Institut für Tierwissenschaften, Abteilung Tierernährung
Rheinische Friedrich-Wilhelms-Universität Bonn
Abteilungsleiter: Prof. Dr. Karl-Heinz Südekum

STUDIES OF PHOSPHORUS REQUIREMENTS IN GILTHEAD SEABREAM (*SPARUS AURATA*) AND OF POTENTIAL USE OF SUPPLEMENTARY PHYTASE IN GILTHEAD SEABREAM AND RAINBOW TROUT (*ONCORHYNCHUS MYKISS*)

Inaugural - Dissertation
zur
Erlangung des Grades

Doktor der Agrarwissenschaften
(Dr. agr.)

der
Hohen Landwirtschaftlichen Fakultät
der Rheinischen Friedrich-Wilhelms-Universität
zu Bonn

vorgelegt am 2. Mai 2005
von
Karen Güthler
aus Sankt Augustin

gefördert durch die H. Wilhelm Schaumann Stiftung, Hamburg

Referent: Prof. Dr. Ernst Pfeffer

Korreferent: Prof. Dr. Dr. Helga Sauerwein

Tag der mündlichen Prüfung: 12. August 2005

Diese Dissertation ist auf dem Hochschulschriftenserver der ULB Bonn http://hss.ulb.uni-bonn.de/diss_online elektronisch publiziert (2005)

Studies of phosphorus requirements in gilthead seabream (*Sparus aurata*) and of potential use of supplemental phytase in gilthead seabream and rainbow trout (*Oncorhynchus mykiss*)

The aim of this study was to test plant feedstuffs as an alternative to fishmeal in diets for rainbow trout and gilthead seabream, focussed on the reduction of phosphorus excretion and the potential use of microbial phytase.

The trout experiments took place in Germany in the Institute of Animal Nutrition of the University of Bonn. The seabream experiments were carried out in the National Center for Mariculture in Eilat, Israel. In all experiments digestibility and/or utilisation of phosphorus was determined by the difference method.

In two growth experiments response of seabream to rising levels of dietary phosphorus was examined. Each trial contained seven diets basing on wheat gluten. Different levels of dietary phosphorus at constant remaining composition were achieved by supplementation of di-calcium phosphate (DiCaP) in the first and mono-calcium phosphate (MoCaP) in the second experiment. In separated experiments the digestibilities of DiCaP and MoCaP were determined in seabream. In a third growth experiment the effect of rising supplementations of microbial phytase was examined. This experiment was carried out in parallel in trout and seabream using the same diets which based on soy protein concentrate.

Weight gain, feed intake, feed conversion efficiency and body composition showed a clear dependence on phosphorus or phytase level at phosphorus deficiency. Exceeding respective dietary phosphorus or phytase concentrations showed no further effects. Phosphorus requirement of seabream was determined to about 6.5 g digestible phosphorus per kg diet at 18 MJ DE/kg. A supplementation of microbial phytase of 1000 FTU/kg was sufficient to enable maximum weight gain in trout. In seabream the requirement of supplemental phytase could not definitely determined, since overall dietary phosphorus concentration was too low for the demand of gilthead seabream.

Additionally several plant feedstuffs were tested for their phosphorus digestibility with and without supplementary phytase: full-fat soybeans, soy protein concentrate and rapeseed oilmeal in trout and rapeseed oilmeal in seabream.

The trout diets based on wheat gluten. Test components were added achieving a dietary phosphorus level below the requirement of trout. In the seabream experiment rapeseed oilmeal was the only phosphorus source.
Phosphorus digestibility of rapeseed oilmeal, soy protein concentrate and soybeans in trout could be increased by supplementation of phytase from 27 to 83 %, from 41 to 93 %, and from 43 to 94 % respectively. In seabream phosphorus digestibility of rapeseed oilmeal was improved from 50 to 84 %.

Untersuchungen zum Phosphorbedarf von Goldbrassen (*Sparus aurata*) und zu Einsatzmöglichkeiten von Phytasezusätzen für Goldbrassen und Regenbogenforellen (*Oncorhynchus mykiss*)

Ziel dieser Arbeit war die Bewertung von pflanzlichen Futtermitteln als Alternative zu Fischmehl in der Ernährung von Goldbrassen und Regenbogenforellen, mit Schwerpunkt auf einer Minimierung der Phosphorausscheidungen und dem Einsatz von mikrobieller Phytase.

Die Versuche mit Regenbogenforellen wurden in Institut für Tierernährung der Universität Bonn durchgeführt, die Versuche mit Goldbrassen fanden im National Center for Mariculture in Elat, Israel, statt. In allen Experimenten wurde die Verdaulichkeit und/oder Verwertung von Phosphor mit Hilfe der Differenzmethode ermittelt.

Der Phosphorbedarf von Goldbrassen wurde in zwei Wachstumsversuchen mit steigenden Phosphorkonzentrationen im Futter bestimmt. Jeder Versuch umfasste sieben Futterischun-gen auf der Basis von Weizenkleber. Als Phosphorquelle wurde im ersten Versuch Dicalcium-phosphat (DiCaP), im zweiten Versuch Monocalciumphosphat (MonoCaP) verwendet. Die Bestimmung der Verdaulichkeiten von DiCaP und MonoCaP erfolgte in einem separaten Experiment. In einem weiteren Versuch wurde der Effekt von steigenden Phytasezusätzen untersucht. Hierbei handelte es sich um einen Doppelversuch mit Regenbogenforellen und Goldbrassen, die dieselben Futtermischungen auf der Basis von Sojaproteinkonzentrat erhielten.

Steigende Phosphor- bzw. Phytasekonzentrationen im Futter bewirkten im Bereich der Unterversorgung mit Phosphor eine Erhöhung der Futteraufnahme, des Gewichtsansatzes, der FCE und des Phosphorgehalts im Körper. Bei einer Steigerung der Phosphor-konzentrationen über den Bedarf hinaus konnten keine weiteren Effekte beobachtet werden.
Bei Zugrundelegung des maximalen Gewichtsansatzes ergab sich für Goldbrassen ein Phosphorbedarf von 6,5 g verdaulichem Phosphor bei einem Energiegehalt von 18 MJ pro kg Futter. Die notwendige Höhe der Phytasesupplementierung für Forellen lag bei 1000 FTU pro kg Futter.

Weiterhin wurden einige pflanzliche Futtermittel mit und ohne Phytasezusatz auf ihre Phosphorverdaulichkeit hin untersucht. Die Futtermischungen für die Regebogenforellen basierten auf Weizenkleber und enthielten Anteile von Sojabohnen, Sojaproteinkonzentrat und Rapsextraktionsschrot. In Goldbrassen kam Rapsextraktionsschrot zum Einsatz, das auch die einzige Phosphorquelle in den Futtermischungen darstellte. Die Phosphorkonzen-trationen im Futter lagen unterhalb des jeweiligen Bedarfs der Fische.
Eine Zusatz von 1000 FTU Phytase pro kg Futter bewirkte eine Steigerung der Phosphor-verdaulichkeit von 27 auf 83 % von Rapsextraktionsschrot, von 41 auf 93 % von Sojaprotein-konzentrat, und von 43 auf 94 % von Sojabohnen.
Bei den Goldbrassen erhöhte ein Phytasezusatz die Phosphorverdaulichkeit von Raps-extraktionsschrot von 50 auf 84 %.

Table of contents

Index of abbreviations

BM:	Basal mix
BW:	Body weight
Cg:	Concentration in live weight gain
CPC:	Corresponding dietary phosphorus concentration to 95 % of the plateau value
CphyC:	Corresponding dietary phytase concentration to 95 % of the plateau value
DC:	Digestibility coefficient
DCP:	Digestible crude protein
DE:	Digestible energy
DE_g:	Digestible energy for growth
DE_m:	Digestible energy required for maintenance
Di-CaP:	Dicalcium phosphate
DM:	Dry matter
FCE:	Food conversion efficiency
FTU:	Phytase unit. One FTU is the activity of phytase that liberates 1 μmol of inorganic phosphorus per minute from an excess of sodium phytate at pH 5.5 and 37 °C.
HCl-IA:	Hydrochloric acid insoluble ash
ht:	Hard tissue
MBS:	Metabolic body size
Mono-CaP:	Monocalcium phosphate
NCM:	National Center for Mariculture (in Eilat)
P_{av}	Available phosphorus
$Pret_D$	Phosphorus retention depending on dietary phosphorus concentration
$Pret_I$	Phosphorus retention depending on phosphorus intake
RS:	Rapeseed oilmeal
SB:	Full-fat Soybeans, pre-cooked
SPC:	Soy protein concentrate
st:	Soft tissue
TM:	Test mix

Indications of quantity not followed by "DM" refer to materials containing their water content.

Index of formulas and calculations

$$\text{Mean body weight [g/fish]} = \frac{\text{Weight of the group [g]}}{\text{Number of fish in the group}} \quad [1]$$

$$\text{Mean weight gain [g/fish]} = \text{Mean final weight [g/fish]} - \text{Mean initial weight [g/fish]} \quad [2]$$

$$\text{Feed intake [g DM/fish]} = \frac{\text{Food eaten by the group [g DM]}}{\text{Number of fish in the group}} \quad [3]$$

$$\text{Feed conversion efficiency FCE} = \frac{\text{Weight gain [g]}}{\text{Feed intake [g DM]}} \quad [4]$$

Concentration of nutrients and energy in gain

$$C_G \text{ [g, MJ/fish]} = \frac{\text{Final biomass [g]} \times \text{final body concentration [g, MJ/kg]} - \text{initial biomass [g]} \times \text{body concentration of control group [g, MJ/kg]}}{\text{Weight gain [g]}} \quad [5]$$

$$\text{Efficiency [\%]} = \frac{C_G \text{ [g/kg]} \times \text{FCR}}{\text{Concentration in diet [g/kg]}} \times 100 \quad [6]$$

FCR on defined on previous page

Growth parameters (weight gain, feed intake, FCE) and concentration of minerals in gain were evaluated by regression analysis using the function

$$y = a(1 - e^{-b(x-c)}) \quad [7]$$

x = dietary phosphorus concentration

a = plateau value at infinite dietary phosphorus concentration

b = curvature parameter

c = intersection point with the x-axis

Lipid concentration in gain was evaluated by regression analysis using the exponential function

$$y = a + be^{kx} \quad [8]$$

x = dietary phosphorus concentration
a = plateau value at infinite dietary phosphorus concentration
b = curvature parameter
k = curve extension parameter

Phosphorus retention fitted best to the function

$$y = \frac{a + [b(1+c) - a]e^{-kx}}{1 + ce^{-kx}} \quad [9]$$

Apparent digestibility coefficients of **nutrients in feed mix**

$$ADC\ [\%] = 100 - 100 \times \frac{\%\ \text{marker}_{\text{feed}}}{\%\ \text{marker}_{\text{faeces}}} \times \frac{\%\ \text{nutrient}_{\text{faeces}}}{\%\ \text{nutrient}_{\text{feed}}} \quad [10]$$

Apparent digestibility coefficients of **phosphorus from the test component**

$$DC_{TC}\ [\%] = \frac{DC_{TM}\ [\%] - DC_{BM}[\%] \times (1 - t)}{t} \quad [11]$$

DC_{TC} = digestibility coefficient of test component [%]
DC_{TM} = digestibility coefficient of test mix [%]
DC_{BM} = digestibility coefficient of basal mix [%]
t = phosphorus part from the test component, determined using the formula

$$t = 1 - \frac{\text{P - concentration in basal mix [g/kg DM]} \times \text{part of basal mix in test mix}}{\text{P - concentration in test mix [g/kg DM]}} \quad [12]$$

Index of Tables

Index of Figures

Synopsis of experiments

Experiment	1	2	3	4	5	6a	6b
Species	trout	seabream	seabream	seabream	seabream	trout	seabream
Test subject	plant feedstuffs ± phytase	dietary phosphorus Di-CaP	dietary phosphorus Mono-CaP	Di-CaP Mono-CaP	plant feedstuff ± phytase	dietary phytase	dietary phytase
No. of diets	8	7	8	6	2	8	8
Replications	3	3	3	2	2	3	3
No. of fish per group	15	27	26	15	15	20	24
Growth experiment		×	×			×	×
Digestibility determination	×			×	×	×	×
Duration [days]	42	54	92	20	14	53	98
Feeding	restricted	to satiation	restricted	to satiation	to satiation	to satiation	to satiation
Initial weight [g/fish]	81.9	28.3	29.9	ca. 350	ca. 350	101	61.4
Final weight [g/fish]		80.5	97.2			251	120

1 Introduction

In the past decades it was realised that excessive nutrient excretion from farmed animals can cause considerable ecological damage. Therefore, nutrition research has been focused on minimizing of nutritional waste output. In comparison to cattle, swine and poultry nutrition this aspect has been neglected in aquaculture (LALL 1991).

However, while demand for fish increases and capture of marine fish reaches the limits of exploitation, fish farming is gaining importance. The European production of rainbow trout (*Oncorhynchus mykiss*) has more than tripled from 102,665 tons in 1980 to 315,983 tons in 2002. Production of gilthead seabream (*Sparus aurata*) in Europe including Israel increased steeply from 4,570 tons in 1990 to 77,081 tons in 2002 (FAO 2004).
Today aquaculture production of trout and seabream is usually carried out in ponds and cages under very intensive conditions. This requires an external feed supply, which is accompanied by a considerable load of nutrients into the water.
The dimension of phosphorus impact from trout feed is illustrated by the following estimation:
Considering a ratio of gain : feed consumption of 1 : 1, a phosphorus concentration of 4.5 g/kg in the body of trout and 10 g/kg in the feed would result in a phosphorus load into the European waters of about 1700 tons annually from trout feed.

In Israel the biggest marine fish production site is located in the Gulf of Eilat 8 km north of the coral reefs. Cage farming started here in the early 1990s and has achieved a fish production of about 2,000 tons annually, 90 % of them seabream (GORDIN 2003).
Since the early 1980s a gradual degradation of the coral reefs in the local nature reserve has been observed (ZAKAI & CHADWICK-FURMAN 2002, LOYA & ZAKAI 2002). Therefore, an International Expert Team was formed by the Israeli government to review the current scientific evidence about the ecological situation in the Gulf. The Expert Team neither confirmed nor excluded an unequivocal connection between cage farming and coral degradation based on limited available data and recommended a fish production limit of approximately 2,500 tons per year (DIAMANT & VON WESTERNHAGEN 2003, ATKINSON *et al.* 2001).

Phosphorus is one of the most responsible factors for water pollution from fish feed in addition to nitrogen. Coral reefs require a threshold phosphorus concentration of less than 0.003 ppm, which is far below the standards for potable water (BELL 1992, LAPOINTE 1992). Above this

concentration, exuberantly growing algae smother the corals reefs and compete with them for space and oxygen.

The use of plant protein instead of fish meal could support a reduction in phosphorus impact, for its considerable lower phosphorus concentration.
In recent years plant feedstuffs have proven to have additional advantages, namely

- plant protein is less expensive than fish meal,
- animal meals were often involved in food scandals.

However, an exclusive use of plant feed may cause a deficiency due to its lower phosphorus concentration and differences in phosphorus availability of the different feedstuffs.

For rainbow trout, the phosphorus demand has been determined quite well. In rainbow trout, several successful approaches at replacing fish meal by plant protein have been proven in recent years.
On the other hand, in gilthead seabream little work has been done on phosphorus requirements and the use of plant feedstuffs. The ecological situation described above requires a special interest in further research in this field.

The primary objective of this study was the determination of the phosphorus requirements in gilthead seabream. The secondary objective was the evaluation of plant protein in trout and seabream, encompassing the use of microbial phytase.

2 Objectives

Phosphorus loss from the fish can be divided into three fractions:

1. Inevitable loss. This part depends only on the physiology of the animal and cannot be influenced. A dietary phosphorus supply below this amount would result in a negative phosphorus balance of the fish.
2. Indigestible phosphorus. Dietary phosphorus can be found in chemical structures that are difficult or impossible for the animal to absorb. Even inorganic phosphates differ in their phosphorus digestibility. Largely indigestible to non-ruminants is phytate phosphorus, which is found as a major part of the phosphorus in plant feedstuffs. It can be utilised partially in non-ruminant animals which harbour phytate-splitting bacteria in parts of their digestive tract. The respective phytate phosphorus contents in different plant feedstuffs were summarized by DÜNGELHOEF & RODEHUTSCORD (1995).
3. Regulatory or homeostatic excretion. This part represents the surplus phosphorus which exceeds the demand of the animal and is excreted as a consequence of an excess.

Feeds for salmonids usually include 40–50 % protein. This component is still often supplied as fish meal, containing approximately 20 g phosphorus per kg. Phosphorus concentrations in present fish feeds appear in the range of 9-11 g per kg diet (HARDY & GATLIN 2002), whereas phosphorus requirements of different fish species have been reported at about 3-8 g per kg diet (NRC 1993). Therefore, an immediate decrease of phosphorus loss could be achieved by the reduction of dietary phosphorus.

The requirement of rainbow trout has been determined at about 5 g digestible phosphorus per kg diet (RODEHUTSCORD 1996, RODEHUTSCORD *et al.* 2000, SUGIURA *et al.* 2000). In a diet containing fish meal as a protein source, it is almost impossible not to exceed this level. A low phosphorus concentration in a protein balanced diet can be attained by replacement of fish meal by plant protein sources, which contain approximately 4-10 g phosphorus per kg. Several studies in rainbow trout using plant feedstuffs revealed comparable performances to fish meal diets (KINZINGER 1992, PFEFFER & HENRICHFREISE 1994, RODEHUTSCORD 1996, BRAUN 1999).

In seabream the substitution of fish meal by plant protein has been reported with varying success. ROBAIANA *et al.* (1995) replaced up to 35 % of the fish meal by soybean meal without losing performance after destroying 85% of the trypsin inhibitors.

NENGAS *et al.* (1996) exchanged up to 40 % solvent extracted soybean meal for fish meal and reported a slightly decreased growth performance as the replacement rate increased. In another experiment the authors observed no decline of performance, when 35 % of the fish meal was replaced by pre-cooked full-fat soybean meal. However, in this study fish were fed restrictively. Potential differences in voluntary feed intake were not recorded.

KISSIL *et al.* (2000) reported a depressed voluntary intake in an experiment using rapeseed protein concentrate and soy protein concentrate with a replacement rate up to 100 %. Phosphorus concentrations from phytic acid in the test components were about 13 g per kg DM for rapeseed and 5.3 g per kg DM for soy protein concentrate. The authors also observed depressed growth performance with increasing replacement rate and noted an inverse relationship between dietary phytic acid and voluntary feed intake. However, they could not exclude other negative factors.

In a more recent study by KISSIL & LUPATSCH (2004), partial to complete fish meal substitution was demonstrated using a mixture of the plant proteins corn gluten, wheat gluten and soy protein concentrate. A blend of the three with an equal contribution of protein from each one, gave better growth and feed utilization in seabream at 25 – 100% fish meal replacement.

The chemical structure of phytic acid or phytine consists of an hexagonal hydrocarbon ring with orthophosphate groups bound to the carbon (Figure 1).

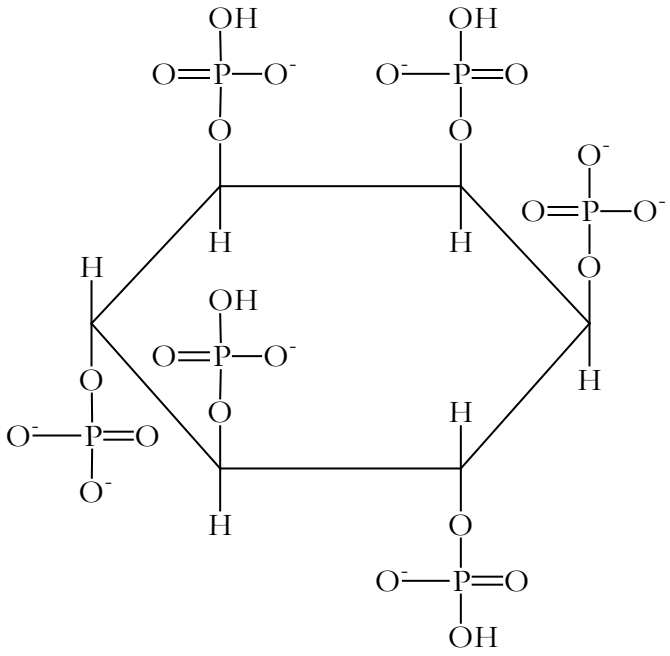

Figure 1: Constitution of phytic acid

The enzyme phytase catalyses the hydrolysis of phytic acid to orthophosphate and inositol. Today microbial phytase is produced by gene technology methods using *Aspergillus niger.* (PALLAUF *et al.* 1992).

In pig and poultry nutrition the increase of phosphorus digestibility by phytase supplementation led to an extensive reduction in the use of inorganic phosphates (DÜNGELHOEF *et al*. 1994, RODEHUTSCORD *et al*. 1996, RODEHUTSCORD *et al*. 1999, SEBASTIAN *et al*. 1998).

Several studies promise considerable effectiveness of supplementary phytase in fish nutrition, mainly in rainbow trout (RODEHUTSCORD *et al.* 1995, LANARI *et al.* 1998, VIELMA *et al*. 1998, SUGIURA *et al.* 2001, CHENG & HARDY 2002), but also in other marine and warm water species (JACKSON e*t al.* 1996, LI & ROBINSON 1997, OLIVA-TELES *et al.* 1998, POWERS HUGHES & SOARES 1998, PAPATRYPHON & SOARES 2001, STOREBAKKEN *et al*. 1998, VAN WEERD *et al.* 1999).

The objectives of this study were

1. the determination of phosphorus requirements in gilthead seabream,
2. the effect of phytase supplementation to plant feedstuffs in gilthead seabream and rainbow trout,
3. a comparison between these two species regarding phosphorus metabolism.

Phosphorus requirement of seabream was determined by dose-response experiments.

Former studies in other fish species showed, that phosphorus digestibility of a diet can be considered as the sum of the phosphorus digestibilities of the individual components multiplied with their respective proportional amounts in the diet. This allows the determination of the digestibility of a single feedstuff incorporated in a diet by use of the difference-method.

In this study the experimental diets consisted of a basal mix containing a phosphorus level far below the demand of the fish. To this basal mix a phosphorus source was added in gradually increasing concentrations. Because of the facilities available, digestibility determinations and growth experiments could not be carried out in parallel. The response of the fish was measured by growth parameters and body composition in comparison to a control group. Phosphorus efficiency could be determined from these data.

For mathematical analysis the response of the fish was regarded as a function of dietary phosphorus concentration. In a Cartesian coordinate system, where the x-axis represents dietary

phosphorus concentration and the y-axis represents performance or phosphorus retention, the experimental data points can be interpreted as an exponential curve, approaching an upper limit (RODEHUTSCORD 1996, ÅSGÅRD & SHEARER 1997). Nonlinear regression will be performed comparing dietary phosphorus (independent variable) versus different response traits (dependent variable) to calculate the phosphors requirement.

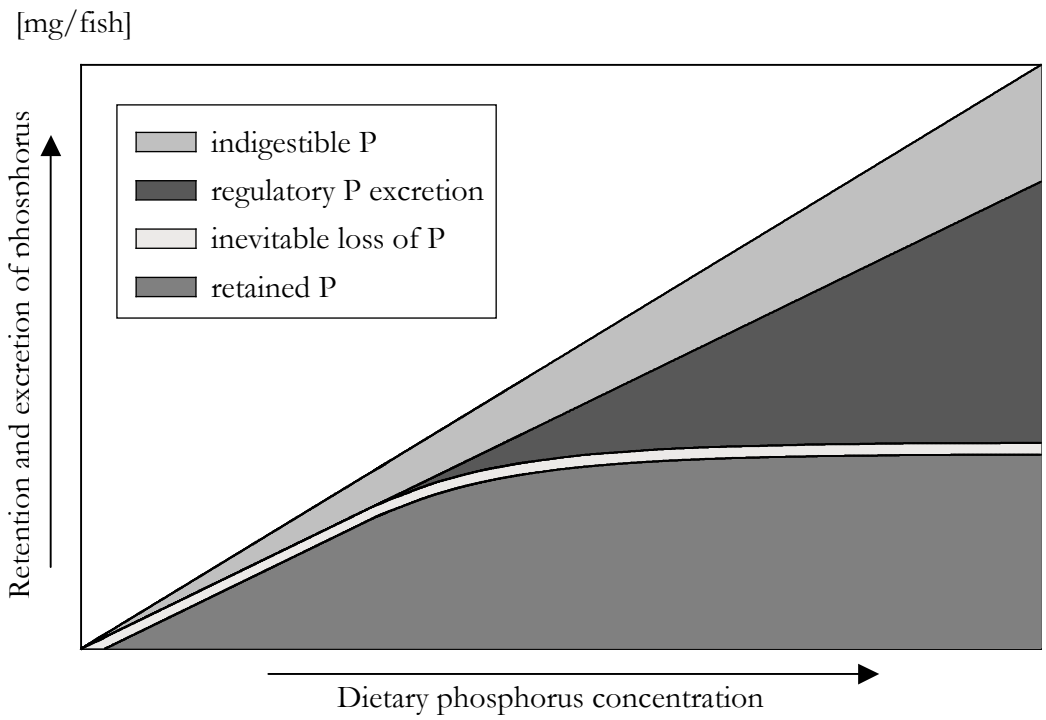

Figure 2: Absolute distribution of phosphorus intake

Figure 2 shows a theoretical example of the expected distribution of phosphorus and the calculation of the curves based on estimated values. The feed intake is assumed constant at rising concentrations of dietary phosphorus.

