimental period.

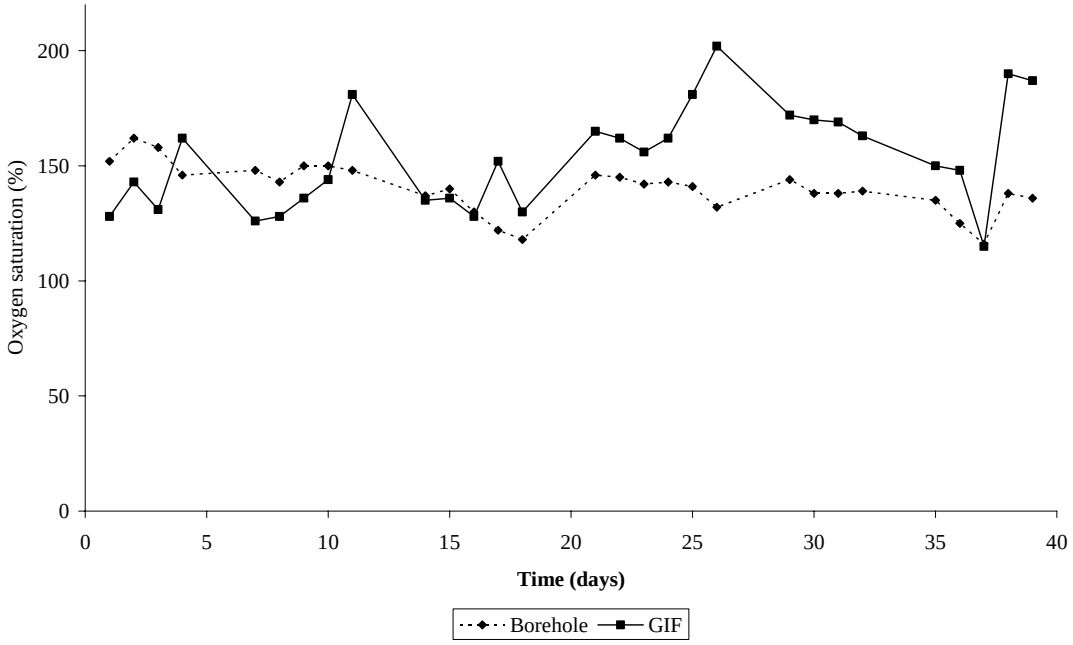

Fig 4.5. Oxygen saturation (%) in the treatments during the experimental period.

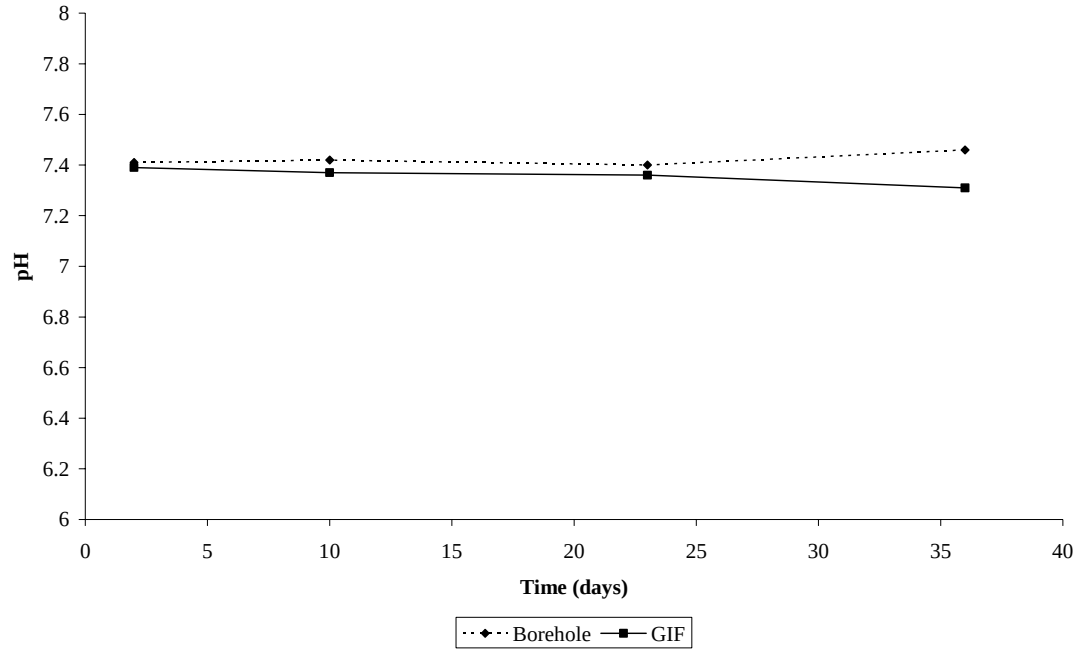

Fig 4.6. pH in the treatments during the experimental period.

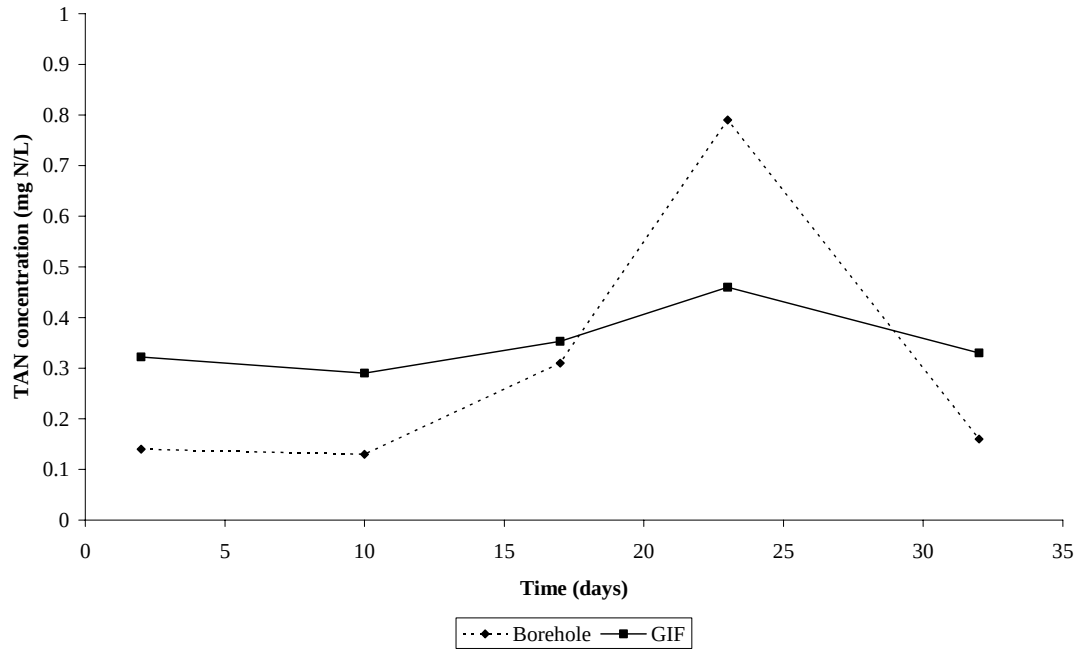

Fig 4.7 Total ammonia nitrogen(TAN) concentration (mg N/L) in the treatments during the experimental period

3.2.4 Results of Experiment 2

Growth

Final mean weight showed a trend towards higher values for the borehole water treatment (Control) than for the GIF treatment (Table 4.3, ANOVA, P = 0.08). The initial mean weights were however not equal between treatments (Table 4.3, ANOVA, P = 0.04): as the final mean weight, the initial mean weight was highest in the borehole water treatment. The resulting specific growth rates (SGR) showed no difference between the treatments (Table 4.3, ANOVA, P = 0.36).

Total feed intake and feed conversion rate (FCR) did not differ between treatments (Table 4.3, ANOVA, P = 0.11 and P = 0.20)
Initial size variation was not different between treatments (Table 4.3, ANOVA, P = 0.25). Final size variation was different between treatments (Table 4.3, P = 0.02) and the increase in size variation during the experiment (ΔCV) showed a trend towards a larger increase in the Borehole water treatment (Table 4.3, ANOVA, P = 0.06).

Water quality
Flow rates ranged from 48.0 to 53.7 L/min for the borehole water treatment and from 48.3 to 56.4 L/min for the GIF treatment. Overall mean flow rates were not different between treatments (ANOVA, P = 0.23). In addition, no differences in flow rates occurred between the treatments on sampling days (repeated measurements ANOVA, P time*treatment = 0.54, Fig 4.8).
Water temperature ranged from 12.6 to 15.4 °C for the borehole water treatment and from 12.3 to 15.4 °C for the GIF treatment. Overall mean (SD) water temperature was 14.2 (0.7) °C for both the bore hole water treatment and the GIF treatment. Water temperature did not show large differences between the treatments during the course of the experiment (Fig. 4.9).
Oxygen saturation range and overall mean (SD) were 120 to 220 % and 156 (22.9) % for the borehole water treatment. For the GIF treatment oxygen saturation ranged from 122 to 195 % and the overall mean (SD) was 153 (15.4). Oxygen saturation did not show large differences between the treatments during the course of the experiment (Fig 4.10).
In the borehole water treatment pH ranged from 7.40 to 7.52 and from 7.11 to 7.35 in the GIF treatment. In the GIF treatment pH was slightly lower than the borehole water treatment during the course of the experiment (Fig 4.11).
Total ammonia nitrogen (TAN) concentration range and overall mean (SD) concentration were 0.13 to 1.30 mg N/L and 0.44 (0.36) mg N/L in the borehole water treatment. For the GIF treatment total ammonia nitrogen concentration ranged from 0.38 to 1.30 mg N/L and the overall mean (SD) was 0.71 (0.33) mg N/L. TAN levels were lower in the borehole water treatment for the largest part of the experimental period (Fig. 4.12).

Table 4.3. Mean (SD) values per treatment for initial weight, final weight, SGR, total feed intake, FCR, initial and final coefficient of variation, the increase in the coefficient of variation (Δ CV) and survival rate. P-values result from One-way ANOVA (n=3), except for flow rate where P-value results from repeated measurements ANOVA (n = 36). Marked mean values marked are different from the Control at a 5% significance level (**) or 10% significance level (*).

		Treatments		
Parameter	Unit	Borehole water (Control)	GIF	P-value
Initial weight	(g)	58.3 (2.1)	47.5 (5.7)**	0.04
Final weight	(g)	92.4 (15.8)	68.8 (8.1)*	0.08
SGR	(%/d)	0.50 (0.01)	0.41 (0.15)	0.36
Total feed intake	(g)	384 (54)	317 (18)	0.11
FCR		1.21 (0.31)	1.49 (0.10)	0.20
Initial CV	(%)	24 (6)	20 (2)	0.25
Final CV	(%)	46 (3)	33 (5)**	0.02
ΔCV	(%)	22 (4)	14 (4)*	0.06
Survival	(%)	100 (0)	100 (0)	
Flow rate	(L/min)	50.3 (1.1)	51.1 (0.2)	0.23

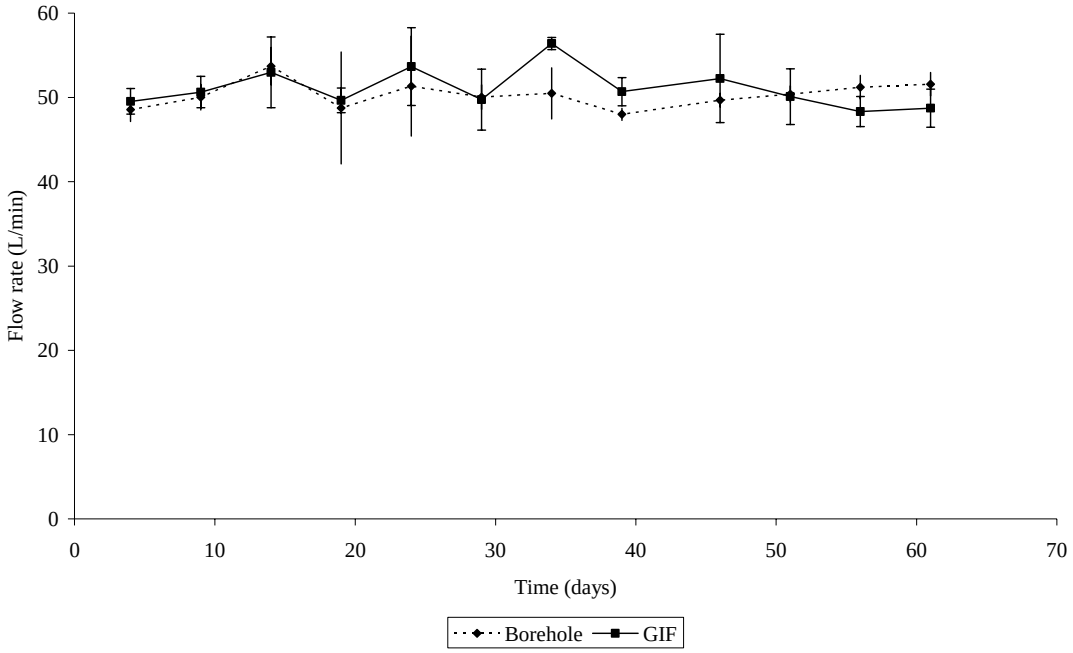

Fig 4.8. Mean flow rate (n = 3)(L/min) of the water supply to the treatments during the experimental period.