The curve of the retained phosphorus agrees with the exponential curve as described above. The regulatory phosphorus excretion is zero with an insufficient supply of phosphorus. When exceeding the phosphorus requirement, it increases almost linearly to the dietary phosphorus concentration.

Figure 3 shows the same theoretical example, representing the fractions as a percentage of total phosphorus intake.

As a characteristic of the feed, indigestible phosphorus appears as a constant percentage of total phosphorus. The inevitable loss makes up the main part at very low dietary phosphorus concentrations. The curve of retained phosphorus shows a typical course for the efficiency of phosphorus retention. With increasing dietary phosphorus concentration the efficiency increases as a

consequence of diminishing parts of inevitable loss. After reaching a maximum, the curve decreases, caused by rising parts of regulatory excretion.

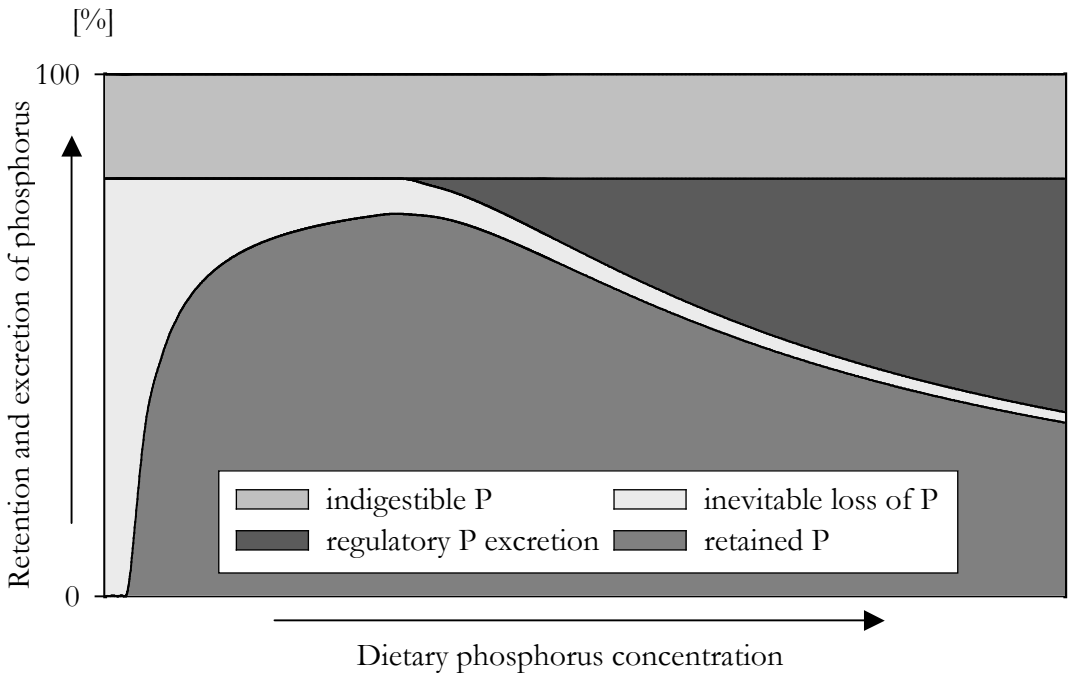

Figure 3: Relative distribution of phosphorus intake

For a determination of the phosphorus requirement and phosphorus digestibility this must be taken into account. Determining the requirement by a dose-response experiment, the maximum efficiency of phosphorus retention should be determined and the requirement should be exceeded clearly. Therefore the dietary phosphorus concentrations should cover a wide range starting from the area of phosphorus deficiency.

In a digestibility determination the diets should contain a phosphorus concentration slightly below the concentration corresponding to maximal phosphorus efficiency. Otherwise there is a risk of underestimating the digestibility.

3 Material and Methods

3.1 Gilthead seabream

3.1.1 Fish and tanks

Fish were taken from a stock spawned and raised at the National Center for Mariculture (NCM). Growth experiments were carried out in 200-L conical outdoor tanks. The tanks tapered at the bottom and were equipped with a drain pipe system in the middle of the tank which resulted in water leaving the tank near the bottom of the water column.

Fish for digestibility trials came from a stock raised at the NCM and were kept permanently in the Nutrition department. During an experiment they were distributed in 600-L outdoor square tanks. For the digestibility experiments duplicate groups of fish instead of triplicate groups were used, since only 16 tanks were available. For each group 2 tanks had to be provided, as fish were moved to a new tank after stripping. A maximum of eight groups of fish were used for stripping at any one time.

Water supply consisted of flow-through seawater, with a salinity of 41 ppm at approximately 8 L per minute.

3.1.2 Feed preparation

Outside temperatures in Eilat in summer can reach 45 °C. Therefore, components sensitive to heat were stored in a cold room at 20 °C. After weighing, all ingredients were mixed for 5 minutes in a 25-L batch mixer, kneaded by hand and mixed again for another 20 minutes.

Composition of vitamin and mineral premixes is listed in Table A 16 and Table A 17.

The feed was pelleted in a steam pellet mill (California Pellet Mill) using a 4 mm screen.

To achieve a homogenous distribution of the phytase, a pre-mixture of 20 g of diet and 250 mg of phytase was prepared. This pre-mixture was then mixed separately with 2 kg diet in a bowl and added to the rest of the diet and mixed in a 5-L batch mixer.

Extruding would have partially destroyed phytase activity, so it was necessary to obtain suitable pellets in a different way. The diets were moistened with water at a ratio of diet : water = 10 : 7. This mixture was then passed through a 3-mm screen and immediately frozen. Diets were kept frozen at –20 °C until use. Oversized pieces were broken by hand before feeding.

Chromium oxide was used as a marker. The use of hydrochloric acid insoluble ash could have overestimated digestibility, since the tanks were located outside where wind blown sand accumulated in them, and the uptake of this sand by the fish could not be excluded.

3.1.3 Faeces collection

In the digestibility trials faeces were collected by stripping. Fish were anaesthetized in a tub containing about 20 L water mixed with a small amount of 20% clove oil in ethanol. Anaesthetized fish held in a towel had slight pressure applied to their abdomen near the anus to empty their bladder. After cleaning the anus, faeces were stripped using the thumb and forefinger into a funnel containing filter paper. Faeces obtained from all fish in each tank were pooled and dried for 24 hours at 60 °C in small glass bowls.

3.1.4 Sample preparation

Dried faecal samples were ground and stored at 20 °C in small glass tubes.
For biomass samples, fish were starved for one day and then sacrificed by immersion in iced water. Fish from each tank were weighed and frozen together and while still frozen homogenized twice through a mincer using a 3 mm screen, dried at 80 °C and finely ground in a blender. From this material about 30 g of each sample was taken and stored at 20 °C in plastic bags. At the beginning of each growth experiment, a control group of 30 randomly chosen fish was frozen and treated like the other fish samples.
Feed was sampled by grinding about 30 g of each diet in a blender, and storing at 20 °C. Feed from experiment 5 (rapeseed/phytase experiment), which contained a high part of water, was dried at 105 °C before grinding and storage.

To evaluate the distribution of P in the body tissue, 10 fish from each tank were dissected into hard and soft tissues in experiment 2 (Di-CaP growth experiment) in the following manner:

1. Fish were cooked in water for six minutes with turning them over after three minutes. Previous trials with test fish had shown, that the fish did not release any water during this short cooking time, therefore, wrapping of the fish was not necessary.

2. After cooking the fish were wiped dry and dissected on a tray.
3. The head and fins were removed using a sharp knife and grouped with the hard tissues.
4. The fish were then opened along the backbone.
5. The skin and muscle tissues were removed and grouped with the soft tissues.
6. All internal organs were grouped with the soft tissues.
7. The fish's skeleton was then removed from the remaining carcass and combined with the hard tissues.
8. Lastly, the remaining muscles and skin were combined with the soft tissues.

The dissection had to be done quickly to avoid loss of water from evaporation. The two fractions of each group were pooled in a dish and wet weights were determined. Then the soft tissue was frozen and later treated like the whole body samples for further analysis. The hard tissue was dried at 105 °C until constant weight . After determining the DM, it was ground in a blender and stored like the other tissue samples.

3.1.5 Chemical analysis

Determination of the major elements Ca, Mg, Na and K were carried out in the Department of Animal Nutrition in the University of Bonn (see chapter 3.2.4). All other chemical analyses of the seabream experiments were carried out in Eilat.

Dry matter was determined gravimetrically by drying the samples at 105 °C until constant weight.

Ash was derived by combustion for 24 hours at 550 °C in a muffle furnace and the organic matter was defined as the weight loss after ashing the samples.

Gross energy was measured as combustion heat in an adiabatic Parr bomb calorimeter using benzoic acid as the standard.

Crude protein was calculated by multiplying N by 6.25. Nitrogen was determined as ammonia after wet ashing in sulfuric acid and distillation using the Kjeldahl technique and titrating to neutral pH.

Lipids were determined gravimetrically after extraction in a mixture of chloroform and methanol, separation and vacuum drying (FOLCH *et al.* 1957).

Phosphorus was extracted from the ash using hydrochloric acid and test solutions were prepared from the extracted material. The orthophosphate was dyed with vanadate-molybdate-solution

and measured photometrically (Kontron instruments, type Uvikon 922) at 435 nm (NAUMANN & BASSSLER, 1976).
Chromium oxide in the digestibility trials was measured photometrically as dichromate at 360 nm after digestion for two hours at 250 °C in a mixture of perchloric acid, concentrated sulphuric acid and Na-molybdate.

3.2 Rainbow trout

3.2.1 Fish, tanks and faeces collection

Experimental trout were taken from a stock spawned and raised in the Department of Animal Nutrition in Bonn. Both experiments were carried out in a partial recirculation unit comprising 24 round 250 L-tanks made of fibreglass. The entire unit contains about 10.000 L of water, in which 40 % for the freshwater is changed daily. Water temperature is maintained by adjusting the warm water supply. Waste water runs through three sedimentation basins and then through a sprinkler. Water is squirted in each tank under high pressure providing a permanent circular current and oxygen supply.
A sedimentation unit is attached to each tank to collect faeces in the following manner: Outflow water from the tank containing faeces leaves the tank through a central drain in the bottom of the tank. The outflow then enters a horizontal pipe 45 cm long and 4 cm in diameter which leads to a larger diameter vertical pipe (70 cm high and 10 cm in diameter) and 30 cm of hose which is clamped off at the end. The water containing faeces leaving the tank slows down from the sudden change in pipe diameter (4 to 10 cm) allowing the faeces to settle in the hose at the end. Faeces is then collected from the hose with a minimum of water by releasing the clamp.
A detailed description of this unit without faecal collection is given by PETRASCH (1981).

3.2.2 Feed preparation

For the Experiment 1 (trout digestibility experiment), components of the basal mix were first mixed by hand and then for an additional half hour in a 50-L drum mixer (Lödige M 200 D).
The basal mix was divided into eight parts and each test component was then added to two of these parts. Diets without phytase supplementation were mixed for 2 minutes in a 5-L batch mixer.

For a homogenous distribution of the phytase in the feed, phytase was supplemented preparing a pre-mixture as described in chapter 3.1.2. and each pre-mixture was mixed with the rest of the diet for 5 minutes in a 5-L batch mixer. The diets were supplied with water in a ratio water : diet = 1 : 3 and mixed in a cutter (EMS, MTZ 10/70). Pellets were obtained by passing the mixture through a mincer (Rewebo RF 8202) using a 4 mm screen, freezing them, and then breaking them into small pieces and storing them in plastic boxes. Required amounts for the trials were left to defrost for about 15 minutes before each feeding.

Hydrochloric acid insoluble ash served as a marker in these diets.

The diets of the two phytase growth experiments were prepared by the BASF for two reasons:

1. The quantity of feed would have been too large for preparation in a mincer and frozen storage facilities were limited.
2. With the addition of water to the diets microbial phytase activity might have started and parts of the dietary phytate would have been already transformed into available phosphorus before feeding.

For these reasons diets were prepared by the BASF in Neumühle/Germany. Extruded pellets were sprayed under vacuum conditions using a solution of phytase and then coated with a layer of fat.

3.2.3 Sample preparation

Approximately 30 g from each feed were taken and ground in a centrifugal mill (Thomas-Wiley Laboratory Mill, Model 4) using a 1 mm screen. Feed from Experiment 1 (trout digestibility experiment) was dried at 105 °C before grinding.

Faecal samples were centrifuged for 10 minutes at 1000 revolutions per minute in a cooling centrifuge (Heraeus Christ, type Varifuge K) and after decanting the water, solid components were freeze-dried and subsequently ground in a mortar and pestle.

Trout were not fed for one day before sampling and were sacrificed in a solution of 4-ethyl-aminobenzoate and frozen. Frozen fish were cut into pieces with a ribbon saw, homogenized by passing two times through a mincer using a 3 mm screen, ground in a cutter and freeze-dried.

3.2.4 Chemical analysis

All chemical analyses of the trout experiments were carried in the Department of Animal Nutrition in the University of Bonn.
For phosphorus analysis a spectrophotometer (Beckmann, type DU-62) was used. Major elements Ca, Mg, Na and K were determined from the ash solution quantitatively using an atomic absorption photometer (Perkin Elmer, type 1100B).
All other nutrients were determined as described in chapter 3.1.5.

3.3 Statistical procedures

The arithmetic mean of each tank was taken as a unit of observation.
All equations were calculated by regression analysis and best fitting parameters were estimated with the iterative non-linear least-squares algorithm of Levenberg-Marquardt. Parameter estimates are listed ± asymptotic standard error, unless otherwise noted. The coefficient of determination expressed as r^2 is the percent of the variation that can be explained by the regression equation.
The one-way analysis of variance (ANOVA) was done by rejection of the null hypothesis with a significance level of 0.05 using Tukey's multiple range test.
All statistical analyses were carried out using SPSS 6.0 for Windows.

4 Experiments

4.1 Experiment 1 – plant feedstuffs in trout with phytase

The field of phosphorus requirement and digestibility of inorganic phosphorus in rainbow trout has been investigated thoroughly (RODEHUTSCORD 1996; GREGUŠ 2000). The aim of this experiment was to evaluate the phosphorus digestibility of plant feedstuffs, including dietary phytase supplementation.

4.1.1 Procedure

Three plant phosphorus sources were tested in rainbow trout: full-fat pre-cooked soybeans (SB), soy protein concentrate (SPC) and rapeseed oilmeal (RS).
To obtain constant phosphorus concentrations in the test diets, a basal mix (BM) (Table 1) with a very low phosphorus concentration was designed (Table A 1). The compositions of the amino acids, vitamin and mineral premix are summarized in Table A 13, Table A 14, and Table A 15.

Table 1: Composition of the basal mix

Components	[g/kg]
Wheat starch	308
Wheat gluten	230
Fish oil	90
Sunflower oil	110
Silicate binder[1]	71
Amino acids	141
Choline chloride	10
Vitamin premix	10
Mineral premix	30

Table 2: Composition of the test diets (total amounts)

	test diets			
[kg]	BM	SB	SPC	RS
Basal mix	3.5	-	-	-
SB	3.5	1.5	-	-
SPC	3.5	-	1.0	-
RS	3.5	-	-	0.75
Phytase[2]	-/+	-/+	-/+	-/+

[1] Sipernat® 50S, Degussa Hüls AG
[2] BASF Natuphos®

The basal mix was divided into four parts. One part served as a control diet. Three plant feedstuffs were added to the respective remaining parts. The added amounts of the plant feedstuffs were chosen in order to increase phosphorus concentrations of the resulting diets to about 2.5 g per kg (Table 2). Table 3 shows the analysed phosphorus concentrations.

Table 3: Analysed concentrations in diets in Experiment 1

	Diets							
	BM-	BM+	SB-	SB+	SPC-	SPC+	RS-	RS+
Phytase [FTU/g]	-	1000	-	1000	-	1000	-	1000
Ash [g/kg]	98.5	99.3	85.2	85.4	89.7	90.1	94.0	93.9
Phosphorus [g/kg]	0.76	0.81	2.28	2.28	2.56	2.41	2.59	2.55
HCl-insoluble ash [g/kg]	81.1	80.4	58.3	58.2	62.5	62.8	68.3	68.2

Each diet was fed with and without supplementation of microbial phytase, resulting in 8 treatments. Each diet was fed to triplicate groups of fish and all groups received restricted doses of diet including identical amounts of basal mix. The intake differed only in the amount of the respective test component.
After an adaptation period of one week, faeces were collected for ten days every morning before feeding as described in chapter 3.2.1

4.1.2 Results

Mean digestibilities of organic matter along with total and partial digestibilities of phosphorus are summarized in Table 4 for the respective test components. The results of the individual determinations are listed in Table A 1.
In all test diets supplementation of microbial phytase increased digestibility of phosphorus significantly. Although the basal mix was supposed to contain no phytate phosphorus, phytase supplementation still caused an effect.
The partial phosphorus digestibilities of the respective test components were calculated using formula [11]. The respective digestibilities of the phytase supplemented diets were calculated using the value of diet BM+ as a control and the digestibilities of the non-supplemented diets were calculated using the value of diet BM-.

Table 4: Total and partial phosphorus digestibilities in Experiment 1

	Diets							
[%]	BM-	BM+	SB-	SB+	SPC-	SPC+	RS-	RS+
Total OM	82 ± 1	81 ± 1	81 ± 0	83 ± 1	85 ± 1	84 ± 0	79 ± 1	80 ± 0
Partial OM	-	-	79 ± 1	88 ± 3	95 ± 4	96 ± 2	63 ± 4	77 ± 1
Total P	72 ± 2	79 ± 0	50 ± 2	90 ± 1	48 ± 1	90 ± 1	37 ± 3	82 ± 1
Partial P	-	-	43 ± 2	94 ± 1	41 ± 2	93 ± 1	27 ± 4	83 ± 1

Differences in partial digestibility of the organic matter can be explained by the individual concentrations of indigestible crude fibre of the test components. Crude fibre concentrations in the test components were not determined, but regarding the concentrations of organic matter they were estimated to about 55 g/kg DM in soybeans, 1-2 g/kg DM in soy protein concentrate and 130-140 g/kg DM in rapeseed oilmeal.

In SB+ phytase supplementation caused a phosphorus digestibility of 94 %, which is comparable to fish meal. The digestibility of the phytase supplemented SPC-diets of 93 % remained shortly below this value. The highest relative effect was observed in RS+, achieving an increase in phosphorus digestibility of 56 percentage points.
Standard deviations of the digestibilities were smaller when phytase was added to the diet. Apparently the treatment with phytase adjusted the amount of digestible phosphorus for all individual fish.

4.2 Experiment 2 – dicalcium phosphate in gilthead seabream

4.2.1 Procedure

In Experiment 2 the phosphorus requirement of gilthead seabream was determined using a series of diets with increasing phosphorus concentrations. The diets consisted of a basal mix and dicalcium phosphate (Di-CaP) supplement, which was exchanged for an inert silica binder (Sipernat 50 S, Table A 18). The basal mix was composed according to the basal mix of Experiment 1, providing a very low phosphorus concentration. Its only phosphorus sources were wheat gluten containing 1.5 g P/kg DM and wheat containing 3.0 g P/kg DM.

Diet composition and the analysed nutrient concentrations are summarized in Table 5. Compositions of the vitamin and mineral premix are summarized in Table A 16 and Table A 17.

Table 5: Composition and analysed concentrations in diets of Experiment 2

	Diets						
	1	2	3	4	5	6	7
Component [g/kg]							
Wheat gluten				500.0			
Wheat				144.3			
Fish oil				200.0			
Vitamin premix				5.0			
Mineral premix				25.0			
Lysine				21.0			
Methionine				5.2			
Arginine				10.0			
Threonine				4.5			
Choline chloride				5.0			
Calcium carbonate	22.0	12.0	7.0	-	-	-	-
Silicate binder	58.0	55.5	48.0	42.5	30.0	17.5	5.0
Di-CaP	-	12.5	25.0	37.5	50.0	62.5	75.0
Analysed concentrations per kg DM							
Crude protein [g]	452	456	441	465	462	470	483
Crude lipid [g]	210	199	208	219	215	200	197
Gross energy [MJ]	22.0	22.2	22.3	22.3	22.1	22.4	22.3
Ash [g]	108	107	106	101	107	106	103
Phosphorus [g]	2.3	3.8	6.1	8.0	11.6	14.0	15.4
Calcium [g]	13.9	11.8	12.6	12.6	15.1	17.5	21.0
Magnesium [g]	1.4	1.5	1.7	1.8	2.1	2.1	2.2
Sodium [g]	3.7	3.5	3.4	3.4	3.5	3.2	3.2
Potassium [g]	7.1	6.4	6.9	7.3	7.0	6.8	7.1

The phosphorus concentrations of Diets 1-7 fulfilled the range from phosphorus deficiency to surplus phosphorus supply. Several body composition analyses done in the NCM of seabream fed commercial diets, revealed a phosphorus concentration of approximately 7.2 g per kg body mass (unpublished), independent of fish size or diet composition. This value was taken as an indicator for the requirement.
Vitamins, minerals and amino acids were added to avoid a lack of any nutrient except phosphorus. Calcium carbonate was added in the first three diets because calcium supply from the Di-CaP had been left out there.

To investigate the influence of dietary phosphorus concentration on feed intake, diets were fed twice a day to satiation.
Fish of each tank were weighed in groups every two weeks with the experiment lasting 54 days at an average water temperature of 26 °C. After 54 days , twenty fish were randomly selected from each tank, ten being used for whole body analysis, and ten were dissected to determine the location of phosphorus in the body.
Two test fish of about 140 g each, that previously had been fed with a standard fish meal diet, were dissected into head, bones, fins, muscles, skin and guts and the individual phosphorus concentrations of each tissue were then determined (Table 6). This was done in order to define the later grouping of soft and hard tissues.

Table 6: Distribution of phosphorus in different body tissues of two test fish

	head	bones	fins	muscles	skin	guts	sum
% of body weight	16.0	5.3	2.4	56.0	11.1	9.2	100.0
g P per kg tissue	17.5	36.4	32.0	2.1	7.1	3.0	
g P per kg body mass	2.8	1.9	0.8	1.2	0.8	0.3	7.8
% of P in total body	36	25	10	15	10	4	100

As a result of the analysis of Table 6, head and fins were put together with the bones as hard tissue and muscles, skin and guts were defined as soft tissue.
The dissection technique is described in chapter 3.1.4 in detail.

4.2.2 Results

During the experiment, 23 fish died of no perceptible reasons. A connection between treatment and mortality was not observed (Table 7, Table A 2).

Table 7: Average survival of seabream in Experiment 2

Treatment	diet 1	diet 2	diet 3	diet 4	diet 5	diet 6	diet 7
Survival [%]	97.5	96.3	93.8	93.8	98.8	96.3	95.0

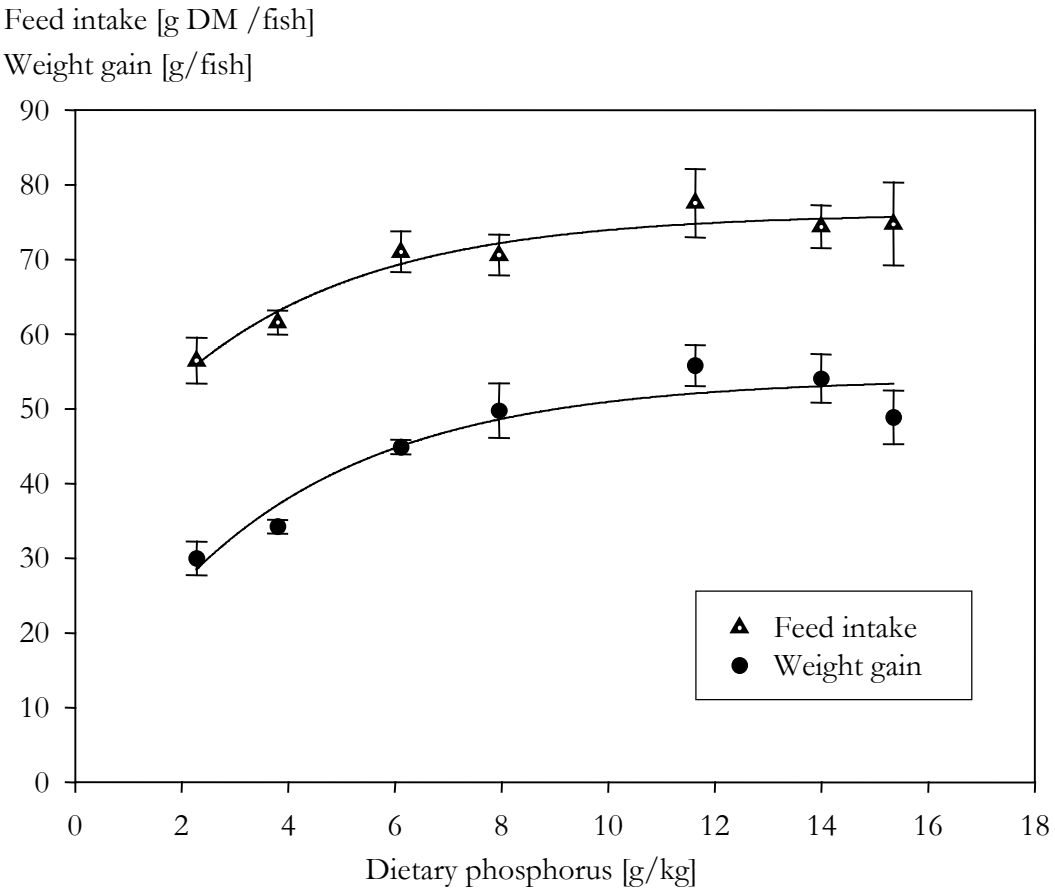

Figure 4: Effect of dietary phosphorus on feed intake, weight gain and FCE in Experiment 2

The curves for feed intake and weight gain were obtained using formula [7]:

$$y=a(1-e^{-b(x-c)}) \qquad [7]$$

Both traits increased with rising dietary phosphorus concentration as shown in Figure 4. No further improvement could be achieved above diet 5 which contained 11.6 g P/kg DM. Feed intake reached a plateau of 76 g per fish and weight gain of 54 g per fish on average.

Lipid concentration in gain was expressed using formula [8]:

$$y = a + be^{kx} \quad [8]$$

x = dietary phosphorus concentration
a = 167 ± 7
b = 208 ± 30
k = -0.269 ± 0.062
r^2 = 0.90

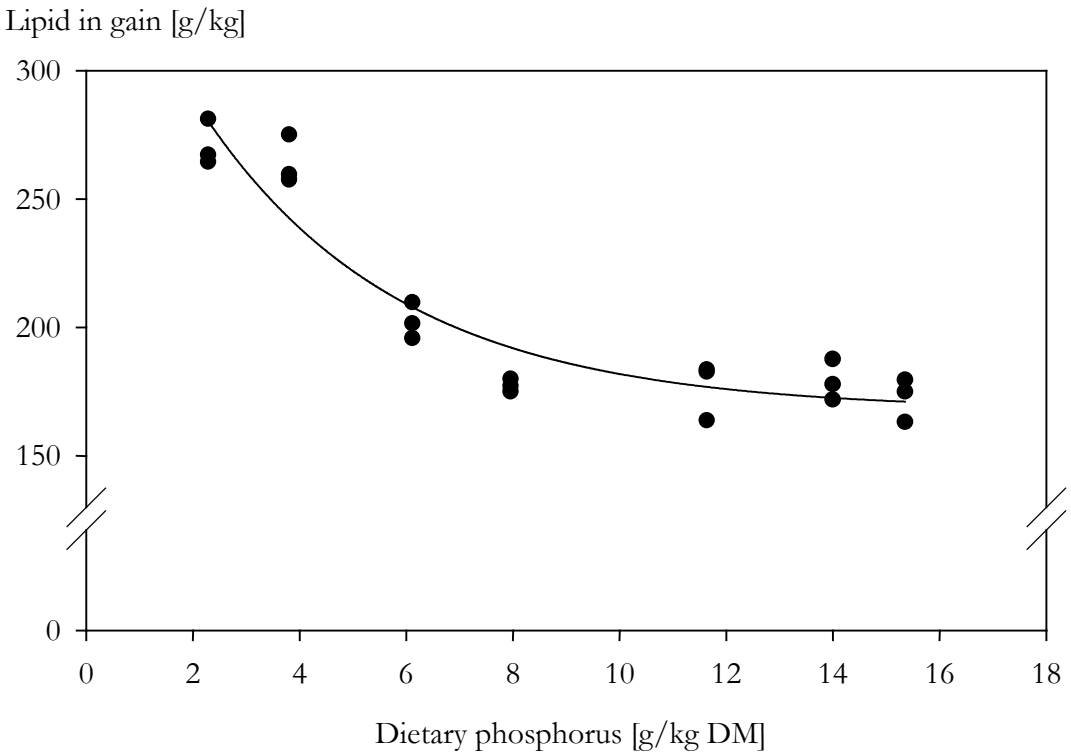

Figure 5: Effect of dietary phosphorus on lipid concentration in gain in Experiment 2

Lipid concentration in the gain showed an inverse dependence on dietary phosphorus concentration (Figure 5), as fish undersupplied with phosphorus gained more lipid in relation to other biomass. No influence of the treatment on protein gain was observed as protein concentration

remained nearly constant over all groups at about 165 g per kg (Table A 3). In the treatments containing sufficient phosphorus, lipid concentrations in the gain approached a plateau value of about 167 g per kg weight gain.

Dietary phosphorus had a strong influence on phosphorus concentration in the gain (Figure 6). Fish fed diet 1 accumulated about 2 g phosphorus per kg weight gain, which is about one fourth of the plateau value of 7.98 g phosphorus per kg gain. The plateau value agrees with collected data from several analyses of phosphorus concentration in the body done at NCM, which ranged from 6.0 to 8.3 g per kg (unpublished).

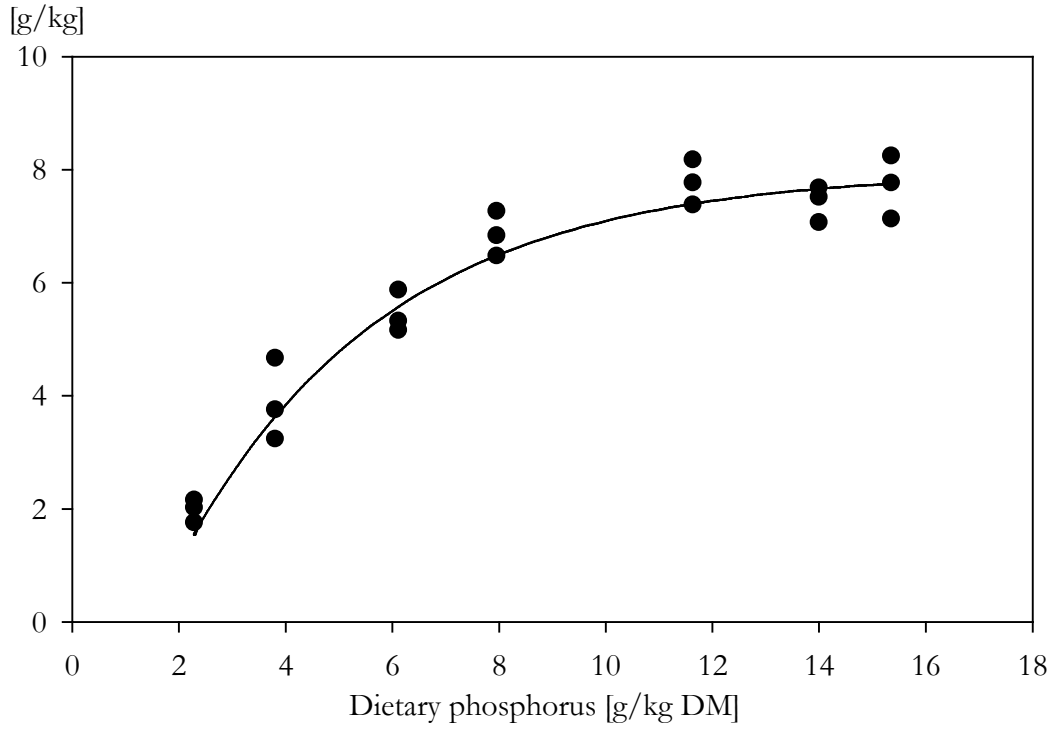

Figure 6: Effect of dietary phosphorus concentration on phosphorus concentration in gain in Experiment 2 (seabream Di-CaP)

In Figure 7 the concentrations of major elements are represented in lipid-free gain, because phosphorus deficiency caused an increased lipid accumulation (Figure 5).

For all major elements a relation to dietary phosphorus level was observed, although their dietary supply was sufficient in all treatments (except phosphorus) (Table 5).

All regression parameters are summarized in Table 8, in which column *a* represents the respective plateau values and column *c* represents the required phosphorus intake for zero mineral gain. Therefore, a minimal phosphorus intake seems to be necessary for any mineral deposition.

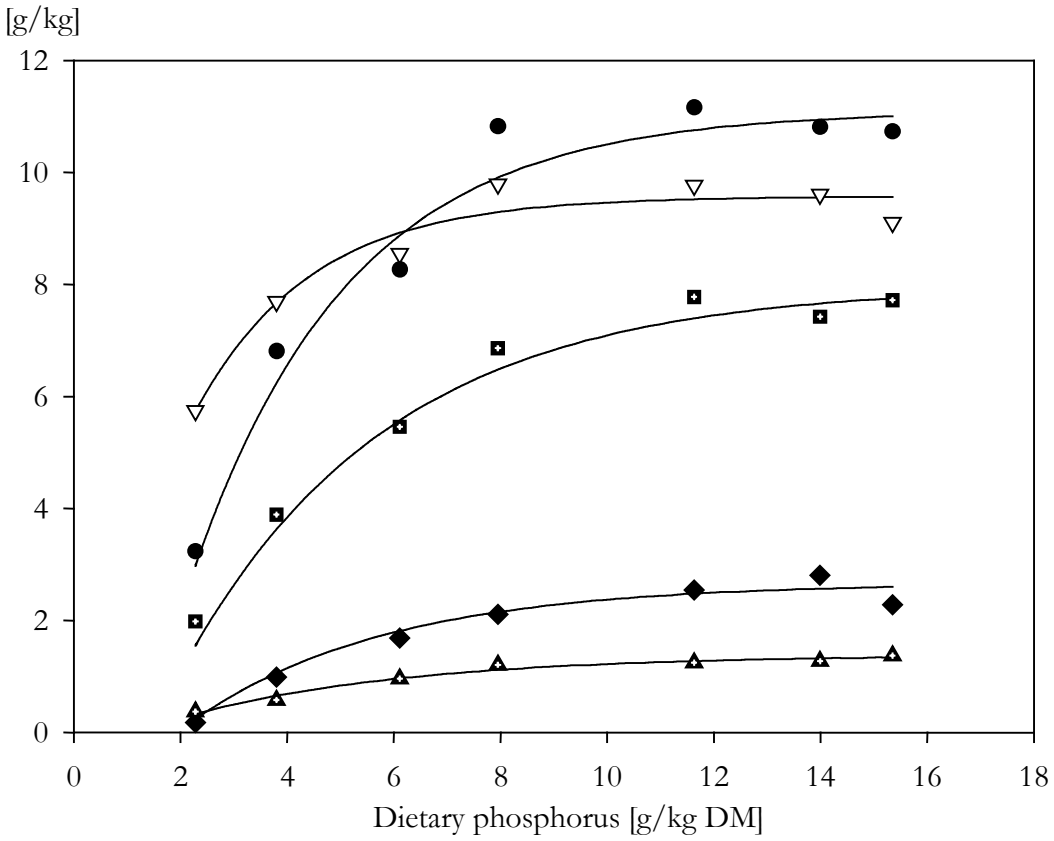

Figure 7: Effect of dietary phosphorus concentration on concentration of minerals in lipid-free gain in Experiment 2 (seabream Di-CaP)

Table 8: Estimated parameters using equation [7] for some traits in Experiment 2

Trait	*a*	*b*	*c*	r^2
feed intake [g DM/fish]	76.18 ± *1.76*	0.29 ± *0.09*	-2.35 ± *1.48*	0.82
weight gain [g/fish]	54.12 ± *1.95*	0.27 ± *0.07*	-0.48 ± *0.81*	0.87
P in gain [g/kg]	7.98 ± *0.26*	0.26 ± *0.04*	1.21 ± *0.25*	0.96
P in lipid-free gain [g/kg]	9.56 ± *0.28*	0.28 ± *0.04*	1.06 ± *0.26*	0.95
Ca in lipid-free gain [g/kg]	13.3 ± *1.0*	0.41 ± *0.21*	1.26 ± *0.76*	0.58
K in lipid-free gain[g/kg]	11.5 ± *0.5*	0.69 ± *0.54*	0.59 ± *1.43*	0.39
Na lipid-free in gain [g/kg]	3.20 ± *0.46*	0.28 ± *0.16*	1.98 ± *0.68*	0.68
Mg lipid-free in gain[g/kg]	1.67 ± *0.07*	0.25 ± *0.50*	0.93 ± *0.38*	0.93

The distribution of phosphorus accumulation between the hard and soft tissues is represented in Table 9, derived from the raw data listed in Table A 4. The undersupply of phosphorus caused a depressed growth, but the ratio between ht and st was not affected. However, the composition of

the tissues differed considerably among the treatments. Phosphorus concentration in hard tissue was up to 10 times higher when it was provided sufficiently as, compared to the group of lowest phosphorus supply. On the other hand, the phosphorus concentration in soft tissue gain was only reduced by half when phosphorus was insufficient. As a consequence the phosphorus accumulated in the hard tissue was reduced to 20 % in the first treatment, in comparison to more than 60 % under sufficient phosphorus supply in diet 3-7 (Table 9, last row).

Table 9: Distribution of phosphorus between hard and soft tissues in Experiment 2

	diet 1	diet 2	diet 3	diet 4	diet 5	diet 6	diet 7
Gain of tissue [g/fish]							
ht	5.2 ± *0.3*	6.0 ± *0.7*	7.9 ± *0.3*	8.9 ± *0.4*	10.9 ± *0.3*	10.3 ± *1.0*	9.8 ± *1.3*
st	23.0 ± *0.5*	26.9 ± *1.1*	33.4 ± *1.9*	37.5 ± *2.3*	44.4 ± *1.4*	38.9 ± *7.3*	37.2 ± *5.6*
P-concentration in tissue gain [g/kg]							
ht	2.2 ± *2.2*	11.1 ± *0.8*	19.0 ± *1.1*	21.7 ± *1.4*	23.0 ± *0.4*	23.9 ± *0.7*	23.1 ± *1.7*
st	1.7 ± *0.1*	2.3 ± *0.2*	2.7 ± *1.1*	3.3 ± *1.4*	3.1 ± *0.4*	3.4 ± *0.7*	3.9 ± *1.7*
P-accretion in tissue [mg/fish]							
ht	11 ± *11*	67 ± *11*	151 ± *13*	193 ± *17*	251 ± *2*	246 ± *22*	225 ± *3*
st	39 ± *4*	61 ± *9*	89 ± *3*	124 ± *12*	138 ± *10*	135 ± *39*	143 ± *12*
% of total P-accretion in ht							
	20 ± *14*	52 ± *4*	63 ± *1*	61 ± *2*	65 ± *2*	65 ± *8*	61 ± *2*

Figure 8 shows the effect of dietary phosphorus level on efficiency of phosphorus retention.
The curve for phosphorus efficiency was obtained by the quotient of the respective curves for retention and intake:

$$y_{Efficiency} = \frac{y_{Retention}}{y_{Intake}}$$

Intake of phosphorus fitted to the function

Intake [g P/fish] = 0.083 (± 0.002)x – 0.058 (± 0.018) $r^2 = 0.99$ [13]

Phosphorus retention was predicted using equation [9]:

$$y = \frac{a + [b(1+c) - a]e^{-kx}}{1 + ce^{-kx}} \qquad [9]$$

x = dietary phosphorus concentration [g/kg]

a = 0.419 ± 0.004

b = -0.020 ± 0.014

c = 6.78 ± 2.01

k = 0.455 ± 0.035

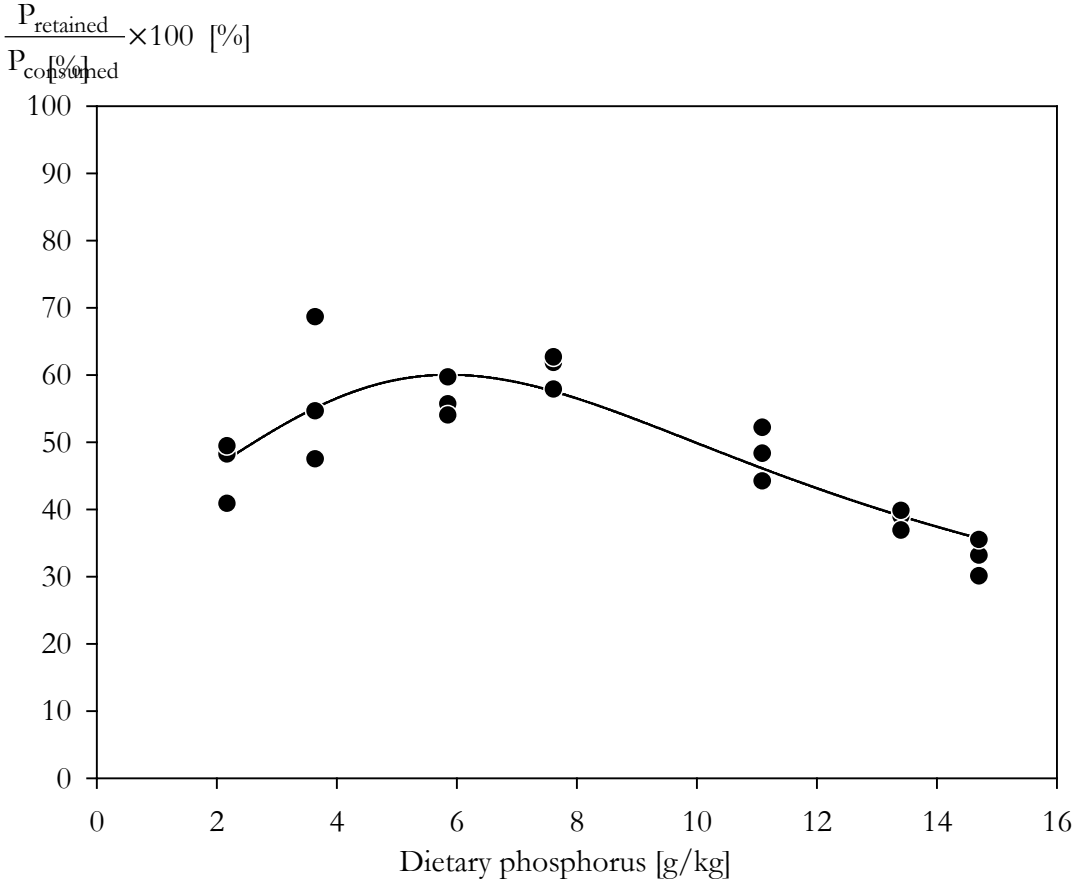

Figure 8: Efficiency of phosphorus retention in Experiment 2

Phosphorus efficiency in Diet 1 was about 47 %. Maximal efficiency of about 60 % corresponds to a concentration of dietary phosphorus of about 4.4 g phosphorus per kg diet (Figure 8).

4.3 Experiment 3 – monocalcium phosphate in gilthead seabream

4.3.1 Procedure

Experiment 3 was carried out nearly in parallel to Experiment 2 (chapter 4.2).
Due to the results of Experiment 2, dissection into hard tissue and soft tissue was not necessary.
Diet composition is listed in Table 10.

Table 10: Composition and analysed concentrations in diets of Experiment 3

	diets							
	1	2	3	4	5	6	7	8
Component [g/kg]								
Wheat gluten				500.0				
Wheat				174.0				140.0
Fish meal								670.0
Fish oil				200.0				180.0
Vitaminmix				10.0				5.0
Mineralmix				25.0				
Lysine				21.0				
Methionine				5.2				
Arginine				4.2				
Threonine				4.5				
Choline chloride				5.0				5.0
Calcium carbonate	8.9	4.8	0.8					
Silicate binder	42.2	39.4	36.6	30.5	23.6	16.8	9.9	
Mono-CaP		6.9	13.7	20.6	27.5	34.3	41.2	
Analysed concentrations per kg DM								
Crude protein [g]	516	515	513	506	503	512	512	499
Crude lipid [g]	213	209	227	234	201	209	202	209
Gross energy [MJ]	23.1	23.3	23.2	22.9	22.6	23.0	23.0	23.0
Ash [g]	79.0	77.2	77.0	77.1	75.9	76.4	72.5	115.6
Phosphorus [g]	2.3	3.1	4.3	6.2	8.0	9.8	11.5	18.2

Supplementation of Mono-CaP followed the same procedure as in Experiment 2 (chapter 4.2.1). Analysis of major elements except phosphorus was not carried out since their concentrations in the diets were supposed to be sufficient and similar to the Di-CaP-diets containing the same components.
The phosphorus digestibility of Mono-CaP was determined to 93 % in rainbow trout (GREGUŠ 2000). This value was taken as a basis for the calculation of the expected limits of digestible dietary phosphorus concentration at supplementation of Mono-CaP.
The experiment lasted 92 days at an average water temperature of 23 °C.

4.3.2 Results

During the experiment 19 fish died. Regarding the mortalities of all groups, no dependence on the treatment could be observed (Table 11).

Table 11: Average survival in Experiment 3

Treatment	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	Diet 6	Diet 7	Diet 8
Survival [%]	91.0	97.4	97.4	100.0	94.9	98.7	97.4	98.7

Although Diet 8 had a completely different composition than Diets 1-7, it will be evaluated and represented in the regression curves together with the other diets, because its content of protein and digestible energy was close to the other diets.
The effect of dietary phosphorus concentration on weight gain and FCE was similar to the Di-CaP growth experiment (Figure 9).
At phosphorus deficiency a dependency of weight gain and FCE on the concentration of dietary phosphorus was observed until it reached a plateau value of about 10 g phosphorus per kg diet.

Regarding the major elements, only phosphorus and calcium concentrations in gain were affected by dietary phosphorus concentration (Figure 10). For concentrations in gain of magnesium, sodium and potassium no clear dependency on dietary phosphorus concentration was observed (Table A 6). However, the regression curves for phosphorus and calcium showed a high coefficient of determination, which indicates a close connection between phosphorus and calcium retention in the body.

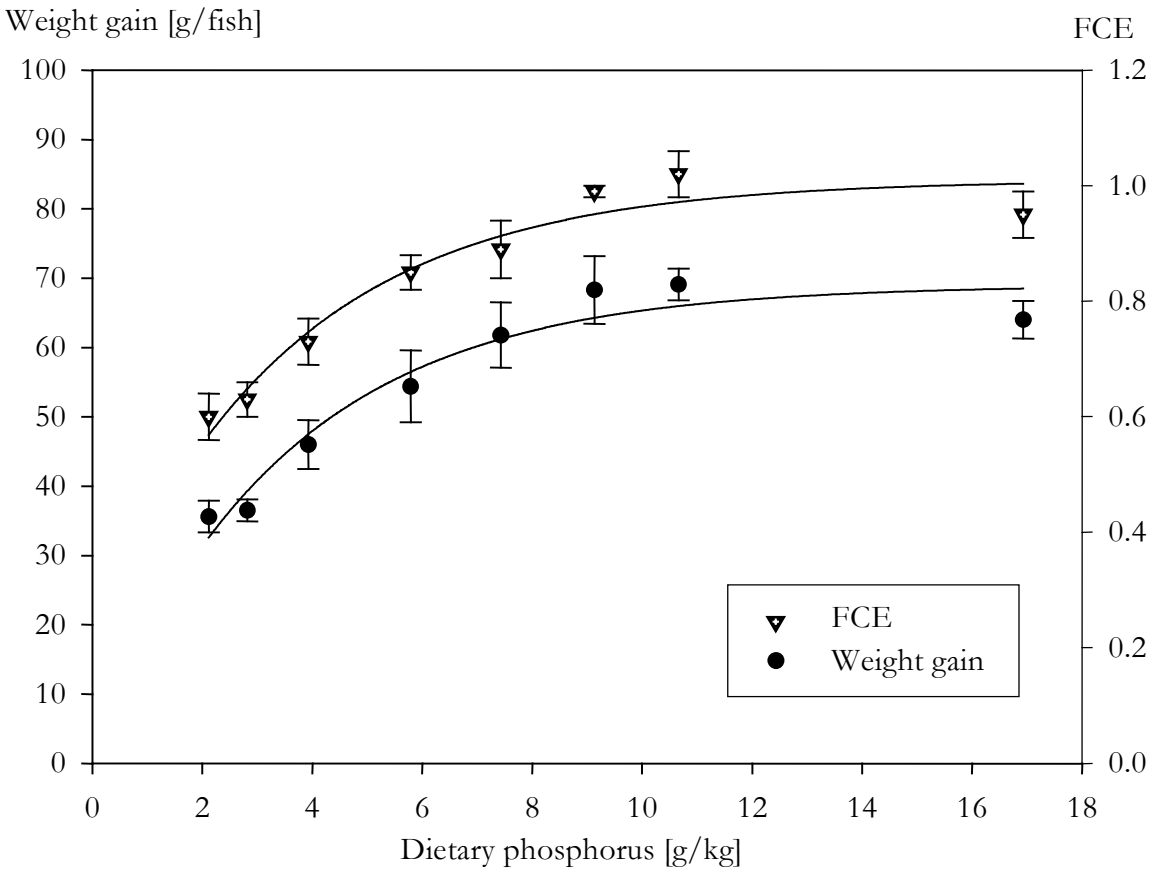

Figure 9: Effect of dietary phosphorus concentration on weight gain and FCE in Experiment 3

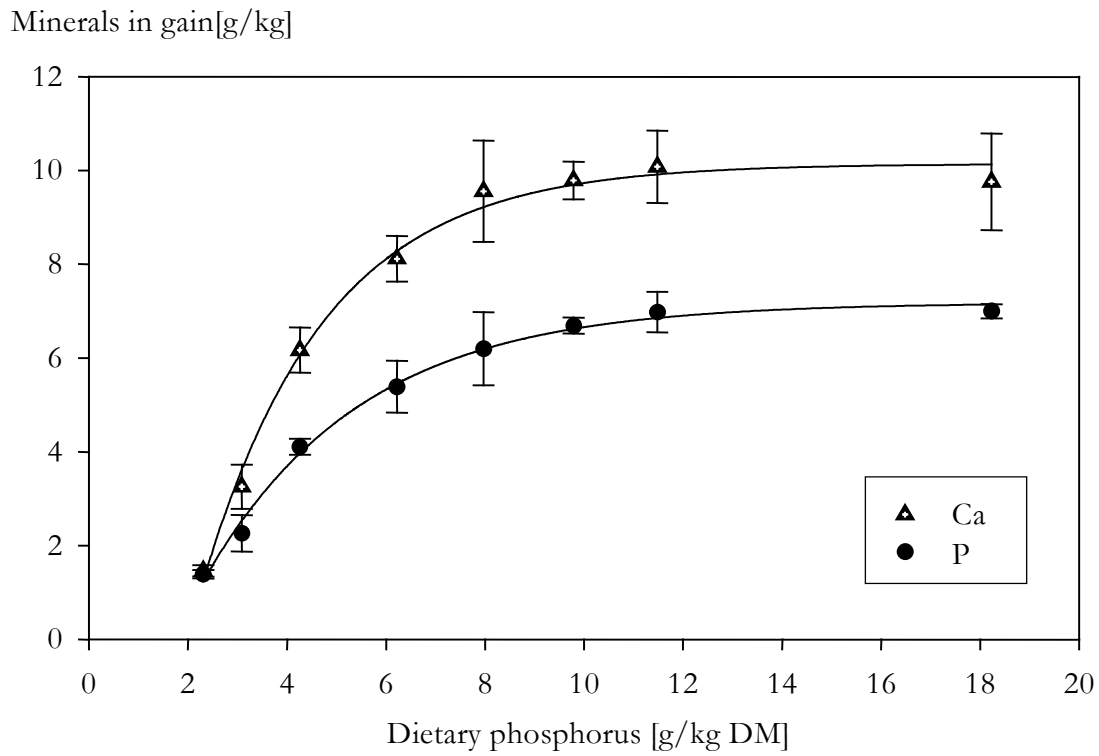

Figure 10: Effect of dietary phosphorus on concentration of phosphorus and calcium in gain in Experiment 3

All regression curves were derived from formula [7]. Like in Experiment 2, the intersection point with the x-axis (parameter c) was negative for weight gain and FCE, and positive for phosphorus and calcium concentration in gain (Table 12).