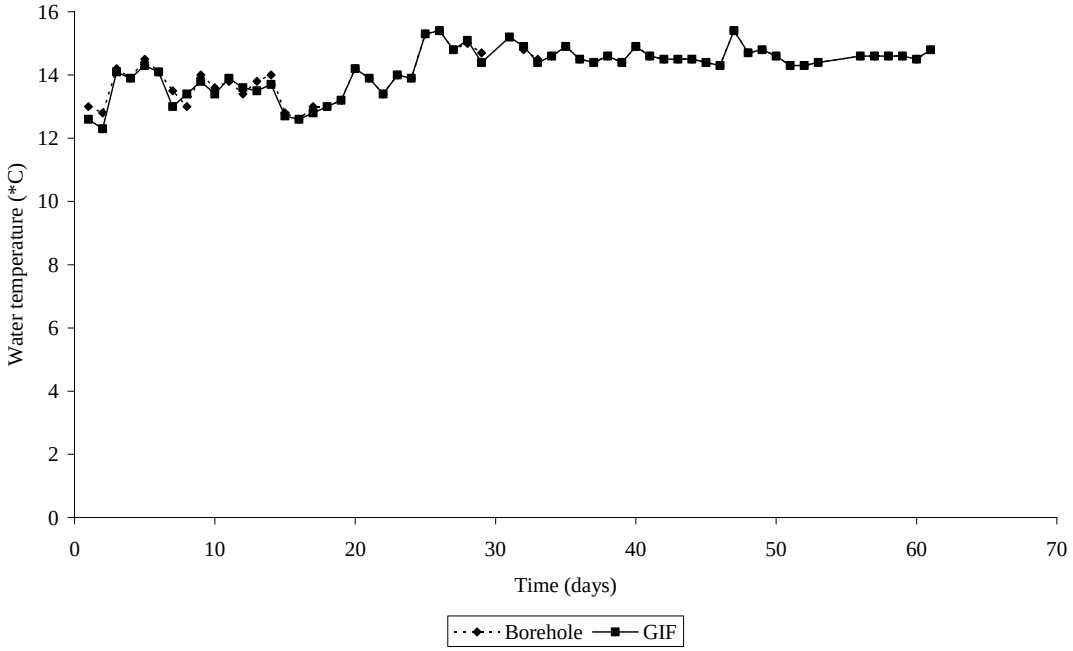

Fig 4.9. Water temperature (°C) in the treatments during the experimental period.

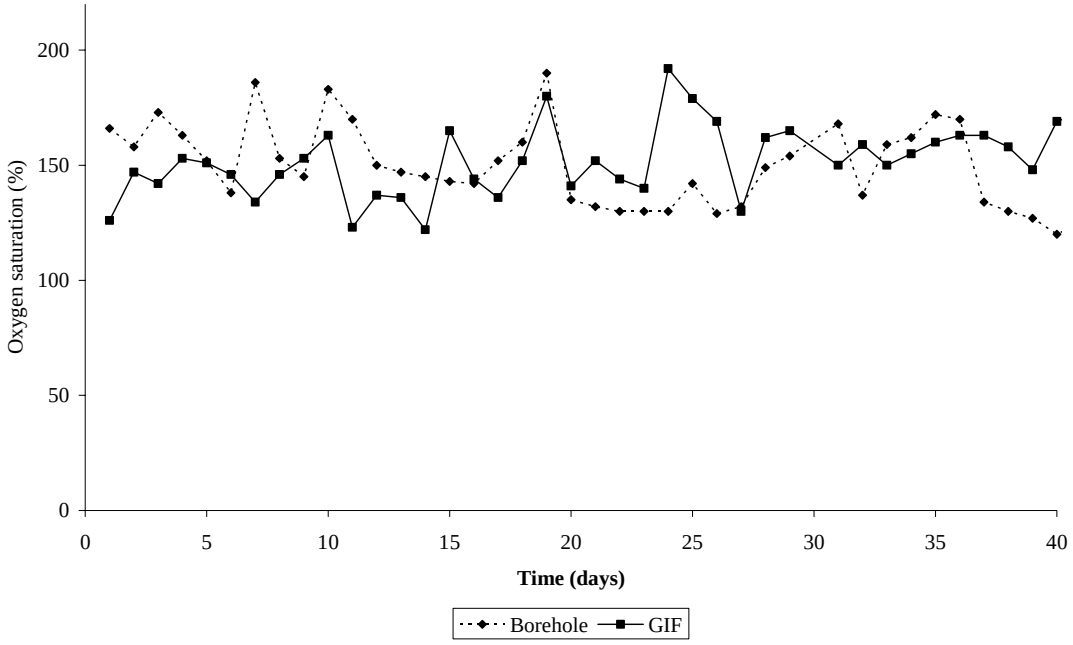

Fig 4.10. Oxygen saturation (%) in the treatments during the experimental period.

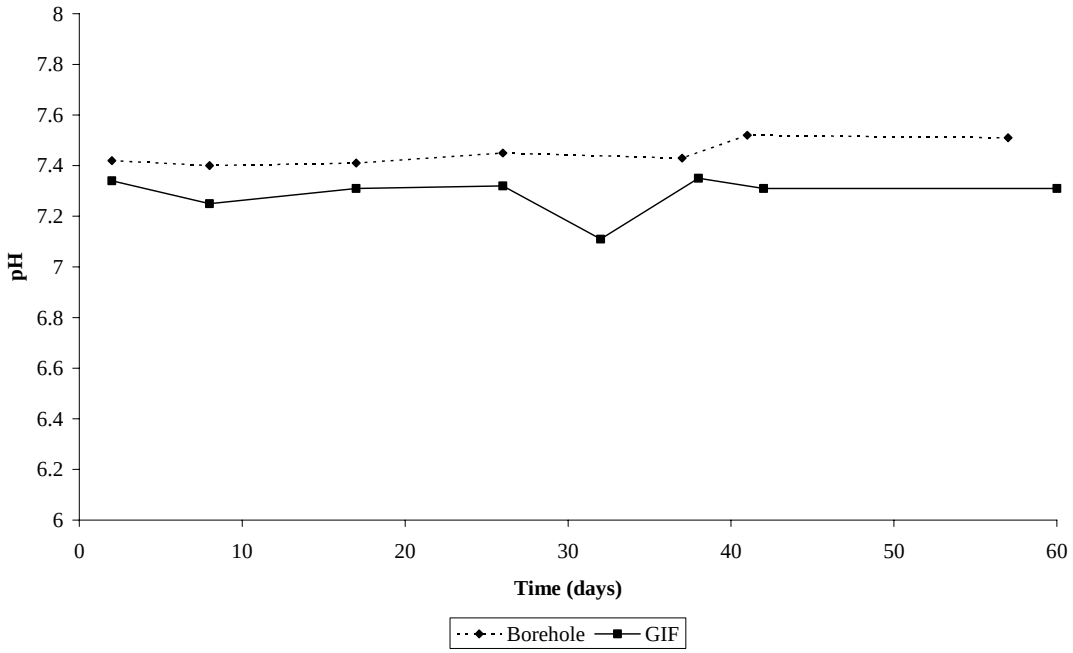

Fig 4.11. pH in the treatments during the experimental period.

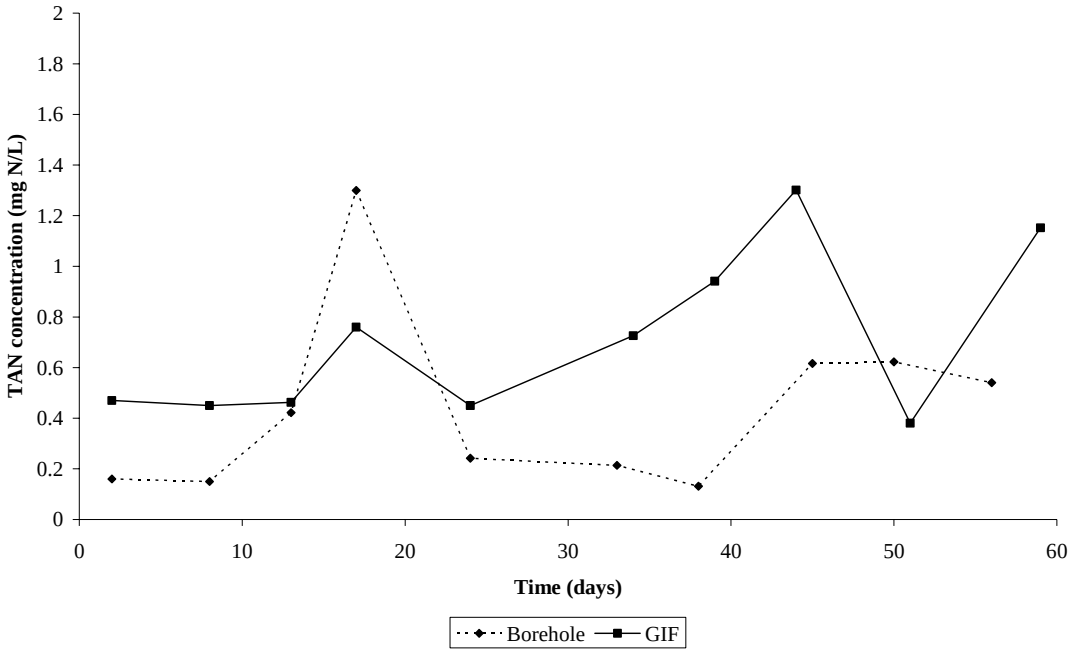

Fig 4.12 Total ammonia nitrogen(TAN) concentration (mg N/L) in the treatments during the experimental period

3.2.5 Results of Experiment 3

Growth

Initial mean weight was equal in all treatments (Table 4.4, ANOVA, P = 0.35). No differences in final mean weight, specific growth rate (SGR), total feed intake and feed conversion rate (FCR) were observed among the treatments (Table 4.4)

Initial and final size variation were equal among treatments (Table 4.4, ANOVA, P = 0.71 and P = 0.14). The increase in size variation (ΔCV) was larger in the GIF treatment compared to the other two treatments (Table 4.4, ANOVA, P = 0.03).

Water quality

Results of water quality measurements are presented in Table 4.5. Overall mean flow rates were different between treatments (ANOVA, P < 0.01). In addition, there was a trend towards differences in flow rates between the treatments on sampling days (repeated measurements ANOVA, P time*treatment = 0.09, Fig 4.13).

Water temperature ranged from 13.0 to 15.2 and was equal for all treatments (Table 4.5, Fig 4.14). Overall mean oxygen saturation levels were not different among treatments (ANOVA, P = 0.20). However, on individual experimental days differences in oxygen saturation levels existed among treatments (repeated measurements ANOVA, P time*treatment < 0.001). Oxygen saturation levels in the borehole water treatment generally differed from the levels in the GIF and GIF + activated carbon treatments (Fig. 4.15) pH increased slightly in all treatments during the course of the experiment and did not show large differences among treatments (Fig. 4.16). Mean total ammonia nitrogen (TAN) concentrations were very similar in the GIF and GIF + activated carbon treatments and slightly lower in the borehole water treatment (Table 4.5). However, the TAN concentration in the borehole water was higher than in the other two treatments for a large part of the second half of the experimental period (Fig. 4.17).

Table 4.4. Mean (SD) values per treatment for initial weight, final weight, SGR, total feed intake, FCR, initial and final coefficient of variation, the increase in the coefficient of variation (ΔCV) and survival rate. P-values result from One-way ANOVA (n=3), except for flow rate where P-value results from repeated measurements ANOVA (n = 36). Mean values marked as (**) are different at a 5% significance level.

Parameter	Unit	Treatments			P-value
		Borehole water (Control)	GIF	GIF + AC	
Initial weight	(g)	77.9 (3.1)	78.1 (4.5)	82.2 (3.4)	0.35
Final weight	(g)	105.6 (3.1)	105.3 (10.1)	109.5 (2.7)	0.68
SGR	(%/d)	0.68 (0.14)	0.66 (0.10)	0.68 (0.13)	0.93
Total feed intake	(g)	338 (2)	311 (35)	334 (24)	0.39
FCR		1.26 (0.25)	1.16 (0.14)	1.26 (0.28)	0.85
Initial CV	(%)	18.6 (6.1)	18.5 (3.0)	15.9 (3.4)	0.71
Final CV	(%)	25.7 (5.2)	35.6 (5.1)	23.9 (6.8)	0.14
ΔCV	(%)	7.0 (2.0)	17.1 (5.1)**	8.0 (3.6)	0.03
Survival	(%)	100 (0)	100 (0)	100 (0)	

Table 4.5 Water quality parameters Experiment 3. Mean flow rates with different letters across rows are different (ANOVA, $P < 0.05$).