The highest FCE value of 1.01 kg weight gain per kg feed intake exceeded the FCE of Experiment 2 by far, where only 0.68 kg gain per kg feed intake were achieved (Table 8).

Table 12: Estimated parameters using formula [7] for some traits in Experiment 3

trait	*a*	*b*	*c*	r^2
Weight gain [g/fish]	68.97 ± 2.49	0.29 ± 0.06	-0.073 ± 0.53	0.90
FCE	1.01 ± 0.03	0.29 ± 0.05	-0.77 ± 0.56	0.92
P in gain [g/kg]	7.17 ± 0.19	0.34 ± 0.03	1.55 ± 0.14	0.97
Ca in gain [g/kg]	10.14 ± 0.26	0.40 ± 0.05	1.98 ± 0.12	0.96

4.4 Experiment 4 (Di-CaP and Mono-CaP digestibility in seabream)

Digestibility of phosphorus in seabream was determined separately in large fish. The treatments consisted of four diets containing different concentrations of Di-CaP, one diet supplemented with Mono-CaP and a control diet. All diets contained the same amount of basal mix. Phosphate sup-plements were exchanged for a silicate binder (Sipernat 50 S, Table A 18).

The level of dietary phosphorus in the Mono-CaP diet was calculated to achieve highest efficiency according to the results of Experiment 3 (Figure 20).

Composition and analysed concentrations of the diets are summarized in Table 13.

Digestibility of protein was very high and showed no large deviations among the treatments. Digestibility of dietary energy was determined only for the control diet and Mono-CaP only, since not enough faeces for energy analysis was obtained in the other treatments. However, an influence of dietary phosphorus concentration on energy digestibility was not observed.

Table 13: Components and analysed concentrations in the diets of Experiment 4

	Diet					
Component [g/kg]	Control	Mono-CaP	Di-CaP 1	Di-CaP 2	Di-CaP 3	Di-CaP 4
Wheat gluten	500					
Wheat	140					
Fish oil	200					
Vitamin premix	5					
Choline chloride	5					
Chromium oxide	8					
Binder	142	122	129.5	104.5	79.5	67
Mono-CaP	-	20	-	-	-	-
Di-CaP	-	-	12.5	37.5	62.5	75
Analysed concentrations per kg DM						
Crude protein [g]	443	444	434	481	468	454
Organic matter [g]	854	844	853	859	856	851
Gross energy [MJ]	20.6	20.5	21.1	21.0	21.4	21.9
Chromium oxide [g]	8.41	7.15	9.32	9.62	9.71	9.32
Phosphorus [g]	2.53	6.70	5.61	9.07	13.98	16.11

The phosphorus digestibility of 44 % in the control agreed with the phosphorus efficiency of 47 % in the control diet of Experiment 2 (Figure 8). Regarding the analysed concentrations of the control diet (Table 13), it provided a concentration of digestible phosphorus of about 1.1 g per kg diet, which excludes regulatory excretion. The low digestibility derived from indigestible phosphorus and from the amount of phosphorus, which is digested but excreted inevitably due to the physiology of the fish. In the phosphate supplemented test diets phosphorus digestibilities were less affected by this inevitable loss. The partial digestibilities of Mono-CaP and Di-CaP 1 were in an almost equal range of about 90 %. With increasing dietary phosphorus concentration the phosphorus digestibilities were influenced by rising regulatory excretion, resulting in a decrease of partial phosphorus digestibility from 91 to 70 % (Diet Di-CaP 1-4).

Table 14: Apparent digestibility coefficients in Experiment 4

	Diet					
Digestibility [%]	Control	Mono-CaP	Di-CaP 1	Di-CaP 2	Di-CaP 3	Di-CaP 4
Phosphorus	44 ± 10	72 ± 2	68 ± 3	69 ± 6	66 ± 1	66 ±1
P from Mono-CaP / Di-CaP	-	88 ± 3	91 ± 6	78 ± 8	71 ± 1	70 ± 2
Organic matter	70 ± 3	75 ± 3	77 ± 2	78 ± 3	77 ± 4	80 ± 2
Crude protein	95 ± 1	96 ± 0	96 ± 1	96 ± 1	97 ± 1	98 ± 0
Energy	79 ± 1	82 ± 1	n. d.	n. d.	n. d.	n. d.

4.5 Experiment 5 – digestibility of rapeseed oilmeal in gilthead seabream

4.5.1 Procedure

Subsequently to the seabream growth experiments a digestibility determination of rapeseed oilmeal with (RS +) and without (RS -) supplementation of microbial phytase was carried out. Rapeseed oilmeal was the only phosphorus source in the diet (Table 15). Therefore, no differentiation in digestibility of test diet and test component was necessary.
Fish were fed to satiation twice a day. After adjusting the fish to the test diets for 5 days they were stripped every second day for three weeks.

4.5.2 Results

The results of the digestibility determinations in this experiment are summarized in Table 16.
Supplementation of microbial phytase increased phosphorus digestibility from 50 % to 84 %. The phosphorus digestibility of Diet RS + is in agreement with the digestibility of rapeseed oilmeal of 83 % performed in trout (Experiment 1, Table 4). Digestibility of organic matter was lower than in Experiment 1 for all treatments. The digestibility of protein and energy of the basal mix in Experiment 1 had been determined at 96 and 73 % (GREGUŠ 2000). In Experiment 5, digestibility of energy was in the same range as in Experiment 1. Protein digestibility was found significantly lower at about 80-84 %, which may be the reason for the lower digestibility of organic matter.

Table 15: Dietary composition and analysed concentrations in Experiment 5

	Diets	
per kg diet	RS -	RS +
Rapeseed oilmeal [g]	782	
Fish oil [g]	200	
Vitamin premix [g]	5	
Choline chloride [g]	5	
Chromium oxide [g]	8	
Phytase[1] [FTU]	-	2000
Analysed concentrations [g/kg DM]		
Phosphorus	9.80	11.90
Chromium oxide	6.46	6.32

[1]BASF Natuphos®

Table 16: Apparent digestibility coefficients in Experiment 5

Digestibility	Treatment	
[%]	RS -	RS +
Phosphorus	50 ± 1	84 ± 4
Organic matter	51 ± 1	52 ± 3
Protein	84 ± 1	80 ± 1
Energy[2]	73	68 ± 2

[2] Sample material of one RS- treatment was not enough for energy analysis

4.6 Experiment 6a and 6b – trout and seabream experiments with phytase

4.6.1 Procedure

Two growth experiments with rainbow trout and gilthead seabream were carried out in parallel using the same diets. The diets were based on soyprotein concentrate and were gradually supplemented with increasing concentrations of supplemental *Aspergillus niger*-phytase.

Targets of this study were

1. the evaluation of soyprotein concentrate supplemented with microbial phytase as an alternative to fish meal
2. a comparison of phosphorus and energy metabolism between the two species rainbow trout and gilthead seabream.

Composition of the experimental diets is listed in Table 18.

Table 17: Comparison between Experiment 6a (trout) and 6b (seabream)

	Rainbow trout	Gilthead seabream
Location	Bonn	Eilat
Beginning and End	19.04. – 10.06.2001	06.06. – 12.09.2001
Duration	53 days	98 days
Water temperature	15 °C	26 °C
Fish per tank	20	24
Initial weight per fish [g]	100	60
Final weight per fish [g]	207 – 256	87 – 120

Table 18: Composition and analysed concentrations in the diets of Experiments 6a and 6b

	diets							
	A	B	C	D	E	F	G	H
Component [g/kg]								
Soyprotein concentrate	520							
Fish oil	190							
Gelatinized wheat starch	200							
Mineral premix	30							
Vitamin premix	5							
Choline chloride	5							
Silicate binder	50							
Phytase[1] [FTU/kg]	0	200	400	600	900	1200	2000	20000
Analyzed concentrations per kg DM								
Crude protein [g]	383	370	376	382	378	381	365	387
Ash [g]	93.9	94.6	94.5	95.0	94.8	93.8	93.9	95.7
Gross energy [MJ]	21.7	21.5	21.7	22.1	22.2	22.1	22.2	21.8
Phosphorus [g]	4.34	4.47	4.65	4.63	4.57	4.65	4.12	4.43
Chromium oxide [g]	3.96	n.d.	n.d.	4.22	n.d.	4.19	n.d.	4.62
Phytase [FTU]	<70	<70	80	330	560	940	1580	18960

[1]BASF Natuphos®

The process of feed production as described in chapter 3.2.2 was developed by BASF especially for this experiment. The main issue during the process was to ensure the stabilization of microbial phytase in the feed. This approach made feed production in large amounts feasible. The diets were finalized in a suitable version for a commercial use.

To investigate a potential detriment of an overdose of phytase, one diet containing a phytase concentration of 20.000 FTU per kg feed was added.

Each diet was fed to satiation to triplicate groups of fish. During the trout experiment faeces were collected three times for two weeks respectively – in the beginning, in the middle and in the end. In Eilat the possibility of faeces collection from the growing fish was not available. The digestibilities of Diets A, C, E and H were determined separately in large fish (chapter 3.1.1 and chapter 3.1.3).

4.6.2 Results

None of the trout, but ten seabream died during the experiment of no perceptible reasons (Table 19). Additionally all the fish in one tank died due to a parasite infection on August 19th so this tank was excluded from the statistical analysis.

Table 19: Average survival in Experiment 6b

Treatment	diet A	diet B	diet C	diet D	diet E	diet F	diet G	diet H
Survival [%]	100.0	100.0[2]	93.1	98.6	98.6	98.6	98.6	98.6

[2]not including tank 31

Neither in trout nor in seabream any negative influence of overdosed phytase was observed.

All curves of the growth parameters were calculated using formula [7].

Trout showed superior growth performances to seabream. In 53 days they doubled their initial weights at least. The curves for feed intake, weight gain and FCE in trout show a steep ascent at low dietary phytase supplementation (Figure 11) but beyond a dietary phytase concentration of about 1200 FTU per kg no further improvement was observed.

Despite the higher water temperature and the longer experimental duration, seabream doubled their initial weights only in the last treatment (Figure 12). The curves of weight gain, FCE and feed intake increase less steeply. Raising the dietary phytase concentration from 2000 to 20000 FTU per kg improved all growth parameters considerably.

The measurements of feed intake and weight gain in the trout experiment are much more scattered than the FCE values and the measurements in the seabream experiment. An explanation may be the arrangement of the tanks, where conditions like light intensity and volume vary evidently among the tanks.

Parameter estimates of the curves are summarized in Table 20.

Table 20: Estimated parameters using formula [7] for some traits in Experiments 6a and 6b

trait	*a*	*b*	*c*	r^2
Rainbow trout				
Feed intake	189 ± 8	0.0020 ± 0.0019	-928 ± 939	0.21
Weight gain	150 ± 7	0.0017 ± 0.0009	-744 ± 414	0.49
FCE	0.80 ± 0.01	0.0013 ± 0.0004	-1398 ± 429	0.74
Gilthead seabream				
Feed intake	115 ± 4	0.00039 ± 0.0001	-3063 ± 1000	0.76
Weight gain	59.1 ± 3	0.00025 ± 0.0001	-2341 ± 858	0.83
FCE	0.51 ± 0.02	0.00026 ± 0.0001	-3800 ± 1317	0.81

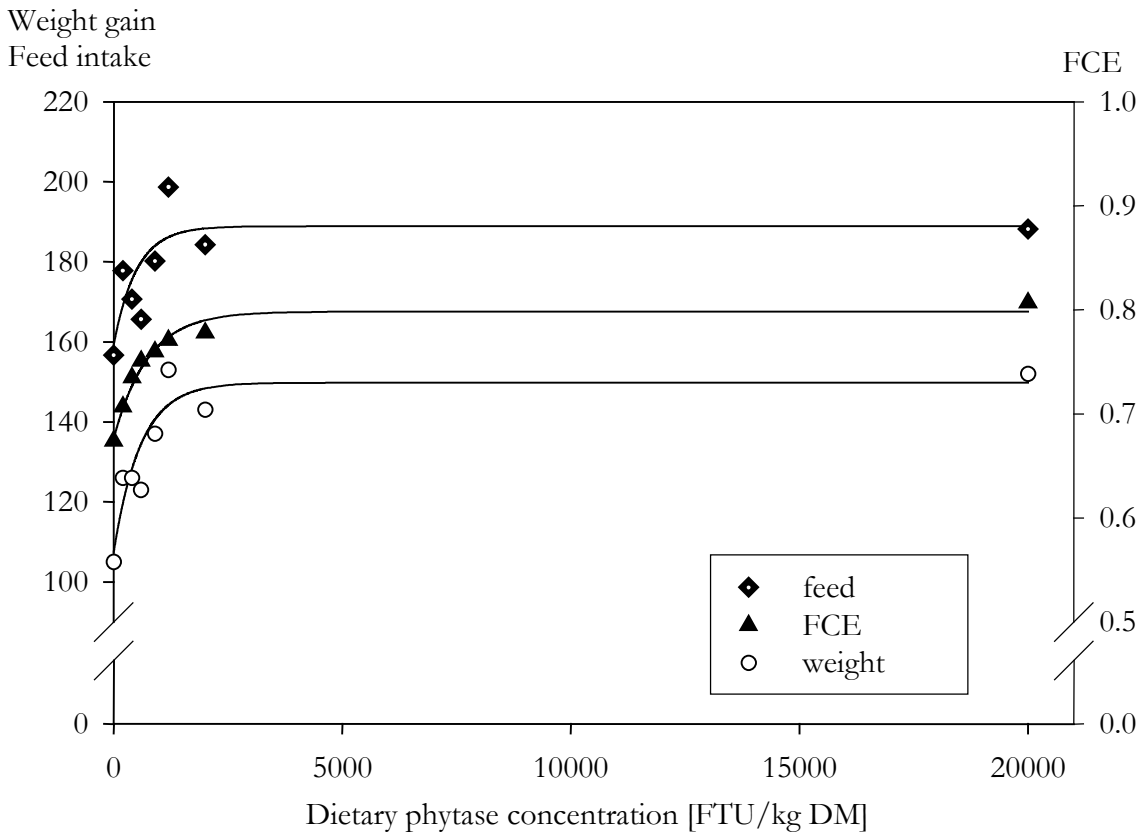

Figure 11: Effect of dietary phytase concentration on growth parameters in Experiment 6a with trout.

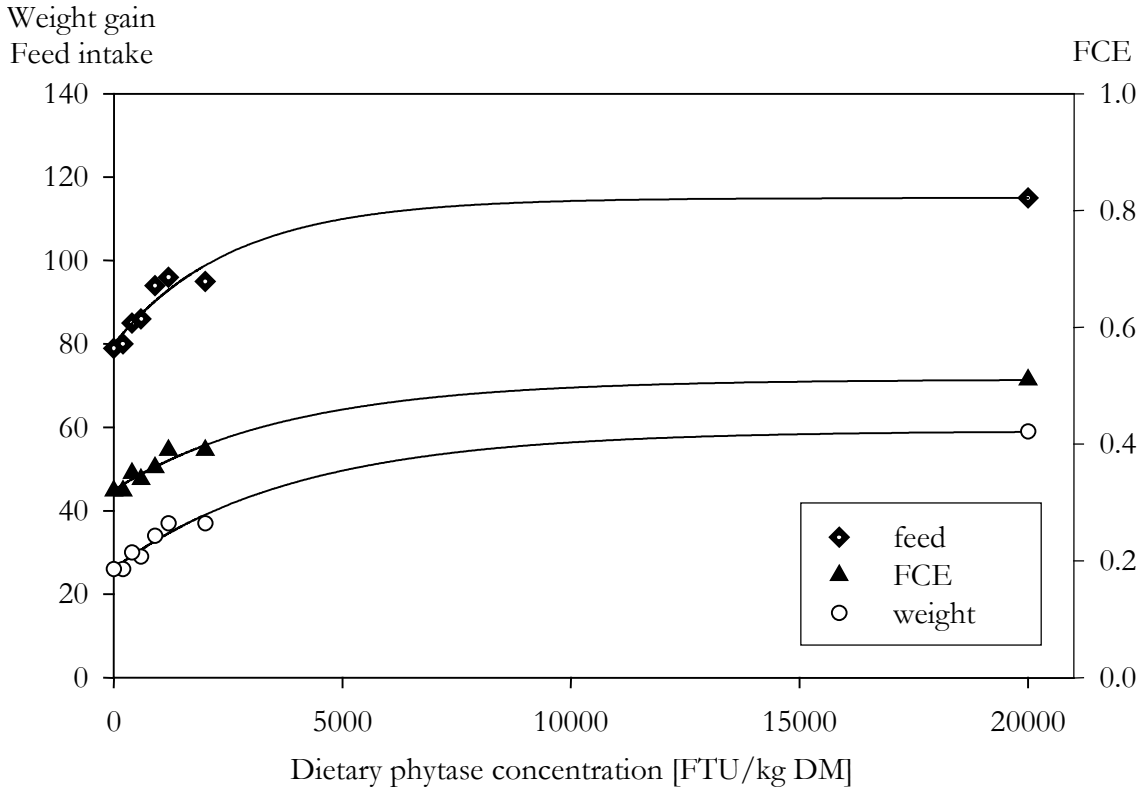

Figure 12: Effect of dietary phytase concentration on growth parameters in Experiment 6b with seabream.

In the trout experiment efficiency of phosphorus retention was determined to be a little higher than phosphorus digestibility in the area of low phytase supplementation (Figure 13). The respective measurements are very close and confirm a parallel increase of these traits in the range of phosphorus undersupply (Rodehutscord *et al.* 2000).

However, an additional way of cleaving the phytate cannot be excluded, since a sprinkler for cleaning the sewage by microbial decomposition is integrated into the partial circulation system in which the trout are kept. These bacteria might be able to cleave parts of undigested phytate and to release orthophosphate into the circulating water. This may explain, why a higher phosphorus efficiency was observed only at low dietary phytase level.

In the seabream experiment phosphorus digestibility was always higher than phosphorus efficiency (Figure 14). Here the large difference between digestibility and efficiency in the area of low dietary phytase is conspicuous. Efficiency of phosphorus retention rose more sharply than phosphorus digestibility. A regression curve for digestibility was not drawn due to the small number of measurements.

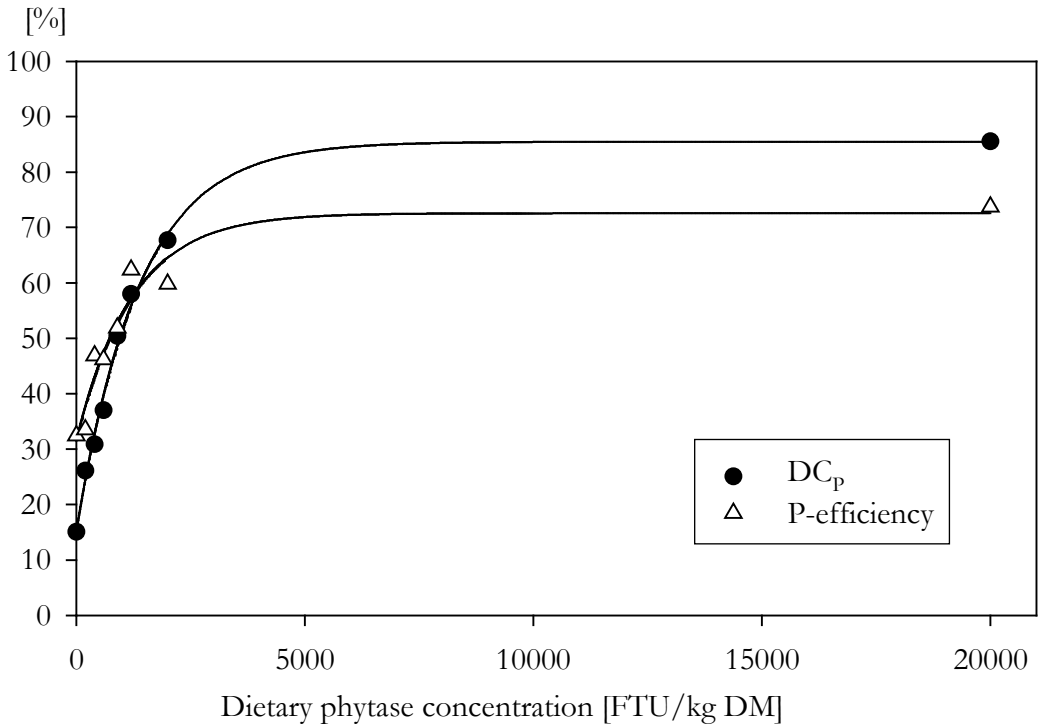

Figure 13: Effect of dietary phytase concentration on digestibility and efficiency of phosphorus in rainbow trout

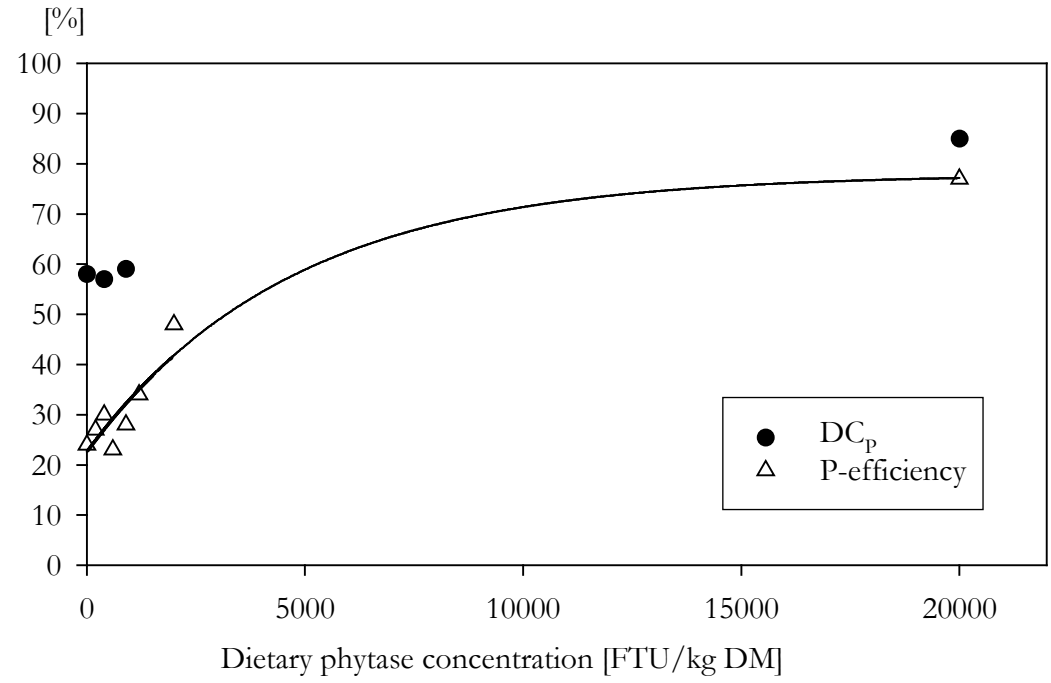

Figure 14: Effect of dietary phytase concentration on digestibility and efficiency of phosphorus in gilthead seabream

5 Discussion

5.1 Validation

Experimental fish were taken from a stock containing fish of identical age and previous feeding. Fish were sorted by size before distributing them randomly among the tanks. Because of the relative homogeneity of the stock, the groups were expected to be of equal weight so the control group was taken as a reference for all groups.