Parameter	Unit	Treatments		
		Borehole water (Control)	GIF	GIF + AC
Flow rate	(L/min)			
Range		47 - 95	86 - 121	105 – 148
Mean (SD)		72 (20)[a]	107 (13)[b]	125 (19)[c]
Temperature	(°C)			
Range		13.0 – 15.2	13.0 – 15.2	13.0 – 15.2
Mean		14.4	14.4	14.4
Oxygen saturation	(%)			
Range		115-183	114-181	115-192
Mean		145 (18)	143 (18)	147 (19)
pH				
Range		7.62-7.77	7.51-7.75	7.47-7.72
TAN	(mg N/L)			
Range		0.11 – 0.98	0.37 – 0.82	0.27 – 0.83
Mean		0.50	0.65	0.61

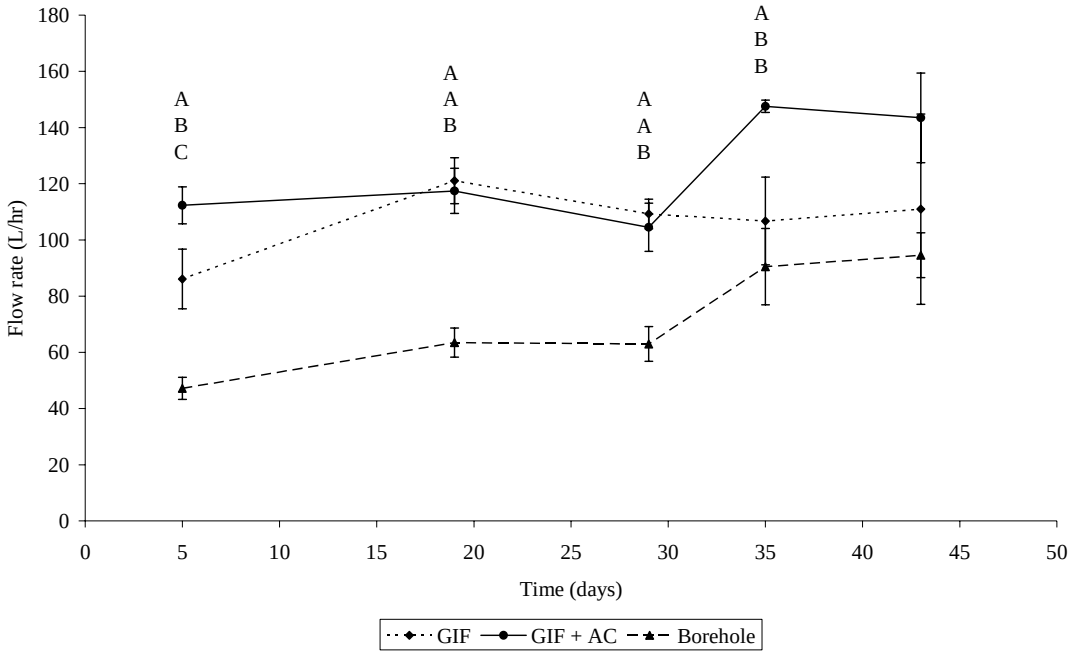

Fig 4.13 Mean flow rate (n = 3)(L/min) of the water supply to the treatments during the experimental period. For each sampling point mean flow rates per treatment with different letters are different at a 10% significance level (repeated measurements ANOVA).

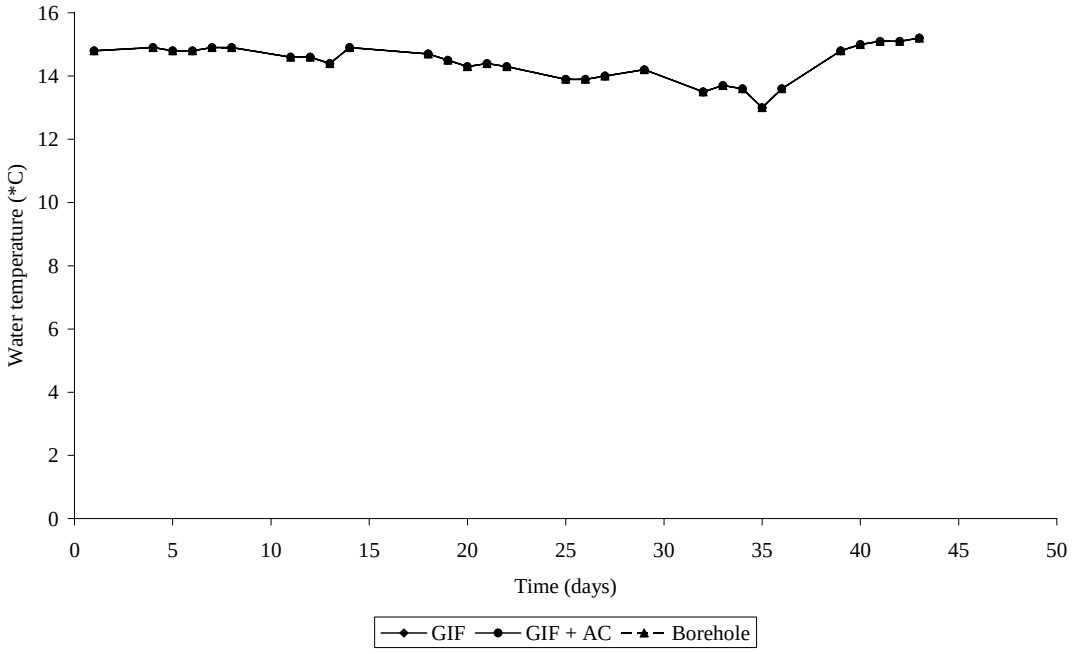

Fig 4.14. Water temperature (°C) in the treatments during the experimental period.

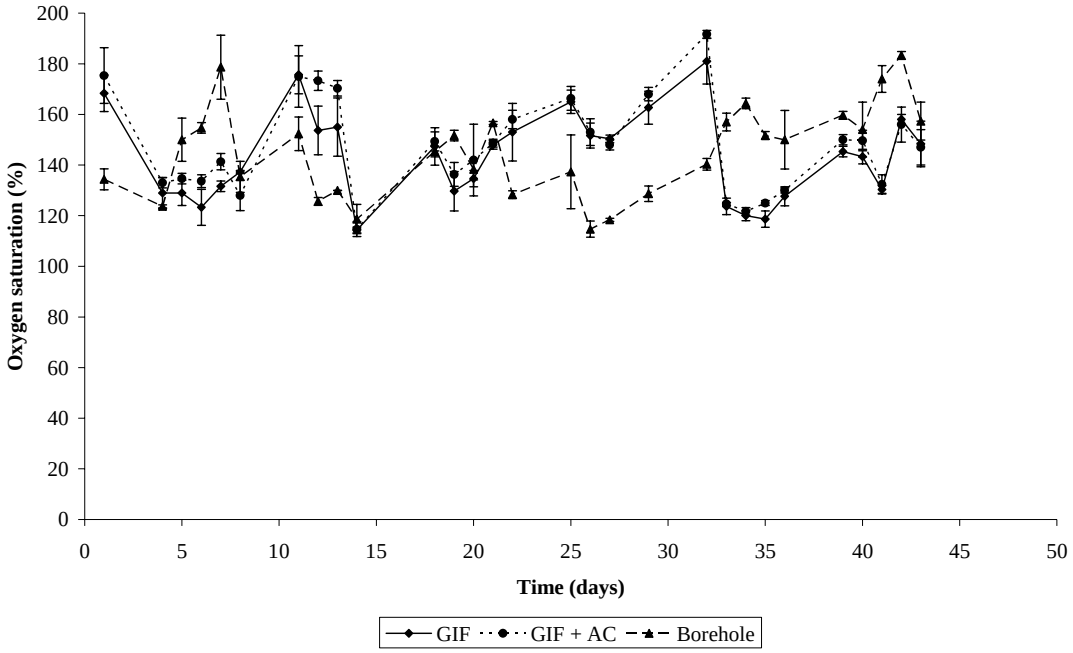

Fig 4.15. Oxygen saturation (%) in the treatments during the experimental period.

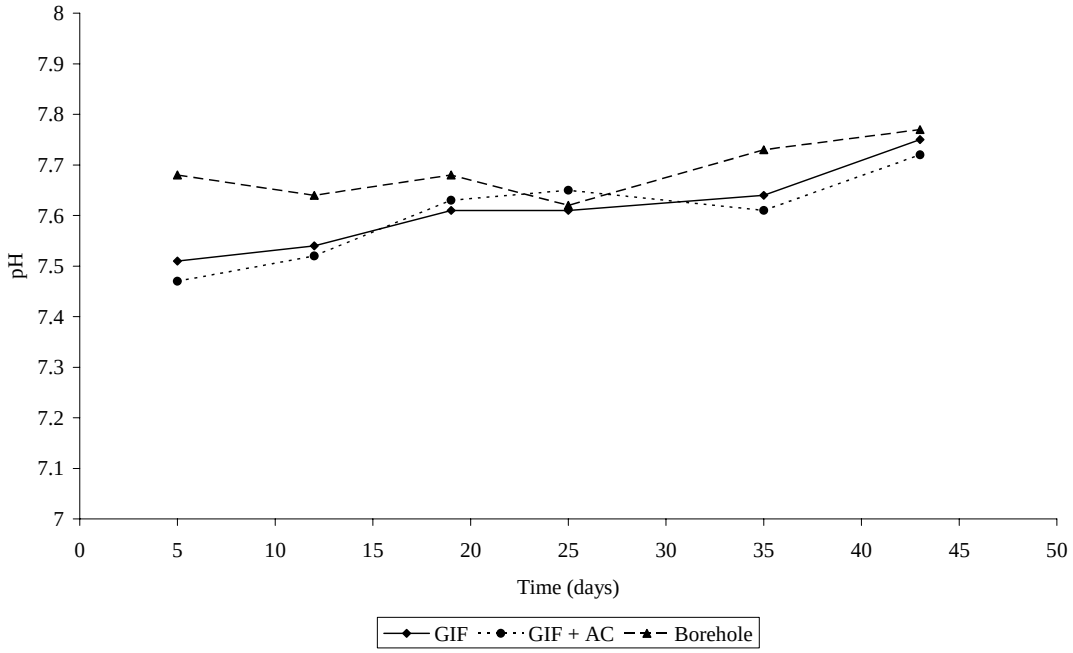

Fig 4.16. pH in the treatments during the experimental period.

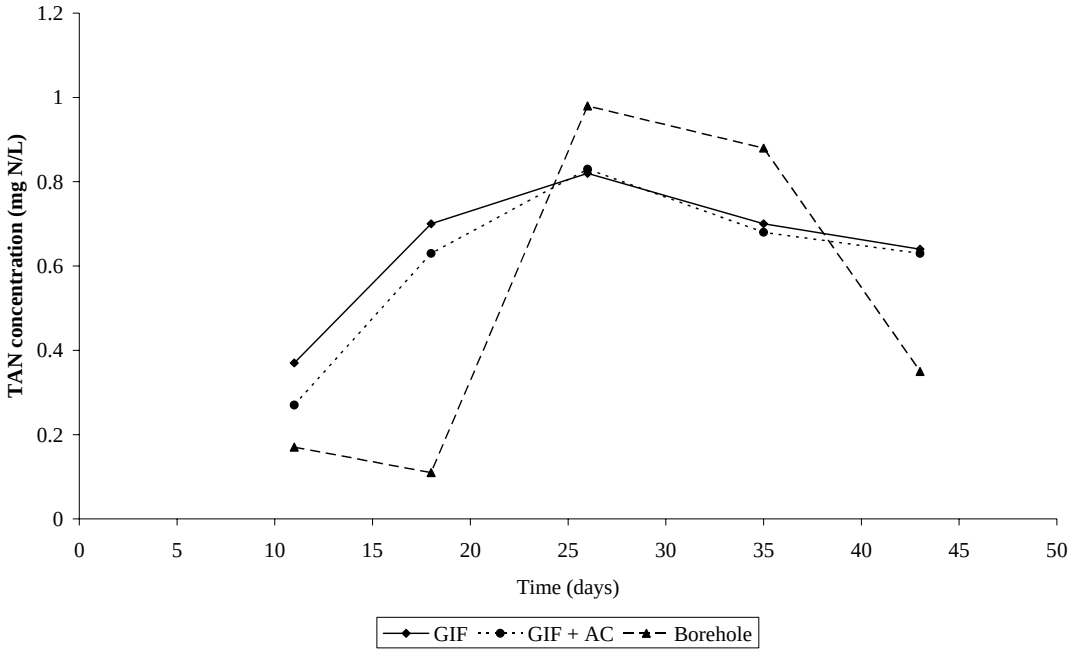

Fig 4.17. Total ammonia nitrogen (TAN) concentrations in the treatments during the experimental period.

3.3 Experiment at Seafarm: the effect of large turbot on growth of juvenile turbot in a commercial shallow raceway (Experiment 4)

3.3.1 Introduction

Our previous findings in Experiment 1, 2 and 3 suggested that it is very well possible that large turbot affect the growth and size variation of juvenile turbot when kept in the same water flow. This finding is relevant in the context of understanding potential interaction between different size classes stocked in the same recirculation system. The direct relevance of our findings on commercial turbot farming in recirculation systems depends on the type of system applied. In many RAS the effluent of the rearing tanks passes through the water treatment facility before it becomes the influent of other tanks, meaning that tanks are connected via the rearing water but always have the water treatment between tanks. The impact of large turbot on juvenile turbot was observed in experiments where the effluent of the tank with large turbot directly served as influent of the tanks with juveniles, without biological treatment in between. In other words, the relevance of the results for commercial RAS where tanks are separated by the water treatment system is yet to be established as for the potential removal of the agents responsible for the observed effects.
However, in RAS using shallow raceways consisting of more than one compartment, different size classes can be stocked in the same water flow without water treatment between stocks. In such cases it is very relevant to be aware of any potential interaction between size classes and effects on growth.
Therefore a fourth experiment was performed to verify our previous findings in shallow raceways on a commercial farming scale level. The experiment was performed by Seafarm BV, The Netherlands.