Fish were always weighed in groups. Before each weighing excess water was allowed to drip off the fish by holding them in a net. Water remaining on the fish was not measured as the quantity was very small and was considered to be equal for all groups.

At each feeding, intake was observed carefully. As soon as pellets sank to the bottom untouched by the fish, feeding was stopped. This ensured the complete intake of given feed. The consistency of the pellets prevented disintegration of the feed before being eaten by the fish.

In the seabream experiments faeces were collected by stripping. The strong green colouring of the used marker, chromium oxide, guaranteed a collection of the "right" material.

In the trout experiments a sedimentation unit was used for faeces collection. After reaching the bottom, within seconds faeces were drawn by the circular flow into the drain, then flowed ca. 50 cm through a horizontal pipe as described in chapter 3.2.1 before sinking into the vertical collection tube. Because of the immediacy of gathering and the short distance travelled the washing out of nutrients from the faeces could be excluded.

5.2 Response of gilthead seabream to dietary phosphorus level

In Experiments 2 and 3, inorganic phosphorus was added to almost identical semi-purified diets, providing a sufficient supply with all nutrients, vitamins and minerals except phosphorus.
Both experiments were comparable in number and initial weight of the fish.
The only differences were the respective phosphorus source and the intensity of feed intake. In Experiment 2 feed was given to satiation, in Experiment 3 it was restricted.

5.2.1 Feed intake, weight gain, FCE

In both experiments a positive relationship between all growth parameters and dietary phosphorus concentration was observed. Under phosphorus deficiency weight gain and feed intake were depressed. Whether a lower feed intake caused a decrease in weight gain or vice versa is still open to discussion.
The decreased FCE values under phosphorus deficiency show a less inhibited feed intake than weight gain (Figure 9). Obviously growth was determined by the level of phosphorus supply, as a minimal phosphorus concentration in the body was required. Reduced feed intake was assumed as a consequence of reduced energy demand.

The relationship between dietary phosphorus concentration and growth parameters can be interpreted as a logistic function (equation [7]) as described chapter 2. Up to a dietary phosphorus concentration of approximately 6 g per kg (diets 1-3) the dependency was almost linear. For phosphorus concentrations higher than 10 g per kg (diet 5-7) no further increase in growth performance was attained. For this reason the following discussion refers to the results of the first four treatments.

5.2.2 Lipid and protein

The response of lipid and protein concentration in gain to dietary phosphorus concentration is represented in Figure 15.
Rising dietary phosphorus concentrations decreased the concentrations of lipids in gain. The statistical analysis resulted in a regression curve beginning at 280 g lipid per kg biomass and then

approaching exponentially to a lower limit of 167 g lipid per kg biomass. The lipid curve was obtained using formula [8]:

$$y = a + be^{kx} \qquad [8]$$

x = dietary phosphorus concentration

a = 167 ± 7

b = 208 ± 30

k = -0.269 ± 0.062

r^2 = 0.90

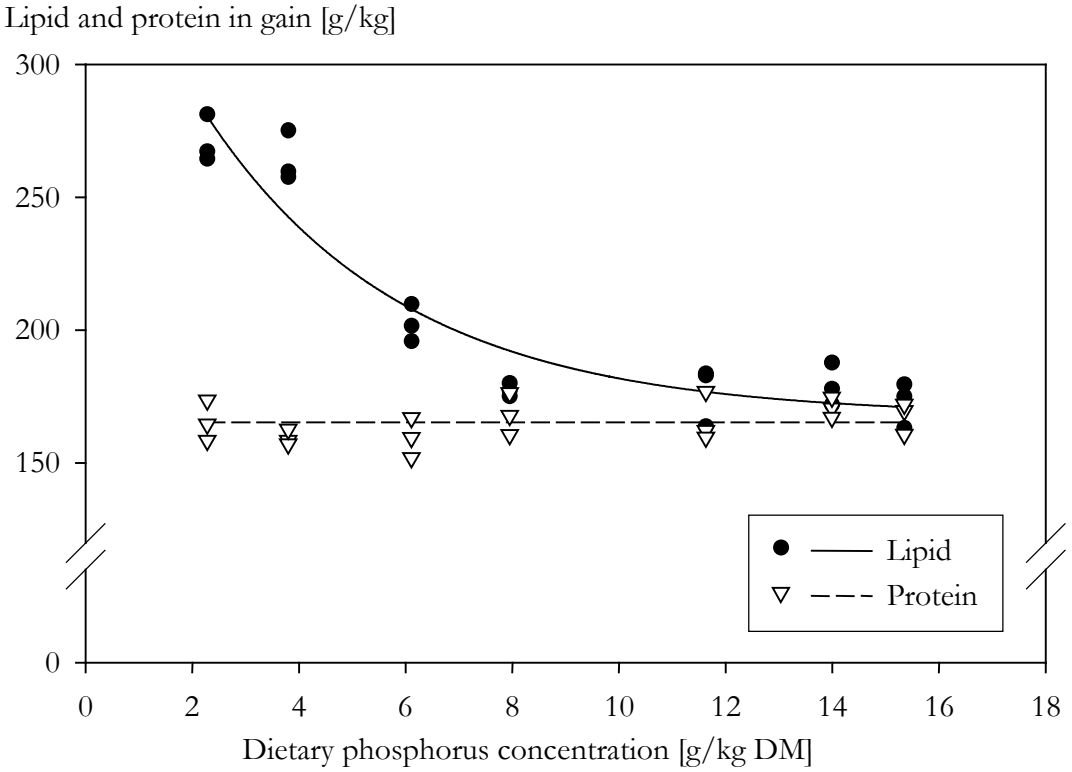

Figure 15: Concentrations of lipids and protein in gain in experiment 2 (DiCaP growth experiment with seabream)

Protein concentration in gain was nearly constant at 165 g per kg biomass and showed no dependency on dietary phosphorus concentration.

This effect indicates a phosphorus deficiency in the intermediary metabolism resulting in superior lipid deposition to total protein deposition. The analyses of hard and soft tissue gain revealed alterations in phosphorus concentration due to phosphorus supply (Table 9). Under phosphorus deficiency, hard tissue contained tenfold lower phosphorus concentrations in comparison to adequate phosphorus supply. In soft tissue, the phosphorus concentrations were only halved.

Almost three quarters of the collected soft tissue consisted of muscle (Table 6). This indicates, that the reduction of phosphorus concentration in protein gain is very limited and protein accretion will be inhibited due to phosphorus deficiency, resulting in a depressed overall growth.
The total depositions of lipid and phosphorus represented in Figure 16 confirm this hypothesis. The regression curve of accreted protein followed equation [7]:

$$y=a(1-e^{-b(x-c)}) \qquad [7]$$

x = dietary phosphorus concentration

a = 9.25 ± 0.49

b = 0.23 ± 0.08

c = -0.75 ± 1.08

r^2 = 0.83

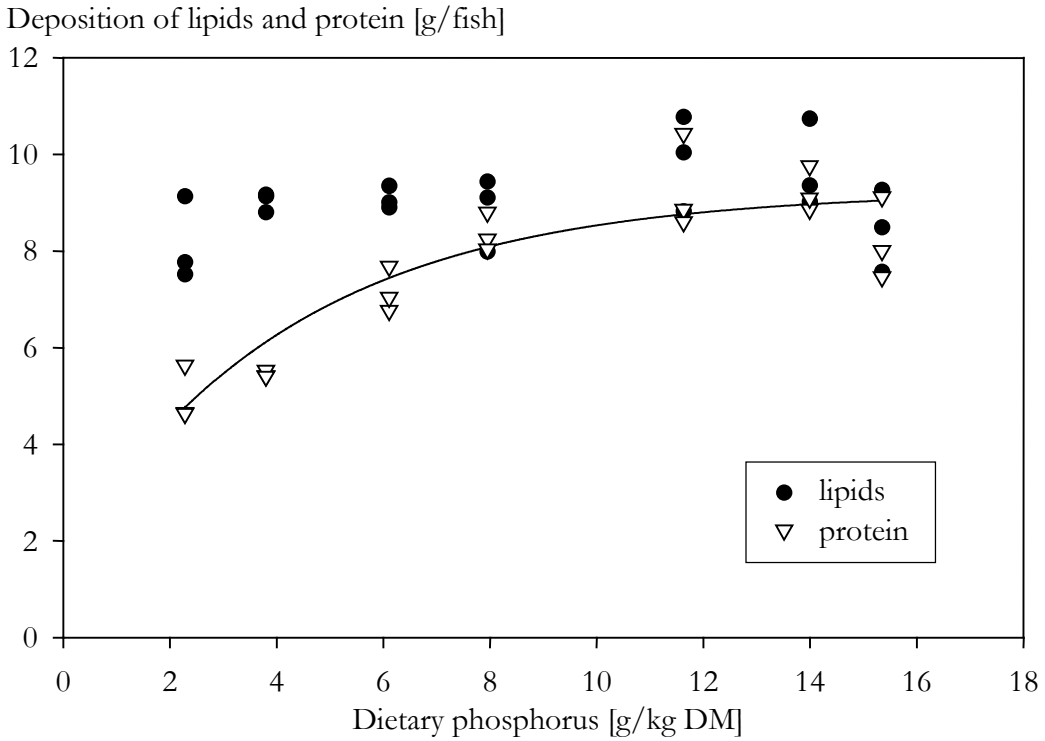

Figure 16: Total lipid and protein deposition in Experiment 2

Lipid accretion was not significantly inhibited under phosphorus deficiency. Feed intake and FCE did not decline equally with depressed protein gain (Table A 2), providing the energy for unabated lipid deposition. The same effect in other fish species had been reported by SAKAMOTO & YONE (1978), CHAVEZ-SANCHEZ *et al.* (2000) and RODEHUTSCORD (1996).

5.2.3 Minerals

Retention of all analysed minerals showed a relationship to dietary phosphorus supply. Decreased phosphorus retention seemed to be a direct consequence of phosphorus deficiency. Also retention of calcium, sodium, potassium and magnesium was impeded at phosphorus undersupply, although these minerals were provided sufficiently (Table 5). Potassium retention was less affected than the other minerals. Calcium, magnesium, and sodium (except the first treatment) retention kept a relative constant relationship with phosphorus retention (Table 21).

Table 21: Relation of phosphorus to calcium in gain in Experiment 2 (seabream Di-CaP)

Treatment	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	Diet 6	Diet 7
P/Ca in gain	0.61	0.57	0.66	0.63	0.70	0.69	0.72
P/Mg in gain	5.35	6.70	5.68	5.68	6.24	5.84	5.62
P/Na in gain	11.0	3.93	3.23	3.26	3.06	2.65	3.38
P/K in gain	0.35	0.51	0.64	0.70	0.80	0.77	0.85

Under phosphorus deficiency, phosphorus was used primarily for other metabolic processes than for ossification. This was confirmed by phosphorus retention in hard tissue and soft tissue (Table 9). Phosphorus retention in hard tissue was substantially more impeded than phosphorus retention in soft tissue. However, the ratio of hard tissue vs. soft tissue did not vary. This indicated a reduction of mineral content in the bones. Obviously the fish were able to compensate for an acute dietary undersupply of phosphorus by reducing phosphorus accretion to the bones. Similar results were observed in rainbow trout by FRENZEL & PFEFFER (1982), RODEHUTSCORD (1996) and GREGUŠ (2000).
However, in Experiment 3 (Mono-CaP experiment) this effect was only observed for phosphorus and calcium retention. Feed restriction may have kept the concentration of retained minerals higher by limiting the whole growth of the fish.

A comparison between the phosphorus concentrations in different body tissues of rainbow trout and gilthead seabream revealed differences of distribution among the individual tissues (Table 22). The values were obtained from fish fed diets adequate in phosphorus before.

Phosphorus concentration in the whole body in seabream was almost twice as high as in rainbow trout. Both species contained approximately equal concentrations of phosphorus in soft tissue. Nevertheless in hard tissue seabream accumulated about a threefold phosphorus concentration in comparison to trout. The relative proportion of phosphorus in soft tissue was twice as high in trout as in seabream.

Regarding the individual fractions, rainbow trout accumulated the largest part of about 40 % of their whole body phosphorus in the muscles. In seabream the main phosphorus part was found in the head (36 %) and bones (25 %).

Table 22: Phosphorus in separate body tissues in rainbow trout and gilthead seabream

	[g P per kg biomass]								
	whole fish	muscles	skin	guts	**st**	head	bones	fins	**ht**
Trout[1]	4.3	1.7	0.4	0.3	**2.5**	0.8	1.0	-	**1.8**
Seabream	7.8	1.2	0.8	0.3	**2.2**	2.8	1.9	0.8	**5.5**
	[% of total phosphorus]								
Trout[1]	100	40	9	8	**58**	20	23	-	**43**
Seabream	100	15	10	4	**29**	36	25	10	**71**

[1] Data from PFEFFER (1978)

5.2.4 Phosphorus requirements

Data of all growth parameters and most of the mineral concentrations in gain can be interpreted statistically in Experiment 2 and 3 by the use of equation [7]. Instead of a definite point for phosphorus requirement this function provides a plateau value in the infinite which represents the theoretical best performance. A recommendation for phosphorus requirements can therefore only be given by deriving a percentage of the respective plateau value. Figure 17 illustrates the corresponding dietary phosphorus concentration (CPC) to 95 % of the plateau value.

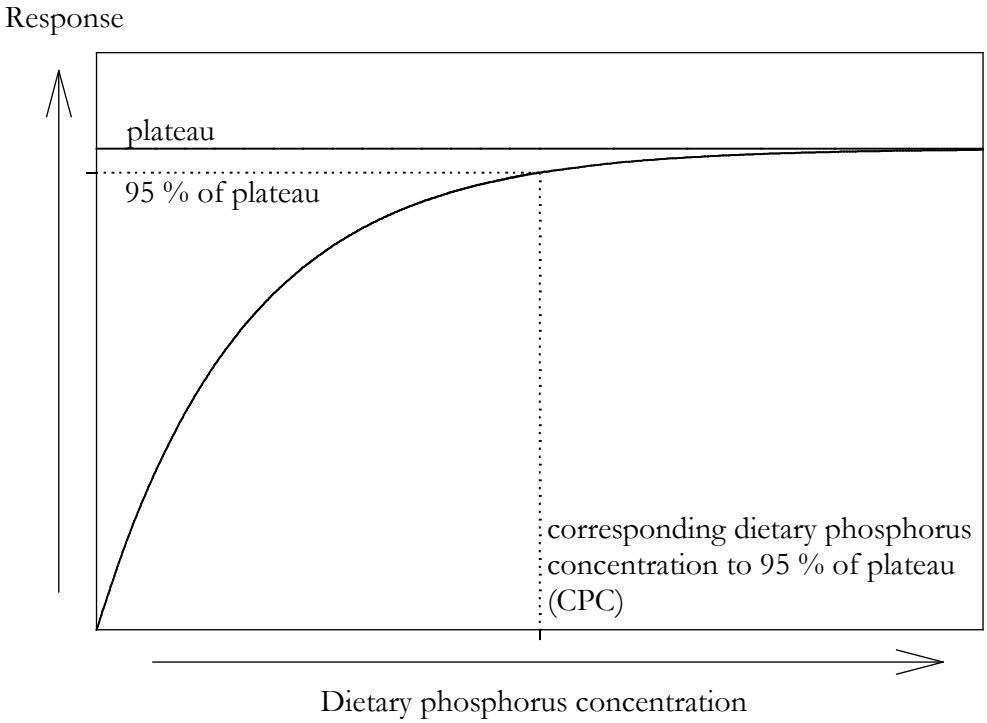

Figure 17: Mathematical approach to determine the phosphorus requirement

In Table 23 the required phosphorus concentrations of 95 % of the plateau values are given.

If 95 % of the plateau value is considered an appropriate level to determine the demand of dietary phosphorus, required dietary phosphorus can be determined for each trait. Table 23 revealed phosphorus requirements depending on the chosen response variable. The respective requirements ranged from about 8.1 to 15.5 g per kg diet, which did not allow a general recommendation for the phosphorus requirement of seabream.

Using Di-CaP as a phosphorus source, a dietary phosphorus concentration of 13 g per kg was necessary for maximum phosphorus gain. In contrast a level of about 11 g per kg was sufficient for maximum weight gain. Thus the phosphorus demand for maximum phosphorus retention exceeded the demand for maximum weight gain. The same effect had been observed in rainbow trout (RODEHUTSCORD 1996).

For the duration of this experiment fish were able to spare phosphorus in their bones up to their individual limit and without any indication of growth depression. However, feeding a diet containing a phosphorus concentration required for maximum growth during a complete grow out period has not yet been tested. The dietary phosphorus concentration for maximum phosphorus gain in soft tissue was determined to approximately 15 g per kg. Providing a lower phosphorus

supply, could eventually cause a cessation of growth or health problems. This issue will be of interest to future research projects.

Table 23: Dietary phosphorus concentrations necessary to attain 95 % of the performance plateau values

Trait	Di-CaP		Mono-CaP	
	95 % of plateau	CPC [g/kg DM]	95 % of plateau	CPC [g/kg DM]
Feed intake	72.4 g	8.1		
Weight gain	51.4 g	10.6	65.5g	10.2
FCE	0.65	8.2	0.96	9.7
P in gain	7.6 g/kg	13.1	6.8 g/kg	11.2
P in hard tissue	22.6 g/kg	9.9		
P in soft tissue	3.6 g/kg	15.5		

In a study reported by PIMENTEL-RODRIGUES & OLIVA-TELES (2001) the effect of rising dietary phosphorus concentration in seabream was analysed, using Di-CaP as a phosphorus source with fish of 5.1 g initial weight over 42 days (Table 24).

Table 24: Effect of dietary phosphorus concentration on weight gain and FCE in gilthead seabream, reported by PIMENTEL-RODRIGUES & OLIVA-TELES (2001)

Dietary phosphorus concentration [g/kg]	3.7	5.7	7.5	9.5	11.0	13.2	15.0
Weight gain[1] [g]	4.1^{a}	6.3^{ab}	8.0^{bc}	7.4^{bc}	8.1^{bc}	9.3^{c}	9.2^{c}
FCE[1]	0.74^{a}	0.90^{ab}	0.92^{b}	0.98^{b}	0.99^{b}	0.99^{b}	1.02^{b}

[1]Figures in the same row followed by a different letter are significantly different at p = 0.05

Since there was no significant increase in weight gain from the third treatment, the authors concluded a demand of 7.5 g phosphorus from Di-CaP per kg diet for gilthead seabream.
By application of the exponential approach, the dietary phosphorus requirement is predicted to be 13.6 g per kg (Table 25), which is much higher than the authors suggested but in agreement with the results of Experiment 2 (Table 23). Obviously there was no change in phosphorus requirement despite the smaller fish size.

Table 25: Estimated parameters using formula [7] for weight gain and FCE and dietary phosphorus requirement to 95 % of the plateau value in the study reported by PIMENTEL-RODRIGUES & OLIVA-TELES (2001)

Trait	*a*	*b*	*c*	r^2	CPC [g/kg]
Weight gain	9.41 ± 0.79	0.24 ± 0.10	1.25 ± 1.11	0.93	13.6
FCE	1.01 ± 0.01	0.36 ± 0.07	-0.05 ± 0.77	0.98	8.36

The predicted requirement depends on the statistical approach (ÅSGÅRD & SHEARER 1997). The authors examined several methods for requirement determination. They considered regression analysis as an objective way to show the relationship between quantitative variables, because regression analysis allows extrapolation. The use of ANOVA is liable to provide lower values for requirements and it is considered a limited expression method for correlated data points.

A dietary phosphorus concentration of about 11 g per kg was necessary for 95 % of the highest growth performance in Experiment 3. At this point the efficiency of ingested phosphorus was calculated to about only 45 %. It was not possible to reduce phosphorus effluents to a minimum without reducing weight gain. The same effect had been observed in rainbow trout (RODEHUTSCORD 1996).

These requirements apply to total dietary phosphorus and not to digestible phosphorus. Phosphorus digestibility differs depending on the nutritional source. Therefore a recommendation should be given as digestible phosphorus. Phosphorus digestibility of Di-CaP was measured at about 90 % resulting in a demand of about 9.5 g digestible phosphorus per kg diet. GREGUŠ (2000) determined phosphorus digestibility of Di-CaP in rainbow trout to 77 % for a dihydride Di-CaP. This difference could not be explained within the scope of this study. However, the respective digestibilities of protein and organic matter confirm a correct measurement (Table A 7).

Mono-CaP was determined to be 88% digestible in seabream, which was close to the respective digestibility of 93 % in rainbow trout (GREGUŠ 2000).

Feeding a phosphorus balanced diet, phosphorus concentration in seabream varied between 6.0 and 8.3 g per kg body weight, irrespective of fish size (unpublished data from the NCM). At least 9 g available phosphorus have to be provided for each kg biomass gain, considering the inevitable loss (see below). This confirms the correctness of the digestibility determinations discussed above.

The phosphorus requirement is determined by growth rate, which is related to the concentration of digestible energy (DE) in the feed. Therefore a recommendation of phosphorus supply should be given in g per MJ DE.

Weight gain and voluntary feed intake can be expressed as functions of dietary DE density and had been calculated by LUPATSCH *et al.* (2000) to be

$$\text{Weight gain [g kg}^{-0.70}\text{day}^{-1}] = -0.033(\pm 0.0064)x^2 + 1.38(\pm 0.21)x - 8.75(\pm 1.58) \quad [14]$$

$$\text{Voluntary feed intake [g kg}^{-0.70}\text{day}^{-1}] = -0.044(\pm 0.0006)x^2 + 1.57(\pm 0.19)x - 6.36(\pm 1.43) \quad [15]$$

These parameters are given per unit metabolic body size ($kg^{0.70}$) so as not to falsify the calculations by deviations caused by different fish size (LUPATSCH *et al.* 1998).
According to weight gain, the required intake of digestible phosphorus corresponds to weight gain × 0.009 [g $kg^{-0.70}day^{-1}$]. In analogy to weight gain it can be expressed as a function of dietary DE density calculating to

$$\text{Required intake of } P_d \text{ [g kg}^{-0.70}\text{day}^{-1}] = -0.298(\pm 0.096)x^2 + 12.39(\pm 3.08)x - 76.9(\pm 23.6) \quad [16]$$
$$r^2 = 0.91$$

From equation [14] and [15] the required dietary concentrations of digestible phosphorus were determined depending on dietary concentration of DE. Further to this the respective P_d/DE ratios were calculated.

In Table 26 some recommendations for the required concentrations of digestible phosphorus per kg diet and per MJ DE are given. Levels of DE in practical diets range usually between 16 and 20 MJ per kg. Lower FCE values would be accompanied by high feed costs and unnecessary water pollution. On the other hand, a dietary DE concentration of more than 21 MJ per kg would lead to undesirable steatosis of the fish. For the sake of completeness borderline values are included in the table.
Depending on the DE level, the P_d/DE ratios revealed between 0.3 and 0.34. The respective FCE values derive from equations [13] and [14]. At a higher FCE the P_d/DE ratios would increase correspondingly.

Table 26: Some recommendations for requirements of digestible phosphorus in the diet depending on dietary concentration of digestible energy

DE [MJ/kg]	14	16	18	20	22
FCE	0.61	0.68	0.73	0.78	0.85
P_d [g/kg]	5.4	6.0	6.5	6.9	7.4
P_d/DE [g/MJ]	0.4	0.4	0.4	0.3	0.3

5.2.5 Retention and efficiency of phosphorus

In Figure 18 accumulated phosphorus retention is shown as a function of dietary phosphorus concentration ($Pret_D$), and in Figure 19 as a function of accumulated phosphorus intake ($Pret_I$). The estimated function for intake was calculated to

Phosphorus intake [g/fish] = 0.0645 x + 0.0017. [17]

Because of the linearity of phosphorus intake, both retention curves have been expressed by formula [9]:

$$y = \frac{a + [b(1+c) - a]e^{-kx}}{1 + ce^{-kx}} \qquad [9]$$

Parameter estimates are given in Table 27.

Phosphorus retention and intake increased in parallel up to a dietary phosphorus concentration of approximately 9 g per kg. Above this concentration the phosphorus retention approaches a plateau value of 0.47 g per fish (Figure 18).

The difference between the intake and the retention curve represents the phosphorus excretion.