3.3.2 Materials and Methods

The experiment was performed in a shallow raceway system consisting of four stacked tanks. The total water flow was pumped to the top tank (Tank 1) from which it cascaded down through the other three tanks (Tank 2, 3 and 4). The water was then treated by drum filtration, a submerged biofilter and a protein skimmer before the water was returned to the top tank. Each tank had a surface area of 16 m^2 and the flow rate was 60 m^3/hr.

Tanks 1 and 3 were stocked with juvenile turbot. Tank 2 was stocked with large turbot, creating two experimental treatments: juvenile turbot stocked either upstream (Tank 1) and downstream (Tank 3) from large turbot (Table

4.6). Tank 4 was not stocked with turbot. The large turbot were stocked in a commercial density of 9 kg/m^2 at which production of growth inhibiting factors (GIF) was expected. The performance of the juvenile turbot in Tanks 1 (upstream) and 2 (downstream) was assessed to investigate the effect of large turbot on juvenile turbot.

Fish in the experimental tanks were fed by hand eight times per day to visually observed satiation using Skretting 5 mm turbot pellets. Care was taken to prevent feed spills to obtain accurate date on feed intake and to prevent unintended supply feed to tanks downstream. Feed administration per tank was recorded on a daily basis.

Oxygen (Hach Lange HQ 40d multi) and water temperature (Hach Lange HQ 40d multi) were measured daily. Total ammonia nitrogen (TAN) (cuvette test of Dr Lange) was measured weekly. Measurements were done downstream in tank 3.

Mean individual weight of the juvenile turbot in the experimental tanks was determined at the start (day 1), at midterm (day 49) and at the end of the experimental period (day 62). Specific growth rates (SGR) were calculated based on mean individual weights as:

Specific Growth Rate (%/day) $SGR = (\ln(W_t) - \ln(W_0)) * \frac{100}{T}$

where: W_0 = mean weight at day 1, W_t = mean weight at day t and T = number of feeding days.

Total feed intake (TFI) resulted from the cumulative daily feed intake per tank. Feed conversion rates (FCR) resulted from the quotient of total biomass increase and the feed intake:

Feed Conversion Rate (kg/kg) $$FCR = \frac{TFI}{(W_t - W_0)}$$

where: W_0 = mean weight at day 1, W_t = mean weight at day t and TFI = total feed intake (g).

Table 4.6. Overview of stocking of the experimental system.

	Tank 1	Tank 2	Tank 3
Treatment/function	Upstream of large turbot	GIF production	Downstream of large turbot
Initial weight (g)	57	977	57
Initial number	430	152	560
Initial stocking density (kg/m^2)	1.53	9.3	1.99

3.3.3 Results

Production parameters

The mortality, specific growth rate (SGR), total feed intake (TFI) and the feed conversion rate (FCR) for the experimental turbot stocked upstream and downstream from large turbot in a shallow raceway system are presented in Table 2. For each parameter the measurements of the first period (day 1 to 49), the second period (day 50 to 62) and the entire experimental period (day 1 to 62) are presented.
Mortality was very low with one fish in each of the experimental tanks. SGR and FCR yield very similar results for the two experimental tanks.

Table 4.7. Production parameters for the experimental turbot stocked upstream (Tank 1) and downstream (Tank 3) from large turbot.

	Tank 1 Upstream	**Tank 3 Downstream**
Mortality (n)	1	1
SGR day 1-49 (%/d)	1.48	1.43
SGR day 50-62 (%/d)	1.43	1.46
SGR 1-62 (%/d)	1.47	1.44
Total feed intake day 1-49 (kg)	22.31	27.65
Total feed intake day 50-62 (kg)	9.06	10.15
Total feed intake day 1-62 (kg)	31.37	37.80
FCR day 1-49	0.85	0.86

FCR day 50-62	0.88	0.75
FCR day 1-62	0.86	0.83

Water quality
Dissolved oxygen saturation ranged from 119 to 128% in Tank 1 and from 109 to 118% in Tank 3. On all sampling days the dissolved oxygen saturation was 10% lower than in Tank 1 (Fig. 4.18). TAN was measured in Tank 3 only and ranged from 0.32 to 0.51 mg N/L.

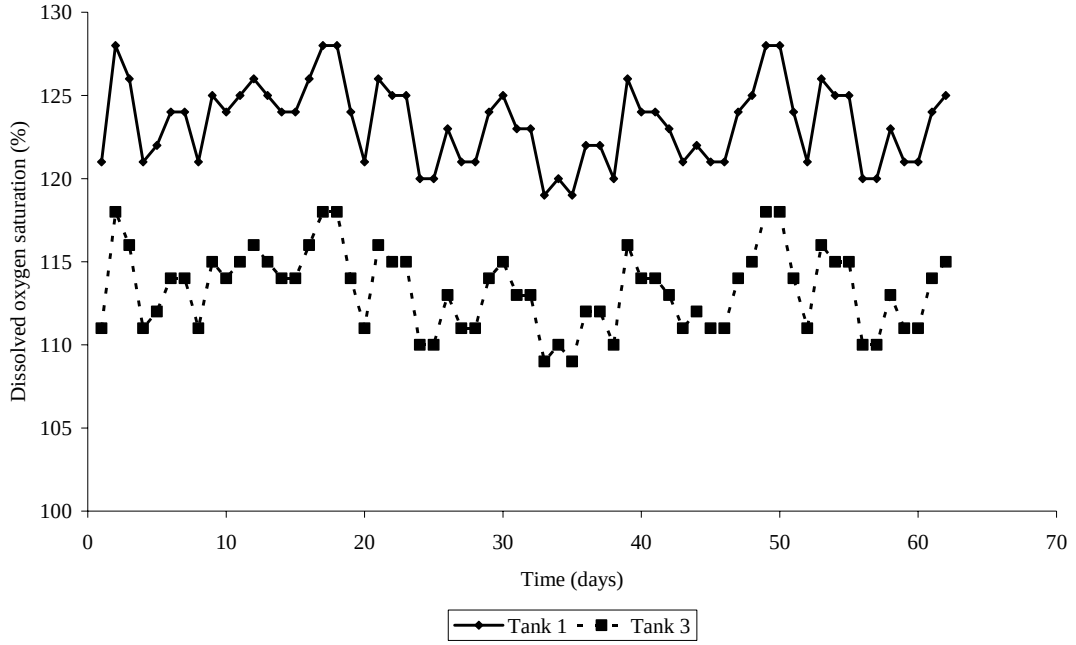

Fig. 4.18
Dissolved oxygen saturation (%) in Tank 1 (upstream from large turbot) and Tank 3 (downstream from large turbot) during the experimental period.

3.4 Discussion

Production parameters in Experiment 1, 2 and 3.
We hypothesized that growth of juvenile turbot is affected when their rearing water was previously in contact with conspecifics and that this effect is due to the excretion of growth inhibiting factors (GIF) to the water. Such a phenomenon is relevant in recirculating aquaculture systems in which different stocks share the rearing water. Locally produced substances can therefore, if not removed in the water treatment unit, eventually reach all other tanks and affect growth of other fish stocks despite the fact that stocks are physically separated in different tanks.
Experiment 1 indeed showed a large effect on specific growth rate (SGR) of water that originated from a tank stocked with large turbot at commercial density (Table 4.2). This result was however not reproduced in Experiment 2 and 3. Experiment 2 showed a similar effect on final mean weight at a 10% significance level, which did not result in a difference in SGR due to the difference in initial mean weight (Table 4.3) and Experiment 3 did not show any effect of treatments on SGR.
Growth performance in the Control treatment of Experiment 1 and all treatments in Experiment 3 was within normal ranges given the size of the fish and the water temperature. In Experiment 2, growth performance was poor in both treatments, indicating that growth was possibly affected by other factors, overruling the effect of the experimental treatments on growth.
Experiment 1 and 3 showed a significantly larger increase of size variability during the experimental periods among the fish receiving water from the GIF production tank (Table 4.2 and 4.4), which is in agreement with earlier findings for *Prochilodus lineatus*, which showed an increase growth variability due to exposure to chemical factors released by conspecifics (Barbosa and Volpato, 2007), while no effect on growth rate was observed for

this species, which is in agreement with our findings in Experiment 3. In Experiment 1 the larger increase of CV of the fish that received the GIF production tank effluent was possible also caused by the higher initial CV and not only the treatment.
A very interesting finding of Experiment 3 is that the large increase in size variation as a result of exposure to water that was previously in contact with conspecifics, seems to be counteracted by treating the water with activated carbon: the GIF+activated carbon treatment yielded the same low increase in size variation during the experiments as the bore hole water treatment (Table 4.4). This suggests that the effect on size variation is caused by chemical factors originating from the GIF production tank which are effectively retained on the activated carbon.

Water quality in Experiment 1, 2 and 3
Water quality and flow rates were monitored in all experiments to establish whether or not these were confounding factors in the experiments. Water temperature showed minimal differences among treatments in all experiments (Fig. 4.4, 4.9 and 4.14) and can therefore be excluded as confounding factor.
Oxygen saturation levels show day to day variations and differences among treatments in all experiments (Fig. 4.5, 4.10 and 4.15), which is due to the difficulty to regulate the supply of pure oxygen with diffusers to individual tanks. Juvenile turbot are reported to display depressed growth at oxygen levels below 5.0 mg/L (~ 60% saturation) (Pichavant *et al.*, 2000), whereas oxygen saturation levels of 147% and 223% did not promote growth of juvenile turbot compared to normoxic conditions within approximately the same size range as the current experiment (Person-LeRuyet *et al.*, 2002). In these experiments overall oxygen saturations ranged from 115 to 220% and it is therefore unlikely that differences in oxygen saturation was a confounding factor in any of the current experiments.
Experimental treatments potentially differed in carbon dioxide levels as for the carbon dioxide production by the turbot in the GIF production tanks and the absence of a carbon dioxide stripping device between the GIF production tank and the recipient experimental tanks, potentially resulting in reduced growth due to higher carbon dioxide levels. Carbon dioxide levels were not measured in the present experiments but higher levels can be expected to be reflected in lower pH.
In Experiment 1 pH was very similar in both treatments (Fig. 4.6), suggesting similar carbon dioxide levels and excluding carbon dioxide as cause for the observed difference growth between treatments. In Experiments 2 and 3 the pH in the treatments receiving water from the GIF production tank was slightly lower than the pH in the bore hole water treatments (Fig 4.11 and 4.16), possibly caused by higher carbon dioxide levels. We expected lower growth in the in the treatments receiving water from the GIF production tank. However, no differences in growth were observed among treatments in both Experiment 2 and 3. Therefore potential differences in carbon dioxide levels (if any) apparently did not affect growth nor acted as confounding factor.
In parallel with carbon dioxide, experimental treatments potentially differed in total ammonia nitrogen (TAN) levels as for the ammonia production by the turbot in the GIF production tanks. To exclude different TAN levels as confounding factor in the experiments, ammonium chloride was added to the bore hole water treatments in all three experiments. As is clear from the TAN levels in treatments, we did not succeed to fully equalize TAN levels. It is however unlikely that differences in TAN levels caused differences in growth among the experimental fish as the highest observed TAN concentration in the current experiments of 1.30 mg N/L (Fig 4.12) concurs with a unionized ammonia nitrogen level of approximately 0.01 mg N/L (Creswell, 1993) which is ten times lower than the lowest observed effect concentration for growth of 104g turbot (0.10 mg unionized ammonia nitrogen) (Person-Le Ruyet, *et al.*, 1997).
Flow rates were equal between treatments in Experiment 1 and 2 (Table 4.2 and 4.3) and can therefore be excluded as confounding factors. In Experiment 3 however significantly different flow rates existed among treatments, with the lowest flow rates in the bore hole water treatment. Increased flow rates promote the growth of juvenile turbot (Schram *et al.* in press) and the expected negative effect on growth of the GIF treatment, based on the results of Experiment 1, was possibly overruled by the higher flow rate in the GIF treatment compared to the borehole water treatment. On the other hand, flow rates were highest in the GIF + activated carbon treatment, which did not result in higher growth compared to the other treatments. In addition, the effect of increased flow rate on water quality is proportional to the production of metabolites in the tank and thereby the biomass in the tank (Schram *et al.* in press). Given the low biomass and stocking density in the experimental tanks, the effect of increased flow rates on water quality is small as is reflected by the equal TAN levels in the GIF and GIF + activated carbon treatments (Fig. 4.17) despite the difference in flow rate (Fig. 4.13) in the second half of the experimental period. In this experiment the effect on growth of different flow rates among treatments is therefore most likely minimal.

All in all we conclude that observed water quality differences among experimental treatments were unlikely to cause differences in the growth of experimental fish in Experiment 1, 2 and 3.

Production parameters in Experiment 4
No effect on specific growth rate (SGR) and feed conversion rate (FCR) was observed in the experiment performed in a commercial shallow raceway setting, which is in contrast with the results of Experiment 1 and 3. It is possible that this observation is attributable to the high water velocity applied in the experimental (and also commercial) shallow raceways at Seafarm.High water velocity reduces the levels to which compounds produced in the tank (such as GIF) can accumulate at the tank level, preventing in-tank accumulation to effective concentrations. It should be noted that GIF accumulation at system level takes place independent from water velocity in the tanks as this is a function of removal in the water treatment unit and the production in the system, meaning that farms applying high water velocities in their tanks can still be affected by growth retardation due to GIF.
We consider it unlikely that water quality affected growth in the experimental tanks. Water quality in shallow raceways is modeled at best with a plug flow approach, as opposed to circular tanks which are considered completely mixed systems. This means that water quality gradient exists along the length of the raceways as a function of production and consumption of compounds by the fishes. Indeed dissolved oxygen saturation was lower in Tank 3 compared to Tank 1, most likely due to oxygen consumption by the fishes or oxygen losses via the water-air interface, resulting in decreasing oxygen levels along the raceway. It is however unlikely that these differences affected growth as oxygen saturation levels of 147% and 223% were reported not to promote growth of juvenile turbot compared to normoxic conditions within approximately the same size range as the current experiment (Person-LeRuyet *et al.*, 2002). Following the plug flow approach it is likely that ammonia levels increased along the raceways as for the ammonia production by the fishes, suggesting that TAN was lower in Tank 1 compared to Tank 3. However, it is unlikely that TAN differences (if any) caused growth differences between tanks as TAN levels in Tank 3 where already within safe limits for turbot growth (Person-Le Ruyet, *et al.*, 1997).

3.5 Conclusions

The results of the present experiments are inconclusive as different effects of preconditioning of rearing water by conspecifics on growth performance of juvenile turbot were found.

The absence of an effect on growth in Experiment 2 is likely attributable to confounding factors overruling experimental treatments, as reflected in the overall poor growth.

The absence of an effect on growth in Experiment 4 is most likely attributable to the high water velocity, preventing GIF accumulation at tank level. Commercial turbot rearing in shallow raceways therefore less likely to be affected by growth retardation due chemical signals released by conspecifics in the same tank.

From Experiment 1 and 3 it is clear that preconditioning of rearing water by large turbot can affect the growth of juvenile turbot. In Experiment 1 and we observed an increased size variation and Experiment 1 we also observed reduced SGR due to preconditioning of the rearing water.

The observed effects are possibly due dissolved substances originating from the GIF production tank, possibly chemical signals released by the large turbot, as the effect on size variation of juvenile turbot was effectively counteracted by pretreatment with activated carbon.

4 The presence of GIF at a commercial turbot farm (Task 2.4)

4.1 Introduction

In this study we aimed to demonstrate the presence of GIF at commercial farms using the same methodology as used for the demonstration of GIF production by turbot, where culture water of commercial turbot farms was extracted and extracts were tested for growth inhibiting properties in juvenile growth trails (chapter 2). Given the inability to demonstrate GIF production by turbot following this methodology, a different approach was chosen for the demonstration of GIF presence at commercial farms.
The presence of (heavy) metals was investigated a commercial turbot farm to get insight into these compounds as potential GIF. In addition, juvenile growth trials to demonstrate GIF activity were performed using test solutions prepared from raw culture water originating from a commercial turbot farm, thereby avoiding uncertainties on the presence of GIF in test solutions related to GIF collection and extraction procedures as discussed in Chapter 2.

4.2 Water quality at GROVISCO

4.2.1 Introduction

As the development of a bio-assay for GIF activity of water samples or extracts was delayed (Chapter 2), it was decided to assess water quality at a commercial turbot farm beyond the routinely measured parameters based on a single measurement per day such as oxygen and ammonia to find possible causes for growth retardation. Dissolved metals can have various effects on fish health and performance, sometimes at relatively low levels. Considering the potential metal sources in aquaculture systems including feed, system renewal water and hard ware of the system itself, it is possible that metals accumulate in RAS to levels at which fish are affected. Accumulation of metals in RAS for turbot has not been addressed previously. In this study we intended to make a quick scan of the levels of eight metals in the culture water of a commercial RAS to find out if metals are a potential threat for fish health and performance or not.
Ammonia production fluctuates during the day as a result of the feeding pattern over the day (Bovendeur *et al.*, 1987). This means that a single daily measurement during the day may not be representative for the exposure of the fish to ammonia, nitrite and nitrate. Therefore the concentrations of ammonia, nitrite and nitrate were measured with one hour intervals over three 24 hours periods.

4.2.2 Materials and methods

Metals
A quick scan for the presence of metals in the culture water of commercial turbot grower GROVISCO, The Netherlands to get insight in accumulation of metals in the culture water of a RAS. Metals in culture water can originate from feed, system renewal water or hard ware of the system itself.
A single water sample was taken on the 15th of may 2008 from the in and outflow of a culture tank and the borehole water. Water samples were conserved by addition on nitric acid (1ml/L). Water samples were analyzed by atomic adsorption spectrometry (AAS) for free arsenic (As), cadmium (Cd), chrome (Cr), copper (Cu), mercury (Hg), lead (Pb), nickel (Ni), zinc (Zn) and aluminum (Al) by OMEGAM laboratories, Amsterdam, The Netherlands.

Nitrogen compounds and other parameters
Water samples were collected during three sampling days with a seven days interval between sampling days. Samples for ammonium, nitrite and nitrate (Hach DR/890 Colorimeter for N) were collected from the in- and outflow of a tank using two auto samplers (Teledyne ISCO 3700 Portable sampler), collecting a 500ml water sample per hour during 24 hours. During each of the three 24 hour sampling periods pH, dissolved oxygen and temperature were measured at intervals of 15 minutes (Hach Lange HQ40d).
Water samples were cooled during sampling, transport and storage prior to analyses.

4.2.3 Results

Metals

Results for the analyses for eight different metals in the renewal water (bore hole) and the in and outflowing water of a tank in a commercial RAS for turbot are shown in Table 5.1. As a reference the legal thresholds for drinking water and the thresholds for trout culture are included. The in and out flowing water of the fish tanks yield the same results and are slightly higher than the renewal water of the RAS.

Table 5.1 Concentrations (µL) of 8 metals in the renewal water (bore hole) and the in and outflow of a tank in a commercial RAS for turbot and the threshold levels for drinking water and trout culture.

Metal	**Bore hole**	**Tank IN**	**Tank OUT**	**Thresholds drinking water***	**Thresholds trout***
Arsenic (As)	< 2	4	4	10	50
Cadmium (Cd)	< 0.1	0.2	0.2	5	6
Chrome (Cr)	< 1	1	1	50	30
Copper (Cu)	2	5	5	2000	300
Mercury (Hg)	0.02	0.03	0.03	1	20
Lead (Pb)	2	< 1	1	10	20
Nickel (Ni)	1	4	4	20	50
Zinc (Zn)	15	27	28		5
Aluminum (Al)	< 50	< 50	< 50		100

* Waterleidingwet (national drinking water law), The Netherlands

** (Post, 1987)

Nitrogen compounds, oxygen, pH and temperature

Results of the water quality measurements are shown in Table 5.2 and Fig. 5.1, 5.2, 5.3 and 5.4. TAN showed some variation during 24 hours periods, but high ammonia peaks were not observed during any of the sampling days (Fig. 5.1 A, 5.2A, 5.3A). The highest TAN levels of 11 mg N/L were measured on sampling day 1, whereas TAN was below the detection limit on sampling day 3, indicating that large differences in TAN levels exist between days.

Nitrate levels varied during 24 hours periods. Concentration differences up to 80 mg N/L (Fig. 5.2 B) were observed on sampling day 2.

Nitrite was lower than 1 mg N/L at all three sampling days (Fig. 5.1C, 5.2C, 5.3C).

pH ranged from 6.05 to 6.95 on the three samplings days and showed a variation during 24 hour periods of approximately 0.3 for all the three sampling days (Fig. 5.1D, Fig 5.2D, Fig. 5.3D). Oxygen levels ranged from 8 to 11 mg/L on sampling day 1 and 2 and showed a diurnal pattern with higher levels at night (Fig. 5.1D, Fig 5.2D). At sampling day 3 oxygen was much more stable around 10 mg/L during the whole 24 hours period (Fig. 5.3D). Water temperature shows variation during and between sampling days (Fig. 5.4).

4.2.4 Discussion

Metals

This study was intended as a quick scan to get insight in accumulation of metals in RAS to potentially problematic levels. Concentrations of measured metals were higher in the tank in and effluent than in the renewal water source of the RAS, suggesting accumulation of metals in RAS. Bioavailability and toxicity of waterborne metals is complex and closely related to other water quality parameters. For example dissociation of metal salts is pH dependent and zinc in estuarine and marine waters, is known to be adsorbed to suspended solids, rendering it less bio available and less toxic than free zinc ion (Neff, 2002). Clearly more research is needed to fully understand the origin and fate of metals within RAS, but this does not appear to be very relevant in the context of growth retardation in RAS. The most important observation of this study is that tank levels of metals are far lower than legally tolerated levels of these metals in drinking water (Table 5.1) and except for zinc also far lower than threshold concentrations for trout culture (Table 5.1). Based on this we conclude that the metals measured in this study are unlikely to be a threat for fish health or be involved in growth retardation. Zinc demands more attention. It is hard to say whether or not the measured zinc level posses a threat for turbot as threshold levels

are lacking. Toxicity of waterborne metals to fish is related to the presence of other cat-ions as for uptake across the gills all ions compete for the same sites. Toxic levels of waterborne metals are therefore lower in ion poor and soft water (Alsop and Wood, 1999) and less of a problem in seawater. This suggests that toxic metal levels are higher for turbot than for trout as for the ion-rich environment that marine waters provide. Taking into account that a decrease of total hardness by ten times increased the toxicity of zinc in rainbow trout by ten times (Bradley and Sprague, 1985 in Alsop and Wood, 1999), the total hardness of hard fresh water of 200-250 mg $CaCO_3$/L and the total hardness of full strength seawater of 6600 mg $CaCO_3$/L, it seems unlikely that a zinc concentration of ca. 30mg/L posses a threat to turbot when the threshold for rainbow trout is 5 mg/L.

Nitrogen compounds, oxygen, water temperature and pH
None of the nitrogen compounds was observed to peak to levels at which turbot growth is likely to be affected, although thresholds for nitrate are unknown. The highest observed TAN levels of 11 mg N/L concur with toxic unionized ammonia levels of 0.02 mg N/L given the ambient pH and water temperature, much lower than the NOEC for juvenile turbot of 0.10 mg unionized ammonia nitrogen (Person-Le Ruyet, *et al.*, 1997).
Daily variation in TAN, pH and oxygen levels in the fish are most likely related to feeding and activity of turbot (Bovendeur *et al.*, 1987). Turbot were fed during the day, resulting in higher oxygen consumption, TAN production and CO_2 production affecting pH, compared to night time periods. Daily variation in water temperature is most likely a function of weather conditions.
The variation observed in water quality parameters during and between sampling days measured is all within safe limits for turbot. It is unlikely that peak levels of water quality parameters measured acted as GIF on the sampling days. However, it has also been observed in this project that water quality differences are reflected in blood chemistry parameters of turbot (Deliverable 10), indicating that water quality changes lead to physiological adaption of the fish. Although within safe limits, it is likely that such adaptations to changes in water quality are energy demanding at the costs of other processes including growth. Given the frequency and amplitude of water quality changes, we expect that the effects on growth can be significant. Therefore future research should be directed towards the effects of short term water quality changes on growth of turbot. Also water quality management at farm level, preventing large fluctuations in water quality deserves more attention, focusing on costs and benefits of investments in water quality control and improved growth.

4.2.5 Conclusions

It is unlikely that accumulation of arsenic (As), cadmium (Cd), chrome (Cr), copper (Cu), mercury (Hg), lead (Pb), nickel (Ni), zinc (Zn) and aluminum (Al) in RAS is related to growth retardation.
Water quality parameters did not exceed safe limits during 24-hours sampling periods. However, large variations of water quality parameters on sampling days and within sampling days were observed and the effect of such fluctuations, although within safe limits, on physiology and growth of turbot should be investigated.