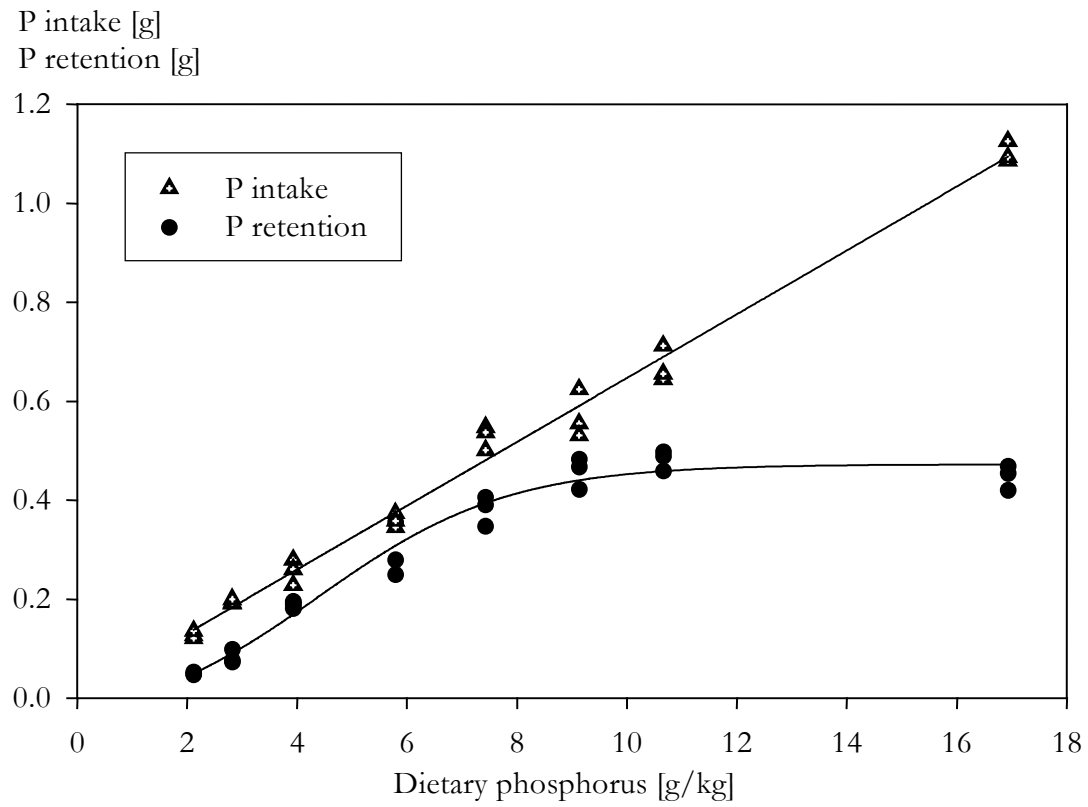

Figure 18: Intake and retention of phosphorus (Pret$_D$) versus dietary phosphorus concentration in Experiment 3

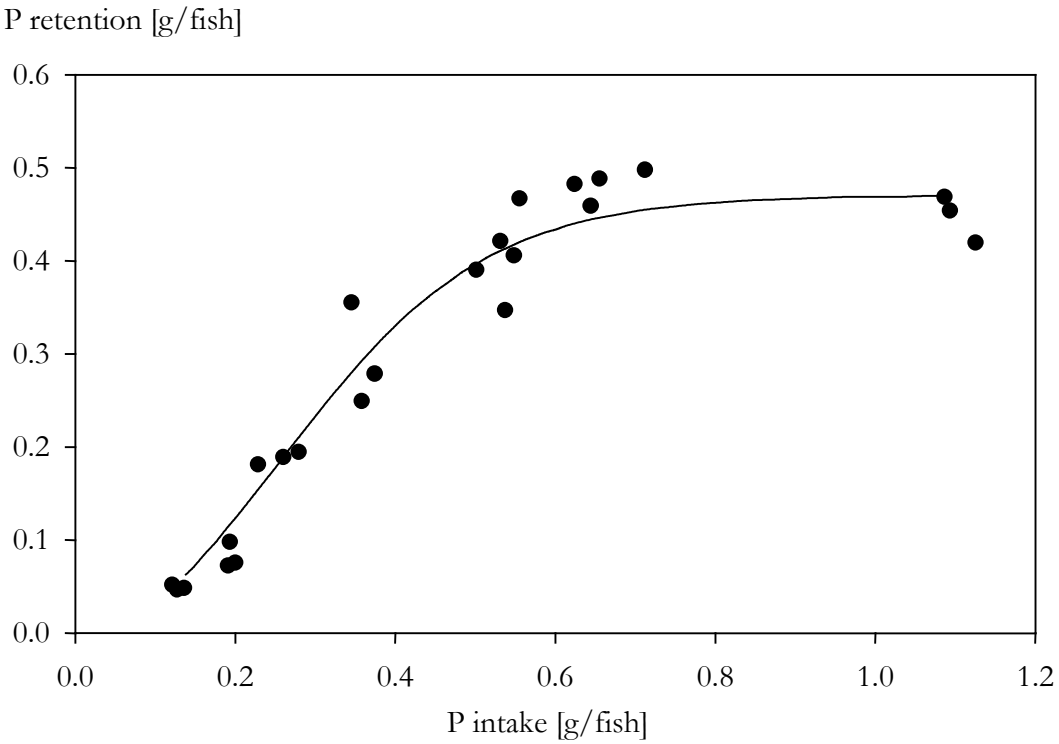

Figure 19: Phosphorus retention (Pret$_I$) versus intake of dietary phosphorus in Experiment 3

Table 27: Estimated parameters for the phosphorus retention curves

P retention [g/fish]	*a*	*b*	*c*	*k*	r^2
$Pret_D$	0.473 ± 0.015	-0.025 ± 0.051	12.7 ± 11.9	0.58 ± 0.13	0.97
$Pret_I$	0.471 ± 0.014	-0.031 ± 0.049	7.52 ± 6.70	7.82 ± 1.83	0.97

The first derivative of equation [9] is equivalent to equation [18]:

$$\frac{\Delta y}{\Delta x} = \frac{ke^{-kx}}{(1+ce^{-kx})^2}(a-b)(1+c) \qquad [18]$$

Completed with the corresponding parameter estimates of the $Pret_I$-function (Table 27, third row), it describes the partial phosphorus retention for each phosphorus intake increment and can be expressed as marginal efficiency (RODEHUTSCORD *et al.* 2000). Using this approach, the efficiency of any phosphorus source can be determined separately from the phosphorus concentration of the basal diet.

Overall efficiency was determined as described in chapter 4.1.2. Its maximum of about 83 % (Figure 20) agrees with the digestibility for Mono-CaP of 88 % determined in Experiment 4 (Table 14).

Maximum phosphorus retention considered as 95 % of the plateau (see chapter 5.2.4) corresponded to a phosphorus intake of approximately 0.63 g per fish (Figure 19).

The maximum of marginal phosphorus efficiency corresponded to a phosphorus intake of about 0.25 g phosphorus per fish (Figure 20). This represented 40 % of the intake required for maximum retention. As a consequence of phosphorus supply close to the requirement, a suboptimal phosphorus utilization must be accepted, although the potential for utilization of the phosphorus source may be very high.

The maximum of the marginal efficiency curve exceeded 100 %, which is theoretically impossible. The curve was estimated by regression analysis based on empirical data. It should be assumed, that slight indefinable influences caused a certain scattering of the observation points, and that their absolute number was too small for a sufficiently valid prediction.

However, little alterations of the estimated parameters within the range of the asymptotic standard errors could provide a curve maximum below the 100 % limit. Therefore a true maximum marginal efficiency close to below 100 % was supposed.

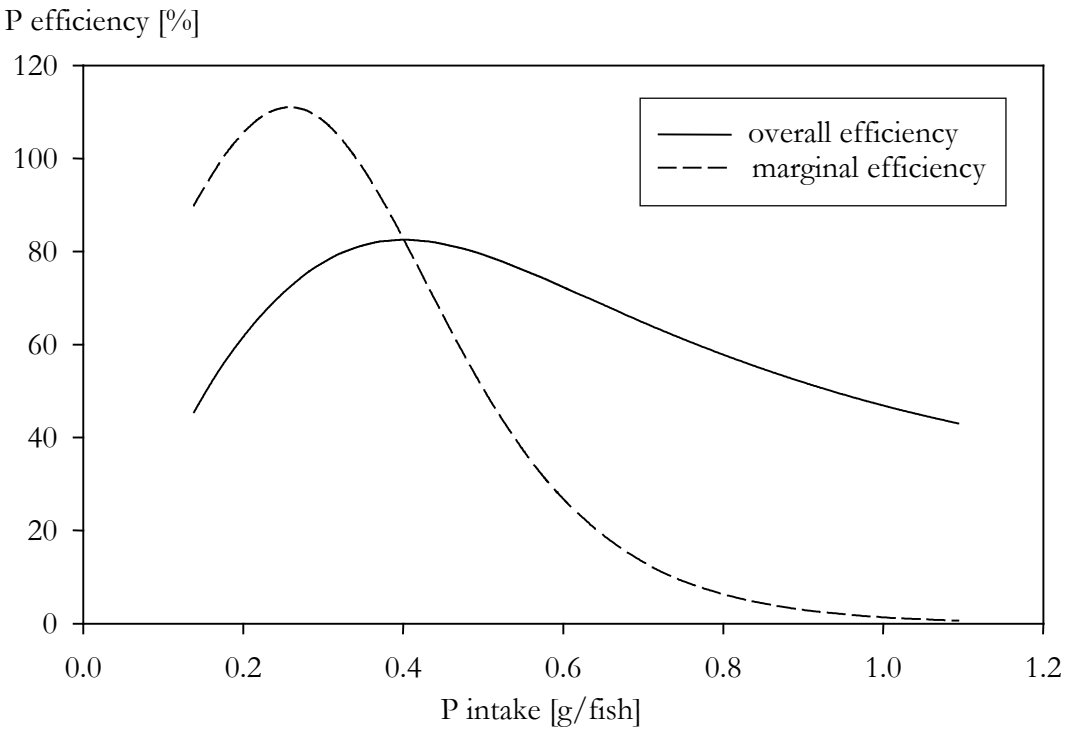

Figure 20: Overall and marginal efficiency for phosphorus from Mono-CaP in seabream (Experiment 3)

5.2.6 Phosphorus excretion

The results of Experiment 3 provided detailed information about the inevitable phosphorus loss. Phosphorus loss was calculated by subtraction of phosphorus retention from phosphorus intake. In Experiment 3 a minimum loss of about 70 mg per fish was observed (Table 28).
As Mono-CaP was not available to seabream for 100 %, the minimum loss consists of the inevitable loss and the indigestible part. Digestibility of Mono-CaP was determined to 88 % in seabream.

Based on the digestibility of the basal diet in Experiment 4, the digestibility of diet 1 in Experiment 3 was assumed to 44 % (Table 14). The calculated excretions of digestible phosphorus (P_d) are summarized in Table 28.

Table 28: Daily phosphorus excretion in Experiment 3

	diet 1	diet 2	diet 3	diet 4	diet 5	diet 6	diet 7	diet 8
P excretion [mg/fish]	0.9	1.2	0.7	0.7	1.6	1.2	2.1	7.1
P_d excretion [mg/fish]	0.4	0.7	0.5	0.5	1.2	1.0	1.6	5.9
P_d excretion [mg/kg fish]	9	15	10	11	23	18	30	110

The observed daily phosphorus excretion was interpreted statistically by use of equation [8]:

$$y = a + be^{kx} \quad [8]$$

x = dietary phosphorus concentration

a = 8.15 ± 3.14

b = 1.32 ± 0.86

k = 0.26 ± 0.04

r^2 = 0.97

Parameter *a* expresses the plateau value, representing the inevitable loss. The daily excretion of digestible phosphorus per kg biomass (fourth row) is shown in Figure 21.

In Experiment 3 a daily inevitable loss of approximately 8 mg per kg live weight was determined.

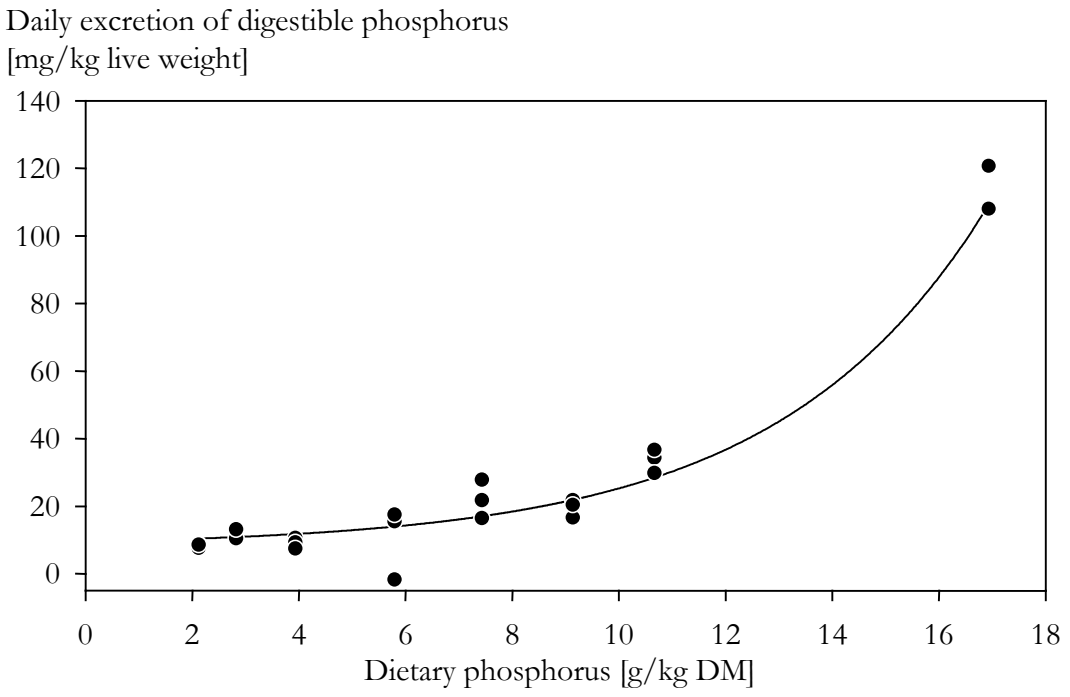

Figure 21: Daily phosphorus excretion per kg live weight in Experiment 3 (seabream Mono-CaP)

5.3 Use of phytase supplementation to diets based on plant feedstuffs in rainbow trout and gilthead seabream (Experiments 1 and 5)

The subject of these two experiments was to investigate the effect of microbial phytase supplementation to plant feedstuffs on phosphorus digestibility in rainbow trout and gilthead seabream. Because of analogous aims and procedures, the experiments will be discussed together. Experiment 1 was carried out in rainbow trout using pre-cooked full-fat soybeans, soy protein concentrate, and rapeseed oilmeal as test components.

In Experiment 5 the test diets were based on rapeseed oilmeal and were fed to gilthead seabream. Concentrations of total dietary phosphorus in the trout experiment ranged between 2.28 and 2.59 g per kg diet, ensuring a suboptimal phosphorus supply as required for digestibility determinations (RODEHUTSCORD *et al.* 2000).

Phytase supplementation increased phosphorus digestibility of all plant feedstuffs considerably. The highest relative increase was achieved in rapeseed oilmeal from 27 to 83 % in trout (Table 4). Highest absolute digestibility of about 95 % was attained in soy protein concentrate with phytase supplementation. This value corresponded to the phosphorus digestibility of sodium monophosphate, which showed the highest digestibility of all examined inorganic phosphates (GREGUŠ 2000).

Similar improvements of phosphorus digestibility of diets based on soybean meal and soy protein concentrate had been observed in previous studies (OLIVA-TELES *et al.* 1998, POWERS HUGHES & SOARES 1998, STOREBAKKEN *et al.* 1998, VIELMA *et al.* 1998, VAN WEERD *et al.* 1999). However, only a few studies allow a direct comparison between single feedstuffs due to the experimental structure. SUGIURA *et al.* (2001) observed an increase of digestibility of phosphorus deriving from defatted soybean meal from 27 to maximal 93 % in rainbow trout. PAPATRYPHON & SOARES (2001) reported improvements from 59 to 87 % of soybean meal and from 52 to 70 % of soy protein concentrate in striped bass at supplementation of 1000 FTU phytase per kg diet. No information was given about the sort of soybean meal, but the phosphorus concentration of about 8 g per kg indicated a reduced fat content.

In seabream the increase of phosphorus digestibility from rapeseed oilmeal remained below the increase in trout, although absolute digestibility of rapeseed oilmeal with phytase supplementation was identical (Table 16). In Experiment 5 (rapeseed oilmeal in seabream) no reference diet was used because of limitations of time and tank facilities. Total phosphorus concentration in the test

diets was 9.8 and 11.9 per kg, respectively. Such a concentration of digestible phosphorus would exceed the demand of seabream and lead to an underestimation of phosphorus digestibility of the test component. In this experiment the diet with phytase supplementation showed the obvious excess of total phosphorus concentration. Therefore the increase of phosphorus digestibility due to phytase supplementation could have been an underestimate.

In both experiments no special measures were taken to avoid hydrolysis of phytate phosphorus before feeding. Diets contained a high amount of water and a time period of about 30 minutes passed between adding the phytase and freezing the diets.
In Experiment 1 concentrations of phytate phosphorus in each test component and in the respective supplemented test diets were analysed by the State Institute for Agricultural Chemistry of the University of Hohenheim. Table 29 demonstrates that large parts of the phytate phosphorus were hydrolysed during this period. In the basal mix no phytate phosphorus was detected.
The exact preparation time had not been measured, but the total amounts of prepared diets were 5 kg, 4.5 kg, and 4.25 kg for soybeans, soy protein concentrate, and rapeseed oilmeal. Little differences in preparation time may have occurred which would partially explain the difference in hydrolysed phytate phosphorus between the test components.
In Experiment 5 the same effect was assumed, as the ambient temperature and the amounts of prepared diets were much higher than in Experiment 1.

Table 29: Dietary phytate-phosphorus concentrations before and after supplementation of microbial phytase in Experiment 1

Treatment	SB	SPC	RS
Phytate-P in test component [g/kg DM]	4.0	6.2	8.7
Phytate-P in test diet, calculated [g/kg DM]	1.20	1.38	1.54
Phytate-P in test diet, analysed [g/kg DM]	0.34	0.70	1.20
Hydrolysed phytate-P [%]	72	49	22

5.4 Use of supplemental phytase in a growth experiment and comparison between trout and seabream

In the growth experiments 6a and 6b the effect of microbial phytase supplementation to a diet based on soy protein concentrate was investigated.
The concentration of total phosphorus in the experimental diets was 4.5 g per kg DM which is below the demand of rainbow trout of 5 g per kg (RODEHUTSCORD 1996). This guaranteed no excretion of surplus phosphorus that would not have been distinguishable from the phosphorus excretion due to phytate phosphorus. In both experiments a distinct improvement of feed intake, weight gain, FCE and phosphorus efficiency was observed with increasing dietary phytase supplementation. In seabream the increase of phosphorus efficiency from 24 to 77 % (Figure 14) was higher than in trout with 32 to 74 % (Figure 13).

As the concentration of total phosphorus was adjusted not to exceed the requirement of trout, it was obviously to low for the seabream. Although the seabream experiment lasted twice as long, they gained relatively less biomass than the trout (Table 17).
The diets were supplemented with a premix providing vitamins and minerals (Table A 16, Table A 17) avoiding a deficiency of other nutrients.
The requirements of protein and energy in seabream increased linear to the respective metabolic body sizes, which are $BW^{0.70}$ for protein and $BW^{0.83}$ for energy (LUPATSCH *et al.* 1998). For maximum weight gain a daily supply of 0.217 MJ DE per $BW^{0.83}$ and 2.68 g DCP per $BW^{0.70}$ was suggested, for maximum protein gain 0.204 MJ DE per $BW^{0.83}$ and at least 3.01 g DCP per $BW^{0.70}$ would have been required (LUPATSCH *et al.* 2000).
In Experiment 6b the mean body weights, determined as the geometric mean of initial and final weight, revealed between 72.3 and 85.3 g (Table A 11). This corresponded to a DCP/DE ratio of about 17.25 for maximum growth and 20.6 for maximum protein retention.
Dietary concentration of DE of about 18.8 MJ per kg was sufficient to ensure high feed intake (LUPATSCH et al. 2000). Digestibilities of dietary protein and energy were determined to about 91 % and 85 % (Table A 12), resulting in a DCP/DE ratio of about 18.5 in the experimental diets. This was close to the required DCP/DE ratio for maximum growth.
The low phosphorus deposition indicated a clear phosphorus undersupply. Phosphorus concentration in the gain varied in diets A-F between 2.7 and 3.7 g per kg, and only in diet G and H did it reach 5.1 g and 6.2 g per kg. Seabream fed diets containing sufficient concentrations of phosphorus had a phosphorus concentration in the gain of about 7.5 g per kg, which agreed with their

initial body phosphorus concentration. Therefore, it should be assumed that phosphorus deficiency was an important cause for the growth depression observed in Experiment 6b. However, other growth depressing influences could not be excluded.

The increase from 2000 to 20000 FTU phytase per kg diet yielded a considerable improvement in efficiency of dietary phosphorus in trout as well as in seabream (Table 30).
POWERS HUGHES & SOARES (1998) and SUGIURA *et al.* (2001) suggested that supplementation of 1000 FTU per kg diet was not sufficient to transform all phytate phosphorus into orthophosphate. The latter observed an increase of apparent phosphorus absorption in rainbow trout with increasing phytase supplementation between 500 to 4000 FTU per kg diet. Supplementation levels in that study were 500, 1000, 2000 and 4000 FTU per kg diet and the respective apparent phosphorus absorptions came to 54, 68, 82 and 90 %.

Table 30: Efficiency of phosphorus retention in % depending on phytase supplementation

	Supplementary phytase [FTU per kg]							
	0	200	400	600	900	1200	2000	20000
Seabream	24.4	27.5	30.2	22.7	28.1	34.0	45.7	76.9
Trout	32.4	33.5	46.9	46.2	51.9	62.3	59.8	73.7

The efficacy of supplementary phytase depends on temperature, pH of the chymus and retention time in the stomach. Phytase from *Aspergillus niger*, as applied here, has one pH-optimum of 2.5 and a second of 5.5 (SIMONS *et al.* 1990). The relative activity of the pH 5.5-optimum amounts to about 50 % of the pH 2.5-optimum. Between both optima the relative activity falls to about 20 % of the pH 2.5-optimum. The supplemented phytase may have been partially inhibited by the pH of the stomach chymus, which can fluctuate in this range (DEGUARA *et al.* 2003).
In Table 31 the required phytase supplementation was determined using the approach of 95 % of the plateau value (chapter 5.2.4, Figure 17). Maximum growth in trout corresponded to a dietary phytase concentration (CphyC) of about 1000 FTU per kg (Table 31).

Table 31: Required phytase supplements to reach 95 % of the respective plateau values

	95 % of plateau	CPhyC [FTU/kg diet]
Trout		
Weight gain	142 g	1021
P in gain	3.67 g/kg	2760
Seabream		
Weight gain	56 g	9709
P in gain	5.89 g/kg	7583

In seabream this approach predicted a phytase requirement of about 9700 FTU/kg for maximum weight gain. This value was due to the moderate slope of the weight gain curve (Figure 12) of seabream.

At 1000 FTU supplementary phytase phosphorus efficiency was calculated to be 54 % in trout and 33 % in seabream. This indicates an insufficient release of inorganic phosphorus in the less supplemented diets due to reduced phytase activity, despite the high water temperature of about 28 °C. Possibly this was caused by the higher phosphorus demand of seabream, which is accompanied by a larger part of inevitable loss.

The results lead to a recommendation of a phytase supplementation in soy protein based diets of approximately 1000 FTU per kg. A further supplementation with inorganic phosphates is not recommended. As in the case with digestible phosphorus, the demand for phytase is likely to depend on the energy content of the diet. Based on this experiment a recommendation of approximately 50 FTU phytase per MJ DE should be given. This relationship agrees with the common feed used in pig nutrition which contains about 16 MJ DE and 500-750 FTU phytase per kg, if we take into consideration, that pig feed often contains high levels of wheat or triticale, which contain appreciable concentrations of intrinsic phytase.

An optimal dietary phytase supplementation for seabream diets could not be determined within the scope of Experiment 6b. The calculated required dietary phytase concentration for maximum weight gain of 9700 FTU per kg diet (Table 31) was obviously too high from an economic point of view. The insufficient concentration of digestible phosphorus coming from soy protein concentrate requires a supplementation of inorganic phosphate. Further research may clarify this issue.

In opposite to rainbow trout, the use of soy protein concentrate as the only protein source yielded previously relative poor performances in seabream. In the literature several antinutritional factors of soy products are discussed to cause negative gastrointestinal effects, which will not be explained here in detail. A review is given by ALEXIS & NENGAS (2001).
Nevertheless it is necessary to continue the research for alternative plant protein sources for seabream. This includes a potential pre-treatment and/or a supplementation with essential amino acids.
In case of a successful exchange of fish meal with a unique plant protein or a plant protein blend, a potential future experiment may be carried out with the diet as suggested in Table 32.

The component "plant protein" refers to a solvent extracted oilmeal or protein concentrate, containing ca. 45-75 % crude protein, as these protein sources are commonly used in animal nutrition. The amino acid pattern of the protein source should be acquainted. Solvent extracted oilmeals and protein concentrates usually contain phosphorus concentrations of 8-14 g per kg.
Considering a phosphorus requirement of about 9.5 g digestible phosphorus per kg feed (chapter 5.2.4), a supplementation of microbial phytase will be inevitable to enhance phosphorus digestibility. A supplementation of inorganic phosphates may be additionally required. The same diet without phytase supplementation may serve as a control.