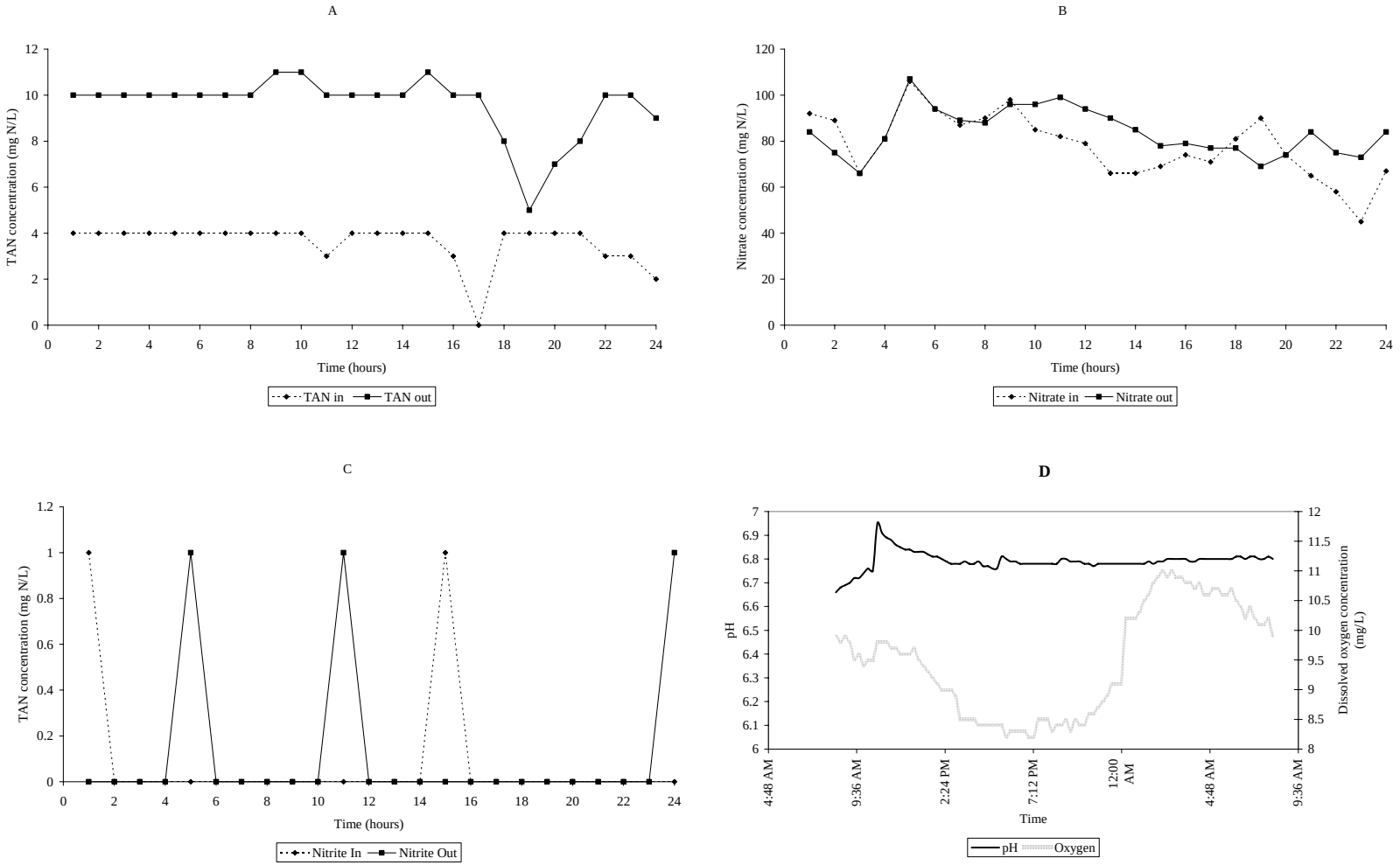

Fig. 5.1 Sampling day 1: TAN (A), nitrate (B) and nitrite (C) concentrations (mg N/L) in the tank influent and effluent and pH and dissolved oxygen (D) in the tank during 24 hours.

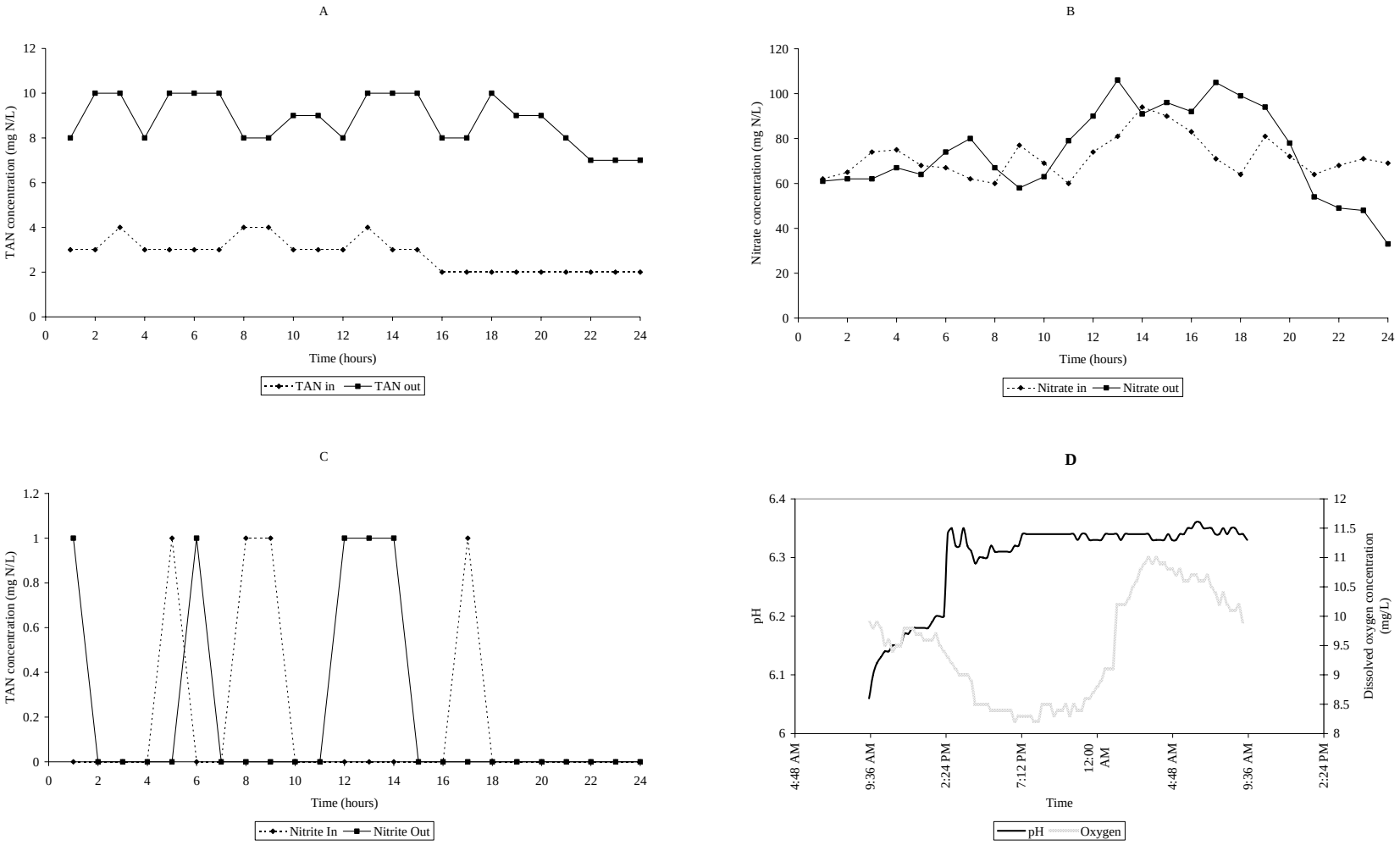

Fig. 5.2 Sampling day 2: TAN (A), nitrate (B) and nitrite (C) concentrations (mg N/L) in the tank influent and effluent and pH and dissolved oxygen (D) in the tank during 24 hours.

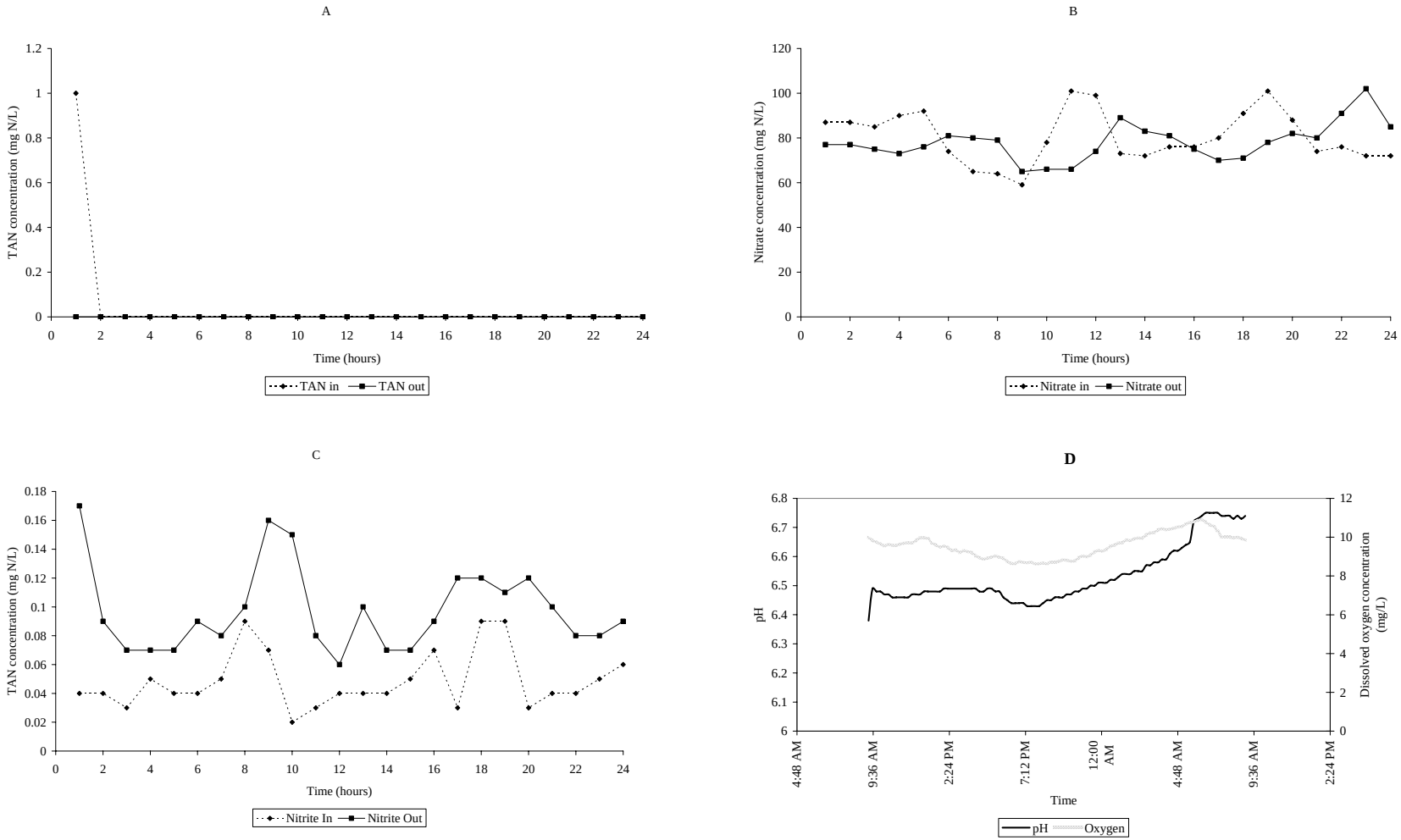

Fig. 5.3 Sampling day 3: TAN (A), nitrate (B) and nitrite (C) concentrations (mg N/L) in the tank influent and effluent and pH and dissolved oxygen (D) in the tank during 24 hours.

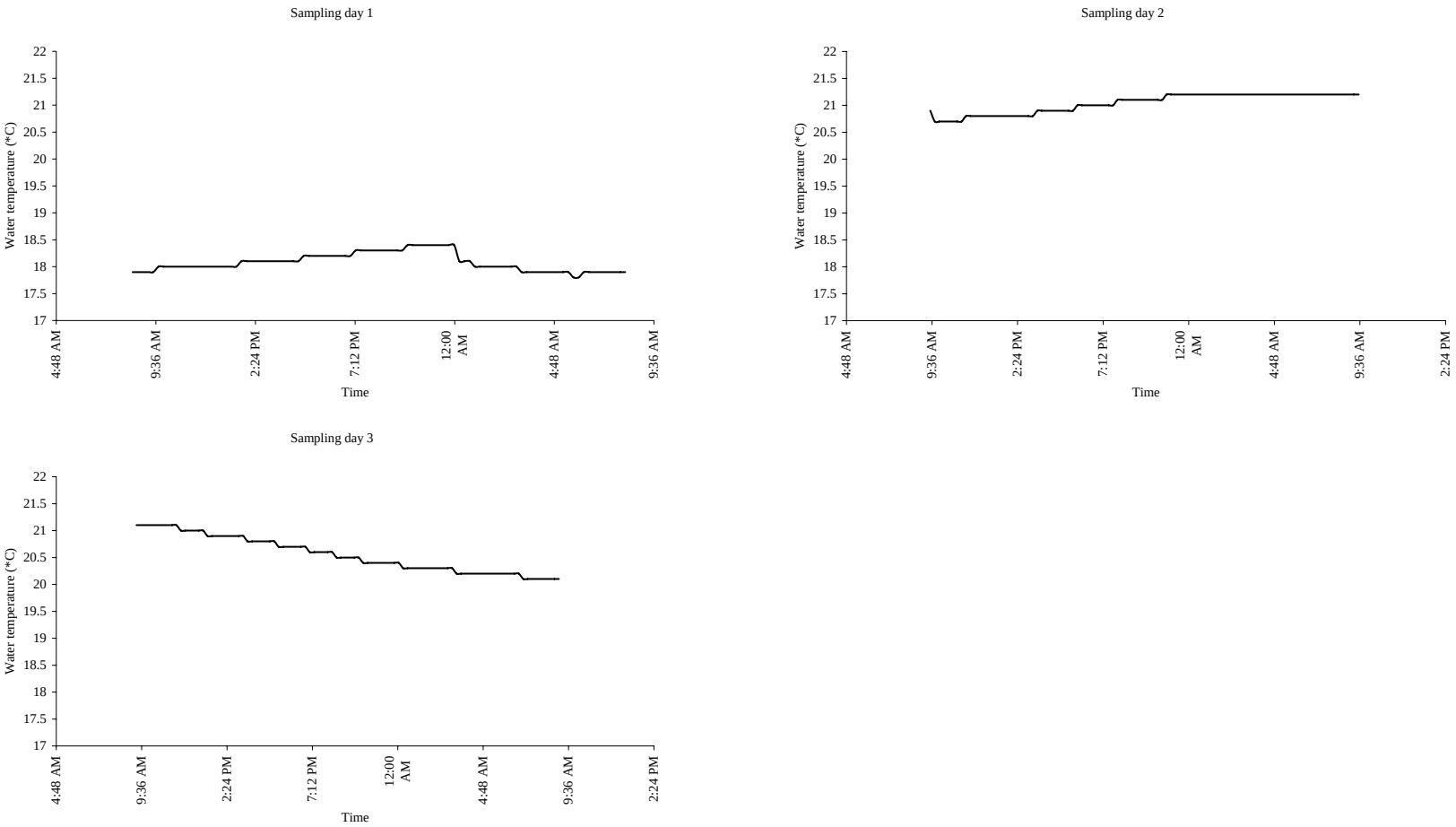

Fig. 5.4 Water temperature on sampling day 1, 2 and 3 in the tank during 24 hours.

4.3 Growth inhibiting properties of farm water Experiment 1

4.3.1 Introduction

The goal of the present experiment was to investigate the presence of GIF in commercial turbot farm water by testing the growth inhibiting properties in a juvenile growth trail

4.3.2 Materials & methods

Treatments
The effect of culture water originating from a commercial turbot farm (farm water) on growth of juvenile turbot was tested. The test solutions were clean seawater (positive control), farm water and a 50-50 mixture of farm water and clean seawater, with three replicates per test solution (Table 5.1).

Table 5.1 Composition of test solutions

Test solution	Farm water content (%)	Clean seawater content (%)
Clean seawater	0	100
Mixture	50	50
Farm water	100	0

Farm water originated from Groente- en Viskwekerij Cornelisse BV, Stavenisse, The Netherlands and was taken from a 35 m^2 circular tank stocked with 2300 kg turbot with a mean weight of approximately 1kg. Upon collection at the farm, the farm water was lead over a 0.2 µm cartridge filter to remove suspended solids. Clean seawater originated from the Oosterschelde estuary, The Netherlands and was filtered (0.2 µm cartridge filter) and UV treated (Dryden Aqua sterilizer IP64) before use. Salinity (25ppt), total ammonia nitrogen (0.95 mg N/L) and nitrite (0.17mg N /L) levels were equalized between the test solution stocks by adding sea salt (Instant ocean) to the farm water and ammonium chloride and sodium nitrite (Sigma Aldrich) to the clean seawater.
The farm water and clean seawater stocks were prepared in advance and stored aerated at 4°C in 120L polyethylene vessels. An aerated stock of 20L for each test solution was kept a 20L glass bottle in the experimental room to renew the test solutions in the experimental jars. These stocks were replenished at least 12 hours before use to allow the test solutions to reach ambient temperature. The stock of the 50-50 mixture of farm water and clean seawater was prepared by mixing 10L farm water and 10L clean seawater.

Set up
Ninety juvenile turbot of 3.65 (0.37) g (mean + SD) were randomly divided over nine 700ml glass jars and placed in a temperature controlled room. Fishes were allowed to acclimatize to the experimental conditions for 5 days, during which all units were filled and renewed with clean seawater. At the start (day 1) of the experimental period each jar was emptied, fishes were weighed as group to determine initial weight and returned to the jar which had been refilled with a randomly assigned test solution.
The experimental system was operated as a semi-static renewal system: 80% of the total test solution volume was replaced twice daily (morning and late afternoon). During draining of the water, uneaten feed and feces were removed with disposable pipets.
Fishes were fed by hand at a level of 5% BW/d. Daily feed portions per group were calculated based on the initial weight and an assumed specific growth rate of 5%/d. During week days daily feed portions were split in four portions and fed during four feeding periods. During weekends daily portions were split in two portions and fed during two feeding periods. First feeding was done 30 minutes after water renewal and last feeding per day was done before the last water renewal. Fish were not fed on the first experimental day and the last experimental day. The used food corresponded to diet Tetramin flocks with 47% protein and 10% fat. Flocks were crushed to obtain small sized flocks before feeding.

Measurements and sampling
Temperature and oxygen levels were measured daily in each jar (Hach Lange HQ 40D). pH (Hach Lange HQ 40D) was measured four times in each during the experiment. Total ammonia nitrogen, nitrite nitrogen and nitrate-nitrogen (Hach DR/890 Colorimeter for N) were measured at day 14 of the experimental period in each jar before the first water renewal.

At the end (day 14) of the experimental period, fishes were group weighed (Mettler AE 163) and counted per jar to determine final mean weight. Specific growth rates per jar were calculated based on initial and final mean individual weight.
Specific growth rate and water quality parameters were tested for significant differences between treatments using One-way ANOVA. The effect of farm water content of test solutions on specific growth rate was analyzed by simple linear regression.

4.3.3 Results

Fish performance
Initial mean weight and specific growth rate were equal among the treatments, whereas final mean weight showed a trend towards a lower final weight for the Farm water treatment (One way ANOVA, Table 5.2). Linear regression shows a significant decrease of the of specific growth rate with increasing farm water content of the test solutions (Fig 5.1). Three fishes died during the experiment out of the total population of 90 fishes: one in two Clean seawater replicates and one in a Farm water replicate. Mortality rates did not differ between treatments (One way ANOVA, Table 5.2).

Table 5.2. Mean (n=3) values per treatment for initial mean weight (Wo), final mean weight (Wt), specific growth rate (SGR) and mortality rate (MR). P-values result from One way ANOVA.

Parameter	Unit	Clean seawater	Mixture	Farm water	P-value
Initial weight	(g)	3.72	3.70	3.54	0.59
Final weight	(g)	4.55	4.52	4.01	0.09
SGR	(%)	2.27	1.67	1.34	0.14
Mortality	(%)	6.7	0	3.3	0.30

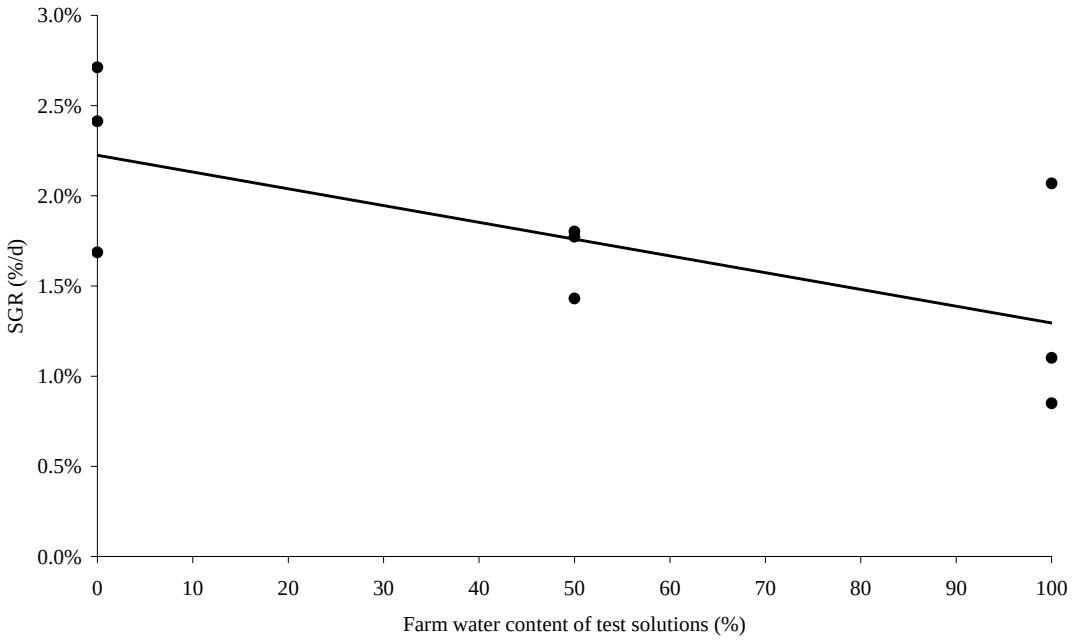

*Fig. 5.1 Specific growth rate (SGR) in relation to the farm water content of the test solutions (X). SGR = 2.23 – 0.0093*X (Simple linear regression, P = 0.04, r^2 = 0.46).*

Water quality
Mean values per treatment are presented in Table 5.3. Temperature, oxygen and total ammonia nitrogen (TAN) levels were equal for all treatments. There is a trend towards higher nitrite levels in the farm water and farm water – clean seawater mixture compared to the clean seawater treatment. Nitrate levels are different between all treatments. The pH in the farm water treatment is lower compared to the other treatments.

Table 5.3 Mean values (n = 3) per experimental treatment for water temperature, oxygen saturation, TAN, nitrite nitrogen, nitrate nitrogen levels and pH. Values with different letters across rows are significantly different at the 5% level (One-way ANOVA).

WQ parameter	Unit	Clean seawater	50-50 mixture	Farm water	P-value	LSD
Temperature		16.8	16.8	16.8	0.19	-
Oxygen saturation	(%)	93	94	90	0.29	
TAN	(mg N/L)	4.3	4.7	5.0	0.58	
Nitrite	(mg N/L)	0.13	1.17	1.00	0.06	0.88
Nitrate	(mg N/L)	8^{a}	69^{b}	144^{c}	<0.001	35
pH		7.75^{a}	7.60^{a}	7.36^{b}	0.004	0.18

4.3.4 Discussion

This experiment demonstrates growth inhibiting properties of farm water originating from a commercial turbot farm applying a recirculation system (Fig 5.1), which is in agreement with growth retardation observed at commercial turbot farms with recirculating water. The effect is however not very strong as no differences in mean specific growth rates were observed (Table 5.2). Maybe a longer experimental period would have allowed for growth differences to develop into more marked and significant differences. In addition the overall growth was rather low, possibly due to suboptimal experimental conditions obscuring growth inhibiting properties of the test solutions.

Next to the potentially different levels of GIF in the test solutions due to different farm water content, other differences in water quality also existed in this experiment which may contribute to the depression of growth (Table 5.3). Nitrite was lower in the clean seawater treatment (be it with a p value of 0.06) compared to farm water and the mixture treatments. Nitrate significantly increased with increasing farm water content of the test solutions. Since thresholds for growth of juvenile turbot are unknown for nitrite and nitrate, it is difficult to conclude to what extend these water quality parameters contributed to the observed effects.

Another possible reason for the observed effect on growth is the sudden transfer from clean seawater during the acclimatization period to the test solutions at the start of the trail. The stress response to this sudden change to a different water quality, may cause temporarily growth reduction, affecting the final weight due to the relative short duration of the trail.

This leads to the recommendation to repeat this experiment with an improved set up. The improvements should include acclimatization of the experimental fish in their test solutions to prevent a stress response due sudden water quality changes at the start of the experiment. The experimental period should be extended to allow growth differences to develop into significant differences. The levels of nitrite and nitrate in the test solutions should also be equalized to exclude these water quality parameters as growth inhibiting factors.

4.3.5 Conclusion

In conclusion, the present experiment demonstrates growth inhibiting properties of water from a commercial turbot farm with recirculating water. It is however not possible to attribute this effect to the presence of GIF or any other single factor. This experiment should be repeated in an improved set up as discussed.

4.4 Growth inhibiting properties of farm water Experiment 2

4.4.1 Introduction

Our previous experiment aiming to demonstrate growth inhibiting properties of culture water from a commercial turbot farm with recirculating water, indeed showed this effect but was inconclusive concerning the causes. We concluded that the experiment should be repeated with an improved set up. In the current experiment the experimental fishes were acclimatized to the experimental conditions in their respective test solutions for all treatments. The levels of nitrite and nitrate were equalized in the test solutions. The experimental period was extended to allow for more growth and thereby potentially larger differences among treatments. The husbandry

systems was improved to improve the overall growth: aeration tubes were suspended above the bottom of the experimental jars, allowing for sedimentation of solids wastes and subsequently better removal when of solids during water renewal. The range of farm water content of the test solutions was extend to four to obtain a better correlation with fish performance.
An extra treatment was introduced to test the effect of activated carbon treatment on growth inhibiting properties of farm water.

4.4.2 Materials and methods

Treatments
The effect of culture water originating from a commercial turbot farm applying a recirculation system (farm water) on growth of juvenile turbot was tested. The experiment consisted of five treatments (test solutions) with three replicates per treatment, yielding a total of 15 experimental units. Test solutions were artificial seawater (positive control), farm water, two mixtures of farm water and artificial seawater and farm water pretreated with activated carbon (Table 5.4).