Table 32: Composition of a potential diet for further research in use of plant feedstuffs in nutrition of gilthead seabream

Components [g/kg]
• ca. 500-700 g plant protein, depending on its protein content
• free amino acids according to the protein source
• 190 g fish oil
• 90 g Premix (Vitamins, minerals, fat binder)
• Di-CaP or Mono-CaP, amount depends on P concentration of the feed
• up to 200 g wheat or wheat starch
• 1000 FTU microbial phytase

In Experiment 6a and 6b phytase was supplemented to the feed avoiding a preliminary enzymatic reaction. The effect of the phytase developed in the digestive tract of the fish.

As conditions in the digestive tract depend on influences which cannot be controlled completely, an alternative approach for making commercially available diets could be the hydrolysis of phytate already in the feed. VAN WEERD *et al.* (1999) observed an increase of phosphorus digestibility from 68 to 79 % in African catfish (*Clarias gariepinus*), when soybean meal was pre-treated with 1000 FTU phytase per kg in contrast to the same diet with phytase incorporated in the diet. In the previously mentioned study by SUGIURA *et al.* (2001) the apparently absorbed phosphorus was enhanced to 93 % when soybean meal was pre-treated with 200 FTU per kg compared to 90 % in the same diet supplemented with 4000 FTU per kg.

In Experiment 6a (trout experiment) phosphorus digestibility increased almost parallel to utilization although the slightly lower figures for digestibility were caused by the partial circulation unit the fish were kept in (chapter 4.6.2).

In seabream measured digestibility was considerably higher than utilization in the low supplemented treatments. The excretion of digestible phosphorus was calculated to be between 19.9 and 8.2 g per kg biomass (Table 33), close to the inevitable loss of about 9 mg per kg biomass mentioned in chapter 5.2.6. These values represent approximate values only, since digestibility was determined in large fish separately and not in the experimental fish.

Table 33: Daily phosphorus excretions in seabream in Experiment 6b

	Supplemental phytase [FTU/kg]			
	0	200	400	20000
excretion of P_d [mg/fish]	1.3	1.2	1.5	0.7
excretion of P_d [mg/kg]	18	17	20	8

Finally this double experiment provided data for a comparison of energy supply between trout and seabream. The respective plateau values were used to reduce the influence of limited phosphorus availability. Table 34 shows the summarized data calculated per fish.

Table 34: Comparison of energy utilization between trout and seabream

per fish	rainbow trout	gilthead seabream
Duration [days]	53	98
Initial weight [g]	101	61
Final weight [g]	251	120
Feed intake [g DM]	170	105
FCE	0.81	0.51
DE in feed [MJ/kg DM]	19.3	18.4
DE intake [kJ/day]	62.0	19.6
Energy retention [kJ/day]	35.8	8.4
k_{Etotal}	0.58	0.43
MBS[1] [g]	0.9	-
[kg]	-	0.135
DE for maintenance [kJ/day]	11.8	7.5
DE for growth [kJ/day]	50.2	12.1
$k_{Egrowth}$	0.71	0.69

[1]Determined as the arithmetic means of initial and final MBS

The relation of DE_g : DE_m revealed a quotient of about 4.2 in trout and 1.6 in seabream, due to the depressed intake and growth in seabream. This explains the lower k_{Etot} in seabream.
No significant difference regarding the $k_{Egrowth}$ was observed. Obviously there was no oversupply of DE_g in seabream. Once ingested, DE was utilized for growth in seabream as efficiently as in trout.
Therefore, differences in growth between trout and seabream cannot be explained by different utilization of digestible energy. The depression in growth and intake of seabream must have been caused by other factors, most likely by the deficiency in phosphorus, but other influences could not be ruled out.

In this study, it was shown that plant protein is an alternative to fish meal in nutrition of gilthead seabream. In Experiment 3 a diet based on wheat gluten and supplemented adequately with minerals, vitamins, amino acids and phosphorus caused the same growth performance as an isonitrogenous and isoenergetic diet based on fish meal (Figure 9).

In Experiment 6b a considerable enhancement of growth performance was achieved by supplementation of microbial phytase in a diet based on soy protein concentrate (Figure 12). However, a sufficient evaluation of this feedstuff could not be carried out because the total dietary phosphorus concentration was far below the requirement of seabream. Further scientific research is necessary to clarify this issue and may focus on the following objectives:

- Evaluation of the suitability of different plant feedstuffs as an alternative to fish meal for gilthead seabream
- Optimal pre-treatment of plant feedstuffs to achieve high nutritional value
- Methods of phytase supplementation to achieve optimal efficacy of the phytase.

The use of plant proteins and highly digestible feed in fish nutrition could have a significant influence on the economics and correspondent ecological effects in fish production.
Especially in the Red Sea region, the most recent data suggest that at best the sea cages in the Eilat area contribute only 2-5% of all the nutrients entering the Gulf. Therefore, the down regulation of phosphorus impact into the water will have an additional positive side effect for the fish farms regarding their public image. An approach of feed development as described in this study for the production of seabream in the Red Sea could avoid future conflicts between the fish producing industry and the local government.

6 Conclusion

In this study the possibility of a diet basing on plant feedstuffs for gilthead seabream and rainbow trout was shown.

Phosphorus requirement of seabream was determined by use of dose-response-experiments. Dietary phosphorus concentration showed a strong effect on growth parameters and body composition.
Requirement of phosphorus could not be defined as a distinct point of dietary phosphorus concentration, but depended on the regarded trait. It seemed appropriate to refer recommended phosphorus concentration to dietary energy, which is the most important factor determining intake and weight gain. A diet with an energy level of 18 MJ DE/kg should contain about 6.5 g P_{av}/kg diet to reach maximum weight gain.
Further it was shown that seabream are able to spare a large amount of phosphorus in their bones under conditions of phosphorus deficiency.

This study continued research in the use of microbial phytase supplementation in plant feedstuffs for trout and seabream.
The effect of microbial phytase depended on diet preparation and on the used feedstuff. Digestibility of phosphorus from soybeans and soy protein concentrate were improved up to 94 %. To take full advantage of microbial phytase, it seems appropriate to split the phytate phosphorus already in the diet before feeding. Further experiments to improve the diet preparation technique are suggested.

In a double experiment with trout and seabream fed the same diets based on soy protein concentrate with different phytase supplementation levels the response of the two species was compared. Seabream showed reduced growth performance in comparison to trout, which could be ascribed largely to the dietary phosphorus concentration, which was sufficient for trout but far too low for seabream. Phosphorus from the plant protein source apparently satisfies the requirement of the trout but not of seabream, even when completely available. A supplementation of inorganic phosphates may be additionally required.

7 Summary

In this study potentialities of reducing phosphorus excretion in nutrition of rainbow trout and gilthead seabream were examined. A reduction of dietary phosphorus was achieved by replacement of fish meal by plant protein sources. Since plant phosphorus is particularly found as phytic acid, which is indigestible to fish, the potential use of phytase supplementation to enhance phosphorus digestibility was investigated.
In seabream the requirements of dietary phosphorus were determined by dose-response-experiments to obtain information about the ideal concentration of digestible phosphorus in the feed. The effect of phytase supplementation to different plant feedstuffs was examined by several digestibility determinations in trout and seabream.

The trout experiments were carried out in Germany in the Institute of Animal Nutrition of the University of Bonn, the seabream experiments in the National Center for Mariculture in Eilat, Israel. In all experiments digestibility and/or utilisation of phosphorus was determined by the difference method.

The response of seabream to rising levels of dietary phosphorus was examined in two growth experiments. The diets based on wheat gluten and were adequate in supply of vitamins, minerals and essential amino acids. Different levels of dietary phosphorus at constant basal mix were achieved by supplementation of dicalcium phosphate (Di-CaP) in the first experiment and monocalcium phosphate (Mono-CaP) in the second experiment. In the second experiment (Mono-CaP) feeding was restricted and an additional isonitrogenous and isoenergetic diet based on fish meal served as a control.
The digestibilities of Di-CaP and Mono-CaP in seabream were determined in separated experiments.

Weight gain, feed intake, feed conversion efficiency and body composition showed a clear dependence on dietary phosphorus concentration at phosphorus deficiency. Exceeding the demand of dietary phosphorus showed no further effects. Phosphorus requirement of seabream was determined to about 6.5 g available phosphorus per kg diet at 18 MJ DE/kg.
In the Mono-CaP experiment, growth performance of the fish fed the fish meal diet was comparable to the fish fed diets based on wheat gluten and sufficient phosphorus supply.

The effect of microbial phytase was tested in trout and seabream using different plant feedstuffs. In trout, full-fat soybeans, soy protein concentrate and rapeseed oilmeal incorporated in a basal diet based on wheat gluten were fed with and without phytase supplementation. Supplementation of phytase increased the phosphorus digestibility from 27 to 83 % in rapeseed oilmeal, from 41 to 93 % in soy protein concentrate and from 43 to 94 % in full-fat soybeans.
In seabream, supplementation of microbial phytase to a rapeseed oilmeal based diet improved phosphorus digestibility from 50 to 84 %.

Finally, in a double growth experiment identical diets were fed to trout and seabream. The diets based on soy protein concentrate contained a phosphorus concentration of about 4.5 g per kg and were supplemented with 0, 200, 400, 600, 900, 1200, 2000 and 20000 FTU phytase per kg diet. In trout a dietary phytase concentration of about 1000 FTU per kg was determined to be sufficient.
However, in the seabream experiment a sufficient supplementation of microbial phytase could not be definitely determined as overall dietary phosphorus concentration was too low for the demand of seabream.
The results of the experiments using microbial phytase indicate that conditions in the digestive tract may inhibit the full hydrolysis of the phytic acid, even when supplemented with sufficient phytase. As a consequence, cleaving the phytic acid during the feed production process may yield better performances.

A comparison between trout and seabream of utilisation of digestible energy for growth resulted in almost identical efficiencies ($k_{Egrowth}$) of 0.71 in trout and 0.69 in seabream.

8 References

Alexis, M. N.; Nengas, I. (2001): Current state of knowledge concerning the use of soy products in diets for feeding sea bass and sea bream. Needs for future research. American Soybean Association, Athens, Greece, 32 pp.

Åsgård, T.; Shearer, K. D. (1997): Dietary phosphorus requirement of juvenile Atlantic Salmon, *Salmo salar* L. Aquaculture Nutrition **3**, 17-23.

Atkinson, M. J.; Birk, Y.; Rosenthal, H. (2001): Evaluation of pollution in the Gulf of Eilat. Report for the Ministries of Infrastructure, Environment and Agriculture, Israel.

Bell, P. (1992): Eutrophication and coral reefs – some examples in the Great Barrier Reef Lagoon. Water Research **26**, 553-568.

Braun, J. (1999): Untersuchungen zur Bedeutung des Aminosäurenmusters sowie zum Einsatz von Fett in der Ernährung von Regenbogenforellen. Diss. agr. University Bonn.

Chavez-Sanchez, C.; Martinez-Palacios, C. A.; Martinez-Perez, G.; Ross, L. G. (2000): Phosphorus and calcium requirements in the diet of the American cichlid *Cichlasoma urophthalmus* (Gunther). Aquaculture Nutrition **6**, 1-9.

Cheng, Z. J.; Hardy, R. W. (2002): Effect of microbial phytase on apparent nutrient digestibility of barley, canola meal, wheat and wheat middlings, measured *in vivo* using rainbow trout (*Oncorhynchus mykiss*). Aquaculture Nutrition **8**, 271-277.

Deguara, S.; Jauncey, K.; Agius, C. (2003): Enzyme activities and pH variations in the digestive tract of gilthead sea bream. Journal of Fish Biology **62**, 1033-1043.

Diamant, A; von Westernhagen, H. (2003): MARS: biological indicators of natural and man-made changes in marine and coastal waters: scientific coordinators' introduction. Helgoland Marine Research **57**, 146-156.

Düngelhoef, M.; Rodehutscord, M. (1995): Wirkung von Phytasen auf die Verdaulichkeit des Phosphors beim Schwein. Übersichten zur Tierernährung **23**, 133-157.

Düngelhoef, M.; Rodehutscord, M.; Spiekers, H.; Pfeffer, E. (1994): Effects of supplemental microbial phytase on availability of phosphorus contained in maize, wheat and triticale to pigs. Animal Feed Science and Technology **49**, 1-10.

FAO (2004): Aquaculture production 2002. Yearbook of Fishery Statistics Vol. 94/2. See also: http://www.fao.org/fi/statist/FISOFT/FISHPLUS.asp, Aquaculture production: quantities 1950-2002.

Folch, J.; Lees, M.; Sloane, G.H. (1957): Simple method for isolation and purification of total lipids from animal tissues. Journal of Biological Chemistry **226**, 497-507.

Frenzel, E.; Pfeffer, E. (1982): Untersuchungen zum Mineralstoffbedarf wachsender Regenbogenforellen (*Salmo gairdnerii* R.). Archiv für Tierernährung **32**, 1-8.

Gordin, H. (2003): Mariculture in Israel. The Israeli Journal of Aquaculture – Bamidgeh **55** (4), 219-221.

Greguš, Z. (2000): Untersuchung zur Verdaulichkeit des Phosphors in Futtermitteln mineralischer oder tierischer Herkunft bei der Regenbogenforelle (*Oncorhynchus mykiss*). Diss. agr. University Bonn.

Hardy, R. W.; Gatlin, D. M. (2002): Nutritional Strategies to reduces nutrient losses in intensive aquaculture. Avances en Nutrición Acuicola VI. Memorías del Sexto Simposium Internacional de Nutrición Acuícola 3 al 6 Septiembre, 2002, Cancún, Quintana Roo, México.

Jackson, L. S.; Li, M. H.; Robinson, E. H. (1996) Use of microbial phytase in channel catfish *Ictalurus punctatus* diets to improve utilization of phytate phosphorus. Journal of the World Aquaculture Society **27** (3), 309-313.

Kinzinger, S. L. (1992): Untersuchungen zur Verdaulichkeitsbestimmung bei Forellen nach der Differenzmethode und Bewertung ausgewählter Einzelfuttermittel. Diss. agr. University Bonn.

Kissil, G. W., Lupatsch, I., Higgs, D. A., Hardy, R.W. (2000). Dietary substitution of soy and rapeseed protein concentrates for fish meal, and their effects on growth and nutrient utilization in gilthead seabream (*Sparus aurata* L.). Aquaculture Research **31**, 595-601.

Kissil, G. Wm.; Lupatsch, I. (2004). Successful replacement of fish meal by plant proteins in diets for the gilthead seabream, *Sparus aurata* L. The Israeli Journal of Aquaculture – Bamidgeh 56(3), 188-199.

Lall, S. P. (1991): Digestibility, metabolism and excretion of dietary phosphorus in fish. In: Cowey, C. B. & Cho, C. Y (Eds.): Nutritional strategies and aquaculture waste. Proceedings of the First International Symposium on Nutritional Strategies in Management of Aquaculture Waste. University of Guelph, Guelph, Canada, pp. 21-36.

Lanari, D.; D'Agaro, E.; Turri, C. (1998): Use of nonlinear regression to evaluate the effects of phytase enzyme treatment of plant protein diets for rainbow trout (*Oncorhynchus mykiss*). Aquaculture **161**, 345-356.

Lapointe, B. (1992): Eutrophication thresholds for macroalgal overgrowth of coral reefs. In: Thacker, K. (ed.): Protecting Jamaica's coral reefs: water quality issues. Negril Coral Reef Preservation Society, Negril, Jamaica, 317-326.

Li, M. H.; Robinson, E. H. (1997): Microbial phytase can replace inorganic phosphorus supplements in channel catfish *Ictalurus punctatus* diets. Journal of the World Aquaculture Society **28** (4), 402-406.

Loya, Y.; Zakai, D. (2002): Status of Coral Reefs in Eastern Africa: Kenya, Tanzania, Mosambique ans South Africa. In: Clive Wilkinson (Ed.): Status of Coral Reefs of the World, Australian Institute of Marine Science. pp. 63-78.

Lupatsch, I.; Kissil, G. W.; Sklan, D. Pfeffer, E. (1998): Energy and protein requirements for maintenance and growth in gilthead seabream (*Sparus aurata* L.). Aquaculture Nutrition **4**, 165-173.

Lupatsch, I.; Kissil, G. W.; Sklan, D.; Pfeffer, E. (2000): Effects of varying protein and energy supply on growth, body composition and protein utilization in gilthead seabream (*Sparus aurata* L.). Aquaculture Nutrition **7**, 71-80.

Naumann, C.; Bassler, R. (1976): VDLUFA – Methodenhandbuch Vol. 3. Die chemische Untersuchung von Futtermitteln. Neumann-Neudamm: Melsungen, Loseblatt-Sammlung.

Nengas, I.; Alexis, M. N.; Davis, S. J. (1996): Partial substitution of fish meal with soybean meal and derivatives in diets for gilthead seabream (*Sparus aurata* L.). Aquaculture Research **27** (3), 147-156.

NRC (National Research Council) (1993) : Nutrient requirements of fish. National Academy Press, Washington D. C

Oliva-Teles, A.; Pereira, J. P.; Gouveia, A.; Gomes, E. (1998): Utilisation of diets supplemented with microbial phytase by seabass (*Dicentrarchus labrax*) juveniles. Aquatic Living Resources **11** (4); 255-259.

Pallauf, J.; Höhler, D.; Rimbach, G.; Neusser, H. (1992): Einfluss einer Zulage mikrobieller Phytase zu einer Mai-Soja-Diät auf die scheinbare Verdaulichkeit von Phosphor und Calcium beim Ferkel. Journal of Animal Physiology and Animal Nutrition **67**, 30-40.

Papatryphon, E.; Soares J. H. (2001): The effect of phytase on apparent digestibility of four practical plant feedstuffs fed to striped bass, *Morone saxatilis*. Aquaculture Nutrition **7**, 161-167.

Petrasch, R. (1981): Beiträge zur Verwertung von Protein und zum Einsatz wasserlöslicher Vitamine in der Ernährung von Regenbogenforellen (*Salmo gairdner* R.) Diss. agr. University Bonn.

Pfeffer, E. (1978): Über die Verteilung von mineralischen Mengenelementen im Körper von Forellen und Karpfen. Zeitschrift für Tierphysiologie, Tierernährung und Futtermittelkunde **40**, 159-164.

Pfeffer, E.; Henrichfreise, B. (1994): Evaluation of potential sources of protein in diets for rainbow trout (*Oncorhynchus mykiss*). Archives of Animal Nutrition **45**, 371-377.

Pimentel-Rodrigues, A. M.; Oliva-Teles, A. (2001): Phosphorus requirements of gilthead seabream (*Sparus aurata* L.) juveniles. Aquaculture Research **32** (Suppl. 1), 157-161.

Powers Hughes, K; Soares, J. H. (1998): Efficacy of phytase on phosphorus utilization in practical diets fed to striped bass *Morone saxatilis*. Aquaculture Nutrition **4**, 133-140.

Robaina, L., Izquierdo, M. S., Moyano, F. J., Socorro, J., Vergara, J. M., Montero, D., Fernández-Palacios, H. (1995): Soybean and lupin seed meals as protein sources in diets for gilthead seabream (*Sparus aurata*): nutritional and histological implications. Aquaculture **130**, 219-233.

Rodehutscord, M.; Becker, A; Pfeffer, E. (1995): Einfluss des Zusatzes einer *Aspergillus niger*-Phytase auf die Verwertung pflanzlichen Phosphors durch die Forelle (*Oncorhynchus mykiss*). Archives of Animal Nutrition **48**, 211-219.

Rodehutscord, M. (1996): Response of rainbow trout (*Oncorhynchus mykiss*) growing from 50 to 200 g to supplements of dibasic sodium phosphate in a semipurified diet. Journal of Nutrition **126**, 324-331.

Rodehutscord, M.; Faust, M.; Lorenz, H. (1996): Digestibility of phosphorus contained in soybean meal, barley, and different varieties of wheat, without and with supplemental phytase fed to pigs and additivity of digestibility in a wheat-soybean-meal diet. Journal of Animal Physiololgy and Animal Nutrition **75**, 40-48.

Rodehutscord, M.; Pfeffer, E. (1999): Maintenance requirement for digestible energy and efficiency of utilisation of digestible energy for retention in rainbow trout, *Oncorhynchus mykiss*. Aquaculture *179*, 95-107.

Rodehutscord, M.; Krause, G.; Pfeffer, E. (1999): Effect of body weight on phosphorus digestibility and efficacy of a microbial phytase in young pigs. Archives of Animal Nutrition **52** , 139-153.

Rodehutscord, M.; Greguš, Z.; Pfeffer, E. (2000): Effect of phosphorus intake in faecal and non-faecal phosphorus excretion in rainbow trout (*Oncorhynchus mykiss*) and the consequences for comparative phosphorus availability studies. Aquaculture **188**, 383-398.

Sakamoto, S.; Yone, Y. (1978): Effect of dietary phosphorus level on chemical composition of red sea bream. Bulletin of the Japanese Society of Scientific Fisheries **39**, 343-348.

Sebastian, S.; Touchburn, S. P.; Chavez , E. R. (1998): Implications of phytic acid and supplemental microbial phytase in poultry nutrition: a review. Worlds's Poultry Science Journal **54**, 27-47.

Simons, P. C. M.; Versteegh, H. A. J.; Jongbloed, A. W.; Kemme, P. A.; Slump, P.; Bos, K. D.; Wolters, M. G. E.; Beudeker, R. F.; Verschoor, G. J. (1990): Improvement of phosphorus availability by microbial phytase in broilers and pigs. British Journal of Nutrition **64**, 525-540.

Storebakken, T.; Shearer, K. D.; Roem, A. J. (1998): Availiability of protein, phosphorus and other elements in fish meal, soy-protein concentrate and phytase-treated soy-protein-concentrate-based diets to Atlantic salmon, *Salmo salar*. Aquaculture **161**, 365-379.

Sugiura, S. H.; Dong, F. M.; Hardy, R. W (2000): A new approach to estimating the minimum dietary requirement of phosphorus for large rainbow trout based on nonfecal excretions of phosphorus and nitrogen. Journal of Nutrition **130**, 865-872.

Sugiura, S. H.; Gabaudan, J.; Dong, F. M.; Hardy, R. W. (2001): Dietary microbial phytase supplementation and the utilization of phosphorus, trace minerals and protein by rainbow trout *[Oncorhynchus mykiss* (Walbaum)] fed soybean meal-based diets. Aquaculture Research **32** (7), 583-592.

Vielma, J.; Lall, S. P.; Koskela, J.; Schöner, F.-J.; Mattila, P. (1998): Effects of dietary phytase and cholecalciferol on phosphorus bioavailability in rainbow trout (*Oncorhynchus mykiss*). Aquaculture **163**, 309-323.

van Weerd, J. H.; Khalaf, K. A.; Aartsen, F. J.; Tijssen, P. A. T. (1999): Balance trials with African catfish *Clarias gariepinus* fed phytase-treated soybean meal-based diets. Aquaculture Nutrition **5**, 135-142.

Zakai, D.; Chadwick-Furman, N. E. (2002): Impacts of intensive recreational diving on reef corals at Eilat, northern Red Sea. Biological Conservation **105**, 179-187.

9 Appendix

Table A 1: Composition of faeces and digestibility of phosphorus in Experiment 1 (Plant proteins in rainbow trout)

Treatment	BM		SB		SPC		RS	
Suppl. phytase [FTU/kg]	0	1000	0	1000	0	1000	0	1000
Composition of faeces [g/kg DM]								
Dry matter	958	946	937	938	966	954	942	975
	954	961	945	941	929	960	959	947
	952	951	961	960	920	702	939	967
Ash	401	374	320	343	302	310	333	346
	405	384	315	320	322	339	340	344
	379	372	311	334	295	360	336	339
Phosphorus	0.83	0.63	4.40	0.98	6.24	1.10	5.57	1.71
	0.84	0.64	4.50	1.04	6.67	1.17	5.66	1.56
	0.72	0.62	4.59	0.96	6.25	1.18	5.39	1.71
HCl-insoluble ash	308	391	233	257	286	304	227	250
	309	301	233	245	321	275	233	253
	292	288	228	261	297	295	241	250
Digestibility of total phosphorus [%]	71.2	78.5	51.8	90.3	46.7	90.6	35.4	81.7
	71.0	78.9	50.7	89.2	49.3	88.9	35.8	83.5
	73.6	78.6	48.6	90.6	48.6	89.6	41.0	81.7
Digestibility of P from test component [%]	-	-	45	94	39	94	24	83
	-	-	43	93	43	92	25	85
	-	-	40	95	42	93	31	83
Digestibility of total organic matter [%]	82.5	80.8	81.4	83.8	84.0	84.7	77.9	80.3
	82.7	81.8	81.3	82.3	85.8	83.9	78.6	80.5
	80.8	80.5	80.8	83.8	84.6	84.6	79.2	80.1
Digestibility of OM from test component [%]	-	-	80	90	91	97	59	77
	-	-	80	85	98	94	63	78
	-	-	78	90	94	97	67	76

Table A 2: Growth data in Experiment 2 (Di-CaP in gilthead seabream)

Treatment	diet 1	diet 2	diet 3	diet 4	diet 5	diet 6	diet 7
Dietary P [g/kg DM]	2.28	3.80	6.11	7.95	11.63	13.99	15.35
Weight gain [g/fish]	29.4	33.3	46.0	51.4	59.0	50.7	53.0
	32.5	35.2	44.6	45.6	54.7	57.2	46.4
	28.1	34.2	44.2	52.4	53.9	54.4	47.2
Feed intake [g/fish]	58.3	62.5	77.5	75.6	85.6	74.6	84.6
	62.9	65.8	72.9	70.5	82.3	80.6	73.4
	56.7	64.6	72.2	75.3	76.1	77.9	76.3
FCE	0.50	0.53	0.59	0.68	0.69	0.68	0.63
	0.52	0.53	0.61	0.65	0.66	0.71	0.63
	0.50	0.53	0.61	0.70	0.71	0.70	0.62
Survival [%]	96.3	100.0	100.0	85.2	100.0	100.0	92.3
	100.0	92.6	92.6	96.3	100.0	100.0	100.0
	96.3	96.3	88.9	100.0	96.3	88.9	92.6

Table A 3: Body composition in Experiment 2 (DiCaP in gilthead seabream)

Treatment	initial	diet 1	diet 2	diet 3	diet 4	diet 5	diet 6	diet 7
Dietary P [g/kg DM]		2.28	3.80	6.11	7.95	11.63	13.99	15.35
Dry matter	308	370	376	354	341	360	356	352
[g/kg]		370	371	355	347	352	357	338
		366	369	356	352	339	349	352
Ash	41.2	27.3	29.8	36.8	37.9	41.9	41.6	41.8
[g/kg]		27.5	32.4	35.4	41.0	40.7	41.0	43.6
		28.8	31.6	34.6	38.3	42.4	39.4	40.7
Crude protein	166	162	164	167	163	174	172	170
[g/kg]		170	161	157	173	164	169	163
		166	162	162	167	162	167	168
Crude lipid	95	181	191	157	149	154	149	147
[g/kg]		194	187	166	145	153	157	137
		179	184	161	151	140	145	147
Gross energy	7.8	10.4	10.7	9.6	9.1	9.5	9.6	9.3
[MJ/kg]		10.5	10.4	9.9	8.9	9.4	9.2	8.7
		10.1	10.1	9.5	9.1	8.7	9.2	9.1
Phosphorus	7.18	4.42	5.07	6.38	6.73	7.58	7.51	7.57
[g/kg]		4.44	5.77	6.04	7.24	7.31	7.41	7.84
		4.72	5.31	5.94	6.96	7.84	7.11	7.15
Calcium	12.4	10.1	10.9	11.2	13.6	12.6	11.7	11.6
[g/kg]		6.7	8.8	9.0	10.4	11.5	11.8	11.3
		6.5	8.3	9.4	10.2	10.8	10.6	11.3
Magnesium	1.78	0.99	1.08	1.24	1.40	1.41	1.46	1.44
[g/kg]		1.07	1.02	1.26	1.37	1.34	1.39	1.50
		1.08	1.19	1.26	1.30	1.39	1.33	1.50
Sodium	6.48	3.66	3.22	3.69	3.65	3.34	3.68	3.71
[g/kg]		3.29	3.18	3.21	3.34	4.38	4.55	3.25
		2.75	3.78	3.52	3.63	3.55	3.57	4.29
Potassium	13.0	9.4	9.7	10.8	10.8	11.1	10.9	11.0
[g/kg]		9.6	10.2	10.5	11.0	10.4	9.7	8.6
		8.4	9.6	8.9	9.7	10.0	10.5	11.3

Table A 4: Hard and soft tissues in Experiment 2 (DiCaP in gilthead seabream)

Treatment	initial	diet 1	diet 2	diet 3	diet 4	diet 5	diet 6	diet 7
Dietary P [g/kg DM]		2.28	3.80	6.11	7.95	11.63	13.99	15.35
Hard tissue [g/fish]	6.1	11.3	11.5	14.3	15.1	17.2	17.2	17.2
		11.5	12.0	13.9	14.5	17.1	16.6	14.7
		11.0	12.8	13.9	15.3	16.7	15.3	15.6
Soft tissue [g/fish]	18.6	41.2	45.9	54.0	57.4	61.4	49.0	61.7
		42.1	44.3	50.3	53.5	64.1	62.0	50.6
		41.5	46.4	51.7	57.4	63.6	61.4	55.0
P-concentration in hard tissue [g/kg]	21.3	11.8	16.1	20.4	22.4	22.2	22.1	22.0
		11.7	16.6	19.2	21.0	22.3	23.4	21.6
		13.9	16.1	20.3	21.1	22.7	22.9	23.6
P-concentration in soft tissue [g/kg]	3.4	2.4	2.7	2.8	3.3	3.3	3.1	3.5
		2.4	2.7	3.0	3.3	3.3	3.5	3.8
		2.5	2.9	3.0	3.5	3.0	3.6	3.8
% of body P in ht	67.2	54.7	57.5	63.5	62.4	63.7	69.7	61.8
		53.7	60.2	61.9	61.3	62.5	62.2	60.0
		56.2	57.9	62.2	59.8	64.6	59.2	61.5
% of body P in st	32.8	45.3	42.5	36.5	37.6	36.3	30.3	38.2
		46.3	39.8	38.1	38.7	37.5	37.8	40.0
		43.8	42.1	37.8	40.2	35.4	40.8	38.5

Table A 5: Growth data in Experiment 3 (MonoCaP in gilthead seabream)

Treatment	diet 1	diet 2	diet 3	diet 4	diet 5	diet 6	diet 7	diet 8
Dietary P [g/kg DM]	2.31	3.09	4.26	6.22	7.97	9.79	11.48	18.23
Weight gain [g/fish]	33.2	35.0	49.7	60.3	64.5	70.5	69.2	65.4
	35.7	36.3	45.7	51.0	64.6	71.7	71.4	65.7
	37.8	38.1	42.6	51.9	56.4	62.7	66.7	60.8
Feed intake [g/fish]	60.0	57.0	64.2	67.9	68.3	70.8	71.0	66.2
	58.1	59.7	64.6	61.8	73.7	72.3	67.4	68.4
	60.8	58.1	60.4	61.5	66.8	64.1	64.5	66.5
FCE	0.55	0.61	0.77	0.89	0.94	1.00	0.97	0.99
	0.61	0.61	0.71	0.83	0.88	0.99	1.06	0.96
	0.62	0.66	0.71	0.84	0.84	0.98	1.03	0.92
Survival [%]	88.5	100.0	96.2	100.0	100.0	100.0	92.3	96.2
	92.3	96.2	92.3	100.0	88.5	96.2	100.0	100.0
	92.3	96.2	103.8	100.0	96.2	100.0	100.0	100.0

Table A 6: Body composition in Experiment 3 (MonoCaP in gilthead seabream)

Treatment	initial	diet 1	diet 2	diet 3	diet 4	diet 5	diet 6	diet 7	diet 8
Dietary P [g/kg DM]		2.31	3.09	4.26	6.22	7.97	9.79	11.48	18.23
Dry matter [g/kg]	310	363	367	379	358	344	347	334	336
		377	361	372	351	338	339	341	335
		369	374	366	353	350	330	340	346
Ash [g/kg]	42.4	25.2	26.3	30.6	36.4	36.4	39.2	38.0	39.9
		25.0	28.8	32.1	35.4	37.7	37.8	39.5	39.2
		23.9	27.5	32.4	33.5	39.5	39.1	40.4	40.4
Crude protein [g/kg]	159	165	165	201	160	167	166	168	167
		166	149	158	174	176	179	175	178
		181	163	170	169	176	172	177	179
Crude lipid [g/kg]	104	183	184	190	164	141	144	132	132
		197	182	185	155	128	125	123	116
		172	191	159	161	141	133	136	137
Gross energy [MJ/kg]	7.5	10.3	10.2	10.4	9.3	8.6	8.9	8.7	8.2
		10.3	9.9	10.1	8.7	8.1	8.0	8.3	8.3
		9.8	10.3	10.0	9.3	9.0	8.7	8.8	8.9
Phosphorus [g/kg]	7.65	4.38	4.61	5.32	6.49	6.73	7.09	6.95	7.32
		4.26	4.93	5.53	6.26	6.09	6.85	7.08	7.15
		4.09	4.48	5.65	5.82	7.17	7.02	7.52	7.15
Calcium [g/kg]	10.6	5.8	6.8	8.0	8.6	9.1	10.4	9.6	9.4
		5.5	6.3	7.6	9.0	10.2	9.8	10.6	9.8
		5.5	6.6	8.1	9.3	10.4	9.9	10.5	10.8
Magnesium [g/kg]	0.52	0.39	0.39	0.43	0.40	0.44	0.46	0.41	0.42
		0.36	0.40	0.36	0.42	0.44	0.45	0.46	0.44
		0.37	0.36	0.41	0.42	0.45	0.44	0.48	0.49
Sodium [g/kg]	1.60	1.42	1.46	1.47	1.20	1.26	1.41	1.33	1.19
		1.45	1.47	1.23	1.29	1.34	1.38	1.41	1.28
		1.25	1.34	1.35	1.34	1.37	1.27	1.46	1.43
Potassium [g/kg]	3.47	2.92	3.25	3.49	3.62	3.48	3.46	3.31	3.29
		3.19	3.17	3.04	3.51	3.55	3.59	3.54	3.38
		3.29	3.15	3.57	3.52	3.64	3.59	3.78	3.80

Table A 7: Composition of diets and faeces and digestibility coefficients in Experiment 4 (DiCaP and MonoCaP in gilthead seabream)

Treatment	Control	Mono-CaP	Di-CaP 1	Di-CaP 2	Di-CaP 3	Di-CaP 4
Diets						
Crude protein [g/kg]	443	444	438	481	468	454
Organic matter [g/kg]	845	844	853	859	856	851
Gross energy [MJ/kg]	20.6	20.5				
Phosphorus [g/kg]	2.53	6.70	5.16	9.07	13.98	16.11
Chromium oxide [g/kg]	8.41	7.15	9.32	9.62	9.71	9.32
Faeces						
Crude protein [g/kg]	50.0 40.3	37.9 37.5	43.3 36.4	49.5 35.7	42.2 34.9	27.7 28.5
Organic matter [g/kg]	459 499	480 499	461 463	449 490	479 478	477 477
Gross energy [MJ/kg]	8.58 7.71	8.73 7.83	- -	- -	- -	- -
Phosphorus [g/kg]	2.45 2.96	4.93 3.84	3.42 4.31	6.23 7.52	10.19 13.28	14.30 17.31
Chromium oxide [g/kg]	16.7 15.5	17.7 15.2	20.6 23.0	24.5 22.5	21.4 26.8	24.7 28.3
Digestibility [%]						
Protein	94.3 95.1	96.6 96.0	95.5 96.6	96.0 96.8	95.9 97.3	97.7 97.9
Organic matter	72.7 68.0	77.0 72.2	75.6 78.0	79.5 75.6	74.7 79.8	78.8 81.6
Energy	79.0 79.8	82.8 82.0	- -	- -	- -	- -
Phosphorus	51.2 36.6	70.2 73.0	70.1 66.2	73.0 64.6	67.0 65.6	66.4 64.6

Table A 8: Composition of faeces and digestibility coefficients in Experiment 5 (Gilthead seabream)

	Rapeseed oilmeal diet	
Suppl. phytase [FTU/kg]	0	1000
Ash	285	269
[g/kg DM]	297	279
Protein	80.0	99.4
[g/kg DM]	69.1	98.8
Energy	8.7	12.2
[MJ/kg DM]	-[1]	12.2
Phosphorus	7.57	3.85
[g/kg DM]	7.59	2.47
Chromium oxide	10.13	10.74
[g/kg DM]	9.81	9.76
Digestibility of	50.5	81.0
dietary phosphorus [%]	48.8	86.6

[1] Sample material was not enough for energy analysis

Table A 9: Growth data and body composition in Experiment 6a (Phytase/Rainbow trout)

Treatment	initial	diet A	diet B	diet C	diet D	diet E	diet F	diet G	diet H
Suppl. phytase [FTU/kg DM]		0	200	400	600	900	1200	2000	20000
Weight gain [g/fish]		128.4	116.1	112.1	147.6	137.3	161.9	139.9	134.8
		86.0	123.1	116.8	107.9	123.1	141.2	136.1	147.2
		102.1	138.1	147.9	138.7	151.1	156.4	153.4	172.8
Feed intake [g/fish]		190.7	167.0	153.3	192.6	177.7	212.6	186.1	166.7
		124.7	185.2	157.6	147.1	171.3	184.1	166.3	179.4
		154.7	181.3	201.3	184.2	191.6	199.5	200.5	218.4
FCE		0.67	0.69	0.73	0.77	0.77	0.76	0.75	0.81
		0.69	0.66	0.74	0.73	0.72	0.77	0.82	0.82
		0.66	0.76	0.73	0.75	0.79	0.78	0.77	0.79
Dry matter [g/kg]	260	372	353	371	370	354	357	355	341
		348	348	363	345	361	347	347	351
		356	360	356	358	349	357	356	356
Crude protein [g/kg]	167	170	158	181	172	168	161	164	166
		172	163	172	172	168	158	173	173
		169	168	164	168	165	163	166	174
Crude lipid [g/kg]	77	185	180	174	178	169	180	172	156
		162	174	175	158	176	166	161	159
		172	176	174	178	166	176	170	166
Gross energy [MJ/kg]	6.7	11.2	10.8	10.9	11.1	10.5	10.7	10.6	10.0
		10.3	10.5	10.8	10.1	10.8	10.3	10.2	10.2
		10.6	10.8	10.7	10.7	10.3	10.7	10.5	10.4
Phosphorus [g/kg]	4.13	2.98	2.85	3.38	3.33	3.44	3.51	3.56	3.71
		3.05	2.95	3.20	3.14	3.44	3.67	3.51	3.92
		3.01	3.01	3.16	3.05	3.25	3.43	3.70	3.99

Table A 10: Composition of faeces in dry matter and digestibility coefficients in Experiment 6a (Phytase/Rainbow trout)

Treatment	diet A	diet B	diet C	diet D	diet E	diet F	diet G	diet H
Suppl. phytase [FTU/kg]	0	200	400	600	900	1200	2000	20000
Composition of faeces per kg DM								
Crude protein [g]	71.4	75.3	90.7	81.0	80.8	90.0	79.7	87.0
	73.2	77.9	76.8	79.8	85.0	78.6	86.8	89.0
	79.0	76.2	82.7	74.8	86.2	89.4	91.4	90.5
Gross energy [MJ]	12.5	12.9	13.1	13.1	13.0	13.3	13.0	13.3
	12.9	13.5	12.5	13.0	13.1	12.9	13.5	13.3
	13.1	12.7		12.9	13.3	13.2	14.0	14.1
Organic matter [g]	671	676	685	688	687	695	688	705
	670	676	669	683	689	687	697	703
	670	679	681	682	688	695	705	716
Phosphorus [g]	18.27	16.14	15.83	13.51	11.14	9.69	8.21	3.72
	18.51	16.85	15.85	14.76	11.85	10.18	8.11	3.82
	18.22	16.22	15.08	13.39	11.98	8.59	6.54	3.44
HCl-insoluble ash [g]	202	196	201	205	215	211	215	226
	197	199	208	207	204	221	208	241
	205	199	209	202	210	208	209	218
Digestibility [%]								
Protein	96.3	95.8	95.1	95.5	95.9	95.5	96.0	95.8
	95.8	95.7	96.1	95.8	95.5	96.3	95.5	96.0
	95.8	95.8	95.9	96.0	95.5	95.1	95.2	95.6
Energy	88.6	87.7	87.9	88.1	88.6	88.2	88.7	89.0
	87.8	87.2	88.8	88.3	87.9	88.3	87.9	89.6
	88.1	88.0		88.1	88.2	88.2	87.4	88.0
Organic matter	85.3	84.3	84.8	84.7	85.3	85.3	85.5	85.9
	84.5	84.4	85.6	85.0	84.8	86.2	84.7	87.0
	85.2	84.6	85.6	84.6	85.4	84.7	84.5	85.3
Phosphorus	18.1	25.8	29.0	33.8	53.6	57.1	66.2	85.6
	5.6	24.1	28.9	37.0	49.0	58.3	64.8	85.7
	21.4	28.5	34.7	40.2	48.6	58.5	72.0	85.3

Table A 11: Growth data and body composition in Experiment 6b (Phytase/Gilthead seabream)

Treatment	initial	diet A	diet B	diet C	diet D	diet E	diet F	diet G	diet H
Suppl. phytase [FTU/kg DM]		0	200	400	600	900	1200	2000	20000
Weight gain		28.8		31.0	38.3	37.9	41.5	35.5	68.9
[g/fish]		25.0	31.1	32.6	24.9	31.9	35.7	38.1	55.8
		22.9	21.2	27.1	24.8	32.1	34.7	36.4	52.0
Feed intake		85.0		85.5	97.9	97.7	103.5	90.6	126.1
[g/fish]		76.9	88.1	84.6	79.5	92.4	92.4	97.2	113.3
		76.1	72.9	86.0	81.5	92.0	91.0	96.9	106.9
FCE		0.34		0.36	0.39	0.39	0.40	0.39	0.55
		0.32	0.35	0.39	0.31	0.35	0.39	0.39	0.49
		0.30	0.29	0.31	0.30	0.35	0.38	0.38	0.49
Survival		100.0	0.0	91.7	100.0	100.0	95.8	100.0	100.0
[%]		100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
		100.0	100.0	87.5	95.8	95.8	100.0	95.8	95.8
Dry matter	334	380		387	378	390	395	386	392
[g/kg]		361	383	371	356	377	381	390	383
		372	389	367	366	378	387	387	385
Ash	41.5	34.2		34.7	32.2	31.9	31.9	36.5	34.4
[g/kg]		33.6	32.5	30.9	32.0	30.7	32.3	34.6	36.3
		37.3	37.6	37.7	33.6	34.6	34.5	33.9	36.9
Crude protein	159	150		166	149	154	153	159	157
[g/kg]		151	148	152	146	155	149	160	151
		157	162	157	158	163	159	158	161
Crude lipid	126	200		191	194	211	206	199	204
[g/kg]		172	194	198	175	196	205	193	195
		187	194	177	182	190	201	199	193
Gross energy	9.2	11.9		11.4	10.8	12.0	11.6	11.3	11.8
[MJ/kg]		10.7	11.1	10.7	9.7	11.1	10.8	11.5	11.0
		11.2	11.5	10.5	11.4	11.4	12.1	11.0	11.6
Phosphorus	7.00	5.70		6.10	5.60	5.50	5.20	6.40	6.10
[g/kg]		6.30	5.90	5.40	5.50	5.40	5.60	6.20	6.80
		5.50	5.80	6.10	5.90	6.10	6.40	6.10	6.80
Calcium	30.5	22.0		22.5	21.2	20.6	22.8	22.3	23.7
[g/kg]		24.6	20.6	20.8	22.3	21.4	21.1	21.7	24.8
		23.0	23.9	23.9	26.6	21.3	21.8	22.6	24.5

Table A 12: Composition of faeces in dry matter and digestibility coefficients in Experiment 6b (Phytase/Gilthead seabream)

Treatment	diet A	diet C	diet E	diet H
Suppl. phytase [U/kg DM]	0	400	900	20000
Composition of faeces per kg DM				
Crude protein	90.0	91.3	93.2	108.1
[g]	104.7	104.9	103.1	93.6
Gross energy	9.8	11.2	10.8	9.4
[MJ]	9.2	9.6	10.6	9.2
Organic matter	613	597	619	603
[g]	621	618	596	578
Phosphorus	5.69	6.57	5.35	2.66
[g]	6.85	5.91	6.09	2.30
Chromium oxide	13.4	12.2	11.7	14.2
[g]	15.2	11.2	12.1	12.4
Digestibility [%]				
Protein	91.8	91.8	91.0	91.9
	91.5	89.7	90.4	92.0
Energy	84.4	82.4	82.8	88.2
	87.0	83.7	83.6	86.8
Organic matter	76.6	77.3	75.3	81.3
	79.0	74.5	77.0	79.4
Phosphorus	59.9	57.0	61.7	85.2
	57.4	58.0	57.7	86.8

Trout experiments

Table A 13: Concentrations of vitamin premix at inclusion level of 10 g per kg diet

Vitamin A	72000 IU
Vitamin D_3	11000 IU
Vitamin E	90 mg
Thiamine	192 mg
Riboflavin	96 mg
Niacin	672 mg
Ca-pantothenate	122 mg
Pyridoxine	42 mg
Folic acid	14 mg
Vitamin B_{12}	0.12 mg
Vitamin K	18 mg
Biotin	3.9 mg
Inositol	1920 mg
Ascorbic acid	288 mg
P-aminobenzoic acid	36 mg

Table A 14: Concentrations of amino acid premix at inclusion level of 141 g per kg diet

Lysine	31.3 g
Methionine	5.1 g
Arginine	4.7 g
Threonine	5.4 g
Tryptophan	0.3 g
Histidine	1.8 g
Valine	8.2 g
Leucine	0.1 g
Isoleucine	6.7 g
Phenylalanine	15.8 g
L-glutamine	50 g
L-asparagine	6.5 g
L-Alanine	5 g

Table A 15: Concentrations of mineral premix at inclusion level of 30 g per kg diet

Ca	49.8 g
K	23.4 g
Na	5.7 g
Mg	3.9 g
Mn	360 mg
Fe	330 mg
Zn	300 mg
Cu	72 mg
Co	54 mg
Se	12 mg
I	9 mg
Mo	2.4 mg

Seabream experiments

Table A 16: Concentrations of vitamin premix at inclusion level of 10 g per kg diet

Vitamin A	16000 IU
Vitamin D_3	1900 IU
Vitamin E	150 mg
Thiamine	30 mg
Riboflavin	45 mg
Niacin	15 mg
Ca-pantothenate	30 mg
Pyridoxine	5 mg
Folic acid	11 mg
Vitamin B_{12}	0.12 mg
Vitamin K	11 mg
Biotin	0.25 mg
Inositol	150 mg
Ascorbic acid	500 mg
Choline chloride	3 mg

Table A 17: Concentrations of mineral premix at inclusion level of 25 g per kg diet

KCl	9000 mg
MgO	6250 mg
FeO_3	638 mg
ZnO	188 mg
MnO	184 mg
$CuCO_3$	144 mg
KI	4.5 mg
$CoCO_3$	1.65 mg
Na_2SeO_3	1.0 mg

Table A 18: Reference of feed components

Trout experiments	
Wheat gluten	Cerestar, Neuss, Germany
Fishoil for trout	Kronen Spezial Tierernährung, Wesel, Germany
Sunflower oil	Food retail trade
Mineral premix	Höveler, Langenfeld, Germany
Vitamin premix	Lohmann Animal Health, Cuxhaven, Germany
Choline chloride	Lohmann Animal Health, Cuxhaven, Germany
Amino acids	Degussa Hüls AG, Hanau, Germany
Sipernat 50S	Degussa Hüls AG, Hanau, Germany
Full-fat Soybeans	Meneba Meel Weert B. V., Netherlands
Soyprotein concentrate	ADM Soya Mainz, Mainz, Germany
Rapeseed oilmeal	Feed trade
Seabream experiments	
Wheat gluten	Amygluten 160, Brenntag N.V., Deerlijk, Belgium
Wheat	Feed trade
Fishoil	Matmor Ldt., Aschdod, Israel
Mineral premix	Koffolk Ldt., Tel Aviv, Israel
Vitamin premix	Koffolk Ldt., Tel Aviv, Israel
Choline chloride	Koffolk Ldt., Tel Aviv, Israel
Chromium oxide	Sigma-Aldrich Chemie GmbH, Munich, Gemany
Arginine	Sigma-Aldrich Chemie GmbH, Munich, Germany
Lysine, Methionine	BASF AG, Ludwigshafen, Germany
Threonine	Kyowa Hakko, Tokyo, Japan
Sipernat 50S	Degussa Hüls AG, Hanau, Germany
Dicalcium phosphate	Matmor Ldt., Aschdod, Israel
Monocalcium phosphate	BASF AG, Ludwigshafen, Germany
Rapeseed oilmeal	Feed trade

Acknowledgements

I sincerely thank my supervisor, Prof. Dr. Ernst Pfeffer, for his guidance and determined support of my thesis as well as continuously offering helpful discussions.

I thank Prof. Dr. Dr. Helga Sauerwein who agreed to be co-examiner.

I would like to thank Dr. George W. Kissil for providing me to carry out the experiments at the National Center for Mariculture in Eilat. He also helped me with several suggestions and revised the English of the manuscript.

Special thanks to Prof. Dr. Markus Rodehutscord for his prompt support in some mathematical problems.

I thank Dr. Jörg Braun for providing the test diets for the double experiment.

I want to thank Dr. Sven Brenner and Kristina Leurs for carrying out the trout part of the double experiment.

I thank Dr. Ingrid Lupatsch for her assistance in carrying out the experiments and for the inspiring discussions we had.

I wish to thank the other Ph.D. students and the staff of the Institute of Animal Nutrition and the National Center for Mariculture for the amicable working atmosphere.

I want to express my gratitude to my family and my friends, especially Martin, for their endless patience and encouragement when it was mostly required.

Finally I would like to thank the H. Wilhelm Schaumann Stiftung for funding my thesis.