Table 5.4 Composition of test solutions

Treatment	Test solution	Farm water content (%)	Artificial seawater content (%)	Treatment with activated carbon
A	Artificial seawater	0	100	No
B	100% farm water	100	0	No
C	66% farm water	66	33	No
D	33% farm water	33	66	No
E	AC-farm water	100	0	Yes

Stocks of test solutions A, B and E were prepared prior to the experiment. Farm water originated from Groente-en Viskwekerij Cornelisse BV, Stavenisse, The Netherlands and was taken from 35 m^2 circular tank stocked with 2300 kg turbot with a mean weight of approximately 1kg. Upon collection at the farm, the farm water was lead over a 2 µm cartridge filter to remove suspended solids and divided over three 120L polyethylene vessels and stored aerated at 4°C one week before the start of the acclimatization period. Two vessel of farm water were used to prepare test solutions B, C and D. Above the water level of the third vessel a column containing 25 kg activated carbon (NORIT GAC 830plus) was installed. The farm water was pumped over of the column and flowing back into the vessel during one week at a flow rate of 1000 L/hr. Mixing of the water was ensured by strong aeration.
Artificial seawater was prepared one week before the start of the acclimatization period from demineralized water and sea salt (Instant ocean) at a salinity of 33‰, equal to the farm water. The artificial seawater was stored aerated in two 120L polyethylene vessels at 4°C.
The nitrite (mg N /L) and nitrate levels in stocks of artificial seawater and activated carbon treated farm were increased to the levels in the farm water by adding, sodium nitrate and sodium nitrite (Sigma Aldrich). Given the low level of ammonia nitrogen in the farm water (0.9 mg N/L) and the expected production and accumulation of ammonia in the experimental units, we decided not to equalize ammonia levels between the test solution stocks.
An aerated stock of 20L for each test solution was kept in five 20L glass bottles in the experimental room for the daily renewal of the test solutions in the experimental jars. These stocks were replenished at least 12 hours before use to allow the test solutions to reach ambient temperature. The stocks of the mixtures of farm water and artificial seawater (treatment C and D) were prepared by mixing 10L farm water and 10L clean seawater upon refill of the 20L stocks.

Set up
Each of fifteen 700 ml glass jars was filled with 650ml randomly assigned test solution, resulting in three jars for each of the five test solutions. Hundred and fifty juvenile turbot of 558 (26) mg (mean + SD) and 43 days post hatch originating from France Turbot, Noirmoutier, France were randomly divided over the fifteen glass jars and placed in a temperature controlled room with a photoperiod of 12L:12D. Fishes were allowed to acclimatize to the experimental conditions for 7 days. During the acclimatization and experimental period, the experimental system was operated as a semi-static renewal system: 80% of the total test solution volume was replaced twice

daily (morning and late afternoon). Draining of the water was sufficient to remove uneaten feed and feces from the jars. Each jar was equipped with an aeration tube to secure sufficient supply of oxygen. The aeration tube was suspended at approximately 1/3 of the water column above the bottom of the jars to create a bottom area with minimal turbulence to allow for sedimentation of solid wastes and to provide a resting area for the fishes. Fishes were fed by hand twice daily prior to water renewal at a rate of 4%/d with Algonorse 3 (EWOS).

Measurements and sampling
Temperature and pH (Hach Lange HQ 40D) were measured on 14 experimental days, oxygen levels (Hach Lange HQ 40D) on 20 experimental days in each jar. Total ammonia nitrogen and nitrite nitrogen were measured in each jar on day 12, 19 and 22 of the experimental period, nitrate nitrogen on day 19 and 22 (Hach DR/890 Colorimeter for N). Salinity (Hach Lange HQ 40D) was measured in each jar on day 22 of the experimental period.
At the start (day 1) and end (day 22) of the experimental period, fishes were weighed (Mettler AE 163) individually and counted per jar to determine initial and final mean weight. Specific growth rates per jar were calculated based on initial and final mean individual weight.
Specific growth rate and coefficients of variation were tested for significant differences between treatments using One-way ANOVA. Water quality parameters were tested for differences between treatments using repeated measurements ANOVA. The effect of farm water content of test solutions on specific growth rate was analyzed by simple linear regression. All statistical procedures were performed in GenStat 10.1.

4.4.3 Results

Fish performance
The initial individual mean weight was equal for all treatments (Table 5.5). No effect of farm water nor an effect of activated carbon treatment of farm water on growth performance was observed: final weight, feed conversion rate and specific growth rate were equal for all treatments (Table 5.5) and no significant relation between SGR and farm water content of the test solutions was found (Fig. 5.2).
The initial coefficient of variation was equal for all treatments and increased during the experiment. No effect of treatments on the coefficient of variation was observed: the final coefficient of variation and the increase in coefficient of variation was equal for all treatments (Table 5.5). No fish died during the experimental period.

Water quality
Results of the water quality measurements are presented in Table 5.6. Water temperature, oxygen level and nitrite nitrogen level were equal in all experimental treatments. The total ammonia nitrogen level was lower in the artificial seawater (A) compared to the other treatments. The nitrate nitrogen level in farm water (B) was lower than the level in the artificial seawater (A) and the test solution with a 33% farm water content (D), but equal to the other treatments. Differences in pH were observed among the experimental treatments, with the activated carbon treated farm water (E) yielding the highest pH and the farm water (B) the lowest. Small differences in salinity were observed among the treatments.

Table 5.5. Mean (n=3) values per experimental treatment for individual initial (Wo) and final (Wt) weight, specific growth rate (SGR), feed conversion rate (FCR), initial (CVo) and final (CVt) coefficient of variation and the change in coefficient of variation (ΔCV). P-values for One way ANOVA.

		Treatment					
Parameter	Unit	A	B	C	D	E	P-value
Wo	(mg)	545	569	562	557	558	0.89
Wt	(mg)	1061	1088	1032	1043	1018	0.97
SGR	(%/d)	3.16	3.07	2.84	2.98	2.86	0.87
FCR	(g/g)	1.05	1.05	1.25	1.10	1.15	0.90
CVo	(%)	19.6	18.9	15.4	15.0	16.7	0.48
CVt	(%)	25.6	26.9	26.7	22.3	22.4	0.72
ΔCV	(%)	6.0	7.9	11.4	7.3	5.6	0.72

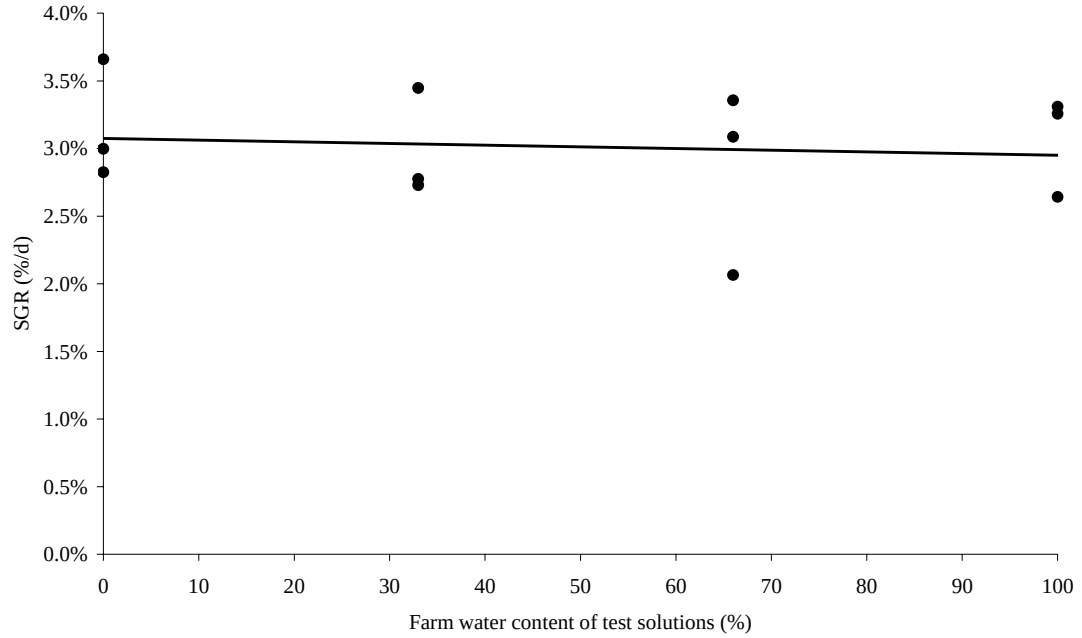

*Fig. 5.2 Specific growth rate (SGR) in relation to the farm water content of the test solutions (X). SGR = 3.05 – 0.0008*X (Simple linear regression, P = 0.80, r^2 = 0.01).*

Table 5.6. Mean values per experimental treatment for water temperature, oxygen, total ammonia nitrogen, nitrite nitrogen, nitrate nitrogen levels, pH and salinity. Values with different letters across rows are significantly different at the 5% level (One-way ANOVA).

		Treatment						
Parameter	Unit	A	B	C	D	E	P-value	l.s.d.
Temperature	(°C)	16.0	16.0	15.9	15.9	16.0	0.95	-
Oxygen	(mg/L)	7.6	7.6	7.6	7.6	7.6	0.97	-
Ammonia	(mg N/L)	8.4^{a}	9.8^{b}	9.3^{b}	9.5^{b}	9.7^{b}	0.01	0.7
Nitrite	(mg N/L)	0.91	1.04	1.22	0.95	0.82	0.62	-
Nitrate	(mg N/L)	164^{a}	129^{b}	144^{ab}	153^{a}	138^{ab}	0.05	23
pH		8.17^{a}	7.94^{b}	8.05^{c}	8.12^{ac}	8.31^{d}	<0.001	0.07
Salinity	(‰)	35.6^{a}	34.8^{ab}	35.2^{ab}	35.5^{a}	34.3^{b}	<0.001	0.9

4.4.4 Discussion

This experiment was performed to verify the results of the previous experiment in which growth inhibiting properties of farm water were demonstrated, but could not be attributed to the presence of growth inhibiting factors. The present study did not confirm our previous findings, in fact no effect of test solutions on fish performance were found (Table 5.5, Fig. 5.2).

The set up of the present experiment was changed compared to our previous experiment to eliminate growth affecting factors other than the presence of growth inhibiting factors, including stress response due to sudden change of test solution at the start of the experiment and differences in nitrite and nitrate levels. In addition the experimental period was longer to allow more time for growth to respond to the test solutions. This approach was successful for nitrite nitrogen which was equal in all treatments (Table 5.6) and nitrate. Nitrate levels were not equal in all treatments but the relative differences were much smaller compared to the previous experiment. Salinity was relatively high in all treatments, compared to full strength seawater, probably due to evaporation of water.

Overall growth was rather low compared to the specific growth rate of turbot in this size class at France Turbot, which varies from 7-9%/d (Lamour, pers, comm.). This indicates that the experimental conditions were not optimal for growth and possibly obscured effects of test solutions.

On the other hand, the specific growth rate in the present experiment was higher than in the previous experiment, despite the fact that in the present experiment initial weights, nitrate and total ammonia nitrogen were higher and temperature was lower. As the husbandry system was the same, the change from Tetra to Aglonorse as feed is the most plausible explanation.
Feed conversion rates were based on feed load (as opposed to feed intake) and were equal for all treatments. The average FCR for all treatments of 1.12 indicates that fishes were not held back in their growth due to a too low feed load nor overfed resulting in excessive deterioration of the culture water.
In this experiment farm water was pretreated with activated carbon to remove potentially present growth inhibiting factors and to counteract negative effects of farm water on growth performance. As no negative effects farm water content of the test solutions on growth was found (Fig 5.2), these effects could not be counteracted by activated carbon. Such a counteracting effect would have supplied indirect evidence for the adsorption of growth inhibiting at the activated carbon.

4.4.5 Conclusion

The present experiment does not show any growth inhibiting properties of farm water originating from a commercial turbot farm using a recirculation system. However, treatment effects may have been obscured by suboptimal experimental conditions for growth.

5 Overall discussion and conclusions

We hypothesized that growth retardation of turbot in recirculating aquaculture systems (RAS) is caused by the production of growth inhibiting factors (GIF) by turbot and the subsequent accumulation of these GIF in the culture water. The literature review revealed that it is quite likely that turbot excrete chemical signals for communication purposes, which are likely to affect growth when accumulating in RAS. However, we were unable to demonstrate GIF production by turbot, despite using a methodology that proved to be successful in other fish species. Also for water originating from a commercial turbot farm using RAS growth inhibiting properties could not be demonstrated. On the other hand growth and size variation of juvenile turbot was found to be affected when fish were reared in the effluent water of a tank containing large turbot, and the effect on size variation was counteracted by pre-treatment of the water by activated carbon. These findings suggest that growth of juvenile turbot can be affected by dissolved substances originating from larger conspecifics. In conclusion: turbot produce compounds that can affect the growth of conspecifics but the importance of GIF in commercial turbot aquaculture in RAS remained unclear.

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7 Quality Assurance

IMARES utilises an ISO 9001:2000 certified quality management system (certificate number: 08602-2004-AQ-ROT-RvA). This certificate is valid until 15 December 2009. The organisation has been certified since 27 February 2001. The certification was issued by DNV Certification B.V. Furthermore, the chemical laboratory of the Environmental Division has NEN-AND-ISO/IEC 17025:2005 accreditation for test laboratories with number L097. This accreditation is valid until 27 March 2009 and was first issued on 27 March 1997. Accreditation was granted by the Council for Accreditation, with the last inspection being held on the 5th of October 2007.

Justification

Rapport C115/08
Project Number: 4394300701

The scientific quality of this report has been peer reviewed by the a colleague scientist and the head of the department of Wageningen IMARES.

Approved: Ir. H.W. van der Mheen
Head Department Aquaculture

Signature: 

Date: December 2008

Number of copies:	7
Number of pages	79
Number of tables:	26
Number of graphs:	28
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